Reproductive Biology of Wild Goldlined Seabream, *Rhabdosargus sarba*, Captive Breeding and Larval Development in the Sultanate of Oman.

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A thesis submitted for the degree of Doctor of Philosophy

By

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Declaration

This work has been conducted exclusively by my own research. Work in this thesis has neither been accepted nor is being submitted for any other degree. Work and analysis in this thesis has been conducted independently unless otherwise acknowledged.

Candidate :.....

Supervisor:.....

Date :.....

بسم الله الرحمن الرحيم

إهداء

إلى أبي العزيز، إلى أمي الغالية. إلى جدتي رحمها المولى

التي أكن لها كل معروف لم ولن أوفيه لها ما حييت.

التي ربتني وغذتني وعلمتني وسهرت علي، فقدتها مشاعل

كانت تضيئ طريقي، فقدتها في الأيام الأخيرة من مشواري

وكانت روحي أنتزعت مني ... إلى أخوتي وأخواتي

وبالخصوص الذين فقدتهم وأنا خارج الوطن رحمهم

الباري.

أهدي هذا العمل إلى زوجتي الغالية، يا من وقفت

بجانبي طوال الطريق، يعجز اللسان عن الوصف. إلى

إبني العزيز عبد العزيز الذي واجه الغربة منذ نعومة أظفاره.

أهدي هذا العمل إلى كل من ساهم في تخفيف معاناتي

من الأصدقاء.. فجزاهم الخالق عني كل الجزاء

إلى وطني رغم الآهات ... عمان

إليكم جميعاً أهدي هذا السفر،

زاهد صالح العجمي

المملكة المتحدة

ديسمبر 2004

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Abstract

This study investigated the, age, growth, mortality and reproductive biology of wild caught goldlined seabream *Rhabdosargus sarba* in the Sultanate of Oman, captive spawning of broodstock and larval development and feeding.

Age was estimated by counting the opaque rings that formed in the sagittal otoliths. It was evident from the marginal zone analysis that one opaque and one hyaline were deposited annually. Length-age and weight-age data were fitted to the Von Bertalanffy growth function (VBGF), which suggested a difference in growth rate between sexes. Males mature at age zero due to the protandrous nature of this species and length at first maturity was $L_{50\%}$= 19.04cm T.L. Females mature at 1+ years of age and length at first maturity was $L_{50\%}$= 23.41cm T.L. Length at first capture was estimated to be (LC_{50}= 13.3cm) which was below the age at first maturity for male. Length converted catch curves gave an estimated instantaneous mortality rate for the sampling site (Z=0.42) and (Z= 0.39) for the fish sampled at the fish market. This total mortality rate gave an exploitation rate of about E=0.15 at both sampling locations which suggested that *R. sarba* was fished below optimal levels of exploitation.

Ovarian development of wild stocks was evaluated histologically. Oocyte development was classified into seven stages including atresia that was subdivided into four stages. These included previtellogenic, vitellogenic, atretic and post-ovulation phases of ovarian development. Testis was classified into four stages based on type of cells and their relative abundance, which included immature, developing, active and post-spawning. Testes were found to mature one month in

advance of ovaries. Based on histological evidence, the natural spawning season occurred in late December to early January and continued until late February. Gonadosomatic index for male and female peaked in January and decreased sharply following spawning activity. Sterology had proven a useful tool to study on the ovarian dynamics of *R. sarba*. The level of plasma calcium (Ca^{2+}) was elevated during maturity stage four (early vitellogenic phase) suggesting higher vitellogenin activity during this stage. Its level was 20% higher than of stage two maturity.

The dynamics of lipid level and composition in female *R. sarba* during the reproductive season were also studied. Females experienced lipid depletion in liver, muscles, carcass and visceral fat as a result of ovarian development during the reproductive season. The level of total lipid correlated well with the ovarian stage of maturity ($p<0.02$). Fatty acid composition and profile of the ovaries at various stages of development also varied during the reproductive season.

Breeding of wild stock of *R. sarba* in captivity was investigated. Spawning occurred after a short captive acclimatisation (two months) period without any artificial induction. Histological and ultrastructural changes in *R. sarba* larva in early life history were also investigated. Morphological and functional differentiation of the digestive tract and the eye of the larvae took place before the completion of yolk and oil globule absorption. Food particles were observed on the third day after hatching in the rectal area of intestine of *R. sarba* larvae at the time when the larval vision system appeared to be functional. Rotifer enrichment with lipid emulsions improved larval growth rate and survival as a result of improvement in the level of HUFA (DHA and EPA). The highest *R. sarba* larval growth ($P < 0.05$) was

obtained while feeding the larvae with rotifers enriched with oil. No significant difference ($P > 0.05$) in larval survival was observed between feeding the larvae with rotifers enriched in sunflower oil and rotifers fed algae. However, highest *R. sarba* larval survival ($P < 0.05$) was obtained while feeding the larvae with rotifers enriched with cod liver oil.

Finally it has to be said that *R. sarba* proven to spawn in captivity and that larval rearing was possible and that it is a candidate species for aquaculture in the Sultanate of Oman. It has to be emphasised that more research could be carried in the future on broodstock management and egg and larval quality in order to reveal more information about the species requirement.

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Chapter 1: General introduction, literature review & aims of research

1.1 General Introduction

The Sultanate of Oman has a coastline of about 1800km consisting of diverse geomorphological and marine habitats (Figure 1.1). The coastal region of Oman is highly populated and many who inhabit the coastal regions engage in capture fisheries activities for their livelihood. Despite the large coastline recent trends suggest fish stocks are declining and the livelihoods of local fishermen threatened. Traditional fishermen use traps, hand lines and bottom gill net as the primary fishing gear for demersal fish. At present there is a shift from pelagic to demersal fishing (Siddeek *et al.*, 1999).

Figure 1.1 A map of the Sultanate of Oman.

Ref.: http://wwp.greenwichmeantime.com

Catch data are difficult to interpret as fish statistics usually includes other sparid fish. According to the Statistics & Information Department, Ministry of Agriculture and Fisheries Resources, Oman the landing of seabream in Oman has increased from 1626 thousand tonne in 1995 to 2213 thousand tonne in 2000 (Figure 1.2,

Oman Fishstat, MAF). FAO statistics for the Western Indian Ocean region showed that the catch in the year 2001 decreased to a level lower than that of 1995 (Figure 1.2). Employment in fisheries, on the other hand, continued to increase side by side with fishery production suggesting an increase in fishing effort, a trend similar to Asia where the number of fisherfolk increased from 9301 in 1970 to 27340 in 2000 (FAO, 2002).

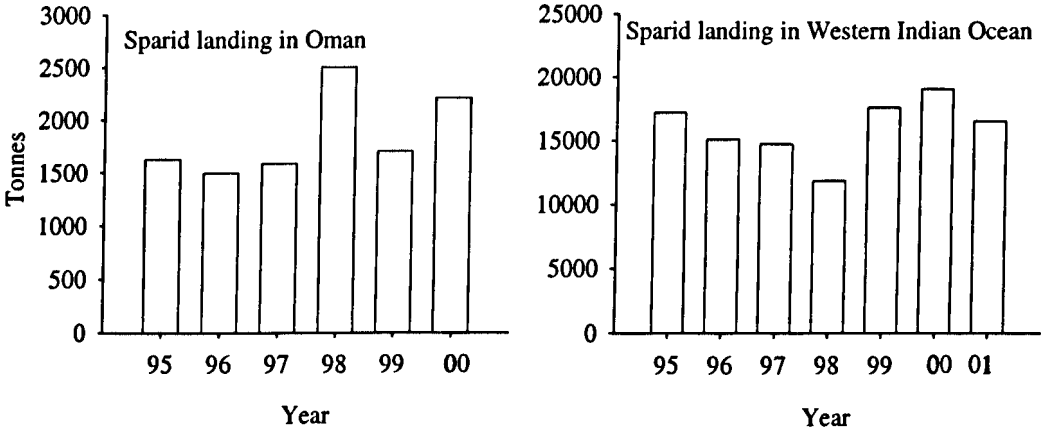


Figure 1.2 Landings of sparid in Oman and in Western Indian Ocean.

Data source for Oman's landing (Oman Fishstat, MAF; electronic source) and for Western Indian Ocean (FAO, Fishery statistic; electronic source).

The increase in landings was encouraged by an ongoing government project in Oman to support certain fisherfolk by providing bigger boats. These developments, which are likely to increase fishing effort further, may lead to a decline in the demersal wild stock including the sparids which account for around 25% of total landing and may jeopardise the livelihood of fishermen.

Therefore, alternative forms of employment for fishermen will need to be explored. In the face of declining fish stocks aquaculture is recognised as a potential substitute.

In recognition of the high probability of decline in fish stocks, the government of Oman through the Ministry of Agriculture & Fisheries Resources is promoting aquaculture of different species in the country mainly by encouraging local and international investment. The aim of the investors at present is to culture Mediterranean finfish species such as *Sparus aurata* since the technology to culture such species has been already developed. It is well known that importing exotic fish has a notable impact on other native species such as disease introductions (Welcomme, 19898; Beveridge *et al.*, 1994) and therefore, in the medium and long term, the feasibility of developing native sparid species is highly desirable. To this end, the goldlined seabream *Rhabdosargus sarba* may be a potential candidate for culture and should be researched.

1.1.1 Taxonomy

Sea breams (family: Sparidae) contain five sub families (Sparinae, Denticinae, Pagellinae, Boopsinae, and Pagrinae), 29 genera, and 100 species in the world (Chen *et al.*, 1999). The body of Sparidae is oblong, deep and laterally compressed. The colour of the Sparidae is variable from pinkish to reddish to yellowish or greyish, and often demonstrates silvery or golden reflections (Fisher & Whitehead, 1974). The seabream that have a red colour inhabit deep sea, while the silver-grey seabream inhabit shallow water (Bromage & Roberts, 1995).

Rhabdosargus sarba (Forskål, 1775) also has another scientific name still in use and that is *Sparus sarba* (Forskål, 1775) (Fisher & Whitehead, 1974). It has two common names in use, the goldlined seabream (Fisher & Whitehead, 1974) and the

silver seabream (Chiba, 1983). The species has a deep body which is laterally compressed with an overall bright grey colour.

1.1.2 Global distribution of the goldlined seabream *R. sarba*

The goldlined seabream (or silver seabream) *Rhabdosargus sarba* (Forskal 1775) inhabits rocky, coastal waters (Hu *et al.*, 1991). It inhabits shallow waters at a depth of generally less than 50m and also can be found in brackish and inshore waters (Okada, 1966; Fowler, 1972; Patnaik, 1973; Berry *et al.*, 1982; Heemstra, 1986). It is found in the seas surrounding Japan, Korea, Taiwan and in the East China Sea (Okada, 1966; Fowler, 1972) and is also found in the Indo-West Pacific (Blaber, 1984; El-Agamy, 1989; Mahboob *et al.*, 1998) and Red Sea (Mihelakakis & Kitajima, 1994). *Rhabdosargus sarba* attains a total length of 60 cm, while the common total length is 40cm (Fisher & Whitehead, 1974).

1.1.3 Food and feeding habit of *R. sarba*

The observations of Patnaik (1973) and El-agamy (1989) were that the feeding habit of *R. sarba* changes with size. Fish of smaller body length, and up to the size of 15cm, feed mainly on euphausids, shrimp larvae and amphipods, while larger ones prey upon other fishes, crustaceans and molluscs. Similar behaviour has been reported for other sparid fish such as the case with black seabream *Acanthopagrus butcheri* (Sarre *et al.*, 2000). Gut content analyses of *R. sarba* larvae (17-31mm) by Patnaik (1973) suggests that copepods represent the major food item and accounted for about 36% and algae was 7.2%. Similar observations have been reported for other sparids such as the black seabream, *Mylio macrocephalus* (Wong & Nip, 2001) who also found that calanoid copepods were the most dominant food item in the digestive tracts. Likewise, the results of Nig *et al.*, (2003) for black seabream,

Acanthopagrus schlegeli and *A. butcheri* (Willis *et al.*,1999) larvae were similar to *R. sarba.*

Some studies suggest that feeding habits may change during the reproductive cycle. According to El-Agamy (1989), the feeding intensity of female *R. sarba* decreases from stage four of maturity onwards. Stage four is considered a mature stage according to Hjort (1910). It is unclear, however, if goldlined seabream shows seasonal differentiation in feeding intensity. El-Agamy (1989) suggests that there is an increase in feeding intensity in fish having stage two and three (premature stage) ovaries. Feeding activity during maturation was found to decrease, although the author suggested that this could be due to biased results citing the fact that fish were caught by traps and gillnets which may result in loss of food through vomiting or due to longer times spent in traps before examination in which case the fish may have emptied their stomach. Patnaik (1973), however, concluded that feeding intensity does not seem to be affected by any seasonal factor such as change in temperature during the reproductive season.

1.1.4 Reproductive biology of *R. sarba*

1.1.4.1 Spawning season of *R. sarba*

The goldlined seabream *R. sarba* has been reported to spawn naturally between December and March in Taiwan (23° 40'N; 119° 20'E) when the water temperature is between 15 and 20° C (Lin *et al.*, 1988). Based on the *R. sarba* ovarian classifications carried out by Patnaik (1973) on wild stock in Chilka Lake, India, the author concluded that mature ovaries are encountered during November to January which facilitates the extension of the spawning season of *R. sarba.* El- Agamy

(1989) conducted a biological study on wild stock *R. sarba* in Qatari waters, Arabian Gulf, and his study showed that this species has a long spawning season which extends for about three months from April to June. He stated this conclusion based on his microscopic observations of the presence of more than one size group of *R. sarba* oocytes in the ovary.

The spawning habit of *R. sarba* under captive conditions was also reported to be fractional and prolonged similar to that of wild stock. Lin *et al.* (1988) observed continuous spawning that started from December to March at a temperature range of 15 – 26°C. Leu (1994) reported that spawning times in Iapan were similar to those of Taiwan. Mahboob *et al.* (1998) reported *R. sarba* spawning to occur in February in Aden, Republic of Yemen. The authors, however, did not manage to spawn *R. sarba* continuously which could be due to the fact that the stocking of wild brooders took place at the beginning of their spawning season and due to the stress imposed on them the spawning event occurred at a later stage and discontinued after few days. But during the period that spawning occurred it was daily and continuous.

1.1.4.2 Gonadal characteristics of *R. sarba*

In most teleosts, the pattern of ovarian development has been categorised as synchronous or asynchronous based on the growth pattern of the oocytes (Scott, 1987). Fish that are considered synchronous ovulators generally have two main stages of developing oocytes in their ovary at any one time; a larger oocyte that will be used in the coming season and a primary oocyte for use at a later phase (Scott, 1987). Fish such as salmonids have two different patterns of spawning behaviour; yearly, such as the case in brown trout, *Salmo trutta* (Bagenal, 1969), or once in the

fish's life as, for example, the Pacific pink salmon, *Oncorhynchus gorbuscha* (Dye *et al.*, 1986). There are many marine teleosts that are asynchronous, whereby, developing oocytes are continuously ovulated and are released in batches throughout the spawning season (Scott, 1987). Some marine species that are well known to aquaculture fall into this category such as sea bream (El-Agamy, 1989; Bromage & Roberts, 1995), sea bass (Carrillo *et al.*, 1991) and halibut (Norberg *et al.*, 1991). There is also one important wild marine species that has been described as an asynchronous ovulator and that is the mackerel (Wallace & Selman, 1990).

There are many families of fish in which hermaphroditism is a common and functional process (Policksansky, 1982). A review of sexuality carried out by Buxton & Garratt (1990) on the Sparidae shows that protandrous, protogynous, simultaneous and rudimentary hermaphroditism has all been reported in the family. As the author suggested, sex inversion is an alternative reproduction strategy which allows individuals to maximise lifetime reproductive success by functioning as one sex when small and as the other at later stages. Pajuelo & Lorenzo (1996) found that high proportions of female red porgy, *Pagrus pagrus* were of smaller sizes and the presence of individuals with regular testes and residues of degenerated ovaries indicated that this species exhibits protogynous hermaphroditism. Protandry has been reported for many other sparids such as *Diplodus sargus* (Micale & Perdichizzi, 1994). This sparid is a protandric hermaphrodite and begins life as a functional male with a testicular zones undergoing active spermatogenesis and an ovarian zone that is arrested at the primary growth (perinucleolar) stage. Similar findings were also reported by Abou-Seedo *et al.* (2003) in a study carried out on yellowfin seabream, *Acanthopagrus latus*. Protandry, however, is considered more

difficult to diagnose due to the fact that testicular structural and germinal features rarely remain, that is why sex-reversed gonads are difficult to distinguish (Sadovy & Shapiro, 1987). Pajuelo & Lorenzo (2003) found that the two-banded seabream, *Diplodus vulgaris* exhibits rudimentary hermaphroditism in which young fish have an immature inter-sexual gonad but mature as either female or male with a low proportion of protandrous sex reversal. There is still debate on some sparids such as Dentex, *Dentex dentex*, as this species is characterised as a protandric hermaphrodite fish (Glamuzina *et al.*, 1989). This early assumption was made on the information that fish in the range of 400g to 580g are either male or immature, whereas fish between 600g and 800g are female, which suggests that *Dentex dentex* is a protandric hermaphrodite. Recent work carried out on this species (reviewed by Rueda & Martínez, 2001) considered *Dentex dentex* as a gonochoristic species, fish that maintain the same sex throughout its entire lifespan.

Evidence derived from histology and biopsies of the species *R. sarba* carried out by Yeung & Chan (1985, 1987b) clearly indicated the occurrence of natural sex reversal from male to female (protandrous hermaphroditism). The authors indicated that the gonad consists of distinct male and female zones and that these zones were clearly separated by connective tissue. The authors have also distinguished four types of gonad. Type I (male), II (intersex), III (female) which has a vestigial testicular tissue and finally type IV (male gonad). The latter was found more commonly in larger specimens and the authors suggested that these functional males might not undergo sex reversal in their life cycle. A study carried out by El-Agamy (1989) in Qatari waters in the Arabian Gulf indicated that sexual maturity was being attained by about 20% of the fish during their second year.

El-Agamy (1989) stated that fecundity generally increases in wild *R. sarba* with increase in fish length or fish weight. The author examined the fecundity of different sizes of fish ranging from 17-26cm (TL) over a period of one year and fecundity varied from 23000 to 99000 eggs per fish. Fertilised eggs were reported to have an average diameter of 950μm (Lin *et al.*, 1988) and 1003μm (Leu, 1994). The average diameter of unfertilised eggs, however, was reported to be about 800μm (Mahboob *et al.*, 1998) and 748μm with a variation of 73μm (Chen *et al.*, 1999). The sizes of unfertilised eggs were similar to sparids such as *Acanthopagrus latus* (824μm), *Acanthopagrus schlegeli* (667μm) and *Pagrus major*s (1019μm) (Chen *et al.*, 1999).

1.1.4.3 Estimating fecundity

As a general practice, fecundity can be estimated using a volumetric method (Simpson, 1951), a gravimetric method (Burd & Howlett, 1974) or by the use of an automated particle counter (Bycroft, 1986; Witthames & Greer Walker, 1987; Annala & Bycroft, 1987). As computer software develops scientists utilise this technology to speed up the process of counting and estimating egg numbers. Most recently computer-assisted image analysis was applied in egg and larval counting and proved to be useful and showed no significant difference from manual counting (Bates & Tiersch, 1997).

Among the chemicals that are used to digest ovarian tissues in order to separate oocytes and ova for fecundity estimation is Gilson's fluid. This chemical, however, is considered harmful and highly toxic, and the digestion process is time consuming and labour intensive. When estimating fecundity using these techniques, knowledge

of the lower size of vitellogenic occytes is essential as counting non-vitellogenic oocytes may overestimate fecundity. Moreover, the existence of atretic oocytes and post spawning follicles may further complicate the process.

A technique known as 'stereology' overcomes these shortcomings and requires only the preparation of a histological slide of the ovary under study. Based on Deleese principle (Deless, 1847), the technique obtains three dimensional information regarding number, length, surface or volume of the object under view from measurements made on 2D microscope sections. The method has been applied and validated on herring, *Clupea harengus*; Dover sole, *Solea solea*; mackerel, *Scomber scombrus* (Emerson *et al.*, 1990; Greer Walker *et al.*, 1994); tilapia, *Oreochromis niloticus* (Srisakultiew, 1993); *Tilapia zilli* (Coward, 1997; Coward & Bromage 2002) and Atlantic northern bluefin tuna, *Thunnus thynnus* (Medina *et al.*, 2002).

1.1.4.4 Steroidogenesis of *R. sarba*

It is now well accepted that oogenesis, especially vitelloginesis and oocyte maturation in many teleost species is regulated by ovarian steroids (Nagahama, 1983, as reviewed in Fostier *et al.*, 1983). During the phase of oocyte growth, ovarian recrudescence begins whereby a secretion of different hormones takes place. For instance, oestrogens (17β-oestradiol, E2 and oestrone E1) dominate the early phase of the reproductive cycle of females (Smith & Haley, 1988). Oestradiol is secreted by the ovarian follicle in response to GtH, which acts on the liver to produce the yolk protein vitellogenin, sequestered into the developing oocytes (Tyler *et al.*, 1991). Kagawa *et al.* (1982) stated that testosterone (T) represents a substrate for 17β-oestradiol (E2), which may play a role in maintaining the oocytes

once vitelloenesis is completed (*Review* Kim, 1993). The author stated that in teleosts that have synchronous ovarian development patterns, levels of E2 and T increased during oogenesis and peaked prior to, or at the beginning of ovulation and fell during final maturation and ovulation of oocytes. The author further added that multiple spawning species such as seabream have been reported to experience short-term fluctuations in plasma E2 and T due to the existence of different size groups of oocytes at different time intervals.

The examination that has been carried out on the annual and short-term cycles of plasma levels of reproductive hormones suggested that 17β-oestradiol (E2) and testosterone (T) were good indicators of vitellogenesis and ovarian recrudescence or development (Pankhurst & Carragher, 1991). McKenzie *et al.* (1989) found that the increase in level of E2 was associated with the first appearance of vitellogenic oocytes in female channel catfish (*Ictalurus punctatus*). Carragher & Pankhurst (1993) found that plasma levels of E2 and T in wild snapper (*Pagrus auratus*) increased in concert with ovarian recrudescence, and the increase in E2 occurred at the same time as the increase in GSI and HSI took place. Yeung & Chan (1987a) found that E2 levels increased in the female of the goldlined seabream, *R. sarba* during the prespawning period. Blythe *et al.* (1994) concluded that the prespawning increase in plasma E2 in striped bass, *Morone saxatilis* coincided with a rapid increase in oocyte growth, and peak E2 levels occurred at nearly the same time as peak oocyte diameters.

Plasma levels of sex steroids in *R. sarba* were investigated by Yeung & Chan (1985) and Chan & Yeung (1989). The study carried out by Yeung & Chan (1985)

concentrated mainly on plasma levels of androstenedione, testosterone (T), 11-oxotestosterone, 11β-hydroxytestosterone, 17β-estradiol (E2) and estrone at different sexual phases of the fish during the spawning season. The study showed that the level of T remained fairly constant throughout the reproductive cycle in all phases except that of the intersexual phase that had a slight increase. The level of E2 in females showed an increase at the prespawning phase. Chan & Yeung (1989) carried out the second study to further investigate the sex steroids in *R. sarba* in comparison to other intersexual fishes. However, although these two studies have explained the change in sex steroids during the reproductive cycles in the three sex phases, they were not related to the development of the oocytes and spermatogenesis in details. In both studies, the authors classified the reproductive cycles in males and females into prespawning, spawning and post-spawn/inactive stages without showing histological evidence of classification.

The gonadal anatomy of the protandrous hermaphroditic *R. sarba* was well described by (Yeung & Chan, 1987b) the authors gave distinguishing features of different types of gonads at different sexual phases.

1.1.4.4.1 Plasma total calcium (Ca^{2+}) and level of vitellogenin

Vitellogenin (VTG) a calcium-binding lipophosphoprotein is produced in the liver, induced by 17β-estradiol and transported to the ovary by the bloodstream where it is incorporated by a receptor-mediated process into the developing ovary (Yaron *et al.*, 1980, Wallace, 1985). Vitellogenin levels in fish plasma are reflected by the level of calcium (Ca^{2+}) since it is a major calcium-binding protein in female fish undergoing oocyte yolk deposition (Tyler & Sumpter, 1990). Several studies have shown that there is a correlation between the level of VTG and Ca^{2+}. Vitellogenin

level in female teleosts is reported to display seasonal variation and elevate simultaneously with ovarian maturation (Whitehead *et al.* 1985, Srivastava & Srivastava 1994, Webb *et al.*, 2002) and in male teleosts, Ca^{2+} levels showed no change at different phases of gonadal maturation during the reproductive season (Balbontin *et al.*, 1978). Mañanós *et al.* (1994) found that VTG levels in females were correlated with the oocyte development in farmed seabass, *Dicentrarchus labrax.*

Estimating plasma vitellogenin (VTG) provides a better way to distinguish fish sex especially for those that cannot be distinguished by external appearance. This avoids the use of surgical biopsies that are labour-intensive, stressful and may cause an infection (Webb *et al.*, 2002, Linares-Casenave *et al*, 2003). Observations of VTG levels in the wild population may provide valuable information on rates of reproductive development and length of ovarian cycles (Linares-Casenave *et al.*, 2003). Estimation of plasma VTG concentrations has been conducted by quantifying the plasma protein phosphorus or total plasma calcium (Doroshov *et al.*, 1997; Srivastava & Srivastava 1994). Plasma calcium (Ca^{2+}) level in *R sarba* during the reproductive season has not yet been estimated.

1.1.5 Culture of *R. sarba*

The sea bream family (Sparidae) is becoming an important species in mariculture. The gilthead seabream, *Sparus aurata*, is one of the most important species for aquaculture purposes in the Atlantic and Mediterranean regions (Ramos & Pereira, 1990) and hold market potential in Europe. Red seabream (*Pagrus major*), silver

seabream (*Rhabdosargus sarba*) and black seabream (*Mylio macrocephalus*) are commonly cultured marine fish in the Western Pacific Rim (Woo & Kelly, 1995). Goldlined seabream *R. sarba* is of commercial interest because of its good flavour (Hu *et al.*, 1991) and has good potential for both restocking and aquaculture (Tsukashima & Kitajima, 1982; Kitajima & Tsukashima, 1983). Thanks to its high commercial value, the fish is a commonly cultured marine food fish in the Western Pacific Rim (Leu, 1994; Woo & Kelly, 1995) and is also cultured successfully in India, Japan and France (Radebe *et al.*, 2002).

1.1.5.1 Culture of *R. sarba* based on wild caught seed

Goldlined seabream *R. sarba* is readily available as juveniles in estuaries, such as in Japan (Okada, 1966; Fowler, 1972), South Africa (Blaber, 1984, Mann, 1995) and Australia (Neira & Potter, 1992). The juveniles of this species has also been commonly observed in mangrove creeks in Oman (Al-Moharami, 1994). Its availability in estuaries during the juvenile stage had supported the initial culture practice of this species which started with the collection of juveniles from local coastal waters (Leu, 1994). This author stated that at the time the supply of seed from natural water bodies was insufficient for mass production. The author further added that seed production capability is the main constraint. Nevertheless, there was a break- through to overcome this constraint and in recent years some progress has been made in artificially spawning the species.

More research is required to understand larval development, feeding and dietary requirements of early life history that may help to increase survival and growth.

Understanding the basic requirement for fish larvae such as food quality with relation to growth is the key for greater survival.

1.1.5.2 Seed production from captive breeding

According to most recent available data on the aquaculture of *R. sarba*, the seed production from around eleven hatcheries in Taiwan reached about 4400 million in 1998 and these are sold at 3cm (TL) to the on growing cages (Yeh, 2001).

1.1.6 Natural spawning of *R. sarba* in captivity

One of the advantages of mass production of the goldlined seabream *R. sarba*, is that it is among the few marine fish that can easily spawn in captivity without the use of hormones or any other induced spawning methods (Leu, 1994). Such an approach helps to avoid or minimise the mortality of the spawners through stress due to stripping and handling (Leu, 1994).

The first natural spawning of *R. sarba* in captivity with the use of hormone treatment and the administration of HCG (human chronic gonadotropin) and without has been observed in Taiwan by Lin *et al.* (1987, 1988, 1989, 1990) and Leu (1994). This was followed by Mahboob *et al.* (1998) who managed to successfully breed *R. sarba* in open-air tanks in Aden in the Arabic Republic of Yemen in 1991. The first natural spawning of the species under captive conditions in Japan, however, was reported in 1995. Mihelakakis & Kitajima (1995) reported their success in spawning *R. sarba* in 1992.

Other sparids such as the white seabream, *Mylio berda* have been demonstrated to spawn naturally in captivity (Mok, 1985). The author mentioned that fertilised eggs of the white seabream could be obtained through natural or artificial spawning, but suggested that these methods are limited to the peak spawning period. The author, however, managed to extend the spawning period from a few weeks to over three months by the use of HCG. Similarly, Faranda *et al.* (1985) reported successfully spawning *Puntazzo puntazzo* in captivity by means of (HCG) hormone injection. The authors compared the spawning behaviour of two spawning stocks, one caught at sea and another reared in the laboratory. They reported that there was no difference of response between the two stocks, which suggests that sparids do not require long acclimatisation periods. Glamuzina *et al.* (1989) also achieved spontaneous spawning under controlled conditions for common dentex, *Dentex dentex* by use of a hormone treatment (HCG). Jug-Dujaković & Glamuzina (1988) reared common two-banded seabream, *Diplodus vulgaris* from juvenile stage until first sexual maturity in captivity, and were able to strip gametes and carry artificial fertilisation that gave positive results. The authors reported that this sparid fish matured spontaneously without the use of hormone treatment.

In Taiwan, following the first attempt to produce *R. sarba* eggs naturally by administration of HCG, the spawning period lasted as long as 97 days at a temperature ranging between 15° to 26°C (Lin *et al.* 1987). Spawning without the use of hormones and/or other inducing methods was observed by Lin *et al.* (1990) and the resulting spawning period of a 4-year-old female *R. sarba* lasted 120 days from November to March at a water temperature ranging between 14.0 and 22.4° C. Leu (1994) had a group of three-year-old *R. sarba* (4 female and 12 male) that

spawned from December to March and over 96 days in water temperatures ranging from 13.8° to 23.5° C, which were similar to those of Lin *et al.* (1990). Mihelakakis & Kitajima (1995) reported that spawning occurred in Japan between April and June within a water temperature range of between 13.5° and 21.3 ° C. and that female *R. sarba* spawn naturally every night in captivity during the spawning season (Lin *et al.*, 1990; Leu, 1994).

1.1.7 Body reserves and their fluctuations during spawning season

Study of the annual cycle of wild fish provides useful information required for evaluating the quality and physiological condition of cultured animals (Knox *et al.*, 1988). Such knowledge can describe environmental and physiological controls of growth and nutrient utilisation that could enhance the growth or final product quality in cultured animals (Craig *et al.*, 2000). The normally occurring biochemical composition of an animal is a useful baseline for comparison to establish the condition of its health and help to understand the dietary requirements of broodstock during the reproduction cycle (Hurwitz & Plavnik, 1986).

The annual cycle in somatic reserves occurs in a variety of species on a continuous basis during the year (Craig, 1977). During the feeding season, species allocate different body compartments to store lipid and other body constituents which vary according to species (Luzzana *et al.*, 1996). Fish then mobilise the stored elements from these compartments to be incorporated into the recrudescent ovary during the reproduction season and/or during the fasting period (Luzzana *et al.*, 1996).

Water, protein and lipid are typically accumulated during somatic growth and, for example, lipid and/or protein can be depleted during gonadal growth (Tanasichuk & Mackay, 1989; Jørgensen *et al.*, 1997). The high energy costs of gonadal development in fish result in redirecting available energy towards gonadal rather than somatic growth such as in perch, *Perca fluviatilis* L. (Craig, 1977), Atlantic herring, *Clupea harengus* L. (Iles, 1984) and Atlantic salmon, *Salmo salar* L. (Rowe & Thorpe, 1990). Reproduction is generally a process which imposes significant metabolic demands on the fish, whatever the pattern of ovarian development (Wiegand, 1996).

Lipid composition and its availability influences a variety of reproductive parameters in fishes (e.g. Craik & Harvey, 1987; Ogata & Murai, 1989) and plays several roles in fish reproductive physiology (Craik & Harvey, 1987). The hepatic synthesis of the yolk precursor, vitellogenin (VTG), requires sufficient supplies of long-chain fatty acids (Sargent *et al.*, 1989), compounds which support the steroid hormones that are necessary for the induction of hepatic VTG synthesis (Emmersen & Petersen, 1976). Cholesterol plays a vital role in the synthesis of steroid hormones, such as testosterone and oestradiol-17β and adequate supply is essential for fish reproduction (Emmersen & Petersen, 1976).

Makarova (1973) concluded that testicular growth in the European Perch, *Perca fluviatilis* is at the expense of liver lipid and mesenteric fat, while ovarian development uses endogenous protein and exogenous lipid. Craig (1977) found that testicular growth in yellow perch, *Perca flavescens* is stimulated by protein and water accumulation, while the lipid weight was relatively same throughout the year.

Ovarian growth in this species, however, took place as a result of continuous accumulation of protein, water and lipid (Tanasichuk & Mackay, 1989).

Jørgensen *et al.*, (1997) and Jobling *et al.* (1998) showed that body lipid in Arctic charr, *Salvelinus alpinus* decreased between 30-40% during maturation. The authors found that the ovaries of mature females held around 25% of the body lipid, while the mature male testes contained less than 3% of the total body lipid. The female, however, is estimated to lose about 80% of its body lipid during spawning. The gonadal development in female Arctic charr and salmonids represents a major drain on lipid stores (Dutil, 1986; Nassour & Léger, 1989; Jonsson *et al.*, 1991). Final oogenesis in Atlantic salmon is followed by depletion in muscle protein and lipid while the muscle water content increases (Aksnes *et al.* 1986).

Triacylglycerols (TAG) were the dominant neutral lipid in both female and male reproductive organs of the Arctic charr (Jobling *et al.*, 1998) and in the roe of orange roughy, *Hoplostethus atlanticus* (Body, 1985), Atlantic herring, and capelin (Tocher & Sargent, 1984).

Sockeye salmon, *Oncorhynchus nerka*, derive the lipid primarily from the carcass (Idler & Bitners, 1960) whereby 8.2% of lipid mobilised during fasting is deposited in the ovaries. Likewise plaice, whereby 10.6% of the lipid is mobilised and about 33% of the protein in the body is directed to egg production (Dawson & Grimm, 1980). The female trout, *Oncorhynchus mykiss*, is reported to mobilise lipid from the carcass and visceral reserves (Nassour & Léger, 1989). Some species, however, do not experience any fall in the body reserves in the period before spawning but

have been reported to decline during gonadal development (Nassour & Léger, 1989). For instance, the northern pike in Lac St.Anne, Alberta, Canada, deposit substantial amounts of lipid in the ovary prior to the spawning season (Medford & Mackay, 1978) and due to low energy at this time there is no depletion of somatic reserves including lipids (Schwalme & Mackay, 1992). Hails (1983) reported the same behaviour in the anabantid, *Trichogaster pectoralis*.

Phospholipids such as phosphatidylinositol, phosphatidylcholine (lecithin), and phosphatidlethanolamine (PE, cephalin) are key components for both the structure and function of cell membranes (Rosenblum *et al.*, 1994). Lipid reserves in teleosts, however, are stored in lipoprotein yolk in some species and a discrete oil globule in others (Wiegand, 1996). Lipids that form lipoprotein yolk are mainly polar lipids, these polar lipids are known also as 'complex' which contain three or more hydrolysis products per mole (Christie, 1982). The main polar lipids in this sense are phosphatidylcholine (lecithin) and phosphatidlethanolamine (PE, cephaline) and these polar lipids are rich in polyunsaturated fatty acids (PUFA), especially 22:6 (n-3) (docosahexaenoic acid, DHA) which is one of the essential fatty acids. The lipid, however, is carried to the oocyte by vitellogenin, which is rich in polar lipids that have triacylglycerol (TAG) (Wiegand, 1996).

Based on the literature available on wild *R. sarba*, information about energy partitioning and lipid dynamics in female *R. sarba* is scarce. It has been demonstrated that lipid and fatty acids (FA) composition of fish eggs reflects the dietary FA provided to the broodstock (Harel *et al.*, 1994, Almansa, *et al.* 1999). It is essential, therefore, to describe quantitative changes in various organs and tissues

especially the ovary at various stages of maturity to have an understanding of the importance of fatty acids and their profiles which may provide the dietary requirement of both the broodstock and the larvae.

The objectives of the present investigation were to quantify seasonal lipid dynamics in the goldlined sea bream, *R. sarba*. Also, no data are available on the FA composition of goldlined seabream *R. sarba*. Information in this respect can be provided through the study of wild females' ovaries during the reproductive season.

1.1.8 Egg and larvae of *R. sarba*

A mature and/or fertilised egg of the silver bream *R. sarba* is spherical, non-adhesive and pelagic (Lin *et al.*, 1988; Lue 1994) with a diameter of between 0.95-1.04mm (Lin *et al.*, 1988; Lue, 1994). The egg has a single oil globule and is pigmented (Mahboob *et al.*, 1998). The average diameter of viable eggs in the various batches was found to decline as the spawning season progressed (Lin *et al.*, 1990; Mihelakakis & Kitijama, 1995). The morphological development of egg and embryo up to early larval stage was described by Lin *et al.* (1988), Mahboob *et al.*, (1998) and early development stages from the hatched larval stage to the juvenile stage is described by Tsukashima & Kitajima (1982) and Mahboob *et al.* (1998). Molecular and biological studies in relation to the development of *R. sarba* larvae have been conducted by Deane *et al.* (2003). The authors showed that the RNA-DNA ratio was highest at one day after hatching (DAH), decreased to low levels between seven and 21 DAH and increased by 28 DAH. The authors found that the yolk-sac is fully absorbed at seven days after hatching (DAH). The authors showed the profile of growth hormone (GH) and insulin-like growth factor I (IGF-I) and

how their levels changed with relation to growth of the larvae as they develop into juveniles. Information on early morphological and ultrastrucure development of *R. sarba* larvae, however, scarce.

Under captive conditions the maximum number of eggs for four-year old female *R. sarba* was estimated to be 198,000 egg.day^{-1} (Lin, *et al.*, 1990), while a three-year-old female has produced a maximum spawning of 77,225 egg.day^{-1} during the spawning season (Leu, 1994).

Broodstock diet, husbandry practice and rearing environment are known to have an affect on the spawning behaviour (Mihelakakis & Kitijama, 1995). Temperature has been found to have the predominant influence on embryonic development of *R. sarba*, while eggs can be incubated at a salinity of between 12-52‰ without affecting the egg viability (Mihelakakis & Kitijima, 1994). The authors concluded that the optimum temperature and salinities for incubation conditions are 18° to 22°C and 24 to 38‰. Eggs found floating were not always fertilised especially in the early days of the spawning season (Mihelakakis & Kitijima, 1994). The authors showed that viable eggs were more numerous amongst buoyant, fertilised eggs, a similar observation to that made earlier by Lin *et al.* (1990).

Scientific and technical interests have focused on native species whose biological cycle can be reproduced using currently available breeding and rearing techniques. On the other hand, techniques for inducing broodstock spawning can be improved, and large quantities of fertilized eggs can be obtained by strengthening research on basic reproduction and physiology of broodstock fish. This includes seasons of

gonadal maturity, age at first maturity, fecundity with relation to age and species environmental requirements. From this point of view, the present research will attempt to cover some aspects of these areas for the *R. sarba* in the Omani waters a candidate for marine culture.

To date, there are very limited studies on the reproductive biology and growth of *R. sarba* in the S. of Oman waters that could form the basis of any research into this species. It was also noted that there is no study that classifies the developmental stages of ovaries and testes in *R. sarba* that can be used as a criteria for gonad classification and maturity staging. Based on cited literature on the goldlined seabream, no report exists on levels of plasma vitellogenin (VTG) during the development of the female ovaries. In addition, little attention has been focused on the morphological development of *R. sarba* larvae and no description has been reported on the ultrastructural changes that occur in the early life history of *R. sarba* larvae.

1.2 Aims of this thesis

1) Investigate the growth and age composition of the wild stock in the Gulf of Oman to determine stock condition and maturity of *R. sarba.*
2) Conduct histological studies on gonadal development and classify the maturity stages of gonads in female and male *R. sarba.*
3) Quantify the various oocyte stages during gonadal development using stereological techniques.
4) Measure plasma calcium concentrations (Ca^{2+}) in female *R. sarba* during the reproductive season to estimate vitellogenin (VTG).

5) Describe the biological characteristics of broodstock for hatchery purposes in the Sultanate of Oman. This will reveal the basic requirement for seed production.

6) Determine the fluctuations of total lipid in muscle, liver and ovary in relation to the status of maturity and the fatty acid profile in female ovaries.

7) Monitor the water temperature over the study period the study site.

8) Investigate the early larval development and ultrastructural changes during the first few days as larvae start exogenous feeding.

9) Evaluate the growth of captive bred larvae fed on lipid enriched rotifers using different enrichment agents.

Chapter 2: Materials and Methods

2.1 General materials & methods

The details given in this chapter refer to the general materials and methods commonly used in this study. Specific details of the materials and methods related to a particular chapter are provided in the relevant chapters. The study was carried out at three locations and these are as follows:

- Institute of Aquaculture, University of Stirling, Scotland, termed hereafter (IoA).
- Department of Fisheries Science and Biotechnology, College of Agriculture, Sultan Qaboos University, Oman. It is termed hereafter (SQU).
- Marine Science and Fisheries Centre, Ministry of Agriculture and Fisheries resources, Oman. It is termed hereafter (MSFC).

2.1.1 Procurement of wild fish

Fish were caught from Albustan beach, which is located along the coast of the capital city Muscat (Figure 2.1) by local fisherman using traditional non-selective demersal traps. Trap inspections and fish collections occurred between 0600 and 0800 hrs on all sampling days. This sampling site was chosen according to accessibility, fish availability and good cooperation with the trap owners. Fish were sampled weekly for reproductive assessment and biometric data collection. During the first month of the study (November) broodstock were also collected. Since the fishing gear is considered non-selective, all *R. sarba* found in the traps during any sampling day were collected for the study.

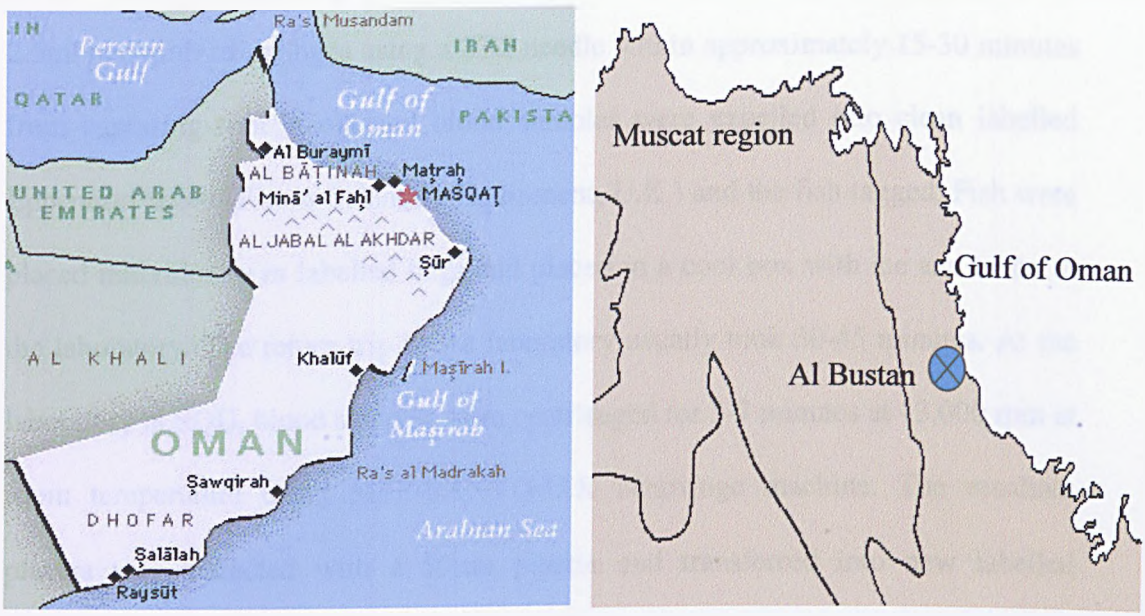


Figure 2.1 Maps showing the sampling site which is located in Albustan along the coast of the capital city Muscat.

Ref. map of Oman: http://wwp.greenwichmeantime.com ; Muscat region: Digital atlas 2000, Ministry of National Economy, Muscat, Sultanate of Oman

2.1.2 Measurement of marine water parameters

Temperature at the fishing ground was measured four times a month at one station where the demersal traps are located (25° 34 N, 58° 36 E). Since the coastal area at this location is an open coast, a decision was taken to sample at one station at a depth of about 15 meters. Both surface and bottom temperatures were recorded. Measurements of water parameters were taken during regular sampling by the Fisheries Science Department, SQU aboard the Aljamiah research vessel using the hydro-lab (Ocean Seven 316 Multiparameters Probe, IDRONAUT Sr1, Italy). Data were fed automatically to a computer and were stored for later analysis.

2.1.3 Blood plasma collection

Fish were removed from traps and transferred immediately to a 50*l* water container aboard the boat. On shore the fish were killed instantly by a strong blow to the head

in order to avoid suffering. Blood samples were drawn from the caudal vessel with 2.5ml heparinized syringes using a 23G needle within approximately 15-30 minutes from capturing time. Collected blood samples were expelled into clean labelled Eppendorf tubes (Fisons Scientific Equipment, U.K.) and the fish tagged. Fish were placed individually in labelled bags and placed in a cool box with ice and taken to the laboratory. The return trip to the laboratory usually took 30-45 minutes. At the laboratory at SQU, blood samples were centrifuged for 5-7 minutes at 13,000 rpm at. room temperature using MSE-SANYO-U.K centrifuge machine. The resultant plasma was extracted with a 50μm pipette and transferred into new labelled Eppendorf tubes and stored in a freezer at -70°C for later analysis.

2.1.4 Biometric data of wild fish

Fish were weighed to the nearest 0.001g (HF-4000 A & D Company Limited, Japan). Total, standard and fork lengths were recorded in mm. Liver, ovaries and visceral fats were removed by dissection and weighed separately to the nearest 0.001g. Gonadosomatic (GSI) and hepatosomatic (HSI) indices were calculated as follows:

Gonadosomatic index: $GSI = 100 (W_G / W_B)$

Hepatosomatic index: $HSI = 100 (W_L / W_B)$

Where:

W_B = Gutted body weight

- For gonad: Total fish weight (Wt) – gonad weight (W_G).
- For liver: Total fish weight (Wt) – liver weight (W_L).

W_G= Weight of gonad (left and right)

W_L = Weight of liver

2.1.5 Biometric data collection from Matrah fish market

The main fish market, 'Matrah Fish market' in the capital city Muscat was chosen for regular sampling to collect data on total length and total weight of goldlined seabream *R. sarba*. This market was chosen for its location as it serves the majority of the temporary and permanent residents of the capital city. Beside this, the fish in this market are sorted and grouped by species or demersal and pelagic which make the sampling more convenient. Prices per kg and number of fish per kg were recorded. Sampling was done over a week twice a month. In each sampling week the sampling was carried out on three separate days; Saturday, Wednesday and Friday.

2.1.6 Collection of fish body tissues and otoliths

At the end of the biometric data recording in the laboratory at SQU, fish were dissected and livers, gonads, lateral muscle sample and visceral fat were removed and stored in test tubes that were kept at -70°C until further analysis. Both right and left sagittal otoliths were extracted, washed and dried. Otoliths were then stored in small labelled Eppendorf tubes until processing.

At the end of the practical work and data collection in Oman, histology samples that were preserved and other biological samples were transported to IoA for further analysis. Prior to sending the cassette that contained ovary sections to IoA they were placed in plastic polyethylene containers. Tissues were saturated with neutral buffered formalin (NBF) and placed at the base of the container. Cassettes were then placed on the tissues inside the container in layers each layer was separated from the other by a layer of wet tissue. It was important to make sure that samples

did not dry out. The containers were sealed with melted plastic glue. On arrival at IoA, cassettes were placed in a suitable container until processing. At the end of the second sampling season, however, tissues were embedded in paraffin wax at the SQU as the facility for doing so became available.

2.1.7 Processing of gonads for histology

A section approximately 0.5mm was cut from the middle part of the right side of each gonad. The section was placed in a plastic cassette used for processing samples for histology work. If tissue was too large for the cassette it was trimmed to a suitable size to fit. The cassette was then placed in 10% neutral buffered formalin (NBF). The volume of formalin ratio to tissue was 10:1. Chemicals used to make 10% (NBF) were as stated in Appendix 1, Table 1.1. However, in the case of hydrated ovaries and active testes, the right ovary and testis was dissected and fixed as a whole.

2.1.7.1 Tissue processing for histology work prior to sectioning

Samples of ovarian tissues were immersed in a graded series of alcohols to dehydrate the tissues. Tissues were then treated with chloroform and embedding agent in order to remove the dehydrating agent. Complete infiltration of paraffin wax was carried out at the latter stage to remove the cleaning agent from the tissues. Processing protocol can be seen in Appendix 1, Table 1.2.

A suitable size metal base mould was selected for each sample and half filled with molten wax. The tissue was placed at the base of the mould and the mould was filled with molten wax using a histo-embedder. The empty cassette was placed on

top of the base mould and was then placed on a cold plate. The block was removed from the mould once it had solidified.

Trimming-in of blocks was carried out to expose the complete surface of the ovary using an old microtome blade. Since the ovary is considered as soft tissue it was not essential to use decalcifying solution. Instead, trimming blocks were soaked in distilled water prior to cutting for approximately 30min. The blocks were then placed on a cold plate face down for no more than five minutes. Blocks were sectioned at 2μm using a new blade and the blade was changed regularly to prevent any damage to the section. Tissues were then placed on a glass slide and allowed to dry on a hotplate. Later, slides were placed into slide racks and dried in an oven at 60°C for at least one hour to fix the section onto the slide. Sections were stained with haematoxylin and eosin (H&E) following the protocol listed in Appendix 1, Table 1.3.

2.1.8 Total calcium analysis

Total calcium in *R. sarba* plasma was analysed to estimate evels of vitellogenin using an atom absorption spectrophotometer (AAS; Perkin-Elmer 2280, Baconsfield, Bucks).

2.1.8.1 Preparation of stock solution (1% Nitric acid)

A plastic container with a capacity of 20*l* was weighed empty. About 10*l* of distilled water was added to the container and weighed again. The amount of 1% Nitric acid (B.D.H/Merck Ltd., U.K.) was calculated (weight of water g. × 0.01) and added to the water in the container. The container was shacken for approximately five

minutes. The volume that was prepared here was more than the required for this study as the solution was also used generally in the laboratory by other students and staff.

2.1.8.2 Preparation of working solution

An empty bottle with a capacity of 5*l* was weighed empty and the desired amount of stock solution was placed inside the plastic container and weighed again to find out the weight of the stock solution. The amount of 1% lanthanum chloride (Fisons Scientific Equipment, U.K.) was calculated (weight of working solution g. $\times$ 0.01) and added to the container. A flea was added to the container and placed on a stirrer for approximately ten minutes.

2.1.8.3 Standard dilution at 2mgl^{-1} concentration

Calcium standards were required for calibration of the atomic absorption spectrophotometer. Working solution at a quantity of 200ml was placed into a volume flask. A quantity of 400μl was removed and replaced with 400μl calcium standard (BDH Ltd., Pooled England). The standard bottle was shaken well prior to the removal of standard solution. The desired amount was poured into a plastic bottle precleaned with working solution.

2.1.8.4 Standard dilution at 4mgl^{-1} concentration

Working solution at a quantity of 200ml was placed into a volume flask. A quantity of 800μl was removed and replaced with 800μl calcium standard (BDH Ltd., Pooled England). The standard bottle was shaken well prior to the removal of

standard solution. The desired amount was poured into a plastic bottle precleaned with working solution.

2.1.8.5 Sample dilution and testing

Test tubes were numbered and plasma samples were allowed to thaw. Plasma tubes were mixed with whirlimixer. Samples were diluted 1:120 to obtain 25μl plasma to 2975μl working solution. Tubes were kept at 4°C over night. Samples were mixed again with whirlimixer prior to testing the following day. The spectrophotometer was calibrated every ten samples and adjusted if required.

2.1.8.6 Calculation of total calcium levels in plasma samples

The readings of the calcium levels that were recorded from the absorption spectrophotometer were multiplied by the dilution used (1:120) and divided by ten to express the total plasma calcium levels in mg%.

2.1.9 Lipid extraction

Lipid was extracted using the Folch-Lee extraction method (Folch *et al.*, 1957) in order to preserve the extract in good condition for further analysis of fatty acids.

2.1.9.1 Preparation of reagents

Three reagents were prepared as required at the following volumes:

- Chloroform: Methanol (2:1): two volume of chloroform to one volume of methanol.

- Chloroform: Methanol (2:1 +PHT): two volume of chloroform to one volume of methanol, both chemicals now contain BHT that helps to prevent oxidation of the lipids.
- KCL (0.88%): distilled water was measured as desired and KCL was added at a concentration of 0.88g .100ml^{-1}. The bottle was shaken well prior to use.

For safety, all the above chemical preparations were performed in a fume cupboard.

2.1.9.2 Extraction procedure

The relevant numbers of glass tubes (50ml) were labelled. The tubes were placed on the scale and approximately 1g of sample to two decimal places was weighed out. To these tubes 20 volumes of C: M (2:1) added, the tubes covered and the samples homogenized using an Ultraturrax^{TM}. Between samples the probe was rinsed in C: M (2:1) to avoid contamination. The tubes were left to set on ice for approximately one hour. After one hour 0.25 volumes of 0.88% KCl was added to the homogenized sample (for 20mls 2:1 volume, 5ml KCl was added) and mixed thoroughly using a vortex machine before leaving to stand for 15 minutes on ice. During this time, the standard tubes (15ml) were numbered and weighed to four decimal places. On these tubes filter funnels were pre rinsed with C: M (2:1) and placed on each tube and left to one side. The glass tubes (50ml) that contained the samples were centrifuged at 1500rpm for two to three minutes. The top layer was then removed by aspiration. The bottom layer was transferred to weighed standard tubes through the pre-washed C: M (2:1) Whatman no.1 filter papers. The large tubes and the filter papers rinsed with C: M (2:1) to remove any remains.

Once the filtrate was totally transferred to the standard tubes, the solvent was evaporated under a stream of oxygen-free nitrogen (OFN) on an N-evap until dry and at the end of the drying process, the tubes were covered with foil and desiccated overnight.

Small glass vials were labelled as desired. And using 1ml Gilsen, 0.9ml C: M (2:1) +BHT was added to each of the glass vials. The vials were then sealed and left until the samples tubes were ready to be weighed.

The following day the tubes now containing the extracted lipid were removed from the desiccator and weighed to four decimal places. The weight of the lipid was calculated by: (weight of tube +lipid) – (initial weight of empty tube). The percentage of total lipid was calculated by: (weight of lipid) / (initial weight of sample)× 100.

To transfer a sub sample of the extracted lipid C:M (2:1) of that amount (weight of lipid × 10) ml was added to the tube and mixed thoroughly using a vortex machine in order to redissolve the lipid at a concentration of $10mgml^{-1}$. Later, 0.1ml was then transferred to the glass vials to provide a total lipid concentration stock solution of $10mgml^{-1}$. The vials were capped with nitrogen and placed in freezer until further analysis.

2.1.10 Methylation of lipid samples for fatty acid analysis

A 1% solution of concentrated sulphuric acid was prepared in methanol. From the $10mgml^{-1}$ total lipid stock solutions, 100μl was removed (this is equivalent to 1mg

of lipid) and placed in a clean small test tube to evaporate off the C:M (2:1) under nitrogen. In the fume cupboard, 1ml toluene and 2ml methylating reagent were added. The tubes were whirlimixed to mix the solvent.

The tubes were then flushed with nitrogen and a piece of filter paper placed into the top of the tubes with the stoppers to prevent the stoppers from blowing off when the tubes were heated. The tubes incubated overnight (16hrs) at 50°C in the hot-block and when removed were allowed to cool in the fridge. The pieces of filter paper were removed and 2ml 2% $KHCO_3$, 1ml isohexane : diethyl ether + BHT (1:1 v:v) and 4mls isohexane : diethyl ether (1:1 v:v) added. The tubes were whirlimixed to mix the solvents. The tubes were centrifuged at 1500rpm for two minutes, the upper organic layers were transferred to clean test tubes (via Pasteur pipette) and a further 1ml isohexane : diethyl ether + BHT (1:1 v:v) and 4mls isohexane :diethyl ether (1:1 v:v) added to the original tubes. The tubes were now whirlimixed, centrifuged and the upper organic layers were transferred to other sets of tubes. All the solvent was evaporated under nitrogen and 100μl isohexane added to the tubes to redissolve the FAME. Methyl esters were purified by TLC on 20x20cm plates. Origins were marked in pencil, 1.5cm from the bottom of the plate, separated by at least 1.2 cm and with 2cm margins at either side of the plate providing four samples per plate.

The samples were loaded using a 100μl Hamilton syringe with an isohexane wash tube for rinsing the syringe between samples. The plate was then run in isohexane: diethyl ether: acetic acid 90:10:1 (v:v) up to 1.5cm from the top of the plate. The solvent tank was rinsed with a little isohexane before use, just in case it had become

dirty on the inside. The plate was then removed from the solvent tank and the solvent evaporated off in the fume cupboard for a few minutes.

The plate was then sprayed with 1% iodine in chloroform to visualise the fatty acid methyl esters (FAME). The outside lanes of the plate were masked off with blank glass plates so that only the very edges of these samples exposed. These edges were then sprayed lightly with the iodine solution. The FAME bands were clearly visible and marked off lightly with pencil. It should be noted that FAME bands were a doublet; saturated and monounsaturated fatty acids form the upper band, polyunsaturates the lowest band and both bands were scraped.

The FAME bands were marked for all samples before being scraped from the TLC plate with a scalpel or a razor blade into clean test tubes. The samples were then eluted with 5mls isohexane: diethyl ether (1:1 v:v). The isohexane: diethyl ether (1:1 v:v) evaporated off under nitrogen and 500μl isohexane was added to each test tube and then transferred to clean, labelled, small vials. The samples were then stored under nitrogen at –20°C in the freezer prior to GLC analysis.

FAME were separated and quantified by gas-liquid chromatography (Fisons GC 8000, Fisons Ltd, Crawley, UK) using a 30 m ×0.32 mm capillary (CP was 52CB; Chrompak Ltd, London, UK). Hydrogen was used as a carrier gas and temperature programming was from 50° to 180°C at 40°C min^{-1} and then to 225 °C at 2 °C min^{-1}. Individual FAMEs were identified by comparison to a known standard made out of well-characterized fish oil.

2.1.11 Methods of statistical analysis

The statistical techniques applied are detailed in the relevant chapters. To test for normal distribution the Anderson-Darling normality test was applied. Where necessary, data were log transformed to ensure normality. Data were tested for correlation using the Pearson correlation test. To compare means between samples, one-way analysis of variance (ANOVA) was applied. The differences between means were compared using Tukey's significant difference test at a probability (p) level of $p<0.05$. Statistical analysis was performed using SPSS release 10. Means are presented in this thesis (±S.E) unless otherwise stated (Sokal & Rohlf, 1995).

Chapter 3: Age and growth of the goldline seabream, *Rhabdosargus sarba*

3.1 Introduction

Age estimations of fish help to provide insight into growth, age at first maturity, recruitment and the age structure of the population under study. These data are essential for developing fisheries yield models (Newman *et al.*, 2000). The age and growth parameters for *R. sarba* have been studied in Chika Lake, India by Patnaik (1973) who estimated *R. sarba* age using length frequency analysis introduced by Petersen (1892). A similar study was carried out in the State of Qatar, the Arabian Gulf (El-Agamy, 1989). The author studied *Sparus sarba* but according to the FAO this species is also called *Rhabdosargus sarba* (Fisher & Whitehead, 1974). El-Agamy, (1989) used scales in his study to estimate the age of the fish, but the use of scales to determine age probably leads to an underestimation of true age (Ashford *et al.*, 2000). Another study carried out in South African waters (Radebe, *et al.*, 2002), estimated age from counts of growth zones (annuli) marked otoliths. The authors attempted to validate the ageing methodology using two methods that showed that the zones were formed annually. The first method was by marginal zone analysis (MZA) that examines the frequency of the deposition of the hyaline and opaque rings that are found at the margin of the otolith in each month. The second method used was an oxytetracycline (OTC) label, which demonstrated that one hyaline band and one opaque band were deposited after the fluorescent marker which proves that deposition of growth rings is annual.

Otoliths grow continually throughout the lifespan of fish. Differences in composition and density of calcium carbonate (mainly otolin) have been suggested as the reason behind daily and annual growth ring formation. This is what makes the otolith a good indicator of fish age.

To date, no estimates of growth are available for *R. sarba* in Omani waters. The present study aimed at ageing this species using the sagittal otolith in order to estimate growth parameters and to investigate growth and reproduction such as size and age at first maturity. It also aimed to develop a validated ageing technique for this species using marginal zone analysis (MZA), which is also known as marginal increment analysis (MIA). This ageing study will also provide information related to mortality and survivorship of *R. sarba* in the Gulf of Oman.

The relationship between otolith weight, length and fish age estimated from sectioned otoliths was also assessed to determine the correlation between these parameters.

3.2 Materials and Methods

Fish samples of *R. sarba* were collected as detailed in Section 2.1.1. Biological and biometric data were recorded as detailed in Section 2.1.4.

3.2.1 Otolith removal from fish

Otolith removal was carried out through the top of the skull where a cut was made horizontally across the top of the eye with a fine hacksaw. This slices through the top of the braincase exposing the brain, which was removed using forceps, and the sagittal otoliths were exposed in the otic capsules. Otoliths were then carefully removed from the capsules. Both right and left sagittal otolith (n= 341) were extracted, washed and dried and the otoliths were stored in small eppendorf tubes until processing.

3.2.2 Processing the otolith

Prior to processing, intact otoliths were measured using a digital caliper (to the nearest 0.01mm) and weighed using an electronic balance (to the nearest 0.001g). The weight and length of left and right otoliths were compared using a *t*-test that showed no significant difference ($p>0.05$); consequently only one sagittal otolith from each fish was processed. The otolith was embedded in epoxy resin (AGAR Scientific Ltd. UK) that was prepared according to the amount desired. For preparation of epoxy resin refer to Table 3.1.

Otoliths were laid flat in epoxy moulds, resin was then added and the moulds were kept in a 60°C oven overnight to polymerize. Resin blocks containing the otoliths were numbered and stored together.

To section the embedded otoliths, blocks were mounted horizontally on a glass slide using Crystalbond™ mounting media so that the surface of the otolith is kept flat with the help of a hot plate. The centrum (nucleus) of the otolith was marked with a pen and a line was drawn on either side of the approximately 0.3mm from the centrum. A fine hacksaw was used to cut the unwanted part of the resin block. The orientation of the remainder of the block now containing the centrum was turned laterally so that the exposed side of the otolith section could be ground to expose the rings. Grinding was carried out on both sides of the section. A grinding wheel fitted with silicon carbide paper was used to polish both sides of the sections. Once the rings were exposed and readable the surface of the section was covered with crystal bond to give better contrast to the rings. In addition slides containing the sectioned otoliths were kept on a thermic plate until the otolith section became dark brown to

further increase the definition of translucent and opaque rings. This process was said to denature the calcium in the otolith (Christensen, 1964). Embedding of the otoliths was carried out at MSC. Processing of otoliths was carried out at SQU. The processing steps are given in Figure 3.1 .

Table 3.1 The composition ratio of the chemical constituents of the epoxy resin used for embedding otoliths.

	Amount (g)			
Chemical	1	2	3	4
ERL 4206	10	20	40	80
DER 736	4.5	8	16	32
NSA	26	52	104	208
DAME	0.4	0.8	1.6	3.2

Where:

ERL 4206 : Vinyle Cyclohexene Dioxide
DER 736 : Diglyoidyl ether of Polypropylene glycol determines the hardness of the block, < amount of DER = hard block and > amount of DER = soft block.
NSA : Nonenyl succinic anhydride
DAME, S1 : Dimethylaminoethanol

3.2.3 Validation

To validate the deposition of growth zones in the otoliths, marginal increment or marginal zone analysis (MZA) was applied (Radebe *et al.*, 2002). The percentage frequency of both hyaline and opaque bands was plotted against the month of the year in which the samples were collected.

Annuli were counted independently on two separate occasions that were at least one month apart. For those whose two counts differ, the second count was used for analysis, since by this time it was assumed that more experience had been gained through reading large numbers of otoliths.

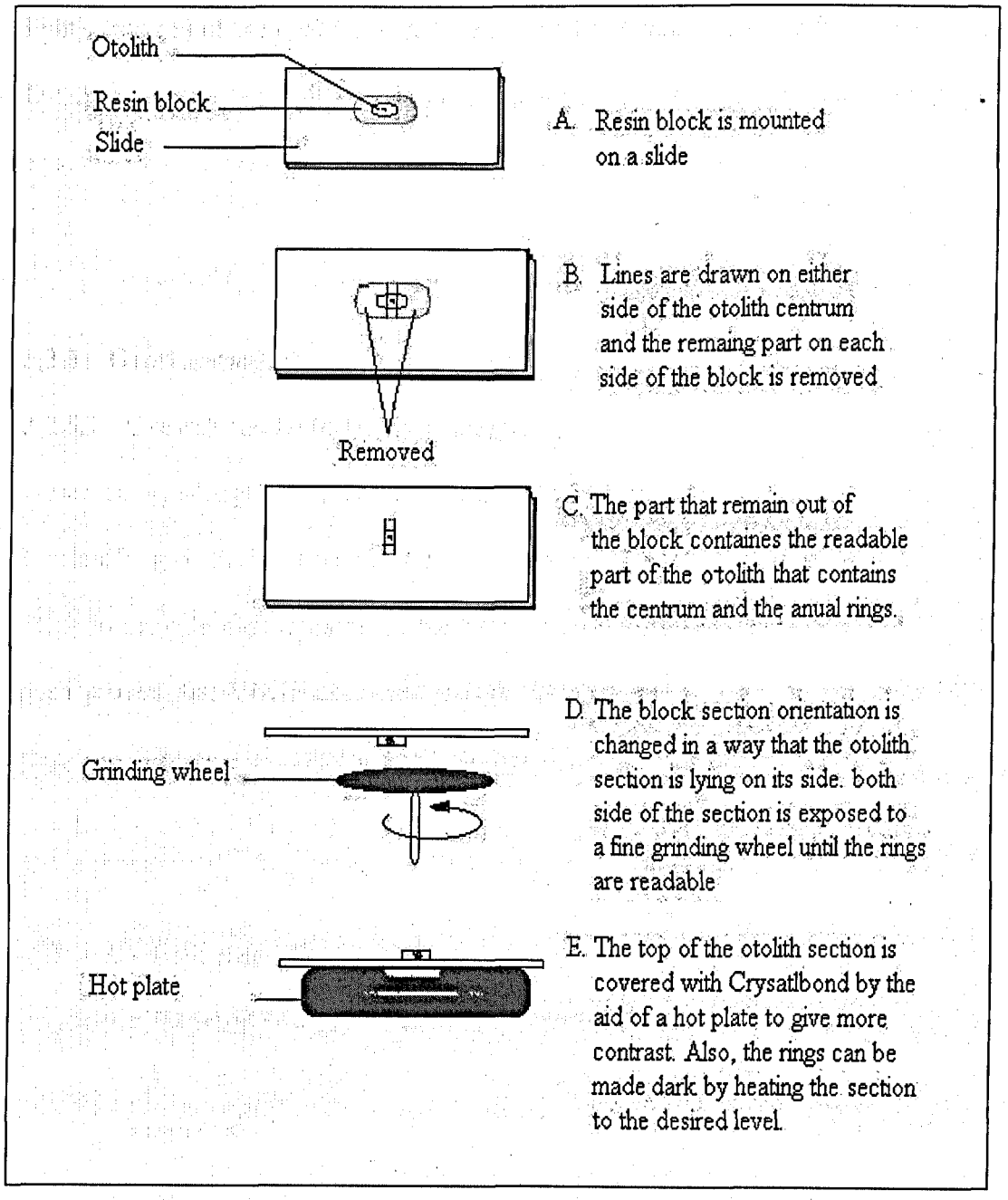


Figure 3.1 The procedure steps of otolith sectioning.

3.2.4 Otolith readings

The otolith sections mounted on slides were placed under a compound microscope (Olympus-SZ60) at 40× magnification with translucent light. Numbers of opaque rings were counted on the smaller side of the sectioned otoliths (Plate 3.1). The margin zone of the otoliths was noted to be opaque (dark) or hyaline (translucent, light). Pictures of the otoliths were taken using an attached digital camera (Olympus DP12) and were then transferred to a computer for processing. Olympus Soft Image System software was used to view the field prior to taking snapshots of the field in view.

3.2.5 Growth models

3.2.5.1 Growth model in terms of length

Observed age-length data for *R. sarba* displayed asymptotic growth. The Von Bertlanffy growth function (VBGF) was applied (Ricker, 1975; Pauly, 1982). It helps to provide a description of the mean growth of a population and it has also been proven that VBGF describes growth data better than many other polynomial functions (Chen *et. al.*, 1992). The VBGF is defined by the equation:

$$L_t = L_\infty \{ 1- \exp [- K (t-t_0)]\}$$

Where:

L_t = length at age t

L_∞ = asymptotic length, the length that a fish will reach had it been allowed to live

K = Brody growth coefficient that defines the growth rate towards L_∞

t = age of fish

t_0 = hypothetical age at which fish would have zero length had it always grown in a manner described by the model

3.2.5.2 Growth model in terms of weight

Observed age-weight data for *R. sarba* was fitted to the von Bertlanffy growth function (VBGF). Since the animal is growing isometrically (increasing in all dimensions in the same rate) and doubles the length this gives a cubic relationship between length and weight and thus has to be added to the equation. The VBGF in terms of weight is defined as:

$$W_t = W_\infty \{ 1- \exp [- K (t-t_0)]\}^3$$

Where

W_t = weight at age t

W_∞ = asymptotic weight, the weight that a fish will reach had it been allowed to live

K = Brody growth coefficient that defines the growth rate towards W_∞

t = age of fish

t_0 = the hypothetical weight at which fish would have zero weight had it always grown in a manner described by the model

3.2.6 Size and age at first maturity

Both male and female gonads were processed as detailed in Section 2.1.7.1. For females, only those that were classified as S4-S6 were considered mature. For males, only those that had a developing and active testes were considered mature and subsequently used in this test. The classification was carried out on 384 animals but only 176 were found to be mature.

For estimation of length at first maturity (50% maturity) a logistic function was fitted to the proportion of mature individuals in each size category using a non-linear regression, (Saila *et al.*, 1988). The calculation of size at first maturity was performed using the equation:

$$P_i = 100/\{1+\exp[-\alpha(L_i - L_{50})]\}$$

Where:

P_i = Proportion of mature fish at length class $_i$

α = constant, a value that increases with the steepness of the selection curve.

L_i = size of individual in cm

L_{50} = length at which 50% mature

The calculation of age at first maturity was performed using the equation:

$$t = t_0 - (1/K) \ln (1-L_t/L_\infty)$$

Where:

t = age of fish

t_0 = the parameter calculated for female age-length model

L_t = the length at age t

L_∞ = the parameter calculated for female age-length model

K = the parameter calculated for female age-length model

3.2.7 Statistical analysis

Analysis of covariance (ANCOVA) was used in nonlinear formulation. An analysis of residual sum of squares (ARSS) was employed to compare VBGF between the sexes (Chen *et al.*, 1992). The ARRS procedures are as follows:

- Residual sum of squares (RSS) and an associated degree of freedom (DF) of VBGF were calculated for each population.
- The resultant RSS and DF of each sample was added to yield summed RSS and DF.
- Data from sampled fish were pooled to calculate the RRS and DF of a total VBGF.

To test whether there was a difference in VBGF between the sexes, the calculated *F* value was compared with F-critical. Maximum and minimum likelihood was also calculated for all the parameters used in the VBGF using Pop Tools 2.6.2, an Excel Add-in developed by CSIRO (http://www.cse.csiro.au/CDG/poptools) that facilitates analysis of matrix population models.

3.2.8 Length-weight relationships

The length weight relationship was investigated. The allometric relationship between length and weight is usually described by the equation:

$W = aL^b$

Where:

W = weight of fish

L = length of fish

a = constant specific to the species (point at which a line will intersect the y- axis)

b = constant specific to the species (an exponent)

The relationship was linearised by the equation:

$Log_{10}W = Log_{10}a + b\ log_{10}L$

The parameters (*a* and *b*) of the equation of the curve were obtained by first calculating the linear regression of the Log_{10} transformed data.

3.2.9 The annual mortality and survival

Annual instantaneous mortality rate (Z) was estimated by linearising the negative exponential relationship between the number of fish in each age class and the age by plotting the natural logarithms of the number of fish in each age class against the age. This produced a straight-line relationship known as the catch curve (Beverton

& Holt, 1957; Ricker, 1975). The slope of the line of best fit (b) equals the total instantaneous mortality rate per year (Z).

The relationship can be given in the equation:

$\ln N_t = \ln N_0 - Z_t$

Where:

N_t = the number remaining at time t,

N_0 = the initial number of individuals at time t=0.

