

Environmental and Endocrine Control of Reproduction and its Manipulation in the Rabbitfish *Siganus canaliculatus* (Park 1797)

A thesis submitted to the University of Wales, Bangor by

Abdulredha Jassim Shams
BSc, MSc

in candidature for the degree of Doctor of Philosophy

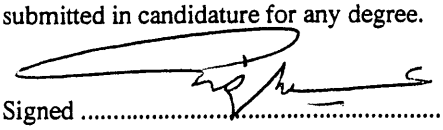
School of Biological Sciences
University of Wales
Bangor
Gwynedd LL57 2UW
U.K.

October 1998

APPENDIX 3 : Specimen Layout for Declaration/Statements page to be included in Higher Degree Theses

DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.



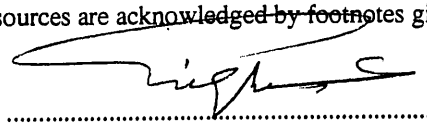
Signed ..... (candidate)

Date 30th September 1998

STATEMENT 1

This thesis is the result of my own investigations, except where otherwise stated.

Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

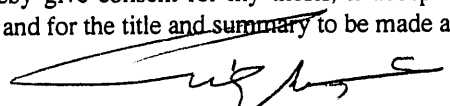


Signed ..... (candidate)

Date 30th September 1998

STATEMENT 2

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.



Signed ..... (candidate)

Date 30th September 1998

NB: *Candidates on whose behalf a bar on access has been approved by the University (see Appendix 2), should use the following version of Statement 2:*

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loans **after expiry of a bar on access approved by the University of Wales on the special recommendation of the Constituent/Associated Institution.**

Signed ..... (candidate)

Date .....

Dedication

To my wife and three children

For all the time we missed when I was away

Abstract

A detailed description is given of the normal seasonal changes in ovarian morphology and oocyte histology in *Siganus canaliculatus* from the previtellogenic stages to the time of completion of vitellogenesis. A histological description is given of the maturational stages observed in oocytes from gonadotropin-treated hatchery fish. Atresia is described for most stages of oocyte development and was observed in both hatchery and wild fish. Seasonal changes in serum oestradiol levels, which correlated closely with vitellogenesis in both hatchery and wild fish, ranged from less than 0.2 ng ml^{-1} from June to November to a peak of approximately 1.0 ng ml^{-1} in the early part of the normal spawning season in April.

Broodstock fish subjected to handling stressors typical of hatcheries (netting, biopsy and blood sampling), can accommodate to moderate levels of stressors. Effects caused by stressors may not be evident until the later stages of oocyte or larval development.

Lunar regimes, including simulated constant full and constant new moon conditions, simulated normal and reversed lunar cycles, did not promote oocyte maturation or spawning in hatchery fish.

Reproduction can be advanced by manipulation of the photoperiod. Photoperiodic regimes which advanced reproduction were those involving a switch to long days prior to or soon after the winter solstice. The extent of the advance (up to 3 months) was not correlated with the date on which the switch occurred. Reproduction was not delayed by any of the photoperiodic regimes which artificially delayed the winter solstice.

A single injection of human Chorionic gonadotropin (hCG) induced final oocyte maturation and spawning in fish which had completed vitellogenesis. The optimum dose was approximately 1.0 IU g^{-1} body weight. There was some evidence that higher and lower doses caused deterioration in gamete and larval quality. Domperidone, a dopamine antagonist, which had no effect alone, potentiated some of the effects of hCG, including a reduction in the interval between injection and spawning. The use of comparatively high doses of either salmon- or mammalian gonadotropin releasing hormone analogues, given as a single injection with and without Domperidone, did not reliably induce spawning.

Table of Contents

Author's Declaration....i

Dedication....ii

Abstract....iii

Table of Contents....iv

Acknowledgements....xi

Chapter One : **General Introduction**

1.1 General Aims....1
1.2 Study Site....2
1.3 General Biology of the Siganidae....2
1.3.1 Taxonomy....3
1.3.2 Reproduction....3
1 Spawning Season & Grounds....3
2 Courtship & Spawning....4
3 Mariculture Importance....6
1.4 Reproductive Physiology of Teleosts....7
1.4.1 Natural Control of Reproduction in Teleosts....7
A Environmental Factors....8
1 Role of Photoperiod....9
2 Role of Temperature....12
B Endogenous Control of Reproduction....14
C Lunar and Semilunar Rhythms....14
D Role of Internal (Endocrine) Factors in the Control of Reproduction....16
1 The Pituitary gland....16
2 The Ovary....18
3 The Hypothalamus....19
1.4.2 Methods of Manipulation of Reproduction....22
A Manipulation of External Factors....23
1 Photoperiod Manipulation....23
2 Temperature Manipulation....26
B Manipulation of Internal (Endocrine) Factors....27
1 Intervention at the Level of the Pituitary Gland....28
2 Intervention at the Level of the Ovary....29
1.5 Plan of Work....30

Chapter Two : **Morphological and Structural Development of the Ovary in *S. canaliculatus***

2.1 Introduction....32
2.1.1 Normal Follicular Development....32
2.1.2 Atresia in Teleosts....35
2.1.3 Ovary of Siganids....36
2.2 Materials and Methods....37
2.2.1 Ovary Sampling....37
A. Wild Fish....37
B. Captive Fish....38
2.2.2 Histological Procedure....38
Ooccyte Measurements....39
2.2.3 Blood Sampling and Oestradiol Assay....40
2.2.5 Gonado-Somatic Index (GSI)....41
2.2.6 Terminology....41
2.2.7 Statistical Analyses....41
2.3 Results....42
2.3.1 The Gonado-Somatic Index (GSI)....42
2.3.2 The Structure of the Follicle of *S. canaliculatus*....42
2.3.3 Oocyte Growth....42
1 Oogonium....43
2 Chromatin Nucleolus Stage....43
3 Early Perinucleolus Stage....43
4 Late Perinucleolus Stage....44
5 Yolk Vesicle Stage....44
6 Early Yolk Granule Stage....44
7 Mid Yolk Granule Stage....45
8 Late Yolk Granule Stage....45
9 Migratory Nucleus Stage....45
10 Oocyte Hydration....46
11 Post-Ovulatory Follicle....46
2.3.4 Follicular Atresia....46
1 Previtellogenic Oocytes (PVO)....47
2 Yolk Vesicle Oocytes (YVO)....47
3 Yolk Granule Oocytes (YGO)....47
4 Migratory Nucleus Oocytes (MNO)....48
10 Hydrated Oocytes (HYO)....48
11 Post-Ovulatory Follicle (POF)....49
2.3.5 The Ovarian Cycle in *S. canaliculatus*....49
2.3.6 Seasonal Changes in Levels of Oestradiol (E_2)....50
2.4 Discussion....51
2.4.1 Pattern of Oocyte Growth in *Siganus canaliculatus*....51
2.4.2 Follicular Atresia....53

Chapter Three : **Effect of Handling Stressors on Ovarian Development and Maturation in *S. canaliculatus***

3.1 Introduction....55
3.2 Materials and Methods....59
3.2.1 Effect of Potential Stressors on Vitellogenesis....59
I Experimental Conditions....59
II Sampling Procedure....60
III Measurements....60
1 Time of Vitellogenesis....60
2 Oocyte Diameter....61
3 Occurrence of Atresia....61
4 Serum Oestradiol (E_2)....61
IV Statistical Analyses....61
3.2.2 Effect of Potential Stressors on Maturation....61
I Experimental Conditions....61
II Sampling Procedure....62
III Measurements....63
1 Oocyte Diameter....63
2 Serum Oestradiol (E_2)....63
3 Spawning Numbers....63
4 Latency Period....63
5 Fertilization Rate....63
6 Hatching and Larval Viability Rates....63
7 Incidence of Atresia....64
IV Statistical Analyses....64
3.3 Results....64
3.3.1 Effect of Potential Stressors on Vitellogenesis....64
1 Time of Vitellogenesis....64
2 Oocyte Diameter....65
3 Incidence of Atresia....65
4 Serum E_2 Profile....65
3.3.2 Effect of Potential Stressors on Maturation....65
1 Spawning....65
2 Latency Period....65
3 Fertilization Rate....66
4 Hatching Rate....66
5 Larval Viability....66
6 Incidence of Atresia....66
7 Serum E_2 Profile....66
3.4 Discussion....67
3.4.1 Vitellogenesis....67
3.4.2 Maturation....69
3.4.3 Serum E_2 Increase During Maturation....72

Chapter Four : **Effect of Lunar Phase on Vitellogenic and Post-Vitellogenic Stages in *S. Canaliculatus***

4.1 Introduction....73
4.2 Materials and Methods....76
4.2.1 Experimental Groups....76
4.2.2 Sampling Procedure....77
4.2.3 Measurements....77
1 Time of Vitellogenesis....77
2 Oocyte Diameter....77
3 Incidence of Atresia....77
4 Gonado-Somatic Indices (GSI)....78
4.2.4 Statistical Analyses....78
4.3 Results....78
1 Time of Vitellogenesis....78
2 Oocyte Diameter....78
3 Incidence of Atresia....78
4 Gonado-Somatic Indices (GSI)....79
4.4 Discussion....79

Chapter Five : **Photoperiodic Manipulation of Reproduction in the Female Rabbitfish *Siganus canaliculatus***

5.1 Introduction....84
5.2 Materials and Methods....87
5.2.1 Experimental Conditions....87
Series 1 - Ambient Temperature....89
Group A : Natural Normal Photoperiod....89
Group B : Simulated Normal Photoperiod....89
Group C : Constant Short Photoperiod....89
Group D : Constant Long Photoperiod....89
Group E : Compressed Rectilinear Short+Long Photoperiod...90
Group F : Switch from Ambient to Long Photoperiod in October....90
Group G : Switch from Ambient to Long Photoperiod in November....90
Group H : Switch from Ambient to Long Photoperiod in January....90
Group I : Switch from Ambient to Long Photoperiod in February....90
Series II - Constant Temperature....91
Group J : Natural Normal Photoperiod....91
Group K: Switch from Ambient to Long Photoperiod in January....91

Group L : Switch from Ambient to Long Photoperiod in February....91
Group M : Switch from Ambient to Long Photoperiod in April....91
5.2.2 Sampling Procedure....92
5.2.3 Measurements....92
1 Time of Vitellogenesis....92
2 Serum Oestradiol (E_2)....93
3 Biopsy Time....93
4 Spawning Readiness....93
5 Macroscopic Observations....93
6 Oocyte Diameter....93
7 Incidence of Atresia....93
5.2.4 Assay of Serum Oestradiol (E_2)....93
1 Procedure....94
2 Sensitivity....95
3 Accuracy....95
4 Specificity....95
5 Precision....96
5.2.5 Statistical Analyses....96
5.3 Results....96
5.3.1 Series 1 - Ambient Temperature....96
1 Time of Vitellogenesis....96
2 Serum Oestradiol (E_2)....98
3 Biopsy Time....99
4 Spawning Readiness....99
5 Macroscopic Observations....99
6 Oocyte Diameter....100
7 Incidence of Atresia....100
5.3.2 Series 2 - Constant Temperature....100
1 Time of Vitellogenesis....100
2 Serum Oestradiol (E_2)....100
3 Biopsy Time....101
4 Spawning Readiness....101
5 Macroscopic Observations....101
6 Oocyte Diameter....101
7 Incidence of Atresia....102
5.4 Discussion....102
5.4.1 The Effect of Photoperiodic Regimes on the Timing of Reproduction....103
5.4.2 Constant Temperature Series....103

Chapter Six : **Endocrine Manipulation of Spawning in *Siganus canaliculatus***

6.1 Introduction....109
6.2 Materials & Methods....116
6.2.1 Hormones and Dopamine Antagonist....116
6.2.2 Experimental Enclosures, Fish and Procedures....116
A - Experiments Conducted....117
Series I- Optimization of the dose of Human Chorionic Gonadotropin (hCG) for the induction of spawning. 117
Series II- Effect of salmon gonadotropin-releasing hormone analogue (sGnRH-A) on the induction of spawning..117
Series III- Effect of mammalian gonadotropin-releasing hormone analogue (mGnRH-A) on the induction of spawning....117
Series IV- Effect of Dopamine antagonist domperidone (DOM) on the induction of spawning....117
Series V- Effect of combining DOM and hCG on the induction of spawning....118
Series VI- Effect of the combination of DOM and sGnRH-A on the induction of spawning....118
Series VII- Effect of the combination of DOM and mGnRH-A on the induction of spawning....118
B - Sampling Procedure....118
C - Measurements....119
1 Oocyte Diameter....119
2 Spawning Percentage....119
3 Latency period....119
4 Time to Oocyte Hydration....119
5 Fertilization Rate....119
6 Hatching and Larval Viability Rates....119
6.2.3 Statistical Analyses....120
6.3 Results....120
Series I- Optimization of the dose of Human Chorionic Gonadotropin (hCG) for the induction of spawning..120
Series II- Effect of salmon gonadotropin-releasing hormone analogue (sGnRH-A) on the induction of spawning..122
Series III- Effect of mammalian gonadotropin-releasing hormone analogue (mGnRH-A) on the induction of spawning....122
Series IV- Effect of Dopamine antagonist domperidone (DOM) on the induction of spawning....122
Series V- Effect of combining DOM and hCG on the induction of spawning....122
Series VI- Effect of the combination of DOM and sGnRH-A on the induction of spawning....123

Series VII- Effect of the combination of DOM and mGnRH-A on the induction of spawning.....123
6.4 Discussion.....123

Chapter Seven : **General Discussion and Recommendations**.....128

References.....132

Appendices.....163

A - Radioimmunoassay Buffer (Phosphate Buffer Saline).....160

Acknowledgments

Praise and gratitude to Allah almighty for giving me strength and faith.

I would like to extend my deepest felt appreciation and gratitude to my supervisor Dr. Andrew S. Grimm for being so understanding and patient, without his guidance none of this would have been possible.

I am also indebted to Mr. Jassim A. Al-Qaseer, Director of Fisheries and a friend, for his committed support and for always finding ways to solve problems encountered during the difficult periods of the study.

And to Dr. Roger Uwate, Fisheries Advisor, thanks for your support, moral and all, couldn't have done it without your constant nagging.

Over the years, the technical and supporting staff of the National Mariculture Center have been kind enough to accommodate me and my experiments amongst their busy schedules, their assistance is greatly appreciated. Special thanks are due to Abdulmajeed Al-Bosta and Amanat Ali for helping me with the running of the experiments. And to the Nutrition Unit for preparing the formulated feeds.

I also thank Mr. Abdulkarim Radhi, Head of Fisheries Statistics Unit, for advise on statistics.

I also extend my thanks to the President of the University of Bahrain for granting me the permission to use laboratory facilities and the library. And to Dr. Hashem Al-Sayed, Chairman of the Biology Department, for extending his support.

In Bangor, I thank Dr. Norma Blackstock for her untiring help with histology work, Dr. Simon Webster for the use and help with the radioimmunoassay equipments, Andrew Davis for his help with the digitizing pad and related software, Head Technician Meirion Thomas and Margaret Greatwich in stores.

This thesis was partly financed by a project of the Food and Agriculture Organization of the United Nations through the office of the United Nations Development Programme (UNDP) in Bahrain, this is acknowledged and greatly appreciated.

Chapter 1

Chapter 1

General Introduction

1.1 General Aims:

The Siganidae, or rabbitfish, are an Indo-West Pacific family of marine, tropical species. They are a popular food in many countries (**Popper & Gundermann, 1975**), the demand for which is rarely satisfied by the small commercial fisheries (**Lam, 1974**). *Siganus canaliculatus* (Fig.1.1), like several other species, is considered to have mariculture potential (**Coche et al., 1979; Lam, 1974**), but suffers from the common disadvantages of a limited spawning season (**Lam, 1974**), and in some areas, a failure to mature and spawn in captivity without intervention.