The annual mortality rate was calculated using the equation:

Mortality rate = $1 \{1- \exp(-Z)\}$

Calculation of natural mortality rate (M) was performed using the multiple regression equation of Pauly (1980), as the equation given by the author takes into consideration growth parameters and average water temperature where the fish live. The author found that M strongly correlated with mean environmental parameters. The mortality equation used:

$\log(M) = -0.0152 - 0.279 \times \ln(L_\infty) + 0.6543 \times \ln(K) + 0.4634 \times \ln(T)$

Where:

M = natural mortality

L_∞ =asymptotic length, the length that a fish will reach had it been allowed to live (cm)

K = growth coefficient that defines the growth rate towards L_∞

T = average water temperature of Muscat Region being 28.3°C

Fishing mortality (F) was estimated by subtraction:

F= Z-M (Z and M are discussed above)

Exploitation rate (E) was estimated from:

E = F/Z (F and Z are discussed above)

Survivorship rate was calculated using the equation:

Survival = 1 exp (-Z)

3.2.10 Calculation of annual mortality and survival on larger population of *R. sarba*.

In order to understand the population dynamics in a broader picture data was collected over a period of a year. Lengths and weights of *R. sarba* landed in Mutrah fish market in the capital city were measured, refer to Section 1.1.5. for details of the sampling procedure.

A total of 1124 fish were used to plot another catch curve in order to find out the slope of the line of best fit (*b*) representing the total instantaneous mortality rate per year (Z). The Z value was calculated in order to compare the value predicted from the data collected at the sampling site. The length data was converted to age using the inverse of the growth equation:

$t = t_0 - (1/K) \ln (1-L_t/L_\infty)$

The values of K, $L_{t \text{ and}}$ L_∞ were obtained from the VBGF age-length model for pooled data of both males and females.

3.2.11 Length at first capture

Probability of capture was estimated by generating a length converted catch curve using the FISAT-II (FAO-ICLARM Stock Assessment Tools) (Gayanilo *et al.*,

1996). The probability curve was smoothed using the logistic transformation of the form (Pauly, 1984):

$P=100/(1+\exp(-r(L-L_c)))$

Where:

P = probability of capture

r = a constant that increases with the steepness of the curve.

L = length of fish (size class)

L_c = mean length at first capture.

Length at first capture (LC_{50}) was estimated from the selection graph generated by the above logistic equation and length-converted catch curve using FISAT-II.

3.3 Results:

3.3.1 Age-length relationships

Von Bertalanffy growth functions (VBGF) for age-length relationships were estimated as L_t = 424.39mmTL $\{1\text{-}e^{-0.16\ (t+3.558)}\}$ for males and L_t = 337.85mmTL $\{1\text{-}e^{-0.37\ (t+2.066)}$ for females. Males were found to grow to a greater asymptotic length (L_∞) than females, while the rate (K.year^{-1}) at which this length was achieved was found to be lower than females (Table 3.2). The relationship between length and age data (observed lengths) and VBGF (predicted lengths) can be seen in Figure 3.2. The VBGF curves varied significantly between sexes and showed that *R. sarba* males and females have significantly different VBGF curves (F = 4; d.f. 336; p ≤ 0 .007). The age-length key of females is listed in Table 3.4 and for males is given in Table 3.5.

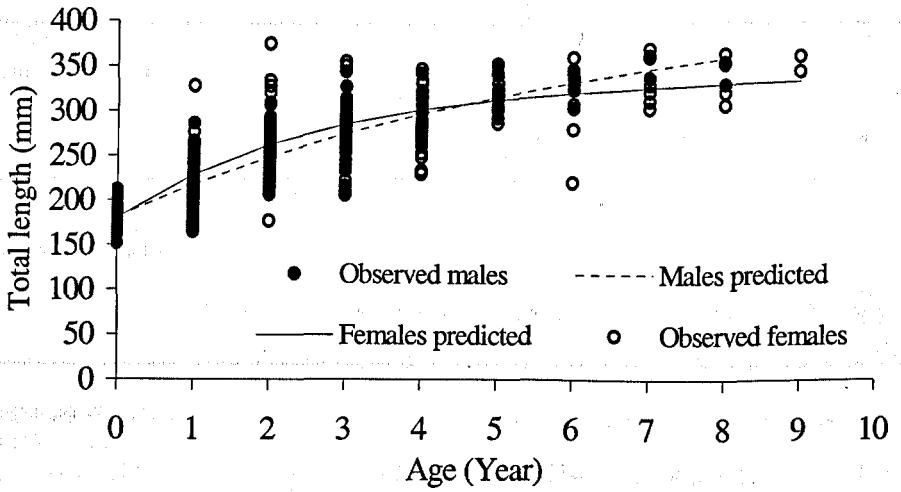


Figure 3.2 The age-length relationships of male and female *R. sarba* calculated by von Bertalanffy growth curve.

Table 3.2 Growth curves parameters estimated by VBGF growth equation for male, female and pooled *R. sarba.*

Parameter	Value	Upper C.I (95%)	Lower C.I (95%)	S.E
VBGF age-length model for male *R. sarba*				
L_∞ (mm) T.L	424.391	619.833	364.404	17.352
t_0 (year)	-3.448	-5.359	-2.403	0.214
K (year)	0.162	0.259	0.070	0.013
VBGF age-length model for female *R. sarba*				
L_∞ (mm)	337.856	368.835	317.859	4.104
t_0 (year)	-2.066	-2.993	-1.476	0.114
K (year)	0.369	0.514	0.246	0.019
VBGF age-length model for male and female *R. sarba* (pooled)				
L_∞ (mm)	364.091	400.528	340.927	7.210
t_0 (year)	-2.694	-3.592	-2.109	0.176
K (year)	0.256	0.335	0.183	0.011

Table 3.3 Parameters estimated by VBGF growth equation for male and female *R. sarba.*

Parameter	Value	Upper C.I (95%)	Lower C.I. (95%)	S.E
VBGF age-total weight model for male *R. sarba*				
W_∞ (g) TW	861.793	1388.488	673.012	54.144
t_0 (year)	-2.785	-4.5001	-1.747	0.2
K (year)	0.232	0.342	0.131	0.156
VBGF age-total weight model for female *R. sarba*				
W_∞ (g) TW	596.273	744.793	514.669	15.615
t_0 (year)	-2.232	-3.733	-1.335	0.168
K (year)	0.3799	0.555	0.236	0.025
VBGF age-gutted weight model for male *R. sarba*				
W_∞ (g)	931.84	765.234	692.208	74.069
t_0 (year)	-3.197	-5.188	-2.039	0.231
K (year)	0.197	0.3	0.103	0.014
VBGF age-gutted weight model for female *R. sarba*				
W_∞ (g)	759.555	948.685	652.844	21.648
t_0 (year)	-2.776	-3.865	-2.010	0.139
K (year)	0.255	0.333	0.182	0.012

Table 3.4 Age-length key for female *R. sarba*

Size category	Age group (year)											Total
TL (mm)	0	1	2	3	4	5	6	7	8	9	11	
150-169	5	1										
170-189	12	4	1									
190-209	2	4		1								
210-229		7	4	2								
230-249		6	5	1	3							
250-269		8	9	8	1							
270-289			9	11	4	2	1					
290-309			6	5	2	2	1	1	1			
310-329			2	4	3	3	1	3	1			
330-349			2	2	5	2	3	2		1	1	
350-369							1		2	1		
370-389												
Total	19	30	38	34	18	9	7	6	4	2	1	168
Average TL(mm)	177.9	225.6	268.6	281.0	296.4	315.6	312.8	332.2	335.8	351.5	321	

Table 3.5 Age-length key for male *R sarba*

Size category	Age group (year)									Total
(TL mm)	0	1	2	3	4	5	6	7	8	
150-169	2	1								
170-189		6								
190-209	5	6	3							
210-229	2	5	18	2						
230-249		4	11	3						
250-269		2	13	9	2					
270-289		1	13	11	5					
290-309			2	9	3	2	2			
310-329				3	4	5	1		1	
330-349				1	2	1	3	1	1	
350-369						2		1		
370-389										
Total	9	25	61	38	16	10	6	2	2	169
Average TL (mm)	192.3	212	247.1	277.5	299.1	323.0	326.1	349.5	340.5	

3.3.2 Age-weight relationships

The age -weight relationships of *R. sarba* were calculated for both total and gutted weights.

3.3.2.1 Age-total weight relationships

The age and total weight of both males and females were found to be significantly correlated (r= 0.996 for males and r=0.958 for females, p=0.001). Von Bertalanffy growth functions (VBGF) for age-weight relationships of *R. sarba* were estimated as $W_t = 861.793\text{gTW}\{1\text{-}e^{-0.232.16\ (t+2.785)}\}$ for males and $W_t = 596.273\text{g TW}\{1\text{-}e^{-0.379\ (t+2.232)}\}$ for females. The VBGF curves in terms of total weight varied significantly between sexes (F = 4; d.f. 336; $p \leq 0$ .007). Males grew to a greater asymptotic weight (W_∞) than females, while the rate (K. year^{-1}) at which this weight was reached was found to be lower than that of females. The relationship between total weight at age data (observed weights) and VBGF (predicted weights) are given in Figure 3.3. The equation parameters are listed in Table 3.3.

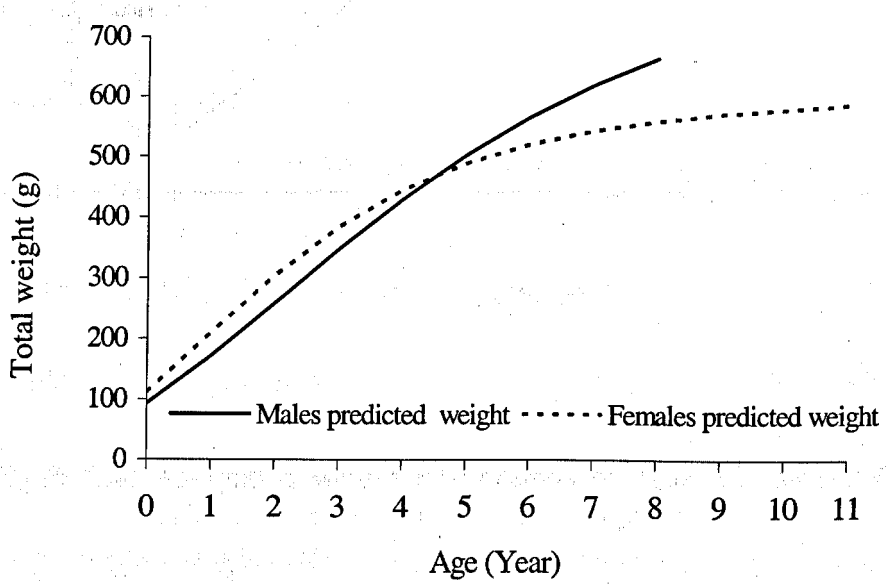


Figure 3.3 Age-total weight relationships of male and female *R. sarba* of a calculated von Bertalanffy age-weight growth curve.

3.3.2.2 Age-gutted weight relationships

The age and gutted weight (GW) were found to be significantly correlated (r= 0.804 for males and r=0.870 for females, p=0.001). Von Bertalanffy growth function (VBGF) for age-gutted weight relationships of *R. sarba* were estimated as W_t = 931.84g GW$\{1\text{-}e^{-0.197(t+3.197)}\}$ for males and W_t = 759.555g GW$\{1\text{-}e^{-0.255\,(t+2.776)}\}$ for females. The VBGF curves in terms of total weight varied significantly between sexes (F = 4.47; d.f. 331; $p \leq 0$ .005). Males grew to a greater asymptotic weight (W_∞) than females, while the rate (K. year^{-1}) at which this weight was reached was found to be lower than that of females. The relationship between weight at age data (observed weights) and VBGF (predicted weights) are given in Figure 3.4. The equation parameters are listed in Table 3.3.

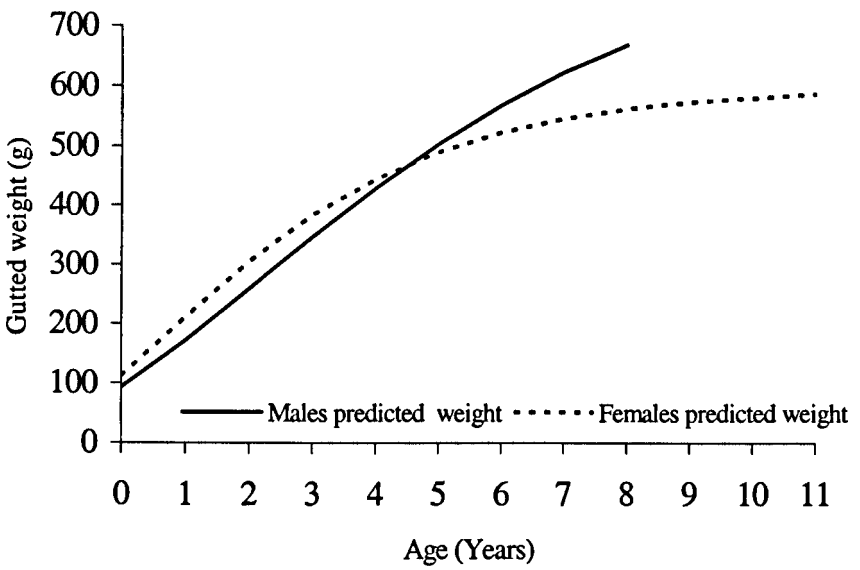


Figure 3.4. Age-gutted weight relationships of male and female *R. sarba* of a calculated von Bertalanffy Age-weight growth curve.

3.3.3 Validation of otolith reading using marginal increment analysis

Marginal increment analysis showed that a higher proportion of the opaque rings were laid out during the winter (January - March). Hyaline growth, however, was found to predominate within the marginal zone of the otolith throughout most of the year (Figure 3.5).

Opaque ring formation was correlated to both changes in temperature and the reproductive season of the species, which was found to fall between January and March. Fish that were sampled during the reproductive season were generally found to have an opaque ring at the margin of the otolith, which was still progressing (Plate 3.1). By the end of the spawning season most fish were beginning to lay down a hyaline band (Plate 3.2). Fish that did not complete their first year, which was considered to be age zero, were generally found with a hyaline band (Plate 3.3). This suggests that one opaque and one hyaline ring were deposited each year.

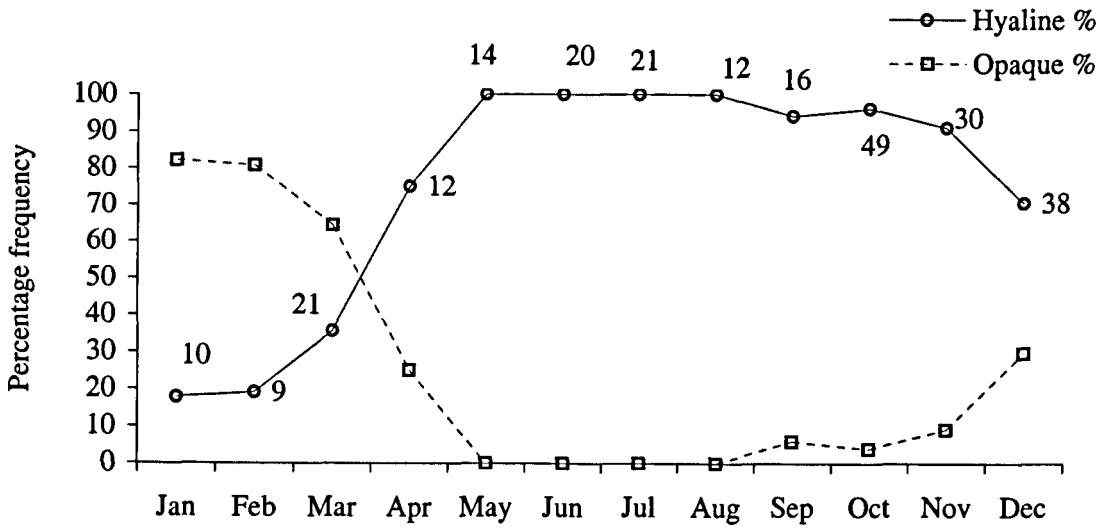


Figure 3.5 Plot of the changes in the marginal zone of *R. sarba* otoliths (n=262) throughout the year.
Numbers in the graph represent the number of fish sampled.

Plate 3.1 Otolith of two-year-old *R. sarba*, 284mm total length and 349g total weight.

The last ring marked on the otolith is opaque. This particular fish was caught during February (in the middle of the spawning season).

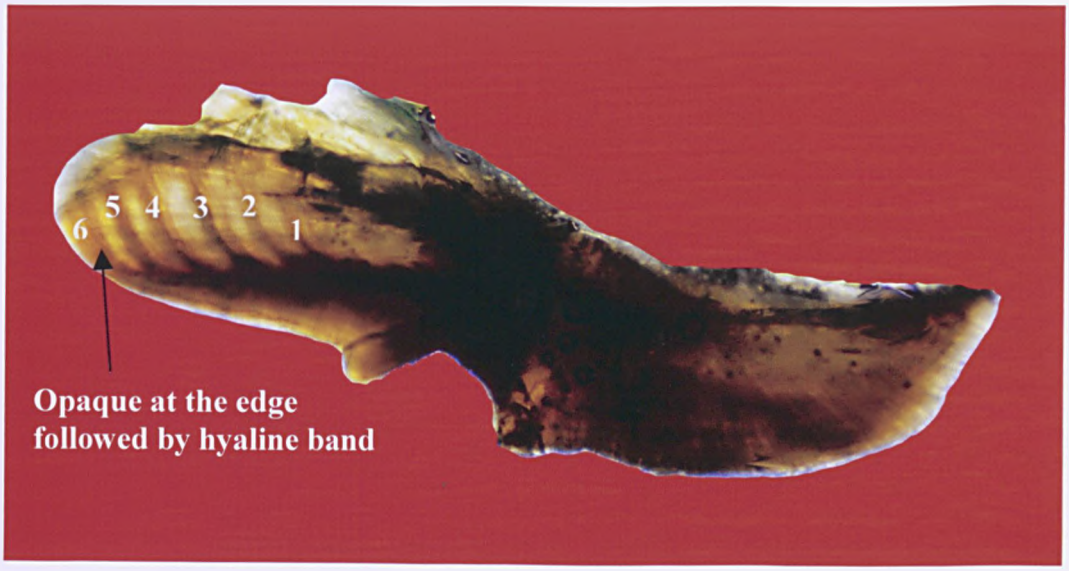


Plate 3.2 Otolith of six-yea-old *R. sarba*, 320mm total length and 515g total weight.

This particular fish was caught during March (at the end of the spawning season). At this time the hyaline ring (translucent) starts appearing.

Plate 3.3 Otolith of zero-year-old *R. sarba*, 166mm TL and 78.47g total weight.

3.3.4 Otolith age-weight and age-length relationships

The relationship between age-otolith length (Figure 3.6) showed that the length increased with age. Age-otolith weight (Figure 3.7) also showed that the weight of the otolith increases with age. Regression analysis between variables is listed in Table 3.6. Comparisons between otolith dimensions and ages for females are listed in Appendix 2, Table 1 and for males in Appendix 2, Table 2.

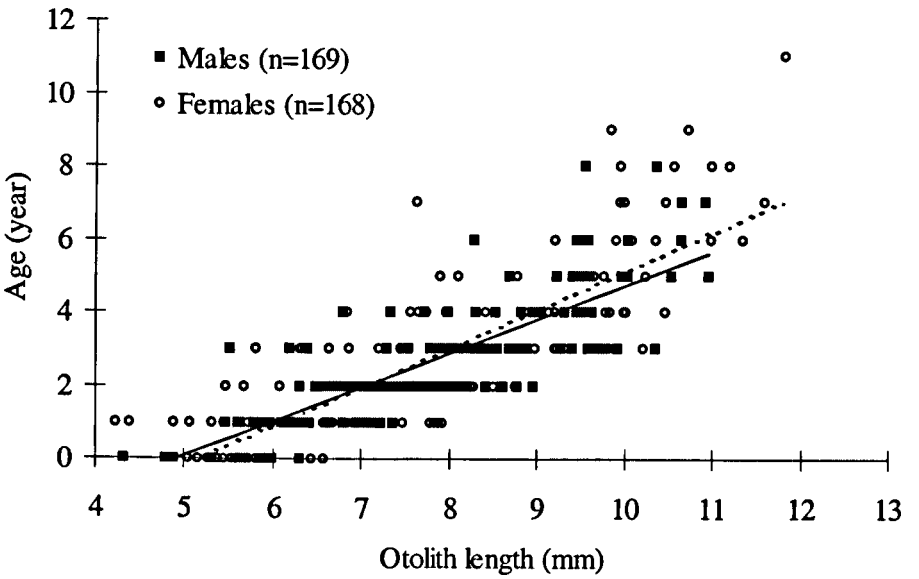


Figure 3.6 Otolith age-length relationships of male and female *R. sarba*.

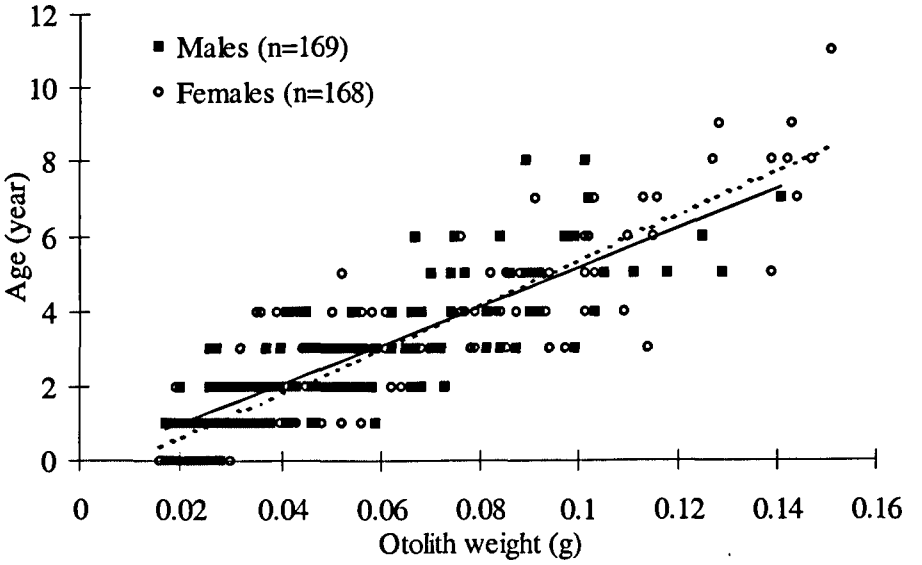


Figure 3.7 Otolith age-weight relationships of *R. sarba*.

Table 3.6 Comparison between otolith dimensions and ages of *R. sarba*.

Sex	*n*	Independent variables	Equation	r^2	S.E.	Significance level
Male	169	Otolith weight	Age=51.34 (Owt)+0.01	0.671	0.91	p<0.001
		Otolith length	Age=0.92 (Ol)-4.52	0.620	1.26	p<0.001
Female	168	Otolith weight	Age=58.79 (Owt)-0.54	0.798	0.96	p<0.001
		Otolith length	Age=1.05 (Ol)-5.46	0.663	1.24	P<0.001
Pooled	337	Otolith weight	Age=55.66 (Owt)-0.30	0.750	0.94	P<0.001
		Otolith length	Age=0.99 (Ol)-5.07	0.660	1.11	p<0.001

Equations are the linear regression form y= $a+bx$. (Owt=otolith weight, g; Ol=otolith length, mm). For all the regression analysis age was the dependent variable. The p value is for regression significance levels. Pooled data is for males and females combined

3.3.5 The catch – age frequency

The catch-age analysis (Figure 3.8) showed that the numbers of *R. sarba* fish caught was higher at the ages of zero to three years, which accounted for about 75% of the total catch. However, while the highest catch, however, was recorded at age zero to two years, the highest percentage of fish caught was at age two years. The catch beyond the age of three years had fallen sharply. It has to be noted here that about 38% of the landed fish had not reached the age at first maturity as calculated in this study for either males or females.

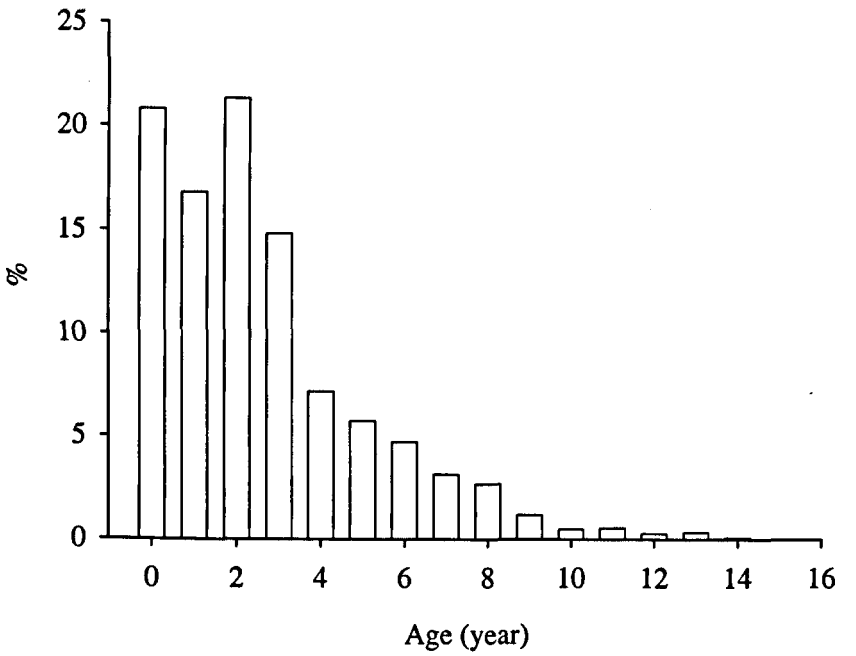


Figure 3.8 Percentage occurring in each age category of *R. sarba*.

3.3.6 Length at first maturity

Male *R. sarba* mature at a smaller length ($L_{50\%}$ = 19.04cm T.L), even before they complete the first year of age. The logistic equation representing the proportion of mature individuals according to size for males is $P = 100/\{1+ e^{-0.99\ (Lt\ -\ 19.04cm)}\}$. Female *R. sarba* mature at a greater length than males ($L_{50\%}$ = 23.41cm T.L) and if converted to age will give 1+ year. The logistic equation representing the proportion of mature individuals according to size for females is $P = 100/\{1+ e^{-0.42\ (Lt\ -\ 23.41cm)}\}$. The logistic relationships between observed percentage matured data (observed data) and predicted percentage matured for both males and females are given in Figure 3.9. The parameters for sexual maturity for both male and female *R. sarba* are given in Table 3.7. The percentage of mature individuals at different length classes of *R. sarba* is listed in Table 3.8. Size at first maturity curves varied significantly between sexes (F = 8.61; d.f. 175; p < 0 .001).

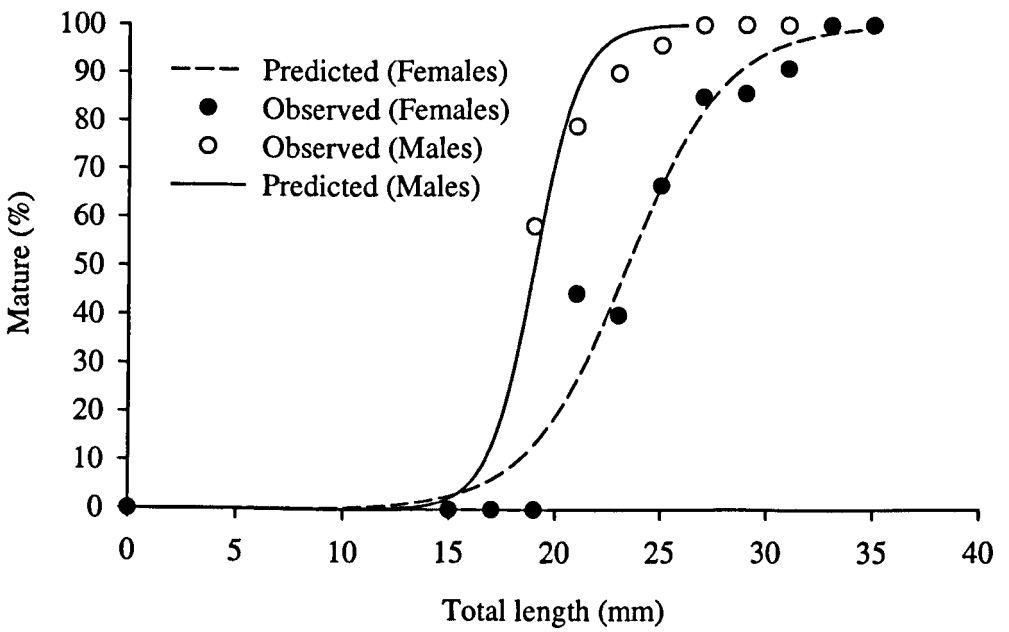


Figure 3.9 Size at first maturity of both male and female *R. sar*ba.

Table 3.7 Parameters of maturity model for male and female *R. sarba.*

Parameter	Value	Upper C.I (95%)	Lowe C.I (95%)	S.E
Size at first maturity for male *R. sarba*				
α	0.99	1.43	0.88	0.05
L_{50}(cm)	19.04	19.31	18.95	0.03
Size at first maturity for female *R. sarba*				
α	0.42	0.32	0.59	0.02
L_{50}(cm)	23.41	24.298	22.53	0.13

Table 3.8 The percentage of mature individuals at different length classes of female and male *R. sarba.*

TL (cm)	% Mature females	% Mature males
0	0	0
15	0	0
17	0	0
19	0	41.67
21	44	86
23	40	90
25	67	96
27	85	100
29	86	100
31	91	100
33	100	100
35	100	100

3.3.7 Length-weight relationships

The linearised power curve ($W = 4.5\ L^{2.84}$) explaining the length-weight relationship of male *R. sarba* and the Log length-weight relationships are shown in Figure 3.10. The linearised power curve ($W = 4.5\ L^{2.88}$) explaining the length-weight relationship of female *R. sarba* and the Log length-weight relationships are shown in Figure 3.10. The linearised power curve ($W = 4.5\ L^{2.87}$) explaining the length-weight relationship of all fish pooled and the Log length-weight relationships are shown in Figure 3.10.

Associated parameters of the total length and total weight are shown in Table 3.9. The log transformed length and total weight data demonstrated a significant difference between the growth pattern of males and females p=0.001 between total lengths and p=0.008 between total weights.

The log transformed length and somatic weight data showed a significant difference between the growth pattern of males and females p=0.001 between the total length and p=0.007 between the somatic weights.

Table 3.9 Parameters of the relationship between total length and total weight for male, female, and pooled sexes.

Sex	*a*	*b*	SE	*n*	r^2
Male	4.390	2.839	0.022	345	0.979
Female	4.501	2.884	0.024	345	0.978
Pooled	4.473	2.869	0.018	690	0.939

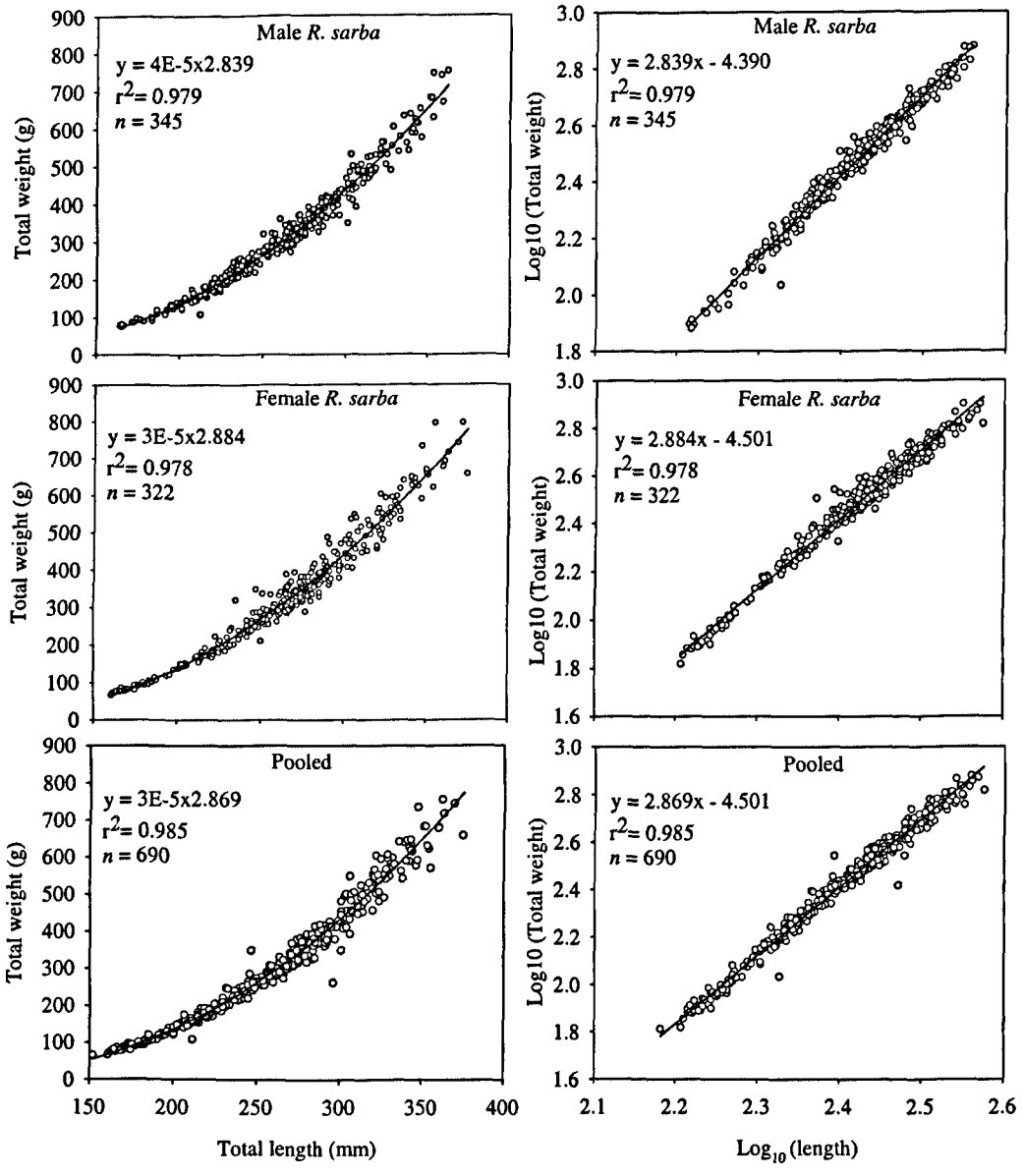


Figure 3.10 Length-weight relationship of male, female and pooled *R. sarba*.

3.3.8 Annual mortality and survival

The length-converted catch curve for the fish collected from the sampling site is shown in Figure 3.11. The length-converted catch curve for the fish measured at the central fish market in Muscat (Mutrah fish market) is shown in Figure 3.12. The instantaneous mortality rate (Z), natural mortality (M), instantaneous rate of fishing mortality (F), annual mortality rate and exploitation rate (E) are presented in Table 3.10.

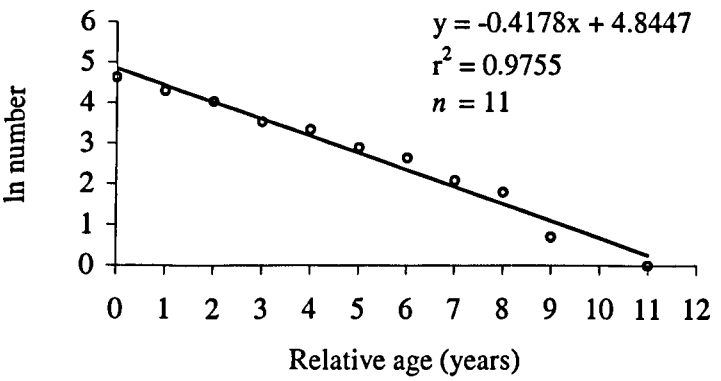


Figure 3.11 Length-converted catch curve of *R. sarba* for the fish collected from the sampling site.

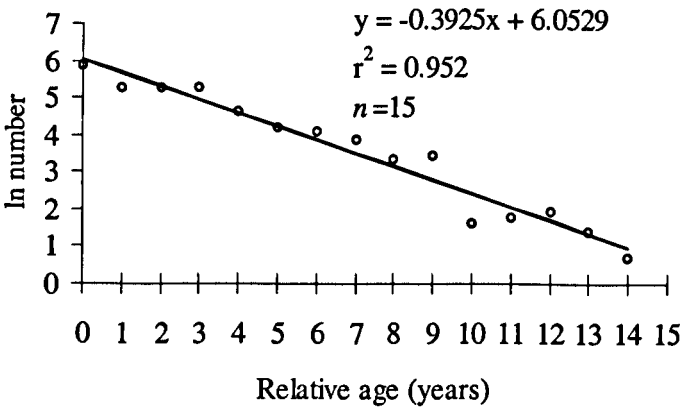


Figure 3.12 Length-converted catch curve of *R. sarba* for the fish measured at the central fish market in Muscat (Mutrah fish market).

Table 3.10 Estimates of instantaneous annual mortality rate, annual mortality rate natural mortality and survivorship.

Parameter	Sampling site	Matrah Market
Instantaneous mortality rate (Z) $year^{-1}$	0.42	0.39
Annual mortality rate. $year^{-1}$	0.34	0.33
Natural mortality rate (M) $year^{-1}$	0.36	0.36
Fishing mortality	0.06	0.03
Exploitation ratio	0.14	0.08
Survivorship rate	0.66	0.67

3.3.9 Length at first capture

Length at first capture (LC_{50}) was estimated to be 13.3cm (Figure 3.13). Both the FISAT II and the logistic curve gave similar values for LC_{50}.

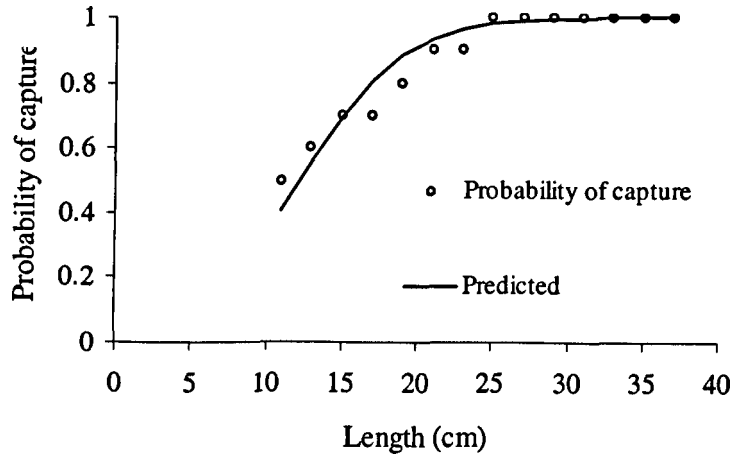


Figure 3.13 A selectivity curve fitted through the probability of capture calculated for each size category by FISAT II.

3.3.10 Landing size at the sampling site and Matrah fish market

The average total length of fish sampled throughout the two years indicated that the fish were at a similar average length during the spawning season (Figure 3.14). The average weight was also shown to be similar during the spawning season (Figure 3.15). Larger and heavier fish were caught in the springtime (March-May). Size frequency distribution of *R. sarba* sampled during both periods of sampling showed trends (Figure 3.18 & Figure 3.19).

The average total length of fish sampled throughout a year at the fish market indicated that the average length of the landed fish was higher in the summer period and relatively similar during winter (January-March) (Figure 3.16). The minimum average weight was recorded in May and September and the highest value was recorded in August. The average weight varied significantly ($p=0.03$) during the spawning season (January-March) (Figure 3.16).

The monthly percentage landing of *R sarba* at Matrah fish market for the two years indicated that the catch was highest in the winter months (December-March), reaching its peak in March of both years. The catch tended to fall during the spring and summer season and started to increase again from September (Figure 3.17). Size frequency distribution of *R. sarba* landed at Matrah market also showed trends (Figure 3.20). Over 30% of the landings recorded in May and June at Matrah fish market were of smaller fish.

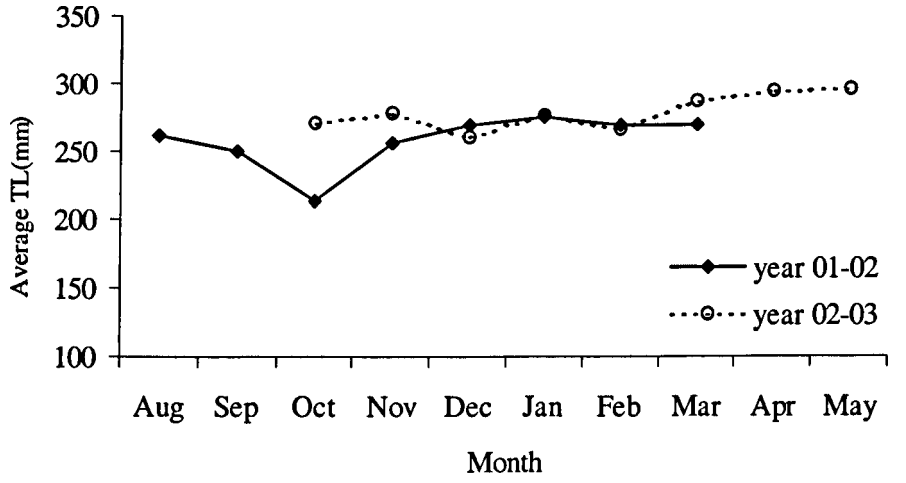


Figure 3.14 Average total length of *R sarba* during the sampling period at the sampling site.

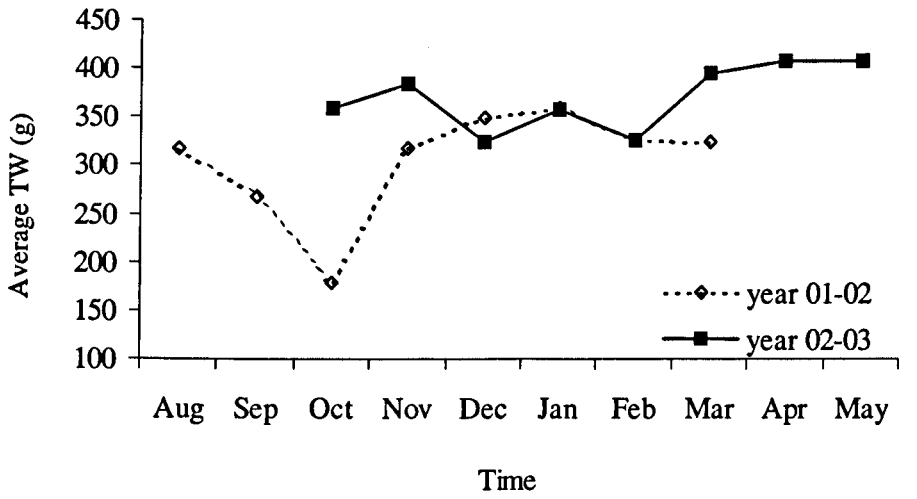


Figure 3.15 Average total weight of *R sarba* during the sampling period at the sampling site.

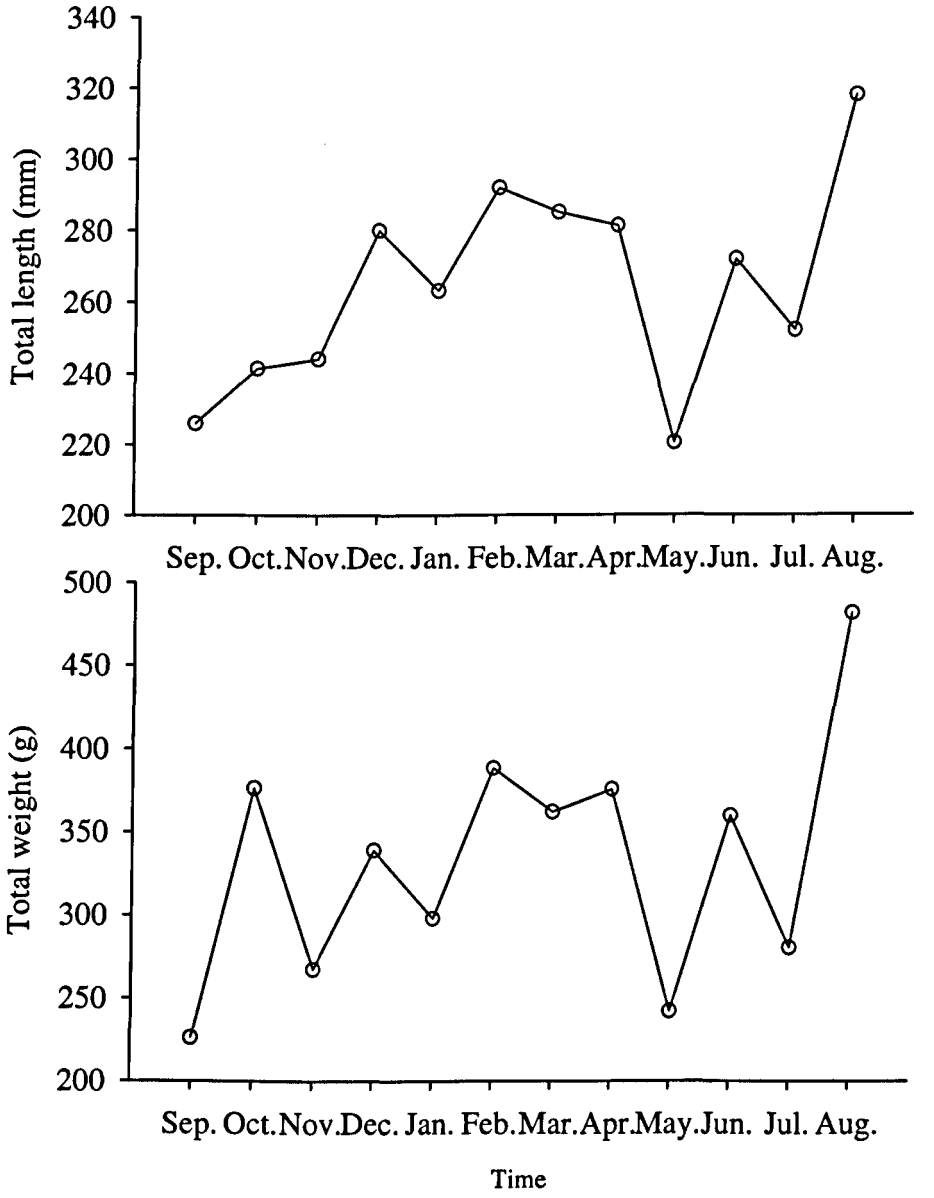


Figure 3.16 Average of total length and total weight of *R sarba* landed at the fish market (Sep. 01 to Aug. 02.

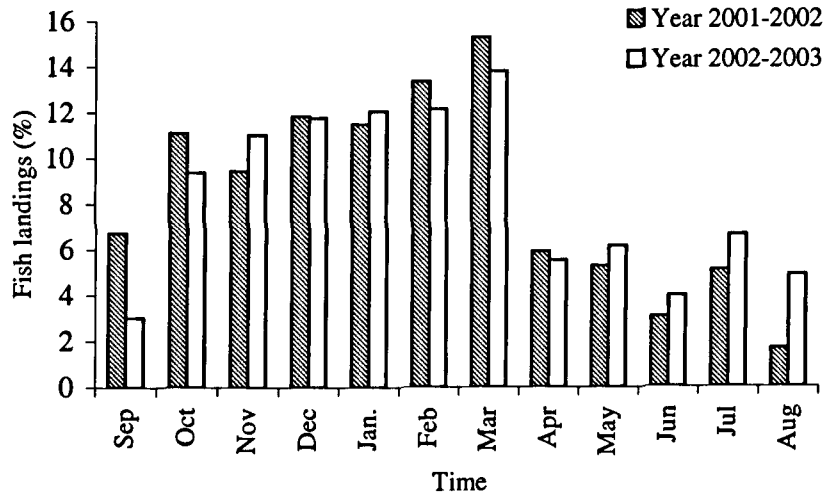


Figure 3.17 Percentage of annual *R. sarba* total landing at the Matrah fish market.

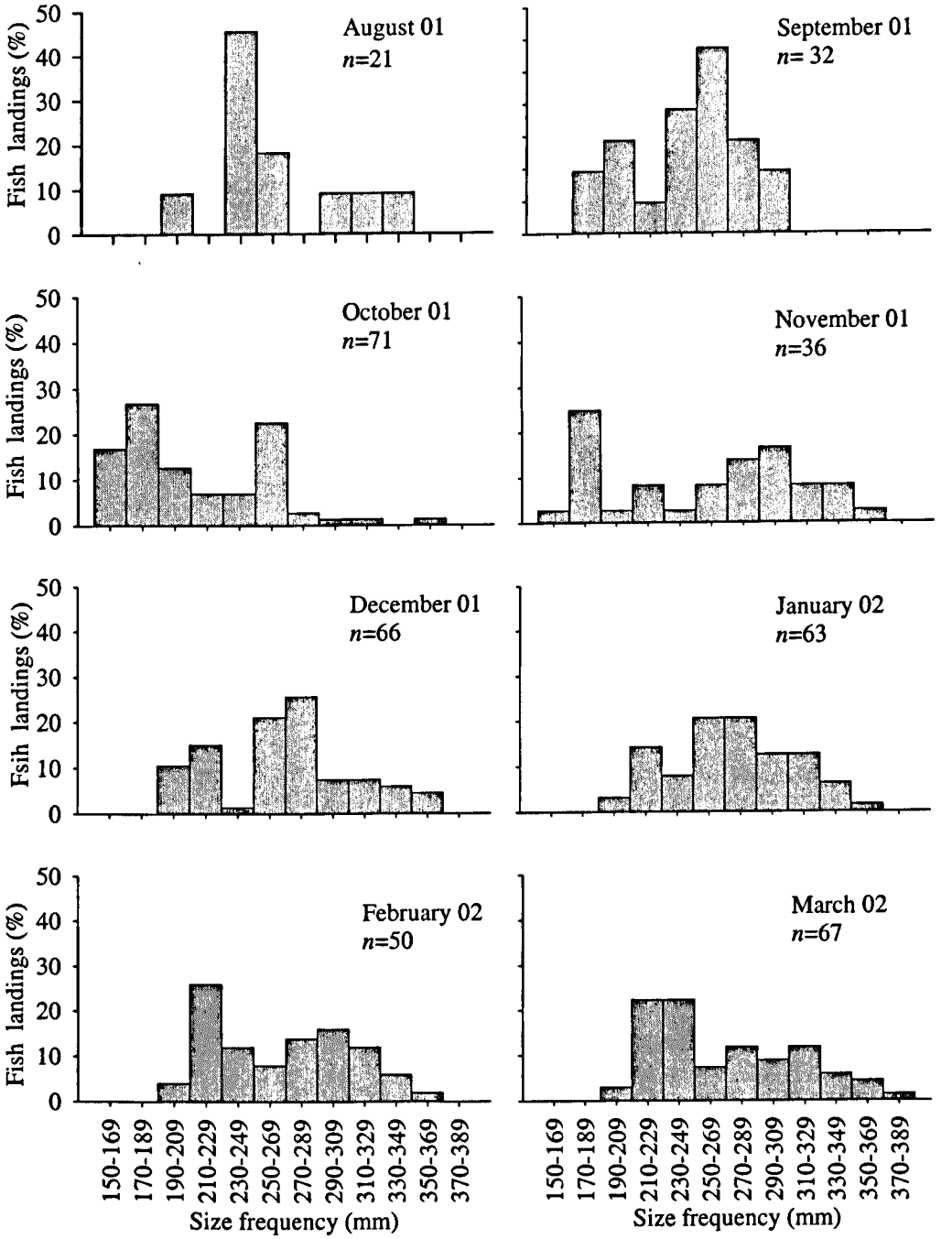


Figure 3.18 Size frequency distribution for the first sampling year (2001-2002).

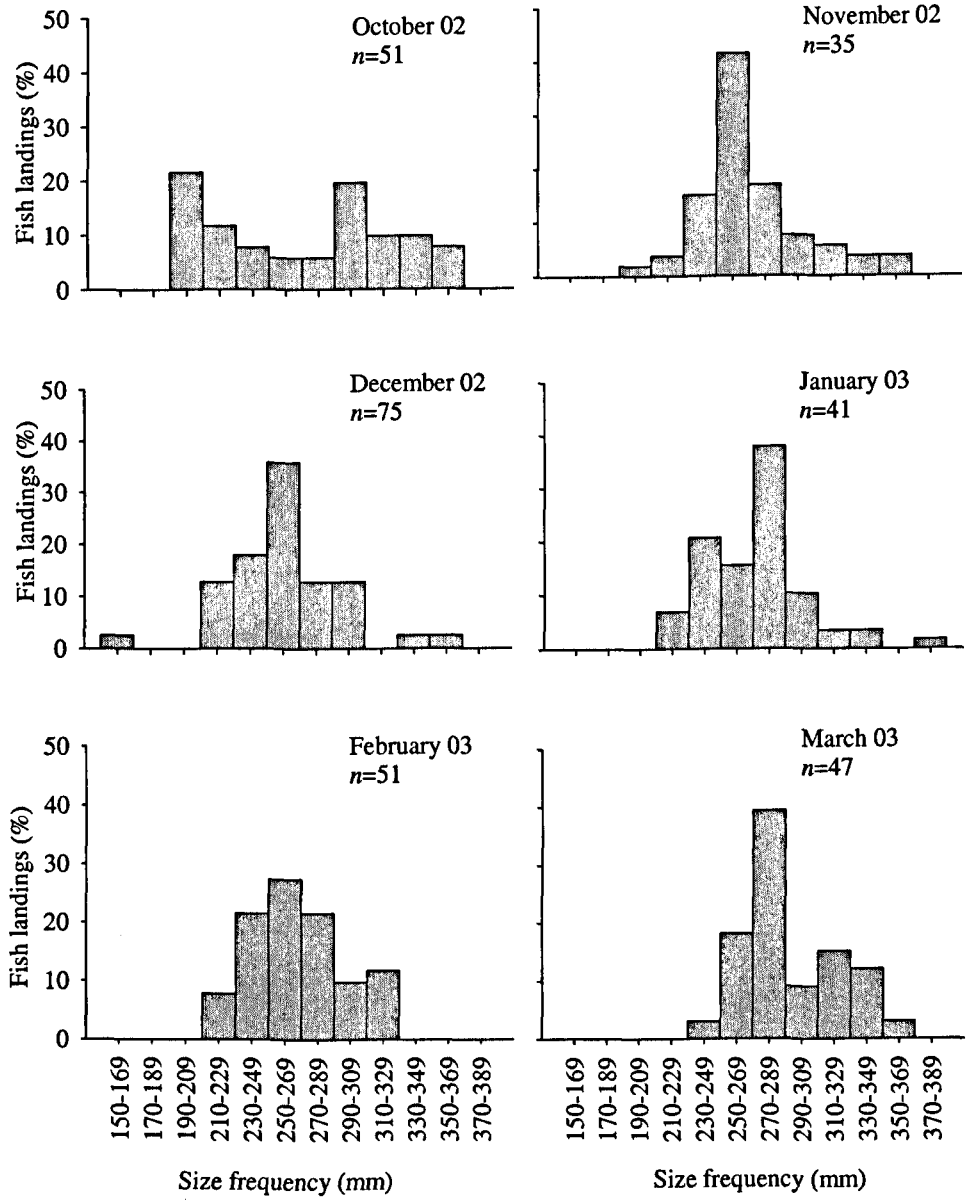


Figure 3.19 Size frequency distribution for the first sampling year (2002-2003).

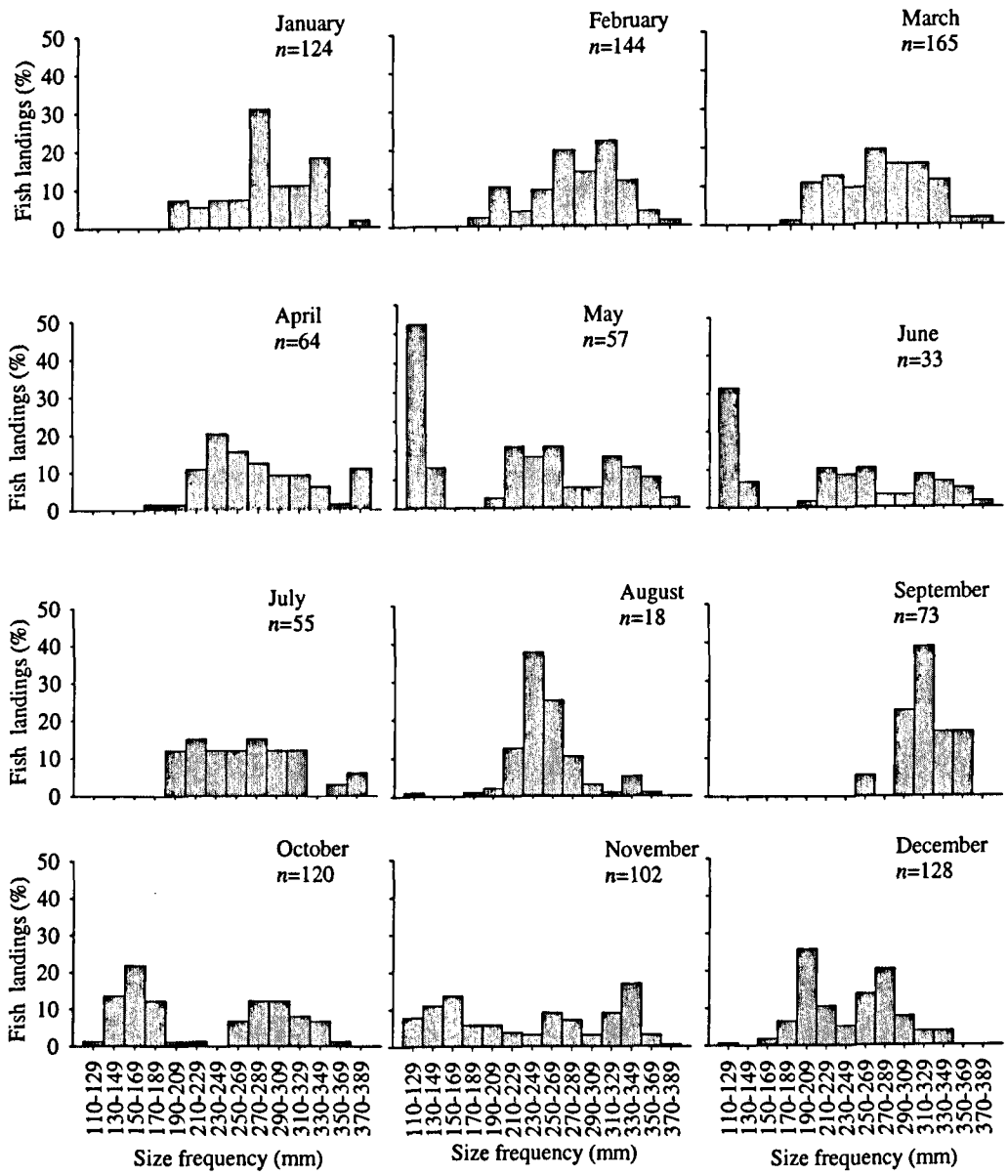


Figure 3.20 Size frequency distribution of *R. sarba* landed at Matrah fish market.

3.4 Discussion

The current study indicated that sectioned otoliths were reliable methods for aging goldlined seabream as ring formation was regular and fish length and otolith size were closely correlated. The data in this study indicated that females grow faster than males. Similar findings reported in other species, for example California halibut, *Paralichthys californicus* (MacNair *et al.*, 2001). The parameters of Von Bertalanffy growth function (VBGF) that were calculated in terms of both total and gutted weight indicated that the males grew to a heavier weight than females at all ages, which could be due to greater energy demand in females than the males thus reducing their capacity for growth. Females also had lower asymptotic mean lengths L_∞ than males for this species. In the study carried out by Pajuelo & Lorenzo (2001) the length-weight relationship of annular seabream, *Diplodus annularis* revealed that females are heavier than males for a given length. The authors related this finding to protandry, because males predominated in smaller size classes and females in larger size classes. Pajuelo & Lorenzo (2003) did not report any differences in the growth parameters of two-banded seabream, *Diplodus vulgaris* which could be due to rudimentary hermaphroditism with a low proportion of protandrous sex reversal. In all studies carried out on sparids using VBGF, including this study, the hypothetical age at which fish would have zero length (t_0) estimation tended to be a negative value and different from zero. Gordoa & Moly (1997) suggested that the VBGF fails to describe fish growth in early stages accurately, therefore, t_0 is negative.

Growth curves for males and females crossed between ages four and five. This result suggests that young male and female goldlined seabream are of similar size

until these ages. Beyond these ages the growth models indicated a shift in growth in favour of males. MacNair *et al.* (2001) attributed the crossover in growth curves to the small sample size for the ages where the crossing occurred. However, in this study this was not the case since the sample size for ages four and five years were at least higher than that of successive ages.

The individual sex parameters of VBGF that were calculated in this study could not be compared with other studies carried out on the same species due to the fact that other studies have worked with either pooled sex (male & female) or unsexed fish. Therefore, pooled data from both sexes in this study were used instead. The calculated parameters of the pooled data (male & female) in this study differed from both the study carried out in the Arabian Gulf (El-Agamy, 1989) and the study that was carried out in South Africa (Radebe *et al.* 2002). In this study $L_{\infty}$was similar to that found in the Arabian Gulf it was, however, different from that of the South African study. The difference in L_{∞} between the current study and the South African study could be attributed to the maximum fish size used in the study. The maximum size used in this study was 33.3cm FL while in the South African study it was > 67cm FL. The other differences in VBGF parameters between the Arabian Gulf study and the current study are possibly related to the use of scales in ageing *R. sarba* in the Arabian Gulf. However, these differences may also related to geographic range of the samples used in these studies. A comparison between these studies is listed in Table 3.11.

The length-weight relationship had shown that the value (b) for both sexes and pooled gave a value (<3) similar to the value found in Chika Lake, India (Patnaik,

1973) and in the Arabian Gulf (El-Agamy, 1989). This value has also been noted for other sparid fish (Pajuelo & Lorenzo, 2001), which reflects the fact that *R. sarba* is lighter in comparison to its unit length.

The presence of specimens smaller than 15cm in landings was due to recruitment as the trap fishing was typically undertaken at a depth of <20m. The oldest fish caught at the sampling site was 11 years; at the fish market, however, the oldest fish was estimated to be at an age of 15 years. These differences in fish size are attributed mainly to selection of fishing gear and the area and depth of the fishing ground. The landing throughout the two study years showed that the *R. sarba* catch was at its highest during the winter months (December-March), which coincided with the spawning season of this species that falls within this period. The reason behind the increase of *R. sarba* catch during this period could be that this species increases its feeding activity during these months and migrates towards shallow waters to graze.

Marginal increment analysis for the otoliths of *R. sarba* collected over one year suggest that there is one opaque and one hyaline ring (annuli) deposited each year. It was generally found that the opaque ring was laid down in winter that coinciding with both a drop in temperature (Ralston & Williams, 1989; Fowler & Doherty, 1992) or an increase in temperature (El-Agamy, 1989; Pajuelo & Lorenzo, 2000). Similar findings have been reported for other sparids such as *Dentex gibbosus*, *D. annularis*, *Pagellus acarne*, *P. erythrinus*, *Pagrus pagrus*, *Spondyliosoma* and *Diplodus vulgaris cantharus* (Pajuelo & Lorenzo, 1995; 1996; 1998; 1999; 2000; 2001; 2003).

The average otolith weight (WO) and otolith length (OL) increased with age throughout the observed ages and the relationship between OW and OL and observed age was linear and statistically significant. This result suggests that these parameters could provide a quick and economic method of aging. Many studies considered otolith weight–age relationships as a viable approach to estimate that could be utilised to derive catch age–frequencies (Boehlert, 1985; Pawson, 1990; Pilling *et al.*, 2003; Pino *et al.*, 2004).

Reproduction was suggested to result in slower growth (Campana & Neilsen, 1985; El-Agamy, 1989). It has been suggested that energy is diverted for gonadal growth rather than somatic growth (Van Der Walt & Beckley, 1997) and reduced availability of prey also restrict fish growth (Ricker, 1971). This study indicated that the formation of most of the opaque rings correlated with spawning season. Similar findings were reported by Radebe *et al.* (2002). The result also agreed with other studies carried out on other sparid fish e.g. *Pochymetopon blochii* (Pulfrich & Griffiths, 1988), *Sarpa salpa* (Van Der Walt & Beckley, 1997; Pajuelo & Lorenzo, 2000).

The age-at-first maturity of *R. sarba* in Oman was at zero years of age for males and one year for females. Males mature at a younger ages than the females; a similar finding was observed by Patnaik (1973) and El-Agamy (1989). Pajuelo & Lorenzo (2001) also reported similar findings for annular seabream, *Diplodus annularis*.The length-at-first maturity for both sexes was greater than that given by El-Agamy (1989) which was 18cm compared to 19cm for males and 23.4cm for females found in this study. It was also different from the result of Wallace (1975). Radebe *et al.*

(2002) suggested that the difference in length at maturity could be due to over fishing which makes the fish mature at a smaller size. In this study however, there was no evidence of over-fishing of this species in Oman, rather, this could be due to the fishing load being concentrated on the younger population.

The catch-age analysis showed that the number of *R. sarba* caught was highest at ages between zero and three years accounting for about 75%. The highest catch was recorded at age zero and two years indicating that a larger population of younger age is taken by the fishermen which could lead to some individuals not being allowed to participate in spawning at all. The percentage of fish caught was at its highest in the two-year-old age group, which means that fish had one to two spawning events. The number of fish in the catch was sharply reduced at ages higher than three years. Also, length-at-first capture $LC_{50\%}$ was found to be 13.3 cm, suggesting that *R. sarba* is vulnerable to fishing gear at a very young age. Given the fact that small-sized individuals dominated the catch of *R. sarba* fish this might be the reason behind the difference in size at maturation between *R. sarba* in Omani waters and South African waters. Differences in size and age-at-first maturity between sexes are related to the protandric nature of sparid fish (Pajuelo & Lorenzo, 2000). Goldlined seabream, *R. sarba* sex ratio and length-frequency distributions were consistent with a diagnosis of protandric hermaphroditism (Yeung & Chan, 1987b; Garrat, 1993). Females smaller than 16cm TL were uncommon, and only one male more than 36cm TL was captured.

The instantaneous mortality rate, Z (Pauly, 1980) was lower when landed fish sampled at the fish market were used in the estimation. Pauly (1987) proposed that

the optimum fishing mortality is calculated by $F_{opt} = 0.4 \times M$. In this study however, $F_{opt} = 0.14$ for all fish sampled ($F < F_{opt}$). The exploitation rate was also lower than E= 0.5. It can be said therefore, that *R. sarba* in the coastal waters of Oman is fished under the optimal level of exploitation. It was evident that the *R. sarba* catch tends to decrease during the spring and summer season a possible indication that fish migrate to deeper waters during the summer period either in search of food or to avoid temperature rise in shallower water. This reduces their vulnerability to fishing gears and reduced catches are reflected in the landings.

The exploitation rate indicates that the stock is not over-fished. However, the direct effects of fishing on the population that could result in future changes in the abundance are from the age or size at first capture. The length-at-first capture ($LC_{50\%}$= 13.3 cm) is less than the length-at-maturity for males which is 19cm and females that is 23.4cm. With this percentage of the total catch below this length there is a danger of recruitment over-fishing. Furthermore, fishing activity increases during the spawning season which could deplete the adult spawners and further add problems to recruitment. Stock–recruit relationships in finfish are maintained by adequate spawning stock that is critical to sustainable fisheries for most exploited finfishes (Myers & Barrowman, 1996). A relationship has been suggested to occur between spawning stock abundance and survival of their young based on trophic interactions and fertilization success (Walters & Kitchell, 2001).

Table 3.11 A comparison between the VBGF growth parameters for *R. sarba* at different localities.

Author	Species	Location	Sex	L_∞ (mm)	t_0 (year)	K (year^{-1})
El-Agamy (1989)	*S. sarba* *	Arabian Gulf	Pooled	375	-1.32	0.162
Radebe, *et al.* (2002)	*R. sarba*	South Africa	Pooled	715	-0.996	0.16
This study	*R. sarba*	Gulf of Oman	M	424.391	-3.448	0.162
			F	337.856	-2.066	0.369
			Pooled	364.091	-2.694	0.256

* A synonyms for the species still used in some locations (Fischer & Whitehead, 1974).

L_∞ = asymptotic length, the length that a fish will reach had it been allowed to live

K = Brody growth coefficient that defines the growth rate towards L_∞

t_0 = hypothetical age at which fish would have zero length had it always grown in a manner described by the model

Pooled data is for males and females combined.

Chapter 4: Gonadal development and recrudescence of ovaries in *R. sarba* during the spawning season.

4.1 Introduction

4.1.1 Ovarian development and spawning patterns in teleosts

In most teleosts, ovarian development has been classified as synchronous or asynchronous and has been classified according to the appearance of oocytes (Scott, 1987). Fish that are considered synchronous ovulators generally have two groups of developing oocytes in their ovary at any one time, a larger oocyte group that will be used in the coming season and a primary oocyte group used in next season (Scott, 1987). Fish such as salmonids have two different patterns of spawning behaviour; yearly as in the case of the brown trout, (*S. trutta*, Bagenal, 1969), or once in the fish's life, for example the Pacific pink salmon (*O. gorbuschus*, Dye *et al.*, 1986).

There are many marine teleosts that are asynchronous ovulators in which developing oocytes are continuously ovulated and are released in batches throughout the spawning season (Scott, 1987). Some well known marine species which fall into this category are seabream (Loir *et al.*, 2001; Bromage & Roberts, 1995), sea bass (Carrillo *et al.*, 1989) and halibut (Norberg *et al.*, 1991). There is also one important marine fishery species described as an asynchronous ovulator and that is the mackerel (Wallace & Selman, 1990). As for *R. sarba*, it has been reported that this species is an asynchronous ovulator (El-Agamy, 1989).

Ovarian development in teleosts is divided into several stages. In the studies that have been conducted to date, oocytes always follow similar patterns of development. These stages have been identified based on biochemical, physiological, histological and morphological criteria (Yamamoto, 1956; Wallace & Selman, 1981; Selman & Wallace, 1983; Bouain & Siau, 1983; Wallace *et al.*,

1987; Bromage & Cumaranatunga, 1988; Selman *et al.*, 1993). The difference among these studies is only in the number of developmental stages of oocytes noted.

The meiotic process by which reproductive cells or mature gametes (oocyte/egg and spermatozoa) are formed is termed gametogenesis. Production of oocytes/eggs is termed oogenesis and production of spermatozoa is termed spermatogenesis.

The primary growth phase that includes oogenesis and folliculogenesis is the first stage in the oocyte development. This stage is generally termed "previtellogenesis" or cytoplasmic growth, a transformation of oogonia to oocytes towards the end of the perinuclear stage. This stage is later followed by the cortical aleveolar stage where the oocyte begins to exhibit some vesicular formation in the periphery of the cytoplasm. Vitellogensis and final maturation is the last developmental stage that the oocyte reaches prior to spawning. The schematic development of oocytes for red seabream, *Pagrus major* is shown in Figure 4.1 (Matsuyama *et al.*, 1988).

Several studies classify oocyte maturation in sparid fish such as *Cheimerius nufar* (Coetzee, 1983); *Chrysoblephus laticeps* and *Chrysoblephus cristiceps* (Buxton, 1990) and *Dentex dentex* (Loir *et al.*, 2001). Some of these studies have reported the germinal vesicle migration as the final stage in the oocyte development (Loir *et al.*, 2001).

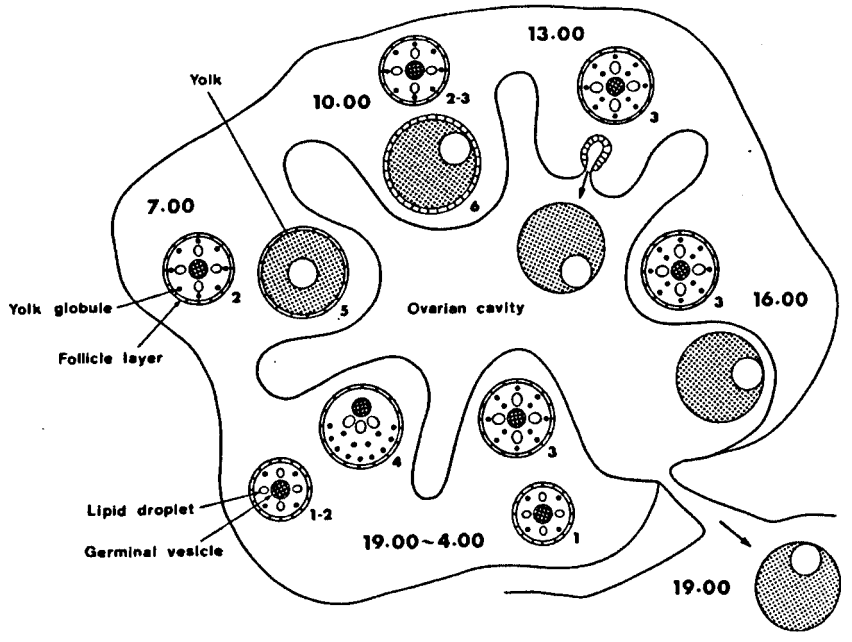


Figure 4.1 Schematic diagram representing the maturation rhythm of the *Pagrus major* (After Matsuyama *et al.*, 1988).

1, Primary yolk stage; 2, secondary yolk stage; 3, tertiary yolk stage; 4 migratory nucleous stage; 5, pre-mature stage; 6, mature stage.

In order to develop detailed classification criteria that would allow the separation of ovarian-developing oocytes in *R. sarba* into distinguishable stages it is important to review the general physiology and oogenesis in teleost ovaries which the following sections describe.

4.1.2 Development of germ cells in the female ovary

This section describes the oocyte developmental stages but will follow the description that is commonly given for oocyte development in teleosts.