There is a large body of literature on the control of reproduction in temperate water fishes. However, information on this field in tropical and semi-tropical species, such as the rabbitfish, is scarce (**Abu-Hakima, 1984; Cyrus & Blaber, 1984; Marino et al., 1995**)).

The general aim of the research program was to study the pattern of ovarian development and maturation and to investigate the environmental and endocrine mechanisms controlling ovarian growth and maturation in the rabbitfish, *Siganus canaliculatus*. A better understanding of the natural control of rabbitfish reproduction would permit the more effective manipulation of reproduction and spawning, with consequent improved predictability and duration of seed production and thus allow better timing of harvest, and



Figure 1.1 - The rabbitfish, *Siganus canaliculatus*.

avoidance of periods of harsh weather conditions. Successful manipulation of the spawning season would be greatly to the advantage of the rabbitfish culture industry in Bahrain in particular and in the region in general.

1.2 Study Site:

The State of Bahrain is located on the southern shores of the Arabian Gulf between latitude $25^0 30'$ and $26^0 20'$ North and longitude $50^0 18'$ and $50^0 55'$ East. The 36 islands comprising Bahrain have a total land area of 706.55 square km. These are mainly low lying islands with a maximum elevation of 134 meters. Except for very limited fertile areas, the land is generally rocky and bare with sand cover of varying depth over a limestone bedrock supporting little desert vegetation. The significant aspects of the climate are cool winters with sparse rainfall, and hot summers with high humidity (**Directorate of Statistics, 1996**). Atmospheric temperatures range from 8 - 42° C. Water temperatures can range from 12 - 37°C (**Directorate of Fisheries, 1996**). Difference in daylength between summer and winter solstice is just under 4 hours (**Annual Meteorology Report, 1996**).

1.3 General Biology of the Siganidae:

The Siganidae inhabit near-shore shallow waters. Adult fish attain a standard length between 20-50 cm (**Woodland, 1990**). In the wild they are essentially herbivorous, feeding on macroalgae, as might be suggested from their single row of flattened, close-set teeth (**Duray, 1990**), but in captivity they will readily accept various non-plant diets (**Drew, 1971, Ben-Tuvia & Kissil, 1973**).

The species vary in their social habits from schooling to solitary, and differ in their habitats from hard corals to estuaries. About half of all species (nine species of the subgenus *Siganus* and all species of the subgenus *Lo*), live as pairs on coral reefs after leading a schooling life when young. Others are usually seen in small schools throughout

life, and of these only *S. vermiculatus*, is estuarine, the remainder move between different habitats (**Woodland, 1990**).

1.3.1 Taxonomy:

The family belongs to the sub-order Acanthuroidei (**Tyler et al., 1989**) and is unique in having pelvic fins with two strong spines separated by three soft rays. All spines on the dorsal, pelvic and anal fins are venomous but not fatal (**Woodland, 1990**). There is a single genus, *Siganus*, and two subgenera, *Siganus* and *Lo*, which are distinguished by the tubulate snout of the latter. The subgenus *Siganus* contains twenty two species while the subgenus *Lo* contains only five species. The phylogenetic relationship within the two sub-genera are still unresolved, mainly because most of the morphometric characters and ornate coloration used by taxonomists are remarkably uniform and make it difficult to distinguish species (**Woodland, 1990**).

1.3.2 Reproduction:

1. Spawning Season and Grounds:

There is a large body of literature on various aspects of the general biology, ecology, pathology and culture of siganids (**Duray, 1990**), but comparatively little on their reproductive biology.

Most of the available data indicate that siganids have a definite spawning season, more marked in some than in others (**Lam, 1974**), and varying in duration from 2-8 months (**Duray, 1990**). *S. canaliculatus* has been reported to have a very distinct spawning season which is somewhat similar in different regions. A spawning period of January-April with a peak in February to March has been reported in Singapore (**Soh & Lam, 1973**), the Philippines (**Lavina & Alcala, 1973**) and Hongkong (**Tseng & Chan, 1982**). In Palau it is February-June with a peak in April-May (**McVey, 1972**). Within the Arabian Gulf the spawning season is shorter. In U.A.E., it is from March to the end of May with a peak in

April (**Al-Ghais, 1993**), and in Bahrain, from late March to early May with a peak in April (**Aradi & Shams, 1993**). A second spawning season for *S. canaliculatus* has been suggested by a few authors (**Drew, 1971; Lam, 1974; Lavina & Alcala, 1973; McVey, 1972**), although there is no direct evidence to support this in any of the above locations.

Information on the characteristics of spawning grounds of siganids is limited. Types of grounds reported include shallow waters in and around corals, sea grass and near mangroves, all of which apparently provide suitable substrates for attachment of eggs and protection for newly hatched larvae (**Drew, 1971; Manacop, 1937; McVey, 1972**).

2. Courtship and Spawning:

In the wild, observations of male-led mating rituals have been reported in the wild for *S. vermiculatus* (**Gundermann et al., 1983; Popper & Gundermann, 1975**) and *S. canaliculatus* (**Manacop, 1937**). In captivity, male-led mating behavior has also been observed in *S. rivulatus and S. luridus* (**Popper & Gundermann, 1975**), and *S. guttatus* (**Hara et al., 1986b**). In the latter species the male starts by chasing and touching the abdomen of the female, followed by repeated nudges on the operculum, anal region, and caudal peduncle, all the time keeping close to the female. The female then releases her eggs, followed by milt from the male. The fish swim actively, apparently to mix eggs and sperm to ensure maximum fertilization. Contrary to the above, **McVey (1972)** and **Avila (1984)** found that in captive *S. canaliculatus,* and *S. vermiculatus* it is the female of these latter species which begin the mating ritual by nudging the male, encouraging it to release milt and then releasing eggs.

Usually, rabbitfish spawn is pairs. It has been suggested that some wild species that live in pairs school to reproduce (**Thresher, 1984**), but considering the territorial behavior of breeding pairs and their aggressiveness towards other pairs, this seems unlikely. Such natural spawning in aggregations has yet to be confirmed, since most of available evidence is from schooling species, in particular those used in mariculture studies (**Woodland,**

1990). In induced spawning trials, up to four females and two males have been seen to spawn at the same time in a limited space (**personal observations**). Sex ratios of one female to one male (**Tseng & Chan, 1982**), and one female to two males (**Gundermann et al., 1983**), have been reported for *S. canaliculatus* during the spawning season.

Spawning is synchronized with the lunar cycle (**Duray, 1990; Lam, 1974**). Many studies on different species of rabbitfish indicate that spawning occurs between the new and full moon, mainly in the first quarter (**Alcala & Alcazar, 1979; Bryan & Madraisau, 1977; Burgan &Zseleczky, 1979; Hara et al 1986b; Hasse et al 1977; Luchavez & Carumbana, 1982; Popper et al., 1976; and Tawada, 1991**).

Natural spawning in captivity has been reported for *S. argenteus* (**Luchavez and Carumbana, 1982; Tobias, 1976;**), and for *S. canaliculatus* (**McVey, 1972**), *S. guttatus* (**Hara et al., 1986a; Juario et al., 1985; Soletchnik, 1984**), *S. rivulatus* (**Popper et al., 1973**) and *S. vermiculatus* (**Popper et al., 1976**). Repeated spawning may occur over 2 to 4 consecutive months in a single spawning season of *S. guttatus* (**Hara et al 1986b**).

Some reports mention that a change in the water level in the holding tanks stimulated the fish to spawn naturally, leading to the opinion that tidal levels act as a trigger for the fish to spawn (**Popper et al., 1973; McVey, 1972**). In Bahrain, where the present study was carried out, trials to trigger spawning in captivity by providing artificial substrates for the attachment of eggs or subjecting the fish to simulated tidal levels by changing water levels in the holding tanks or both were not successful (**personal observations**).

Fecundity of rabbitfishes is high, 250,000 to 500,000 eggs per spawning season (**Lam 1974; Gunderman et al 1983**). Some reports give higher figures of up to 1.2 million eggs for a single female in one spawning season (**Hara et al 1986a; and Al-Ghais 1993**). Eggs are adhesive, small and spherical with many oil globules (**Duray, 1990**), with one known exception, *S. argentus* (**Burgan & Zseleczky, 1979, Luchavez & Carumbana, 1982**) in which eggs are free-floating. Hatching occurs 24 to 72 hours later, depending on water

temperature and species, after which yolk absorption takes from three to four days. The pelagic planktonic larva feeds on plankton (**Lam, 1974**) for 3-4 weeks prior to metamorphosis to the juvenile stage, which adopts a shoaling life style (**Woodland, 1990**). Information on early development is restricted to the few schooling species used in aquaculture. There have been reports on limited description of embryonic development in a few species like *S. vermiculatus* (**Avila, 1984**), *S. argenteus* (**Burgan and Zseleczky, 1979; Luchavez and Carumbana, 1982**), *S. rivulatus* (**Popper et al., 1973**), *S. fuscescens* (**Fujita and Ueno, 1954**), but a very detailed account for *S. guttatus* was given by **Hara et al. (1986b)**. Embryonic development for *Siganus canaliculatus* from fertilization of the egg to 72 hours has been described in a few studies from as early as 1937 (**Akatsu et al., 1984; Manacop, 1937; May et al., 1974; McVey, 1972; Soh & Lam, 1973; Westernhagen & Rosenthal, 1976a**). In all the above reports, description of the embryonic development was essentially the same, with an incubation period of 18-35 hours (62 hours in **Manacop 1937**). Newly hatched larvae measure 1.5-2.6 mm in total length with a straight gut, pigment-free eyes and no mouth. In *S. canaliculatus*, the yolk sac is ellipsoid with one oil globule, the opening of the mouth, formation of olfactory buds and pigmentation of the eyes occurred on day two (**Akatsu et al., 1984**).

3. Mariculture Importance:

Siganids are herbivores, tolerate a wide range of temperature and salinities, are highly palatable, and command a good market price. Although these characteristics are well recognized and have stimulated many investigations on their mariculture potential (**Lam, 1974**), a fact that has been acknowledged repeatedly by many other workers (**Bwathondi, 1981 & 1982; Hasse et al., 1977; Horstmann, 1975; Lichatowich & Popper, 1975; Lichatowich et al., 1984b; Nelson et al., 1992; Parazo, 1989; Pongsuwana et al., 1984; Tseng & Chan, 1982; Westernhagen & Rosenthal, 1976b**), the global production of siganids is still very low, the entire Asian production amounting to only 14 tonnes (**FAO Fisheries Information, 1995**).

Mariculture of rabbitfish was proposed in the Philippines in the early 1960's (**Ablan and Rosario 1962**). In 1972 an informal working group was formed at a meeting in Hawaii to investigate the culture of siganids (**May, 1973**). Five major and one subsidiary problems were identified as areas requiring investigation before siganid mariculture can be fully achieved. These were a species survey, juvenile-to-adult farming, fry production, production economics, spine irritation and ciguatera poisoning, and transportation techniques and diseases. Many of these problems continue to be worked out. Problems with species identifications have been largely resolved (**Woodland, 1990**), but surveys are needed to identify factors affecting market value and acceptability of different species. Work is still needed to develop culture techniques using cages and pens, more efficient feeding regimes, viable mass production of seed of various species, and more information on economics and socio-cultural aspects of rabbitfish farming (**Duray, 1990**).

1.4 Reproductive Physiology of Teleosts:

The ovaries of teleosts, like other vertebrate ovaries, are usually paired structures. They are generally hollow as in amphibia and reptiles, and unlike the solid structures characteristic of cyclostomes, elasmobranchs, birds, and mammals. The cavity in the teleost ovary is formed as a result of the embryonic development of longitudinal ovarian folds that fuse to enclose part of the coelomic cavity (cystovarian condition) which is lined by peritoneal epithelium. This is in contrast to the solid ovary characterized by large, irregular central cavities lined by mesothelium. Cystovarian ovaries often extend posteriorly as functional oviducts which open into the cloaca. The formation and development of ova is remarkably similar in all groups of vertebrates, the variations occurring in later stages being mainly dependent on the reproductive strategy adopted by the female (**Pudney, 1987**).

1.4.1 Natural Control of Reproduction in Teleosts:

Teleost fishes display a wide array of reproductive modes (**Breder & Rosen, 1966; Hoar, 1969; Balon, 1975**). At one end of the spectrum there is oviparity and external fertilization, which is adopted by the majority of the species. Some of these species show

no adaptations for care and protection of the eggs while others exhibit parental care in the form of breeding territories, nest-building, and mouth brooding. At the other end of the spectrum are fishes exhibiting internal fertilization and a range of post fertilization strategies from simple oviposition, through ovo-viviparity to viviparity. In ovo-viviparity development of the egg is carried a step further, embryogenesis occurring within the ovary and the young being released just before or soon after hatching. In such cases the egg yolk is the only nutritional source for the embryo. In more advanced true viviparous species, the young are retained in the ovary of the female and are nourished fully or partially by her, and may be born at later stages of development or even after sexual maturity (**Stacey, 1983**). In addition to this range of adaptations concerned with the timing of fertilization and the handling of fertilized eggs, many teleosts display other reproductive adaptations such as protandrous, protogynous or simultaneous hermaphroditism. There are even rare examples of parthenogenesis (**Smith, 1975**).

Most teleost species manifest an annual rhythm of breeding, which necessitates precise regulation of the reproductive system, especially in females where large numbers of yolked ova are produced. The growth and maturation of oocytes is a major complex biological process requiring synthesis and transfer of immense quantities of materials to the future gamete (**de Vlaming, 1983**). The control of the reproductive system in fishes is effected naturally by environmental and endogenous mechanisms, both of which are mediated through neuro-endocrine secretions. The influence of these factors has been reviewed in several fish species (**Crim 1982; Wootton 1984; Zohar & Billard 1984; Munro et al 1990; Bromage et al., 1993; Carrillo et al., 1993; Thomas & Arnold, 1993).**

A. Environmental Factors:

In many teleosts, gonadal recrudescence is protracted. It usually extends over several phases of the annual cycle, during which time fish may be exposed to a range of environmental factors. Many such factors have been correlated with the reproductive cycle including water turbulence, water level and rainfall (**Beumer, 1979; Bruton, 1979; Springate et al., 1985**), but most studies have concentrated on investigating the influence

of light and temperature (**de Vlaming, 1972b; Breton & Billard, 1977; Scott, 1979; Baggerman, 1980; Whitehead & Bromage, 1980; Lam, 1983; Bromage et al., 1984; Elliot et al., 1984; Bromage & Duston, 1986; Morrison & Smith, 1986; Duston & Bromage, 1987; Bromage 1987; Henderson-Arzaplo & Colura, 1987; Bromage & Cumaranatunga, 1988; Carrillo et al., 1991; Davies et al., 1991; Kelly et al., 1991; Bromage et al., 1993).**

1. Role of Photoperiod:

In many fishes, whether inhabiting temperate or tropical latitudes, reproduction is annual and timed in such a way that release of offspring occurs at a time when favorable conditions prevail. For fish to program stages of their reproductive cycle towards favorable release of progeny in spring, they must be able to measure and predict external seasonal changes. In all salmonids investigated, this synchronization is thought to be primarily achieved by using changes in ambient daylength as the predictive environmental cue (**Bromage et al., 1993**).