4.1.2.1 Primary growth phase: (Previtellogenesis or cytoplasmic growth)

A) Oogonia

Cell division occurs in order to create oogonia made up of a large nucleolus containing one large nucleolus situated within the cytoplasm (Braekevelt & McMillan, 1967). Commonly, these oogonia are found singularly or in small oogonial nests (Bruslé, 1980; Bromage & Cumaranatunga, 1988). Continuous division of oogonia in teleosts is believed to be driven by meiosis in order to form oocytes throughout the life of the fish through periodic mitotic division (Tokaz, 1978). Following this division, oogonia develop into primary and secondary oogonia (Khoo, 1975). Oogonia proliferation is reported to exist at various stages during ovarian development and has been reported to appear before the onset of the spawning event, during the spawning and post-spawning phases (Franchi *et al.*, 1962). In most cases it has been found that oogonia appear in large numbers immediately after spawning (Bromage & Cumaranatunga, 1988; Matsuyama *et al.*, 1988) but in some it is rarely observed and has therefore been suggested to be a short transitory step in young immature females (Loir *et al.*, 2001). Nevertheless, oogonia are present in ovaries containing all stages of oocytes and they are available for recruitment (Bromage & Cumaranatunga, 1988).

B) Stage one oocyte: (chromatin nuclear stage)

As the oogonia develop and pass through mitotic proliferation they enter the early stages of the first meiotic division (chromatin nucleolar stage). During the early stages of meiotic proliferation (prophase) chromosomal DNA replication takes place (leptotene) and this process is followed by the pairing of homologous

chromosomes (zygotene). The homologous chromosomes then shorten and thicken to form synaptonemal complexes (pachytene) and subsequently, the chromosomes divide into "lampbrush" configurations (diplotene). During this stage of development, the oocyte contains a singular nucleus at the centre with one nucleolus and the oocytes are frozen in meiotic prophase (Wallace & Selman, 1981; Nagahama, 1983). As mentioned earlier, this stage of oocyte development is rarely found and it has been suggested that this is due to the rapid transformation from stage one to stage two (Bromage & Cumaranatunga, 1988).

C) Stage two oocyte: (primary stage)

This stage is also known as the "perinucleolar" stage. The growth of primary oocytes is characterised by intense RNA synthesis (Wallace & Selman, 1990). The oocyte grows in size progressively due to the major cytoplasmic process which occurs at this stage of development. In some cases, the oocyte increases in volume from around 1000 to 5000-fold (Nagahama, 1983; Sumpter *et al.*, 1984). The oocytes start to develop a cellular vitelline envelope and granulosa cells around themselves. This layer further develops at a later stage of oocyte development and is termed "zona radiata" (Anderson, 1967; Tesoriero, 1978; Riehl, 1984). The nucleus multiplies into several nucleoli as the ribosomal genes in the nucleus of the oocytes at this stage begin to enlarge (Anderson & Smith, 1978). Transportation of RNA and mRNA from the nucleus to the cytoplasm of the oocyte forms organelles which appear as basophilic-dense material known as the 'Balbiani body', 'yolk nuclei' or mitochondrial cloud (Guraya, 1979; 1986). The formation of these bodies contributes greatly to the increase of oocyte size during this developmental stage (Guraya, 1979; Coello & Grimm, 1990). The Balbiani body is composed of two

parts, the yolk nucleus and the pallial substance (Guraya, 1986). It has been suggested that the yolk and nucleus starts developing during the chromatin-nucleolus stage as a non-basophilic structure (Maridueña, 1984). The pallial substances form during the early perinucleolar stage as a basophilic ring but less basophilic than the surrounding cytoplasm. The Balbiani bodies have also been observed to be composed of RNAs, mitochondria, Golgi bodies, endoplasmic reticulum, ribosomes and vesicular bodies (lipid droplet) (Guraya, 1979, 1986; Selman & Wallace, 1989) and it has been proposed that these bodies function as a centre for the biogenesis of cell organelles prior to the deposition of yolk material into oocytes (Guraya, 1986).

4.1.2.2 Stage three oocyte: Secondary growth phase (cortical aleveolar)

This oocyte stage is characterised by the appearance of numerous vesicles. These vesicles increase in size and number as the oocyte progresses in its development and moves to the periphery of the oocytes. This aggregation of vesicles is termed the "cortical alveoli". Several terminologies have been used to refer to this vesicle aggregation, but cortical vesicle and cortical alveoli have become widely used (Wallace *et al.*, 1987; Bromage & Cumaranatunga, 1988). The cortical alveoli have been shown to contain glycoproteins and associated enzymes that may contribute to the hardening of the vitelline envelope after ovulation and the prevention of polyspermy (Kitajima *et al.*, 1994). These yolk vesicles have been suggested to be synthesised within the oocyte (Shackley & King, 1977). During this stage, a vitelline envelope or chorion termed zona radiata and zona pellucida starts forming between the oocyte ooplasm and the granulosa cells. In some sparid fish e.g. *C.*

cristiceps the oocyte diameter at the end of this stage reaches up to 158 -180μm (Buxton, 1990).

The appearance of lipid vesicle oocytes in the gonads is considered to be a sign of ovarian maturation by some authors (e.g. Barr, 1963,1968). Reinboth (1970) suggested that it occurs in the protandric sparids once a change of sex has taken place.

4.1.2.3 Stage four oocyte: exogenous vitellogenic stage

Vitellogenesis is a phase of rapid ovarian growth that is distinguished by the hepatic production and ovarian uptake of the yolk protein precursor vitellogenin (VTG). The growth during this stage is prolonged and is controlled by the pituitary gonadotropins (Wallace, 1978). In some species such as salmonids, vitellogensis extends for up to six months (Tyler *et al.*, 1991) and in batch spawners the period is approximately five to seven weeks as for the striped bass, *Morone saxatilis,* for example (Blythe *et al.*, 1994). Matasuyama *et al.* (1988) reported that the red seabream, *Pagrus major* has a diurnal ovarian maturation rhythm in which a clutch of eggs is spawned daily suggesting an even shorter period. The oocyte at this stage grows enormously due to vitellogenesis, which may reach up to 95% of the final egg size (Tyler, *et al.*, 1991b, Tyler *et al.*, 2000). In batch spawners such as *Sparus aurata* vitellogenic material accounts for about 16% of the final egg size (Carnevali *et al.*, 1992).

Choriogenin H and vitellogenin (VTG) are proteins primarily produced from liver cells in females and are transported to the ovary by means of the blood stream. Choriogenin H is required for the formation of egg chorion and VTG is a

phospholipoprotein complex required for oocyte growth and used as a source of energy for larvae (Murata *et al.*, 1997). Oestrogens, mainly estradiol (E_2) have been suggested to act at the gonad to stimulate oocyte development and to induce these proteins in the liver (Murata *et al.*, 1997). Exogenous oestrogen increases serum calcium concentrations in female goldfish (Bailey, 1957) and salmonids (Bjornsson *et al.* 1989). Plasma VTG and calcium has been found to increase after the onset of yolk deposition in most fish such as the white sturgeon *Acipenser transmontanus* (Linares-Casenave *et al.*, 2003).

4.1.2.4 Stage five & six oocyte: Germinal vesicle migration and final maturation

Stage five oocyte development is characterised by the migration of the germinal vesicle (nucleus) towards the animal pole of the ooplasm that is situated near to the micropyle (Wallace & Selman, 1981). This stage is considered as stage seven by some authors (e.g. Bromage & Cumaranatunga, 1988) but considered as stage five in the case of sparid fish (Loir *et al.*, 2001). The VTG sequestration continues until the point of germinal vesicle breakdown (GVB) (Wallace & Selman, 1985).

Hydration of eggs plays an important role for those fish that produce buoyant pelagic eggs such as marine fish. This hydration transforms stage five oocytes to stage six oocytes as a result of rapid water uptake and successive water-hardening (Hirose *et al.*, 1976; Bromage & Cumaranatunga, 1988). A process termed 'ovulation' takes place soon after the oocytes become mature (eggs). During this process, the eggs are expelled by a contraction of the ovarian follicle layer into the ovarian cavity or ovarian lumen ready to be released into the surrounding water (oviposition). Granulosa and thecal layers that were formally surrounding the

oocyte are left in the ovary once the oocyte is released into the ovarian lumen, leftover tissue are termed "post ovulatory follicles" (POF). Post ovulatory follicles (POF) are observed to contain large numbers of oogonia as a sign of oogonia proliferation due to the degeneration process of the granulose layer (Kagawa *et al.*, 1981, Matsuyama *et al.*, 1988).

4.1.2.5 Atresia: resorption of oocyte

A degeneration and resorption process that occurs at various stages of oocytes development in many vertebrates (Saidapur, 1978) is known as atresia (Macer, 1974; Nagahama, 1983) and it has been suggested that this has a significant effect on fish fecundity (Bromage & Cumaranatunga, 1988).

Stress arising from various factors such as starvation, unfavorable temperature and photoperiod regimes, sub-optimal water quality and over crowding has been linked to atresia (Kazanskii, 1963; Dettlaff & Davydova, 1979). Such stress leads to insufficient gonadotrophic stimulation especially during the vitellogenic phase (Micale *et al.*, 1997). Insufficient gonadotrophic stimulation could cause stress-induced change in the steroid production of the follicle (Clearwater & Pankhurst, 1997). Insufficient gonadotrophic stimulation, however, has lower occurrence in wild fish (Witthames & Greer Walker, 1991).

Atresia is divided into four stages α, β, γ and δ (Bretschneider & Duyvene de Wite, 1947). The following short review will describe the characteristics of the common atresia stages.

During the first degeneration and resorption process (α-stage), the oocyte shrinks due to loss of water. This step in atresia is also associated with the presence of increased macrophages within the follicle. Transformed granulosa cells appear to be the only cells involved in the initial phase of digestion of the oocyte envelope. The yolk content of the oocyte liquefies and loses its acidophilic nature; at the same time the nucleus bursts releasing its content into the ooplasm.

During the β-stage multiplication of the follicle cells increases. During this stage numerous blood vessels and blood-born cells have been observed among the follicle cells. It has been suggested that these blood-born cells participate in the process of recycling some energy invested in the production of yolk materials by the female (Linares-Casenave, 2002). Transformed granulosa cells remove the egg envelope and oocyte contents by lysosomal digestion.

The γ-stage marks completion of the disintegration and resorption of the atretic oocyte. δ-stage is the final stage of atresia in which black-brown residual bodies are found, commonly known as "brown bodies" (Chan *et al.*, 1967). These bodies are said in some cases to remain until the next spawning season (Webb *et al.*, 1999). The fifth stage of atresia (Σ stage) is given by Khoo (1975) who suggested its involvement in oogenesis as numerous oogonia were found in postovulated ovaries.

A literature search revealed that there is no study that classifies ovarian development of *R. sarba*. The only study that is available based on histological morphology is the study carried out by Yeung & Chan (1987b). This study focused on the gonadal anatomy of the protandrous hermaphroditism in *R. sarba* in which

the authors gave distinguishable features of different types of gonads at different sexual phases in this respect. However, the study carried out by the authors has explained the change in sex steroids during the reproductive cycles in the three sex phases, but were not related to the development of the oocytes and spermatogenesis in an intensive way. The authors classified the reproductive cycles in male and female into prespawning, spawning and post-spawn/inactive stages according to the maturational state of the male and female germ cells, but no detailed histological evidence was given.

4.1.3 Hermaphroditism in Sparid

Hermaphroditism is not only common among some teleosts fish families but is also functional (Tyler & Sumpter, 1996). A review of sexuality carried out by Buxton & Garratt (1990) on sparids shows that protandrous, protogynous, simultaneous and rudimentary hermaphroditism have all been reported in the family. Schematic diagrams of hermaphroditism in Sparidae are given in Figure 4.2. As the author suggests, sex inversion is an alternative reproduction strategy that is undertaken in order to allow fish individuals to maximise lifetime and reproductive success by functioning as one sex when small and as the other at an older age.

Protandry has been reported for many sparids such as *Diplodus sargus* (Micale & Perdichizzi, 1994) and yellowfin seabream, *Acanthopagrus latus* (Abou-Seedo *et al.*, 2003). In these studies the authors found that fish begin life as a functional male with a testicular zone undergoing active spermatogenesis and an ovarian zone that is arrested at the primary growth (perinucleolar) stage. Sparids like red porgy, *Pagrus pagrus* exhibit protogynous hermaphroditism in which a high proportion of females

were found at a younger age (Pajuelo & Lorenzo, 1996). Some sparids are also considered as a gonochoristic species such as *Dentex dentex* in which case fish maintain the same sex throughout their entire lifespan. (Rueda & Martínez 2001). Pajuelo & Lorenzo (2003) found that the two-banded seabream, *Diplodus vulgaris* exhibits rudimentary hermaphroditism in which young fish have an immature inter-sexual gonad but mature as either female or male with a low proportion of protandrous sex reversal.

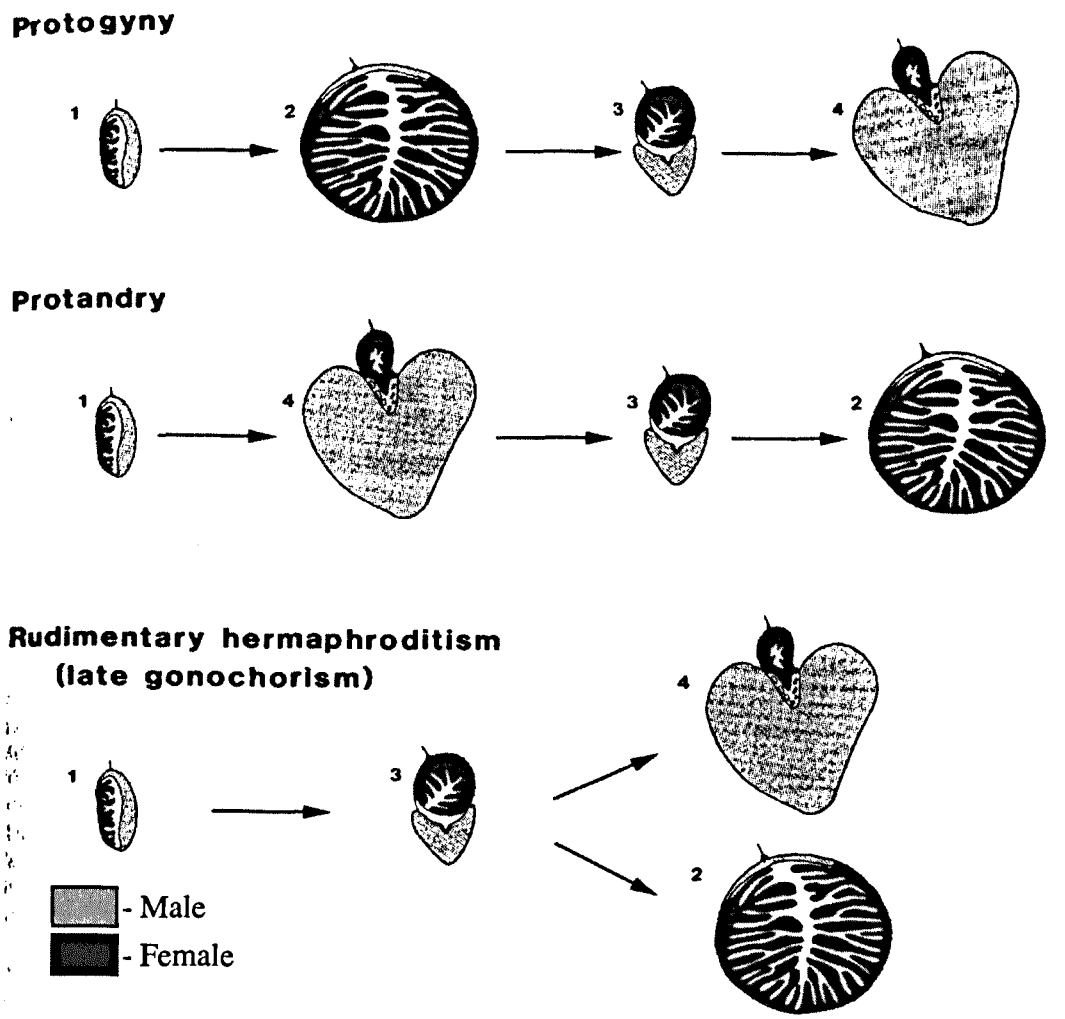


Figure 4.2 Schematic diagram of hermaphroditism in Sparidae.

After Buxton & Garratt (1990), Permission granted thankfully by the authors.
1, juvenile; 2, functional female; 3, intersex (non functional); 4, functional male.

Evidence derived from histology and biopsies in the *R. sarba* carried out by Yeung & Chan (1985, 1987b) indicated clearly the occurrence of natural sex reversal from male to female (protandrous hermaphroditism). The authors indicated that the gonad consists of distincts male and female zones and clearly separated by connective tissue. The authors have also distinguished four types of gonads. Type I (male) which is in the juvenile and adult, II (intersex), III (female) which has a vestigial testicular tissue and finally type IV (male). The latter has been found more commonly in larger specimens and the authors suggest that these functional males may not undergo sex reversal in their life cycle.

4.1.4 Application of stereology in the study of fish physiology

Stereology has been used to quantify and study the properties of ovarian oocytes in many fish such as herring, *Clupea harengus* and Dover sole, *Solea solea,* (Emerson *et al.*,1990). It has also been applied to tilapia *Oreochromis niloticus*, (Srisakultiew, 1993) and *Tilapia zilii* (Coward, 1997; Coward & Bromage 2002). More recently, stereology has also been applied to Atlantic northern bluefin tuna, *Thunnus thynnus* (Medina *et al.*, 2002). Emerson *et al.* (1990) state that stereology has been proven to provide similar results to other methods used to estimate ovarian oocytes such as the volumetric methods (Simpson, 1951), gravimetric methods (Burd & Howlett, 1974) or by the use of automated particle counters (Witthames & Greer Walker, 1987). Stereology is carried out without the use of any chemicals that might be harmful and is also based on correct classifications of oocytes using histo-morphometric data provided by the processed histology slides.

Therefore, the present study attempted to develop detailed classification criteria for *R. sarba* oocytes that could be used to classify the stage of maturity of the female's ovaries. It also considers spawning biology in Omani waters where traditional demersal trap fishing is taking place. It is the intention here to study the ovarian condition of wild caught *R. sarba* in more detail with relation to the condition of the various oocyte stages during development of ovaries through the use of stereology.

4.1.5 Morphology of testis in teleosts

In teleosts the testis appears as a paired organ located on the dorsal part of the abdominal cavity of the fish above the intestine. It is attached to the dorsal wall by the mesogonium. In most teleosts, testis is a structure that is prolonged at the posterior and contains numerous lobules, white/cream in colour. In *R. sarba*, each part of the gonad occupies approximately two-thirds of the entire length of the abdominal cavity (Yeung & Chan, 1987b). The two lobes of the gonad join together posteriorly and end at the level of the genital papilla between the rectum and the urinary ducts. Fish testes has large number of lobules that are separated by thin layers of connective tissue which extend from the testicular capsules (Roosen-Runge, 1977).

Two types of testicular structures have been reported; tubular and lobular (Billard *et al.*, 1982) (Figure 4.3). The first type has regular form with no lumen and is generally not common. This form is limited to the atheriniform fish (Grier *et al.*, 1980). Lobular type testes are more common in teleosts. This type basically appears histologically like a lobe, its diameter and shape is inconsistent. The

spermatogenetic cells are found scattered along the lobule. Commonly, two types of cells are found within the lobular component: distinct somatic and germ cells.

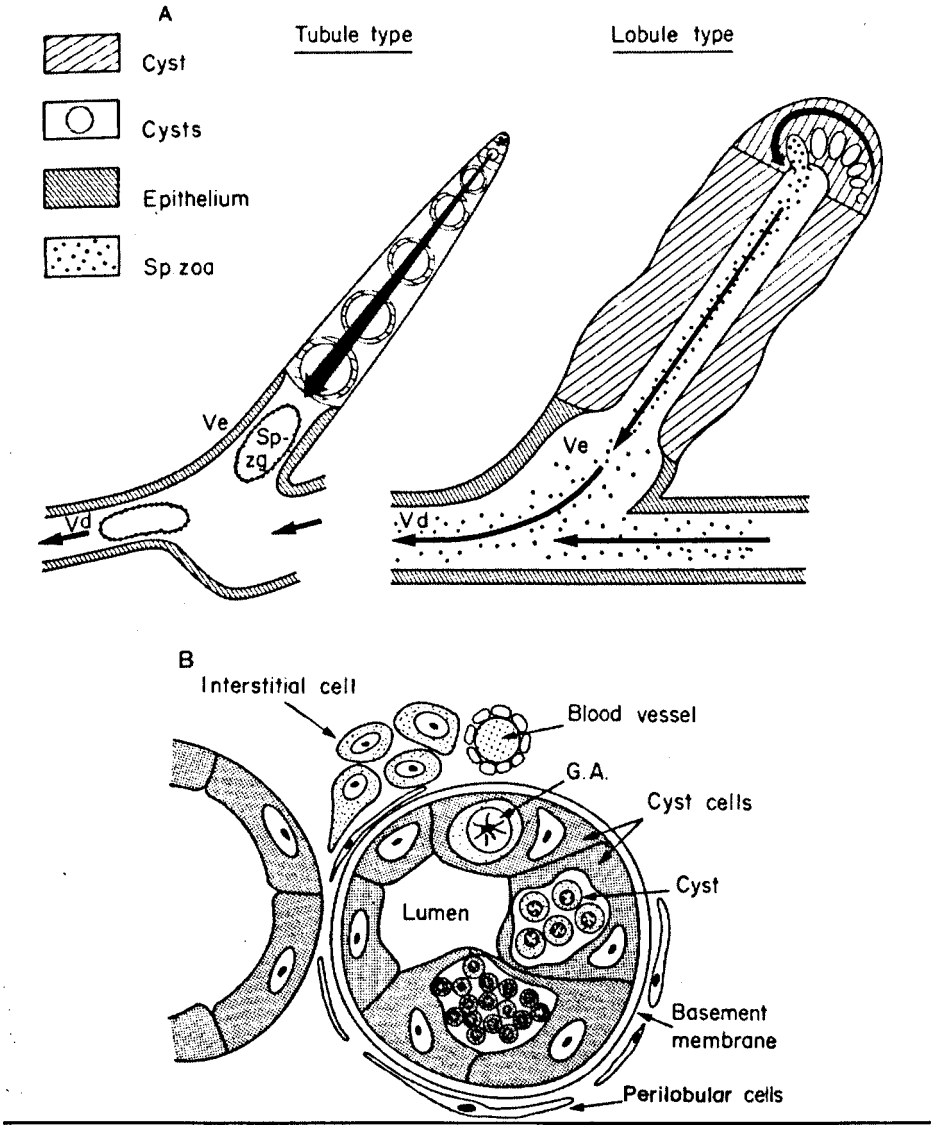


Figure 4.3 (A) Schematic diagrams represent types of testis found in teleosts. (B) Cross section of a lobule. After Billard *et al.* (1982).

Ve, vas efference; Vd, vas deferens, Spzg, spermatozeugma; GA, type A spermatogonia

4.1.6 Spermatogenesis in teleosts

The germ cells undergo a series of developmental stages termed "spermatogenesis" which leads to formation of spermatozoa (Billard *et al.*, 1982) (Figure 4.3). The

germ cells in the lobule tend to develop asynchronously in order to provide a continuous supply of spermatozoa during the reproductive season. Spermatogenesis involves initial proliferation of spermatogonia cells through repeated mitotic division followed by a growth period that leads to the formation of primary spermatocytes. Spermatocyte cells undergo reduction division (meiosis) to form secondary spermatocytes that eventually lead to formation of spermatids. Spermatids undergo metamorphosis and as their development proceeds, it results ultimately in the release of spermatozoa into the lobular lumen. The spermatozoa accumulate in the sperm duct (vas deferens) through a series of contractions that occur to the vas deferens. Spermatozoa are released by abdominal musculature contraction during the reproductive season. The spermatozoa of fish with external fertilization are simple, with a round head, rudimentary middlepiece and are poor in mitochondria (Jones & Butler, 1988; Lahnsteiner *et al.*, 1997). According to Bromage & Roberts (1995) the spermatozoa in teleosts have no acrosomes and this characteristic may be attributed to the existence of a micropyle in the oocyte.

During the development of the testes many structural changes take place. The width of tunica has been found to become thin as the fish approaches its maturation and thickened during the spent phase soon after the reproductive season ends (Brown-Peterson *et al.*, 2002). Lobule surface of the testes has been found to increase as spermatogenesis takes place and increases in its activity especially during the reproductive season (Gill *et al.*, 2002).

Little attention has been given to the reproductive cycle and seasonal testicular changes of male goldlined seabream. The aim of the present study, therefore, is to

describe the developmental stages in male goldlined seabream testes in order to determine criteria that can be used to define the maturity of male *R. sarba.*

The aims of this chapter:

10) Conduct histological studies on gonadal development and classify the maturity stages of gonads in female and male *R. sarba.*

11) Quantify the various oocyts stage during gonadal development using stereological techniques.

12) Measure plasma calcium concentrations (Ca^{2+}) in female *R. sarba* during the reproductive season to estimate vitellogenin (VTG).

4.2 Materials and methods

4.2.1 Wild fish sampling and histology

Fish samples that were used in this study were collected during two periods. The first was from August 2001 to April 2002 and the second from September 2002 to April 2003. A total of 321 females and 337 males were captured during the two periods. Sampling was terminated when the females and males were observed to have resting gonads (gonads found at the end of spawning season). Following biometric data measurements as detailed in Section 2.1.4 fish were dissected and the right lobe of the gonads removed and fixed in 10% buffered formalin. A piece of approximately 5mm thick tissue was removed from the middle section of the right gonad and placed in a histological cassette. For stage 6 ovaries the whole ovary was fixed individually in small plastic containers since it was too difficult to cut open the ovary while the oocytes were at their final stage of maturation. Similarly, for active testes whole testis was fixed individually. The tissues were processed, sectioned and stained at the Institute of Aquaculture, Stirling University, UK as detailed in Section 2.1.7.

For ovary, the germ cells were classified based on protocols by Selman & Wallace (1983; 1989), Cumaranatunga (1985), Kokokiris *et al.* (1999) and Loir *et al.* (2001). For testis, the germ cells were classified according to the protocol of Buxton (1990) and Loir *et al.* (2001).

Slides were examined and micrographs were taken under a compound microscope (4 -100× power) using a digital camera (ZEISS AKSIOSKOP.2 MOT). Slides were

viewed on a computer monitor using KS300 version 3.0 image software (Imaging Associates Ltd, UK).

4.2.2 Estimate of developing occyte from a histological slide of an ovary.

Stereology or three-dimensional reconstruction is used to estimate 3D properties of the target from random two-dimensional plane sections or projections (Weible, 1979). Those stereological techniques that have been developed and are applied to date are based upon the 'Delesse Principle' (Delesse, 1847). The principle is fundamental in this respect and shows that random two-dimensional observations could be used to quantify the three-dimensional information of an object.

4.2.2.1 The principle of Delesse & theoretical stereology

Stereology systems require that a test system (e.g. grids, points, lines) be placed over the photograph of a section cut through a specimen with a view to estimating geometrical parameters such as volume, surface area, length and total curvature.

The workings of the Delesse principle can be easily explained. A cuboid structure such as a cube of an ovary tissue containing oocytes of various (irregular) shapes is placed into an *x,y, z* coordinate system and sliced parallel to the *x,z* plane into thin slices of thickness d_y (Figure 4.4). The total sectional area (A_{st}) of each histological slide and sectional object area (A_{so}) evidently contains a certain amount of volume of sectional object (V_{so}) and the total volume of section (V_{st}) as described in the equations (1) and (2).

$$V_{so} = A_{so} \times D_y \quad \text{.....(1)}$$

$$V_{st} = A_{st} \times D_y \quad \text{.....(2)}$$

Total sectional volumes (V_{st}) and total sectional object volumes (V_{so}) are added to obtain total volumes of the cuboid and the object. The total object volume (ΣV_{so}) is then divided by the total sectional volume (ΣV_{st}) to calculate volume density (V_{vo}), see equation (3).

$$\frac{\Sigma V_{so}}{\Sigma V_{st}} = \frac{V_{so}}{V_{st}} = V_{vo} \quad \text{.....(3)}$$

The value calculated for the volumes V_{so} and V_{st} is replaced by the products of area multiplied by the thickness calculated as shown above in equation (1) and (2) and the following calculation is performed as shown in equation (4).

$$\frac{\Sigma(A_{so} \times dy)}{\Sigma(A_{st} \times dy)} = \frac{(dy \Sigma A_{so})}{(dy \Sigma A_{st})} = \frac{A_{so}}{A_{st}} = A_{ao} \text{.....(4)}$$

It is apparent from the above equation that V_{vo} in equation 3 and A_{ao} in equation 4 are equal, thus it has been proved that

$$V_{vo} = A_{ao} \text{.....(5)}$$

If the measurements of A_{ao} are performed using a point counting technique (P_{po}) with random grid point procedure suggested by Weibel (1979) with a view to measuring the area fraction of an object (P_{so}) contained in a total area (P_{st}) of the histological section, then:

$$P_{po} = \frac{P_{so}}{P_{st}} \text{.....(6)}$$

Finally we arrive at the conclusion that:

$$V_{vo} = A_{ao} = P_{po} \quad \text{.....(7)}$$

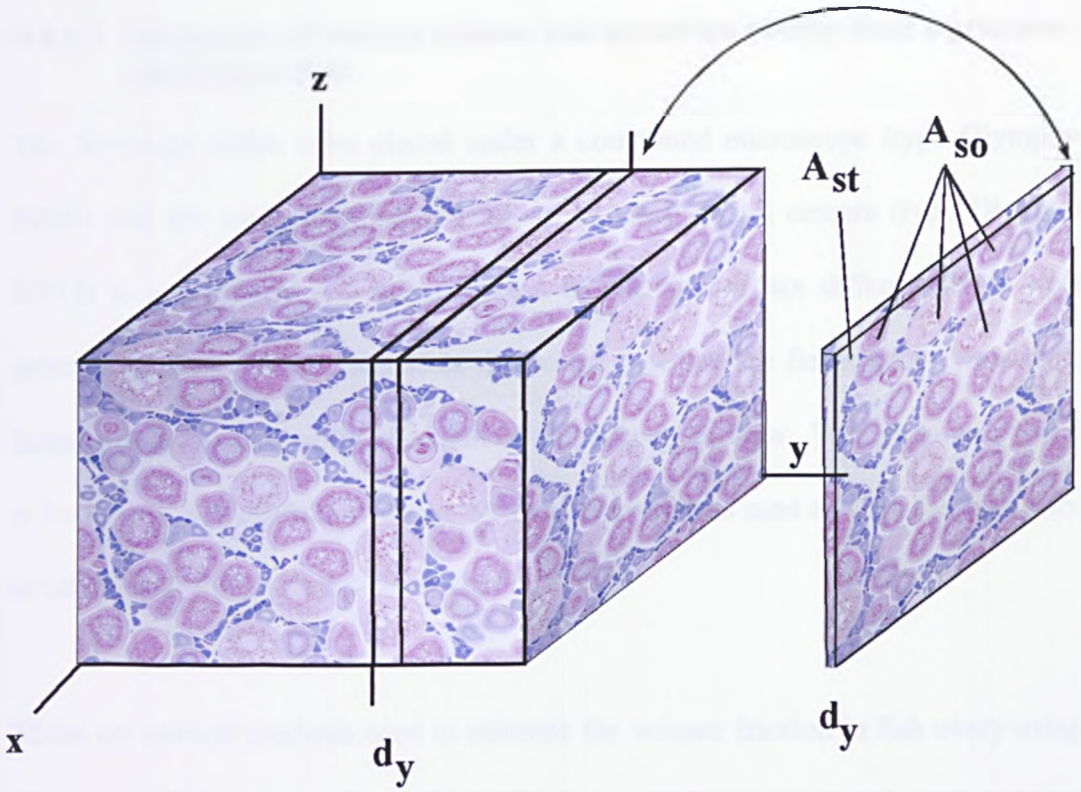


Figure 4.4 Model illustrating the Delesse principle modified from Weibel (1979).

A_{so} - area of the image (oocyte)

A_{st} - area of the section,

d_y - thickness of the section

Amount of material that is actually examined in the microscope is often a tiny fraction of the whole object of interest, therefore, stereology can successfully quantify target objects, surface area and density, target volume and volume ratio and quantify the number of objects that are of interest to the researcher. It has been proven to provide information about the unknown properties of specimens such as cell nuclei, mitochondria and liver cells.

4.2.2.2 Estimation of volume fraction and numerical density from a prepared histological slide

The histology slides were placed under a compound microscope (type Olympus-SZ60) and the images transferred via an attached digital camera (type Olympus DP12) to a computer at a magnification of ×4. At least six different fields were selected randomly from each slide due to the fact that the fields under view were large and covered a considerable part of the slide under view. Total field area was 5 × 3= 1.5cm². Olympus Soft Image System software was used to view the area prior to taking snapshots of the field in view.

There are several methods used to estimate the volume fraction in fish ovary using stereological methods such as the graphical method (graph paper), mass method (plastic weighing) and intersection method (point counting) (Weible, 1979). For more details in this area refer to (Srisakultiew, 1993). Using a stereology grid such as the Weibel multipurpose grid, however, is common but requires adaptors fitted between the camera and the microscope (Emerson *et al.*, 1990; Srisakultiew, 1993; Coward, 1997; Coward & Bromage 2002). For this study an alternative method was developed in view of such constraints. Canvas version 7.0.2 image software was used to create a series of grids (n= 252) represented as circles in Plate 4.1 which was then superimposed on the section image.

The area fraction (volume fraction) of stage 'x' oocytes (A_{ax}=V_{vx}) on each of six histological sections for the three examined ovarian stages was computed as follows: The field (ovarian section picture) was downloaded to the monitor. The grids field (n=252) was pasted on the ovarian field so that it overlies the picture field. Each oocyte stage was counted separately, identifying which of the 252 grid

points overlap a specific oocyte stage and the total number of these points found overlying each development stage was noted. The grid points that were found not to be overlying any oocyte were ignored. All stages of atretic oocytes (α, ß and γ) stages were counted and pooled. The above procedure was repeated for all the randomly selected fields in each of the slides used in this study. This allowed the calculation of mean±S.E.

a) Area fraction (volume fraction) of stage 'x' oocyte (A_{ax}=V_{vx}) of ovarian development was calculated using equation (8):

$$A_{ax} = A_{sx} / A_{ts} \quad \text{.....(8)}$$

Where:

A_{ax} = area fraction of stage 'x' oocytes.

A_{sx} = number of grid points occupied by stage 'x' oocytes in the field.

A_{ts} = total number of grid points on the field (i.e. the total grid points).

4.2.2.3 Calculation of ovarian numerical densities (N_{ax})

From micrographs of histological slides for female *R. sarba* ovary sections, the numerical density (N_{ax}, number of oocyte per unit volume) was calculated from the area fraction (volume fraction) in stage two to stage six ovary according to the formula developed some time ago by Weibel & Gomes (1962), see equation (9). The formula was later modified by Weibel *et al.* (1966) to be known as:

$$N_{vx} = (K_x N_{ax}^{1.5}) (\beta_x V_{vx}^{0.5})^{-1} \quad \text{.....(9)}$$

Where:

N_{vx} = number of stage 'x' oocyte per unit volume

N_{ax} = number of stage 'x' per unit area

V_{vx} = volume fraction occupied by stage 'x' oocyte

K and β are coefficients specific to the species (Weibel, 1969)

β_x = related to the shape of the x' oocyte, see equation (10)

K_x = related to the size (diameter of oocytes) distribution of x' oocyte see equation (11).

Micrographs for the histological slides were taken as detailed in section (4.2.2.2). Graph paper was photographed for calibration purposes using the same magnification that was used in taking the histological view of the fields in order to know the filed size. The total area of each field was 1.5cm² on each histological section and the total numbers of each oocyte developmental stage studied here that fell within the boundary imposed by the field shown in Plate 4.2 were counted in at least four fields randomly selected from each histological slide. Since the field that was selected covered the majority of the slide it was not possible to capture more than four fields. To be consistent, any oocytes found crossing the left and / or the top of the field (i.e. the drawn square) were included in the count, whilst those oocytes found crossing the right or bottom of the field boundaries were excluded from the counting.

The total number of oocytes of each developmental stage in a unit volume (N_{vx}) can be used to quantify the total number of each oocyte size category in the whole ovary by multiplying (N_{vx}) value (derived from equation 9) by the entire ovarian volume.

4.2.2.4 *K* and β coefficients

The formulas used to calculate other elements in order to estimate N_{vx} in equation (9) are given below:

Firstly, the shape of the x' oocyte (β_x) is calculated by equation (10):

$$\beta_x = (l_x)(s_x)^{-1} \quad \text{.....(10)}$$

Where:

β_x = shape of the x' oocyte

l_x = length of long axis of oocyte stage 'x' (mm)

s_x = length of short axis of oocyte stage 'x' (mm)

Secondly, the oocyte size (diameter of oocytes) distribution of stage 'x' oocyte was calculated using equation (11):

$$K_x = (M_3 M_1^{-1})^{1.5} \quad \text{..... (11)}$$

Where:

K_x = size of the 'x' oocyte distribution

M_1 = first moment of the size distribution calculated by equation (12)

M_3= third moment of the size distribution calculated by equation (13)

$$M_1 = (D_{x1} + D_{x2} + D_{x3} + \ldots D_{xn})(n^{-1}) \quad \text{.....(12)}$$

$$M_3 = (D_{x1}^3 + D_{x2}^3 + D_{x3}^3 + \ldots D_{xn}^3)(n^{-1}) \quad \text{.....(13)}$$

Where:

n = total number of oocytes counted

Dx = mean diameter of stage 'x' oocytes calculated by equation (14)

$$D_x = (l_x + s_x)(2^{-1}) \quad \text{..... (14)}$$

4.2.2.5 Validation of the stereology method

At least two fish from age two, three and four years old mature female *R. sarba* ovaries that contained hydrated eggs were examined using both direct counting under a compound microscope and numerical density counting (N_{vx}/N_{ax}) using the stereology technique. This was carried out on stage six oocytes that could be identified by their appearance of being hydrated and having a single oil globule. Beside oocyte morphological appearance, average diameter of stage six oocytes was also checked before counting the oocytes.

4.2.2.6 Estimation of ovarian volume

Ovarian volume was estimated from the equation given by the linear regression of the relationship between the ovary weight (g) and the total volume of the ovary (ml).

4.2.2.7 Statistical analysis

Weible (1979) demonstrated that if the diameter distribution K of the particles to be counted is between 1.0 and 1.07 then the distribution is normal. In this study, however, K ranged between 1.01 and 1.13. The K values of stage three to atretic oocytes were within the range of the normal distribution except for stage two oocytes. Therefore, the data was logarithmically transformed prior to statistical analysis.

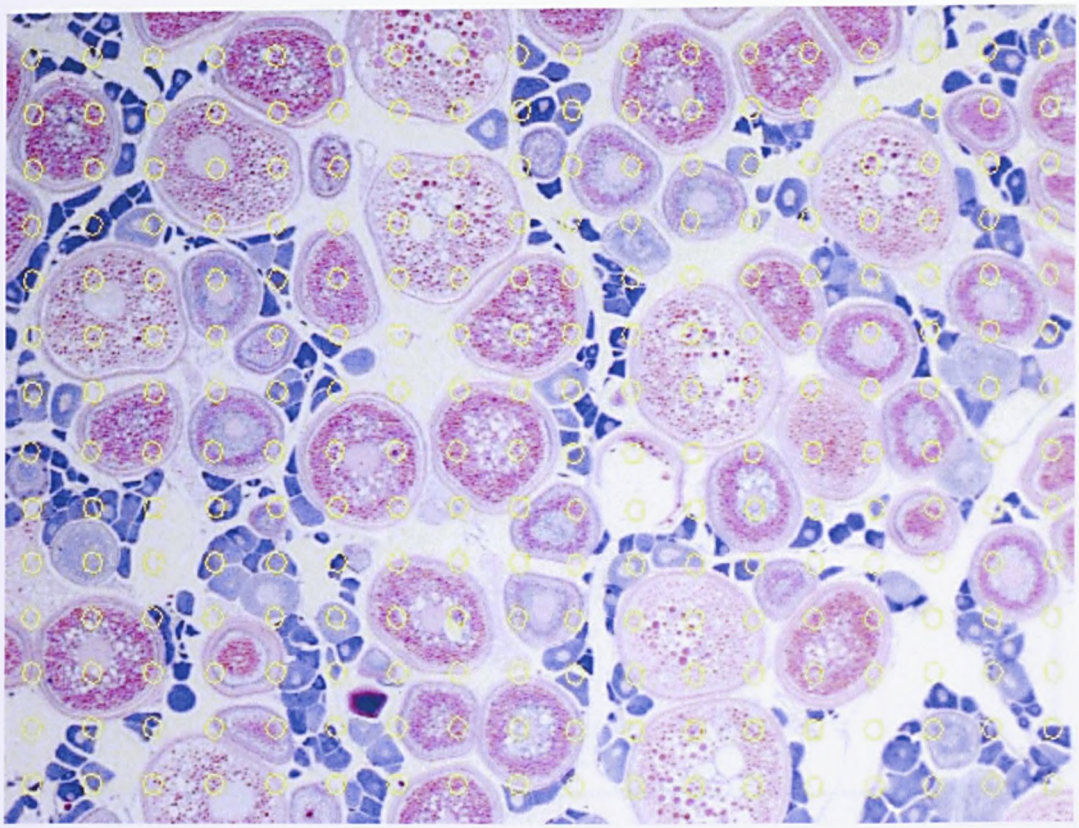


Plate 4.1 An example of the estimation of area fraction of stage x oocytes (A_{ax}) of different developmental stages in a unit volume of *R. sarba*.

The counting listed below was carried out from the field shown above. The numerical density (N_{ax}) for the above field is shown in Plate 4.2. Any differences that may have occurred between the calculation below and what is in the field are due to changes that took place during the transformation of the above picture from one software to another.

Total number of grid points on field = 252

Ovary area fraction of x oocytes (A_{ax}) calculated as follows:

A_a stage 2 oocytes = 40/252 = 0.158×100 = 15.8%

A_a stage 3 oocytes = 8/252 = 0.032×100 = 0.32%

A_a stage 4 oocytes = 106/252 = 0.420×100 =42%

A_a stage 5 oocytes = 54/252 = 0.214×100 =21.4%

A_a atretic oocytes = 0/252 = 0×100 =0%

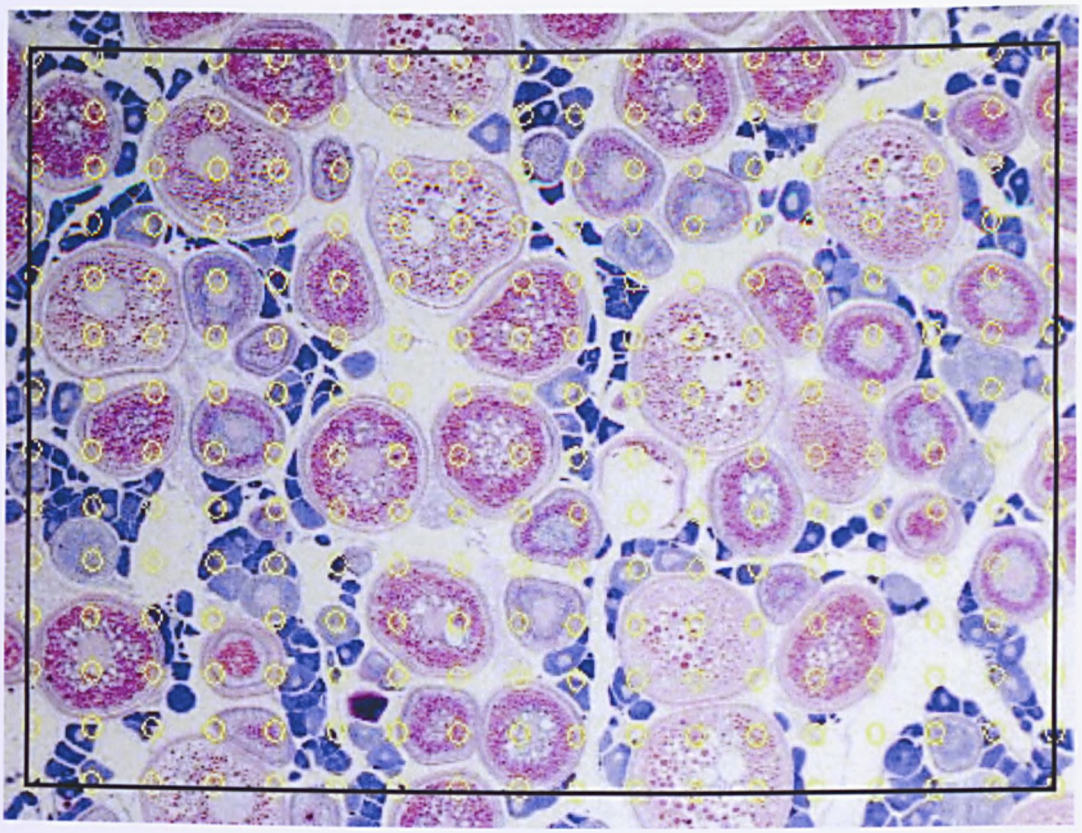


Plate 4.2 An example of the estimation of numerical density of stage 'x' oocytes (N_{ax}) of different developmental stages of *R. sarba*.

The counting listed below was carried out from the field shown above. Any differences that may occur in the calculation below and what is in the field are due to changes that took place during the transformation of the above picture from one software to another.

Total area = 1.5 cm^2 / field.
Ovary numerical density of x oocytes (N_{ax}) calculated as follows:
N_a stage 2 oocytes = 165
N_a stage 3 oocytes = 6
N_a stage 4 oocytes = 32
N_a stage 5 oocytes = 8
N_a stage 6 oocytes = 0
N_a atretic oocytes = 0

4.2.3 Ovarian follicles measurements

Ovarian follicle diameters, testis germ cells diameters, tunica width and lobular lumen measurements were taken using calibrated ocular scales (Eyepiece micrometers, Graticules Limited, U.K) in a compound microscope Olympus BH-2 Japan. The maximum and minimum axis was measured for each oocyte development stage. The measurement was carried out only on the oocytes that were sectioned through the nucleus and was conducted on at least 500 oocytes selected randomly from each developmental stage. Tubule surface of testis was calculated from the formula $\pi ab/4$ where *a* and *b* are the minimum and maximum diameters of each tubule (Gill *et al.*, 2002).

4.3 Results.

4.3.1 Gonadosomatic index (GSI)

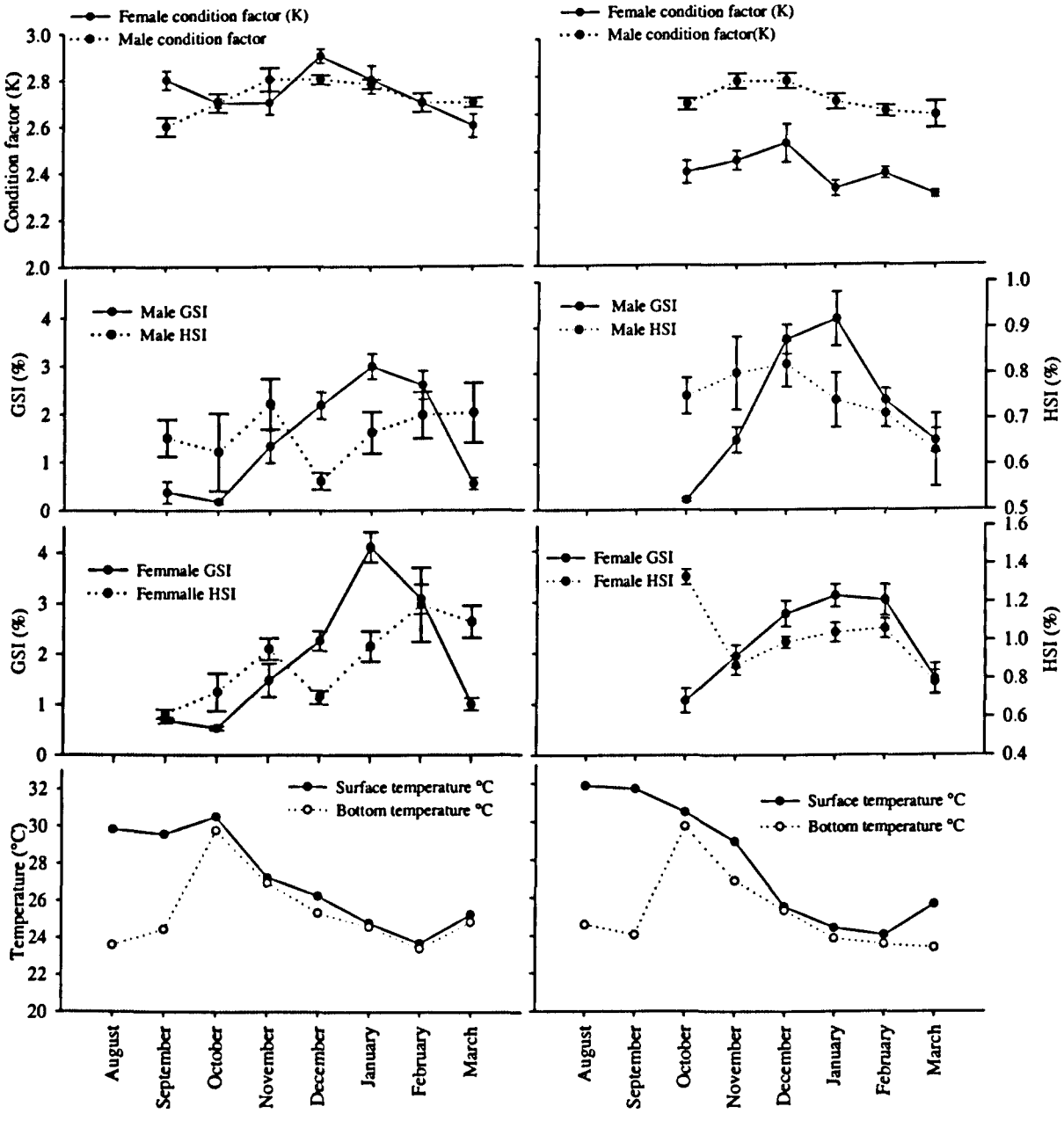
Variation in average GSI for females and males during the two study periods is shown in Figure 4.5. Average GSI for both male and female value increased to a peak in January and then continued to decrease until March. The spawning period began in late December and lasted until the end of February and it was further evident from histological data that revealed the existence of resting ovaries in late February marking the end of the spawning season (Figure 4.6). The changes in GSI of *R. sarba* were in line with the change in water temperature. Bottom and surface water temperature started falling in October and the GSI was found to increase starting from this month. As the water temperature rose up again in March, the GSI fell to a minimum.

4.3.2 Hepatosomatic index (HSI)

For females, the liver, or hepatosomatic, index (HSI) was low before the spawning season (Figure 4.5). Generally it began to increase from December onwards following an increase in GSI and reached its maximum peak during the month of February, decreasing at the end of the season during the month of March. For males, HSI was not in line with increased GSI. Male HIS over the two years did not show the same trends before and after spawning season. The index started to increase following the onset of spawning during the first sampling period, however, in the second sampling period the index decreased when testes reached the active stage (Figure 4.5).

4.3.3 Condition factor "K"

In males condition factor (K) reached a peak in November in both years while females peaked in the following month. The K values for both sexes began to fall beyond December and continued to fall to the point where the minimum values for both sexes were recorded in March (Figure 4.5). Correlation analysis between GSI and K for females showed a significant relationship ($r=0.821$, $p=0.013$). Correlation analysis between GSI and K values of males, however, showed that the two variables were not correlated ($r=0.106$, d.f.=355, $p=0.089$). The index for males before and after the spawning season did not differ significantly ($p>0.05$), for females, however, there was a significant difference between the two periods ($p=0.02$). During the first sampling season, no significant difference was observed in K value between sexes, however, during the second sampling season, significant difference was observed between males and females ($p=0.03$).



First sampling period (August 01 - March 02) Second sampling period (August 02 - March 03)

Figure 4.5 Indices for GSI, HSI and condition factor (K) of female and male *R. sarba* during the first and second sampling season shown with the average temperature of seawater at the sampling site.

4.3.4 The development and cycle of the female ovary

Female ovarian developmental stages of female *R. sarba* (Figure 4.6) were classified according to the most advanced oocyte stages found in the ovary. The classifications were carried out taking into account the histological morphology of the oocyte. Eight stages of ovarian condition or activity were identified during the reproductive period of *R. sarba* (Figure 4.6 & Table 4.1).

In October the ovary remains in the primary growth phase (perinucleolar stage). The ovary contained mainly nests of oogonia and oocytes at the beginning of the meiotic prophase and numerous early primary oocytes (Plate 4.3). Stage two ovaries accounted for approximately 100% and 80% in October in the first year and the second year respectively (Figure 4.6). For the same period in the second year of sampling, however, stage three, the cortical alveoli stage (Plate 4.4) and stage four, (the early vitellogenic stage Plate 4.5) were also present. Females that were classified as having stage two ovaries during the period that fell between January and February were caught immature and probably did not contribute to spawning.

An increase in stage four ovaries (Plate 4.5) was noted from November until spawning commenced which is in late December to early January. The percentage of stage four ovaries increased from 37% in November to 55% in December during the first season. During the second season, however, stage four ovaries were at their highest in November; reaching 80%, and during December the percentage was similar to that of previous year. The percentage of stage four ovaries decreased gradually as the spawning season progressed and at the end of the season in March stage four ovaries were not recorded.

Stage five ovaries (Plate 4.6) were observed from December during both sampling periods. They were observed until March during the first sampling period and until February during the second sampling period. Percentage stage five ovaries was generally lower than those of stage four and stage six during the reproductive season.

Stage six ovaries (Plate 4.7) were found in fish sampled towards the end of December and increased between January and February. Postovulatory follicle envelopes (termed hereafter POF) which were evidence of recently spawned females started to appear at the end of December but they were sparse (Plate 4.8). However, ovaries with POF had increased in January and February and ovaries that were found containing POF did not contain oocytes greater than stage three (S3), (Plate 4.8).

Spawning activity took place at the end of December and continued throughout January to the beginning of March when resting ovaries started to appear. Two different ovaries at this stage were distinguished. The first type was found with numerous atretic oocytes of various stages (Plate 4.9) while the second type was at the final stage when all the atretic oocytes were fully absorbed and the ovary contained POF that were formerly occupied by oocytes and degenerated oocytes or 'oogonia'. At this stage the ovaries did not contain any oocytes greater than stage two (S2), (Plate 4.10) and females that were found with resting ovaries in March accounted for approximately 80% and 90% in the two sampling seasons respectively.

Atretic oocytes were not found in immature gonads but were observed in 10-20% of the gonads of sexually maturing and mature females and started to appear in late December. They were mostly seen to affect the vitellogenic oocytes. The atretic oocytes that were found in stage five ovaries were all at the 'γ' stage compared to stage four ovaries that contained all three stages of atretic oocytes (i.e. α, β & γ). So-called 'brown bodies' can be seen in spent ovaries (recent spawning) (Plate 4.9) as well as those ovaries found to be resting at the end of the spawning season (Plate 4.10). Atretic oocytes were present during both the spawning and post-spawning seasons.

Generally, ovarian developments started one month earlier in the second sampling period compared to the first.

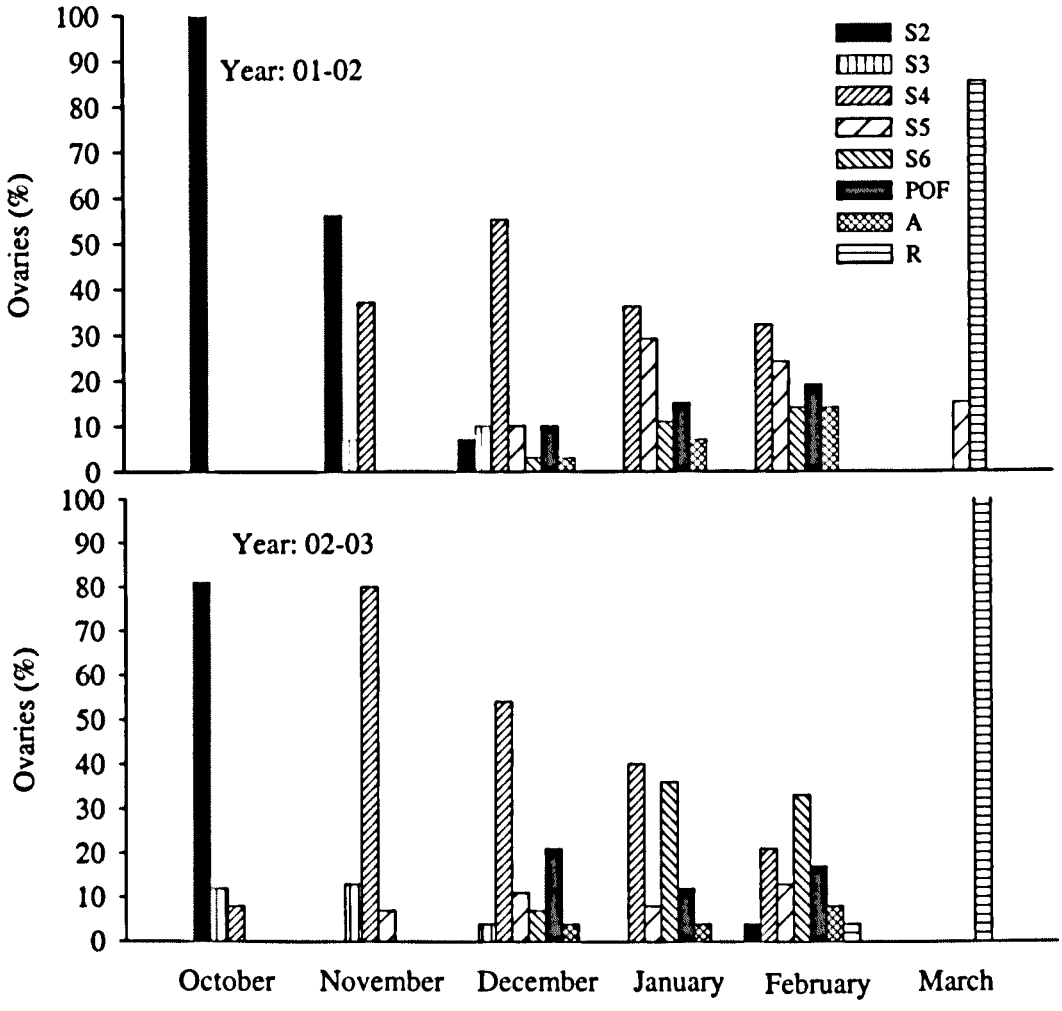


Figure 4.6 Percentage of ovarian stages of development of female *R. sarba* during the reproductive season in the first and second sampling season.

S2 to S6 - stage two to stage six ovaries
POF - postovulatory follicles (evidence of recent spawning).
A - only atretic oocytes were found in the entire ovary (mainly α, β and γ)
R - resting ovary

Table 4.1 Histological characteristics used to classify oocytes in *R. sarba* using haematoxylin and eosin stain.

STAGE		ELL CHARACTERISTICS
S1 Oogonial		Oogonia, small primary oocytes
S2 primary oocytes		Homogeneous cytoplasm within the oocytes. Small, translucent ovarian follicles. Nucleus is circular with numerous nucleoli. Cytoplasm is dark blue.
S3 Cortical alveoli (previtellogenic)		Peripheral cortical alveoli are visible in the oocyte, nucleus and numerous nucleoli.
S4 Vitellogenesis		Pigmented ovarian follicles, number and size of yolk granules and oil droplets is increasing. This stage is divided into (S4I) and S4II. The earlier stage still have a cytoplasm that is stained dark blue.
S5 Prespawning (migratory nucleus)		vitellogenic oocytes, that have a germinal vesicle near the membrane. Evidence of oil droplet fusion prior to germinal vesicle breakdown.
S6 Oocyte maturation		hydrated oocytes with a single oil droplet and yolk plates.
Postovulatory		ovaries contain empty postovulatory follicles of oocytes that contain oogonia and the next stage of small primary oocytes.
Atresia	(α):	Cytoplasm and yolk plates are disorganised along with the breakdown of the nucleus.
	(β)	Follicle cells continue to multiply. Their invasion to the oocyte progress and the oocyte envelop by these cells continues until it breaks down.
	(γ)	Zona radiata disappear, liquefied yolk is resorbed until it disappears completely.
	(δ)	Brown bodies start appearing as a dark brown colour beside a fraction of the remainder of the liquefied yolk and among the postovulatory follicles.

4.3.4.1 Ovary weight and fish length of mature female *R. sarba*

Ovary weight (OW) of *R. sarba* that were classified as mature (S4) tended to increase with fish length (Figure 4.7). The OW and fish length of females displayed a significant correlation ($r=0.687$, $t = 8.392$, d.f. = 79, $p<0.001$).

The relationship could be described by the equation:

$$OW = -18.99 + 0.106\ TL$$

Where:

OW is gonad weight in gram

T.L = total length in mm.

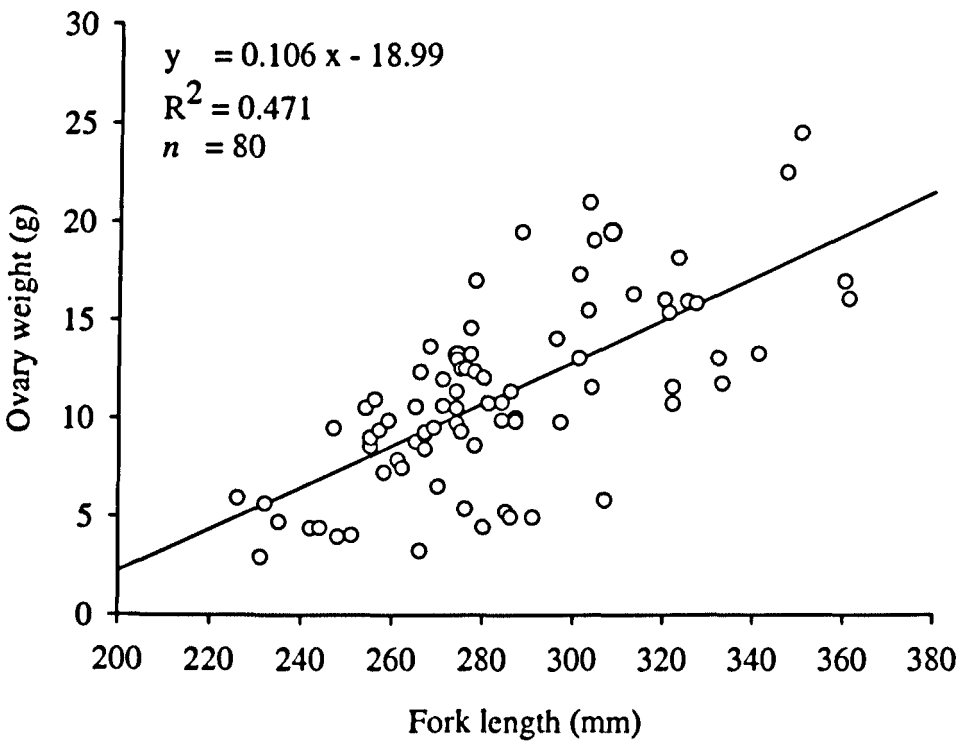


Figure 4.7 Relationship of weight of mature stage four (S4) ovaries to fork length of *R. sarba.*

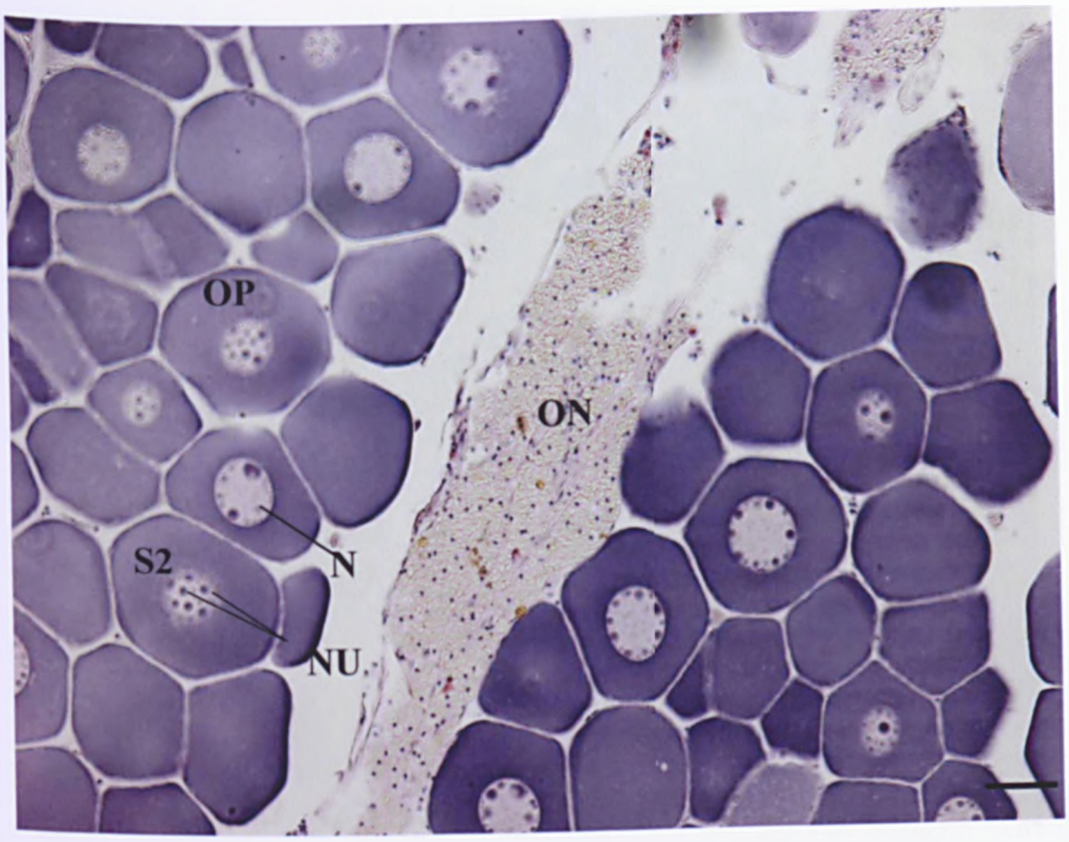


Plate 4.3 Transverse section (3µm thick) of *R. sarba* ovary (633g in weight & six-year-old) sampled in August showing oogonia nest and primary oocytes (S2).

Mag. ×20; scale bar 25µm.

N - nucleus
NU - nucleolus
ON - oogonia nest
OP - ooplasm
S2 - stage two oocyte

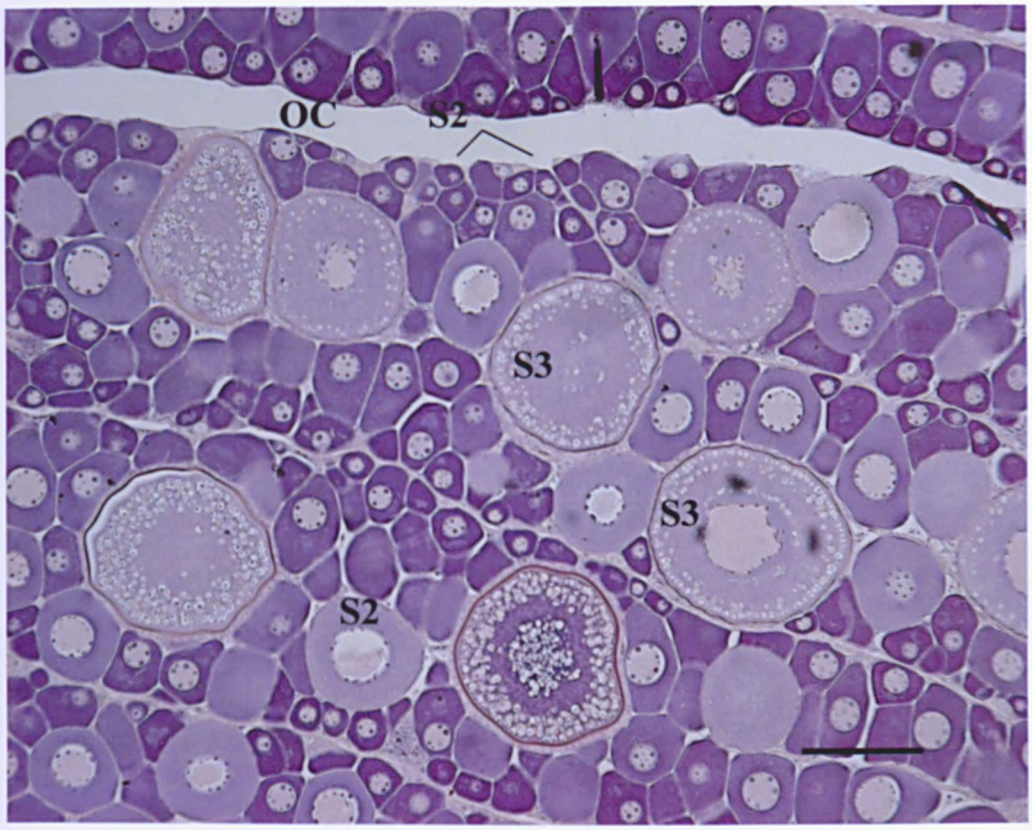


Plate 4.4 Transverse section (3μm thick) of *R. sarba* ovary (246g in weight & one-year-old) sampled in late August showing ovary at stage three.

Mag. ×10; scale bar 100μm.
OC - ovarian cavity
S2 - stage 2 oocyte
S3 - stage 3 oocyte

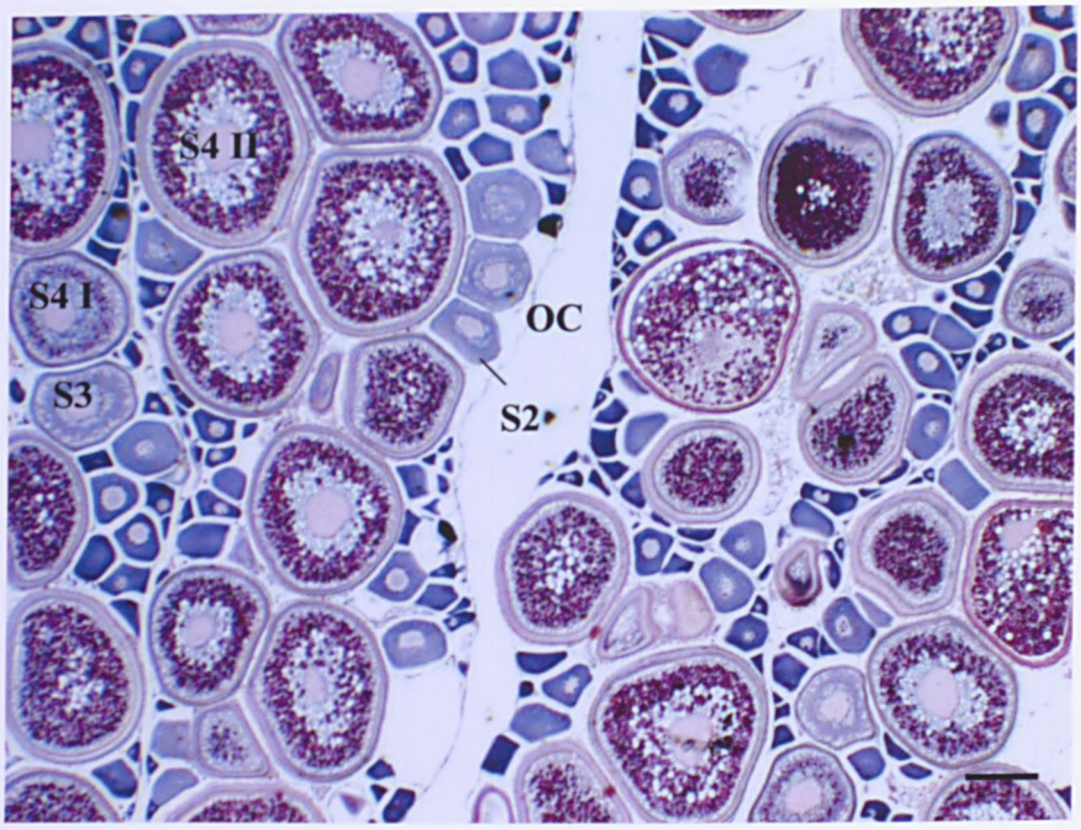


Plate 4.5 Transverse section (3µm thick) of *R. sarba* ovary (733g in weight & four-year-old) sampled in late November showing ovary at stage four (S4).

Mag. ×10; scale bar 100µm.
OC - ovarian cavity
S2 - stage two oocyte
S3 - stage three oocyte
S4 I - early stage four oocyte
S4 II - late stage four oocyte

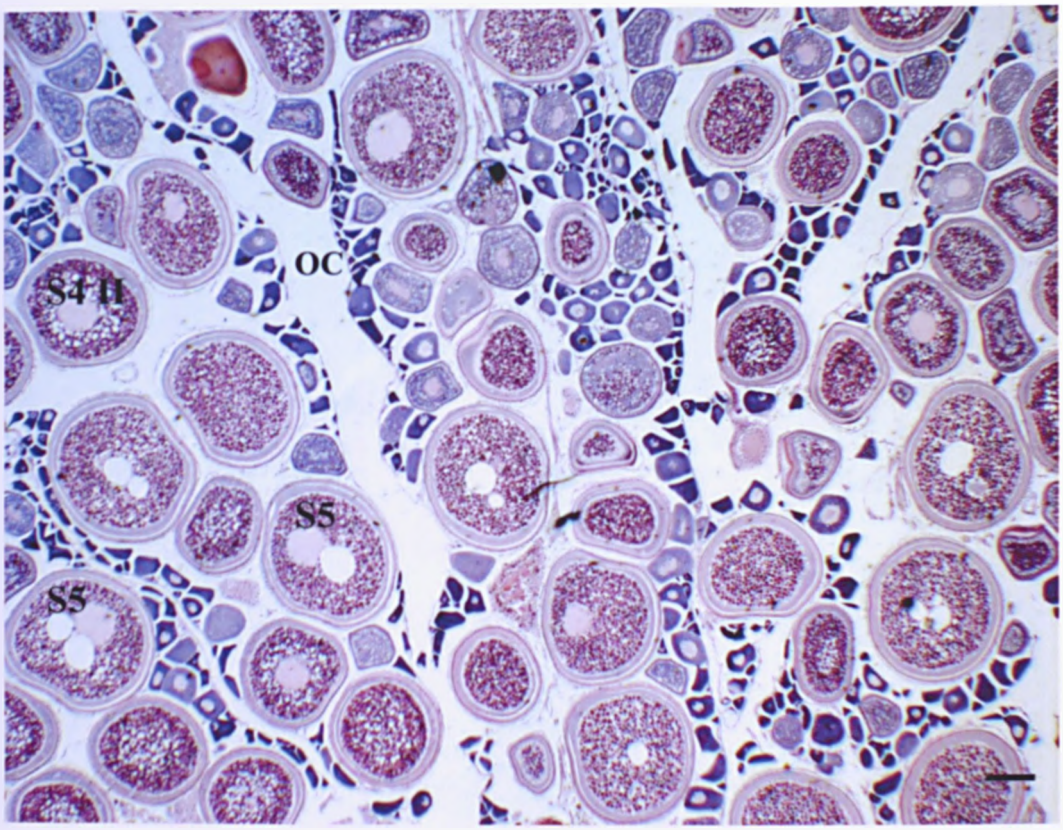


Plate 4.6 Transverse section (3µm thick) of *R. sarba* ovary (573g in weight & five-year-old) sampled in mid February. This ovary is at stage five (S5).