Since gonad growth is dependent on sufficient nutrient supply or sufficient nutrient reserves (**Springate et al., 1985**), it has been proposed that nutritional status or readiness is somehow assessed before gonad development commences. In rainbow trout and salmon it has been proposed that the decision to commence gonadal development is based on the coincidence of a critical nutrient-dependent developmental stage and the environmental conditions prevailing at a precise stage(s) of the annual cycle (**Duston & Bromage, 1987 and 1988; Thorpe et al., 1989**). In salmonids, day length is undoubtedly the main environmental agent controlling reproductive development, but there still is a controversy with regard to how daylength is utilized and whether the influence of photoperiod is direct or simply used for entrainment of an endogenous biological clock. An answer was sought by looking into possible similarities between fish and higher vertebrates, especially the more studied birds. This led to the labeling of fish as either long or short day spawners but this still did not give a satisfactory answer the original question and did little towards the understanding of these processes in fish (**Bromage et al., 1993**). The concept of either

long or short day breeding fish was misleading. In the rainbow trout, the final stages of maturation and spawning occur under decreasing or short daylengths, hence their original designation as short-day breeders (**Breton & Billard, 1977; Peter, 1981**), but further investigations showed that the earlier stages of development are initiated under and influenced by longer daylengths, which led to their reclassification as long-day breeders (**Whitehead & Bromage, 1980; Bromage et al., 1982a; Scott et al., 1984; Sumpter et al., 1984**). It is now known that maturation in the rainbow trout is affected by both short and long photoperiods (**Bromage et al., 1984; Bromage & Duston, 1986**).

Studies to determine the effect of photoperiod on the rates of maturation and times of spawning in salmonids indicate that a seasonally-changing light cycle is not essential for reproductive development (**Whitehead et al., 1978; Bromage et al., 1982a, and 1984; Bromage & Duston, 1986; Duston & Bromage, 1986**). In fact, a shift in the time of reproduction can be effected by exposing fish to long or short days or a combination of both.

Long days applied early in the reproductive cycle generally advance maturation and spawning, the maximal advance (5-7 months) being observed in fish treated immediately after spawning (**Bromage et al., 1984; Scott et al., 1984**), whereas subsequent treatments until the summer solstice have been suggested to show diminishing effects (**Bromage et al., 1993**). Subjecting the fish to the same conditions after the summer solstice delays the process (**Lundqvist, 1980; Whitehead & Bromage, 1980; Bromage et al., 1982a, 1984; Bourlier & Billard, 1984).**

The effect of short days also varies depending on when it is applied and the time of natural spawning of the treated fish. Maximal delay of 5 months has been reported for an early December spawning stock when exposed to short days in mid-January (**Duston & Bromage, 1987**). But exposing a December-January spawning stock to the same photoperiod in early January induced a significantly shorter delay of 2-3 weeks (**Bromage et al., 1984**). A January-February spawning stock treated in mid-February showed a 12-14

week delay in the next spawning (**Whitehead & Bromage, 1980; Bromage et al., 1984**). Application of short days 3-4 months into the reproductive cycle of the rainbow trout advances the spawning time, with the effect diminishing as the treatment time becomes nearer to the winter solstice. Again, the response is also influenced by the time of natural spawning of the treated fish (**Bromage et al., 1993**).

The effect of combinations of long and short daylengths also varies depending on time of application. In November-spawning rainbow trout exposed to long days early in the cycle followed by short days in May, spawning is advanced by 3-4 months. Reversing the treatment induces delayed spawning.

In spite of the fact that all of the various light regimes discussed above can be used to alter the reproductive cycle, synchronization of spawning of individual fish within the same stock is improved in fish exposed to altered seasonal light cycles and long/short day combinations than those exposed to constant long or short light regimes (**Bromage & Duston, 1986; Bromage et al., 1993**).

The concept of long and short days discussed earlier refer to daylengths of over 16 hours and under 6 hours of light per day, respectively. This concept has changed and it is well established now that it is not the magnitude of change in the length of day that is the critical factor but rather the direction of the change. So any daylength can be considered long or short provided that it is preceded by daylengths which are shorter or longer, respectively (**Bromage et al., 1993**).

The pineal body is the most likely receptor and translator of photoperiodic signals. In the mullet, *Liza ramada*, investigations on the regulatory role of photoperiod on ovarian development before, during, and after the breeding season and the part played by the pineal body showed that the pineal body is both photosensory and secretory, its photosensory traits being the photoreceptor cells which have close contact with axons. The "interstitial" or "supporting cells" were presumed to be secretory. Pinealectomy accelerated ovarian growth in fish exposed to short photoperiod but had no effect on fish

exposed to long photoperiod. Injections of melatonin, a presumed pineal hormone, in multiple doses of 50 or 250 μg, inhibited gonadal growth, the inhibition increasing with the increase in dosage; this finding was believed to support the concept that the pineal body inhibits gonadal development in this fish under short photoperiod conditions (**Abraham and Sagi 1984**).

Some insight on how external information is processed and interpreted by the central nervous system comes from hypotheses or models put forward to explain how refractoriness develops physiologically in birds. One model developed by **Hamner (1968)** postulates that exposure to continuous long days renders the bird unable to measure daylength. A second model suggests that cyclic changes occur in hypothalamic sensitivity to steroid feedback (**Cusick & Wilson, 1972**). A third proposes the existence of an inhibitory hormone which suppresses gonadotropin release, while another advocates a more simple explanation that some form of exhaustion develops in the neural or endocrine mechanisms regulating the secretion of LH and FSH (**Storey and Nicholls, 1976**). None of these hypotheses have total support, instead, there is evidence form various species that either support or contradict each of them.

Other species in which it has been shown that photoperiod affects the time of spawning include the milkfish (**Lee et al., 1987**), the striped bass (**Henderson-Arzapalo and Colura, 1987**) and cyprinids (**Hontela & Stacey, 1990; Poncin, 1991**).

Most studies on the role of environmental factors have been conducted on species from high latitudes, at which there are marked differences in seasonal day length. However, photoperiod may not be the only environmental factor affecting the reproductive cycle, it may be acting in conjunction with other factors.

2. Role of Temperature :

Determining the precise role of temperature in the control of reproduction is made difficult by the fact that in many species the reproductive cycle is correlated with regular seasonal

changes in both photoperiod and temperature. Such a correlation has been shown to exist in *Lepomis gibbosus* (**Smith, 1970**), *Gambusia affinis affinis* (**Sawara, 1974**), *Notemigonus crysoleucas* (**de Vlaming, 1975**), cyprinid fishes (**Okuzawa et al., 1989**), *Mugil cephalus* (**Kelley et al., 1991**) and the female Ayu (**Chang et al., 1993**). There are a few examples where temperature has been shown to be the major proximate factor, although in at least one species, the longjaw goby (*Gillichthys mirabilis*), low temperatures promote sexual maturation regardless of photoperiod (**de Vlaming, 1972b**). In most species temperature is considered as exerting a permissive role through its rate controlling action on the metabolic processes in poikilotherms (**Kelley et al., 1991**).

In salmonids, temperature is known to affect reproductive development and the timing of spawning (**Morrison & Smith., 1986; Nakari et al., 1988; Bromage & Cumaranatunga, 1988**). Very low temperatures have been shown to inhibit the final maturation and ovulation of trout, whereas higher temperatures can enhance development (**Goryezko, 1972; Titarev, 1975; Morrison & Smith, 1986; Nakari et al., 1987, 1988**). Low temperatures have also been known to depress yolk sequestration by the developing oocyte (**Tyler et al., 1987**). However, there is little evidence to show that it is able to act as a proximate cue (**Bromage et al., 1993**).

Temperature may play a great part in the final stages of maturation in many teleosts, even though the underlying endocrine mechanisms may be poorly understood (**Stacey, 1984**). Its influence on the reproductive cycle may be through action on: (1) gametogenesis (**Lofts et al., 1968**), (2) rate of secretion and clearance of gonadotropin (**Breton & Billard, 1977; Cook & Peter, 1980; Peter, 1981**), (3) daily cycles of GtH (**Hontela & Peter, 1978**), (4) the responsiveness of the liver to estrogen in the production of vitellogenin (**Yaron et al., 1980**), and (5) responsiveness of the gonad to hormonal stimulation (**Jalabert et al., 1977; Bieniarz et al., 1978**).

However, the majority of scientists play down the importance of temperature, and favour light as the major environmental factor controlling the reproductive cycle. The effect of

temperature is thought to be a direct one on the rate of such physiological processes as yolk synthesis and sequestration, which will effect the time at which the oocytes attain full maturity and/or size (**Tyler et al., 1987**). Rainbow trout exposed to long days followed by short days showed a four month advance in spawning timing, irrespective of whether they were on constant or seasonally changing temperature. It was concluded that temperature did not act as a major environmental cue in the control of seasonal reproduction.

B. Endogenous Control of Reproduction:

There is compelling evidence that endogenous rhythms control reproduction in many fishes, including sea bass (**Devauchelle, 1984**), rainbow trout (**Bromage & Duston, 1986; Duston & Bromage, 1986**), and the threespine stickleback (**Baggerman, 1989**). According to **Baggerman (1989)**, in the threespine stickleback, spontaneous maturation occurs even in fishes reared under constant short days from the egg stage. The cause must be some endogenous factor, most likely to be an endogenous annual reproductive rhythm. A similar mechanism in sea bass, *Dicentrarchus labrax* was suggested by **Carrillo et al. (1989)**, who reported spawning in fish maintained under constant short days. This indicated that a normal photoperiodic cycle is not required for completion of reproduction, and strongly suggests that endogenous mechanisms may be involved in the timing of reproduction. The existence of a similar mechanism was demonstrated in the barbel maintained under constant environmental conditions (**Poncin, 1991**).

It may be that, as in the threespine stickleback and perhaps other species, seasonal breeding is based on an annual interplay between endogenous rhythms and environmental factors (**Baggerman, 1989**). Indeed **Bromage et al. (1993)** suggest that the endogenous rhythms are fundamental in controlling the reproductive cycle, and that the environmental factors such as photoperiod are used as mere reference points for entrainment of these endogenous rhythms.

C. Lunar and Semilunar Rhythms:

Cycles of maturation and spawning with a lunar or semi-lunar periodicity have been reported for many teleost species, particularly marine estuarine and euryhaline species, under both natural and laboratory conditions, and similar cycles have also been observed in many invertebrates (**Taylor, 1984**). Suggested advantages of lunar or tidal spawning cycles are: (i) the synchronization of gamete release from many individuals (**Colin et al., 1987; Dotan, 1990)** and (ii) optimization of time of release in relation to development, predation, and dispersal of eggs and larvae (**Lobel, 1978; Pressley, 1980; Doherty, 1983; Foster, 1987**).

Of several hypotheses put forward to explain semi-lunar cycles, the major one proposes that the maturation and spawning cycle is controlled by an endogenous rhythm with a semi-lunar or lunar periodicity which free-runs under constant conditions in the laboratory (**Hsiao & Meier, 1992**). It is suggested that this endogenous rhythm is entrained by geophysical exogenous factors like lunar light intensity or lunar dependent tidal cycles (**Taylor, 1986; Rossiter, 1991**), although other more exotic factors such as magnetism, electrostatic fields and gamma radiation have also been suggested (**Grau et al., 1981; Hsiao & Meier, 1992**). However, it is difficult to understand the mechanism by which these more exotic factors could influence the reproductive cycle, unless it can be shown that they have a lunar or semi-lunar periodicity.

The lunar tidal cycle as an external cue has enjoyed a fair amount of support from many workers. The coincidence of spawning with tides has been documented for barramundi, *Lates calcarifer* (**Moore, 1982**), cyprinodonts (**Greeley, 1984**), and damselfishes (**Foster, 1987**).

The lunar influence occurs only during the spawning season after vitellogenesis is well advanced, i.e., it probably affects only the terminal events of the reproductive cycle. This is supported by observations in the killifish *Fundulus heteroclitus,* which show that during

the lunar cycle in which spawning occurs, the only changes in the ovary are in the extent of ovarian hydration and in the incidence of mature oocytes (**Taylor & DiMichelle, 1980**).

In the killifish *Fundulus heteroclitus* (**Taylor et al., 1979**), and the Hawaiian labrid, *Thalassoma duperrey* (**Ross, 1983**), gonadal maturity and spawning readiness are greatest at times coincident with new and full moons, suggesting that they are synchronized with the semilunar cycle of spring tides. Another study on the killifish also showed a cyclic pattern of serum oestradiol levels which paralleled the cycle of gonadal maturity and spawning (**Bradford & Taylor, 1987**).

Although much evidence supports the existence of a lunar-mediated reproductive rhythm, there are unresolved questions, e.g., What is the synchronization or triggering mechanism that regulates this behavior?. Is it environmental stimuli?, or is it an endogenous mechanism? The persistence of the semilunar reproductive cycle in *Fundulus heteroclitus* in constant moonlight and other conditions support the existence of an endogenous mechanism. The role of moon light or tidal factors may be a means for synchronizing spawning with spring tide cycle. It was also possible to entrain the spawning to the spring tide cycle by using artificial moonlight (**Taylor 1991**).

D. Role of Internal (Endocrine) Factors in the Control of Reproduction:

Environmental factors mediate their control over reproduction through the nervous and endocrine systems. In fish and other vertebrates, it is the central nervous system-hypothalamic-pituitary-ovarian axis which plays the key role in the transduction of environmental information into the appropriate reproductive action, leading to the production of ripe oocytes. Although at its simplest, the axis may be viewed as a sequence of control levels which feed forward to the next element in the sequence, the axis also shows evidence of self regulation through the operation of feedback loops.

1. The pituitary gland:

Most of the important phases in gonadal development are controlled by a hormone or hormones secreted by the proximal or rostral pars distalis of the pituitary gland and referred to as gonadotropins (**Scott, 1987**). A single gonadotropin was first isolated from the pituitary of the common carp (**Burzawa-Gerard, 1971**), and found to be a glycoprotein composed of covalently linked α- and β-subunits, similar to the general structure of glycoprotein hormones in other vertebrates (**Burzawa-Gerard, 1982**). The partial sequencing of its subunits was carried out by **Jolles et al. (1977)** who demonstrated the resemblance of the carp β-subunit to that of human LH.

Until recently, it was considered that the teleost pituitary secreted only one gonadotropin, even though the concept that the teleost pituitary contains more than one gonadotropin was postulated some time ago (**Idler, 1982**). This is in contrast to the long established evidence in mammals of two- follicle stimulating hormone (FSH) and luteinizing hormone (LH). More recently, the concept of duality of gonadotropin was revived by the isolation from some salmonids of two chemically distinct glycoprotein hormones: GtH-I and GtH-II (**Kawauchi et al., 1987; Swanson et al., 1987b,c; Suzuki et al., 1988a,b,c; Swanson et al., 1989**). These differ both in structure and in their action. GtH-I shares a single α-subunit with GtH-II. The β-subunit of GtH-I and that of GtH-II differ markedly and have only 30% homology in amino acid sequence (**Kawauchi et al., 1989**). A similar duality of gonadotropins was also established in bonito (**Kawauchi et al., 1991**) and *Fundulus heteroclitus* (**Lin et al., 1992**).

GtH-I and GtH-II stimulate oestradiol secretion from isolated salmon follicles to the same extent, but the action of GtH-I, which is low in carbohydrates, appears to be restricted to the early vitellogenic phase where it also stimulates vitellogenin uptake in females. GtH-II, on the other hand, has a high carbohydrate content and a broader range of actions affecting oocyte maturation and ovulation, as well as vitellogenin uptake by the oocyte. It predominates during final reproductive maturation and is more potent in stimulating the

secretion of 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP) from postvitellogenic follicles (**Suzuki et al., 1988c,d; Swanson et al., 1991).** In the rainbow trout, only GtH-I stimulates oestradiol secretion from early vitellogenic follicles (**Sumpter et al., 1991**) and the incorporation of vitellogenin into oocytes *in vivo* and *in vitro* (**Tyler et al., 1991**).

2. The Ovary:

All vertebrate oocytes are surrounded by a complex multi-layered follicle made up of a number of cell layers. Two of these are always present-an inner *granulosa* layer, which is separated by a basement membrane from an outer *theca* layer, which may be subdivided into a theca interna and a theca externa. Special cell types in the theca interna and the predominant cell type of the granulosa have both been implicated in steroidogenesis (**Scott, 1987**).