Mag. ×4; scale bar 200µm.
OC - ovarian cavity
S4 II - early stage four oocyte
S5 - stage five oocyte (note the coalescence of lipid droplets to form one single lipid droplet)

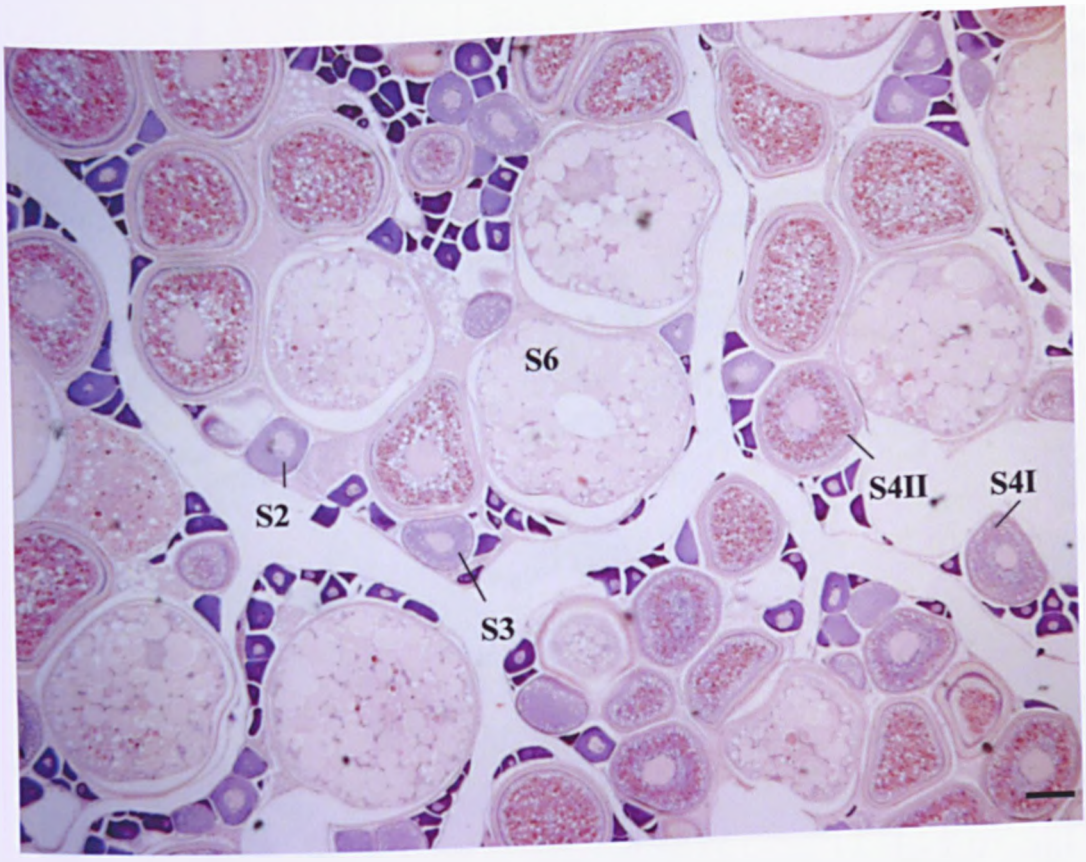


Plate 4.7 Transverse section (3µm thick) of *R. sarba* ovary (414g in weight & three-year-old) sampled in mid January showing ovary at stage six (S6).

Note that during hydration stage of ovary development, stage five oocytes were not present.
Mag. ×4; scale bar 200µm.
S2 - stage two oocyte
S3 - stage three oocyte
S4 I - early stage four oocyte
S4 II - late stage four oocyte
S6 - stage six ovary

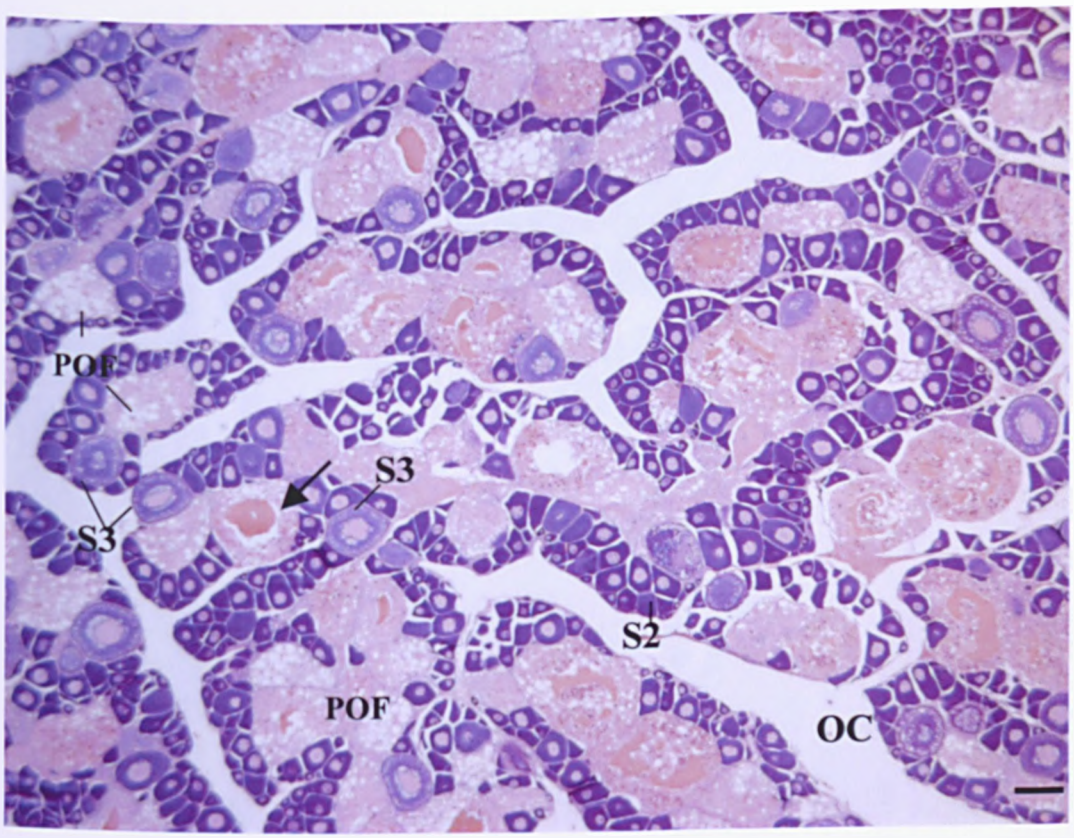


Plate 4.8 Transverse section (3µm thick) of *R. sarba* ovary (417g in weight & three-year-old) sampled early January and showing evidence of recent spawning.

Note that there are no oocytes greater than stage three oocyte.
Mag. ×4; scale bar 200µm.
OC - ovarian cavity
POF - postovulatory follicle envelop
S2 - stage two oocyte
S3 - stage three oocyte
Arrow: indicates yolk materials, the remains of an atretic oocyte

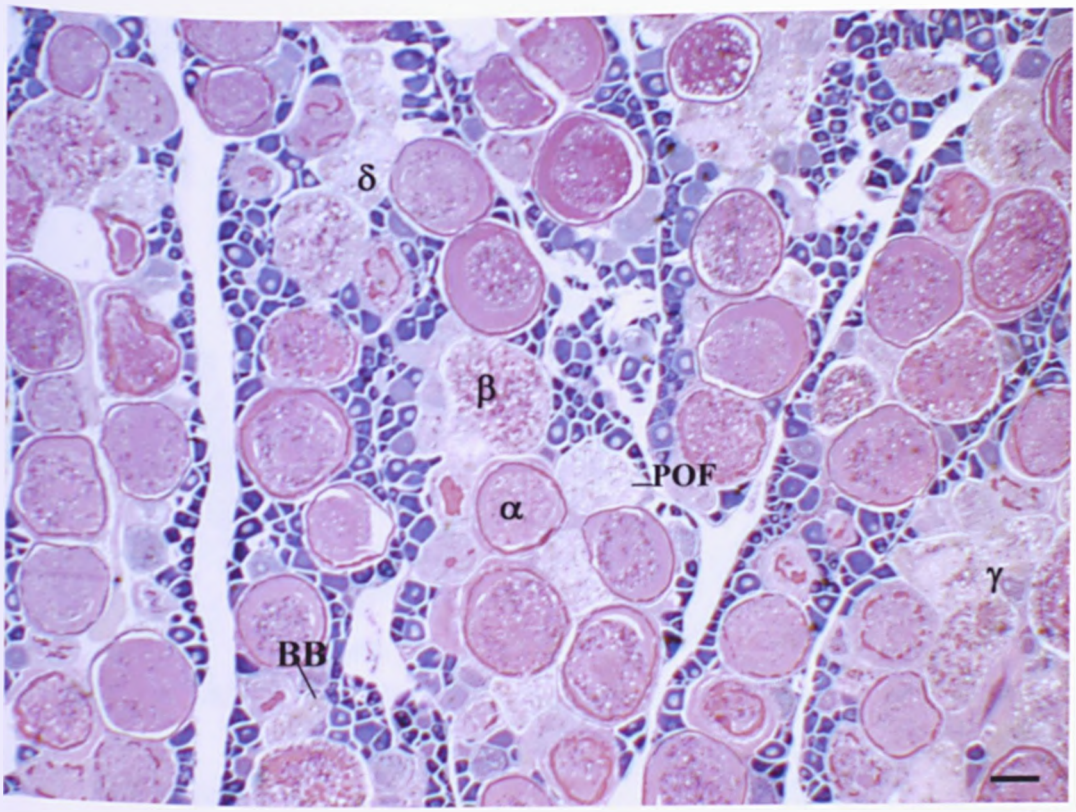


Plate 4.9 Transverse section (3μm thick) of *R. sarba* ovary (548g in weight & seven-year-old) sampled in early March (end of spawning season) showing the ovary with numerous atretic oocytes and postovulatory follicles.

Note that there are no oocytes greater than stage two oocytes. All oocytes that contained yolk materials have disintegrated. This type of ovary was also seen during the active spawning period.
Mag. ×4, scale bar 400μm
α - atretic oocyte stage I
β - atretic oocyte stage II
γ - atretic oocyte stage III
δ - atretic oocyte stage IIII
BB - brown bodies (residual bodies resulting in the final stage of atresia, δ-stage)
POF - postovulatory follicle envelop

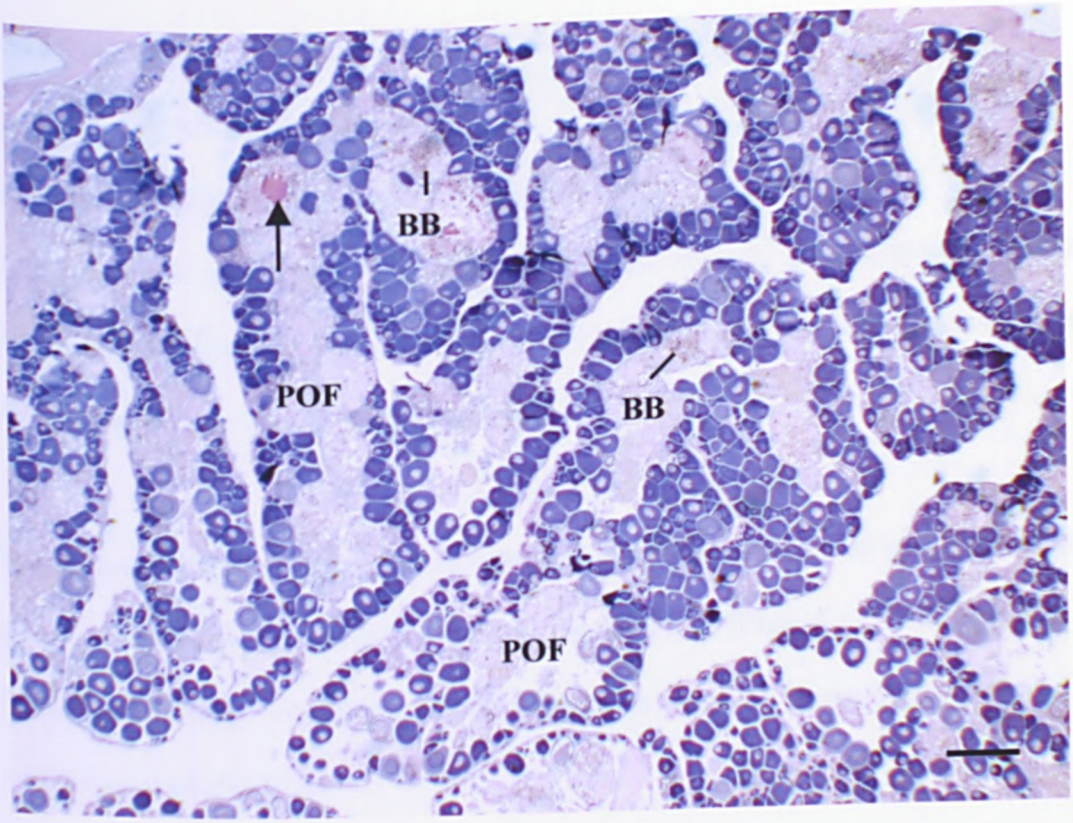


Plate 4.10 Transverse section (3µm thick) of *R. sarba* ovary (677g in weight & nine-year-old) sampled in early March showing the ovary at rest at the end of the spawning season.

Note that there are no oocytes greater than stage two oocytes.
Mag. ×4; scale bar 400µm.
BB - brown bodies (residual bodies resulting in the final stage of atresia, δ-stage)
POF - postovulatory follicle envelop
Arrow: indicates the remains of an Atretic oocyte (liquefied yolk material).

4.3.4.2 Oocyte development in *R. sarba*

4.3.4.2.1 Previtellogenic stage

A) Stage one (chromatin nucleolar stage)

Oogonia were rarely observed and were found only in post spawning ovaries. However, oogonia nests (pools) were found embedded in the germinal tissues and only found in stage two ovaries (Plate 4.3).

Oogonia consisted of a small nucleus stained dark blue surrounded by a slightly thin layer of cytoplasm stained light blue to pink in colour (Plate 4.11). The appearance of POF at the stage of oogonia degeneration was found to be similar to the ovarian connective tissue stroma.

B) Stage two oocyte (primary stage)

These oocytes had homogeneous cytoplasm stained deeply with haematoxylin (Plate 4.12). The oocytes were characterised by the presence of many basophilic nucleoli of various sizes in the peripheral regions of the nucleus. The nucleoli tend to be close to the nucleus membrane during the last developmental stages of stage two oocytes. The diameter of the nucleus was about 50-75% of the total oocyte diameter and the oocytes diameter at this stage ranged between 45μm and around 200μm. The Balbiani bodies appeared as translucent bodies in the cytoplasm (ooplasm) of the oocytes, and were found to increase in size as the oocyte developed (Plate 4.13) and found to disperse later, refer to Plate 4.12.

C) Stage three oocyte (cortical alveoli stage)

The ooplasm of stage three oocytes was not stained uniformly due to the presence of lipid droplets within the ooplasm (Plate 4.14) and the ooplasm stained less basophilically than for stage two. There were two layers of vacuoles formed in stage three oocytes. The layer of vacuoles was found surrounding the nucleus which was located in the central area of the oocyte. The second layer of vacuoles formed a row around the periphery of the ooplasm and are known as cortical alveoli. Thecal and granulosa layers started to appear at this stage forming a follicular layer around the oocyte. The outside envelop of the oocyte 'zona radiata' had started to form but was rather thin compared to those of the following stages of development. A comparison between oocytes envelopes of (S3) and (S4) is given in Plate 4.19. The oocyte diameter at this developmental stage ranged between 168 and 300μm.

4.3.4.2.2 Vitellogenic stage

A) Stage four oocyte (yolk stage)

There were two distinguishable levels of development in stage four that were S4I and S4II. In stage S4I the ooplasm in the oocyte was still visible (Plate 4.15). Small yolk granules appeared in the periphery of the oocyte which marked the beginning of vitellogensis. Numerous vacuoles were found throughout the ooplasm and in S4 II the ooplasm had disappeared completely (Plate 4.16). Large numbers of vacuoles were found surrounding the nucleus which was situated at the central region of the oocyte. Yolk granules clustered together to form a layer surrounding the vacuoles around the periphery of the oocyte while small more dense vacuoles were found at the periphery of the oocyte. More defined zones were apparent in the structure of the follicular layer, zona externa (ZE) and zona interna (ZI) were more developed

than stage three oocyte and the development of the zona radiata was more advanced (Plate 4.19). Oocyte diameter at this developmental stage ranged between 312 and 587μm.

B) Stage five oocyte (migratory nucleus stage)

Stage five oocytes (S5) were characterised by germinal vesicle migration (Plate 4.17). During this stage the nucleus progressively migrates towards the animal pole where the micropyle was located. This was followed by the coalescence of the oil droplets (the small vacuoles) which formed a single lipid globule as oocyte development progressed (Plate 4.6). Other cytoplasm changes also took place, such as the fusion of yolk globules. Those globules were found distributed throughout the ooplasm towards the end of this developmental stage. The oocyte diameter at this stage ranged between 512 and 706μm.

4.3.4.2.3 Hydration stage

A) Stage six oocyte (Mature stage)

Oocytes at this stage were hydrated (Plate 4.18). Zona radiata appeared to be thinner compared with those of stage four and had clearer radial striations (Plate 4.20) and at this stage thecal cells were visible. Granulosa was still seen but was less apparent compared to earlier stages. Generally, the intercellular spaces that were formed by the follicular layer in the previous stage of development were lost here and yolk globules had fused to form yolk plates which were then distributed throughout the cytoplasm. The oocytes were found to contain one lipid plate. The oocyte diameter at this stage of development ranged from 630 to 980μm.

4.3.4.2.4 Atretic oocyte (α, β , γ & δ)

Atresia was initiated by the disorientation of the nucleus and the oocytes surrounding layers (Plate 4.21). This was followed by the disorganisation of the cytoplasm and the yolk plates and the breakdown of the nucleus as it released its content into the cytoplasm (Plate 4.22). As the zona radiata (ZR) continue to break up, the follicle granulosa cells underwent hypertrophy, increased and invaded the cytoplasm of what was formally known as the oocyte (Plate 4.23 & Plate 4.24). This process allowed the liquefied yolk to escape outside the oocyte through ZR as it broke and disappeared completely. The oocyte yolk content was resorbed and finally what is known as 'Brown bodies' started appearing as a dark brown stain beside a fraction of the reminder of the liquefied yolk (Plate 4.9). The diameter of the atretic oocytes at this stage ranged between 437 to 710μm.

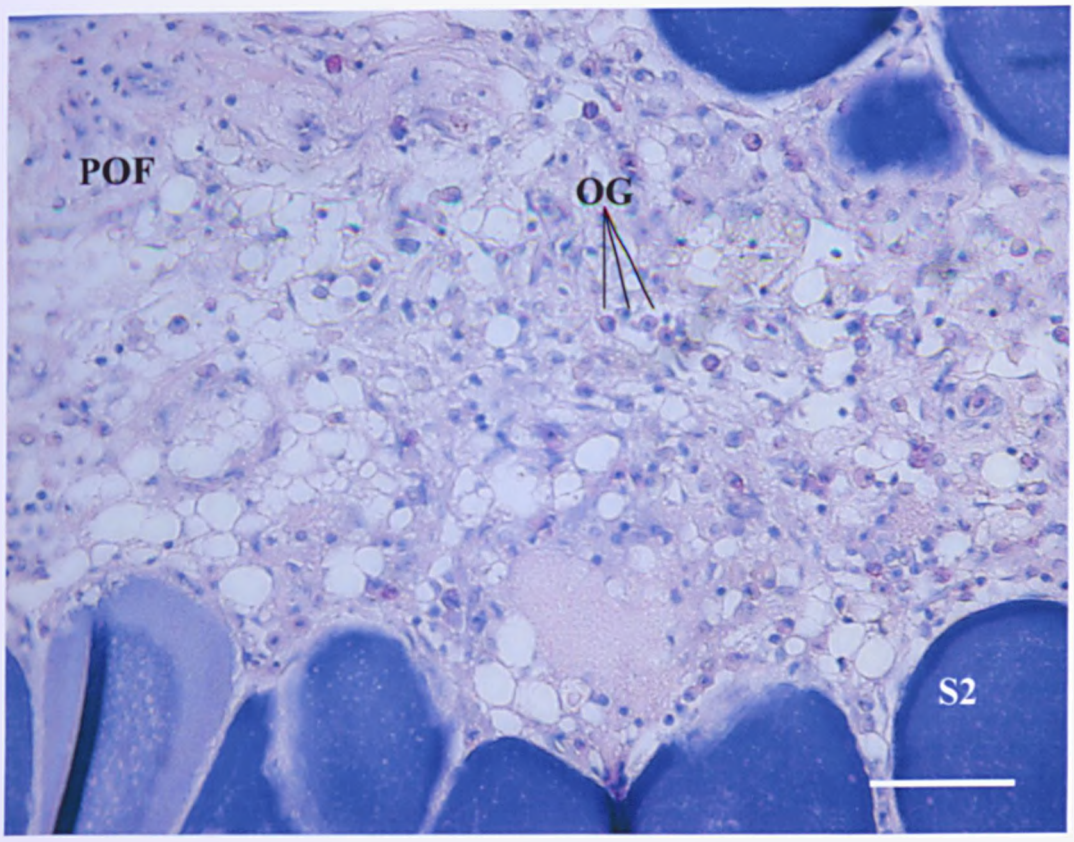


Plate 4.11 Transverse section (3µm thick) of *R. sarba* ovary (540g in weight & three-year-old) sampled in early January showing postovulatory follicles (POF) containing oogonia.

Mag. ×40; scale bar 100µm.
OG - oogonia
POF - postovulatory follicle envelop
S2 - stage two oocyte

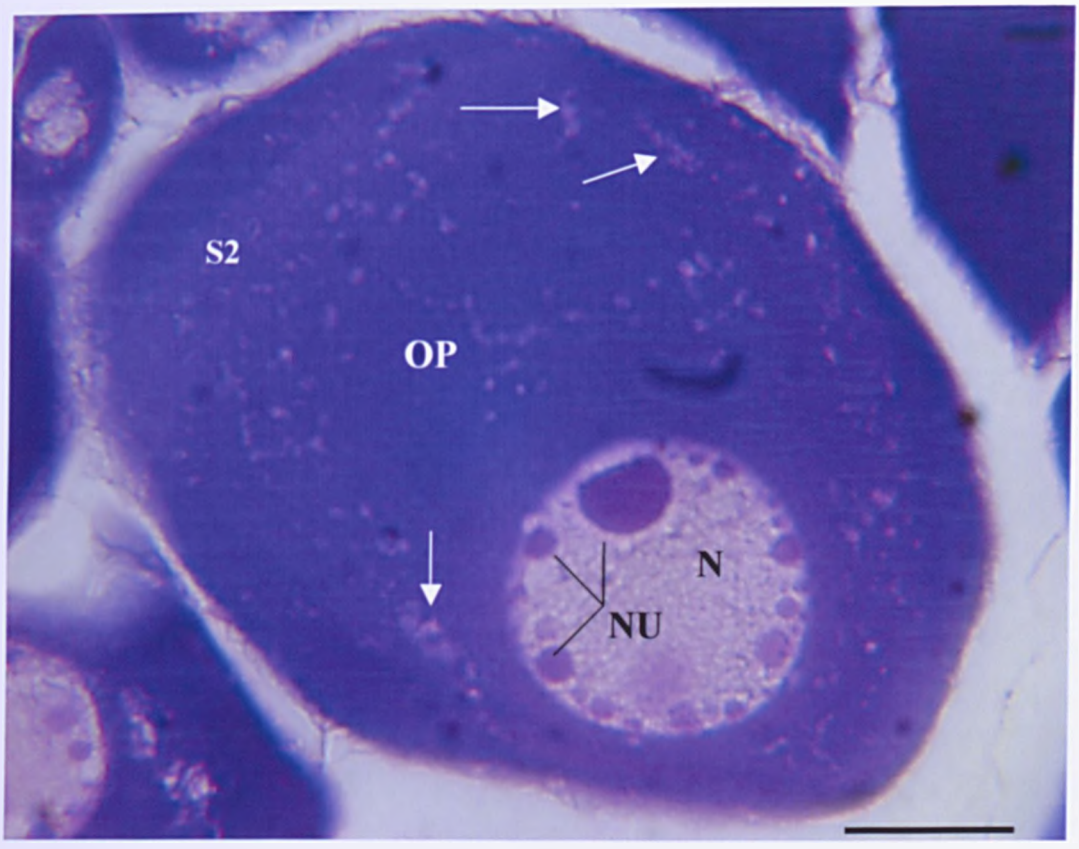


Plate 4.12 Transverse section (3µm thick) of *R. sarba* ovary (495g in weight & one-year-old) sampled in early November showing primary oocyte (S2).

Mag. ×100; scale bar 20µm.
N - nucleus
NU - nucleolus
OP - ooplasm
S2 - stage two oocyte
Arrows: indicate the Balbiani's body.

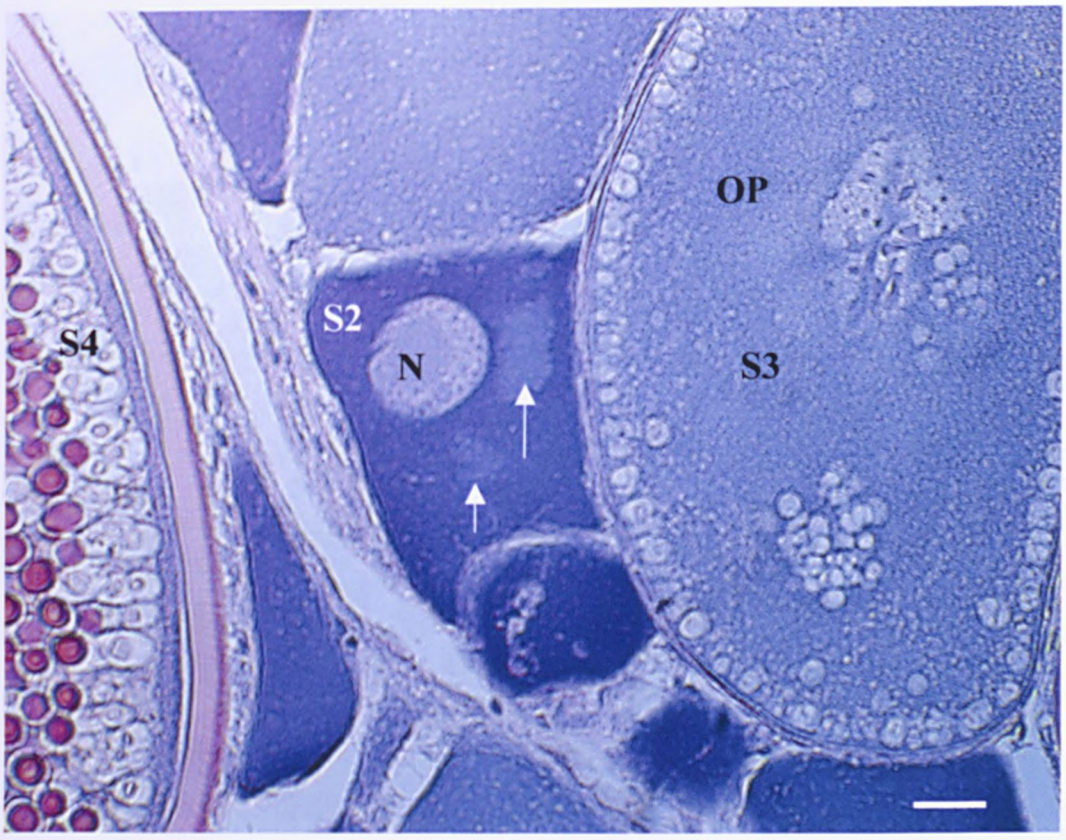


Plate 4.13 Transverse section (3µm thick) of *R. sarba* ovary (495g in weight & one-year-old) sampled in early November showing primary oocyte (S2) containing Balbiani's bodies.

Mag. ×40; scale bar 40µm.
N - nucleus
OP - ooplasm
S2 - stage two oocyte
S4 - stage four oocyte
Arrows: indicate the Balbiani's body.

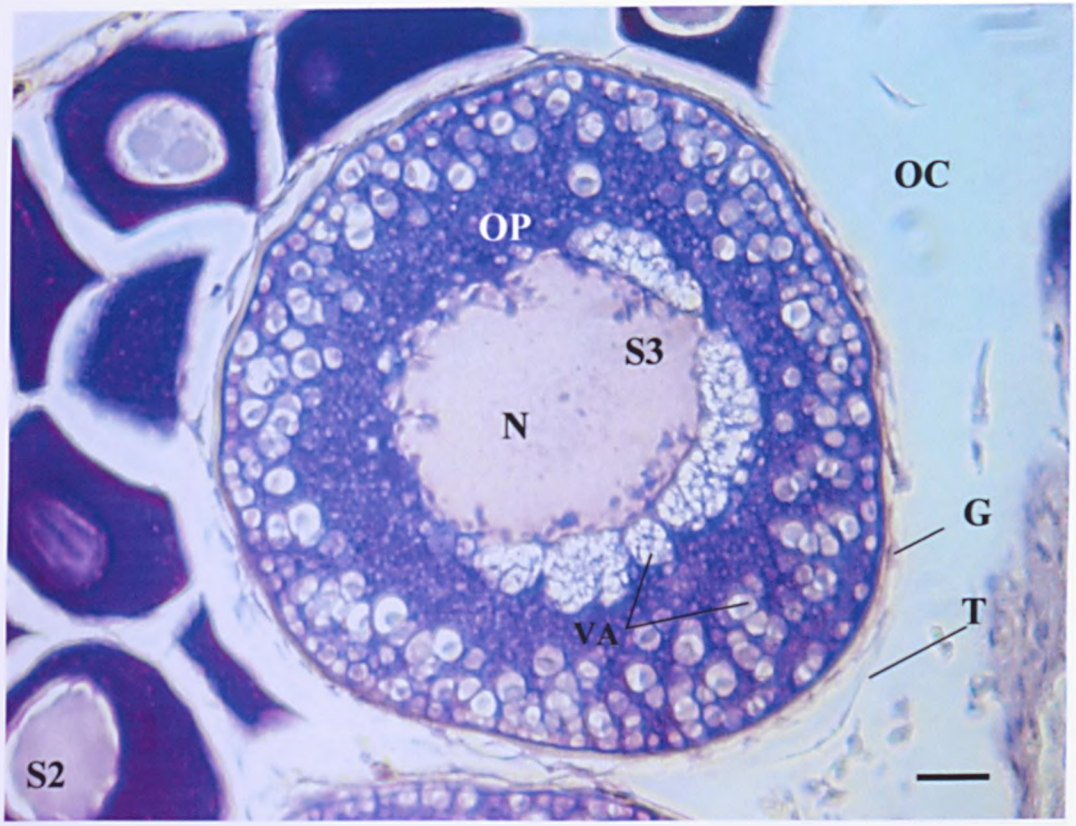


Plate 4.14 Transverse section (3µm thick) of *R. sarba* ovary (293g in weight & one-year-old) sampled in early February showing stage three oocyte (S3).

Mag. ×40; scale bar 50µm.
G - granulosa
N - nucleus
OP - ooplasm
OC - ovarian cavity
S2 - stage two oocyte
S3 - stage three oocyte
T - thecal layer
VA - vacuoles (lipid)

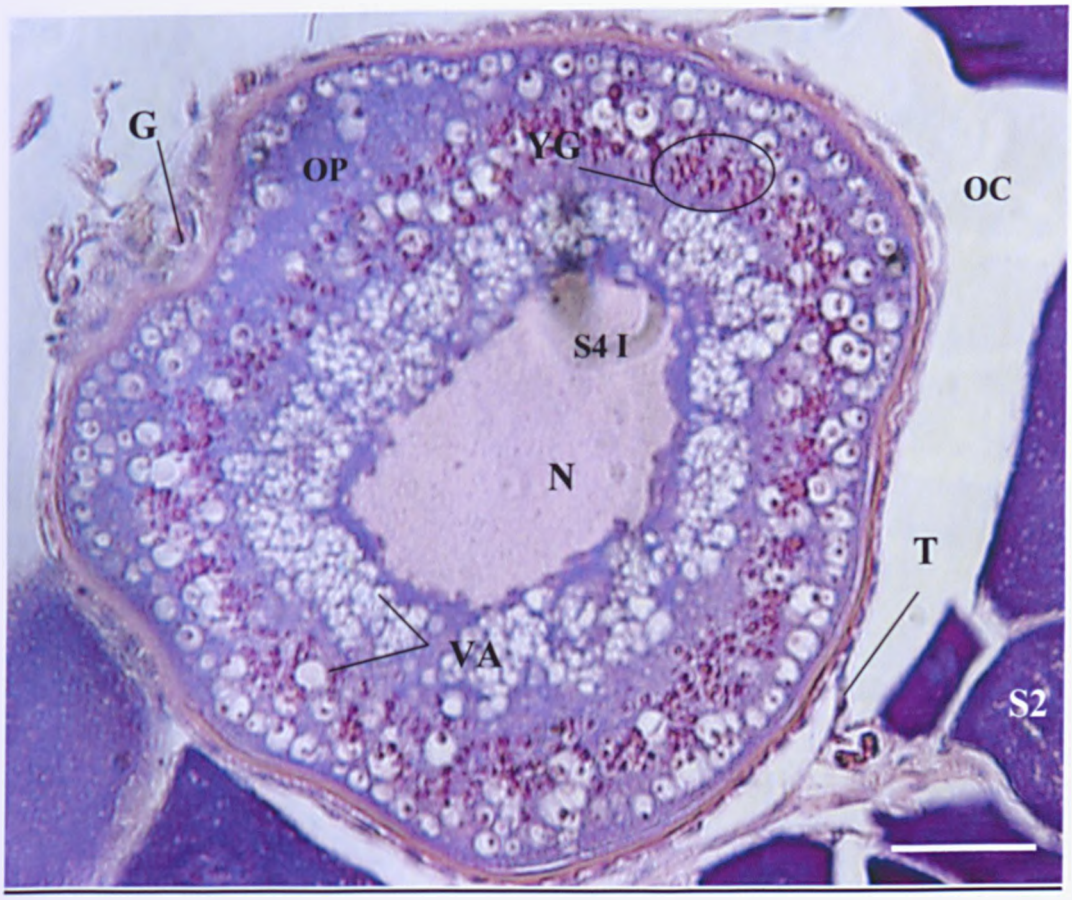


Plate 4.15 Transverse section (3µm thick) of *R. sarba* ovary (310g in weight & three-year-old) sampled in late November showing early stage four oocyte (S4I).

Mag. ×40, scale bar 50µm.
G - granulosa layer
N - nucleus
OC - ovarian cavity
OP - ooplasm
S2 - stage two oocyte
S4 I - early stage four oocyte
T - thecal layer
VA - vacuoles (lipid)
YG - yolk globules

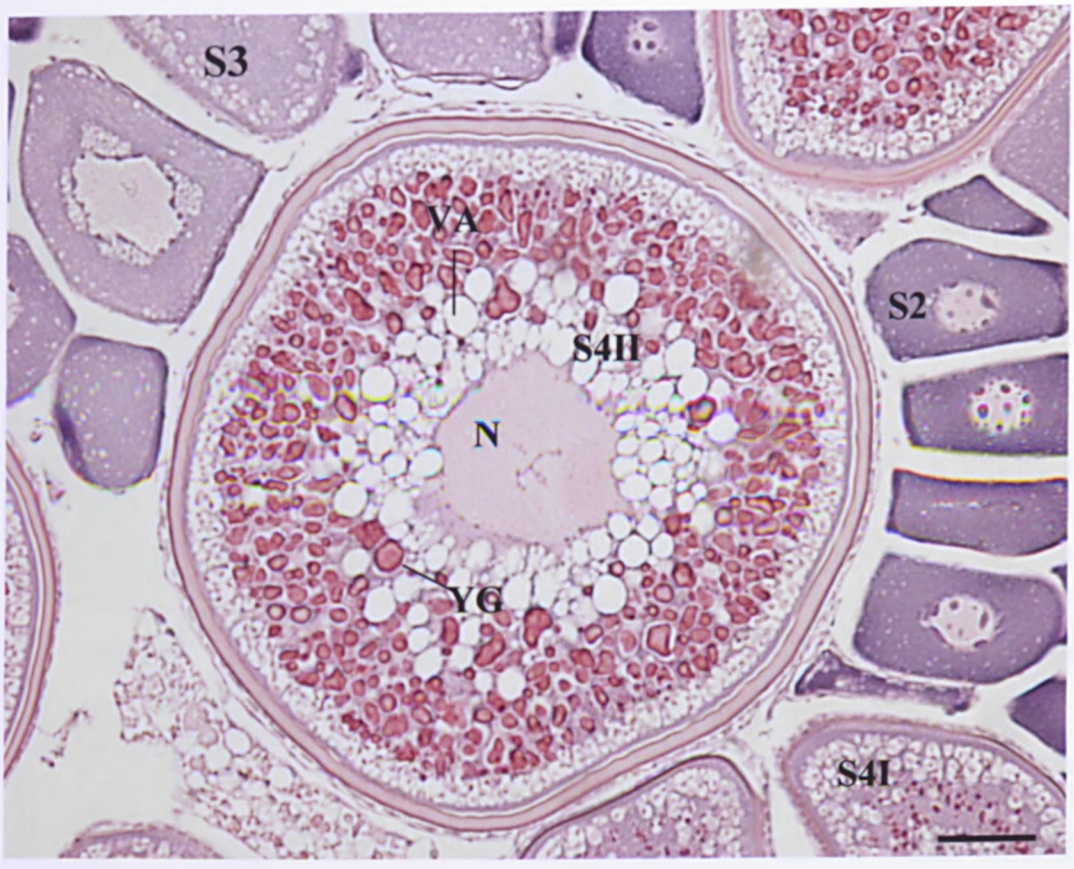


Plate 4.16 Transverse section (3µm thick) of *R. sarba* ovary (733g in weight & four-year-old) sampled in late November showing stage four oocyte (S4).

Mag. ×20; scale bar 50µm.
N - nucleus
S2 - stage two oocyte
S3 - stage three oocyte
S4 I - early stage four oocyte
S4 II - late stage four oocyte
YG - yolk globules
VA - vacuoles (lipid)

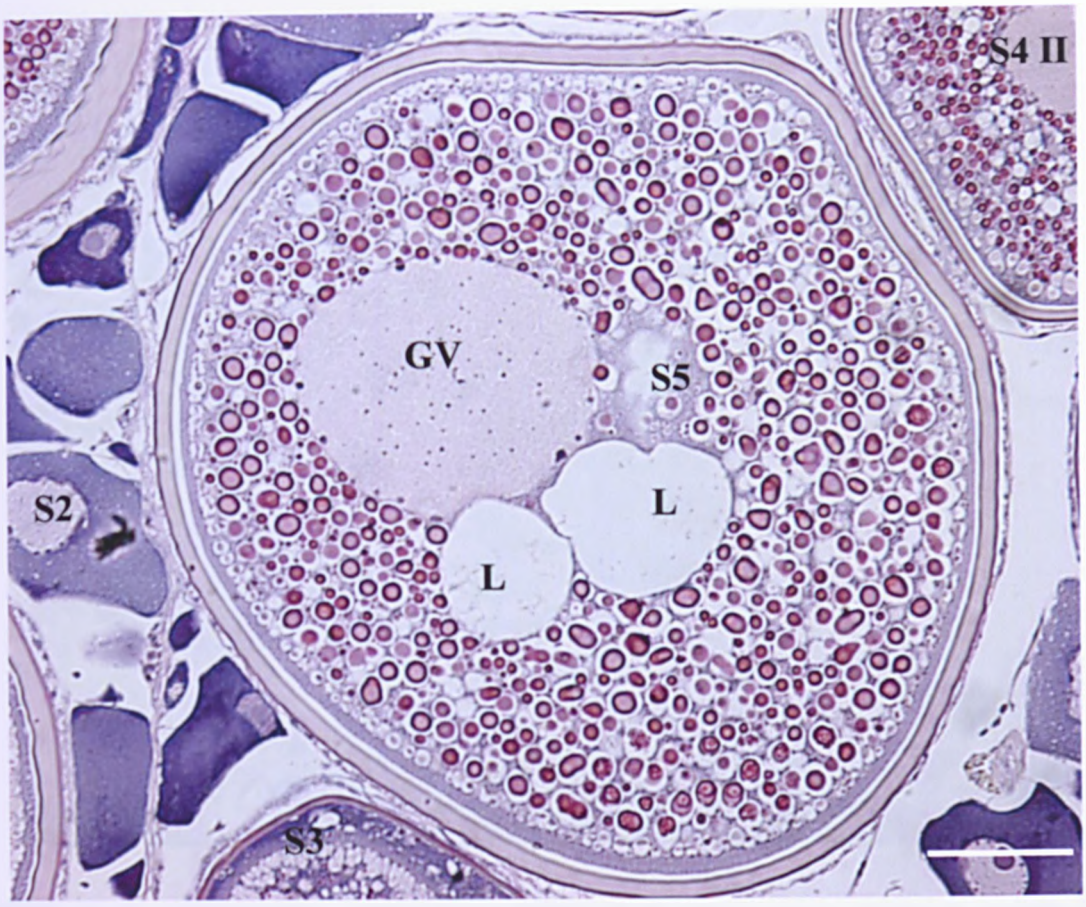


Plate 4.17 Transverse section (3µm thick) of *R. sarba* ovary (573g in weight & five-year-old) sampled in early February showing the stage five oocyte (S5).

Mag. ×10; scale bar 100µm.
GV - germinal vescicle
L - lipid globule
S2 - stage two oocyte
S3 - stage three oocyte
S4 II - late stage four oocyte
S5 - stage five oocyte

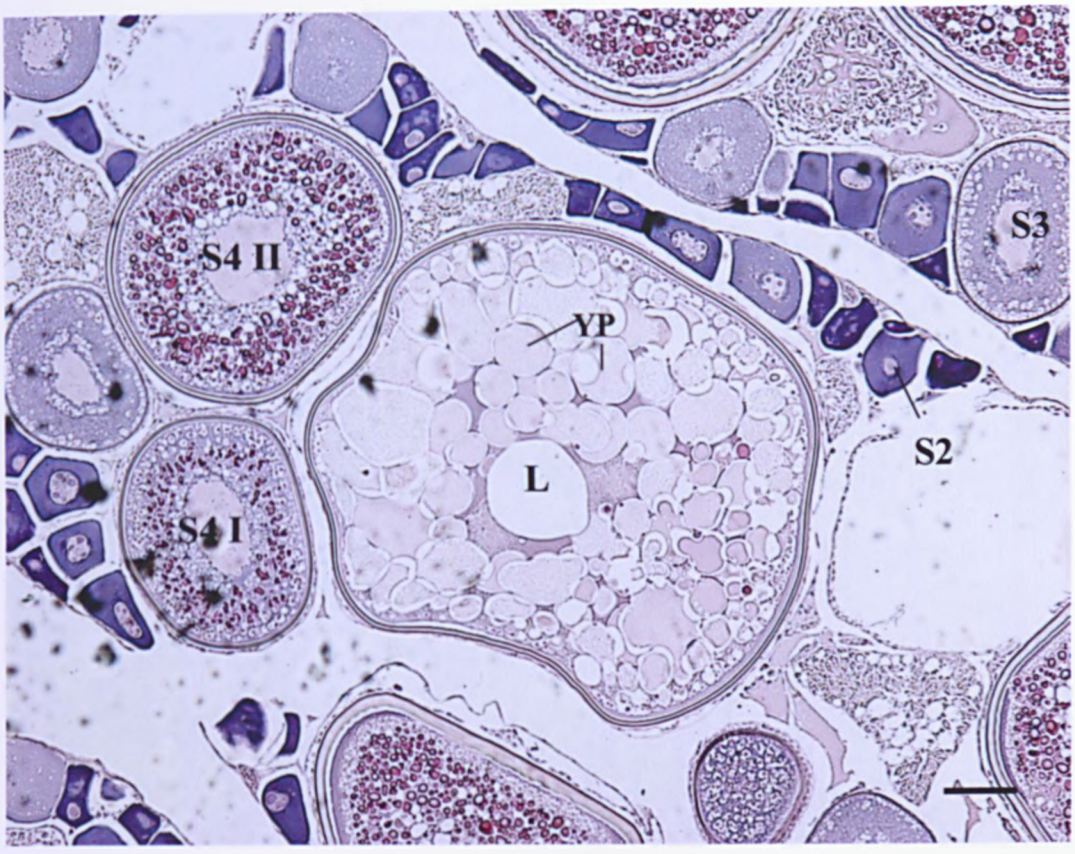


Plate 4.18 Transverse section (3µm thick) of *R. sarba* ovary (414g in weight & three-year-old) sampled in early February showing the stage six oocyte (S6).

Mag. ×10; scale bar 50µm.
L - lipid globule
S2 - stage two oocyte
S3 - stage three oocyte
S4 I - early stage four oocyte
S4 II - late stage four oocyte
S6 - stage six oocyte
YP - yolk plates

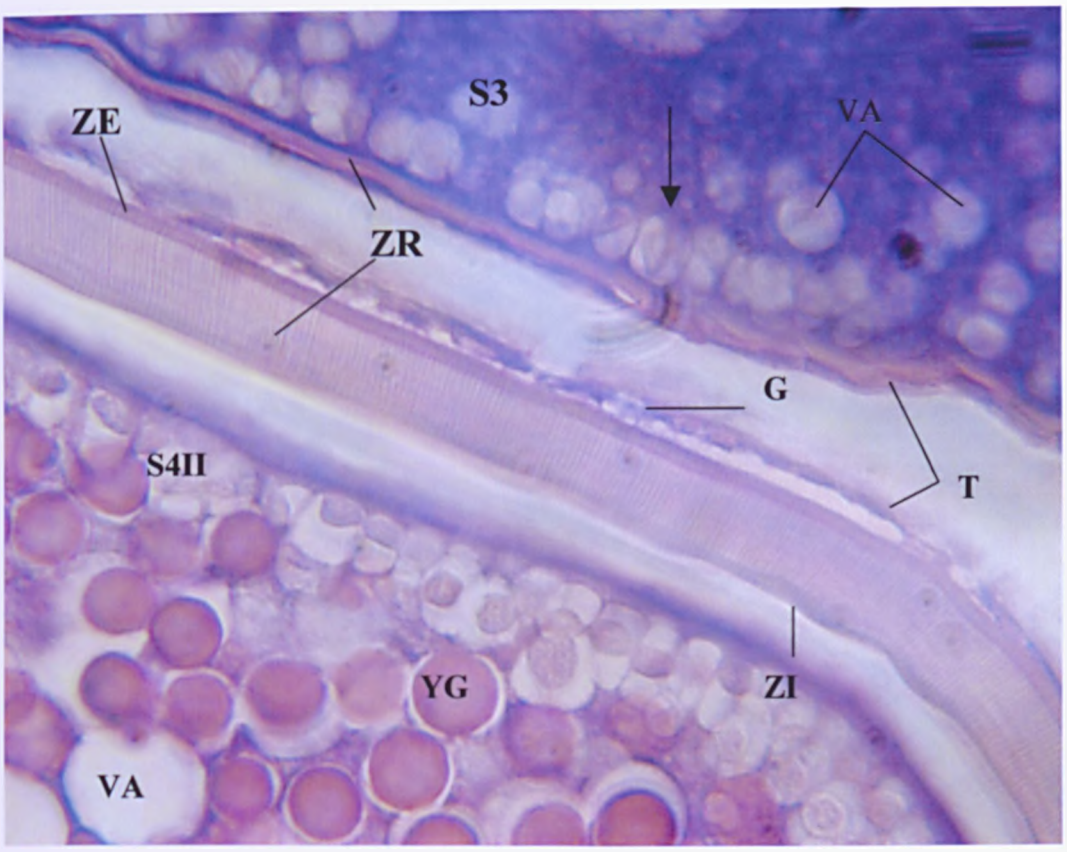


Plate 4.19 Transverse section (3µm thick) of *R. sarba* ovary (293g in weight & one-year-old) sampled in early February showing the stage six oocyte (S6).

Mag. ×10.
G - granulosa layer
S3 - stage three oocyte
S4 II - late stage four oocyte
T - thecal layer
VA - vacuoles (lipid)
YG - yolk globules
ZR - zona radiata
ZI - zona radiata interna
ZE - zona radiata externa
Arrow: indicates the cortical alveoli

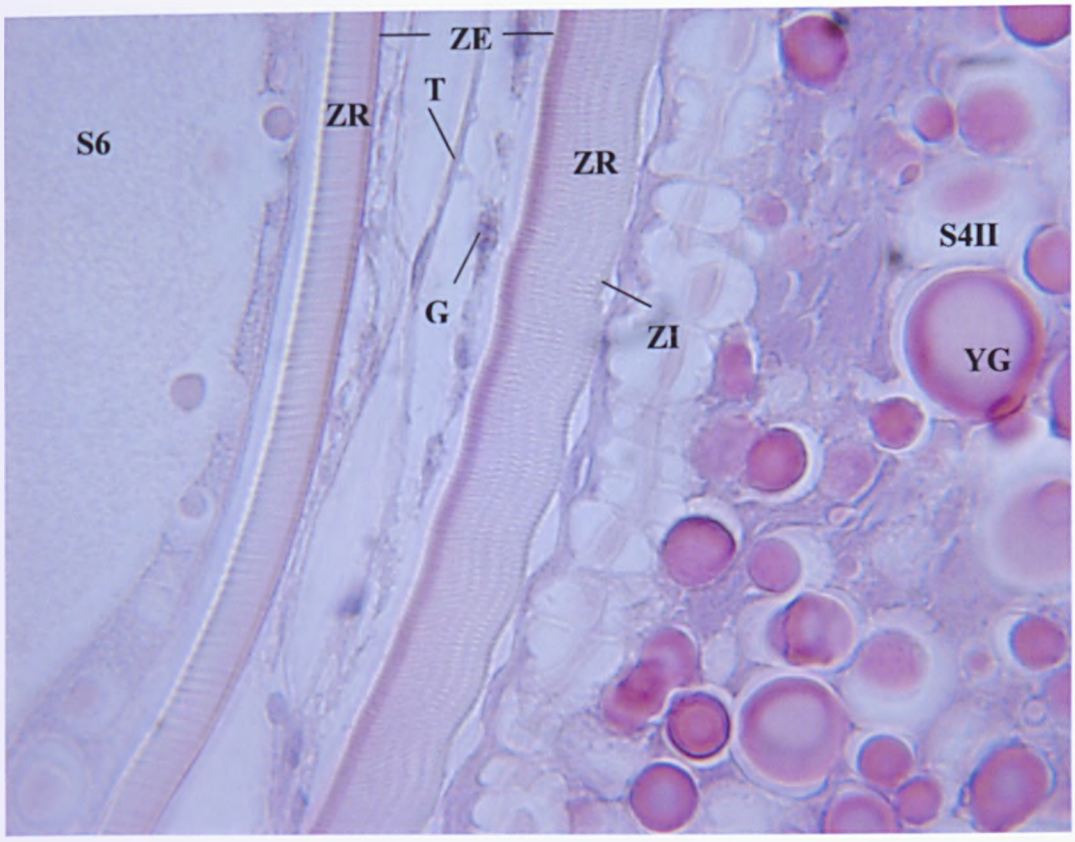


Plate 4.20 Transverse section (3µm thick) of *R. sarba* ovary (414g in weight & three-year-old) sampled in early February showing the fine structure of the follicular layer of stage four (S4) and stage six (S6) oocyte.

Mag. ×100.
G - granulosa layer
S4 II - late stage four oocyte
S6 - stage six oocyte
T - thecal layer
YG - yolk globules
ZR - zona radiata
ZI - zona radiata interna
ZE - zona radiata externa

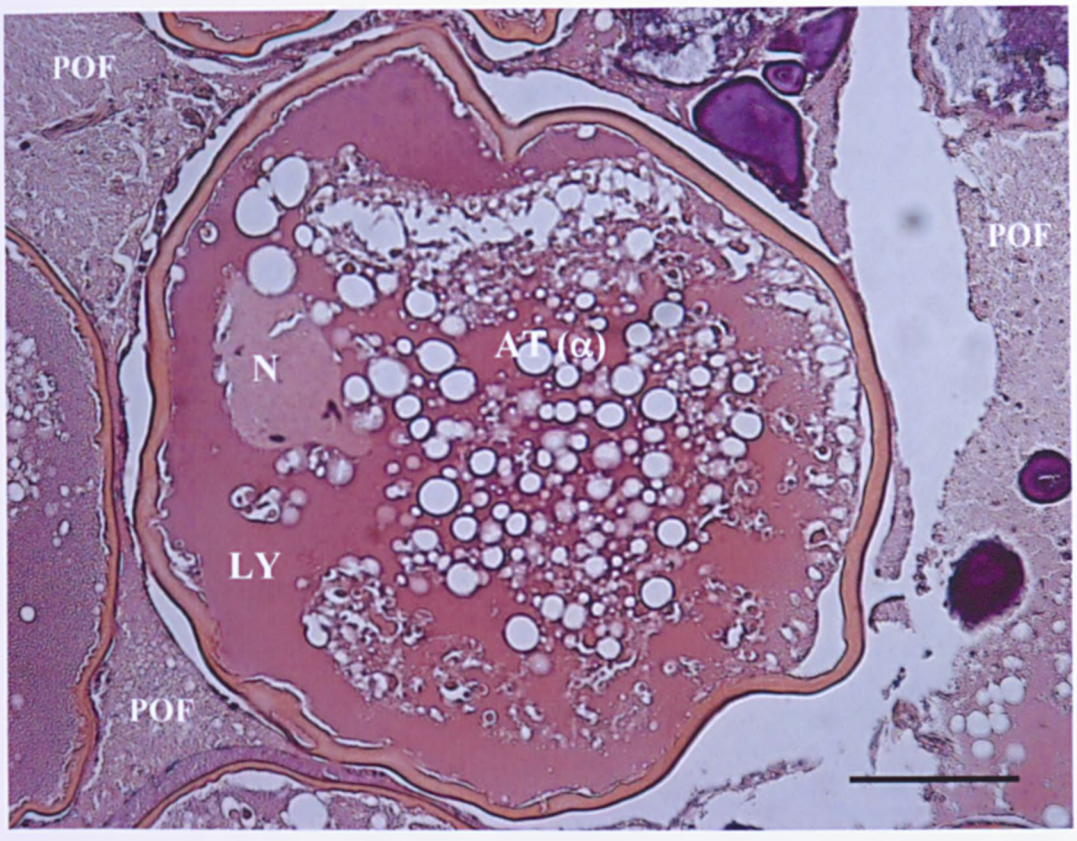


Plate 4.21 Transverse section (3µm thick) of *R. sarba* ovary (342g in weight & two-year-old) sampled in early January showing α atretic oocyte.

Mag. ×20, scale bar 100µm.
AT (α) - atretic oocyte α stage
LY - liquefied
N - nucleus
POF - postovulatory follicle layer

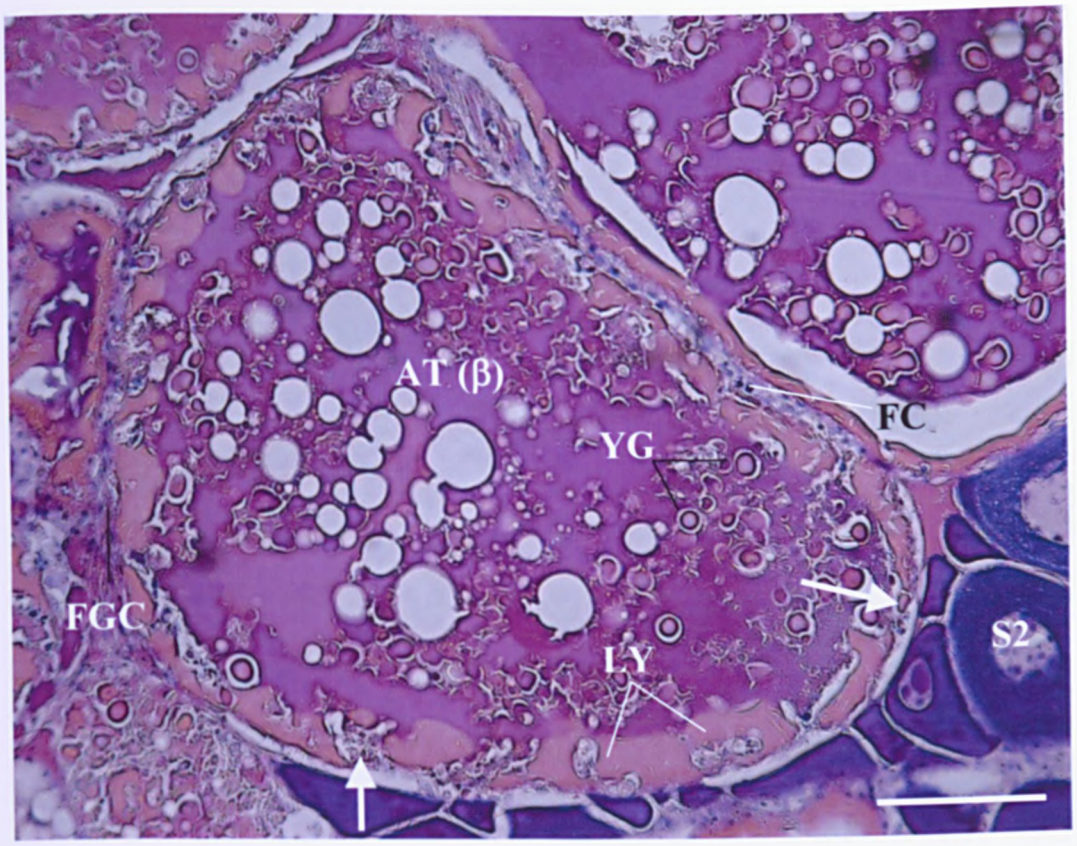


Plate 4.22 Transverse section (3µm thick) of *R. sarba* ovary (246g in weight & one-year-old) sampled in early January showing α atretic oocyte. Note that the nucleus has disappeared completely.

Mag. ×20, scale bar 50µm.
AT (β) - atretic oocyte β stage
FGC - follicle granulosa cells
LY - liquefied yolk
N - nucleus
S2 - stage two oocyte
YG - yolk globule
Arrows: indicate the fragmentation of the ZR and invasion of follicle granulosa cells.

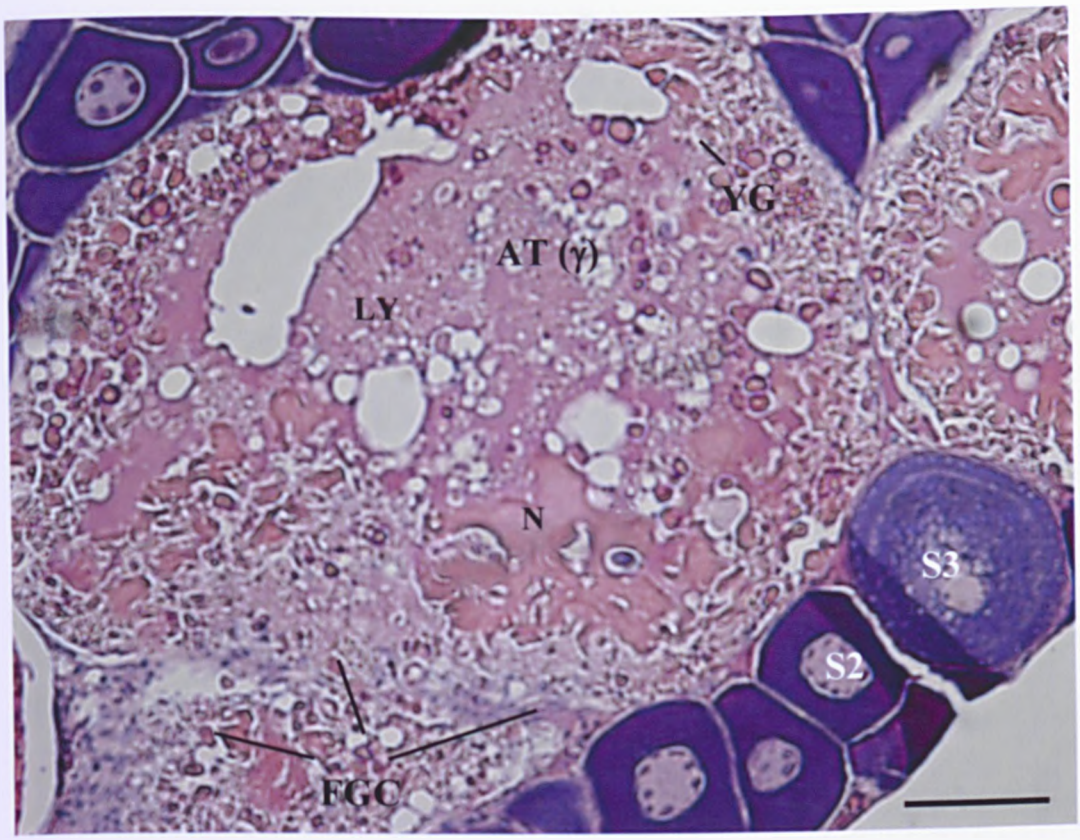


Plate 4.23 Transverse section (3µm thick) of *R. sarba* ovary (355g in weight & four-year-old) sampled in late January showing γ atretic oocyte. The yolk material is being released and resorbed. Follicle cells further invade the cytoplasm of the oocyte.

Mag. ×20, scale bar 50µm.
AT (γ) - atretic oocyte γ stage
FGC - follicle granulosa cells
LY - liquefied yolk
N - nucleus
S2 - stage two oocyte
S3 - stage three oocyte
YG - yolk globule

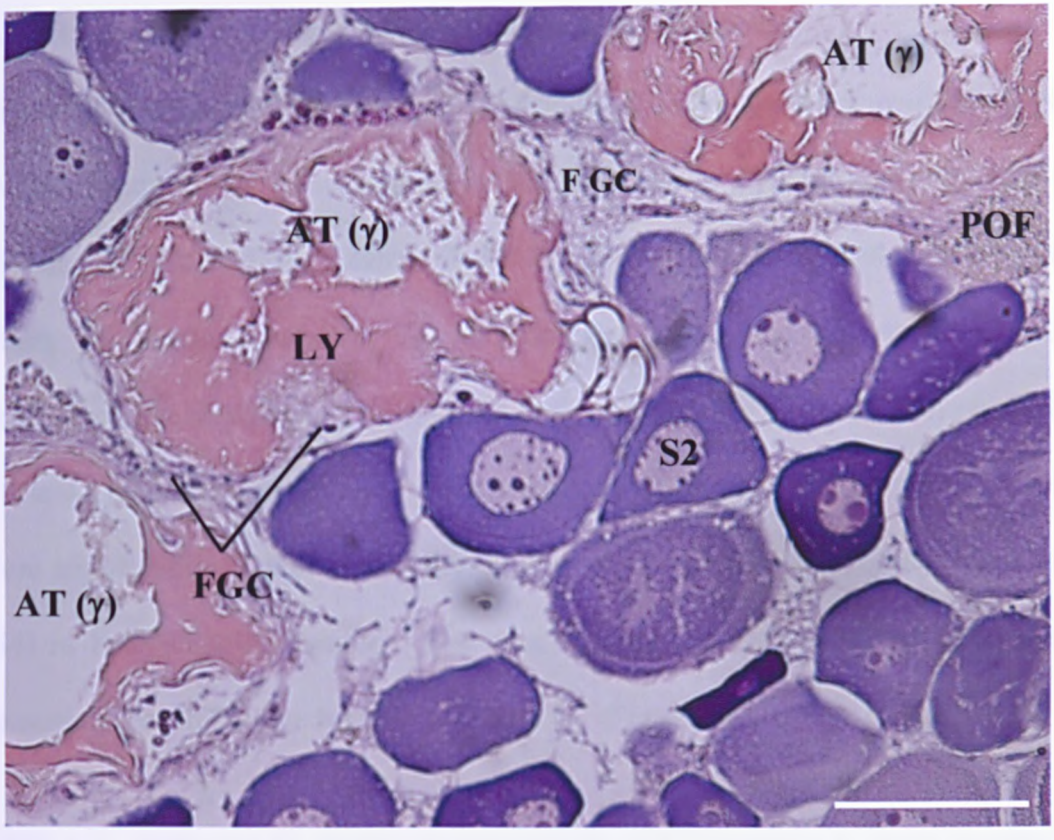


Plate 4.24 Transverse section (3µm thick) of *R. sarba* ovary (355g in weight & four-year-old) sampled in late January showing γ atretic oocyte as it continues to be resorbed.

Mag. ×20, scale bar 50µm.
AT (γ) - atretic oocyte γ stage
FGC - follicle granulosa cell
LY - liquefied yolk
POF - postovulatory follicles
S2 - stage two oocyte

4.3.4.3 Plasma calcium (Ca^{2+}) level of the female *R. sarba*

Levels of plasma calcium (Ca^{2+}) of the female *R. sarba* increased gradually as the ovaries matured. In fish sampled in the first season (01-02) concentrations of plasma Ca^{2+} differed significantly ($F = 2.93$; $df = 6$; $p = 0.01$; Table 4.2) between the stages of ovarian maturity. In fish sampled in the second season (02-03) concentrations of plasma Ca^{2+} differed significantly ($F = 2.79$; $df = 6$; $p = 0.015$; Table 4.2) between the stages of ovarian maturity. It was evident that the Ca^{2+} levels were significantly higher at the beginning of the exogenous vitellogenic stage four (S4) in both sampling seasons. Fish that were at resting stage at the end of the spawning season had the lowest levels of Ca^{2+} (Figure 4.8).

Table 4.2 Mean (±S.E) of total plasma calcium (Ca^{2+}) levels in *R. sarba* females at various stages of maturity.

Ovary stage	Total calcium (mg%) Year (01-02)	Total calcium (mg%) Year (02-03)
Stage two	1.79 ± 0.10^{a} (n = 40)	1.87 ± 0.10^{a} (n = 15)
Stage three	1.63 ± 0.15^{a} (n = 15)	1.80 ± 0.09^{a} (n = 8)
Stage four	2.08 ± 0.11^{b} (n = 31)	2.18 ± 0.27^{b} (43)
Stage five	1.8 ± 0.10^{a} (n = 6)	1.81 ± 0.13^{a} (n = 8)
Stage six	1.88 ± 0.16^{a} (n = 8)	1.83 ± 0.07^{a} (n = 18)
POF	1.84 ± 0.13^{a} (n = 10)	1.82 ± 0.10^{a} (n = 11)
Resting	1.53 ± 0.04^{a} (n = 27)	1.48 ± 0.05^{a} (n = 8)

Mean values that are not significantly different within column ($p > 0.05$) share common superscripts.

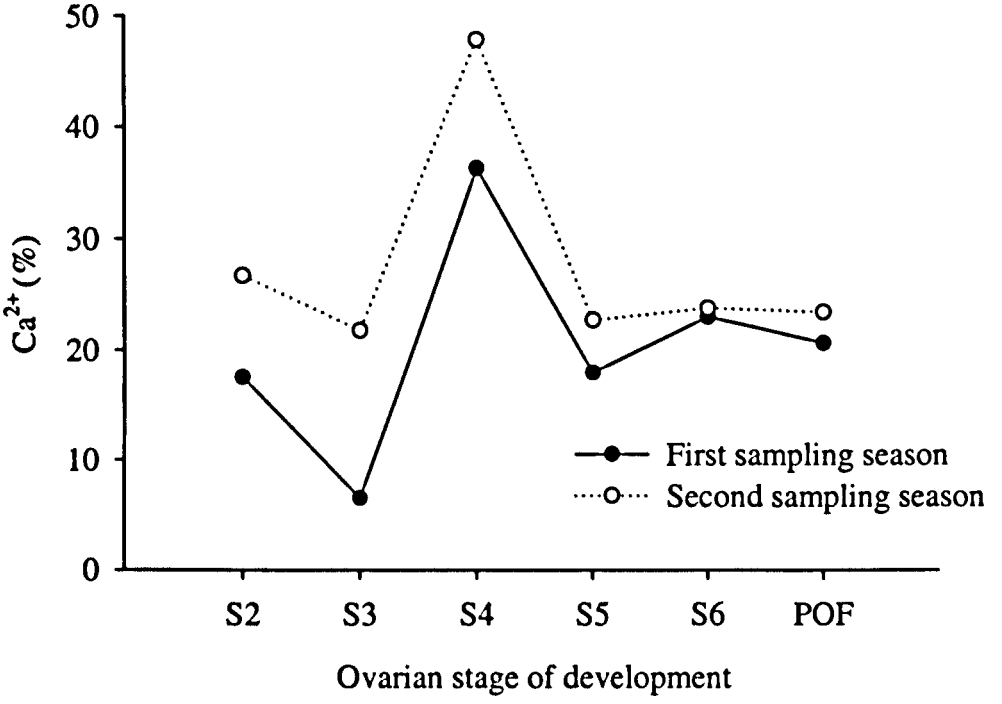


Figure 4.8 Illustrates the changes in plasma Ca^{2+} levels between the ovarian developmental stages.

The Ca^{2+} levels of resting females were used as a base level (100%) and the levels of Ca^{2+} at each ovarian stage were compared with this level. The calculation was performed as follows:

$$\frac{Ca^{2+} \text{ in distinct ovary stage} - Ca^{2+} \text{ in resting females}}{Ca^{2+} \text{ in resting females}} \times 100$$

4.3.4.4 Ovarian follicle measurement

Mean oocyte shape (β) in relation to diameter at various *R. sarba* developmental stages is shown in Figure 4.9. Ovarian oocyte mean diameters, shapes and other associated coefficients are shown in Table 4.3. The coefficients suggest that as the oocytes progressed in their development they became more spherical and the (β) value approached 1. However, stage two (S2) and atretic (α) oocytes tended to have an ellipsoid nature.

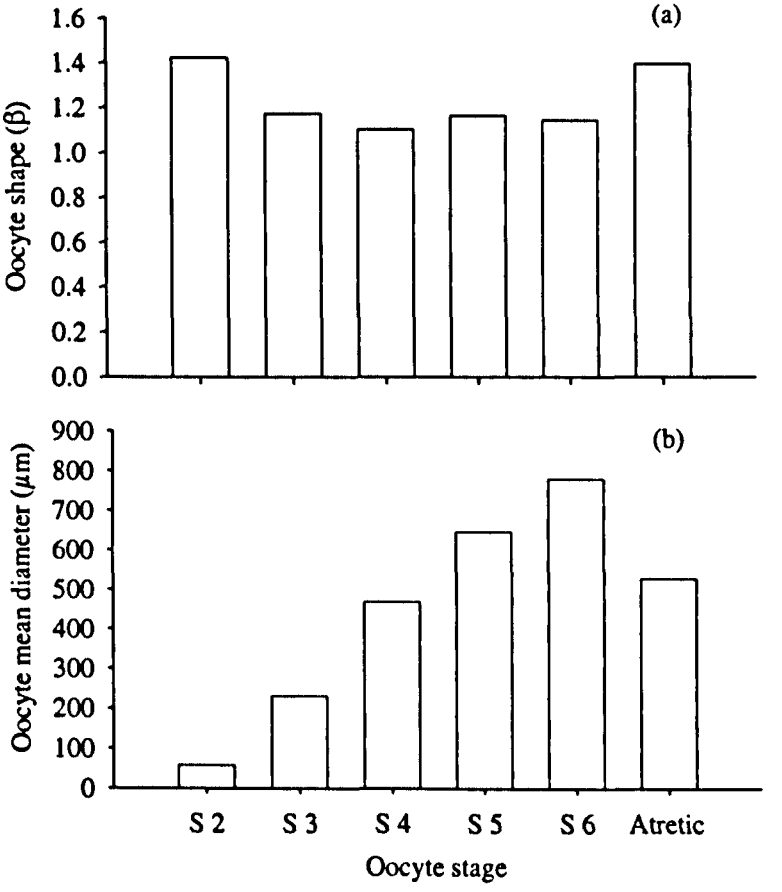


Figure 4.9 The (a) mean oocyte shape (*β*) and (b) mean oocyte diameter at various developmental stages of *R. sarba*.

Oocyte shape (*β*) that has a value of 1.0 indicates a spherical shape while (*β*) value of >1.0 indicates a progressively ellipsoid nature.

Table 4.3 Oocytes sizes and coefficients in relation to shape and size distribution of developmental stages of *R. sarba* oocytes taken from a prepared ovarian histological slide.