Neither ultrastructural studies or steroid enzyme histochemical studies have been sufficient to clarify the steroidogenic role of the thecal and granulosa cells (**Scott, 1987**). Resolution of the steroidogenic capacity of these cell types was made possible by techniques which permitted the separation and culture of relatively pure cell layers of the follicle of the amago salmon (*Oncorhynchus rhoduras*). Stimulation with salmon gonadotropin (0.1-1 μg/ml) induced 17β-oestradiol production by intact follicles and by co-cultured layers, but not by isolated theca or granulosa layers. Similar treatment with gonadotropin also stimulated testosterone production by the theca layers by up to 80 times but hardly at all by the granulosa layers. Incubation of granulosa tissue with testosterone resulted in enhanced 17 β-oestradiol production (**Kagawa et al., 1982**).

Such findings suggested a two-cell type model of ovarian steroidogenesis, similar to that in mammals, whereby the theca layer synthesizes the androgens, which are then transferred to the granulosa layer to be aromatized to estrogens (**Scott, 1987**). A similar model has been proposed for the synthesis of 17α,20β-dihydroxy-4-pregnen-3-one by ovarian follicles of mature salmonid oocytes, in which the thecal layer, under the influence

of GtH, produces 17α-hydroxyprogesterone that traverses the basal lamina and is converted to 17α,20β-dihydroxy-4-pregnen-3-one by the granulosa layer, where GtH enhances the activity of the key enzyme 20β-hydroxysteroid dehydroxygenase (**Young et al., 1986; Nagahama, 1987a,b**). Isolated thecal and granulosa layers stimulated with GtH-I and GtH-II showed that GtH-II was more potent in enhancing 17α-hydroxyprogesterone production by thecal layers and 17α,20β-dihydroxy-4-pregnen-3-one production by granulosa layers in the presence of 17α-hydroxyprogesterone (**Kawauchi et al., 1989**).

An example of this two-cell type model is seen in the rainbow trout, where plasma levels of GtH-I and GtH-II in pre-gametogenesis are low, but during early vitellogenesis GtH-I levels become predominant, acting on the follicular wall leading to exogenous vitellogenin incorporation into developing oocytes. Plasma GtH-II levels are elevated during final reproductive maturation and can stimulate oocyte maturation and ovulation, spermiation, gonadal steroidogenesis, and ovarian adenosine monophosphate activity, as well as the uptake of vitellogenin by the oocytes (**Scott, 1987; Kawauchi et al., 1989**).

3. The Hypothalamus:

In the early 1970's it was established that in mammals the pituitary gonadotropic hormones are regulated by a factor or factors synthesized and secreted by peptidergic cell bodies and neurons in the hypothalamus (**Schally et al., 1973; Vale et al., 1973**). The short decapeptide that was eventually extracted was at first called Luteinizing Hormone-Releasing Hormone (LH-RH), but later re-named Gonadotropin-Releasing Hormone (GnRH). It was later established that mammalian GnRH (mGnRH) stimulates GtH release and ovulation in teleosts, suggesting a structural similarity between the GnRH of fish and mammals (**Breton et al., 1972; review by Peter, 1982&1983**). Confirmation of this suggestion has shown that the teleostean GnRH (tGnRH) and mammalian GnRH (mGnRH) differ only in the amino acids at position 7 and 8 (**King & Millar, 1980**; **Barnett et al., 1982**). The primary structure of this tGnRH, which was determined in the chum salmon (*Oncorhynchus keta*) is pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH_2

(**Sherwood et al., 1983**), whereas in mGnRH Leu and Arg replace Trp and Leu at positions 7 and 8, respectively. The widespread distribution of salmon GnRH (sGnRH) among teleosts was suggested based on comparisons of the structure of GnRH in *Salmo gairdneri, Carassius auratus and Solea solea* (**Breton et al., 1984**). This was further supported by HPLC and cross-reactivity studies on the brain extract of mullet (*Mugil cephalus*), milkfish (*Chanos chanos*) and rainbow trout (**Sherwood et al., 1984**).

The work of **Idler & Crim (1982), Barnett et al. (1982),** and **Sherwood et al. (1984),** suggested that two forms of GnRH may coexist in teleosts, a situation already established in birds. The two forms are the [Trp^7,Leu^8]-GnRH (salmon GnRH or sGnRH) and [His^5,Trp^7,Leu^8]-GnRH (chicken-II type GnRH or cGnRH-II). However, the brain of eels (**King et al., 1990**) and of a number of other primitive bony fish (**Sherwood et al., 1991**), contains mGnRH, whereas the catfish brain contains a catfish-specific form of GnRH, [His^5, Asn^8]-GnRh, cfGnRH), in addition to cGnRH-II (**Bogerd et al., 1992; Ngamvongchom et al., 1992a**). In the gilthead seabream, *Sparus aurata*, **Powell et al. (1994)** identified three native forms of GnRH, namely sGnRH, cGnRH-II and new seabream GnRH (sb-GnRH) with the structure of Ser^8 mGnRH. The possibility exists of further, as yet unidentified, forms of GnRH in milkfish and mullet (**Sherwood et al., 1984**), winter flounder (**Idler & Everard, 1987**), snook (**Sherwood et al., 1993b**), platyfish (**Magliulo-Cepriano et al., 1994**), and *Haplochromis burtoni* (**White et al., 1994**).

Evidence for the presence in the brain of a factor which inhibits the release of gonadotropins in teleosts came from studies on the lesioning of the Nucleus Lateralis Tuberis (NLT) of goldfish, in which the pituitary is effectively isolated from the hypothalamus, and which resulted in an increase in serum GtH levels and ovulation. This factor was called GtH-Release Inhibitory Factor (GnRIF) (**Peter et al., 1978; Peter & Paulencu, 1980**). It has since been identified as dopamine, an amine which counteracts the effect of GnRH, to varying degrees, in different species of teleosts (**Lin & Peter, 1996**). Dopaminergic inhibition is very significant in goldfish and cyprinids (**Peter et al.,**

1985, 1986, 1987b; Sokolowska et al., 1985a&b; Lin et al., 1987a), but less pronounced in the Chinese loach (**Lin et al., 1988**), salmonids (**Van der Kraak et al., 1986**) and tilapia (**Gissis et al., 1991**), the catfish (**Alok et al., 1993**) but is entirely absent in the *Micropogonias undulatus* (**Copeland & Thomas, 1989**).

Additional brain hormones and neurotransmitters which are implicated in the regulation of GtH release from the pituitary include neuropeptide Y (NPY) (**Peng et al., 1990**), γ-aminobutyric acid (GABA) (**Kah et al., 1990**), taurine, glutamate, aspartate, norepinephrine and serotonin (**Chang & Peter, 1984; Somoza et al., 1988**). Cholinergic nerves are also suspected of participating in this regulation (**Mikolajczyk et al., 1993**). Nevertheless, current knowledge indicates that neuroendocrine regulation of GtH secretion in teleosts is mainly under a dual neurohormonal system in which GtH release is stimulated by GnRH and inhibited by dopamine, which acts as a gonadotropin release-inhibitory factor (GRIF) (**Lin & Peter, 1996**). Dopamine action at the level of the pituitary is multi-faceted-by modulation of GnRH action, by alteration of spontaneous release of GtH and by inhibition of release of GnRH (**Peter et al., 1986,1991**). The surge in GtH secretion may be a combined result of stimulation of GnRH and release from inhibition by dopamine (**Lin & Peter, 1996**).

Although there is no obvious portal system in teleosts comparable to the median eminence-portal system of other vertebrates, the blood supply to the adenohypophysis passes through the neurohypophysis, which contains fibres originating in two areas of the hypothalamus, the nucleus lateralis tuberis (NLT) and the preoptic region. Fibres from these regions also penetrate the adenohypophysis, touching the GtH cells directly. Neurosecretory activity in the NLT and preoptic region is associated with reproductive activity. Both of the nuclei comprising the preoptic region, namely the nucleus preopticus (NPO) and nucleus preopticus paraventricularis (NPP), send fibres to the pituitary gland (**Scott, 1987**). Many different hypothalamic nuclei have been described in teleosts, but only three have been studied in detail and in a representative range of species. These are NPO, NLT and Paraventricular organ (PVO). All have been implicated in hypothalamic

control of the pituitary (**Batten & Ingleton, 1987**). The PVO is sometimes divided into two parts, the nucleus recessus lateralis (NRL) and nucleus recessus posterioris (NRP), both of which send aminergic fibres to the neurohypophysis and to the NPO/NPP and NLT regions, suggesting a possible functional interaction in steroid feedback control of GtH synthesis and secretion (**Scott, 1987**).