Oocyte stage	Mean oocyte shape ratio $(\beta_x)^{(*1)}$	Diameter distribution $(K_x)^{(*2)}$	$M1^{(*3)}$	$M3^{(*4)}$	Mean K/β
2	1.42±0.05	1.13	0.06	0.06	0.83±0.02
3	1.17±0.05	1.04	0.23	0.24	0.90±0.02
4	1.10±0.01	1.02	0.47	0.50	0.94±0.01
5	1.16±0.02	1.05	0.65	0.67	0.91±0.01
6	1.14±0.02	1.01	0.78	0.80	0.90±0.01
Atretic (α)	1.31±0.03	1.02	0.55	0.56	0.80±0.02

The above measurements and coefficient values were used to determine the numerical densities (N_{vx}) of stage 'x' oocyte beside the volume fraction (V_{vx}). For calculation of the above values refer to section (4.2.2.4). Numbers of oocyte analysed for each category were 200.
*1 -calculated using equation (10)
*2 -calculated using equation (11)
*2 -calculated using equation (12)
*4 -calculated using equation (13)
Note: all the above mentioned equations are shown in Section 4.2.2.4.

4.3.4.5 Volume fraction/area fraction (V_{vx}) of oocyte stages in the vitellogenic and hydrated ovaries.

(a) Stage two (S2) oocytes (refer to Figure 4.10)

The mean ovarian volume fraction (V_{vx}) of stage two oocytes was found to be significantly higher in stage four ovaries ($p<0.001$) while no significant differences were found between the levels of S2 in stage five and six ovaries ($p>0.05$). The mean volume fraction V_{vx} percentage of stage two oocytes appeared to be generally higher in the early vitellogenin stage (Stage four ovaries, 27%) compared with the final ovarian stage of development (stage six ovaries, 13%).

(b) Stage three (S3) oocytes (refer to Figure 4.10):

No significant differences ($p > 0.05$) were detected in the mean volume fraction V_{vx} of stage three oocytes between stage four and five ovaries. Stage three oocytes volume fraction V_{vx} in stage six ovaries were found to be significantly lower than

that of stage four & five ovaries (p=0.006). Generally the mean volume fraction V_{vx} of this stage of oocyte development appeared to be comparatively lower compared to the rest of the oocyte stages of development including atresia.

(c) Stage four (S4) oocytes (refer to Figure 4.10):

Significant differences (p<0.001) were detected in the mean volume fraction V_{vx} of stage four oocytes at all ovarian stages. However, the mean V_{vx} of stage three oocytes was lowest in stage six (hydrated ovaries). The volume fraction V_{vx} percentage of stage four oocytes appeared to be generally higher in the early vitellogenin stage (Stage four ovaries, 45%) compared with the final ovarian stage of development (Stage six ovaries 23%).

(d) Stage five & six (S5 & S6) oocytes (refer to Figure 4.10):

The mean volume fraction V_{vx} of stage five oocytes was found to be about 20% in stage five ovaries while the mean volume fraction V_{vx} of stage six oocytes was around 25% in stage six ovaries.

(e) Atretic oocytes (refer to Figure 4.10):

Atretic oocytes mean volume fraction V_{vx} in stage four ovaries was found to be significantly higher (p<0.001) than those of stage four & five ovaries. No significant differences were detected in the mean volume fraction V_{vx} of atretic oocytes in stage five & six ovaries. The mean volume fraction V_{vx} of the atretic oocytes was generally found to decrease as the ovaries progressed to stage six. The levels were 6.5% in stage four ovaries compared to 1% in stage six ovaries.

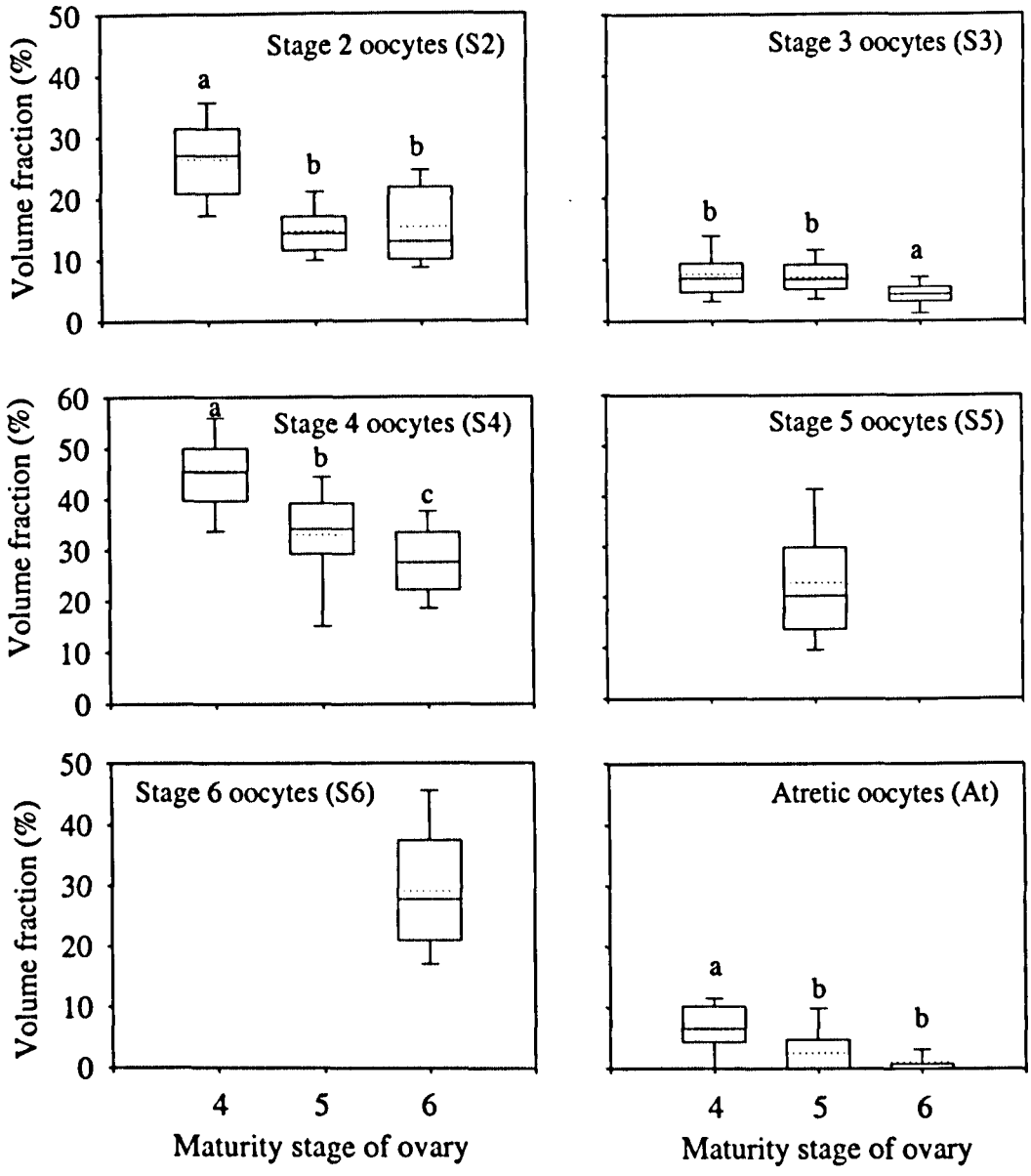


Figure 4.10 Percentage of volume fraction (area fraction) of oocyte stages in vitellogenic and hydrated ovaries. At- (atretic oocytes α, β and γ).

Box-plots show average (solid line) and median (dotted line). The box represents the interquartile range which contains 25% and 75% of values. Values that are not significantly different (P>0.05) share common superscripts. Lines that extend from the box represent the highest and lowest values.

4.3.4.6 Numerical density (N_{vx}) of oocyte stages in the vitellogenic and hydrated ovaries.

(a) Stage two (S2) oocytes (refer to Figure 4.12):

The mean numerical density of stage two oocytes per unit volume (N_{vx}) was significantly higher ($p=0.005$) in stage four ovary but no significant differences ($p>0.05$) were detected between stage five & six ovaries. There were, however, significant differences between ages ($p>0.001$) and middle age tends to be the highest.

(b) Stage three (S3) oocytes (refer to Figure 4.12):

No significant differences ($p>0.05$) were found between mean numerical density N_{vx} of stage three oocytes in stage four & five ovaries. However, numerical densities N_{vx} were observed to be significantly lower ($p=0.003$) in stage six ovaries. Once again, there were significant differences between ages ($p>0.001$).

(c) Stage four (S4) oocytes (refer to Figure 4.12):

No significant differences ($p>0.05$) were detected in mean numerical density N_{vx} of stage four oocytes between stage five & six ovaries. Stage four oocyte mean numerical densities N_{vx} in stage four ovaries were found to be significantly higher than that of stage five & six ovaries ($p=0.008$).

(d) Atretic oocytes (refer to Figure 4.12 & Figure 4.13):

Atretic N_{vx} levels were found to be significantly higher ($p<0.001$) in stage four ovaries. No significant differences ($p>0.05$) were found between the level of atretic oocytes in stage five & six ovaries. The mean numerical density N_{vx} levels of atretic oocytes in stage six ovaries were found to be the lowest.

Table 4.4 The numerical density per unit area/volume (N_{vx}).

Ovarian stage	N_{vx} of distinct oocyte stage per unit area					
	S2	S3	S4	S5	S6	Atresia (α)
4	780.21	22.02	48.5	-	-	3.80
	±28.50	±0.75	±0.91			±0.27
5	446.7	20.14	28.24	10.28	-	0.67
	±15.79	±0.88	±1.41	±0.54		±0.11
6	404.39	8.10	26.2	-	6.0	0.43
	±10.21	±0.40	±0.70	(Not found)	±0.15	±0.30

N_{vx} values were calculated using equation 2 in vitellogenic and hydrated ovaries. The N_{vx} values can be multiplied by the total volume of the ovary to estimate the numerical density of a distinct oocyte stage in the entire ovary of the female. Stage five oocyte (S5) did not exist among stage four ovaries.

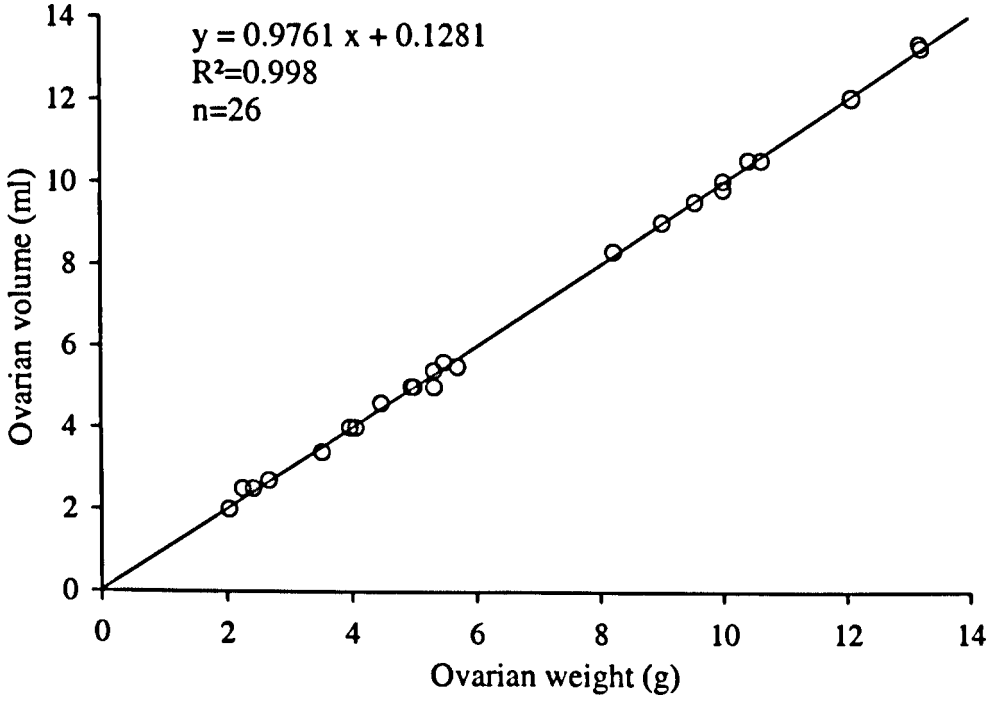


Figure 4.11 Regression between *R. sarba* ovarian weight and ovarian volume.

The equation that describes the relationship is (y = 0.9761x + 0.1281), d.f. = 25, p≤0.001. The relationship was used to quantify the numerical density of distinct oocyte stage in the entire ovary of the female.

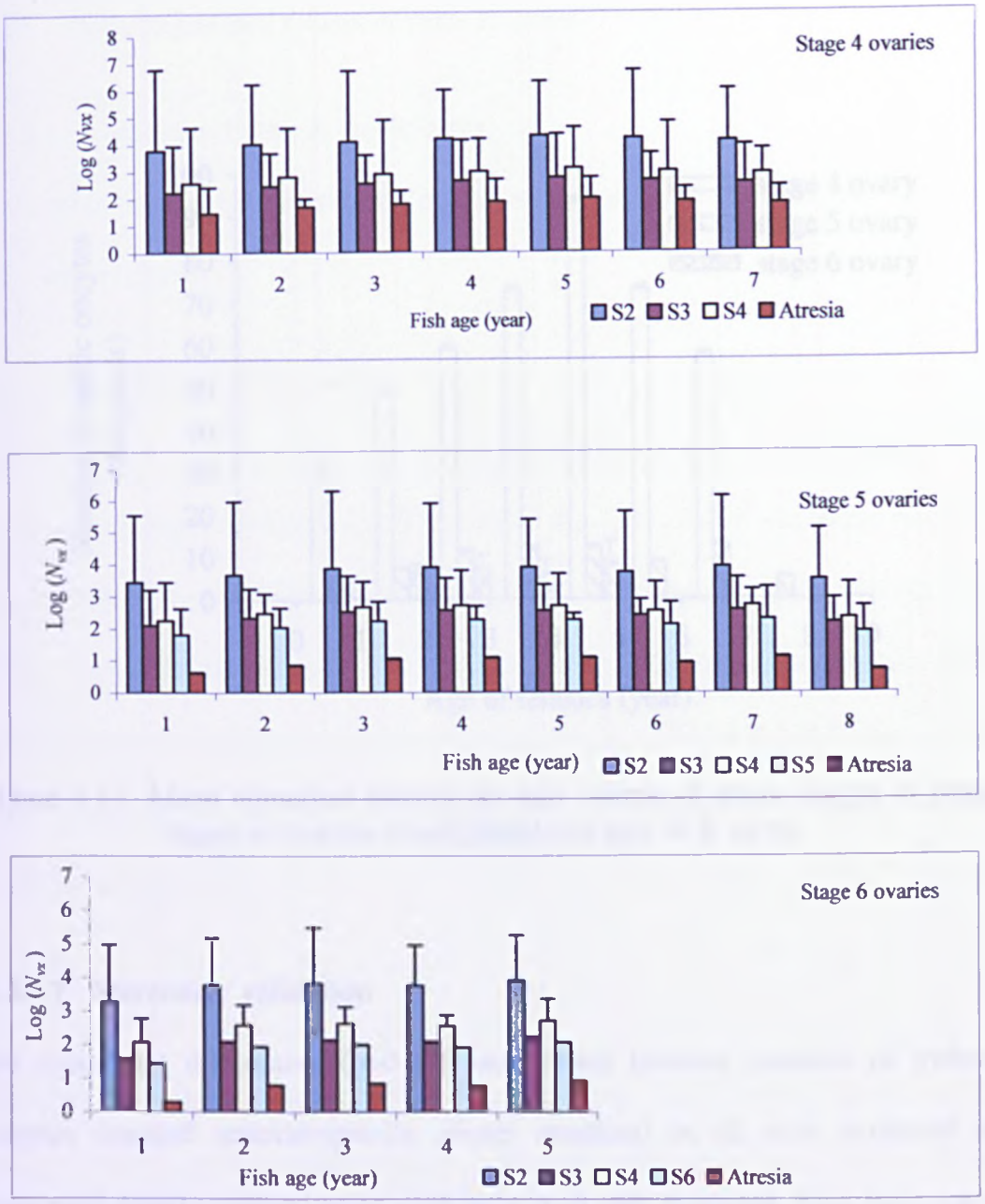


Figure 4.12 Mean numerical density per unit volume of stage four - six ovaries.

In instances where S.E. is not visible, it was found to be very small for presentation purposes.

S2 - stage two oocyte
S3 - stage three oocyte
S4 - stage four oocyte
S5 - stage five oocyte
S6 - stage six oocyte

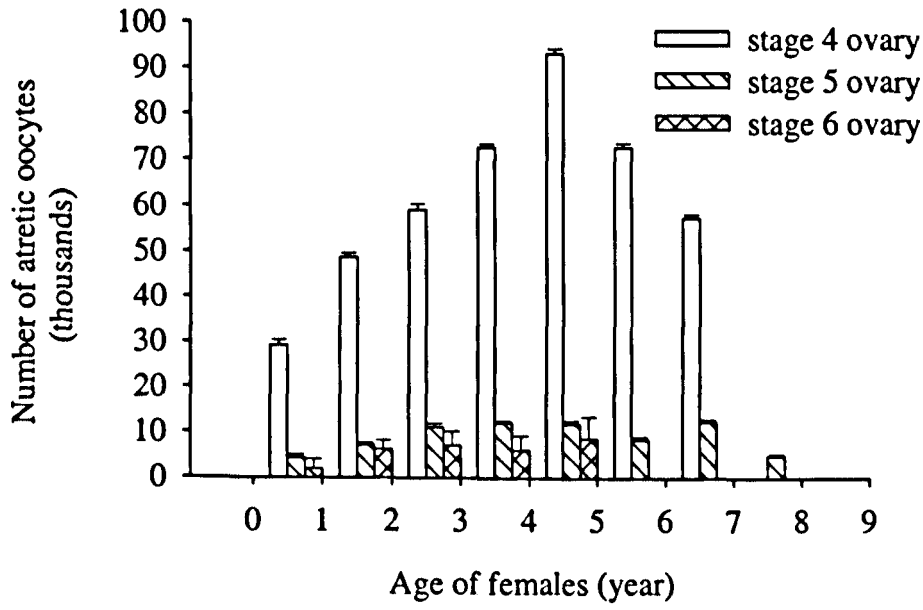


Figure 4.13 Mean numerical density per unit volume of atretic oocyte in various stages of ovarian development and ages of *R. sarba*.

4.3.4.7 Stereology validation

No significant differences ($p>0.05$) were found between numbers of hydrated oocytes counted macroscopically (direct counting) in all ages examined and numerical density counted using stereology in all ages examined. Also, there was no significant variation ($p>0.05$) between the two lobes of the ovaries in terms of the number of hydrated oocytes (S6) counted macroscopically (direct counting).

4.3.5 Maturity classification of male *R. sarba*

4.3.5.1 Cell types in the *R. sarba* testis

Four spermatogenetic cell types were identified in goldlined seabream testes and they are as follows:

A) Spermatogonia (a and b):

Spermatogonia (*a* stage) had a basophilic cytoplasm that was stained lightly with slightly large nuclei (Plate 4.25). The size of this type of cell was larger than other spermatogenetic cells. The diameter of the cell averages 16μm. Spermatogonia (*b* stage) stained deeply (Plate 4.25) and the diameter of the cell averaged 6μm. As the spermatogonia cells developed from stage *a* to *b* the cells tended to congregate and form larger nests. These nests were found near the periphery of the tubule and were stained more deeply.

B) Spermatocytes:

Smaller cells stained deeply with H&E. The cells had irregular shapes and the diameter of the cell averaged 4.04μm. The germ cells were clustered (nests) and their clusters were scattered along the tubules and along the lumen (Plate 4.27 & Plate 4.28).

C) Spermatids:

Spermatids cells were small in size compared to spermatocyte cells. The cytoplasm of the cell was stained uniformly with H&E (Plate 4.27 & Plate 4.28). The cell typically measured 3μm in diameter.

D) Spermatozoa:

Spermatozoa appeared round and stained uniformly. The cells were stained dark blue with H&E (Plate 4.27 & Plate 4.28). The diameter of the head of the cell averaged 2μm. With occasional exceptions, the tail was indistinct under light microscopy.

4.3.5.2 Tunica of the testes

The testis of *R. sarba* was surrounded by a thin tunica albuginea (capsule). The fibre septs of the tunica were projected to the inside of the organ. The tunica width of *R. sarba* testis was observed to decrease with fish maturity (Figure 4.15). The width of tunica and testis maturity displayed a significant correlation ($r=0.227$, d.f. = 355, $p=0.022$). Significant differences were found between maturity groups in means of tunica width ($p<0.001$), however, there was no significant difference between developing and active testes in terms of tunica width ($p>0.05$). Generally, tunica width appeared to be comparatively smaller during the active phase of development compared to that of the developing stage. During the post-spawning phase of testis development tunica width increased sharply and was found to be significantly different ($p<0.001$) from other stages of development (Figure 4.15).

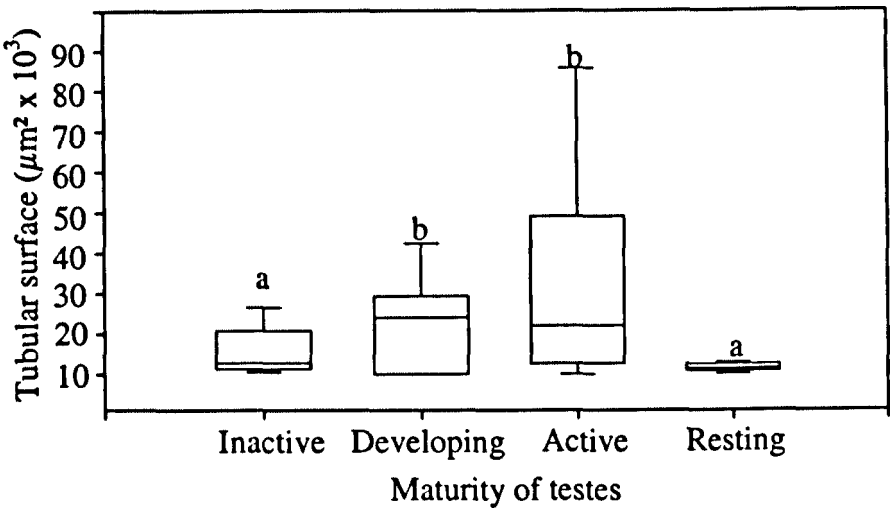


Figure 4.14 Box-plot shows the average (solid line) tubular lumen surface versus the maturity stage of testes.

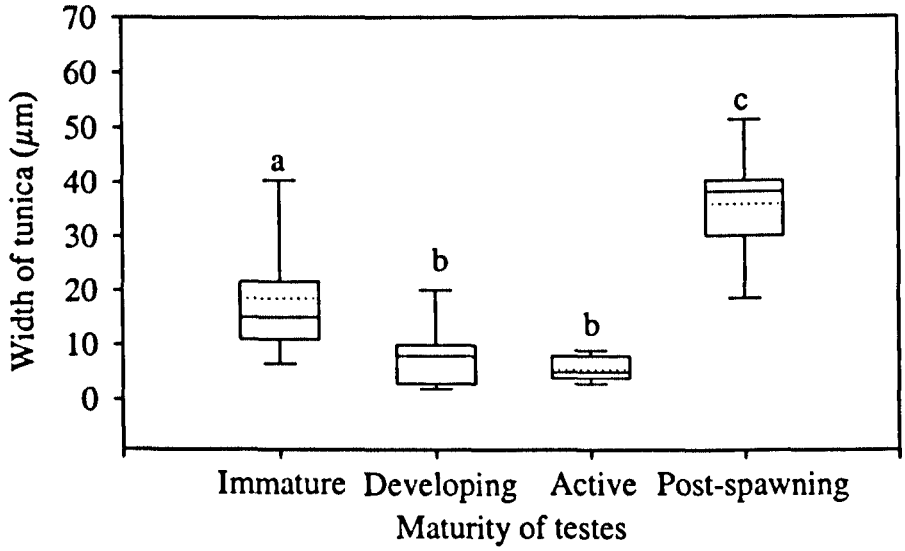


Figure 4.15 Box-plot shows the average (dotted line) and median (solid line) of width of tunica verses maturity stage of testes.

The box for the two graphs represents the interquartile range which contains the 25% and 75% of the values. Values that are not significantly different (P>0.05) share common superscripts. Lines that extend from the box represent the highest and lowest values.

4.3.6 The development and cycle of the male testis

Testis development stages for *R. sarba* during the first and second sampling seasons were classified. The classifications were carried out taking into account histological morphology and the most advanced cell stage found in the testis and their relative abundance. Four stages of testis condition or activity were identified during the reproductive period of *R. sarba* (Table 4.5). The histological characteristics and macroscopic appearance of *R. sarba* testes during maturation are shown in Table 4.5.

Monthly changes in testicular development in *R. sarba* examined in this study during the two periods are shown in (Figure 4.16). Advancement in testicular development did occur in the second year and spermatogonial proliferation occurred during the summer. All fish that were sampled in October during the first sampling period were found to be in the immature stage. During the second sampling period, however, the percentage of immature testes in October was about 96 %. As the surface and bottom temperature started to decrease after October early spermatogenesis started to increase. In November of the first sampling period the testes that were found at the developing phase were about 50%. Due to the advancement in testicular development, which occurred in the second sampling period, developing testes started to appear in October and acccounted for approximately 5% of fish sampled in this month. As maturation proceeded, increasingly more active testes were found from November onwards and in both sampling periods all fish that were sampled in January and February were at the active phase of development. From February onwards the water temperature started to rise, during which resting testes started to appear. In the first sampling period

resting testes appeared in March and represented about 51% of those sampled. During the second year however, resting testes were seen in February and accounted for approximately 4% of the sampled testes and increased during March to reach around 47%.

Table 4.5 Histological chatacteristics used to describe testis reproductive stages in *R. sarba* collected in Gulf of Oman.

STAGE	HISTOLOGICAL CHARACTERISTICS	MACROSCOPIC APPEARANCE
Immature	Both type A & B (SG) cells were present in high density. SC cells were present in some individuals. SD cells were rarely observed. SZ present in both lobules and main sperm duct at lower density.	Testes small and thin, greyish-white.
Developing	Spermatogonial proliferation took place followed by spermatogenesis. All types of spermatogenic cells existed. SG cells decreased, SC and SD were much abundant. SZ increased in both the lobules and ducts and surface area of lobules had increased.	Testes enlarged in length, greyish-white colour. Creamy-white colour in more advanced testes.
Active	SG cells decreased to a minimum. SC and SD cells were abundant but less than previous stage. SZ increased in both the lobules and ducts and due to this both lobules and sperm duct surface area increased greatly.	Testes creamy-white, elongated and milt expelled from the sperm duct with slight pressure and when duct is cut.
Resting	SG started to increase. SC decreased greatly. SD cells were present only in some individuals. SZ were present in some lobules. Sperm duct collapsed but still contained small amount of SZ.	Noticeable decrease in size of testes, reddish-grey in colour.

SC - spermatocytes
SD - spermatids
SG - spermatogonia
SZ - spermatozoa

Table 4.6 Histological characteristics of male *R. sarba* gonads in relation to the histological appearance of the gonads.

STAGE	CHARACTERISTICS OF GERM CELL			
	Spermatogonia	Spermatocytes	Spermatids	Spermatozoa
Immature	+ + + + +	(+ / -)	(+)	++
Developing	++	+ + + + +	+ + + + +	+++
Active	+	++++	++++	+ + + + +(MS)
Resting	++	++	(+ / -)	++

+ to + + + + : shows the relative abundance of each cell type
(+ / -) : cell present in some individuals
(+) : rarely found
MS : milt stripping possible

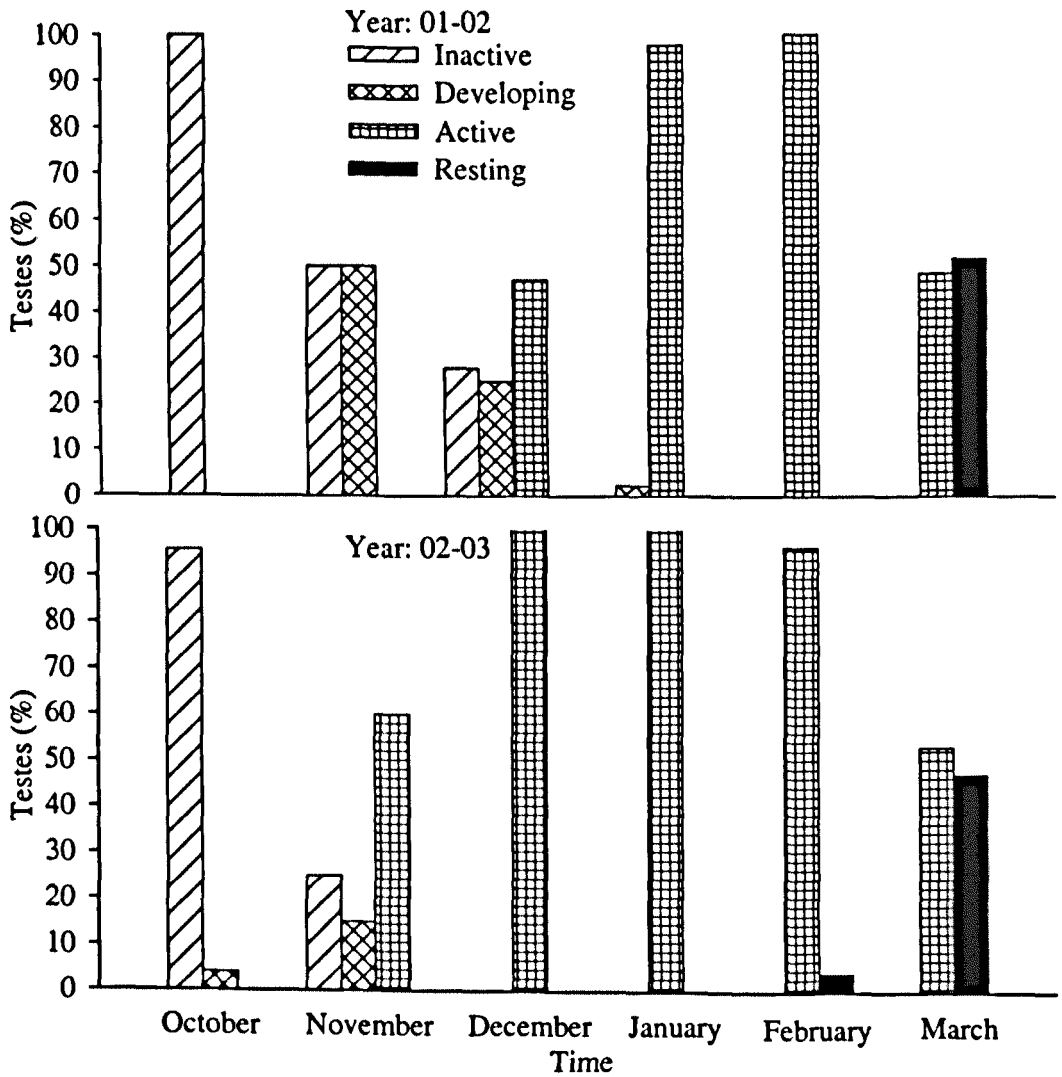


Figure 4.16 Percentage of testes at various stages of development in *R. sarba* during the reproductive season in the first and second sampling season.

4.3.7 Testes weight and fish length of mature male *R. sarba*

The testes weight (TW) of *R. sarba* that were classified as mature (active) tended to increase with fish length (Figure 4.17). The TW and fish length of *R. sarba* displayed a significant correlation ($r=0.81$, $t = 20.251$, d.f. = 215, $p<0.001$).

The relationship could be described by the equation:

$$TW = -37.72 + 0.204\ F.L$$

Where:

TW = gonad weight in gram

F.L = fork length in mm.

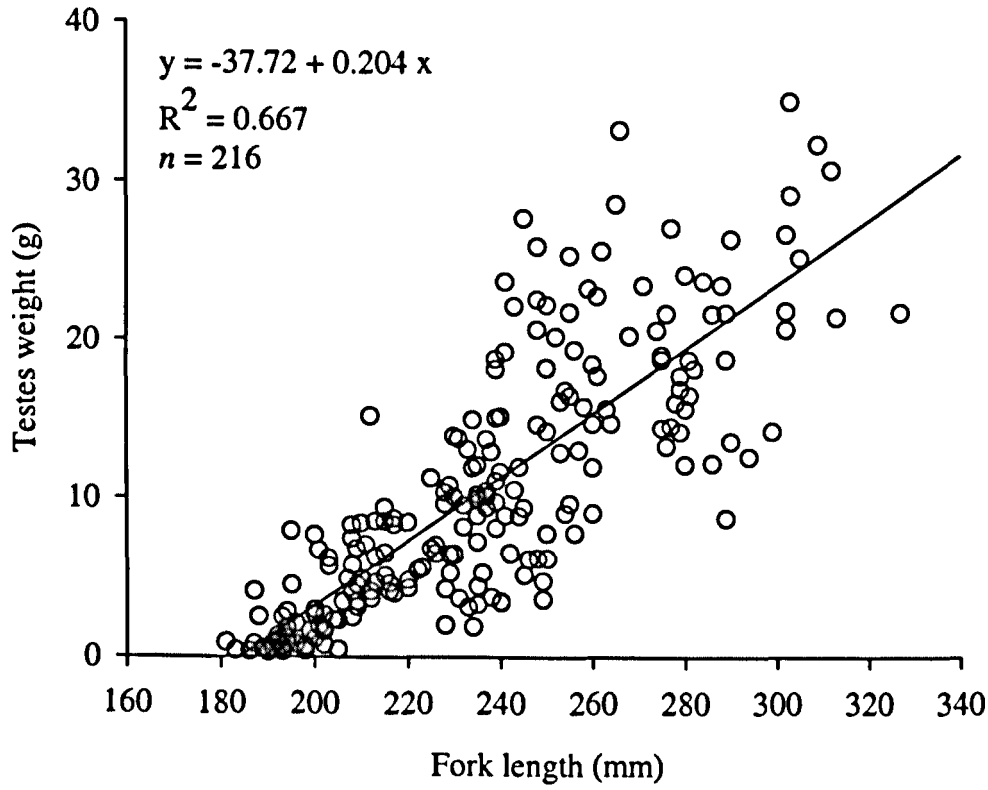


Figure 4.17 Relationship of weight of mature testes (active) to fork length of *R. sarba*.

4.3.8 Classification of male *R. sarba* testes

4.3.8.1 Immature testes

Spermatogonia (SG) were found to predominate in the seminiferous tubules of the testes. Small numbers of residual spermatozoa were present in some lobules especially those in the central part of the tenses (Plate 4.25). The tubular lumen had less surface area when compared with that of the active stage testes (Figure 4.14). Significant differences were found between tubular lumen surface area of immature testes and active and developing testes ($p=0.001$). No statistical difference was found between this stage and the post-spawning stage ($p>0.05$) in terms of surface area of the lumen. In most samples, the main sperm duct had gametes (residual spermatozoa) (Plate 4.26). The testes occasionally contained small cysts of primary spermatocytes. Spermatid cells were rarely found during this stage of development and at this stage tunica width averaged about 18μm (Figure 4.15) and was found to be significantly different from other maturity stages ($p<0.001$).

4.3.8.2 Developing testes

Different stages of male testis germ cells (spermatocytes, spermatids and spermatozoa) were found synchronously developing in this testicular stage of development and these cells became more frequent (Plate 4.27). Spermatogonia, however, were less apparent at this stage. The tubular lumen had greater surface area compared to the previous stage of development and increased as the amount of gametes (spermatozoa) also increased (Figure 4.14). At this stage lumen surface area was significantly different from immature and post-spawning stages ($p=0.001$) but no significant difference was found between the developing and active stage ($p>0.05$) in terms of lumen surface area (Figure 4.15).

The development of spermatogenesis was observed to take place in the central part of the testes and progress towards the most external tubules as the testis proceeded in its development. The testis walls (tunica) at this stage were thinner than the previous stage and tunica width averaged about 8μm. Here, the tunica width was found to be significantly different from immature and resting stages ($p<0.001$). However, no significance difference was found between the developing and active stage ($p>0.05$) in terms of tunica width (Figure 4.15).

4.3.8.3 Active testes

All spermatogenic stages were present (Plate 4.28). Spermatogonia numbers, however, were lower during this stage. Spermatozoa filled the lumen lobule in the entire testis. The sperm duct was full of sperm and the amount of spermatozoa was much greater than at the previous phase of development (Plate 4.29). Release of sperm was possible by slight pressure on the fish abdomen.

The lumen of the lobule had the largest surface area compared to other stages of development and was significantly different from the immature and resting stages ($p<0.001$) (Figure 4.14). However, no significant difference was found between the developing and active stage ($p>0.05$) although the surface area during the active phase was generally larger.

During this stage tunica width averaged about 5.6μm and was thinner than at the previous (developing) stage (Figure 4.15). Here, the tunica width was significantly different from immature and resting stages ($p=0.001$) and no significant difference

was noted between the active and developing stages ($p>0.05$) in terms of tunica width.

4.3.8.4 Resting testes

Spermatogonia and spermatocytes concentrations were similar to that of the immature stage and spermatids were rarely observed at this stage. Generally the intermediate stages (spermatocytes and spermatids) were at lower concentrations (Plate 4.30) and some lobules contained residual sperm (Plate 4.30 & Plate 4.32). The main sperm duct also contained some residual spermatozoa but the amount of sperm was lower than immature stage (Plate 4.31).

The tubular lumen had less surface area than all other stages of development (Figure 4.14) and significant difference was found between the tubular lumen surface area of the developing and the active testes ($p=0.001$). No statistical difference was found between this and the immature stage ($p>0.05$) and here the tunica width averaged about 36μm and was significantly different from other maturity stages ($p=0.001$) (Figure 4.15).

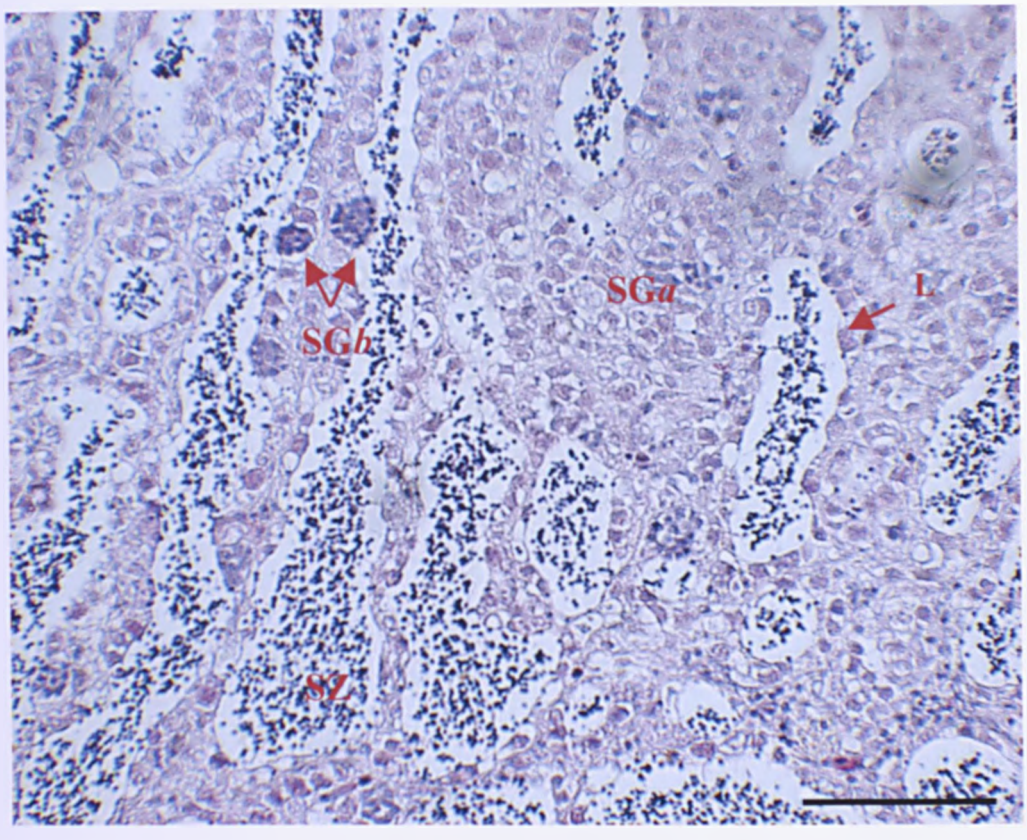


Plate 4.25 Transverse section (3μm thick) of *R. sarba* (246g in weight & two-year-old) testis at immature phase sampled in late August.

Mag. ×40; scale bar 100μm
L - lobular lumen
SG*a* - spermatogonia type A cells
SG*b* - spermatogonia type B cells
SZ - spermatozoa

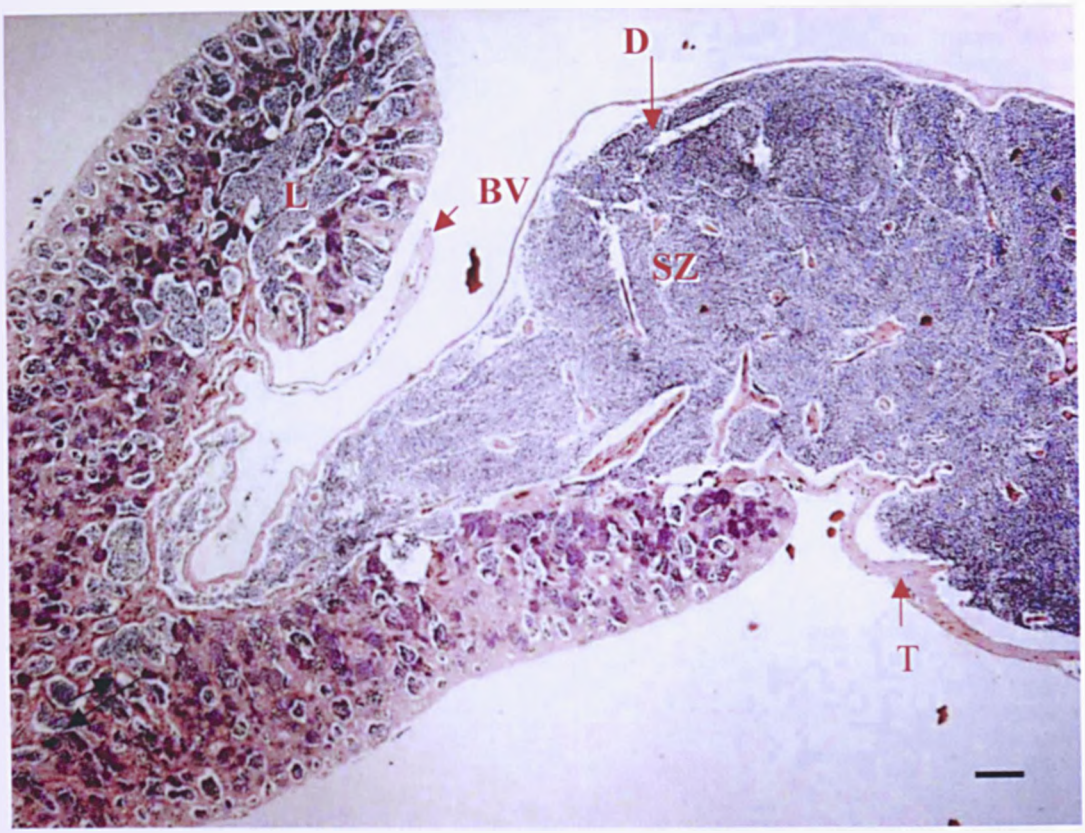


Plate 4.26 Transverse section (3µm thick) of *R. sarba* (246g in weight & two-year-old) testis at immature phase of development sampled in August.

Mag. ×4; scale bar 100µm.
BV – blood vessel
D - duct (main sperm duct)
E - epithelium
L - lobular lumen
SZ - spermatozoa
T - tunica

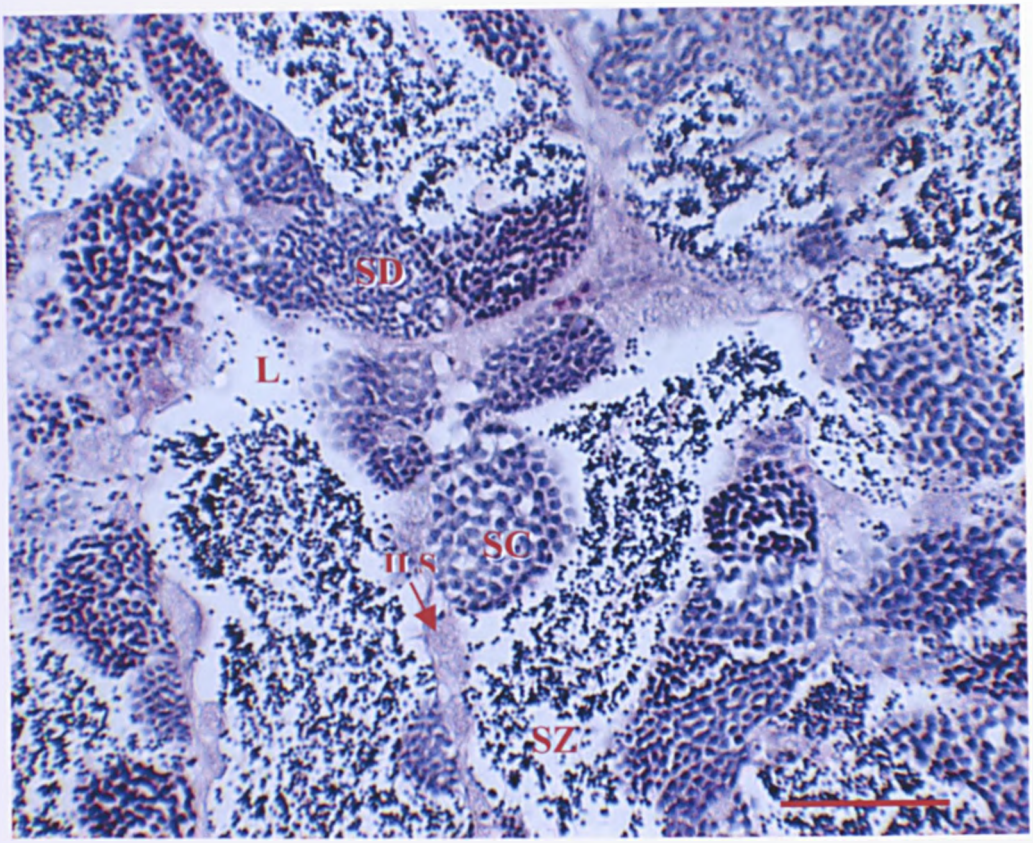


Plate 4.27 Transverse section (3µm thick) of *R. sarba* (485g in weight & four-year-old testis at development phase sampled in late November.

Mag. ×40; scale bar 100µm.
ILS - intra-lamellar stromal strands
L - lobular lumen
SC - spermatocytes
SD - spermatids
SZ - spermatozoa

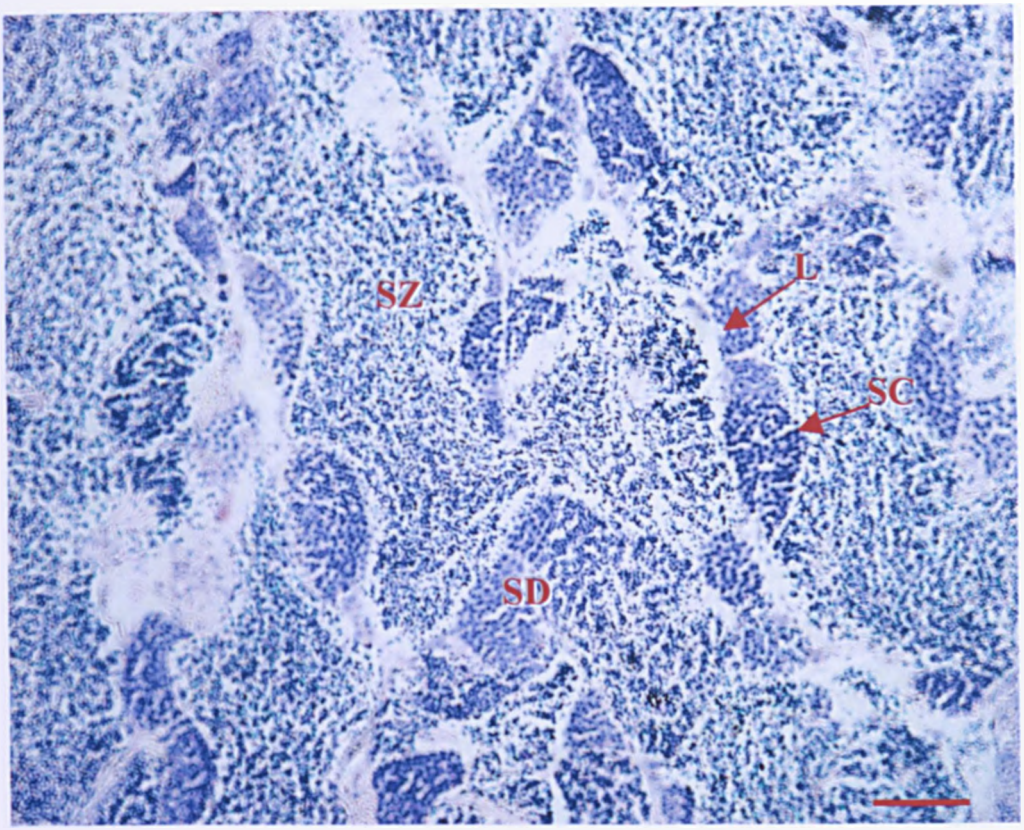


Plate 4.28 Transverse section (3µm thick) of *R. sarba* (588g in weight & five-year-old) testis in active phase sampled in January.

Mag. ×10; scale bar 100µm.
L - lobular lumen
SC - spermatocytes
SD - spermatids
SZ – spermatozoa

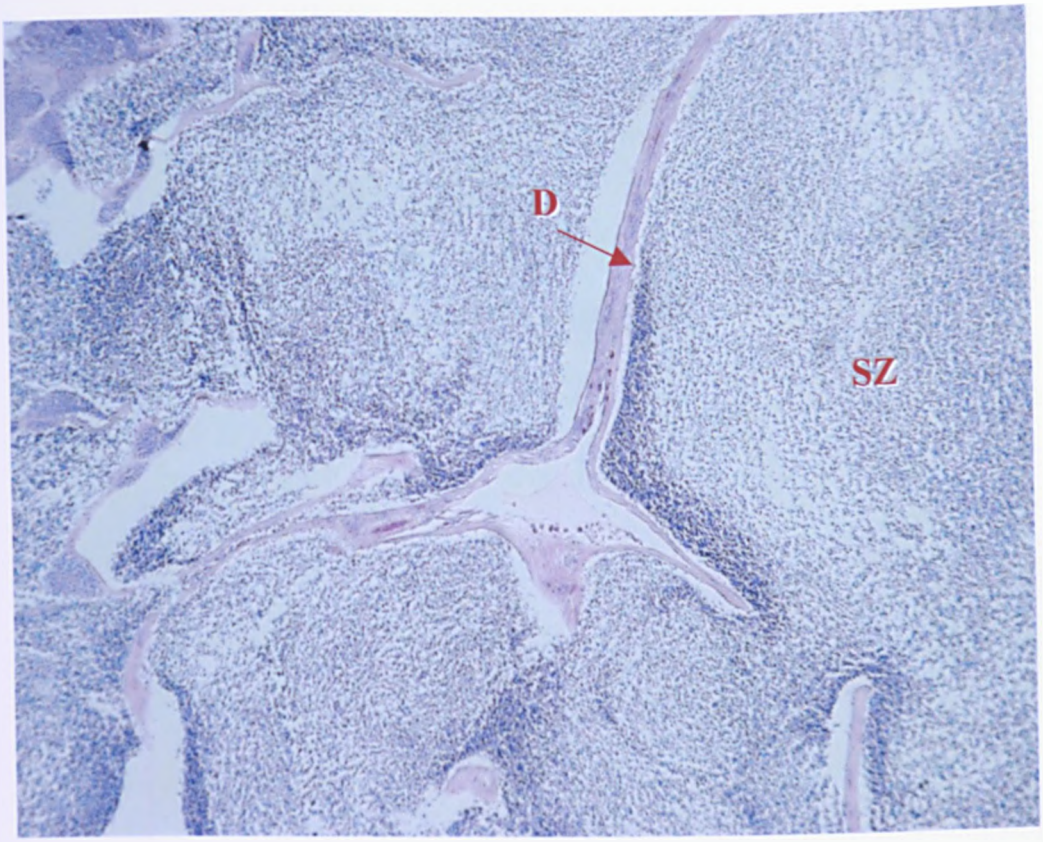


Plate 4.29 Transverse section (3µm thick) of *R. sarba* (588g in weight & five year-old) testis in active phase sampled in January. This plate shows the main duct full of spermatozoa.

Mag. ×10; scale bar 100µm.
D - duct (main sperm duct)
SZ – spermatozoa

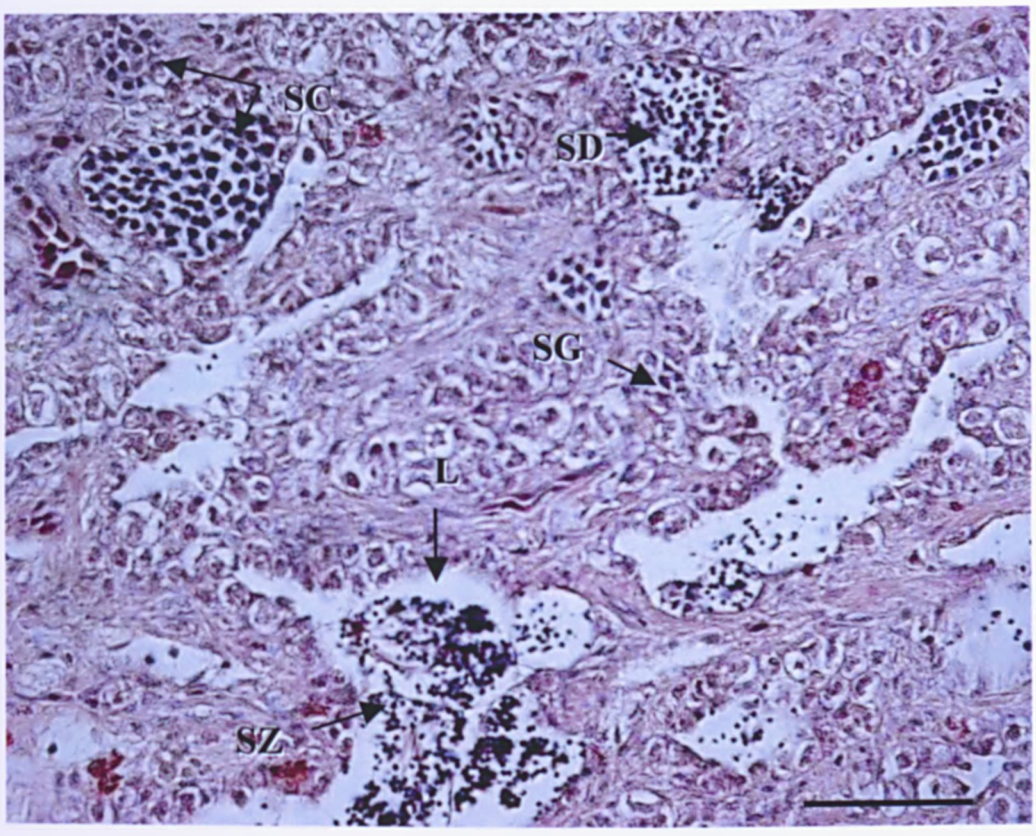


Plate 4.30 Transverse section (3µm thick) of *R. sarba* (320g in weight & three year-old) testis at post-spawning phase sampled in March.

Mag. ×40; scale bar 100µm.
L - lobular lumen
SC - spermatocytes
SD - spermatides
SG - spermatogonia
SZ -spermatozoa

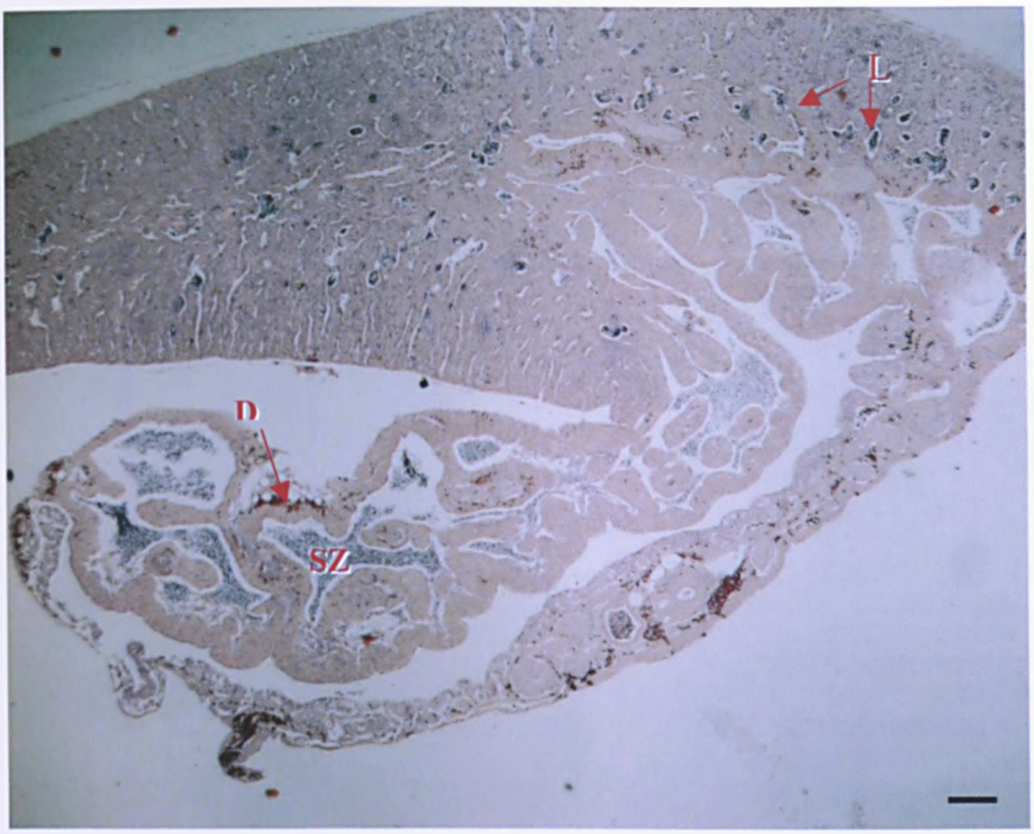


Plate 4.31 Transverse section (3µm thick) of *R. sarba* (320g in weight & three year-old) testis at post-spawning phase sampled in March.

Mag. ×4; scale bar 100µm.
D - duct (main sperm duct)
L - lobular lumen
SZ -spermatozoa

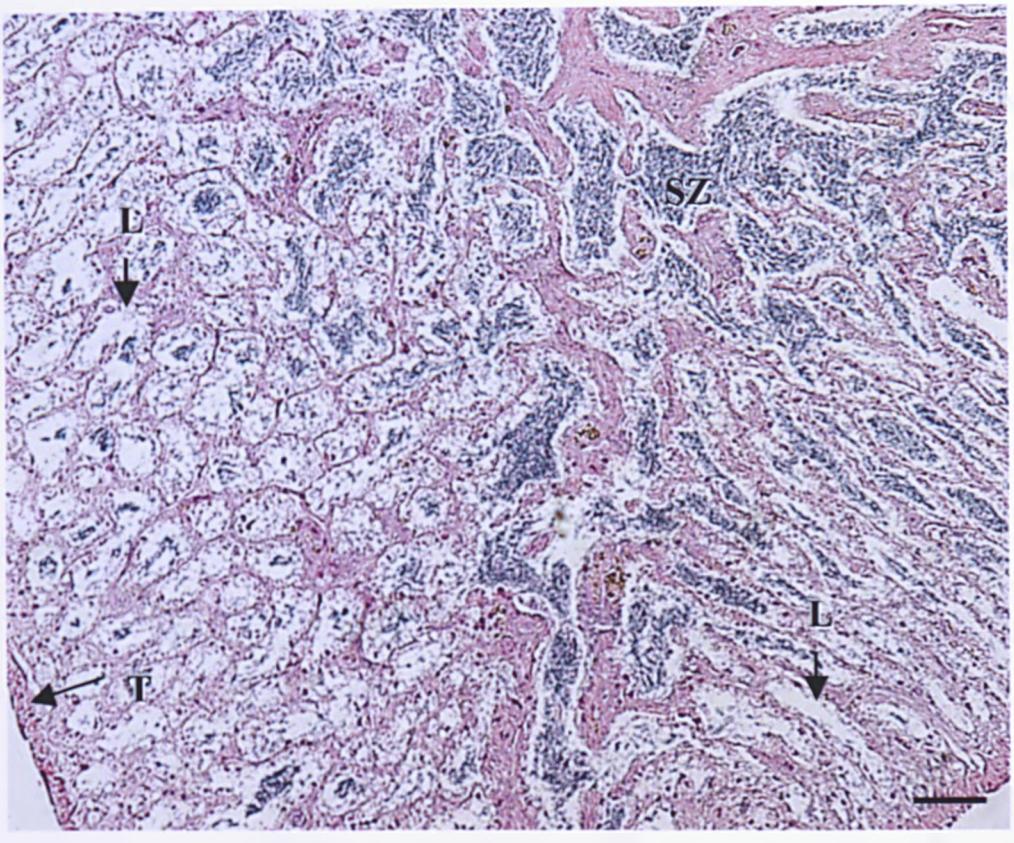


Plate 4.32 Transverse section (3µm thick) of *R. sarba* (334g in weight & three year-old) testis at post-spawning phase sampled in March.

Mag. ×10; scale bar 100µm.
L - lobular lumen
SZ - spermatozoa
T - Tunica

4.3.9 Intersex frequency in *R. sarba*

Intersex (transaction) gonads (Plate 4.33) appeared more frequently in the zero and two years of age classes. Beyond the age of three years, the intersex frequency declined to a minimum (Table 4.7). Intersex gonads in *R. sarba* were found up to the age of seven years and beyond this age no intersex gonads were observed.

Table 4.7 The intersex frequency of *R. sarba at* age class.

AGE (year)	FORK LENGTH (mm)	TOTAL NUMBER IN EACH AGE CLASS	NUMBER OF FISH
0	192	30	1
1	225	38	2
2	237.14	34	7
3	247.6	18	5
5	263	9	1
6	289	7	1
7	303	6	1

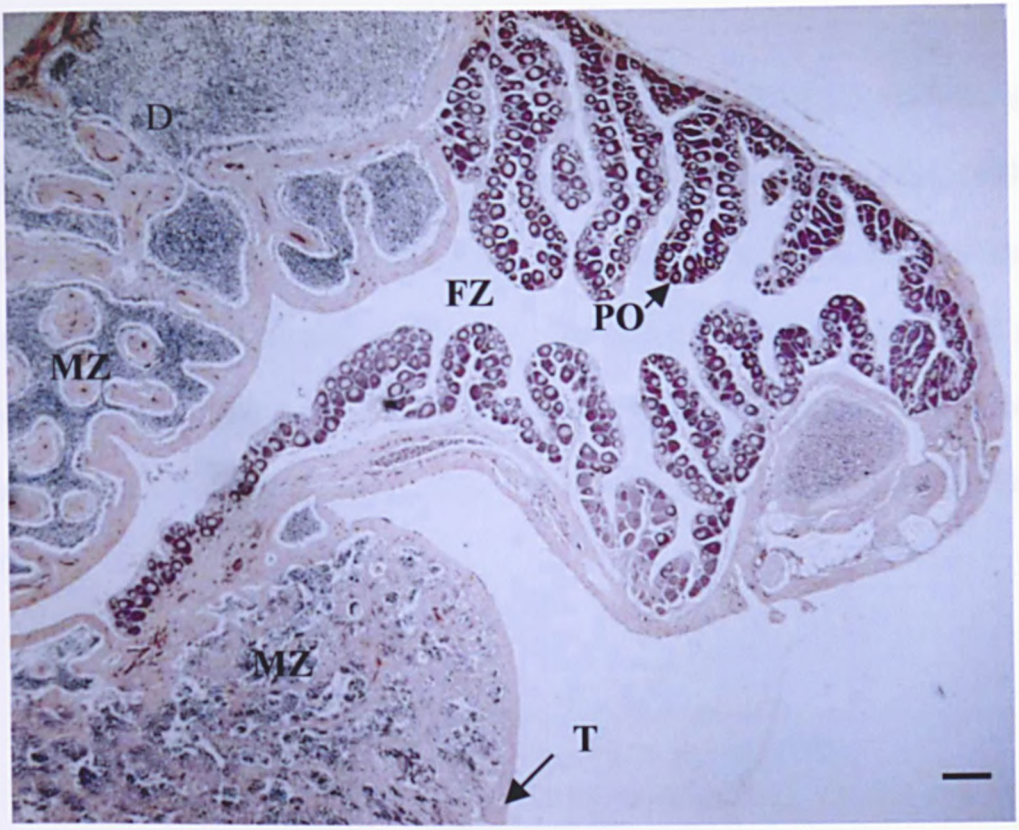


Plate 4.33 Transverse section (3µm thick) of *R. sarba* (246g in weight & two-year-old) intersex gonad sampled in late August showing spermatozoa in sperm duct and lumens of the male zone, and stage two oocytes in the female zone.

Mag. ×10; scale bar 100µm.
D -duct (main sperm duct)
FZ - female zone
MZ - male zone
PO - primary oocytes
SZ - spermatozoa
T - tunica

4.4 Discussion

The spawning cycle is partially determined by the drop in GSI, particularly for those fish that have one spawning season each year over a short period (Le Clus, 1989). In the Gulf of Oman, *R. sarba* presented a single spawning season that falls during winter between January and March. This was apparent from the single GSI peak that was seen in January and started to fall afterwards. The spawning behaviour is similar to that reported in some other localities cited in the literature (see Figure 4.18).

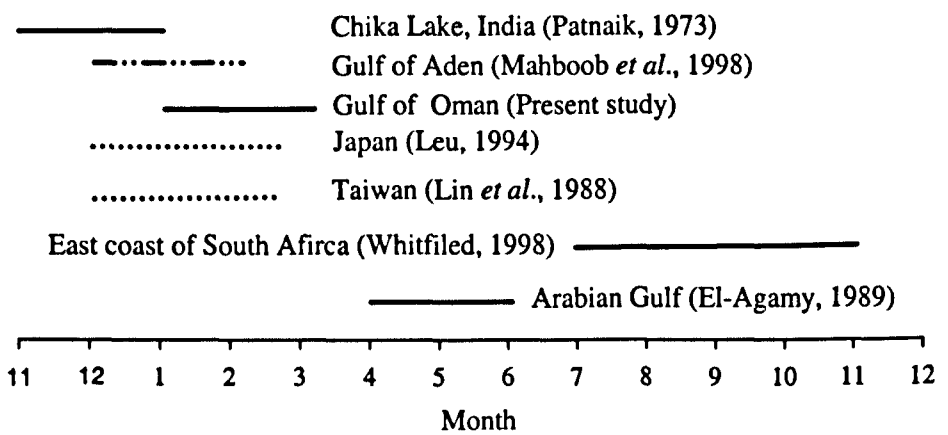


Figure 4.18 Spawning season of *R. sarba* at different geographical regions.

Solid lines represents studies conducted on wild fish, dotted line represents studies conducted on captive broodstock without control, dotted and dash represents studies conducted on captive broodstock where spawning did not continue during spawning season.

Differences in the onset of the spawning season of *R. sarba* at various localities as shown in Figure 4.18 were probably related to environmental influences such as the photoperiod and water temperature which trigger the reproductive behaviour (Bromage *et al.*, 1993). Despite the advance of one month that existed in the onset of the vitellogenin developmental stage between the two spawning seasons, the reproductive season (spawning event) took place at almost the same time as was

evident from the appearance of postovulatory follicle (POF) in the ovaries. The reason for such advancement could be related to environmental triggers such as water temperature and photoperiod. Tropical fish species are generally observed to spawn daily on a regular basis provided temperature and day-length are constant and at appropriate levels (Bauer & Bauer 1981).

Sexual maturation in teleosts is driven by endogenous rhythms controlled by environmental cues such as water temperature and photoperiod of which the latter is reported to have more affect (Bromage *et al.*, 1993). A hormone known as melatonin secreted by the pineal gland as a responce to light intensity has been suggested to play an important role in maturation (Porter *et al.*, 1999). In the current study, the increase in GSI and the development of the gonads was in line with the change in temperature similar to other sparid fish cited in the literature for example, *Diplodus sargus nufar* (Coetzee, 1986), *Chrysoblephus laticeps* and *C. cristiceps* (Buxton, 1990). Currently fish spawning in captivity, through temperature and photoperiod manipulations which simulate seasonal changes that advance gonadal maturation has been successfully achieved in several species of commercial interest. These species includes, rainbow trout, *Oncorhynchus mykiss* (Duston & Bromage, 1986), Atlantic salmon, *Salmo salar* (Hansen *et al.*, 1992), haddock, *Melanogrammus aeglefinus* and cod, *Gadus morhua* (Buckley *et al.*, 2000; Martin-Robichaud & Berlinsky, 2004; Norberg *et al.*, 2004). It has been also implemented on Mediterranean species such as gilthead seabream, *Sparus aurata* and seabass, *Dicentrarchus labrax* (Carrillo *et al.*, 1995; Kissil *et al.*, 2001). Environmental manipulations, however, have their own limitations and affect gamete quality fecundity and egg viability (Carrillo *et al.*, 1995).

Changes in gonadosomatic index of females during the reproductive season is due to the occyte development that takes place inside the ovary (Tyler & Sumpter, 1996). Female *R. sarba* increased GSI from about 0.5 to 4% during vitellogenesis, similar to other asynchronous ovulatory species that have small oocyte volume. Other seabream species also exhibit similar trends such as sheepshead, *Archosargus probatocephalus* which display a change of GSI from about 0.6-5.5% (Render & Wilson, 1992), sharpsnout seabream, *Diplodus puntazzo* from about 1-3.5% (Micale *et al.*, 1996) and striped seabream, *Lithognathus mormyrus* from about 0.5-5.5% (Türkmen & Akyurt, 2003). Synchronous ovulatory fish that have large oocyte volume such as rainbow trout have been observed to increase GSI from 0.4% to 20% at ovulation (Tyler *et al.*, 1990), similarly, Atlantic salmon, *Salmo salar* which increases GSI from 0.5% to 17% during vitellogenesis (King & Pankhurst, 2003). During the breeding season, however, the ovary experiences fluctuation in its size and volume. Martin-Robichaud & Berlinsky (2004) suggest that variation in GSI during the reproductive season might be due to the rate of development of oocytes batches within the ovary that alters its dimensions.

Gonadosomatic index (GSI) of male *R. sarba* peaked at the same time as that of the female. Increased GSI reflected the advancement of testis to the active phase and the large increase in number of spermatogenic cells of all stages during the active phase of testis development. Also, during active phase the sperm duct was filled with spermatozoa to a level greater than at previous. This increase in spermatozoa possibly derived by sperm competition between individuals to maximise sperm production for the purpose of a higher fertilisation rate which has been suggested to increase the testes mass and hence raise GSI (Buxton & Garratt, 1990; Taborsky,

1998). In the current study, GSI averaged in both sexes was at a similar rate. Buxton & Garratt (1990) showed that the differences in GSI in sparidae also depend on the reproductive styles of the species, but it is generally observed that male GSI is lower than that of females (Pajuelo & Lorenzo, 2000).

The index of Fulton's condition factor (K) is considered a direct and quantitative measure of relative robustness of the body and was developed by Hile (1936). The index reflects the well-being and the condition of the fish at any particular moment in time. For *R. sarba*, K value peaked before the spawning season, and may have been raised by the nutritive condition of the species during the period that precedes the spawning season. Regardless of sex, most fish tend to maximise their K value during the pre-spawning period for example dolphin fish, *Coryphaena hippurus* (Wu *et al.*, 2001), sardine, *Sardina pilchardus* (Zwolinski, 2001) and cultured sharpsnout seabream, *Diplodus puntazzo* (Hernández *et al*, 2003).

As *R. sarba* females utilised their stored energy reserves during the reproductive phase, K value decreased sharply, this demand for greater energy for reproduction is a characteristic of asynchronous spawners (Hsiao *et al.* (1994). Due to demands for energy in the reproductive season, strong correlation is therefore found between gonadosomatic index (GSI) and condition factor (K) index. Females have been suggested to contribute more to the developing embryo than males and hence the need for greater energy (Murua & Saborido-Rey, 2003). The decrease in female K index during the spawning season may have also contributed to the increasing levels of atresia in mature ovaries as a method of reallocation of some of the energy reserved in the ovaries. Óskarsson *et al.* (2002) carried out a study on Norwegian

spring-spawning (NSS) herring, *Clupea harengus*, in which they observed the highest level of atresia to occur at the time when K value is low, during which major increases in vitellogenic activities are taking place. The authors further added that fish that are found with poorer K value had the lowest number of developed oocytes and the lowest fecundity. Fish experience loss in energy during reproduction and food availability or fasting may impose further pressure on the energy storage sites in the fish. Henault & Fortin (1991) suggested that cisco or lake herring, *Coregonus artedii* utilises fat stored in the muscle to meet its energy requirements during spawning season and this occurs in post-autumn when feeding conditions are poor.

Male *R. sarba* K value peaked one month ahead of females. Since males utilise less energy in reproduction process than females (Buxton, 1990), their energy reserves may not decrease significantly; therby causing little effect K value. Consequently, no correlation was observed between gonadosomatic index (GSI) and condition factor (K). Nevertheless, poor condition of both males and females could influence the fertilisation such as in cod, *Gadus morhua* (Rakitin *et al.*, 1999) and hence the quality of progeny (Trippel, 2003). The higher condition factors observed for males than for females during the second sampling period could be attributed to differences in fish size. Average fish weight recorded during the first season (2001-2002) was generally lower than that of the second sampling season (2002-2003). Also, as discussed in Chapter three, males were found to be heavier than females particularly after the age of four years. Furthermore, males GSI values were higher than those of females, differences in GSI values between sexes have been suggested to affect K value in silver crucian carp, *Carassius gibelio* (Balik *et al.*, 2004). In

some species where the spawning event takes place in more than one period, the K value is found to be higher in instance where food is more abundant, for instance in silver fish, *Odontesthes bonariensis* (Barros & Regidor, 2002). These authors did not find a correlation between female K value and GSI and they attributed this to the optimum feeding conditions that the species has throughout the year which compensate the energy loss encountered during the spawning season. Low K values for *R. sarba* have also been reported by El-Agamy (1989) who suggests that poor living conditions are the reason for low K values observed in this species. Condition factor (K) has been suggested to fluctuate in response to various factors such as gonadal development, age and seasonal changes in growth of fish (Ricker, 1975; Henault & Fortin, 1991).

The present study provides a description of the histology of oogenesis in *R. sarba* in Omani waters. Stages of development for female ovary were classified into two, three, four, five, six, atresia and postovulatory ovaries. The latter includes ovaries found at the end of the season that were described in this study as resting ovaries. Oocyte development recognised in *R. sarba* was divided into seven stages including atresia which was further divided into four distinct stages. Ovarian classification criteria developed in this chapter was used to classify female stage of maturation wherever needed in this study.

Goldlined seabream *R. sarba* are multiple asynchronous spawners, characterised by the existence of ovarian oocytes at different stages of development during the reproductive season in its ovary. Development of oocytes in *R. sarba* was similar to other asynchronous spawners such as *Pagrus major* (Matsuyama *et al.*, 1987 &

1988), *Chrysoblephus laticeps & C. cristiceps* (Buxton, 1990), *Dentex dentex* (Loir *et al.*, 2001) and *Thunnus thynnus* (Corriero *et al.*, 2003). Despite the fact that there are differences in the number of stages of oocyte development in teleosts, in general they follow similar pattern of development. This pattern or distinct stages can be summarised into six main ones according to Bromage & Cumaranatunga (1988) and Selman & Wallace (1989) and these are the oogenesis, primary growth stage, cortical alveoli stage, vitellogenic stage; final maturation and ovulation stage, for review refer to Coward *et al.* (2002).

Small numbers of ovaries during this study were observed to contain oogonia nests. Oogonia, however, were rarely observed and were limited to post ovulatory follicles that were seen in post-spawning ovaries (PSO). Oogonia nests were observed about four months prior to the reproductive season, and have also been observed in seabass, *Dicentrarchus labrax* (Mayer *et al.*, 1988) and seabream common dentex, *Dentex dentex* (Loir *et al.*, 2001). During the current study, oogonial proliferation was observed to occur in PSO and oogonia were at higher levels, this was in agreement with (Mayer *et al.*, 1988). Loir *et al* (2001) also observed oogonia at the beginning of the meiotic prophase in the sparid common dentex, *Dentex dentex* but, as the author suggested these oogonia were rarely observed and are limited to immature females. Bromage & Cumaranatunga (1988) suggested that transformation of oogonia to the next two stages of oocyte development in rainbow trout is a quick event and happens over a short period of time. The authors observed oogonia at a higher rate in PSO, but they are present throughout ovarian development stages with lesser numbers than that of the PSO ovary and ready for recruitment to the next stage when required. Similarly (Coward, 1997) also reported

presence of oogonia throughout ovarian developmental stages of tilapia, *Tilapia zillii.* As oogonia proliferation occurs by mitotic division, oogonia enter the early stages of the first meiotic division (chromatin nucleolus stage) after which they develop to the primary stage oocyte (S2).

During the resting stage, before the spawning season, ovaries of *R. sarba* generally contained primary stage two (S2) oocytes. Stage two oocytes were ellipsoid and mean oocyte shape β value was in the vicinity of 1.4. Balbiani bodies were found and appeared translucent in the cytoplasm of S2 oocytes, and these bodies increased in size as oocytes progressed in development. In late primary oocyte growth Balbiani bodies were observed to move to the periphery of the cytoplasm where they dispersed, this is in agreement with Coello & Grimm (1990). Guraya (1986) suggested that Balbiani bodies are composed of yolk nucleus and pallial substance, yolk nucleus starts developing during the chromatin-nucleolus stage as a non-basophilic structure (Maridueña, 1984). The pallial substances form during the early perinucleolar stage as a basophilic ring but less basophilic than the surrounding cytoplasm. Balbiani bodies have also been observed to be composed of RNAs, mitochondria, Golgi bodies or apparatus, endoplasmic reticulum and vesicular bodies (Guraya, 1986; Selman & Wallace, 1989; Wallace & Selman, 1990). It has been proposed that these bodies function as a centre for the biogenesis of cell organelles prior to the deposition of yolk material into oocytes (Guraya, 1986). Balbiani bodies have been observed in many fish species including Nile tilapia, *Oreochromis niloticus* (Hussein, 1984, Srisakultiew, 1993), rainbow trout, *Oncorhynchus mykiss* (Bromage & Cumaranatunga, 1988), sea bass, *Dicentrarchus labrax* (Mayer *et al.*, 1988) and Atlantic mackerel, *Scomber scombrus* (Coello &

Grimm, 1990). As oocytes develop they progress to oocyte stage three (S3), the cortical alveoli stage.

In the current study, many changes were observed in stage three (S3)oocyte, including development of various layers and deposition of vacuoles (lipid). Thecal and granulosal layers appeared in *R. sarba* oocytes during S3 of oocyte development, the cortical alveoli stage. The appearance of these two layers has been suggested to be important for oocyte maturation since steroidogenic activities increase during the active vitellogenic phase (Kagawa *et al.*, 1982, Nagahama, 1994). Gonadotropin-I (GtH-I) stimulates the production of testosterone (T) in the thecal layer, which is the outer layer of the developing oocyte. Testosterone is then converted to 17β-estradiol (E_2), produced mainly in the ovary by the granulosa layer which is the first layer surrounding the zona radiata (Wallace, 1978, Nagahama, 1994). Following the development of various layers that form what is known as 'oocyte envelope', oocyte growth is accelerated by the uptake of vitellogenin (VTG) (Tyler *et al.*, 1988 & 2000). Vitellogenin itself is synthesised in the liver and synthesis is initiated by ovarian oestrogens and pituitary gonadotropins. Vitellogenen is then transported to the developing oocytes by means of bloodstream (Wallace & Selman, 1985; Tyler *et al.*, 1988 & 2000). In *R. sarba* the oocyte diameter increased from 200μm during the cortical alveoli stage (CAS) which is classified in the current study as stage three (S3) to about 620μm during the migratory nucleus stage (MNS) which is classified in the current study as stage five (S5). Oocyte of other teleosts also exhibit an increase in size as they progresses in development from pre-vitellogenic to vitellogenic stage and the energy content in the oocyte undergoing vitellogenesis increased during the period of yolk deposition

(Eldridge *et al.*, 2002). Loir *et al.* (2001) reported that the oocyte diameter of common dentex, *Dentex dentex* increased from a maximum diameter of 220μm during CAS to a maximum diameter of 700μm during vitellogenesis. Oocyte sizes of red seabream, *Pagrus major* increased from a maximum diameter of 200μm during CAS (S3) to a maximum diameter of 530μm during MNS (S7) (Matsuyama *et al.*, 1988). In tilapia, *Tilapia zillii*, oocyte diameter was observed to increase from 280μm during CAS, (S4) to about 964μm during MNS (S7) (Coward & Bromage, 1998). In bluefin tuna, *Thunnus thynnus*; the oocyte diameter changed from 220μm during CAS (S2) to 650μm during MNS, (S5) (Corriero *et al.*, 2003).

As vitellogenin is accumulated in the oocyte it forms yolk bodies that are rich in glycoproteins, mainly lipovitellin and phosphovitin, which are rich in lipid and phosphorus (*Reviews*, Tyler & Sumpter, 1996; Coward, *et al.*, 2002; Arukwe & Goksøyr, 2003). Prior to deposition of VTG into the growing oocyte, it is sequestered in follicles by receptor-mediated endocytosis at the oocyte outer layer (Wallace *et al.*, 1987; Tyler *et al.*, 1988; Wallace & Selman, 1990). In the current study during stage four (S4), the vitellogenic stage, oocyte diameter averaged 430μm and oocytes became spherical than at other stages of oocyte development, as β value (the oocyte shape) was closer to one. Lipid vacuole and yolk globule numbers increased in the oocyte as it progressed to the following stage, which is the migratory nucleus stage (MNS).

First meiotic division takes place during the migratory nucleus stage (MNS), which initiates germinal vesicle breakdown (GVBD) whereby the nucleus moves towards the micropyle and breaks down, a phenomenon occurring in order for fertilisation to

take place (Wallace & Selman, 1981; Guraya, 1986; Bromage & Cumaranatunga, 1988). During this stage VTG sequestration discontinues at the point of germinal vesicle breakdown (GVB) (Wallace & Selman, 1985). The MNS in many studies, however, is classified as stage seven, which includes, rainbow trout, *Oncorhynchus mykiss* (Bromage & Cumaranatunga, 1988), tilapia, *Tilapia zillii* (Coward & Bromage, 1998) and red seabream, *Pagrus major* (Matsuyama *et al.*, 1988). During stage five (MNS) of oocyte development in *R. sarba*, lipid vacuole fusion was faster than of yolk globules and a double or a single of what is termed lipid globule was common. Regardless of the staging criteria used in all the available studies on oocyte classification of teleosts, fusion of lipid vacuoles and yolk globules occur during MNS (e.g. Matsuyama *et al.*, 1988, Srisakultiew, 1993), and MNS is considered the beginning of the oocyte maturation process (Guraya, 1986).

Following germinal vesicle breakdown (GVBD) during stage five (S5) the oocytes experience many changes in size and structure as they become mature during stage six (S6). During (S6), oocytes underwent hydration resulting from rapid uptake of fluid through the follicle. Yolk platelets of various sizes were observed in the oocytes as a result of yolk globule fusion, and a single oil globule also resulted from fusion of lipid vacuoles (Matsuyama *et al.*, 1988). Yolk platelets were also observed in *Oreochromis niloticus* (Srisakultiew, 1993). Single oil globules are typical in marine fish that produces pelagic eggs, these are of nutritive value and have been shown to be composed of neutral lipid: triacylglycerol (TAG) and steryl and/or wax ester (*Review*, Tocher, 2003). Since oil is less dense than the surrounding water, it aids egg and post-hatch larva buoyancy (Ehrlich & Muszynski, 1982; Buxton, 1990; Rønnestad *et al.*, 1992). Stage six oocytes reached an average diameter of 750μm,

and their shape was closer to being spherical shape than stage five (MNS, S5) of oocyte development, as the β value was closer to one. During hydration (ovulation), zona radiata also became denser and compact, a preparation in order to act as "eggshell" that is important during fertilisation and embryonic development (Grierson, & Neville, 1981). Zona radiata (*Zr*) are suggested to consist of yolk protein (*Zr*p) that is also synthesised in the liver during vitellogenesis, for further details refer to the review of Arukwe & Goksøyr (2003).

Following hydration, a process called "ovulation" takes place; the ovulated egg is delivered to the ovarian lumen ready for release into the outside environment (oviposition). As the oocyte leaves the follicular lumen that it used to occupy, it leaves the granulosa and thecal layers behind and these are termed "post ovulatory follicles" (POF). During the current study, however, the stage at which POF were seen could have been at the second stage of the degenerative process (Matsuyama *et al.*, 1988) since what is termed 'oocyte lumen' was not observed. Also, in the current study POF were seen containing large numbers of oogonia which meant that oogonia proliferation had already taken place as has been suggested by Kagawa *et al.* (1981). The reason for not seeing what is termed as collapsed follicle lumen (Kagawa *et al.*, 1981) may possibly be attributed to sampling time. Matsuyama *et al.* (1988) showed the existence of a large follicular lumen immediately after the spawning event for red seabream, *Pagrus major*. These authors suggested three stages in the degenerative process in which empty lumen that was formally occupied by the oocytes become filled with ovarian connective tissue "stroma" and degenerated oogonia. Post ovulatory follicles (POF) have been reported in other fish species such as white spotted char, *Salvelinus leucomaenis* (Kagawa *et al.*, 1981),

Pacific sardine, *Sardinops sagax* (Chong *et al.*, 1991), tilapia *Oreochromis niloticus* (Srisakultiew, 1993) and *Tilapia zillii* (Coward & Bromage, 1998).

In the current study atretic oocytes were found in mature and post spawning fish, on some occasions atresia was found during the reproductive season. The diameter of the atretic oocyte (α) stage was 500μm and was similar to that of stage four oocytes (S4). Its shape, however, was ellipsoid and β value (oocyte shape) was about 1.4 similar to that of stage two oocytes. Atresia was not common in immature ovaries (pre-vitellogenic stage). Similar observation have been reported in other teleosts such as tilapia, *Oreochromis niloticus* (Srisakultiew, 1993); *Tilapia zillii* (Coward & Bromage, 1998) and bluefin tuna, *Thunnus thynnus* (Corriero *et al.*, 2003). Many explanations have been suggested for atresia, including feeding (Bromage & Cumaranatunga, 1987; Ma *et al.*, 1998), condition of hormone in the fish (Bromage & Cumaranatunga, 1988) which may be caused by overcrowding and other environmental effects such as water temperature and age of the fish (Guraya, 1986). All the given conditions may contribute to stress and hence unsuccessful completion of the maturation phase (Mylonas *et al.*, 1997). Taking these factors into account, there are studies, however, which have suggested that atresia is not directly related to animal condition when sampled such as in Atlantic cod, (Kjesbu *et al.*, 1996) and Atlantic herring, *Clupea harengus* (Kurita *et al.*, 2003). While others have suggested that providing fish with well-balanced rations may reduce atresia (Bromage & Cumaranatunga, 1987) or eliminated (Tyler & Sumpter, 1996). At the end of the reproductive season in the current study, all vitellogenic oocytes that were not developed to final stages of maturation were resorbed. The incidence of atresia was at its highest during this time, in agreement with Hunter *et al.* (1986).

During the current study a factor that might have contributed to atresia could be the stress imposed on fish during the time spent in the fishing traps (Dettlaff & Davydova, 1979). During the (δ) stage of atresia, brown bodies (Chan *et al*, 1967) were observed but were restricted to post-spawning ovaries. The reason could be that no female was sampled during the reproductive season that contained brown bodies, or this event may be a transitional phase.

The stereological study of female ovaries revealed that stage two oocytes of development had the highest volume fraction/area fraction (V_{vx}/V_{ax}) in all ovarian stages during the reproductive season. This could be related to the fact that those ovaries had experienced spawning and were experiencing the development of oocytes at a higher rate than at a later stage of ovarian development.

Stage three oocytes V_{vx}/V_{ax} were found to be smaller in all ovaries studied. Their V_{vx}/V_{ax} did not exceed 7%, as found in stage four ovaries in early vitellogenic stages tending to decrease as ovaries progressed in development towards the hydration process. This may be due to the fact that the S3 oocyte stages of development were quick events experienced by ovaries and soon after this stage oocytes progressed to the vitellogenin stage. Stage four oocytes (S4) V_{vx}/V_{ax} levels were highest in all ovarian stages, due to the fact that this event in the oocyte developmental stage is a prolonged one (Yamamoto, 1956). Stage five oocytes (S5) did not exist in hydrated ovaries (stage six ovaries) and this again could be attributed to the fact that this event is also a rapid one among oocyte developmental stages. 20%-60% of the vitellogenic oocytes reached the hydration stage and the percentage varied between individuals, this could be attributed to the atretic levels that this species experiences.

Atretic oocytes were found to occupy higher V_{vx}/V_{ax} in stage four ovaries; this could be due to the fact that during this stage the oocytes were more vulnerable to atresia than at other stages of development, this agrees with Micale *et al* (1997). V_{vx}/V_{ax} was found to be minimal in hydrated ovaries and mainly stage-γ. The numerical density per unit area/volume (*Nvx/Nax*) study showed that the N_{vx} of atretic oocytes (stage-α) was highest during the early vitellogenic stage (stage four ovaries) and declined substantially as the ovaries developed into the final stage of maturation. Similar observations were reported in Atlantic mackerel (Greer Walker *et al.*, 1994). It has been suggested that this is due to some feedback mechanism that may exist to use the yolk resources more efficiently at this stage of ovarian development in order to allow minimum resorption in spent ovaries (Greer Walker *et al.*, 1994). The numerical density *Nvx/Nax* of all oocytes stages declined beyond the age of five years which suggests that fecundity decreases after this age.

Validation of stereology did not show significant differences between numerical density using stereology techniques and direct counting carried out microscopically on stage six oocytes (S6, hydrated oocytes). The direct counting method did not reveal any significant differences between the two lobes of the ovaries with regard to the number of hydrated oocytes (S6) an observation in agreement with El-Agami (1989). Despite the fact that validation was carried out on only S6 oocytes, but stereological techniques have been validated in earlier work carried on asynchronous ovaries by Srisakultiew (1993) and Coward & Bromage (2002) who did not find any significant difference between direct oocyte counting from Gilson's fluid and stereological methods. Similarly, Srisakultiew (1993) did not observe any significant differences among stereological methods (graphical, mass and

intersection methods) used to estimate the volume fraction of oocytes. in earlier work carried out by Emerson *et al.* (1990) a comparison was made between stereological methods and volumetric and automated particle counter methods in both sole and herring. The authors did not find any significant differences between these three methods used to estimate fish fecundity, which indicate the effectiveness of stereology.

Total plasma calcium in *R. sarba* increased after the onset of yolk deposition in the oocytes (S4) above the basal level, the levels measured during the resting stage of the ovarian development. This reflected the vitellogenin level since Ca^{2+} is a major calcium-binding protein in female fish undergoing oocyte yolk deposition (Fleming *et al.*, 1964; Tyler & Sumpter, 1990). Linares-Casenave *et al.* (2003) also reported that Ca^{2+} elevates after the onset of yolk deposition in the oocytes of cultured white sturgeon, *Acipenser transmontanus*. It has to be pointed out that quantifying plasma Ca^{2+} is indirect method to estimate plasma VTG. However, plasma VTG is measured by more direct and accurate method such as radial immunodiffusion (Fujii *et al.*, 1991) or by an enzyme-linked immunosorbent assay (ELISA) (Cuisset *et al.*, 1991). Mosconi *et al.* (1998) carried out purification and validation of (ELISA) for gilthead seabream, *Sparus aurata*, they concluded that ELISA can be used for a rapid and reliable measurement of plasma VTG in this species. Nevertheless, plasma VTG and Ca^{2+} shown to have a linear relationship as it have been witnessed by (Linares-Casenave *et al.*, 2003). Calcium levels therefore, are used to differentiate maturity stage and sex without the need to use surgical biopsies (Webb *et al.*, 2002). Calcium is necessary for the formation of vitellogenin molecules (de Vlaming *et al.*, 1980; Yeo & Mugiya 1997).

The present study also provides a description of the histology of the testes of *R. sarba* in the Gulf of Oman. Stages of development of testes were classified into immature, developing, active and resting.

A change in the type of cell division that occurs in the germinal epithelium of the testis during the reproductive cycle helped to assess the maturity stage of male *R. sarba*. Spermatogonial proliferation (mitosis) and subsequent intensive sperm production (meiosis) correlated well with change in water temperature. Increase in water temperature at the end of the spawning season drove the testes back to the mitosis phase of development and spermatogonial proliferation started in early winter. Changes in environmental cues such as water temperature have been found to initiate breeding (Rubenstein & Wikelski, 2003).

Early spermatogenesis was observed from November onwards during both sampling periods and active testes were at their highest level during January and February. Fully active testes accounted for about of 96% of sampled testes during the breeding season (January- March). Spermatogenesis continued until the end of February when the percentage of active testes started to decrease while resting testes started to appear and increased towards the end of March. The stripping of individuals was only possible at the active stage when testes became mature and all lobules and the main sperm duct were filled with spermatozoa. Distinct differences between the onset of the gonad development between males and females existed. Sexually active males were observed in November, that is two months prior to the spawning season that started in late December to early January during the two spawning seasons. Variations of gonads development timing in fish between males and females, and

the advancement of male gonads compared to female has been noted elsewhere (Carrasson & Bau, 2003).

The gonads walls (tunica albuginea) became thin as a result of progressive expansion of the testes with maturation. Thickness of tunica in resting testes was greater than at other stages of development and thickness was correlated with maturity. Tunica thickness in *R. sarba,* therefore, was a good indicator of maturity as *R. sarba* had exhibited significant changes in tunica thickness during the developmental stages of reproduction. Similar observations have been reported in other tropical fishes, for example Nassau grouper, *Epinephelus striatus* (Sadovy & Colin, 1995) and cobia, *Rachycentron canadu* (L.) (Brown-Peterson *et al.,* 2002). Enlargement of the surface of the lobular lumen during the reproductive season is attributed to the fact that more spermatids cells burst and releasing active spermatozoa into the lumen, resulting in expansion. Brown-Peterson *et al.* (2002) concluded that classifications of testicular development made on the bases of the nature and changes that take place in the germinal epithelium provide much better classification criteria than using the traditional terminology.

Spermatozoa were present in all testes with different quantities depending on the maturity status of the testes. The existence of sperm in the lobular lumen and the duct particularly in immature testes outwith the breeding season, is natural (Htun-Han, 1978; Coetzee, 1986).

Sex change has been suggested as an alternative reproduction strategy carried out by sparid species to maximise lifetime reproductive success by functioning as one sex

when small and as the other at later stage (Pajuelo & Lorenzo, 1996). Sex changes in *R. sarba* in the Gulf of Oman occurred between 200 and 300mm FL. Yeung & Chan (1987, b) have shown that the sex change of *R. sarba* occurs at a smaller size than 200mm F.L. Intersex (transaction) gonads were higher during the zero and two years of age, during this age the percentage of individuals with intersex gonads was above 30%. Beyond the age of two years, this frequency declined. The intersex gonads in *R. sarba* were found up to the age of seven years and beyond this age no intersex gonads were observed. Garrat (1993) reported that *Acanthopagrus berba* sex changed at a length similar that found in this study. Matsuyama *et al.* (1988) observed higher hermaphrodite numbers among captive reared *Pagrus major* juveniles compared to wild. The authors relate this to differences in food availability and higher water temperatures for reared populations as opposed to their wild counterpart. Intersex *R. sarba* were not discussed in detail since it is already described earlier with histological evidence (Yeung & Chan (1987, b).

Chapter 5: Body lipid dynamics of female *R. sarba* and fatty acid profile in the ovary during the reproductive season

5.1 Introduction

The reproductive cycles of tropical species are seasonal being driven by changes in environmental factors such as water temperature and photo-period (Robertson, 1990). It has been suggested that fish feed extensively in the early stages of maturation with a view to increasing energy reserves to meet future requirements for sexual maturation and subsequent gonadal development (Love, 1980). Many studies have indicated that fish deposit fat that leads to a rise in body condition (Fishelson *et al.*, 1987). Fish attempt to reduce their feed intake during the reproductive season and during the low feeding period tend to mobilise energy, essential fatty acids and other nutrients from various body compartments to meet the requirement for maintenance, physical activity and sexual maturation (Love, 1980; Nassour & Leger 1989).

Eliassen & Vahl (1982) and Tocher & Harvie (1988) suggest that growth of gonads before the onset of the spawning season in cod, *Gadus morhua* leads to a decrease of about one-third in lipid reserves in liver and muscle. Female rainbow trout *Oncorhynchus mykiss* (Walbaum) mobilize mainly visceral lipid reserves (Nassour & Leger, 1989). In Arctic charr, *Salvelinus alpinus* the carcass accounts for about 50% of the total lipid (TL) content as the fish move into the freshwater environment for reproduction (Jobling *et al.*, 1998). The authors suggest that the TL of the body decreases 30-40% during maturation. Batch spawners such as gilthead seabream continue to feed throughout the spawning season producing a total egg biomass greater than their own body mass and therefore some of the energy deposited in the egg is derived from their diet (Almansa *et al.*, 2001). The authors found that in this species lipid reserves were stored in the liver and muscle before vitellogenesis.

These reserves, however, were mobilised to meet the demand for energy in egg production and/or energy substrate for specific lipoprotein synthesis.

Lipids and their constituent fatty acids (FA) have a major effect on reproductive success, egg viability and survival of offspring (Wiegand, 1996). The level of FA composition must satisfy embryonic nutrition needs for development and subsequent growth. It is essential that lipid deposited in the egg fulfils the nutritional requirements of larvae during their early stages of development until they physically start exogenous feeding (Craik & Harvey, 1987).

Lipids and their fatty acids have a number of biological functions in fish such as acting as a substrate for catabolism, a structural component in cell membrane and as a precursor for chemical messengers (Tocher, 2003, Tveiten *et al.*, 2004). Besides the fact that fatty acids are the major source of metabolic energy in fish throughout its life cycle, they also act as a major source of metabolic energy for reproductive activity (Henderson *et al.*, 1984, Watanabe *et al.*, 1984).

Marine fish contain large amounts of saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA), particularly highly unsaturated fatty acids (HUFA) of the n-3 series (n-3 HUFA). The lipid content in fish eggs is found in two main reserves, the lipoprotein yolk (LPY) which predominantly contains phospholipid beside some neutral lipids and the oil that exists in the form of droplets or large oil globules. The oil droplet is composed of neutral lipid: triacylglycerol (TAG) and steryl and/or wax ester. For a review of lipid and fatty acids in teleosts, see Sargent *et al.* (1995), Wiegand (1996) and Tocher (2003).

It has been demonstrated that PUFA composition of the diet can affect the pituitary of teleosts and gonadal hormone levels, which in turn affect reproductive performance (Sorbera *et al.*, 1996). In the n-3 and n-6 series there are fatty acids of biological importance, particularly docosahexaenoic acid (22:6n-3, DHA), eicosapentaenoic acid (20:5n-3, EPA) and arachidonic acid (20:4n-6, AA). Marine fish are not able to synthesise DHA and EPA from shorter chain precursors of PUFA such as linoleate (18:2n-6) and linolenate (18:3n-3) due to weakness in the activity of Δ5- desaturase. DHA and EPA, therefore, are converted to the functionally active HUFA and thus they are termed essential fatty acids (EFA) (Sargent *et al.*, 1995).

Studies on the EFA (essential fatty acid) requirements of marine fish species have shown that the n-3 EFA requirements can only be met by EPA (20:5n-3) and DHA (22:6n-3) (Tocher, 2003). EFA contents are one of the nutritional factors that greatly affectss spawning quality in fish (Watanabe *et al.*, 1984). EPA is a precursor of prostaglandins (PG) from series III, one of the major PG components synthesised in marine fish (Sorensen *et al.*, 1988). Fernandez-Palacios *et al.* (1995) found a positive correlation between egg quality in gilthead seabream (*Sparus aurata* L) and dietary n-3 HUFA. The authors stated that numbers of unfertilised eggs were significantly reduced by an increase in dietary 20:5n-3 (EPA) fatty acid.

Sufficient DHA level is essential for marine fish larvae especially in its rapid growing and developing phase, as larvae at this stage have a high percentage of neural tissue in their relatively small body mass (Tocher & Harvie, 1988; Bell &

Dick, 1991). DHA deficiency has been demonstrated to weaken vision at low light intensities in juvenile herring (*Clupea harengus* L.) (Bell *et al.*, 1995). DHA, however, was suggested to be of greater importance in embryonic development than EPA (Watanabe, 1993). This, it has been suggested, is due to the fact that marine fish are unable to elongate and desaturate EPA to DHA at a sufficient rate to fulfill requirements (Watanabe, 1993, Sargent *et al.*, 1995).

Arachidonic acid (20:4n-6, AA) is an essential fatty acid and is the precursor of PG from series II. Some PGs produced by the female goldfish, such as PGFs, have an important role as pheromones, stimulating male sexual behavior and synchronizing male and female spawning, thus directly affecting fertilization success (Sorensen *et al.*, 1988). Castell *et al.* (1994) demonstrated that AA is important in growth, development and eicosanoid production.

As more attention is paid to absolute amounts of fatty acids, it is of similar importance to look at the relative proportions of n-3 n-6 DHA EPA, and AA EPA (March, 1993). It is important that eggs contain the correct balance of DHA:EPA to provide better larval development on hatching (Bell *et al.*, 1997). Marine fish have a limited ability to convert EPA to DHA (Sargent *et al.*, 1995) which makes it important that the dietary ratio of DHA:EPA is optimal to supply sufficient 22:6n-3 (Bell *et al.*, 1997).

Information about energy location and lipid dynamics in female *R. sarba* is lacking. The objectives of the present investigation were to quantify seasonal lipid dynamics in goldlined sea bream, *R. sarba* and thereby describe quantitative changes in

various organs and tissues during maturation. Also, it has been well demonstrated that FA profile and composition of lipids from fish eggs reflect dietary FA provided to broodstock (Harrel *et al.*, 1994, Almansa, *et al.* 1999). No data are available on the FA composition of goldlined seabream *R. sarba.* Information in this respect can be provided through the study of wild female ovaries during the reproductive season.

5.2 Materials and methods

5.2.1 Description of the samples

Fish specimens were sampled as detailed in section 2.1.1. Fish were dissected and the following tissues and organs carefully separated with a scalpel: visceral fat, stomach and intestine, liver, gonad, sub-sample of lateral muscle and carcass. The carcass was minced whole, homogenised and a sub sample collected. All the visceral fat mass in the abdominal cavity was carefully removed and the weight was recorded to the nearest 0.001g. Sub samples were taken from the lateral muscle under the first dorsal fin. All tissues and organs were stored in test tubes at –60°C until further analysis.

5.2.2 Extraction of total lipid

Approximately 1g of wet tissue were weighed to four decimal places and used for total lipid extraction. Total lipid extraction was performed using the Folch-Lee extraction method (Folch *et al.*, 1957) as detailed in section 2.1.9.

5.2.3 Fatty acid analysis

The fatty acid composition of the total lipids of each female's ovary was determined by gas-liquid chromatography of the methyl ester derivatives prepared as described in section 2.1.10.

5.3 Results

5.3.1 Total lipid in the ovaries

Percentage mean total lipid (TL) in ovaries was found to be significantly different between maturity stages ($p<0.001$). As ovaries progressed during maturity, mean TL increased (Figure 5.1). TL in mature ovaries (stage four, five, and six) was higher than in stages two, three and the post-spawning stage. TL in mature ovary (stage 4) increased about 63% over that found in immature ovary (S2). Significant differences between stages of maturity in terms of percentage of TL in the ovaries are given in Figure 5.1.

Mean percentage TL in ovaries was found to be significantly different between months ($p<0.001$). TL in ovaries began to increase from November until January; thereafter as the spawning continued TL in ovaries started to decline (Figure 5.1). Ovaries that were sampled during the spawning season in January-February had the highest level of TL compared to other months, with the highest value being in January when TL increased 75% from that recorded in October. Significant differences between months are given in Figure 5.1.

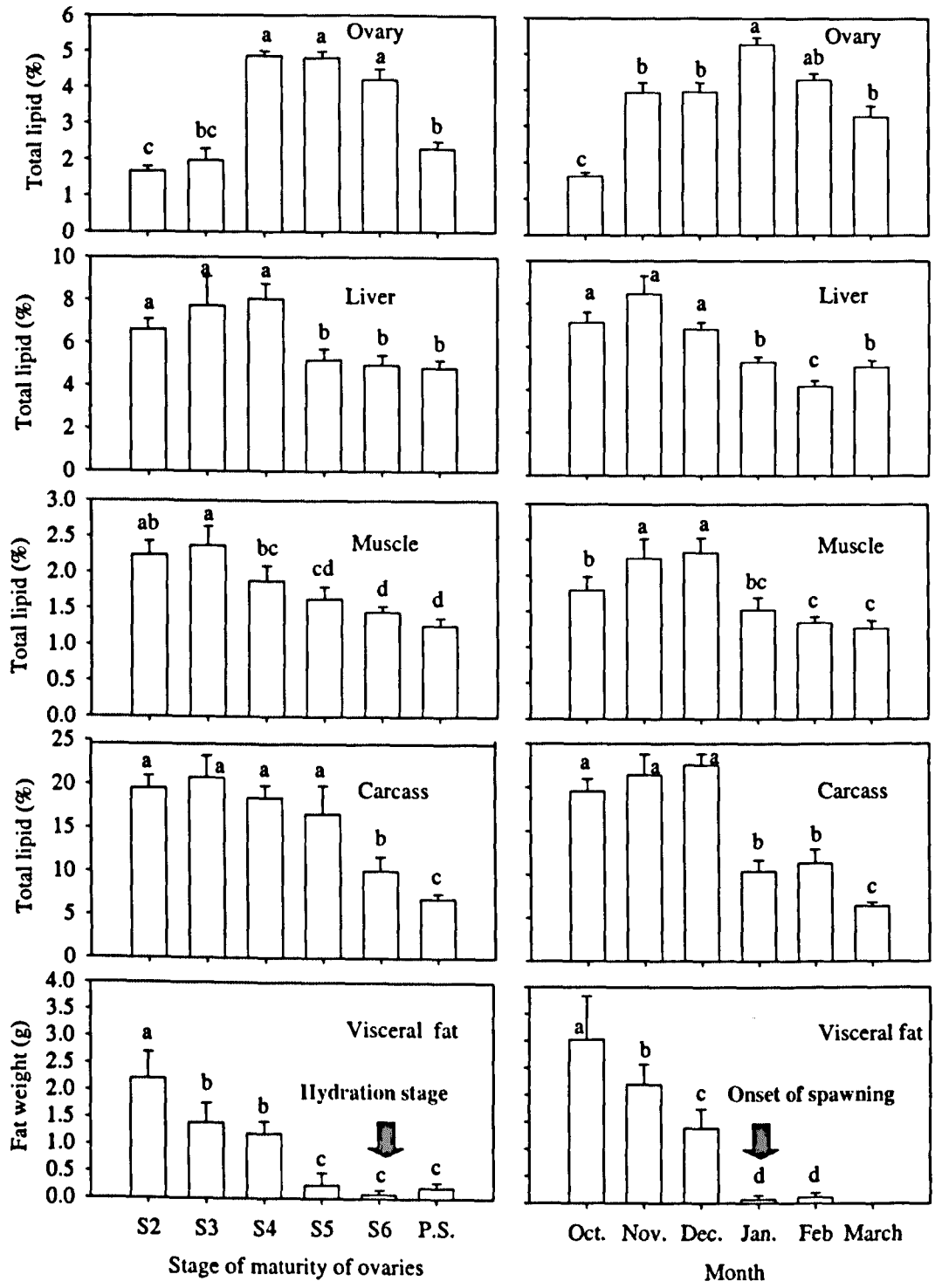


Figure 5.1 Changes in total lipid in ovary, liver, muscle, carcass and perivisceral fat of female *R. sarba* during the reproductive season in relation to stage of maturity and time (2002-2003).

Data is presented as average value ± S.E. Values that are not significantly different ($p>0.05$) share common superscripts. Arrow indicates the onset of spawning.

Percentage of TL in ovaries was significantly correlated with stage of maturity, fish length and fish weight, Table 5.1. The content of TL lipid in the ovary was closely related to female size. Ovaries of larger and heavier females had higher TL while smaller and lighter females had lower ovarian TL.

Table 5.1 Correlation coefficients calculated for the percentage of total lipid (TL) in female *R. sarba* during the reproductive season.

	Total lipid (%)				
PARAMETER	OVARY	LIVER	MUSCLE	CARCASS	PERIVISCERAL FAT
Maturity stage	r = 0.307 p =0.001	r = -0.218 p=0.017	r = -0.265 p=0.01	r = -0.424 p = 0.001	r = -0.403 p=0.001
Fish length (FL)	r = 0.343 p=0.001	r = 0.091 p>0.05	r = -0.141 p>0.05	r = -0.275 p=0.005	r = -0.028 p>0.05
Fish weight (TW)	r = 0.161 p=0.047	r = 0.038 p>0.05	r = - 0.109 p>0.05	r = -0.212 p=0.031	r = 0.066 p>0.05

5.3.2 Total lipid in the liver

The percentage of TL in the liver of female *R. sarba* was significantly different between maturity stages (p=0.03). The level of TL in the liver decreased significantly during stage five and onwards. The percentage of TL of stage two, three and four females was highest compared to other stages of maturity and significantly different from stage five, six and post-spawning. TL levels in livers of stage five, six and post-spawning (PS) fish were similar. Significant differences between stages of female maturity in livers TL percentage are given in Figure 5.1.

No significant differences were found in liver TL during the months before the spawning season. As reproduction commenced in January and February liver TL

started to fall, and TL level of TL differed significantly between these two months. Liver TL started to increase again to a level similar to that of January (Figure 5.1). Mean percentage liver TL was found to be significantly different between months ($p<0.001$). Significant differences between months in terms of mean of percentage TL are given in Figure 5.1.

Liver TL in the liver was negatively related to maturity stage of the fish, as females progressed in maturity level liver TL decreased. In contrast, ovarian TL and female size were unrelated, Table 5.1.

5.3.3 Total lipid in the lateral muscle

Percentage of mean TL in the lateral muscle was significantly different between maturity stages of females ($p=0.02$). Generally, as females matured, the mean percentage muscle TL decreased (Figure 5.1). Females at post-spawning stages had the lowest mean percentage TL which was significantly different to stage two, three and four.

TL in the muscle began to increase in November, peaked in December then started to decline in January during the onset of the spawning season (Figure 5.1). Mean percentage muscle TL was found to be significantly different between months ($p<0.001$). Fish that were sampled during the spawning season in January-February had a lower TL level compared to the period that fell before the spawning season (October-December). However, muscle TL level at the end of the spawning season was lowest. Significant differences between months are given in Figure 5.1.

Percentage lateral muscle TL was significantly correlated with female maturity. The negative relationship suggested that TL decreased with advancement of ovarian development. Muscle TL of females during the reproductive season, however, was not correlated with fish length and weight, Table 5.1. TL in the lateral muscle fluctuated with the advancement of the ovarian maturation regardless of the female size.

5.3.4 Total lipid carcass

TL in the carcass was significantly different between maturity stages of females ($p<0.001$). Immature females had the highest level of TL, and as the females matured the level of TL started to decrease significantly reaching its lowest level at the post-spawning stage (Figure 5.1).

TL in the carcass continued to increase until December and from January onwards it started to decline (Figure 5.1). Mean percentage carcass TL was found to be significantly different between months ($p<0.001$). TL decreased approximately 13% during the spawning season in January-February from the level that was recorded before spawning (November-December) in immature fish. However, carcass TL level at the end of the spawning season was lowest and decreased approximately 19% from the immature level. The significant differences between months are given in Figure 5.1.

TL in the carcass was negatively correlated with maturity of females. Carcass TL was maturity dependent rather than female size dependant (Table 5.1).

5.3.5 Weight of the visceral fat

Percentage mean weight of viscera (VF) fat was significantly different between female maturity stages ($p<0.001$). Immature females (S2) had the highest level and as fish matured VF decreased significantly. In females that had stage six ovaries, VF was lowest and decreased by approximately 97% from that of the immature stage (S2) (Figure 5.1). During the post spawning stage, however, females started accumulating fat in the viscera again and hence the level rose (Figure 5.1).

VF in females continued to decrease from November onwards whilst there was concomitant increase in ovary TL. For the duration of the spawning event, which took place during January and February, VF was at the minimum level. During this period the level decreased up to 98% from the level recorded in October (Figure 5.1). Mean VF was found to be significantly different between months ($p<0.001$). No VF was observed in post-spawning females sampled in March (Figure 5.1).

Percentage VF was significantly correlated with female maturity stage, a negative relation which showed that as females matured VF decreased. No correlation, however, was found between VF and fish length and weight, and therefore, size of fish had no influence on VF, Table 5.1.

5.3.6 Fatty acids composition in ovary

The major fatty acids in the total lipids from *R. sarba* ovary were myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0), palmitoleic acid (16:1 n-7), oleic acid (18:1 n-9), linolic acid (18:2 n-6), arachidonic acid (20:4n-6) eicosapentaenoic

acid (20:5 n-3), docosapentaenoic acid (22:5n-3) and docosahexaenoic acid (22:6 n-3).

The composition of fatty acids (FA) in the ovary during stage two to six including the post-spawning stage is shown in Table 5.3. The profile of the total fatty acids in each lipid class is given in Figure 5.2,a. Data on the composition of FA for pooled immature (stage two & three), mature (Stage four, five & six) and post-spawning (PS) is given in Table 5.3 and the profile of the total fatty acids in each lipid class is given in Figure 5.2, b.

Table 5.2 Total lipid content (% of wet weight) and fatty acid composition (% of fatty acids) of total lipid from ovary of goldlined seabream *R. sarba* during the reproductive season.

FATTY ACID	STAGE 2	STAGE 3	STAGE 4	STAGE 5	STAGE 6	PS
Lipid content	1.5±0.5	2.0±0.7	4.9±1.0	4.8±0.8	4.4±0.8	2.3±1.0
14	2.7±0.5^{a}	1.7±0.6^{bc}	1.8±0.2^{bc}	2.3±0.5^{ab}	2.0±0.2^{ab}	1.3±0.2^{c}
15	1.2±0.2	1.1±0.4	1.3±0.6	1.1±0.3	1.0±0.2	0.8±0.1
16	25.6±3.8^{a}	23.5±2.2^{a}	22.5±2.5^{b}	21.4±2.5^{b}	22.9±1.7^{b}	17.8±3.2^{c}
18	12.3±0.1^{a}	10.1±1.8^{b}	6.3±0.8^{c}	6.2±0.8^{c}	6.4±0.8^{c}	8.3±1.2^{bc}
20	0.6±0.1^{a}	0.4±0.2^{ab}	0.2±0.0^{c}	0.2±0.1^{bc}	0.1±0.0^{c}	0.3±0.1^{bc}
22	0.5±0.2^{a}	0.4±0.1^{ab}	0.2±0.1^{bc}	0.2±0.1^{bc}	0.1±0.1^{c}	0.4±0.1^{ab}
Σ Saturated	42.9±4.1^{a}	37.2±2.8^{ab}	32.2±3.1^{bc}	31.4±2.7^{bc}	32.5±1.5^{bc}	28.9±4.2^{c}
16:1n-9	0.1±0.0^{a}	0.1±0.0^{a}	0.9±0.2^{bc}	0.8±0.4	1.1±0.4^{c}	0.3±0.1^{ab}
16:1n-7	4.6±0.9	3.6±0.5	4.2±0.8	4.6±0.4	4.1±0.4	2.9±0.4^{a}
18:1n-9	17.3±4.4	14.0±3.2	18.7±5.6	15.4±3.8	16.6±1.6	13.6±3.4^{a}
18:1n-7	3.8±0.4	2.7±1.4	3.6±0.7	3.1±0.5	3.1±0.3	3.2±0.7
20:1n-11	1.2±0.4^{a}	0.8±0.6^{ab}	0.6±0.5^{ab}	0.5±0.3^{ab}	0.3±0.2^{b}	0.3±0.1^{b}
20:1n-9	0.8±0.1^{a}	0.7±0.1^{ab}	0.6±0.1^{bc}	0.5±0.2^{bc}	0.4±0.0^{c}	0.6±0.1^{ab}
20:1n-7	0.5±0.1^{a}	0.3±0.1^{ab}	0.3±0.2^{ab}	0.5±0.1^{ab}	0.2±0.1^{b}	0.2±0.1^{b}
22:1	0.5±0.1	0.4±0.2	0.3±0.1	0.2±0.1	0.4±0.3	0.3±0.1
24:1n-9	0.6±0.3^{ab}	0.9±0.2^{bc}	0.4±0.1^{a}	0.5±0.2^{ab}	0.4±0.1^{a}	1.2±0.3^{c}
Σ Monoenes	29.4±5.0	23.4±2.9	29.7±4.7	25.8±3.1	26.6±3.6	22.6±4.2
18:2n-6	4.1±1.6	3.3±3.0	5.4±4.2	5.4±2.5	5.8±2.3	3.6±2.7
18:3n-6	0.3±0.2	0.4±0.3	0.2±0.1	0.2±0.1	0.3±0.1	0.4±0.1
20:2n-6	0.6±0.1	0.5±0.1	0.5±0.1	0.4±0.1	0.4±0.2	0.4±0.1
20:3n-6	0.3±0.1	0.3±0.2	0.2±0.1	0.2±0.1	0.2±0.0	0.3±0.2
20:4n-6 AA	4.5±1.7^{a}	10.6±2.8^{bc}	6.9±3.0^{ab}	5.7±1.2^{a}	5.3±1.7^{a}	11.7±1.2^{c}
22:4n-6	1.4±0.7	1.2±0.6	1.4±0.5	1.3±0.5	0.9±0.3	2.0±0.6
22:5n-6	0.6±0.2^{ab}	0.9±0.1^{bc}	0.9±0.2^{bc}	0.9±0.3^{bc}	0.7±0.2^{bc}	1.3±0.5^{c}
Σ n-6 PUFA	11.7±1.7^{a}	17.3±3.2^{ab}	16.8±5.3^{ab}	14.2±3.4^{ab}	13.4±2.7^{ab}	19.5±1.9^{b}
18:3n-3	0.5±0.2	0.3±0.2	0.6±0.4	0.8±0.2	0.8±0.3	0.3±0.2
18:4n-3	0.4±0.1	0.3±0.1	0.4±0.1	0.3±0.0	0.3±0.1	0.2±0.1
20:3n-3	0.1±0.0	0.2±0.0	0.1±0.1	0.2±0.1	0.1±0.1	0.1±0.0
20:4n-3	0.2±0.1^{ac}	0.2±0.1^{ac}	0.3±0.1^{ac}	0.3±0.1^{ac}	0.3±0.1^{ab}	0.1±0.0^{c}
20:5n-3 EPA	2.7±1.1^{a}	3.6±1.0^{ab}	3.3±1.5^{ab}	4.2±1.0^{ab}	4.7±1.0^{ab}	5.1±0.8^{cb}
22:5n-3	3.3±1.3	3.3±1.4	3.9±1.7	4.2±0.4	4.8±1.3	4.0±1.4
22:6n-3 DHA	6.5±3.3^{a}	11.0±3.9^{ab}	12.2±3.8^{ab}	18.6±3.8^{b}	15.9±3.5^{b}	14.9±7.1^{ab}
Σ n-3 PUFA	13.7±5.7^{a}	18.8±6.3^{ab}	20.8±6.8^{ab}	28.0±4.9^{b}	26.4±5.2^{b}	24.7±7.4^{ab}
Σ PUFA	25.4±7.4^{a}	36.0±3.5^{ab}	37.6±4.9^{b}	42.8±4.7^{b}	39.8±4.3^{b}	44.2±7.2^{b}
n-3:n-6	1.2±2.0^{a}	1.2±0.6^{a}	1.5±0.3^{ab}	2.2±0.4^{b}	2.1±0.3^{b}	1.3±0.4^{a}
DHA:EPA	2.3±0.4^{a}	3.0±0.7^{b}	4.0±1.0^{b}	4.4±0.5^{b}	3.2±0.2^{ab}	3.0±1.6^{ab}
AA:EPA	1.7±0.2^{ae}	3.1±1.1^{be}	6.9±2.0^{c}	1.4±0.1^{de}	1.1±0.1^{d}	2.3±0.4^{e}

Means given with ±S.D. (n=5). Values within rows sharing common superscript are not significantly different (p≥0.05). PS, post spawning; PUFA, polyunsaturated fatty acids; DHA, Docosahexaenoic acid; EPA, eicosapentaenoic acid, AA, Arachidonic acid.

Table 5.3 Levels of fatty acid composition (% of fatty acids) of total lipid in immature, mature and post-spawning *R. sarba* ovaries during the reproductive season.

FATTY ACID	IMMATURE OVARIES	MATURE OVARIES	POST-SPAWNING OVARIES
Lipid content	1.6±0.6[a]	4.8±1.0[b]	2.3±1.0[a]
14	2.2±0.8[a]	2.0±0.4[a]	1.3±0.2[b]
15	1.2±0.3	1.1±0.4	0.8±0.1
16	24.5±3.2[a]	22.3±2.2[a]	17.8±3.2[b]
18	11.2±1.8[a]	6.3±0.8[b]	8.3±1.2[c]
20	0.5±0.2[a]	0.2±0.1[b]	0.3±0.1[b]
22	0.4±0.2[a]	0.2±0.1[b]	0.4±0.0[a]
Σ Saturated	40.0±4.5[a]	32.1±2.4[b]	28.9±4.2[b]
16:1n-9	0.1±0.0[a]	1.0±0.4[b]	0.3±0.1[a]
16:1n-7	4.1±0.8[a]	4.2±0.6[a]	2.9±0.4[b]
18:1n-9	15.6±4.1	17.1±0.4	13.6±3.4
18:1n-7	3.2±1.1	3.3±0.6	3.2±0.7
20:1n-11	1.0±0.5[a]	0.5±0.4[b]	0.3±0.1[b]
20:1n-9	0.8±0.1[a]	0.5±0.1[b]	0.6±0.1[ab]
20:1n-7	0.4±0.1	0.3±0.2	0.2±0.1
22:1	0.4±0.3	0.3±0.3	0.3±0.1
24:1n-9	0.8±0.3[a]	0.4±0.1[b]	1.2±0.3[c]
Σ Monoenes	26.4±5.0	27.6±4.1	22.6±4.2
18:2n-6	3.7±2.3	6.1±2.6	3.6±2.7
18:3n-6	0.4±0.2[a]	0.2±0.1[b]	0.4±0.1[a]
20:2n-6	0.5±0.1	0.4±0.1	0.4±0.1
20:3n-6	0.3±0.2	0.2±0.1	0.3±0.2
20:4n-6 AA	7.5±3.7[a]	6.0±2.2[a]	11.7±1.2[b]
22:4n-6	1.4±0.6[ab]	1.2±0.5[a]	2.0±0.6[b]
22:5n-6	0.7±0.3[a]	0.8±0.2[a]	1.3±0.5[b]
Σ n-6 PUFA	14.5±3.8	15.0±4.1	19.5±1.9
18:3n-3	0.4±0.2[ac]	0.7±0.3[b]	0.3±0.2[c]
18:4n-3	0.3±0.1	0.3±0.1	0.2±0.1
20:3n-3	0.1±0.1	0.1±0.1	0.1±0.0
20:4n-3	0.2±0.1	0.3±0.1	0.1±0.0
20:5n-3 EPA	3.2±1.1[a]	4.0±1.3[a]	5.1±0.5[b]
22:5n-3	3.3±0.8	4.3±1.3	4.0±1.4
22:6n-3DHA	8.7±4.1[a]	14.9±4.3[b]	14.9±7.1[b]
Σ n-3 PUFA	16.2±6.3[a]	24.7±6.5[b]	24.7±7.4[b]
Σ PUFA	30.7±7.8[a]	39.7±4.8[b]	44.2±7.2[b]
n-3:n-6	1.2±0.4[a]	1.8±0.3[b]	1.3±0.4[ab]
DHA:EPA	2.7±0.7[a]	3.9±0.6[b]	3.0±1.6[ab]
AA:EPA	2.4±1.1	1.7±1.0	2.3±0.4

Results are means ±S.D. (n=5 for immature and mature ovaries and n=10 for PS). Values sharing common superscript within rows are not significantly different ($p \geq 0.05$). PUFA, polyunsaturated fatty acids; DHA, Docosahexaenoic acid; EPA, eicosapentaenoic acid, AA, Arachidonic acid. Immature ovaries, stage two & three; mature ovaries, stage four, five & six.

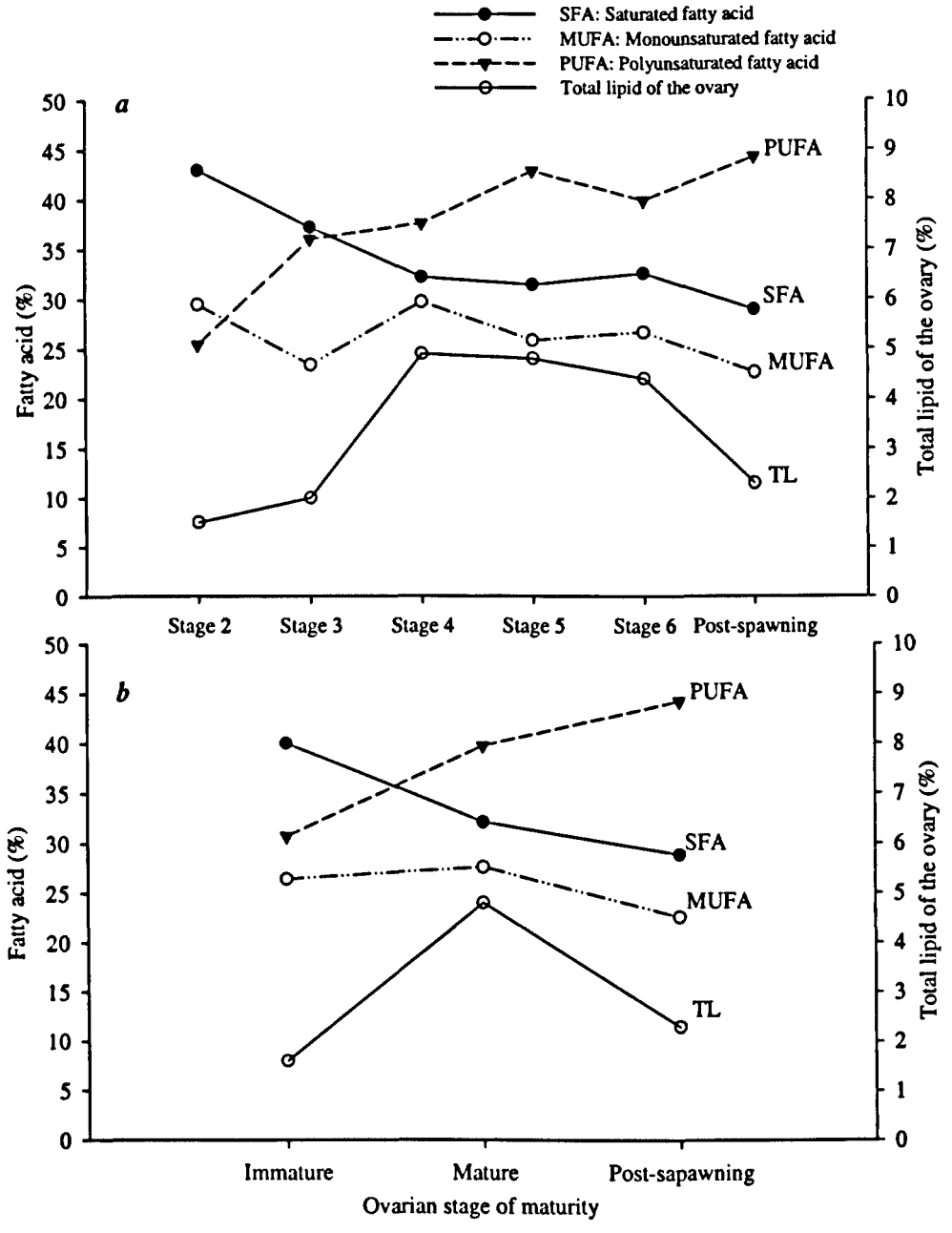


Figure 5.2 Fatty acid profiles in *R. sarba* ovaries during the reproductive season.

Classified ovaries from stage two-six and post spawning stage (*a*). Pooled levels of immature (stage two & three), pooled mature (stage four-six) and post-spawning stage (*b*).

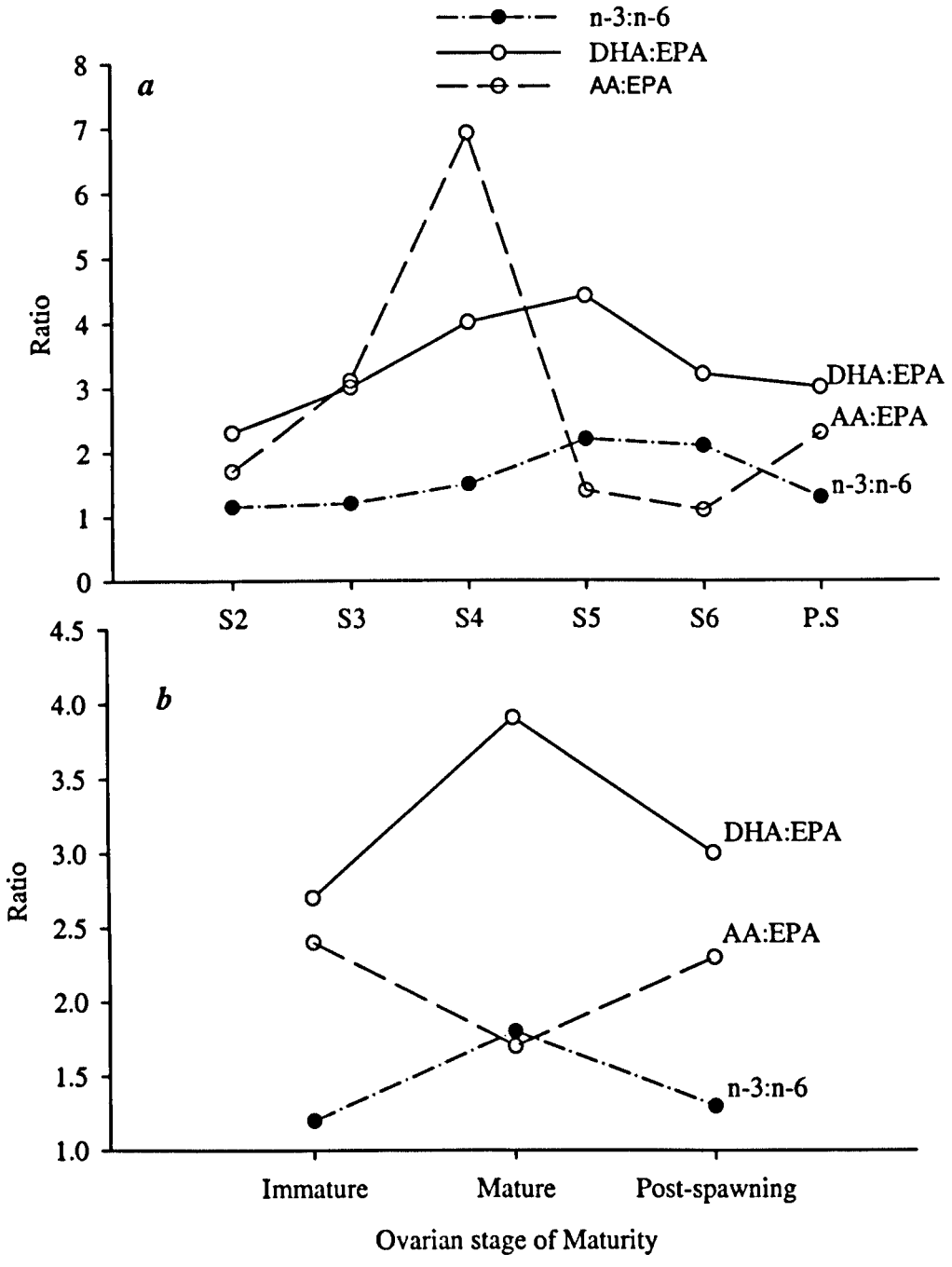


Figure 5.3 The ratio of n-3:n-6, EPA:DHA and AA:EPA in the ovary of *R. sarba* during the reproductive season.

Classified ovaries from stage two-six and post spawning stage (a). Pooled levels of immature (stage two & three), pooled mature (stage four – six) and post-spawning stage (b).

SFA in all stages was dominated by 16:0 and moderate levels of 18:0. All other SFA were present in minor levels particularly the 20:0 and 22:00. Generally, the

levels of 16:0 and 18:0 were lower during the mature stage. Level of 18:0 in particular was significantly lower during maturity from other stages of development including the PS. The MUFA were dominated by 18:1n-9 in all stages and levels were not significantly different between maturity stages.

The levels of n-3 and n-6 classes of PUFA also varied significantly according to the state of maturity of the ovary. The level of n-3 fatty acids was higher than of n-6 of fatty acids at all stages of maturity. Levels of n-3 fatty acids increased significantly as the ovary matured.

5.3.6.1 Fatty acids levels in stage two ovary

Levels of FA were mostly saturated (SFA) rather than monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) during this stage of ovarian development. The levels of 20:5n-3 (EPA) were not significantly different from other stages with the exception of the PS stage. No significant difference was observed in the level of 22:6n-3 (DHA) between stages two, three and four, while its level was found to be significantly different from stages five & six, the final stages in ovarian maturation.

5.3.6.2 Fatty acids levels in stage three ovary

As the ovary developed to stage three, the cortical alveoli stage, SFA levels started to decrease and MUFA also declined. Total PUFA increased and, in particular, n-3 PUFA, there was a notable increase in levels of EPA and DHA.

5.3.6.3 Fatty acids levels in stage four ovary

During stage four of ovarian development, which is classified as a mature ovary, the SFA level continued to decline and was significantly lower than that of the stage two ovary. No significant difference was detected in the level of MUFA between this stage and the previous stages of ovarian development. Total PUFA increased, particularly n-3 PUFA, although the level of EPA did not rise from that measured in stage three. The level of DHA, however, continued to increase.

5.3.6.4 Fatty acids levels in stage five ovary

SFA and MUFA levels declined from the previous stage of maturity and total PUFA increased significantly from the level measured in stage two ovary, particularly n-3 PUFA. EPA and DHA continued to rise and the level of DHA was significantly different from stage two ovary.

5.3.6.5 Fatty acids levels in stage six ovary

During stage six of ovarian development, which is classified as the final stage preceding oviposition, total SFA increased slightly, but not significantly, from stage five. Again, there was no significant difference detected in the level of MUFA between this stage and other stages of ovarian development. Total PUFA decreased and the level of EPA increased by a fraction from the level that was measured in stage five while the level of DHA declined.

5.3.6.6 Fatty acids levels in post-spawning stage

During the post-spawning phase, SFA levels continued to decline and were found to be significantly lower than those of stage two and three ovarian development. No

significant difference was detected in the level of MUFA between this stage and the previous stages of ovarian development and the level of total PUFA increased, particularly n-6 PUFA. EPA increased by an insignificant fraction and DHA was not significantly different from previous stages of ovarian maturity.

5.3.6.7 Fatty acids levels in the immature, mature and pooled ovaries

Fatty acids in stage two and three ovaries (immature) were pooled and stage four; five and six (mature) were also pooled to provide a general picture and a quick comparison of the FA profile during these two stages of ovarian development.

Total SFA in immature ovary was significantly higher than the level measured in mature and post-spawning (PS) stages due to the higher levels of 16:0 and 18:0. Total MUFA in immature ovary was not significantly different from that of mature and PS stages. Total PUFA level in immature ovary was significantly lower than in mature or PS stages. Notably, n-3 HUFA were significantly lower than in preceding stages. EPA in immature ovary was not significantly different from the mature stage. The level of DHA was significantly lower immature ovary from that of the mature and PS stages.

Fatty acids levels in vitellogenic and hydration stages (mature) showed variations from the immature stage in particular. Total SFA was significantly lower than the level measured in immature stages. The significant drop in the levels of 18:0, 20:0 and 22:0 fatty acids drives the decrease in the level of SFA from the levels recorded in the immature stage. Total MUFA was not significantly different from that of immature and PS. Total PUFA was significantly higher than immature stage. This is

due to the significant rise in the level of n-3 HUFA from that of the former stage. The level of EPA did not show any significant rise from immature stage. The level of DHA was significantly higher from that of the immature stage.

Total SFA was significantly lower than the level measured in immature stage, while no significant difference was detected between post-spawning (PS) and mature stages in terms of SFA. Total MUFA was not significantly different from that of immature and mature stages. Total PUFA was not significantly higher than mature stages, but there was a significant difference between PS and immature stage. n-3 HUFA was significantly higher than that of immature stage. EPA was significantly higher from immature and mature stages. DHA was similar to that of mature stage, but significantly higher than the level measured in PS stages.

5.3.6.8 The ratios of n-3:n-6, DHA:EPA and AA:EPA in *R. sarba* ovary

The ratios of (n-3) to (n-6), DHA to EPA and AA to EPA in the ovaries during stages two to six including the post-spawning stage are shown in Table 5.2, Figure 5.3, *a*. Ratios of pooled immature (stage two & three), mature (Stage four, five & six) and post-spawning (PS) are given in Table 5.3, Figure 5.3, *b*.

Significant differences were observed in the ratio of n-3:n-6 between the final stages of maturity (stage five and stage six) and other stages of ovarian development. The level tends to rise as the ovary matures reaching its highest level during stage five of ovarian maturity. The ratio declined during the PS stage to a level not significantly different from that of stage two, three and four of ovarian development. For pooled data, the ratio of n-3:n-6 showed a significant increase

during the maturation stage compared to the immature stage. At the PS stage the ratio declined to a level not significant different from that of the immature stage.

The DHA:EPA ratio showed a significant increase starting from the cortical alveoli stage (stage three) throughout the vitellogenic stages of development (stage four & stage five). However, no significant difference was detected between the hydration stage of maturity (stage six) and the immature stage (stage two) despite the fact that the ratio was slightly higher in stage six than stage two. The ratio was generally found to be not significantly different from the early stages of ovarian maturity starting from stage three-stage five.

For pooled data, the DHA:EPA ratio showed a significant increase during the maturation stage compared to the immature stage. At the PS stage the ratio declined to a level not significantly different from both the immature and mature stages.

The ratio of AA:EPA (20:4n-6 and 20:5n-3) in stage two ovary was not significantly different from that of stage three, stage five and PS. The highest value of this ratio was in stage four the early vitellogenic stage; during this stage the ratio was found to be significantly different from all other stages of ovarian development. The ratio was significantly lower in stage six, the hydration stage, than other stages of maturity with the exception of stage five.

For pooled data, AA:EPA ratio showed no significant difference between immature, mature and PS stages. However, the ratio was lower during the maturation stage.

5.4 Discussion

Gonadal development can lead to considerable drain on energy reserves in fish (Rijnsdorp & Ibelings, 1989). Levels of lipid in *R. sarba* ovaries showed great differences between maturity stages. Total lipid (TL) in mature ovaries increased by about 63% from that level measured in immature ovaries, reflecting lipid deposition in mature ovaries. As the numbers of mature ovaries increased at the onset of the spawning season in January, levels of TL increased by 75% compared with levels measured in October. At the end of the spawning season (March) TL in the ovaries decreased by 45% showing great energy loss. Almansa *et al.* (2001) showed that ovarian TL in gilthead seabream, *Sparus aurata* decreased by 80% after the reproductive season. In this study, ovarian samples that were analysed in March contained atretic oocytes which may have contributed to the level of TL. If sampling had been carried out beyond March, levels of TL may have been lower and similar to that recorded in October.

As a result of ovarian development, fish body mass can change during the pre- and post-spawning periods as lipid reserves are utilised for reproduction (Rijnsdorp & Ibelings, 1989; Jonsson *et al.*, 1991). Female *R. sarba* deposit fat prior to the spawning season as reserves to meet the high energy and nutrient requirement for gonadal development and throughout the active spawning season. Since *R. sarba* are batch spawners, continuous recruitment of maturing eggs throughout the spawning season demands higher lipid reserves to sustain these needs.

During ovarian stages three and four, the cortical and early vitellogenesis stages, levels of liver TL reached a maximum which may be due to the production of

vitellogenin that is synthesised in the liver and transported to the developing oocyte (Arukwe & Goksøyr, 2003). The liver could be a storage organ as the maximum fat concentration in this organ was found during the period of minimum number of stage four ovaries (the mature ovaries). Liver lipid levels of some fish are high. It has been found, for instance, that an increase in hepatosomatic index of Atlantic cod, *Gadus morhua* by about 13% was accompanied by an increase in liver lipid level by about 70% (Lie *et al.*, 1986). Muscle and liver have also been suggested to experience fat loss during reproduction in other sparid fish, such as gilthead seabream, *Sparus aurata* (Almansa, *et al.*, 2001) and sharpsnout seabream, *Diplodus puntazzo* (Hernández *et al.*, 2003). Similar observations were reported for free-ranging red drum, *Scianops ocellatus* (Craig *et al.*, 2000).

Liver TL at ovarian stages five & six and the post spawning (PS) stage was not significantly different suggesting that the liver maintains a certain level of lipid during these stages.

Carcass TL of post-spawning stage females decreased by about 70% the level recorded during the period preceding vitellogenesis. The liver showed a reduction of about 38% during PS stage from the level measured prior to vitellogenesis. Lateral muscle TL showed a reduction of about 51% during the post spawning stage and the weight of the visceral fat (VF) reduced by about 80% from the weight of VF found during the period preceding vitellogenesis. This significant reduction in TL from the carcass and VF reserves suggests that muscle and visceral lipids are used as a main source of energy in *R. sarba* and that this species relies heavily on fat for both reproductive and metabolic energy.

Rijnsdorp & Ibelings (1989) reported that female North Sea plaice experiences encounters a loss of about 44% of their energy at the end of the spawning season as opposed to the male, which loses only about 27%. It was also evident that fish deposit larger amounts of visceral fat (VF) in the viscera during the pre-spawning stage and utilise it during the reproductive season which suggests that this site represents an important reserve for reproductive energy as well as nutrients. A similar finding was reported by Gakichko & Dubrovskaya (1970). Generally, increase in ovarian weight throughout maturation led to a major depletion in the carcass, muscle and VF TL and to a lesser extent liver TL.

The fatty acid (FA) composition of the ovarian total lipid goldlined seabream, *R. sarba* showed general similarities with other marine fish such as cod, *Gadus morhua* (Lie *et al.*, 1992), Atlantic halibut, *Hippoglossus hippoglossus* (Evans *et al.*, 1996), gilthead seabream, *Sparus aurata* (Almansa *et al.*, 2001) and white seabream, *Diplodus sargus* (Cejas *et al.*, 2003). In the present study ovarian fatty acid showed some clear, significant differences between stages of ovarian maturity. The high levels of the major fatty acids in the total lipids from *R. sarba* ovary 14:0, 16:0, 18:0, 16:1 n-7, 18:1 n-9, 18:2 n-6, 22:5n-3 AA, EPA, and DHA demonstrate the importance of these fatty acids in creating an important energy store for future embryonic development (Almansa *et al.*, 2001).

Total PUFA increased during the period of vitellogenesis and maturation. PUFA levels have been related to reproductive success especially for n-3 PUFA (Watanabe & Kiron, 1995; Navas *et al.*, 1997) and considered as a major energy source during

early larval development (Falk-Peterson *et al.*, 1989). An increase in n-3 HUFA in broodstock diet is proven to increase egg viability (Fernandez-Palacios *et al.*, 1995).

Navas *et al.*, (1997) demonstrated that levels of both DHA and EPA increased during vitellogenesis. The major long chain PUFA was DHA and it was elevated significantly during the stages of vitellogensis, a similar finding to that reported for seabass (Navas *et al.*, 1997) and other sparid fish (Mourente & Odrizola, 1990; Almansa *et al.*, 2001). Almansa *et al.* (2001) has suggested that mature ovaries contain high amounts of DHA-rich vitellogenin deposited into mature oocytes prior to spawning. DHA levels were higher than EPA during the reproductive period which suggests that DHA as an EFA has a more important role in the enzyme activity of the cell membrane and in physiological balance (Watanabe *et al.*, 1989, Takeuchi *et al.*, 1990). Deficiencies in DHA lead to behavioural impairment in larvae (Sargent, 1995). Other studies have suggested that DHA has a more important biochemical function in the lipid than EPA (Bell *et al.*, 1985a, b) and that larvae of gilthead sea bream had conserved DHA over EPA during deprivation (Koven *et al.*, 1993). Generally, these two fatty acids are considered essential fatty acids in marine fish. The rise in HUFA and other fatty acid levels during the PS stage could be related to the high level of atresia during this stage and the existence of liquefied yolk which is which is rich in vitellogenin in the ovary despite ovarian lipid depletion.

In this current study arachidonic acid (AA) was the predominant egg polar lipid (n-6) PUFA, which serves as the precursor for the II-series prostaglandins and other eicosanoids (Sargent 1995). Bessonart *et al.* (1999) found that the growth and

survival of gilthead seabream larvae can be improved by elevating AA levels in the diet. This higher level also improved fertilisation and hatching rate of halibut (Mazorra *et al.*, 2003). In the present study, the levels of AA in *R. sarba* ovary showed a significant increase during the cortical alveoli stage, a significant decrease during the vitellogenesis period and a significant increase during the PS stage suggesting considerable utilisation during the reproductive process (Falk-Petersen *et al.*, 1989).

Palmitic acid (16:0) was the predominant saturated fatty acid; it has been suggested that this fatty acid is important as an energy source and structurally in the *sn*-1 position of membrane phospholipids (Sargent, 1995). Oleic acid (18:1n-9) was predominant in the MUFA; it has been suggested that this fatty acid confers fluidity on membranes when inserted in the *sn*-1 position of phosphatidyl ethanolamine (PE) and is an important energy supply for embryos (Dey *et al.*, 1993).

Ratios of n-3:n-6 and DHA:EPA showed significant increases during the spawning season while AA: EPA showed a decrease during the same period. DHA:EPA contents in eggs were positively correlated with egg symmetry and viability (Pickova *et al.*, 1997). However, some of these ratios such as AA:EPA are species specific, that is, subjective to the environment the species inhabits (Bell & Sargent, 2003). It can also be suggested that there is no general requirement for a high AA:EPA in order to increase the survivability of the eggs of *R. sarba*. A similar finding has been reported by Tveiten *et al.* (2004) for spotted wolf-fish *Anarchichas minor.*

In summary, accumulation of fat reserves in various compartments of *R. sarba* before the onset of the spawning season and the depletion of these reserves during the period of increased vitellogenic activity, the composition of fatty acids and its profile suggest the importance of these elements in the broodstock diet.

Chapter 6: Spawning, early larval development and feeding of goldlined seabream *R. sarba*

6.1 Introduction

6.1.1 Captive spawning

The need to develop farming technologies for a native species for aquaculture and perhaps stock enhancement is becoming increasingly important for the Sultanate of Oman. Developing sustainable technologies for new species in culture must include captive production of seed and stock for propagation.

At present, there is greater government support or overseas investment for the development of aquaculture in Oman using a variety of introduced marine species. None of these investors have focused on the parallel research of native species that has potential commercial value for Oman.

Countries developing aquaculture using introduced species should consider the potential impact it may have on the natural ecosystem. Organisations and investing agencies should consider three major issues prior to introduction of new species in aquaculture. These three considerations are economic, biotechnical, and environmental impacts (Ross & Beveridge, 1995). Economic and biotechnical issues are species specific and will depend on the production level required by aquaculturists. These issues are linked with the environment and any advancement in each of these issues will have additional implications.

In addition, it is vital to examine the impact an aquaculture venture can have on wild populations within and around the culture facility. Some of the environmental considerations one must consider before establishing a system: can disease impact

on wild stocks? Would escape of the species cause environmental or genetic problems?

Kottelat (1990) suggested that the reason behind the decline of indigenous fish populations in Sulawesi, Indonesia might be the introduction of exogenous species. According to Fernando (1991) Asia has encountered some loss of native species due to the introduction of fish. Escape of introduced (exogenous) fish has been reported by several authors to have a negative effect through crossbreeding (Taggart & Ferguson, 1986; Nickelson *et al.*, 1986; Welcomme 1988; Beveridge *et al.*, 1994).

In early stages of aquaculture, fingerlings or juveniles were collected from the wild and reared in captivity until they reached marketable size for example, *Rhabdosargus sarba* (Leu, 1994). Likewise, wild broodstock collected from spawning grounds during the spawning season have been utilized in many cases for gamete procurement by direct stripping and use in seed production such as the case with red grouper, *Epinephelus akaara* (Tseng, 1983) and seabass, *Lates calcarifer* (Tookwinas, 1990). Egg production has also been acquired by natural spawning in captivity, for example with silver seabream, *Sparus sarba* (Lin *et al.*, 1988). For those species that fail to mature in captivity, however, hormone treatment has been given to induce maturation and ovulation, for instance striped bass, *Morone saxatilis* (Mylonas *et al.*, 1998) for comprehensive review on this issue, refer to Zohar & Mylonas (2001). Other species may require environmental manipulation such as the case with red drum, *Sciaenops ocellatus* (Lawson *et al.*, 1989).

In order to improve and achieve successful aquaculture practice, life cycle and reproduction habits of species of interest have to be controlled and well understood. Once this is achieved, continuous production of fish can be attained by off-season spawning (Zohar & Mylonas, 2001). Such an approach has opened the window for greater numbers of species being cultured. In the last decade breeding of marine fish has advanced greatly in many regions around the world, such as in the Pacific region (Hong & Zhang, 2003; Le Moullac *et al.*, 2003) and Mediterranean regions (Conides *et al.*, 2002; Gordin, 2003). Recently, artificial production of red drum fry has reached over 10 million annually (Hong & Zhang, 2002). The authors also attributed the rise in fry production of red seabream, *Pagrosomus major*, Japanese seabass, *Lateolabrax japonicus*, and black porgy, *Sparus macrocephalus* in China to levels higher than 10 million to the continuous advancement in breeding techniques of marine fish in China since the 1990's.

6.1.2 Early larval development

Generally, most fish larvae are visual feeders (Blaxter, 1986; Batty, 1987). Therefore, the development of a functional visual system is important for prey capture (Porter & Theilacker, 1999). Vision is also important in orientation, schooling and eluding predators (Blaxter, 1986). Besides environmental parameters such as temperature and light conditions (Paul, 1983; Blaxter, 1986), larval survival and growth depends also on their ability to feed successfully (Heath, 1992). Those larvae with a relatively small yolk-sac that provides limited nutritive reserves, must start exogenous feeding soon after they hatch (Mani-Ponset *et al.*, 1996; Calzada *et al.*, 1998). For several days fish larvae utilise nutrients provided by the yolk-sac as well as that from exogenous sources; this phase of development is termed the 'endo-exotrophic phase' (Mani-Ponset *et al.*, 1996). As the authors suggested, for larvae to

pass through this critical phase, successful synchronisation must occur between the exhaustion of indigenous reserves and first feeding.

In order for larvae to utilize ingested food, they must also develop a digestive system that is able to absorb food (Govoni *et al.*, 1986). Inadequate physical ability to feed due to poor development of such important organs has been reported to be the major cause of larval starvation and mortality (Avila & Juario, 1987). Studies that have been carried out on early larval development showed that the yolk absorption event is accompanied by the differentiation and development of many organs, particularly the digestive system (Johns *et al.*, 1981).

In sparids, it has been reported that the major and important changes in the vision system occur in the lecitotrophic phase as preparation for onset of exogenous feeding that involves prey searching and capturing (Kawamura, 1984; Pankhurst, 1996; Roo *et al.*, 1999). Development of vision was studied in relation to gut development in red porgy, *Pagrus pagrus* (Roo *et al.*, 1999). The author demonstrated that vision was developed before the onset of exogenous feeding.

Study of the development of the digestive function of the gut in fish larvae determines the dietary requirements of the larvae for the specific developing stage of its digestive system (Vegetti *et al.*, 1999). This is accomplished by a better understanding of the intestine structure through obtaining data on the topological features provided by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Cataldi *et al.*, 2002).

Development of the digestive tract, morphology and histochemistry in seabream have been studied by Sarasquete *et al.* (1993; 1995), Moyano *et al.* (1996), Diaz *et al.* (1997) and Calzada *et al.* (1998). The early development of the digestive tract and the intestinal convolutions of goldlined seabream larvae *R. sarba* are described by Tsukashima & Kitajima (1982). The description of the development and external morphology of *R. sarba* larvae is given by Tsukashima & Kitajima (1982), Lin *et al.* (1988) and Mahboob *et al.*, (1998). Dean *et al.* (2003) provided a molecular and biochemical study in relation to larval development of goldlined seabream. In all these studies, the authors used the other synonym for the species that is *Sparus sarba*. Knowledge of functional development of the various organs in *R. sarba* larvae is lacking.

6.1.3 Enrichment of live feed

Fish larvae require a suitable diet that has the essential nutritional value needed for growth and survival and diets that have the appropriate size. Rotifers *Brachionus plicatilis* and *Artemia* nauplii have been widely used as live food for fish larvae due to the many advantages that these microorganisms have. For rotifers, some of these advantages have been that its production can be controlled in order to have a mass culture that provides rapid production of rotifers (Watanabe *et al.*, 1983). Also, *B. plicatilis* have an appropriate size as first feed for fish larvae that is characterised as the intermediate size between microlagae and *Artemia* (Watanabe *et al.*, 1983; Tamaru *et al.*, 1993). This live feed remains important due to lack of artificial feed for first feeding of marine fish larvae (Dhert *et al.*, 2001).

Marine fish contain large amounts of saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA) particularly highly unsaturated fatty acids (HUFA) of the n-3 series (n-3 HUFA) such as eicosapentaenoic acid (EPA, 22:5n - 3) and docosahexaenoic acid (DHA, 22:6n – 3) (Tocher, 2003). Therefore, sufficient DHA and EPA is essential for marine fish larvae especially in their rapid growth and development phases, as larvae at this stage have a high percentage of neural tissue in their relatively small body mass (Tocher & Harvie, 1988; Bell & Dick, 1991). Inappropriate levels of these fatty acids has been demonstrated to have a negative effect on the development of fish larvae and may affect their survival (Koven *et al.*, 1989; Rodríguez *et al.*, 1993; Bell *et al.*, 1995).

Despite the fact that cultured rotifers have been proven to synthesise some n-3 HUFA, their contents of DHA and EPA are still relatively poor which does not promote good growth and survival rates in fish larvae (Lubzens *et al.*, 1985). Therefore, enrichment of rotifers with ω-yeast, oil-based emulsions, fish by-products, silage and microencapsulates to boost the level of essential fatty acids are common practice in many hatcheries (Kitajima *et al.*, 1980; Walford & Lam, 1987; Dhert *et al.*, 1990; Kanazawa, 1993; Tocher *et al.*, 1997; Estévez *et al.*, 1999; Dhert *et al.*, 2001).

This study aimed to investigate the following:

- The breeding of native seabream species *R. sarba* to evaluate feasibility of artificial seed production in Oman.
- The morphological development of *R. sarba* larvae in early days post-hatching in order to determine timing and development of photoreceptors in the retina with relation to gut development and the onset of exogenous feeding.
- The effects of lipid and fatty acid level using enriched rotifers on the growth and survival of *R. sarba* larvae for the first 20 days after hatching

6.2 Materials and Methods

6.2.1 Broodstock acquisition and maintenance

Broodstock fish were collected from the sampling ground. Following removal from fishing traps fish were transferred immediately to containers provided with portable aerators. On arrival, fish were kept in 800*l* tanks provided with air stones and good water exchange at 20*l*min^{-1} (Figure 6.1). Fish remained in this system for about three days in order to separate non-injured from injured fish, as some fish tended to be damaged during handling and also due to time spent in the trap. Following this, MS222 (Sigma-Aldrich Canada, Oakville, ON, Canada), 100mg*l*$^{-1}$ (Esteban *et al.*, 1998) was used to reduce stress while transporting them to 2500*l* tanks (Figure 6.1). Broodstock tanks were supplied with a good air supply and water exchanged at a rate of 50*l*min^{-1} during high tide and 30*l*.min^{-1} during low tide as the water supply from the water well was affected by tidal changes. Water in broodstock tanks was aerated with air stones and water supplied from a well source provided water at a salinity of 30 to 32‰ depending on the tidal condition. Water quality monitoring was carried out on a daily basis and temperature, DO, and salinity were monitored. The outdoor tank facility was shaded by a black polyethylene sheet to provide 100 % shade to reduce sunlight (Figure 6.1).

Goldlined seabream were divided into two tanks, female to male ratio was 2:1 and in the range of 500g wet body weight. Broodstock were fed trash fish (approximately 5% wet body weight), this included sardine and squid daily and once a week they were offered clam meat. Tank cleaning was carried by siphoning out every morning to remove uneaten food and other biological waste that had accumulated.

The water outlet of each tank was covered with a fine mesh (100μm), referred to hereafter as egg-collector, to ensure that no eggs escaped from the tanks in the drainage pipe (Figure 6.2). Egg-collectors were placed starting from early December one month prior to the spawning season in the afternoon and were removed every morning before feeding and tank cleaning for spawning evaluation. A water sample was also collected to check for spawning every day, in the early morning, and spawning behaviour noted.

Eggs were removed from the egg-collector and placed in a plastic container that had the same water temperature as that of the broodstock tank. Remaining eggs found floating in the breeding tank were collected with a fine dip net (100µm mesh size). Unfertilised or dead eggs, that had sunk to the bottom of the tank, were removed by siphoning during routine cleaning. The oxygen level in the broodstock tank was 6.0mgl^{-1} and salinity varied between 30 and 32‰.

Figure 6.1 Broodstock tanks used for housing *R. sarba* at the MSFC.

Figure 6.2 Egg collection mesh was placed at the outflow in the broodstock tank (arrow).

6.2.2 Egg incubation

Once eggs were collected from the spawning tank they were kept in a 20*l* container that contained filtered water and was aerated to create upwelling. The eggs remained in this container for five minutes and the air was then switched off in order to separate eggs from unwanted particles collected from the spawning tank. The fertilised eggs remained floating and detritus and unfertilised eggs settled at the bottom of the container. Floating eggs were collected with a beaker and rinsed with clean filtered water to avoid the introduction of contaminants. Following the rinsing process, a quick check was carried out on the eggs to check for fertilisation. Fertilised eggs were distributed between the incubation flasks at $100l^{-1}$ (Figure 6.3). The incubators were supplied with a mild air supply to create upwelling in order to keep the eggs in suspension.

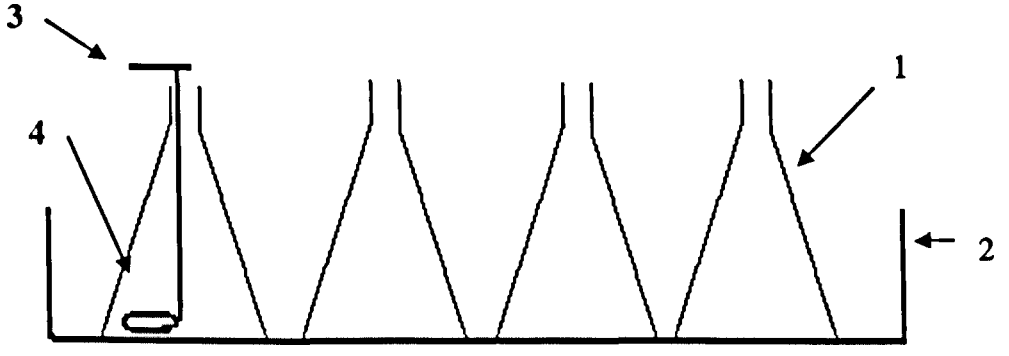


Figure 6.3 Schematic diagram showing the layout of the egg incubator facility that was used to incubate the *R. sarba* egg.

1. Laboratory glass flask (capacity 2*l*), 2. Vessel containing water to keep the temperature similar in all the flasks, 3. Air control valve to control air flow to facilitate suitable egg lift in the water, 4. Air stone, fixed at the bottom of the flasks.

6.2.3 Larval rearing system

Transparent plastic containers (5*l*) capacity were covered with black plastic sheets to minimise disturbance to the larvae (Figure 6.4). Containers were provided with an air supply using aquarium air pumps. One battery-operated air pump with many outlets was kept aside in case of a power cut. Each container was provided with a

fine air stone and an air control valve. The system was placed in an air-conditioned room to provide temperature control.

Figure 6.4 Larval rearing containers equiped with air supply.

6.2.4 Micro-organism culture method

Algae *Tetraselmis suecica* was used as feed for the rotifer cultures. It was essential to start the production chain starting from the stock level. *T. suecica* was acquired from the Marine Resources Research Center (MRRC) in Umm Qaiwain, UAE and transported in a 250ml flask to MSC in Oman by road for a distance of approximately 500km. The flasks were kept in a thermal box provided with a continuous air using a portable air pump. Crushed ice was placed in one side of the foam box to cool the temperature to at least <27°C. On arrival, a new stock was prepared at MSC by inoculating into sterilised flasks containing sterile media (Figure 6.6, a).

6.2.4.1 Algae (*T. suecica*) culture

A batch culture technique was applied to produce *T. suecica*. A schematic diagram for batch production in this study is given in Figure 6.5. Each inoculation step involved addition of 20% fresh algae cells to the following volume. The steps from 200ml to 20*l* were produced indoor in a culture shelf (Figure 6.6, *a*) and from 100-600*l* were produced outdoors. During outdoor culture the 100*l* containers were illuminated with light that promotes plant growth (Figure 6.6, *b*) while the 600*l* glass tank was not illuminated at night in order to slow down algae growth (Figure 6.6, *c*). Due to high temperatures and dusty environments, all outdoor algae containers were covered; and three times the volume required was produced as a safety measures. Algal tanks were continuously aerated and algae were produced at a cell density of 2.5×10^6. A haemocytometer was used to count cell concentration.

6.2.4.1.1 Stock culture media

The stock culture media that was used was supplied by the Marine Resources Research Center in Umm Qaiwain, UAE. Stock media was prepared from a combination of several chemical components (Appendix 3, Table 3.1). The quantities listed in Appendix 3, Table 3.1 were diluted in 1*l* salt water. To prepare for inoculation, 1ml was removed from the prepared solution and added to a 1*l* culture medium. The medium prepared was then divided into 250ml flasks. The 200ml flasks were then sterilized.

6.2.4.1.2 Grow-out fertilizer

Due to availability constraints and cost involved with the suggested chemical used to fertilize algae an alternative, agricultural fertilizer (Grow More®) readily available in the local market at a very low cost was used to fertilize algal cultures.

Initially, a culture trial was made using different fertilizer concentrations to find out levels that gave good results. The chemical contents of this fertilizer are listed in Appendix 3, Table 3.2.

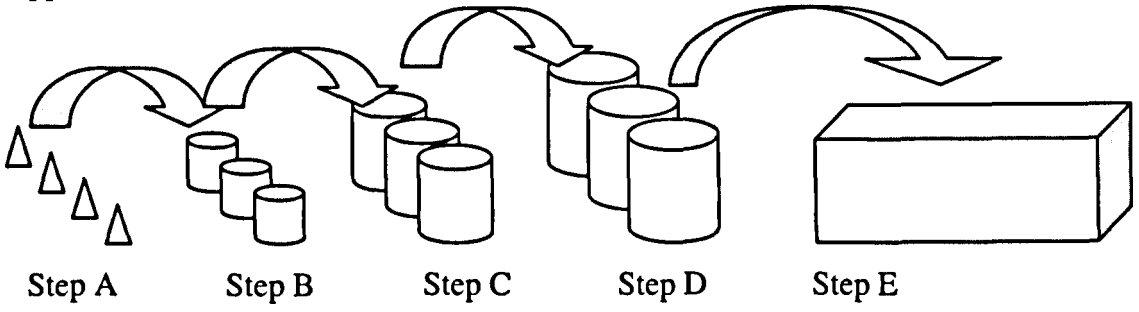


Figure 6.5 Schematic diagram showing the production steps for batch culture of algae.
Step A, stock culture indoors
Step B, 2*l* containers indoors
Step C, 6*l* containers indoors
Step D, 100*l* containers outdoors
Step E, 600*l* glass tank outdoors.

6.2.4.2 Rotifer (*Brachionus plicatilis*) culture

A stock of rotifers *B. policatilis* (S-type) was acquired from MRRC and batch cultured at MSFC. The stock was acquired approximately three months prior to the reproductive season of *R. sarba*, and during this time it was culture only up to 10*l* containers from a stock level in 2*l* plastic containers indoors. In early December, rotifer culture extended to 800*l* FRP tanks outdoors (Figure 6.1). Rotifer tanks were supplied with algae once daily in order to minimise growth of rotifer. The density and the condition of the culture was checked regularly and production density was estimated to be around $100\pm20ml^{-1}$.

6.2.4.3 Quality control

In order to prevent contamination between algae and rotifer tanks, separate equipment was used for each culture. Stock culture was thinned regularly. All

containers and equipment were disinfected with bleach. Monthly average temperatures of algal and rotifer culture water outdoors are given in Table 6.1.

Table 6.1 Monthly average temperature of algae and rotifer culture water outdoors.

MONTH	ALGAE (°C)	ROTIFER (°C)
November	25	24.3
December	23.5	24.2
January	23.5	24
February	26.0	25.5

Figure 6.6 Algal culture facility at MSFC.

a -stock algal culture indoors.
b - 100*l* outdoors under light illumination
c - 600*l* culture outdoors without light illumination at night to control algae growth.

6.2.5 Rotifer enrichment experiment

6.2.5.1 Emulsions

The two experimental emulsions used included cod liver oil and corn oil and the controll group was fed algae *T. suecica.* Lipid emulsions were made following the protocol given by Rodríguez *et al.* (1996), except that egg yolk was not added as it may contain anti nutrient factors. The emulsion ingredients are given in Table 6.2 .

Table 6.2 The lipid emulsion content.

Component	Amount
Soya lecithin (Sigma-Aldrich®)	0.5g
Enrichment oil	8g (SFO, CLO)
Saline water (30- 32‰)	150ml
Anti oxidant	300μl

SFO, sunflower oil; CLO, cod liver oil.

The emulsion (150ml) was then mixed in a household blender and fed to the rotifers stocked at $150ml^{-1}$ in three litre inverted, conical, plastic containers which were aerated vigorously, the enrichment period was six hours. Two batches of enriched rotifers were prepared at an interval of six hours in order to keep the concentration of the rotifers constant in the larval tank and to optimise feeding of larvae. The emulsion (150ml) was added to the rotifer containers twice, once at zero hours and again after three hours.

Plankton mesh (50μm) was used to harvest and rinse the enriched rotifers. Prior to feeding the larvae, rotifers were rinsed to avoid contaminats. Sub-samples of enriched and non-enriched rotifers were collected in duplicate once every five days

rinsed in fresh water, dried on a filter paper and stored in small tubes at -70°C for further analysis.

6.2.5.2 Larviculture for feeding experiment

Goldlined seabream *R. sarba* broodstock were maintained as described in Section 6.2.1. Eggs for this experiment were collected from a natural spawning of captive broodstock and incubated as detailed in Section 6.2.2. Hatched larvae were reared as described in the system given in Section 6.2.3. Approximately one third of the rearing water was siphoned out every morning starting from two-day-after hatching (DAH) and replaced with filtered, aerated water maintained in a container in the rearing room.

Room temperature was controlled by an air-conditioner that maintained the rearing water temperature at 23.2±0.3°C. Dissolved oxygen was 5.24±0.3 mgl^{-1} and 12L: 12D lighting regime was used. Water salinity averaged 30.0±2.0‰, as this is the salinity provided by the well used for the water supply to the culture facility at MSC.

Rearing containers were stocked with 50 larvae (10 larvae l^{-1}) in triplicate per diet. Larvae were reared following the procedure described by Leu (1994). Larvae were fed with three different diet, rotifer fed algae (control diet), rotifers enriched with sunflower oil (SFO) and rotifers enriched with cod liver oil (CLO). Larvae were fed enriched rotifers for the 20 days experiment; rotifers were added to the rearing containers at a density of 10±5 individuals ml^{-1}. Algae was added to the rearing water at a density of about 30-50× 10^6 cell ml^{-1}.

6.2.5.3 Larval measurements and survival rate

Larval total length was measured at the beginning, at day ten and at the end of the 20 day experiment period. Measurement was conducted using an ocular micrometer (Wild Heerbrugg Switzerland, mounted on a ZEISS compound microscope, model AKSIOSKOP.2 MOT). On day ten of the experimental period nine larvae were removed from the rearing containers of each diet and treated with an overdose of MS222.

6.2.5.4 Total lipid and fatty acid analysis of the rotifers

Approximately 1g of wet rotifers were weighed to four decimal places and used for total lipid extraction. Total lipid extraction was performed using the Folch-Lee extraction method (Folch *et al.*, 1957) as detailed in Section 2.1.9. Fatty acid composition of total lipids of rotifer determined by gas-liquid chromatography of the methyl ester derivatives prepared as described in Section 2.1.10.

Due to unforeseen circumstances, larvae were lost and, therefore, no material was available for measuring the total lipid and fatty acid composition of larvae.

6.2.6 Transmission electron microscopy (TEM)

6.2.6.1 Larval samples

Larval samples from this study were obtained from natural spawning in captivity. Larvae were reared on rotifers, *Brachionus plicatilis* produced as detailed in Section 6.2.4.2. Larvae were fed rotifers twice daily at a density of 10 ± 5 individuals ml^{-1}.

The rearing water parameters were temperature 22.8-23.4°C; salinity 30-32 ‰ and DO 85-90% saturation. Light regime was 12L: 12D.

6.2.6.2 Larval sample processing

Samples of *R. sarba* larvae for EM were taken over the first six days. Five individuals were sampled daily from day zero to day six. Whole larvae were fixed in Karnovsky's fixative, at 4° C for at least two hours and placed in cacodylate buffer (pH 7.5) at 4° C until further possessing. Prior to placing in the automatic processing machine as detailed Appendix 4, Table 4.1, larvae were placed on a plastic disc and the tail was removed under Leica stereo microscope (Leica CA-L2). Further processing steps are detailed in Appendix 4, Table 4.1. Processing of samples was carried out in the Electron microscopy laboratory, College of Medicine, SQU. Preparation and ingredients of Karnovosky's fixative is listed in Appendix 4, Table 4.2 and ingredients of washing cacodylate buffer pH. 7.2-7.4 is listed in Appendix 4, Table 4.3.

6.2.6.3 Block sectioning

Trimming was carried out by glass knife until the sample surface was exposed. Semi thin sections (0.5 μm) were also taken using the same type of knife and were stained with toluidine blue. A diamond knife was used to take ultrathin sections of about 60nm thickness. Ultrasections were mounted on a copper grid and were stained with metal stain.

6.2.6.4 Metal stain protocol for ultrathin sections

A drop of saturated uranyle acetate was placed on a piece of dental wax and the grid was floated over the drop for 30 minutes in the dark. The grid was then rinsed firstly with 50% alcohol and then with distilled water and floated on a drop of lead citrate for 25 minutes. Once again the grid was thoroughly rinsed, dried on filter paper and immediately removed to avoid dust accumulation on the grid. The section was then ready to screen. All the processing steps of EM sections that were carried out at SQU are in line with the method used at the IoA. Sections were then viewed in a JEOL JEM-1230 transmission electron microscope operated at 80 kV.

6.2.7 Statistical analysis

Statistical analysis was performed as detailed in Section 2.1.11. Growth and survival performance was examined as follows:

1- Survival rate (%) = (final larval number/initial larval number)×100

2- Larval length gained at the end of the experiment (mm) = final larval length – Initial larval length

3- Specific growth rate (% day) = $100\ \frac{LnL_t - LnL_0)}{\Delta_t}$. Where L_t is the final length, L_0 is the initial length, Ln is normal log and Δ_t time (days)

6.3 Results

6.3.1 Captive spawning of the native goldlined seabream, *R. sarba*

6.3.1.1 Spawning of brood stock

Female and male *R. sarba* were observed to reach the water surface before the spawning event. After two months of maintenance natural spawning took place in the holding tanks during December. Captive broodstock had four spawning events, and after these events spawning discontinued. Spawned eggs were found inside the mesh bag that was placed at the water outlet of the holding tank. Some spawned eggs remained floating in the broodstock holding tanks and were scooped out later. Water temperature of broodstock tanks during the study period is given in Figure 6.7.

6.3.1.2 Fertilisation rate, incubation and hatch rate

Fertilisation rate, defined as spawned eggs of *R. sarba* that had normal cell division, averaged 80±15%. Viable hatch averaged 70± 10%. Water temperature of the incubators ranged between 23.2°-24.4°C and averaged 23.9°±0.5°C. Larval hatching started to occur 23-24 hours from the blastula stage.

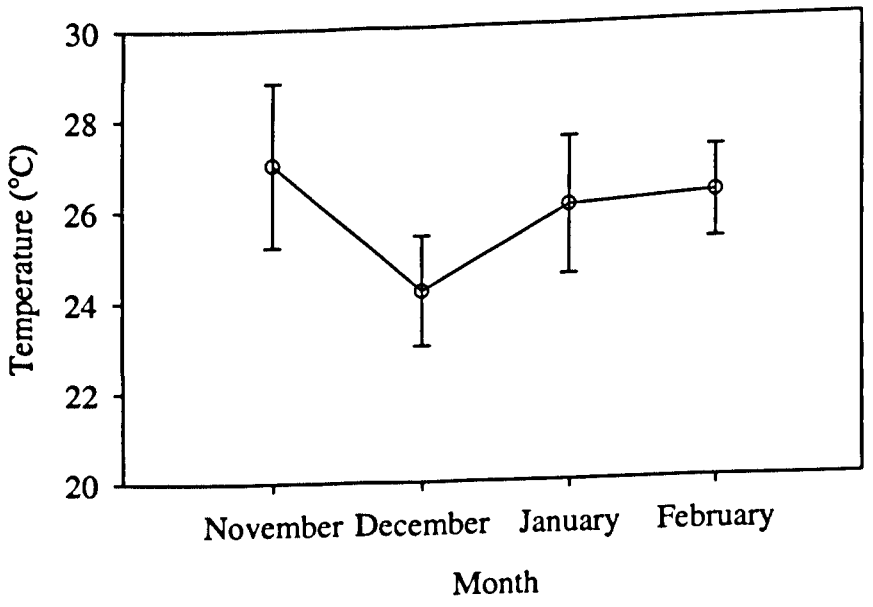


Figure 6.7 Average monthly water temperature (°C) during breeding trial of *R. sarba* broodstock. Data presented are means with ± SD.

6.3.2 Early development of goldlined seabream, *R. sarba*

6.3.2.1 Larvae at zero day after hatching (zero DAH)

At hatching the eye was undifferentiated and had no obvious shape (Figure 6.8, *a*). At 20 hours post hatching the eye had a spherical shape and consisted of numerous retinal cells, with some form of zonation to the retina. (Plate Plate 6.1& Plate 6.2). Retina cells were not arranged radially and the outer nuclear layer (ONL) had started to form at most parts of the eye and its width was approximately 5μm. Columnar nuclear bodies (CNB) were found in the outer nuclear layer (ONL) probably precursors of nuclei of the photoreceptors, while photoreceptors themselves were not yet developed (Plate 6.2). The outer plexiform layer (OPL) that separates the inner plexiform layer (IPL) and inner neuclear laye (INL) was visible during this time. The pigment epithelial cells did not exist at this stage of

development, but the outer limiting membrane was rather thick (Plate 6.1). The lens of the eye was spherical and developed within the first 20 hours post hatching and lens epithelial cells (LEC) were well established and differentiated (Plate 6.3 & Figure 6.8, *b*). A development of what could be the optic fissure was observed at 20 hours post hatching while the optic nerve was not yet developed (Plate 6.4)

At hatching, the larvae had a large yolk-sac that was present in the abdominal cavity with a single oil globule positioned at the posterior end of the yolk mass. The gut at this time was undifferentiated; it appeared as a simple straight tube or lumen that was narrow (Figure 6.8, *a*).

At zero DAH (20 hours after-hatching) the yolk-sac still existed with its oil globule. The digestive tube, mouth and anus of the larvae were still undifferentiated and were not yet distinguishable (Figure 6.8, *b*). The ultrastructural investigation at zero DAH showed that the gut was lined by columnar epithelial cells that had microvilli at their apical surface. The microvilli that were lining the surface of the epithelial cells in the mid-region of the digestive tract had an irregular shape and some regions of the epithelial lining were lacking microvilli (Plate 6.11 - Plate 6.15). During this stage of larval development ciliated cells were also observed in the gut lumen (Plate Plate 6.12, Plate 6.14 & Plate 6.15). A few small mitochondria of irregular shape and some endoplasmic reticulum were present in the epithelial lining of the digestive tract (Plate 6.13). Microvilli were more numerous at the posterior region of the digestive tract (rectal area) 20 hours post-hatching (Plate 6.13). In the midgut region (antero-median intestine), the nucleus occupied a large section of the cells (Plate 6.12).

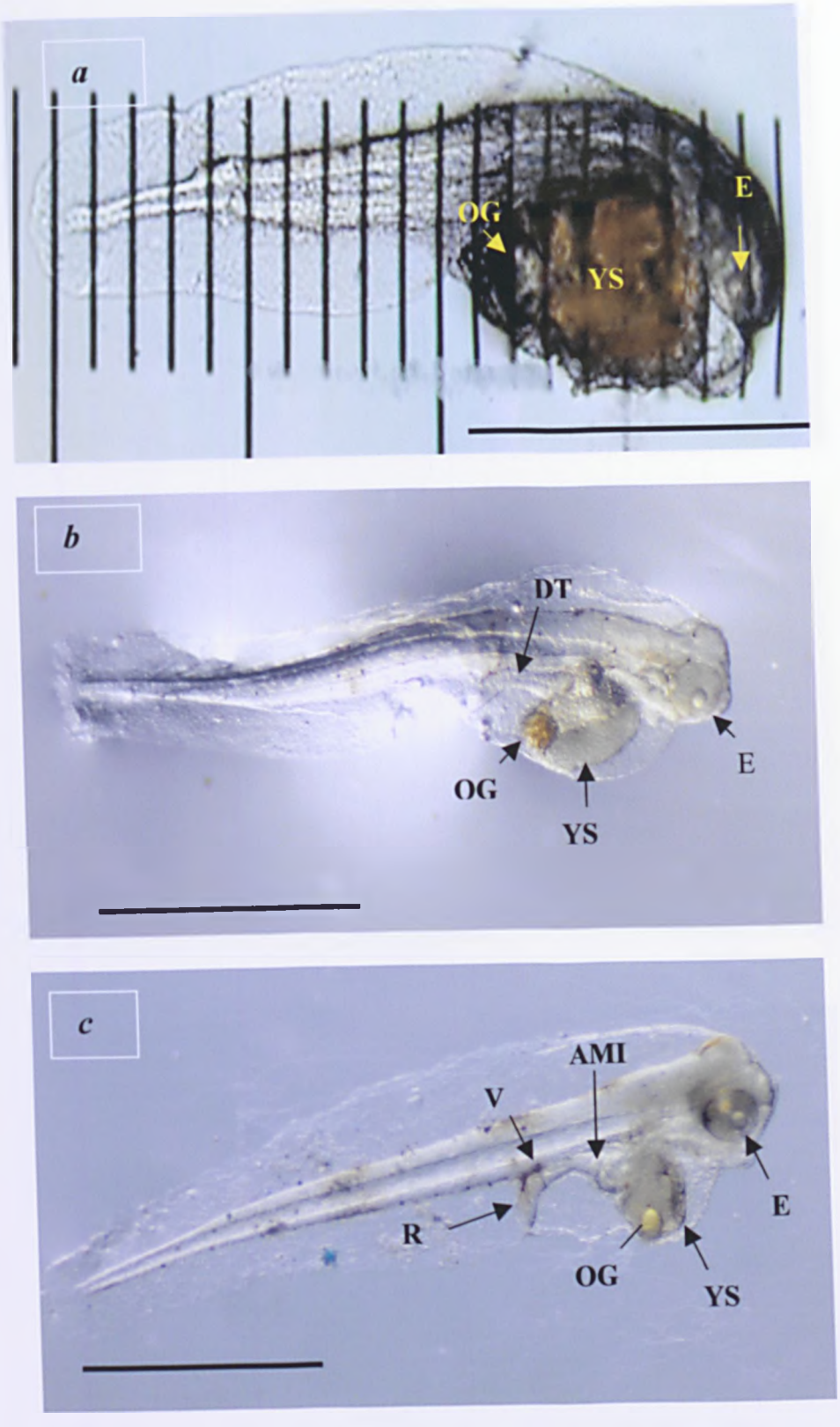


Figure 6.8 Larvae of *R. sarba* from hatching until D5 post-hatching. These larvae were fixed for EM, and those photographs were taken prior to EM processing. Scale bar 1mm.

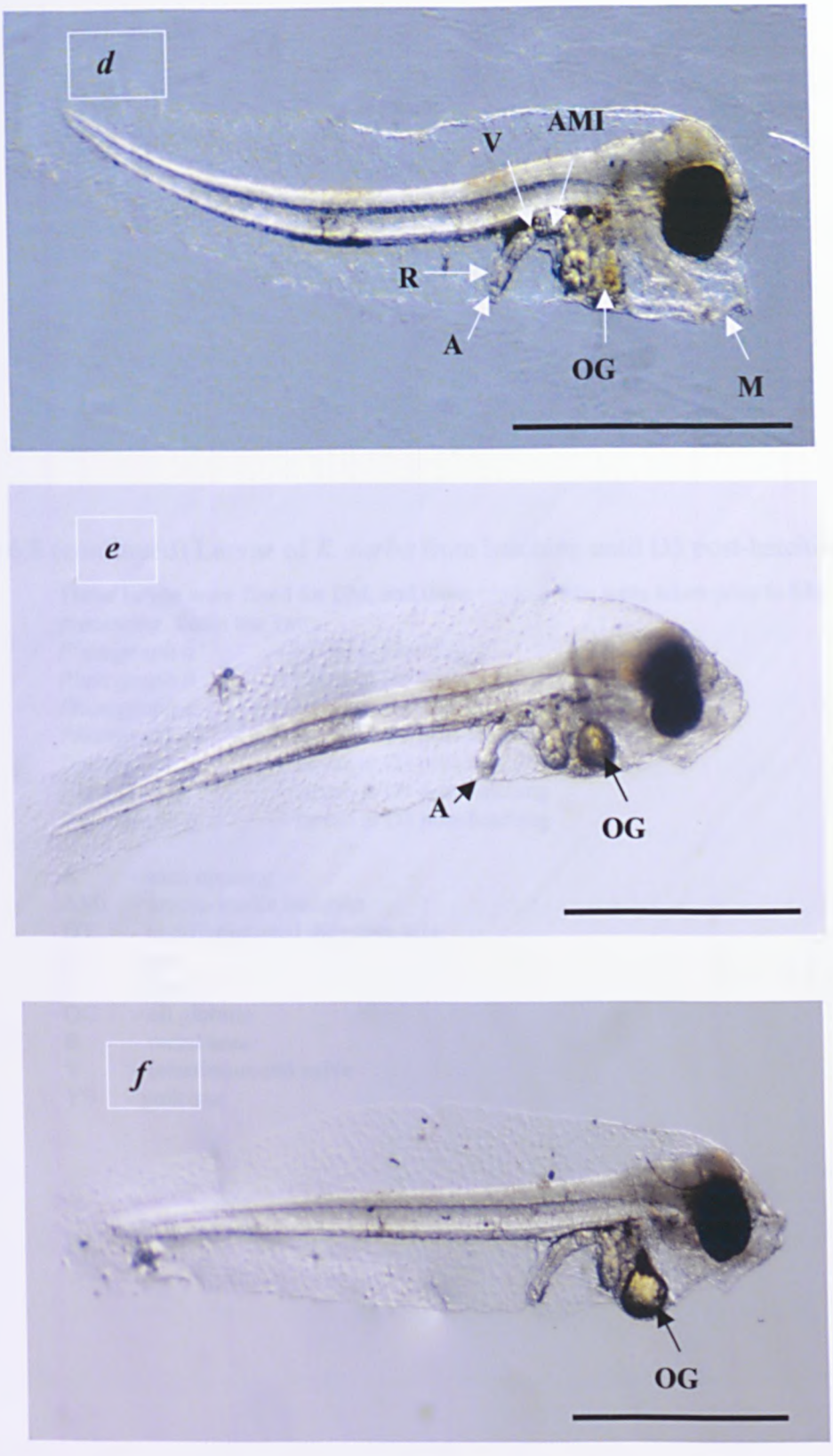


Figure 6.8 (continued) Larvae of *R. sarba* from hatching until D5 post-hatching. These larvae were fixed for EM, and those photographs were taken prior to EM processing. Scale bar 1mm.

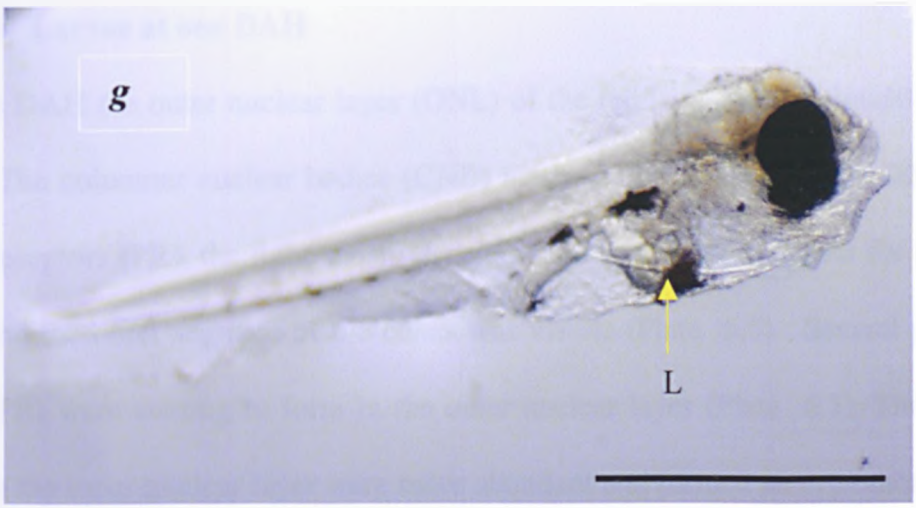


Figure 6.8 (continued) Larvae of *R. sarba* from hatching until D5 post-hatching.

These larvae were fixed for EM, and those photographs were taken prior to EM processing. Scale bar 1mm.

Photograph a - larvae at hatching
Photograph b - larvae at D0 post-hatching
Photograph c - larvae at D1 post-hatching
Photograph d - larvae at D2 post-hatching
Photograph e - larvae at D3 post-hatching
Photograph f - larvae at D4 post-hatching
Photograph g - larvae at D5 post-hatching

A - anus opening
AMI - antero-media intestine
DT - undifferentiated digestive tube
E - eye
L - liver
OG - oil globule
R - rectal area
V - intestino-rectal valve
YS - yolk-sac

6.3.2.2 Larvae at one DAH

At one DAH the outer nuclear layer (ONL) of the retina was approximately 15μm thick. The columnar nuclear bodies (CNB) increased in numbers in the ONL. The photoreceptors (PR), the light-sensitive cells, were beginning to appear for the first time and the outer segment of the cones was visible (Plate 6.5). Several pigment cells (PE) were starting to form in the outer nuclear layer (Plate 6.5). The retinal cells in the inner nuclear layer were more abundant and formed an even more radial shape than at the previous stage of larval development (Plate 6.6).

During this stage of larval development external morphology showed that the gut lumen widened and began to separate into a long midgut (antero-median intestine) and short hindgut (rectal area) (Figure 6.8, *c*). The two regions were separated by an intestino-rectal valve that appeared as a straight line across the digestive tube (Figure 6.8, *c*). The size of the yolk-sac had greatly shrunk but the oil globule was still visible in the abdominal cavity. Both the mouth and anus had not yet differentiated.

The ultra structure of the digestive tract in the rectal area of the intestine (Plate 6.15) and antero-median intestine area (Plate 6.16) showed the presence of some ciliated cells in the lumen. Microvilli were more regular during this stage, and their number had increased compared to the previous stage of larval development. Still no food particles were observed in the lumen at this stage of larval development.

6.3.2.3 Larvae at two DAH

During this time, pigment epithelium cells (PE) of the eye were visible and were well defined (Plate 6.7). They were organized as a single layer of cuboidal cells and the eye resembled its final colour for the first time; also refer to Figure 6.8, *d*. The photoreceptor (PR) cells were completely differentiated in the outer nuclear layer (ONL) and were segmented (Plate 6.7). The external (PRES) segment of the photoreceptors (PR) was pigmented (Plate 6.7). During this time the larval vision system appeared to be complete and functional.

The intestino-rectal valve that separates the antero-median intestine from the hindgut was more advanced during this stage of larval development. The digestive tract was more convoluted. The mouth and the anus were more advanced, and there was a possibility that the anus opened during this or the following day (three DAH). The yolk-sac was largely absorbed but the oil globule was still visible in the larval abdominal cavity (Figure 6.8, *d*). Ciliated cells were not observed in the intestine during this stage, microvilli were regular and abundant, and epithelial cells were not observed to be lacking of microvilli. (Plate 6.17).

6.3.2.4 Larvae at three, four & five day after hatching

At third DAH the outer nuclear layer (ONL) in the eye was approximately 20μm thick. The number of photoreceptors (PR) in the ONL increased both in length and number; the cone-type photoreceptors were clearly distinguished. During third DAH the pigment epithelium layer was found to be thicker (Plate 6.8) and the photoreceptors had an oil droplet in the outer segment (Plate 6.9). By four DAH, the eyes of larvae were well established and the layers of the retina were clearly differentiated. The eyes at four DAH (Plate 6.10) were similar to the adult stage.

Little change was observed in the external morphology of the gut during this period. The mouth during these three days became more advanced. The oil globule was still visible during three DAH and four DAH (Figure 6.8, *e* & *f*); however, it was not visible during five DAH (Figure 6.8, *g*). The ultrastructure of the intestine during three DAH showed that the mitochondrion had increased in number and the microvilli were more compact, food particles were observed in the rectal area of the intestine (Plate 6.18).

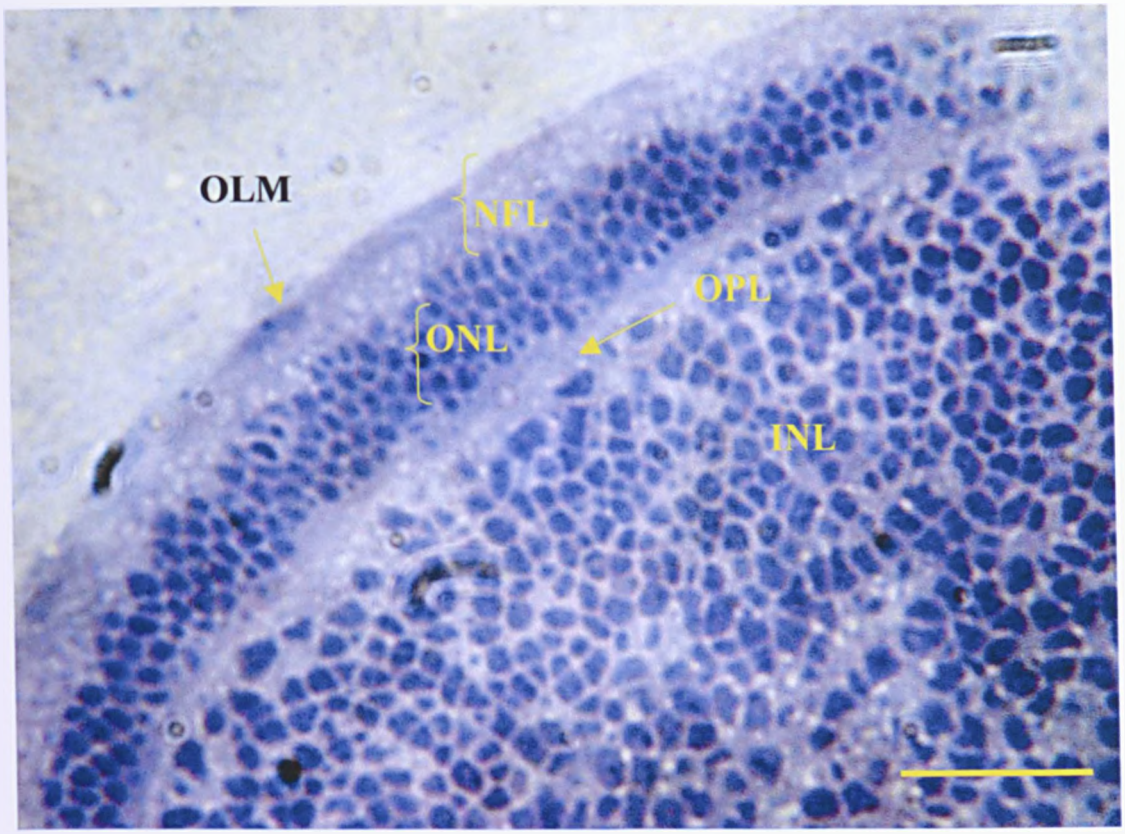


Plate 6.1 Semi-thin section of eye of zero DAH (20 hrs post-hatching) *R. sarba* larvae showing general view of retinal layers.

Scale bar 20 µm.
INL - Inner nuclear layer
NV - Nerve fibers
OLM - Outer limiting membrane
ONL - Outer nuclear layer
OPL - Outer plexiform layer

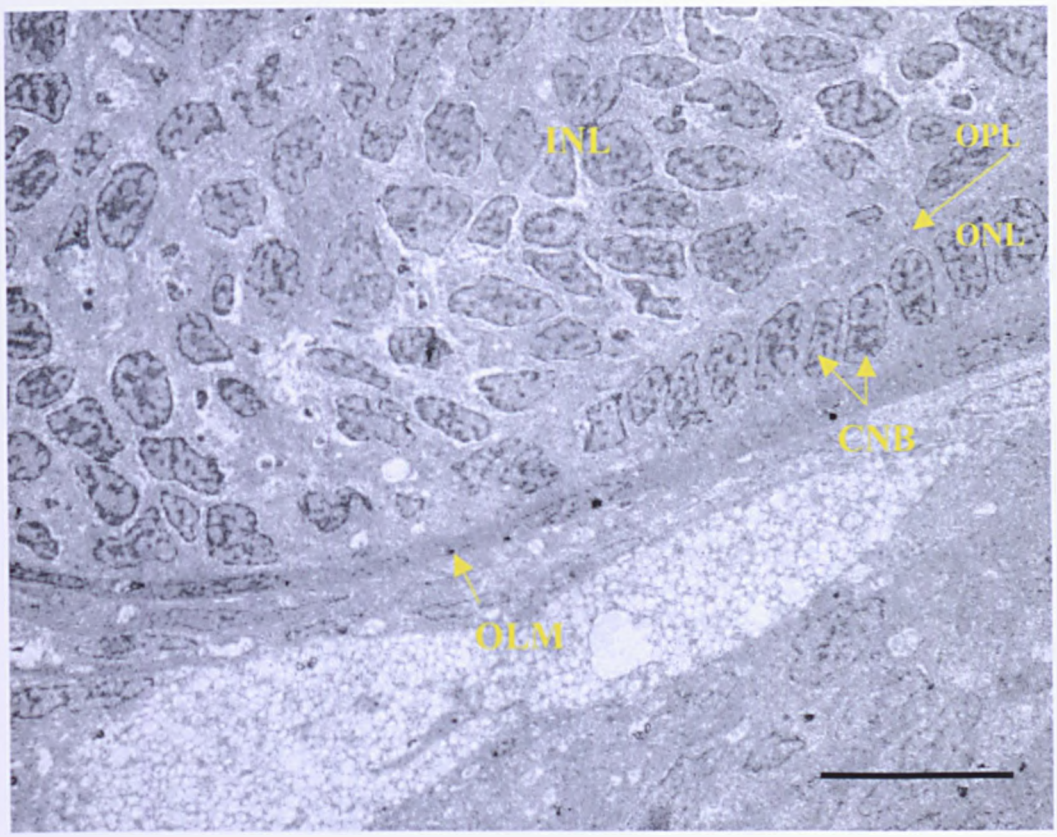


Plate 6.2 Electron micrograph of longitudinal section through eye of *R. sarba* at zero DAH (20 hrs post-hatching).

Scale bar 10µm.
CNB - columnar nuclear bodies (nuclei of photoreceptors)
INL - inner nuclear layer
ONL- outer nuclear layer
OPL - outer plexiform layer

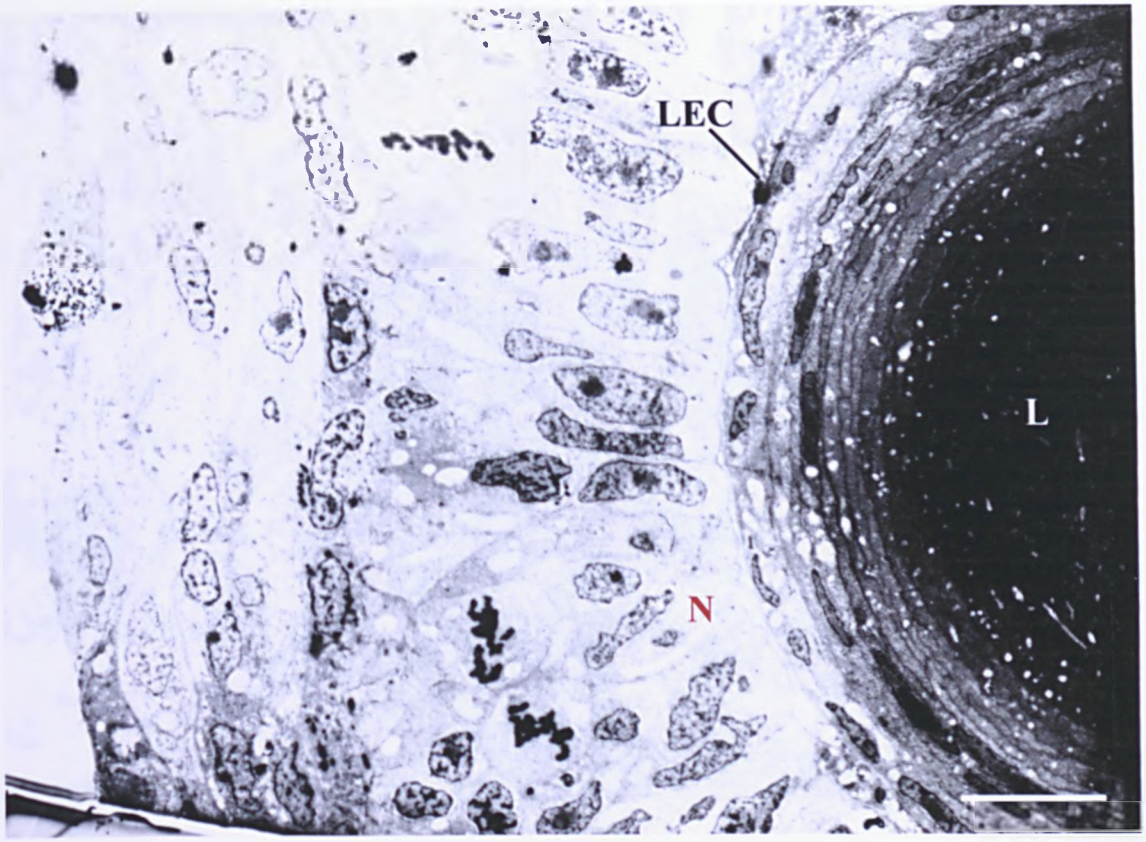


Plate 6.3 Electron micrograph of cross section through eye of *R. sarba* at zero DAH.

Scale bar 4µm.

L - lens

LEC - lens epithelial cell

N - neurons

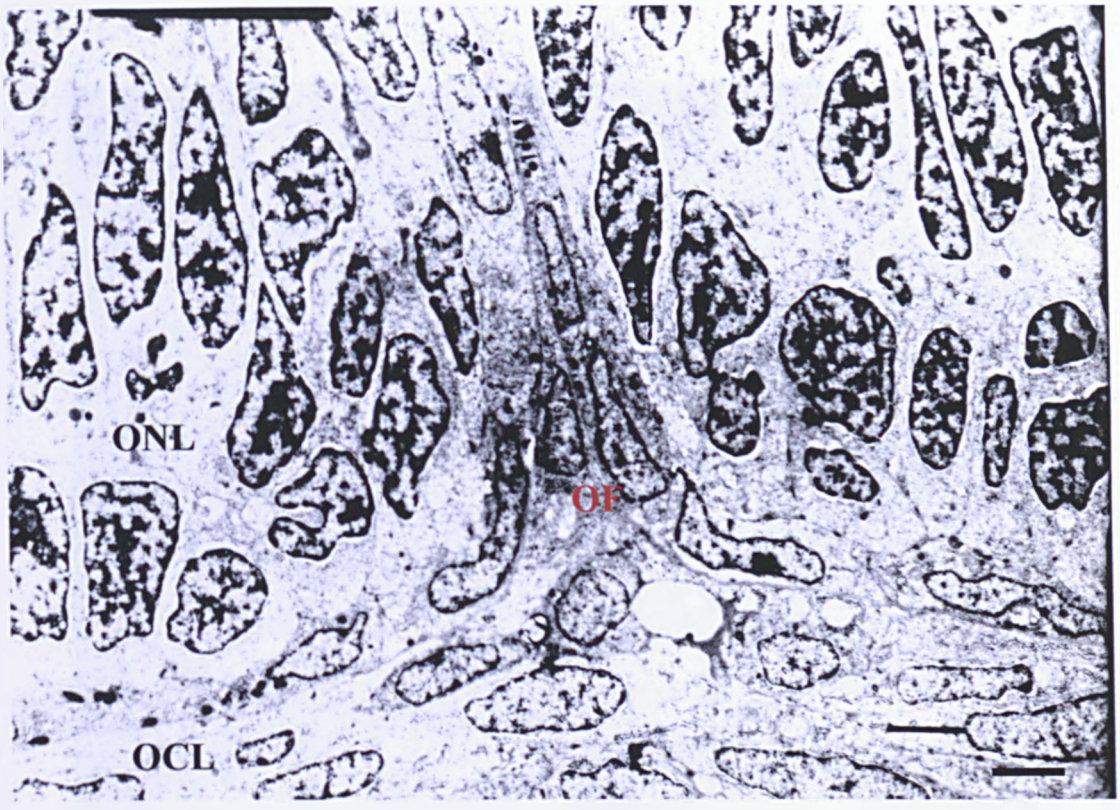


Plate 6.4 Electron micrograph of cross section through eye of *R. sarba* at zero DAH.
Scale bar 2µm.
OCL - outer capillary layer
OF - optic fissure
ONL - outer nuclear layer

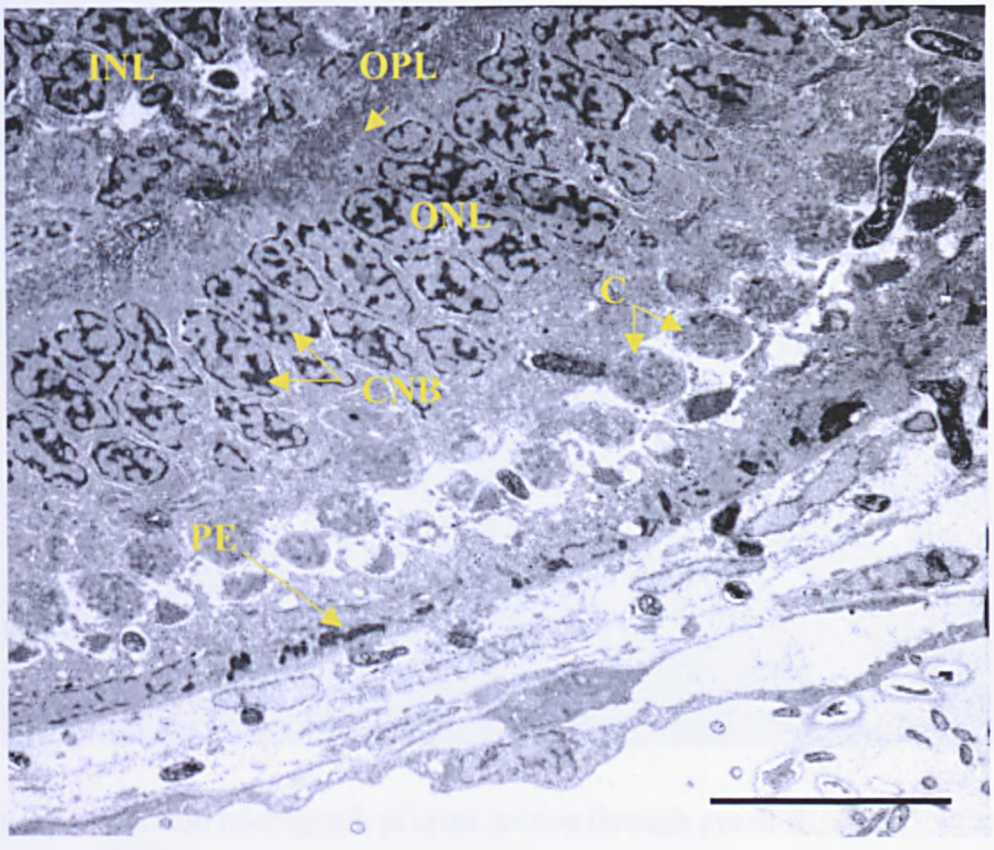


Plate 6.5 Electron micrograph of longitudinal section through eye of *R. sarba* at one DAH.

Scale bar 10µm.
C - cones (outer segment)
CNB - columnar nuclear bodies (nuclei of photoreceptors)
INL - inner nuclear layer
ONL - outer nuclear layer
OPL - outer plexiform layer
PE - pigment epithelium

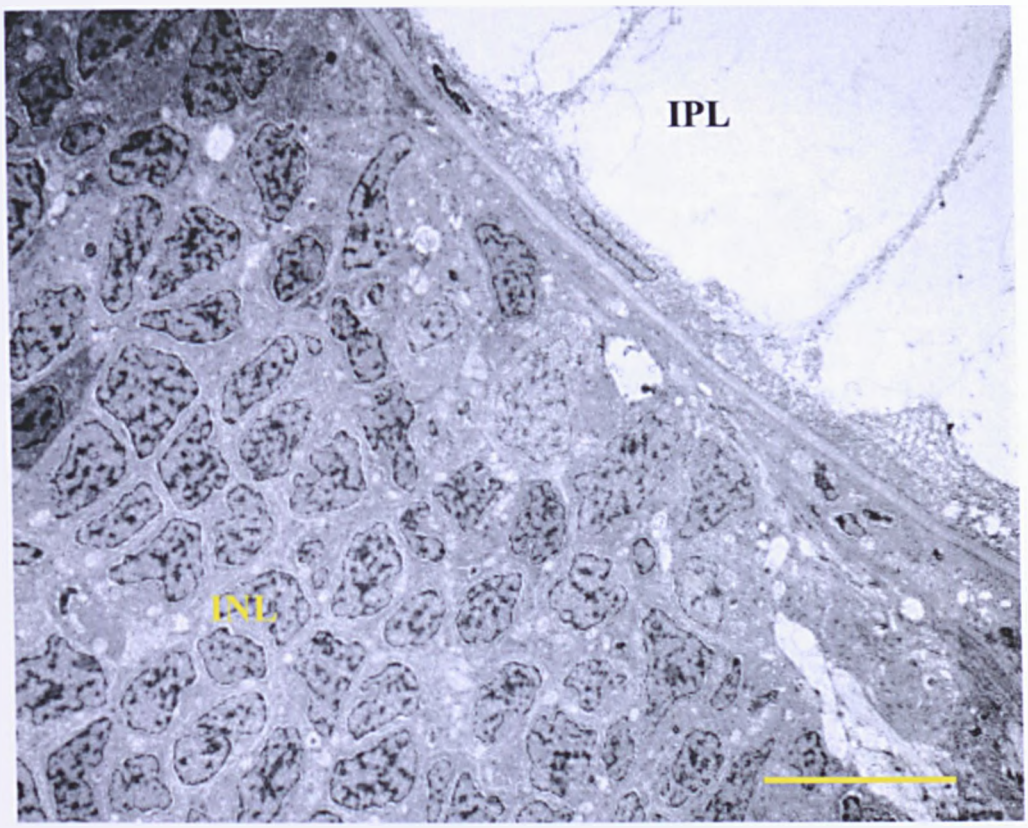


Plate 6.6 Electron micrograph of cross section through eye of *R. sarba* at one DAH.

Scale bar 10µm.
INL - inner nuclear layer
IPL - inner plexiform layer

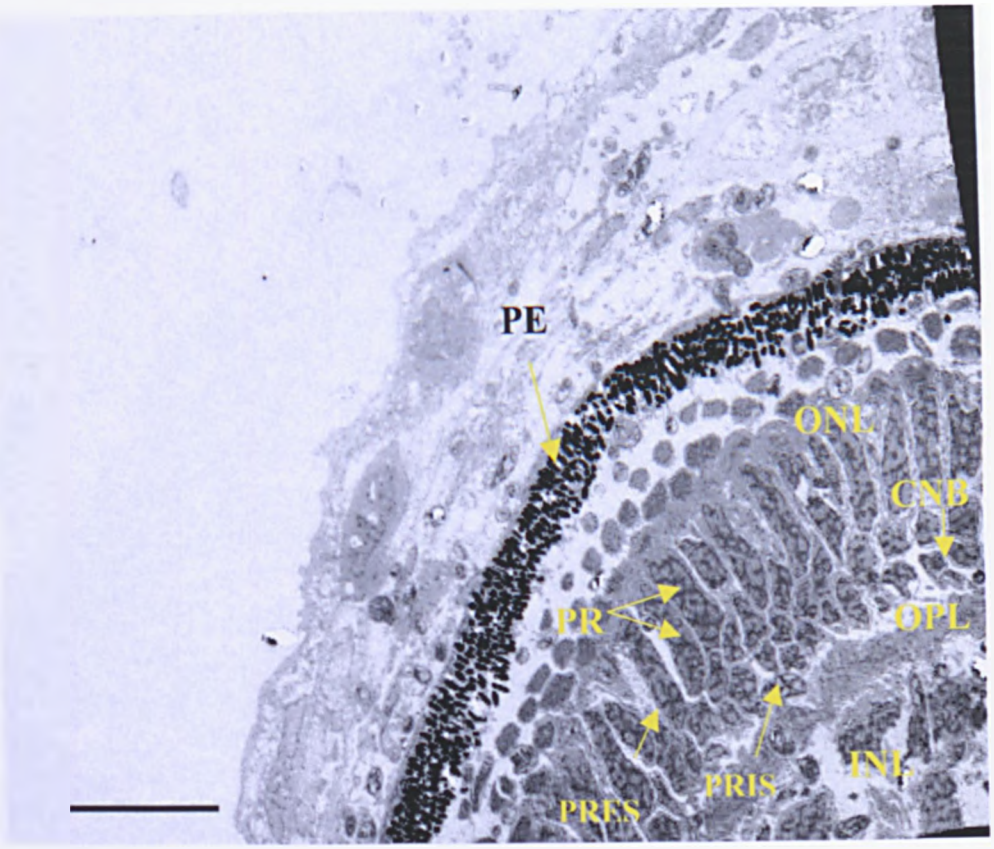


Plate 6.7 Electron micrograph of cross section through eye of *R. sarba* at two DAH.

Scale bar 10µm.
C - cones (outer segment)
CNB - columnar nuclear bodies (nuclei of photoreceptors)
INL - inner nuclear layer
ONL - outer nuclear layer
OPL - outer plexiform layer
PE - pigment epithelium
PR - photoreceptors
PRES - photpreceptor outter sigment
PRIS - internal segment

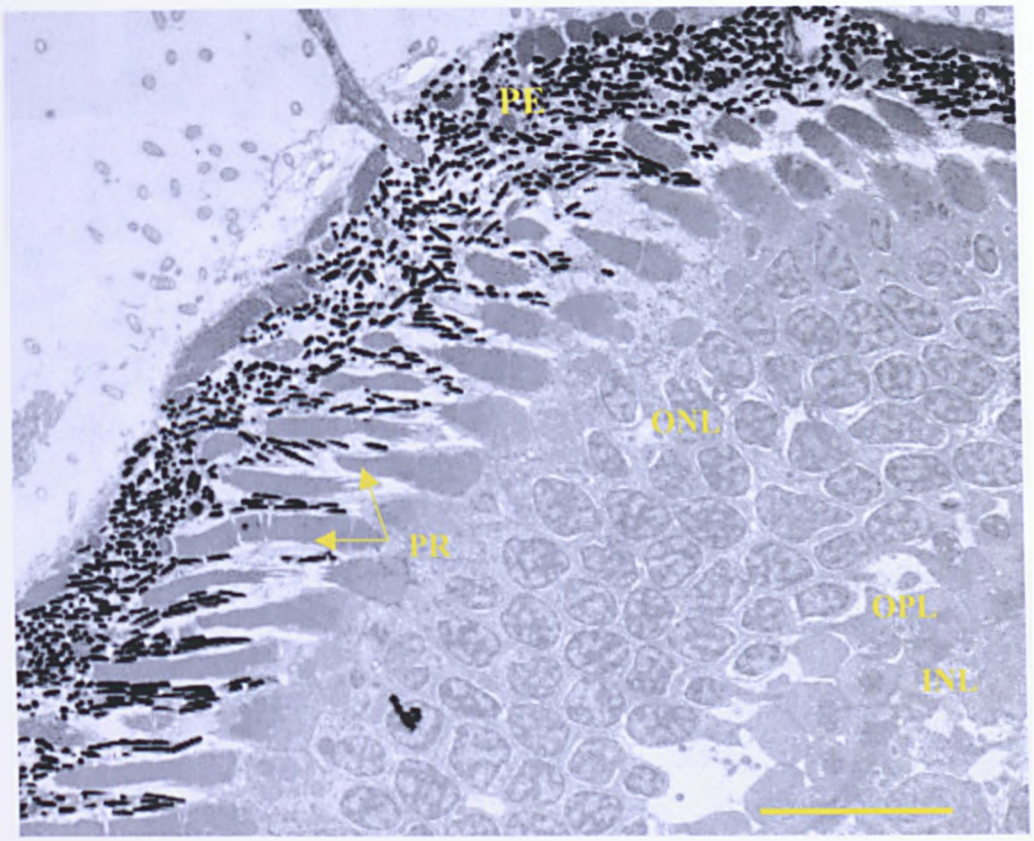


Plate 6.8 Electron micrograph of cross section through eye of *R. sarba* at third DAH.

Scale bar 10μm.
CNB - columnar nuclear bodies (nuclei of photoreceptors)
INL - inner nuclear layer
ONL - outer nuclear layer
OPL - outer plexiform layer
PE - pigment epithelium
PR - photoreceptors

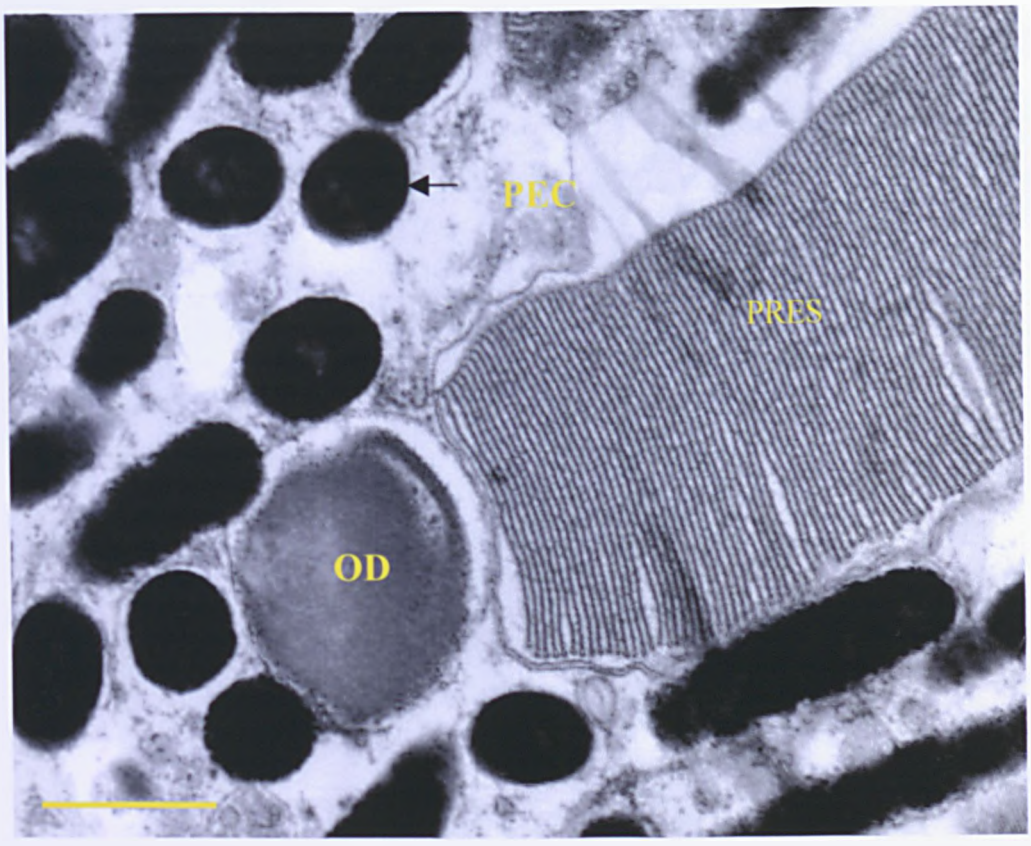


Plate 6.9 Electron micrograph of cross section through the eye of *R. sarba* at third DAH showing existence of an oil droplet in outer segment of rod-type photoreceptor.

Scale bar 0.5µm.
OD - oil droplet
PEC - pigment epithelium cell granule
PRES - photoreceptor outer segment

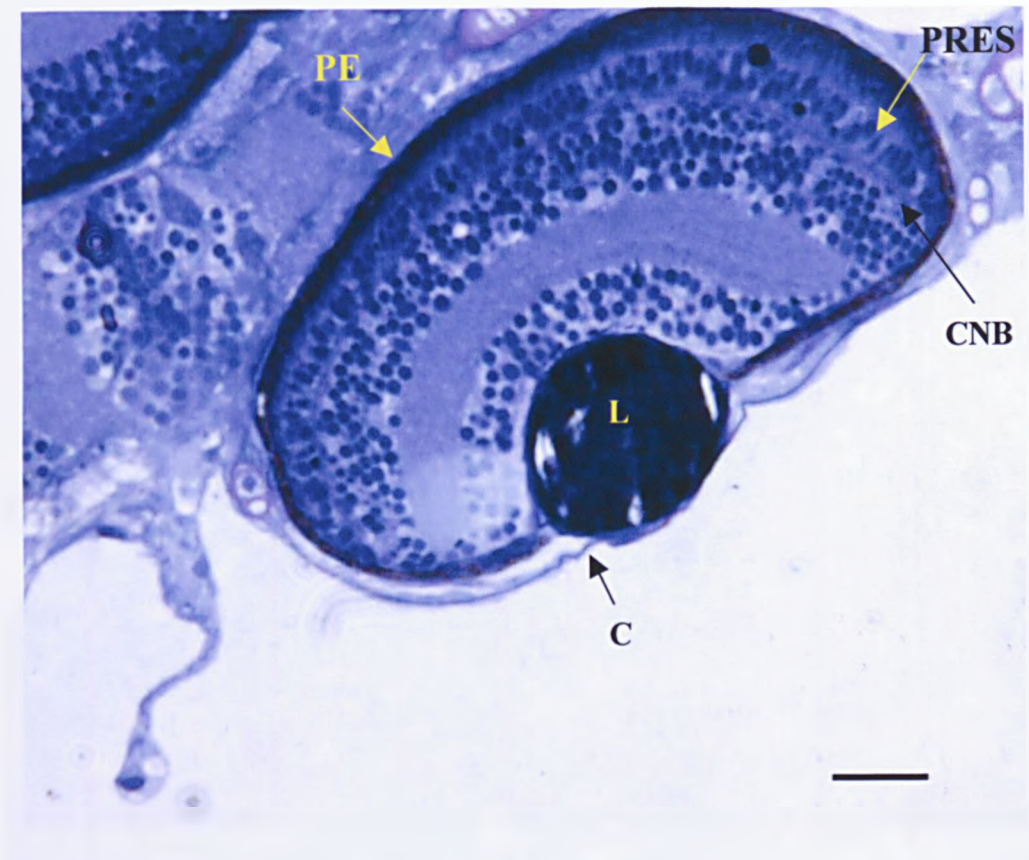


Plate 6.10 Semi-thin section of eye of four DAH *R. sarba* larva showing general view of retina layers.

Scale bar 20µm.
C - cornea
CNB - columnar nuclear bodies (nuclei of photoreceptors)
L - lens
PRES - photpreceptor outter sigment
PE - pigment epithelium
R - retina

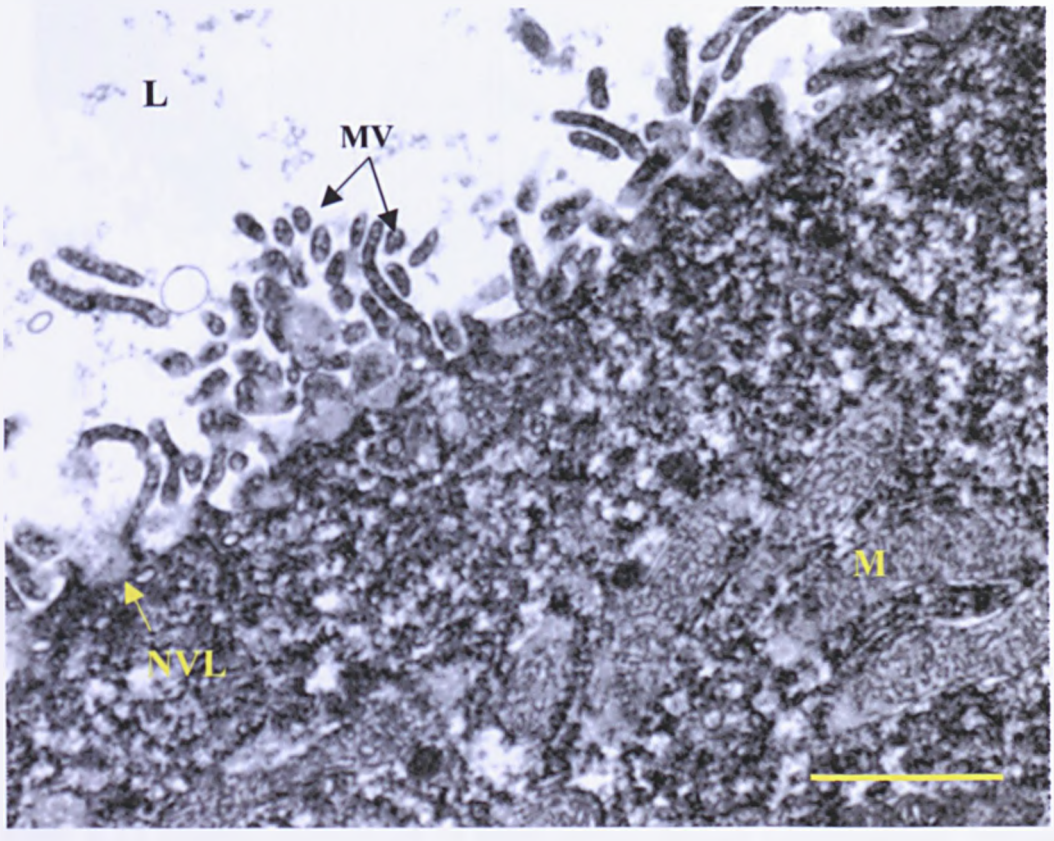


Plate 6.11 Electron micrograph of cross section through antero-median intestine of *R. sarba* at zero DAH (20hrs post-hatching).

Scale bar 1µm.
L - lumen
M - mitochondrion
MV - microvilli
NVR - non-villous region

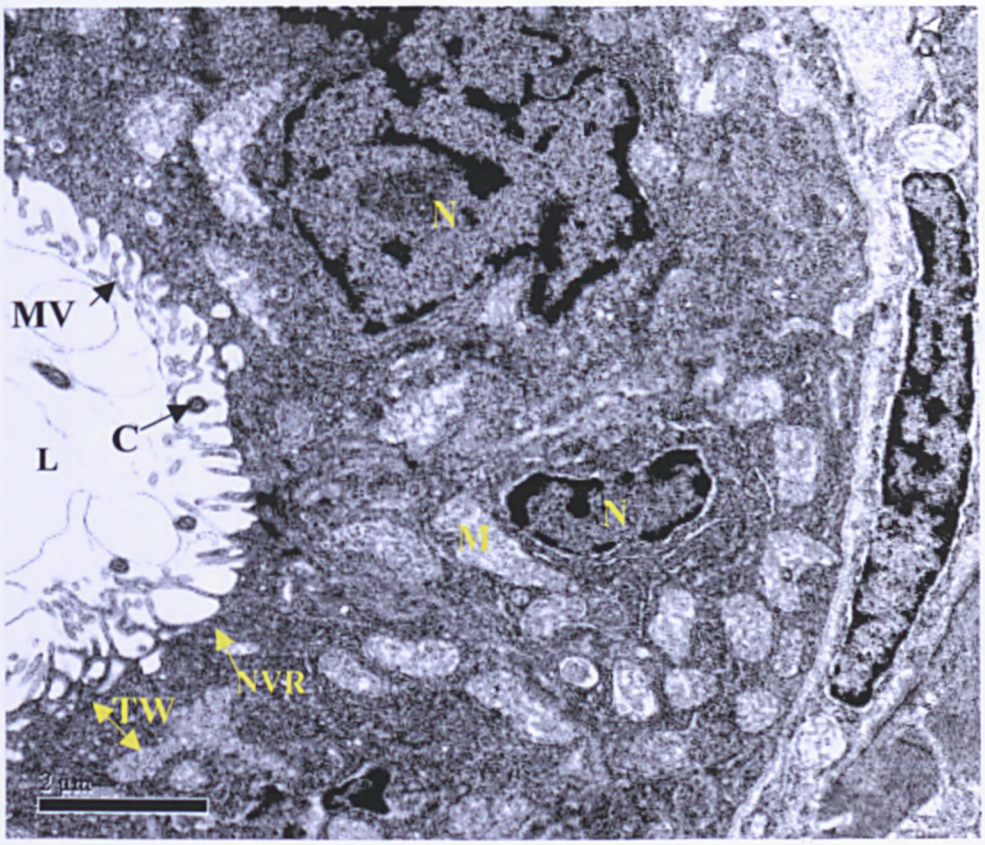


Plate 6.12 Electron micrograph of cross section through antero-median intestine of *R. sarba* at zero DAH (20 hours post-hatching).

Scale bar 2µm.
C - cilia
L - lumen
M - mitochondrion
MV - microvilli
N - nucleus
NVR - non-villous region
TW - terminal web (two-headed arrow indicates the thickness of the terminal web)

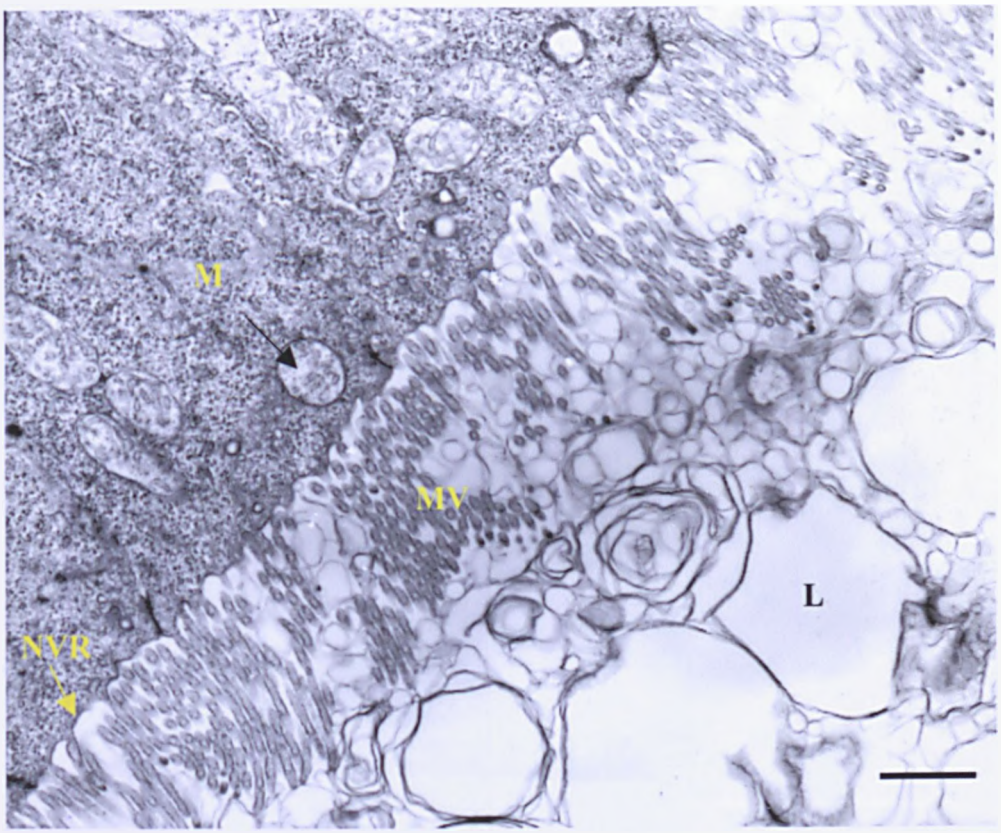


Plate 6.13 Electron micrograph of cross section through rectal area of intestine of *R. sarba* at zero DAH.

Scale bar 1µm.
L - lumen
M - mitochondrion
MV - microvilli
NVR - non-villous region

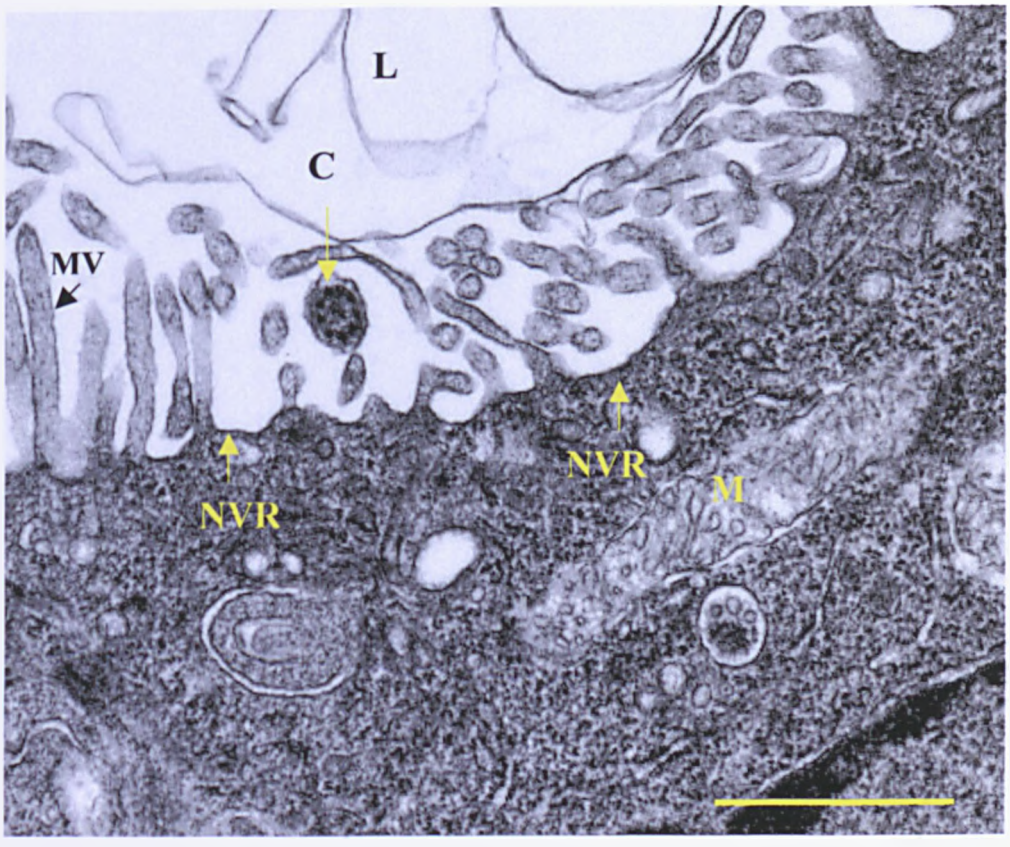


Plate 6.14 Electron micrograph of cross section through antero-median intestine of *R. sarba* at zero DAH.

Scale bar 1µm.
C - cilia
L - lumen
M - mitochondrion
MV - microvilli
NVR - non-villous region

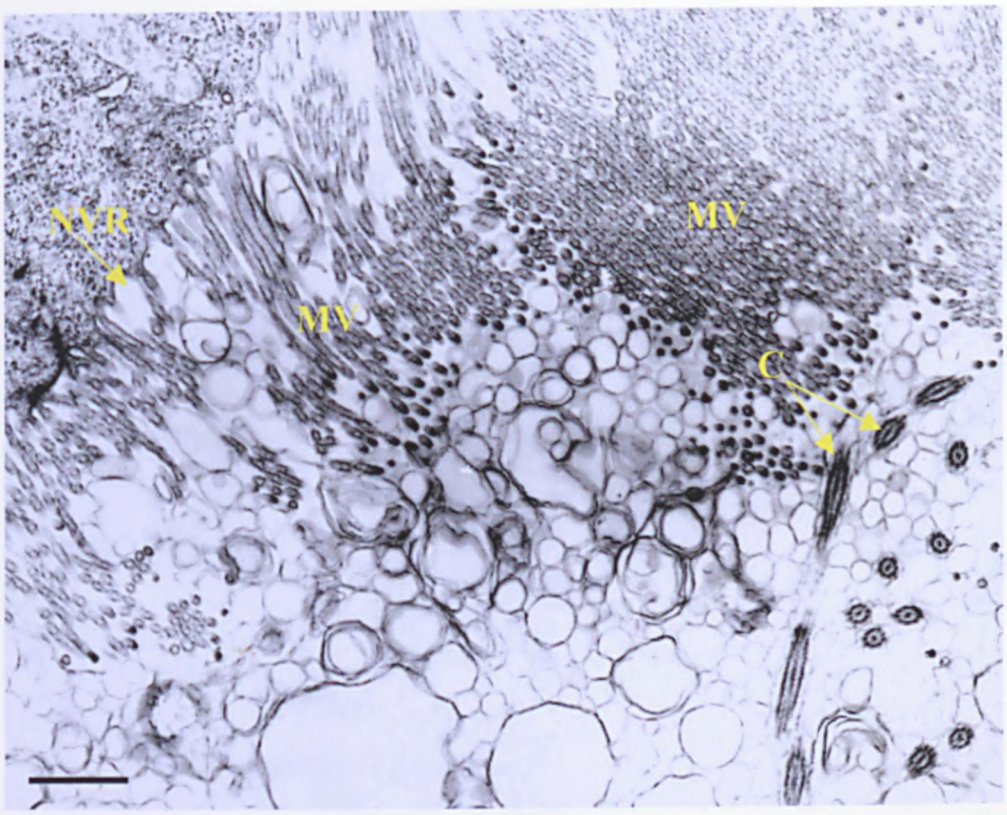


Plate 6.15 Electron micrograph of cross section through rectal area of intestine of *R. sarba* at one DAH.

Scale bar 1µm.
C - cilia
L - lumen
M - mitochondrion
MV - microvilli

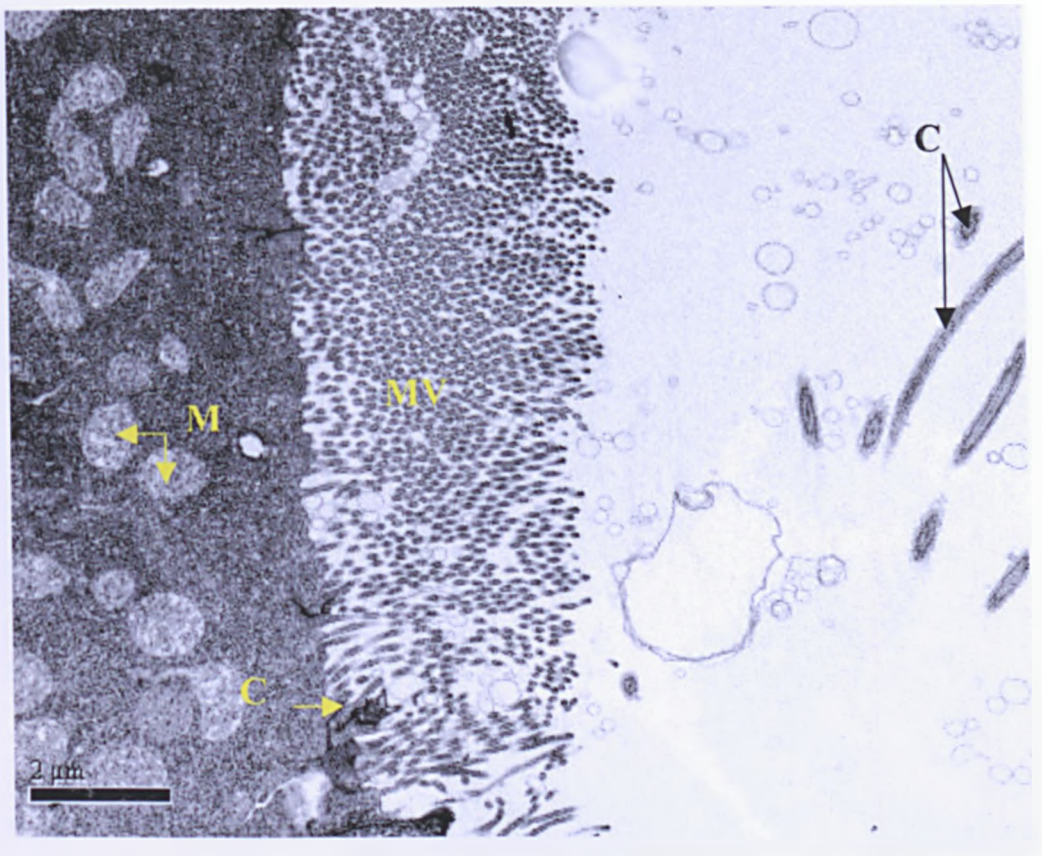


Plate 6.16 Electron micrograph of cross section through antero-median intestine of *R. sarba* at one DAH.

Scale bar 2µm.
C - cilia
L - lumen
M - mitochondrion
MV - microvilli

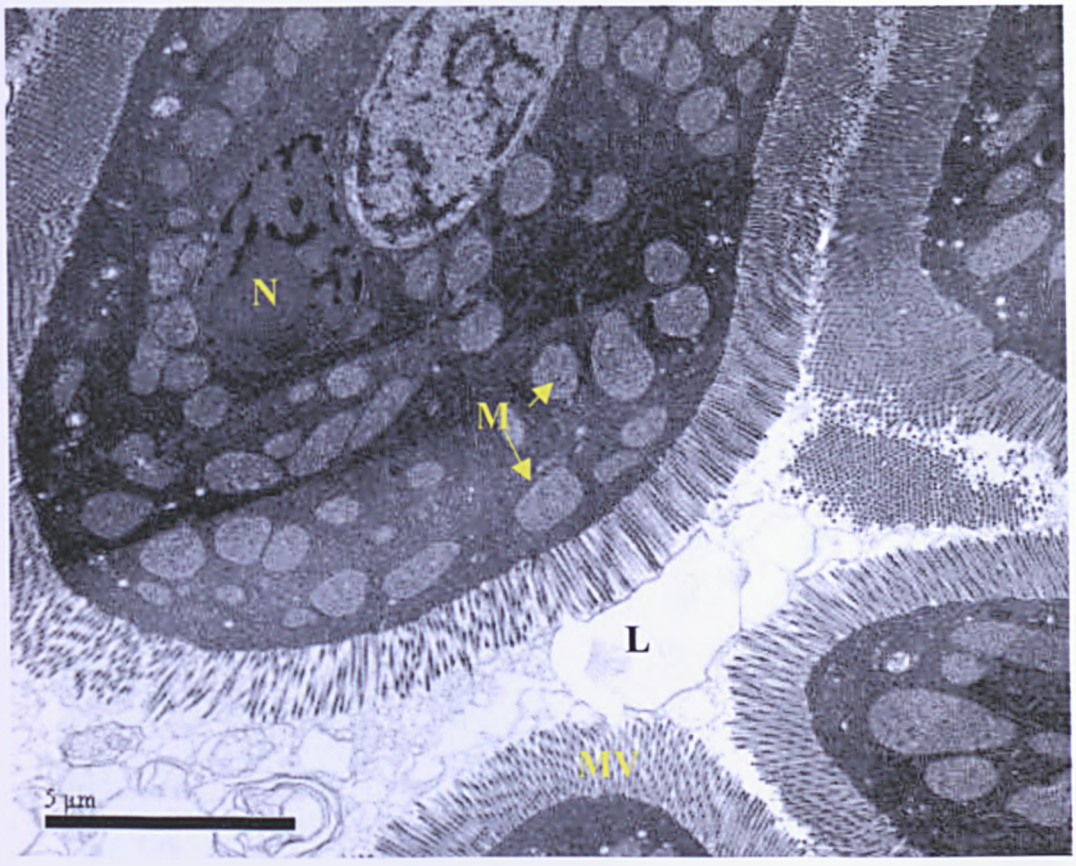


Plate 6.17 Electron micrograph of cross section through rectal area of intestine of *R. sarba* at two DAH.

Scale bar 5µm.
L - lumen
M - mitochondrion
MV - microvilli
N - nucleus

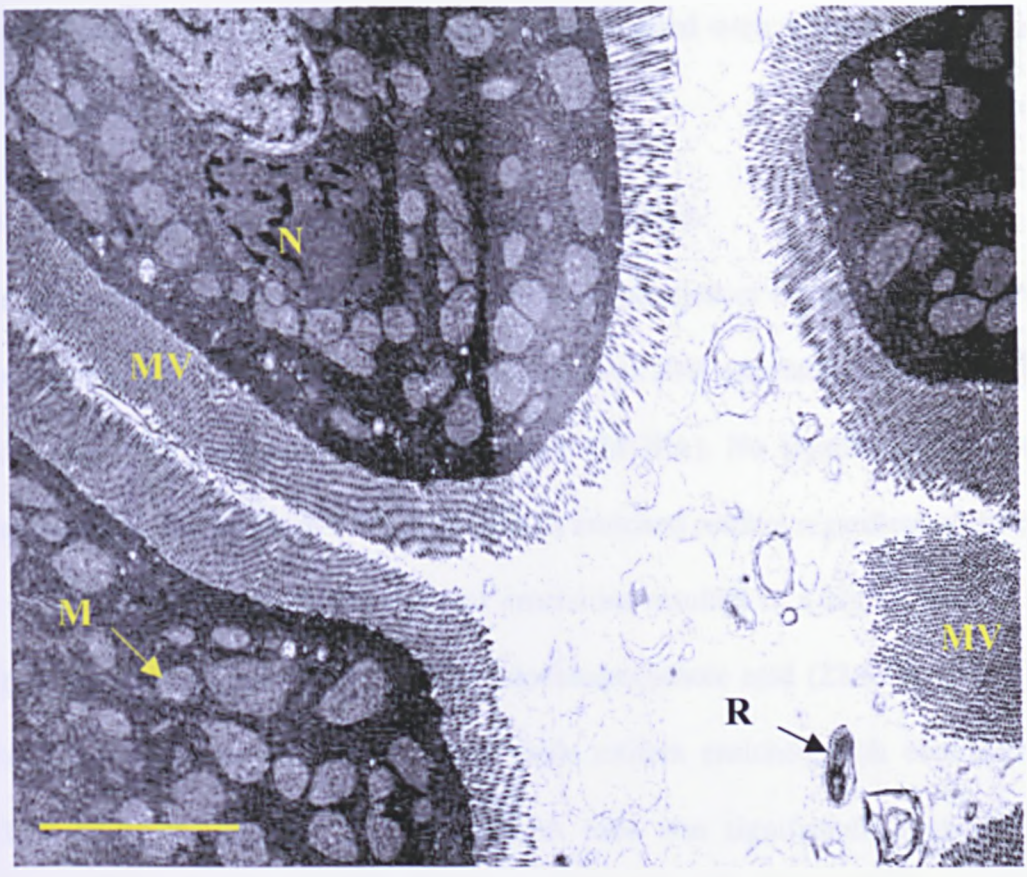


Plate 6.18 Electron micrograph of cross section through rectal area of intestine of *R. sarba* at third DAH.

Scale bar 5µm.
M - mitochondrion
MV - microvilli
N - nucleus
R - rotifer

6.3.3 Feeding and growth of *R. sarba* larva treated with different enrichment agents

6.3.3.1 Lipid and fatty acid compositions of rotifers

The percentage of total lipid and fatty acid composition of the enriched rotifers is given in Table 6.3. All three diets contained over 20% saturated fatty acids (SFA) and about 35% monounsaturated fatty acids (MUFA). No significant differences were found in the levels of SFA & MUFA in enriched rotifers regardless of the type of oil used. Rotifers enriched with oil emulsions resulted in a higher level of n-3 highly unsaturated fatty acids (HUFA) docosahexaenoic acid (22:6n-3, DHA) and eicosapentaenoic acid (20:5n-3, EPA) with rotifers enriched with cod liver oil (CLO) being the highest. The DHA: EPA ratio was significantly higher in the rotifers enriched with cod liver oil (CLO) compared with sunflower oil (SFO) and rotifers fed algae. Similar results were found for (22:6n-3, DHA) and (20:4n-6, AA), DHA: AA ratio. The EPA: AA ratio was not significantly different among the three diets but slightly higher in the enriched rotifers (diet 2 & 3).

6.3.3.2 Larval growth and survival

Growth of *R. sarba* larvae fed differently enriched rotifers for 20 day differed significantly ($p=0.03$). Feeding larvae with enriched rotifers resulted in better growth, especially those fed rotifers enriched with CLO which contained high EPA and DHA (Figure 6.9). No significant differences were observed in total length of larvae for all diets at ten DAH. However, significant differences were detected in final length gained and specific growth rate (SGR) between groups fed enriched rotifers and the control group that was fed *Tetraselmis* (Table 6.4).

Survival rate, however, was improved with larvae that were treated with high levels of DHA and was found to be significantly higher in the groups that were fed rotifers enriched with CLO. At the end of the 20 day experiment survival was 26% for CLO and 15% for sunflower oil (SFO) compared to 7% in the control group (Figure 6.10).

Table 6.3 Fatty acid composition of rotifers enriched for six hours using two different oil emulsions.

FATTY ACID	DIET 1(Control) (Rotifer-algae)	DIET 2 (Rotifer-SFO)	DIET 3 (Rotifer-CLO)
Lipid content	0.7±0.0[a]	1.3±0.1[b]	1.6±0.2[b]
14	0.58±1.0[a]	3.02±0.8[b]	3.26±1.2[b]
16	21.14±2.0[a]	13.83±1.5[b]	14.30±2.5[b]
18	5.64±1.0[a]	5.21±0.5[a]	3.01±1.0[b]
Σ SFA[1]	27.95±2.0[a]	22.97±1.2[b]	21.66±1.8[b]
16:1n-7	2.36±1.0[a]	0.46±0.2[b]	6.2±1.5[c]
18:1n-9	11.38±1.7[a]	28.86±3.0[b]	14.68±2.0[a]
18:1n-7	2.98±0.5	1.29±0.8	3.52±1.0
20:1n-9	2.85±±0.6[a]	0.9±0.1[b]	5.86±0.5[c]
ΣMUFA[2]	27.30±2.0[a]	35.09±1.8[b]	35.61±2.0[b]
22:1n-9	0.24±0.0[a]	0.45±0.1[ab]	0.74±0.1[bc]
18:2n-6	19.91±3.0[a]	27.49±4.2[b]	24.15±4.8[b]
20:4n-6 (AA)	0.20±0.1[a]	0.62±0.1[b]	0.67±0.1[b]
Σ n-6 PUFA[3]	22.96±1.5[a]	28.87±2.5[b]	25.87±3.0[b]
18:3n-3	6.08±1.0	6.30±0.8	5.16±1.0
20:5n-3(EPA)	1.09±0.2[a]	3.81±0.5[b]	4.32±1.0[b]
22:6n-3(DHA)	0.28±0.0[a]	1.3±0.5[b]	3.9±0.5[c]
Σ n-3 PUFA[4]	9.0±2.0[a]	13.07±0.5[b]	16.82±1.0[b]
DHA:EPA	0.25±0.1[a]	0.34±0.5[a]	0.91±0.1[b]
DHA:AA	1.38±0.2[a]	2.10±0.6[a]	5.84±1.0[b]
EPA:AA	5.46±1.0	6.14±0.8	6.4±1.5

1 - Includes 15:0 and 20:0
2 - Includes 16:1n-9, 20:1n-11, 20:1n-7, 22:1 and 24:1n-9.
3 – Includes 18:3n-6, 20:2n-6, 20:3n-6 and 22:4n-6.
4 – Includes 18:4n-3, 20:3n-3, 20:4n-3 and 22:5n-3.
Dry weight (DW mg^{-1}±SD) of rotifers was 85.0±5.0 for rotifers fed with algae, 87.0±2.0 for rotifers enriched with SFO and 84±3.0 for rotifers enriched with CLO.

Table 6.4 Growth performance of *R. sarba* larvae fed rotifers treated with different emulsions.

Feed type	Initial size (mm)	Final size (mm)	Size Gained (mm)	Gain relative (%)	SGR (% day^{-1})
Tetraselmis	1.90±0.20	5.70±0.25[a]	3.8±0.22[a]	200.30±2.8[a]	5.50±0.06[a]
SFO	1.90±0.20	6.20±0.10[b]	4.3±0.20[b]	226.32±2.1[b]	5.9±0.07[b]
CLO	1.90±0.20	6.40±0.30[b]	4.5±0.21[b]	236.84±1.8[b]	6.1±0.09[b]

Data presented as mean ±S.D. Mean values that are not significantly different within column ($p>0.05$) share common superscripts.

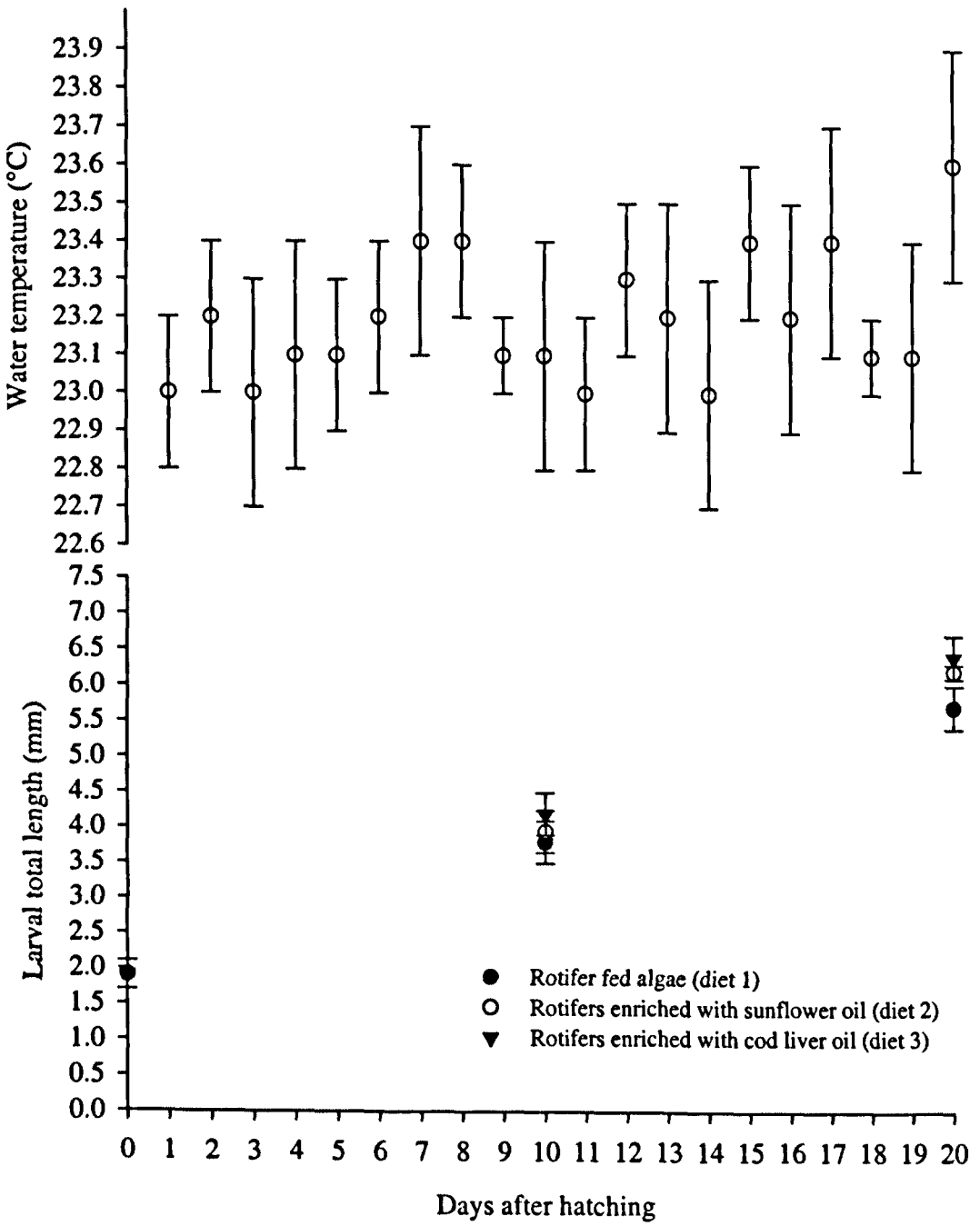


Figure 6.9 Growth of larval *R. sarba* in total length and food items given during the first 20 days of its life.

Data presented as mean ±S.D.

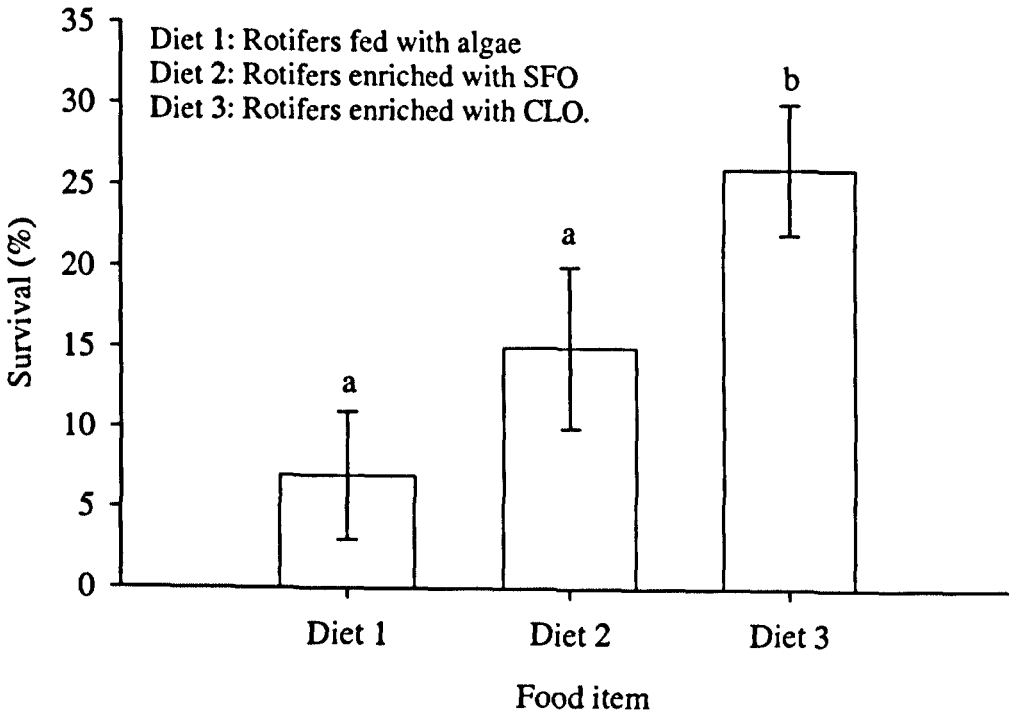


Figure 6.10 Survival of *R. sarba* larvae for each diet at the end of the experiment (20DAH).

Data presented as a mean±S.D.

6.3.4 Discussion

6.3.4.1 Captive spawning of wild stock *R. sarba*

Wild goldlined seabream *R. sarba* were successfully spawned in captivity in the Sultanate of Oman. The results of this study indicated that *R. sarba* responds well to various aspects of culture. The species spawned naturally in captivity, without the use of hormones or environmental manipulation. Several marine species spawn naturally in captivity without control such as the bluefin trevally, *Caranx melampygus* (Moriwake *et al.*, 2001), black sea turbot, *Psetta maxima* (Shiro *et al.*, 2002), spangled emperor, *Lethrinus nebulosus* (Yongzhong *et al.*, 2002) and mangrove red snapper, *Lutjanus argentimaculatus* (Leu *et al.*, 2003).

Goldlined seabream adapted well to captive conditions and tolerated transportation and handling stress, two months was needed for acclimatisation. Wild broodstock were brought in November 2002 and spawned in late December 2002. Similar behaviour was reported by Mahboob *et al.* (1998) and Leu (1994). In early November it was possible to identify the sex, especially males, by applying gentle pressure to the abdomen of the fish. Males produced milt as they matured one month ahead of females. This allowed for sex identification without the need for biopsy, but it is acknowledged that it may not be entirely accurate.

Mihelakakis & Kitajima (1994) reported a higher hatching rate of *R. sarba* egg occuring at salinity between 20 and 36‰ at 18.5°C and maximum yield of normal larvae at 31.4‰. The supplied water in this study had a salinity of 30‰ and had no affect on broodstock behaviour in terms of spawning. It also had no affect on the incubation time and hatching performance as the incubation and embryonic

development was similar to that reported by Lin *et al.* (1988) and Mahboob *et al.* (1998). The spawning occurred at night, a similar finding has been reported in earlier work (Leu, 1994); a behaviour reported for most tropical marine fish species. Johannes (1978) suggested that this behavior is a strategy to minimize egg and larval predation. Tait & Hickman (2001) found that gamete collection of New Zealand turbot, *Colistium nudipinnis* and brill, *Colistium guntheri*, by means of stripping and artificial fertilisation at sea was largely successful when carried out within two to three hours before or after sunset.

The egg-collector that was placed at the holding tank outlet preserved spawned eggs well until removal the following morning. Since spawning of *R. sarba* occurred at night, the placement of egg-collector was not essential until early evening, as water exchange rate tended to slow when it was in position.

Previous studies carried on goldlined seabream suggest that the hatching starts between 24-38 hours post fertilisation (Table 6.5) and is temperature dependant with the longest being a temperature range of 19.2-22.3°C (Leu, 1994). Mahboob *et al.* (1998) reported a hatching rate of 81% to occur after 24-26 hours post-spawning at a temperature range of 23.5-24.5°C. Since eggs were collected the following morning, most eggs had reached the blastula stage. Therefore, based on the status of cell division of the fertilised eggs at the time of egg collection during the current study and the information reported by Lin *et al.* (1988) the hatching period was estimated to be 30 hours at a temperature range of 23.2-24.4°C. Goldlined seabream showed similarity with other sparid species in terms of time required for hatching such as the sheepshead seabream, *Archosargus probatocephalus* that require 28

hours post-fertilization at 23°C (John & Tucker, 1987). However, hatching of other sparids species was observed to take longer than *R. sarba*. Hatching of red porgy *Pagrus pagrus*, for instance, was observed to occur 50 hours post-fertilization, at 18°C water temperature (Machinandiarena *et al.*, 2003).

Table 6.5 Comparison between hatching time of *R. sarba* eggs observed in different studies.

TEMPERATURE (°C)	TIME TO HATCH (h:min)	Author
20.3-32.03	32.03	(Lin *et al.*, 1988)*
23.5-24.5	24-26	(Mahboob, *et al.*, 1998)*
19.2-22.3	30-38	(Leu, 1994)
23.2-24.4	30	This study

* Named by the synonym *Sparus sarba* Forsskal 1775.
For comprehensive study on the effect of salinity and temperature on incubation period refer to (Mihelakakis & Kitajima, 1994).

Spawning of the broodstock was related to water temperature and failure of continued spawning could be attributed to the rise in water temperature that changed the spawning behaviour. The well that supplied water to the culture facility was affected greatly by the tidal system and always had higher water temperature than seawater. During low tide, the quantity of supplied water decreased and its temperature increased. Furthermore, the water exchange in the holding tanks was slow during low tide, which may have contributed further to the increase of water temperature. Abou-Seedo *et al.* (2003) reported that temperature is one of the environmental factors that triggers spawning in captive yellowfin seabream, *Acanthopagrus latus*, while change in temperature are known to delay spawning

periodicity (Hilder & Pankhurst, 2003). Generally, environmental and feeding conditions are evidently the key to success in broodstock management (Shiro *et al.*, 2002).

To study the aquaculture feasibility of any fish species, it is essential to understand the entire behavioural cycle of reproduction such as spawning behaviour and mating (Mercy *et al.*, 2003). During the current investigation of the captive breeding *of R. sarba*, there were constraints that might have affected broodstock performance. Further research is needed in a more suitable environment to overcome these constraints to achieve continuous spawning at least during the natural spawning season for a better understanding of the culture requirements of *R. sarba* in Oman. Understanding the processes involved in reproduction will provide baseline knowledge to aid in designing the optimal breeding system to maximise the production of eggs and allow sustainable production of high quality fry (Tait & Hickman, 2001).

Taking into account reproduction considerations, ability of species of interest to spend most of their life cycle under artificial conditions should be also studied. This research includes larval rearing, their ability to accept and consume artificial diets, their ability to grow in captivity and their growth at high stocking densities. Therefore, study of morphological characteristics provides useful knowledge that helps to improve growth and nutrition during early life stages (Aguilera *et al.*, 2002).

6.3.4.2 Early larval development

Sight plays an important role in initial feeding success and therefore, its development is of fundamental importance to survival. The eye of *R. sarba* at hatching was not functional and was undifferentiated. Similar observations have been reported for other species of seabream such as *Lates calcarifer* (Walford & Lam, 1993), *Sparus aurata* (Sarasquete *et al.*, 1995), *Pagrus pagrus* (Roo *et al.*, 1999) and sand bass *Paralabrax maculatofasciatus* (Pena *et al.*, 2003).

At zero DAH (20 hours after-hatching) the eye was spherical and the retina had a zonation with undifferentiated cells. The eye also lacked differentiated photoreceptors, while columnar nuclear bodies, the nuclei of photoreceptors were observed in the external part of the retina (the outer nuclear layer). Up to this stage of larval development the eye was not yet pigmented. Similar observations have been made on other sparids such as *Pargus major* (Kawamura, 1984) and *Pagrus pagrus* (Roo *et al.*, 1999). Lack of eye pigmentation at hatching has been reported for many teleosts (Blaxter, 1986, Bailey & Stehr, 1986, Porter & Theilacker, 1999, Kaji *et al.*, 2002). At zero DAH (20 hours after-hatching) optic fissure of what could be the entrance of optic nerve was observed while optic nerve itself was not yet developed.

As in most fish that rely on vision for feeding, the eye has to develop to support prey searching and capturing activity. At one DAH the eye in *R. sarba* larvae had the basic elements required for vision, but the eye was still lacking photoreceptors. The nuclei of photoreceptors however, were more advanced during this stage of larval development. These developmental signs have been suggested as an

indication that vision is about to function (Kawamura, 1984, Roo *et al.*, 1999). The present study has demonstrated that retinal photoreceptors increased in length and in number as the yolk-sac was absorbed. By two DAH the eye was fully pigmented, suggesting that the larval vision system is functional (Kawamura, 1984; Porter & Theilacker, 1999) as it has also been reported that pigmentation cells are responsible for photon absorption (Roo *et al.*, 1999). An oil droplet was present in the outer segment of the photoreceptors, an observation also reported for the sparid red porgy, *Pagrus pagrus* (Roo *et al.*, 1999) which further demonstrates the importance of fatty acids for vision of fish larvae (Bell & Dick, 1993). The authors showed that rod-type photoreceptors had more docosahexaenoic acid (DHA) than cone-type photoreceptors in the eyes of herring. Goldlined seabream larvae had a pure cone retina at the onset of the exogenous feeding, similar to that reported for other sparids such as *P. major* (Kawamura, 1984), *P. auratus* (Pankhurst, 1996) and *P. pagrus* (Roo *et al.*, 1999). A pure cone retina was also reported in other teleost fish such as haddock *M. aeglefinus* (Blaxter & Staines, 1970) and walleye pollock *T. chalcogramma* (Porter & Theilacker, 1999). The time of the appearance of rod-type photoreceptors in the *R. sarba* eyes is not known. Those larvae that are reported to have a similar retina at the onset of exogenous feeding, however, are reported to start developing rods at metamorphosis as is the case with sole *S. solea* (Sandy & Blaxter, 1980). For sparids such as red seabream *P. major* (Kawamura, 1984) it has been reported that the rods appear at a later stage in larval development when the larvae reach a total length of 11mm. Pankhurst (1996) reported that rod precursor cells appear in the retina at eighteen DAH in New Zealand snapper, *P. auratus*. A similar observation was also reported for red porgy *P. pagrus* (Roo *et al.*, 1999). Rods have been suggested to especially enhance the vision in deeper water as the

light intensity decreases (O'Connell, 1981). Therefore, rods become useful as the larvae complete metamorphosis and migrate to deeper waters where visual sensitivity has to increase (Roo *et al.*, 1999).

In present study newly hatched *R. sarba* larvae had a large oil globule at the anterior end of the yolk-sac, similar to other marine fish larvae. At hatching, the digestive system was histologically undifferentiated. Furthermore, the digestive tract was a straight tube attached to the dorsal end of the yolk-sac and was not connected to either mouth and anus, similar to observations reported in other seabream *L. calcarifer* (Walford & Lam, 1993), *S. aurata* (Sarasquete *et al.*, 1995) and *P. pagrus* (Roo *et al.*, 1999). A similar digestive system was also reported in other species of fish larvae such as the Atlantic menhaden, *L. xanthurus* (Govoni, 1980) and the sand bass *P. maculatofasciatus* (Pena *et al.*, 2003). The separation of the digestive tract into anterior and posterior by the intestino-rectal valve was observed at one DAH. At two DAH the digestive system was well differentiated and the separation of mid and hindgut by the intestino-rectal valve became more advanced. It has been suggested that this separation keeps the digestive enzymes in the gut in order for them to be re-utilised (Pedersen & Hjelmeland, 1988). Food particles were observed in the rectal part of the intestine at three DAH as the mouth completed its development, similarly, food was observed at the same time in gilthead seabream larvae, *Sparus aurata* (Calzada *et al.*, 1998). Mouth development for other sparid species, has been reported to occur at similar time such as in sheephead seabream, *Archosargus probatocephalus* (John & Tucker, 1987) and *Sparus sarba* (Deane *et al.* 2003). Machinandiarena *et al.* (2003) observed mouth development in red porgy *Pagrus pagrus* to occur between three DAH and four DAH.

In this study, by two DAH, the yolk-sac was completely absorbed. In sheephead seabream, *A. probatocephalus* the yolk-sac is exhausted at four DAH (John & Tucker, 1987) and at two DAH for seabass, *Lates calcarifer* (Walford & Lam, 1993). Oil globule was observed until four DAH, beside its nutritive value (*Review*, Tocher, 2003), it also aids the buoyancy of post hatched larvae (Ehrlich & Muszynski, 1982; Buxton, 1990; Rønnestad *et al.*, 1992) possibly until the swim bladder is developed.

Ultrastructural observations of the gut showed that the layer of gut epithelium at some regions of the luminal surface was straight and microvilli were not present at zero DAH. However, these straight borders were not observed at one DAH onwards as microvilli increased in number on luminal surfaces and became more regular. Also at some regions ciliated cells were found in the epithelium lining. The existence of simple type luminal surface during the early part of larval life has been reported for other fish larvae (Avila & Juario, 1987). Ciliated cells were present in the digestive tract of *R. sarba* larvae and were observed at zero DAH and one DAH but those cells were not found in the gut at two DAH and onwards. Ciliated cells are uncommon in the adult stage, but have been reported in many teleosts larvae and juveniles (Iwai, 1967; Iwai & Rosenthal, 1981; Govoni *et al.*, 1986; Loewe & Eckmann, 1988; Calazda *et al.*, 1998). In Calazda *et al.* (1998) study on *Sparus aurata* larvae, the authors reported that cilia were found to run parallel to the lumen longitudinal axis and similar observation were made in *R. sarba* larvae in the current study. In the absence of smooth muscle in the early larval intestine, peristalsis is not possible as a mean of moving ingesta. Therefore, these cilia may

contribute to the circulation of the intestinal contents (mainly yolk) at the time when the anus is still closed in order to facilitate absorption (Iwai, 1967).

Generally, morphological and functional differentiation of the digestive tract and the eye of the *R. sarba* larvae precedes the completion of the yolk and oil globule absorption. The separation of mid and hindgut, observation of food particles and the absorption of the yolk-sac all took place as vision became fully functional. Such development prevents the larva from starvation, as the endogenous nutrition reserves become depleted (Avila & Juario, 1987). Food particles were observed at three DAH and the oil globule was exhausted at four DAH, these findings were also reported for seabass, *L. calcarifer* (Khono *et al.*, 1986; Walford & Lam, 1993), seabream, *A. probatocephalus* (John & Tucker, 1987) and halibut, *H. hippoglossus* (Kjørsvik & Reiersen, 1992). John & Tucker (1987) reported that vision and digestive systems in sheephead seabream, *Archosargus probatocephalus* were functional at three DAH but no histological evidence was provided.

6.3.4.3 Growth and feeding of *R.Sarba* larva

At the end of the 20 day experimental period, the *R. sarba* larval growth in terms of length and survival rate were significantly higher in groups fed enriched rotifers and resulted in higher specific growth rate (SGR). Survival rate was higher in those groups that had higher levels of n-3 highly unsaturated fatty acids (n-3HUFA), particularly docosahexaenoic acid (22:6n-3, DHA) and eicosapentaenoic acid (20:5n-3, EPA). Watanabe *et al.* (1989) showed that the survival rate improved significantly as the level of eicosapentaenoic acid (EPA) increased for red seabream. El-Dakar *et al.* (2001) also reported the same results for seabass. Abu-

Rezq *et al.* (2002) demonstrated that feeding the blue-fin seabream larva, *Sparidentex hasta* with rotifers high in DHA did not improve growth, but rather improved survival rate significantly. Survival may also be attributed to the increase in the levels of arachidonic acid (20:4n-6, AA) in enriched rotifers compared to the control. This particular fatty acid has been shown to improve larval survival (Bell *et al.*, 1985b; Castell, *et al.*, 1994) particularly in the case of handling stress (Koven *et al.*, 2001).

The level of EPA in the CLO resembled that of the wild female's ovaries, particularly at the final stage of maturity (S6). As discussed earlier in Chapter 6, the level of EPA in the females' ovaries at this stage was 4.7%. However, the enrichment of rotifers has certainly increased their level of total lipid, although some of the essential fatty acids such as DHA and AA were not similar to that level found in the ovaries of the wild population during the final stage of maturity. Rodríguez *et al.* (1996) suggested that rotifers incorporate EPA better than DHA regardless of their ratios in the diet. However, levels of EPA and DHA were significantly higher in rotifers enriched with CLO; this may have contributed to better growth and survival rate.

It has been suggested that EPA and DHA are the most important n-3 family fatty acids that should be incorporated in the diet of fish larvae at appropriate levels (Rainuzzo *et al.*, 1997). No significant differences were observed between levels of total saturated fatty acids (SFA) and total monounsaturated fatty acids (MUFA) among rotifers enriched with CLO and SFO. Also, the level of total lipid (TL) in

these treatments was not significantly different from each other; these findings were also observed by Rodríguez *et al.* (1998).

Deficiency in essential fatty acids (EFAs) such as EPA and DHA has been demonstrated to affect the growth, survival and susceptibility to stress and disease of many fish species. These species are for example, red seabream, *Pagrus major* (Watanabe *et al.*, 1989; Furuita *et al.*, 1996), seabass, *Dicentrarchus labrax* (El-Dakar *et al.*, 2001), milkfish, *Chanos chanos* (Gapasin & Duray, 2001), striped bass, *Morone saxatilis* and palmetto bass, *M. saxatilis* (Tuncer & Harrel, 1992), rainbow trout *Oncorhynchus mykis* (Kiron *et al.*, 1995) and catfish, *Ictalurus punctatus* (Lingenfelser *et al.*, 1995).

Several authors have demonstrated that the level of essential fatty acids such as EPA and DHA could be improved by increasing the enrichment time (Rodríguez *et al.*, 1996; Reitan *et al.*, 1997; Mingri *et al.*, 2002). In this study, the total lipid and the level of fatty acids might have been improved had the enrichment time been longer than 6 hours.

It is worthwhile noting here that *Tetraselmis* microlagae provides better growth and survival rate when compared with other species of microlagae such as *Chlorella* (El-Dakar, *et al.*, 2001).

Chapter 7: General discussion

This study was carried out with the view to address the possibility and importance of utilising native goldlined seabream in marine aquaculture in the Sultanate of Oman. In order to contribute to this goal various fundamental biological information that was deemed necessary for such an evaluation was researched and discussed in the relevant chapters.

The current study investigated the basic reproductive biology of wild goldlined seabream *R. sarba* in Omani waters. The study addressed age, growth, age at maturity and mortality rate. The biological data was supported with histological evidence of the gonads of the species that provided more accurate data related to maturity and gonadal recrudescence. Changes in body energy reserves, mainly lipid in female *R. sarba* during the reproductive season were investigated to address the importance of lipid in the development of ovaries. Fatty acid composition and profiles in the ovaries at various stages of development during the reproductive season were investigated and showed a trend. The study also investigated the spawning of captive broodstock and focused on the development and feeding of *R. sarba* larvae during early life history.

In chapter one of this study utilisation of *R. sarba* otoliths proved a useful and reliable method to age the species. During the current study, it was noted that the ring formation in the otolith showed in Chapter three that an opaque ring (dark ring) was formed mainly during the reproductive season and coincided with the development of the gonads as indicated from the gonad histology reported in Chapter four. Opaque ring formation correlated well with the condition and well-being of *R. sarba*. It was apparent that during the reproductive season the condition

of maturing fish became poorer possibly due to depletion of energy reserves. Lipid reserves in females in particular during the study reported in Chapter five decreased during the reproductive period, which may have contributed to slow growth (Iles, 1984; Rowe & Thorpe, 1990; Wiegand, 1996) and hence the formation of an opaque ring during this period (Pulfrich & Griffiths, 1988; Van Der Walt & Beckley, 1997; Pajuelo & Lorenzo, 2000; Radebe, *et al.*, 2002).

The exploitation rate indicated that *R. sarba* wild stock was not overfished. However, the direct effects of fishing on the population and future changes in the abundance of the species could result from the age or length at first capture. The length at first capture estimated from the artisanal fishery was $LC_{50\%}$= 13.3cm total length (TL) which was smaller than the length at first maturity for males which was $L_{50\%}$=19cm TL and females which was 23.4cm TL. With the current $LC_{50\%}$ being below length at first maturity for both sexes one can predict a danger of recruitment overfishing. Furthermore, the fishing activities increased notably during the reproductive season which could deplete the adult spawners and further add problems to recruitment. Stock–recruit relationships in finfish are maintained by adequate spawning stock that is critical to sustainable fisheries (Myers & Barrowman, 1996).

A relationship has been suggested to occur between spawning stock abundance and survival of their young based on fertilization success (Walters & Kitchell, 2001). Goldlined seabream *R. sarba* sex ratio and length-frequency distributions were also consistent with a diagnosis of protandric hermaphroditism (Yeung & Chan, 1987b; Garrat, 1993). Females smaller than 16cm TL were uncommon, and only one male

more than 36cm TL was captured. Concentration of catch at smaller age, therefore, will remove individuals from the population before change of sex takes place, such action will make the species mature at lower ages and hence lower fecundity. There is no fishery regulation which control the size of demersal finfish landed, therefore, whatever sizes were caught were displayed in the market. During the current study, a newly recruited cohort of *R. sarba* were observed three months after the spawning season and these individuals were displayed for sale in the fish market. Since *R. sarba* feed near to the shore and can be found in shallow waters the source of these individuals (<60mm) were mainly from recreational fishing and by-catch from beach seine fishery. It is worthwhile noting here that in general the recreational fishermen and part-time fishermen (individuals engaged partly in the fishery) also contribute to the landings of *R. sarba* of all sizes. There is no data available about the contribution of these two types of fishermen in terms of landings of demersal species, which could be significant. In the absence of regulation that controls the sizes of demersal finfish supplied to the fish market, individuals who practice fishing will still continue fishing without paying attention to the problem that is arising from such practice. On the other hand, however, the market will accept any fish landed regardless of size. It is unlikely that fishermen realise by themselves the effect they are imposing on the fish population. In the view of these problems, it is recommended therefore, that a minimum landing size is imposed and its implementation is monitored so that individuals of *R. sarba* can have the opportunity to spawn more than one spawning season. Also, since *R. sarba* is mainly caught by demersal traps, these traps are size selective to some extent by the mesh and funnel size (Al-Masroori, 2002). Therefore, modification is required in order to eliminate small individuals from being caught. Regulation of fishing gear,

may have been successfully applied to certain fishery practices in Oman but neglected on others (Siddeek *et al.*, 1999).

The environmental data about sea water temperature reported in Chapter four and the water temperature measured in broodstock tanks described in Chapter six during this study indicated the importance of temperature in gonadal development of *R. sarba*. Development of gonads of wild *R. sarba* collected from the fishing grounds indicated a correlation with decrease in water temperature, and increase of water temperature marked the end of the spawning season. With regards to the wild broodstock held in captivity, spawning occurred as temperature decreased and spawning activity was ceased due to increase in water temperature in the holding tanks. The current findings indicate the importance of this environmental parameter in reproduction of fish and therefore low temperature should be maintained in order to achieve continuous spawning during the reproductive season (Bromage *et al.*, 1993).

As it was reported in Chapter five, female *R. sarba* tended to maximise lipid reserves during the period that precedes the reproductive season. The lipid was stored in various body tissues such as liver, muscles and in the viscera as visceral fat. High vitellogenic activity during vitellogensis was observed from the histology sections reported in Chapter four of mature ovaries, which suggested high demand for lipid. Vitellogensis is suggested to be the most important stage in oocyte development (Tyler & Sumpter, 1990; Coward & Bromage, 2002; Kwon *et al.*, 2001). Depletion of body lipid reserves, therefore, was correlated with ovarian development, which showed that stored lipid was mobilized and incorporated into

growing oocytes. At the end of the reproductive season in March, female *R. sarba* had negligible or no visceral fat, and level of fat in the liver started to recover to levels not significantly different from what was measured at the beginning of the reproductive season. Muscle and carcass also experienced a significant reduction in total lipid from that level measured during the period that preceded the reproductive season. The present study, therefore, showed the importance of lipid in broodstock diets for both the broodstock itself and the progeny as some of the stored energy may be utilised for body maintenance (Love, 1980; Jonnsson *et al.*, 1991; Almansa *et al.*, 2001; Kwon *et al.*, 2001).

Total lipid (TL) and fatty acid (FA) composition and profile in wild *R. sarba* ovaries reported in Chapter five provided guidelines regarding the dietary requirement of broodstock, which may assist improving survival of progeny (Almansa *et al.*, 2001). As these authors suggest, the level of fatty acids in the ovaries is reflected by the diet of broodstock. Total polyunsaturated fatty acids (PUFA) accounted for about 40% which is typical of wild marine teleosts and reflects the importance of these FA's for the developing embryo and hatched larvae during the endogenous feeding phase (Tocher & Sargent, 1984). The level of n-3 PUFA was higher than n-6 PUFA in maturing ovaries, which reflect the importance of n-3 PUFA in the survival of marine teleosts during early life stages. The dominating FA in (n-3) PUFA was docosahexaenoic acid (DHA, 22:6n-3), which comprised about 15% of mature ovaries, reflecting the importance of this FA during early ontogeny (Ashton *et al.*, 1993; Silversand *et al.*, 1996; Bruce *et al.*, 1999). Eicosapentaenoic acid (20:5n-3, EPA) in (n-3) PUFA was also comparatively high

in mature ovaries which also reflects the importance of EPA for marine fish larvae (Silversand *et al.*, 1996).

No control was applied to the level of fatty acids provided in the enrichment emulsions supplied to the live prey of *R. sarba* larvae during the 20 days of post-hatching reported in Chapter six. Despite this, oil emulsions proved to improve the FA profile and composition of the rotifers compared with non-enriched rotifers. Growth and survival rate was higher in those groups that had higher levels of n-3 highly unsaturated fatty acids (n-3HUFA), particularly docosahexaenoic acid (22:6n-3, DHA) and eicosapentaenoic acid (20:5n-3, EPA). The levels of these FA's were also high in the mature ovaries reported in Chapter five, and shown to improve survival of marine fish larvae (Watanabe *et al.*, 1989, Abu-Rezq *et al.*, 2002). Arachidonic acid (20:4n-6, AA) in the enriched rotifers was higher compared to the control, which may also have contributed to the increase in survival (Bell *et al.*, 1985b; Castell, *et al.*, 1994; Koven *et al.*, 2001). Arachidonic acid (AA) was also found at comparatively high levels in mature ovaries of *R. sarba* discussed in chapter five, and has been demonstrated to improve larvae growth and survival (Bessonart *et al.*, 1999).

As reported in Chapter six, the level of EPA in the cod liver oil (CLO) resembled that of the wild female's ovaries, particularly at the final stage of maturity (stage six, S6) discussed earlier in Chapter five. The level of EPA in the females' ovaries at this stage was 4.7% and was 4.2% in the rotifers enriched with CLO. However, the enrichment of rotifers has certainly increased their level of total lipid, although some of the essential fatty acids such as DHA and AA were not similar to levels

found in the ovaries of the wild population during the final stage of maturity. The absolute dietary FA requirements of *R. sarba* larvae are not known and were not researched during this study, therefore, further research is needed in order to determine optimum levels of essential FA's for *R. sarba* larvae.

Larvae of *R. sarba* were observed to start feeding on the third day after hatching which coincided with the development of vision and digestive organ. Despite the fact that vision was probably functional during two DAH, larvae did not commence feeding at this time. Feeding commenced during three DAH when yolk sac was largely absorbed. Therefore, it is recommended that food should not be offered to the larvae soon after hatching especially when the costs involved in preparation of live feed, such as enrichment of rotifers are taken into account. It is also important to reduce risk of contamination especially during early life stages when larvae are still depending on endogenous food and where high mortality could occur due to factors such as poor environmental condition. In this study, the number of larval samples used in monitoring feeding incidence reported in Chapter six was low and not be reliable. Therefore, more research is required to accurately estimate feeding intensity or feeding incidence when *R. sarba* larvae start exogenous feeding since individual larvae usually develop at different rates. It has to be taken into account however, that fish larvae attain better growth if food is available prior to completion of yolk absorption such as in Atlantic salmon, *Salmo salar* (Koss & Bromage, 1990), Siberian sturgeon, *Acipenser baeri* (Gisbert & Williot, 1997). Busch (1996) suggested that external food should not be provided for Ruegen spring herring, *Clupea harengus* until the yolk sac is mostly exhausted when makes the oesophagus is sufficiently open for easy passage of prey.

The price of wild *R. sarba* in Matrah fish market was affected by supply, high price occurred at lower supply (Figure 7.1). The price of *R. sarba* in Matrah fish market varies between about $2.2 to $3.0 at an exchange rate of (1$=0.386 OR/kg). Price of *R. sarba* was also monitored in one of the popular superstores in Oman and remained at ($1.6, O.R. 0.6/kg) throughout the year. Higher prices found in the fish market were due to the fact that fish were not sold by weight but instead by the piece depending on the size of fish displayed and judged by personal perception of the fish seller. Nevertheless, it was bought no matter what the price was at the fish market. Based on the data provided by the Oman Fisheries Company (OFC), a leading company in the fishery sector in Oman, the price of *R. sarba* in international markets is around ($1.4, O.R. 0.56/kg)(Personal communication). According to the information supplied by the company *R. sarba* is popular in international fish markets particularly Europe. The production of *R. sarba* by OFC, however, was not known due to the fact that the company added goldlined seabream, *R. sarba* production together with some other bream.

Goldlined seabream, *R. sarba* is among the seabream family (Sparidae), which are becoming important for marine aquaculture especially in the Western Pacific Rim (Woo & Kelly, 1995). Currently, gilthead seabream, *Sparus aurata* is cultured in Oman, whilst the international market for species such as seabream, *S. aurata* and sea bass, *Dicentrarchus labrax*, is already saturated (Rad & Koeksal, 2000). Therefore, it is essential to explore other finfish species that have a good perception locally, regionally and internationally.

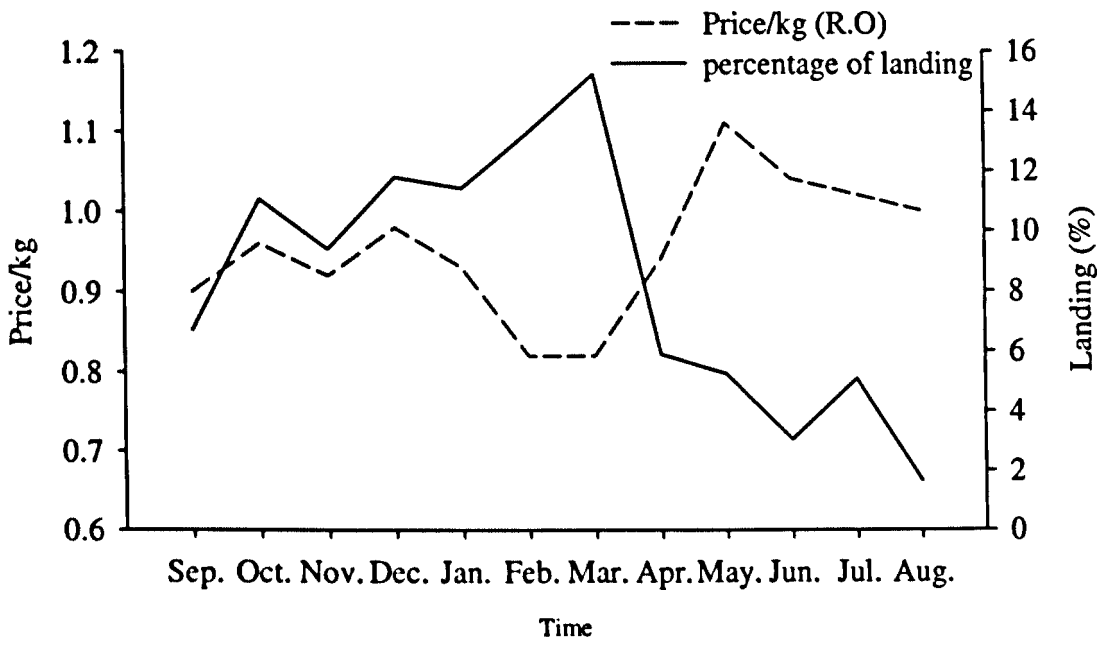


Figure 7.1 Price and landings of goldlined seabream *R. sarba* at Matrah fish market.

Price was monitored from September 01 to August 2002.

To ensure long-term development of aquaculture, national aquaculture will need to strengthen and the development of local species of commercial importance in the fishery market should take place. Rad & Koeksal (2000) reported that culture of other alternative species in Mediterranean has already been implemented and other species of seabream such as *Pagrus pagrus* is currently cultured as an alternative source of income and providing good penetration in Mariculture. National institutions and related governmental organizations, therefore, need to provide new opportunities in aquaculture from local indigenous source (Ross & Beveridge, 1995).

It has to be noted here that this study lacked proper market research evaluation of *R. sarba* as opposed to current cultured seabream *Sparus aurata* especially in the

international market. Therefore, future research may focus on such issues in order to evaluate market perception especially for a cultured commodity. Future research may also include production cost evaluation to find out whether *Sparus sarba* could be produced at lower cost than that of current species.

The breeding of *R. sarba* in captivity is the first attempt to breed a native finfish species in the Sultanate of Oman. The current study is a novel contribution to the knowledge of one of the important demersal finfish species in Oman. It should open the door to more research on this species and indeed other species that are in high demand in the market. According to the author's personal perception, *R. sarba* could be utilised as a model species for further research on various biological and physiological aspects on marine finfish.

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Appendix 1.

Table 1.1. Chemicals and solutions used to prepare 10% NBF

CONTENT	AMOUNT
sodium dihydrogen phosphate (monohydrate)	4.0 g
disodium hydrogen phosphate (anhydrous)	6.5g
formaldehyde	100ml
distilled water	900ml

Table 1.2. Processing protocol for wax paraffin wax embedding.

MEDIUM	TIME (h:min)
50% Methylated spirit	00:30
80% Methylated spirit	01:30
100% Methylated spirit	01:30
100% Methylated spirit	01:30
100% Methylated spirit	01:30
100% Ethanol	01:45
100% Ethanol	01:30
chloroform	00:50
chloroform	00:50
molten wax	01:45
molten wax	01:45
molten wax	01:30
molten wax	01:30

Table 1.3. Staining protocol for H&E stain.

MEDIUM/STEP	TIME (minutes)
xylene (dewaxing)	5.0
absolute Alcohol I	2.0
methylated spirit	1.5
wash in tap water	0.5-1.0
mayers Haematoxylin	5.0
wash in tap water	0.5-1.0
1% Acid alcohol	3 quick dips
wash in tab water	0.5-1.0
scott's tap water substitute	1.0
wash in tab water	0.5-1.0
wosin	5.0
quick wash in water	o dip
methylated spirit	0.30
absolute alcohol II	2.0
absolute alcohol I	1.5
xylene (clearing)	5.0
xylene (coverslip)	until slides were covered.

Appendix 2

Table 2.1 Biometrics data for female *R. sarba* otoliths.

	AGE										
DATA	0	1	2	3	4	5	6	7	8	9	11
Otolith $\underline{W}$(g)	0.02	0.03	0.04	0.06	0.07	0.09	0.10	0.11	0.14	0.14	0.15
Max of otolith *W*(g)	0.03	0.06	0.06	0.11	0.11	0.14	0.12	0.14	0.15	0.14	0.15
Min of otolith *W* (g)	0.02	0.02	0.02	0.03	0.04	0.05	0.08	0.09	0.13	0.13	0.15
SD	0.00	0.01	0.01	0.02	0.02	0.02	0.01	0.02	0.01	0.01	0.0
Otolith $\underline{L}$ (mm)	5.63	6.54	7.37	8.27	8.91	9.24	10.17	9.92	10.66	10.27	11.81
Max otolith *L* (mm)	6.57	7.92	8.74	10.22	10.45	10.24	11.34	11.58	11.17	10.71	11.81
Min otolith *L* (mm)	5.04	4.22	5.46	5.79	6.83	7.90	9.19	7.63	9.95	9.82	11.81
SD	0.39	1.07	0.74	1.09	1.01	0.88	0.71	1.44	0.54	0.63	0.0

$\underline{W}$ = Average weight
$\underline{L}$ = Average length

Table 2.2 Biometrics data for male *R. sarba* otoliths.

	AGE								
DATA	0	1	2	3	4	5	6	7	8
Otolith $\underline{W}$(g)	0.02	0.03	0.04	0.06	0.07	0.09	0.09	0.12	0.10
Max of otolith *W*(g)	0.03	0.06	0.07	0.10	0.10	0.13	0.13	0.14	0.10
Min of otolith *W* (g)	0.02	0.02	0.02	0.03	0.04	0.07	0.07	0.10	0.09
SD	0.00	0.01	0.01	0.02	0.02	0.02	0.02	0.03	0.01
Otolith $\underline{L}$(mm)	5.86	6.44	7.40	8.19	8.77	9.59	9.75	10.78	9.94
Max otolith *L* (mm)	6.47	8.21	8.96	10.35	10.39	10.96	10.64	10.91	10.34
Min otolith *L*(mm)	3.98	4.35	6.30	5.50	6.79	8.01	9.28	10.64	9.53
SD	0.80	0.82	0.67	1.16	0.99	0.80	0.50	0.19	0.57

$\underline{W}$ = Average weight
$\underline{L}$ = Average length

Appendix 3

Table 3.1 The type and amount of fertilizer used in the stock media.

CHEMICAL	QUANTITY (g)
KNO_3	150
FE-EDTA	2
NaH_2PO_4	30
Clewat-32	10

Table 3.2 Composition of the fertilizer used in algae culture.

CHEMICAL/ELEMENT	CONCENTRATION (%)
total nitrogen (N)	20
ammonical nitrate	8
nitrate nitrogen	5.9
urea nitrogen	6.1
available phosphoric Acid	20
soluble potash	20
boron	0.02
copper	0.05
iron	0.10
manganese	0.05
molybdenum	0.0005
zinc	0.05

The above percentages are derived from ammonium phosphate, potassium nitrate, potassium sulfate, urea, boric acid, copper EDTA, ammonium molybdate and zinc EDTA.

Appendix 4

Table 4.1 Processing protocol for TEM specimens.

CHEMICAL /MATERIAL		TIME (hr:min)
primary fixation in Karnovsky's, at 4°C, pH 7.2		02:00
rinse in cacodylate Buffer at 4°C twice		01:00 each
* post-fix in 1% osmium in distilled water		01:00
* rinse in distilled water twice		00:10 each
* en-bloc stain: 2% uranyl acetate in 30% Acetone		01:00
* dehydrate through acetone series at room temperature		
	25%	00:10
	75%	00:10
	95%	00:10
	100% (4 changes)	00:10 each
* intermediate rinse with propylene oxide (2 changes)		00:05 each
* infiltrate with araldite epoxy resin		
	propylene oxide : Araldite 1:1	01:00
	propylene oxide : Araldite 1:3	00:30
	araldite 100% at 37°C	01:00
	araldite 100% at 60°C	00:30
	araldite 100% at 60°C	00:30
place both the embedding resin and the tissue in vacuumed oven at 60°C to eliminate air bubbles.		00:05
embed tissues in green block moulds		
polymerise in oven at 60°C		16:00 (overnight)

Steps marked with * are carried out inside an automatic processing machine (Leica, EM-TP).

Table 4.2. Ingredient of Karnovosky's fixative.

CHEMICAL /COMPONENT	AMOUNT
paraformaldehyde	2.0 gm
25% glutaraldehyde	8.0 ml.
1 nutral NaOH.	Enough to dissolve paraformaldehyde
1 nutral HCl.	Buffer to pH 7.2
1 molar Na Cacodylate	10 ml.
sucrose	2.0 g
1% MgCl.	1.0 ml
1% CaCl.	1.0 ml
distilled water	100 ml

Table 4.3 Ingredient of washing cacodylate buffer pH. 7.2-7.4.

CHEMICAL /COMPONENT	AMOUNT
1 molar Na. Cacodylate	15 ml.
1 normal Hcl.	1.0 ml
1% MgCl.	1.0 mg
1% CaCl.	1.0 mg.
sucrose	2.5 gm
distilled water to	100 ml