Most of the knowledge on the localization of GnRH secretion in the brain comes from immunocytochemical studies based on antibodies to LH-RH, and despite some discrepancies, there is now a good evidence for the presence of immunoreactive perikarya and nerve fibres in several brain locations (**Kah, 1986**). Immunoreactive perikarya have been identified in the area dorsalis pars medialis (ADPM) of the telencephalon in the rainbow trout (**Goos & Murathanoglu, 1977**), in the ventral lateral border of the NPP and posterior portion of the NLT in the goldfish (**Kah et al., 1984a**), and in the NPP, NLT, nucleus olfactoretinalis (NOR), and dorsal midbrain of the platyfish, *Xiphophorus maculatus* (**Münz et al., 1981&1982; Halpern-Sebold & Schreibman, 1983; Schreibman et al, 1983, 1984**). In addition, fibres immunostained with anti-LH-RH sera have been found in locations all over the brain of many teleosts, forming tracts linking the NPP, NLT, and NOR regions with each other, in the pituitary, ADPM, optic and olfactory nerves, and pineal complex (**Scott, 1987**). In the goldfish, immunoreactive fibers were detected in many parts of the brain, mostly along the midline, one fibre tract could be traced from the cells located in the olfactory bulbs to the pituitary stalk. Large electrolytic lesions placed in several sites in the forebrain showed that most of the fibres detected in the pituitary gland originate from the small bipolar perikarya in the ventrolateral preoptic region (**Kah et al., 1986a**). Such a pattern of distribution is similar to what has been described for mammalian LH-RH (**Merchenthaler et al., 1984**).

1.4.2 Methods of Manipulation of Reproduction:

Most teleosts are seasonal spawners, and only a few breed continuously (**Peter & Hontela, 1978; Lin & Peter, 1996**). In the wild, fry are usually available for only a

limited part of the year. In captivity many species do not spawn, and those that do, spawn in an unsynchronized manner or in an inappropriate season for the optimal management of resources (**Yaron & Zohar, 1993**). The extension of the spawning season in fish either by accelerating or retarding the reproductive cycle is essential to aquaculture (**Yaron et al., 1984**). This can be achieved by manipulation of the reproductive cycle of the fish. In the beginning, the manipulation of external zeitgebers such as photoperiod and temperature appeared to solve part of the problem. More recently the induction of spawning through endocrine intervention has become popular in fish culture, due to its relative ease and efficiency (**Yaron & Zohar, 1993**).

A. Manipulation of External Factors:

Of all the possible environmental factors which may control the reproductive cycle of fishes, photoperiod and temperature have received extensive attention. This may simply reflect the heavy bias of research effort towards fishes of temperate regions which are exposed to pronounced annual cycles of photoperiod and temperature. For tropical species subject to only slight annual changes in photoperiod and temperature, other environmental factors associated with changes in rainfall, may be more reliable agents for the control of the reproductive cycle. However, in any review of external factors, photoperiod and temperature will inevitably dominate the discussion.

1. Photoperiod Manipulation:

Various photoperiod regimes have been shown to affect the reproductive development of fish in different ways. Such effects can be brought about by a seasonally-changing light cycle or by photoperiods of constant long or short duration (**Takashima & Yamada, 1984; Duston & Bromage, 1987; Prentice & Thomas, 1987; Roberts, 1987; Davies et al., 1991; Kelly et al., 1991**).

The major source of knowledge on the effect of photoperiodic manipulation on fish gonadal development comes from the work on salmonids. The fact that salmonid

reproduction is susceptible to changes in daylength was observed quite some time ago (**Hoover & Hubbard, 1937; Corson, 1955; Breton & Billard, 1977; Whitehead et al., 1978; Whitehead & Bromage, 1980; Bromage et al., 1982a**). Since then a number of studies have shown that exposure of fish to modified seasonal light cycles leads to changes in reproductive cycle timing (**Zanuy et al., 1986; Devauchelle, 1987; Carrillo et al., 1989; Duston & Bromage, 1991**). Advanced or compressed seasonal cycles of photoperiod induce early gonadal development, whereas delayed or extended ones result in later spawning (**Billard, 1985**). Recently, it has also been shown that compressed cycles can also advance the time of first spawning (**Bromage, 1987**).

Generally speaking, long days or continuous light can advance maturation and spawning time if applied during the earlier part of the reproductive cycle. Advances of 5-7 months have been reported for the rainbow trout (**Bromage et al., 1984; Scott et al., 1984**), while the same exposure from the summer solstice onwards delays the spawning time (**Whitehead & Bromage, 1980; Bromage et al 1982a&1984; Bourlier & Billard, 1984**). Such a marked difference in the effect of the same photoperiod applied at different times in the reproductive cycle has been interpreted in the following way. Early in the reproductive cycle the fish are already on a naturally increasing daylength, and exposure to long days at that time produces phase advances in the endogenous rhythm, with considerable temporal variation in the responsiveness of this rhythm, with the maximal advance occurring when treatment is done immediately after spawning and with the effect diminishing until the summer solstice, after which the fish experience a naturally decreasing photoperiod and the same photoperiod produces a phase delay (**Bromage et al., 1993**).

The maturational response of salmonids to short days vary considerably over the year-long cycle. Exposure of fish to short days during the first 2-3 months after spawning leads to a delay in the time of the next spawning. The magnitude of this delay depends on two factors, the point at which the photoperiod is applied and the time of natural spawning of the stock being treated. For example, it was reported that in the rainbow trout, 12-14 week delays were observed when a January/February spawning stock was exposed to short days

in the middle of February (**Whitehead & Bromage, 1980; Bromage et al., 1984**). A longer delay of 5 months was observed when an early December spawning stock were exposed to short days in the middle of January (**Duston & Bromage, 1987**). However, the same photoperiod, when applied from early January in a December/January spawning stock, resulted in a 2-3 week delay only (**Bromage et al., 1993**).

A distinct change in the effects of short days occurs when applied in the period from 3-4 months after spawning to the summer solstice. Within that period, short days cause an advance in spawning time. The earlier the treatment within this period, the greater the advance in time of spawning. The time of natural spawning of the treated fish also has some influence on the effect of short days, with later spawning fish stocks (Jan/Feb.) showing more marked advances than autumn spawning stocks (**Bromage et al., 1993**).

The application of a combination of long and short days in a November spawning strain of rainbow trout results in a 3-4 month advancement in spawning time. The long days were applied early in the cycle and short days in May. The exposure to two different photoperiods has a cumulative effect, resulting in at least two phase advances (**Bromage et al., 1993**).

Similar modifications of the reproductive cycle were also reported in some other fishes like the milkfish (**Lee et al., 1987**), the sea bass (**Carrillo et al., 1989&1991**), the barbel (**Poncin, 1991**), and the murrel (**Srivastava & Singh, 1991**).

Female rainbow trout maintained under constant conditions of LD 6:18, temperature and feed ration for a period of over four years exhibited a rhythm of gonadal maturation and spawning that was self-sustaining for at least three cycles, and which had a varying circannual periodicity (**Duston & Bromage, 1991**). These results and those obtained from previous studies (**Whitehead et al., 1978; Bromage et al., 1984; Duston & Bromage, 1987&1988**) support the concept that the rhythm is entrained by changes in photoperiod, and that it is driven by an endogenous mechanism exhibiting three criteria: (1) a rhythm with a periodicity which approximates to, but differs significantly from, one year, which

(2) persists for more than two cycles under constant environmental conditions, and (3) is capable of entrainment to normal or altered light cycles (**Gwinner, 1981&1986**). The irregularity of such circannual rhythms has been used as evidence that they are not driven by a true oscillator akin to circadian phenomena, but rather by a "sequence of linked stages" (**Mrosovsky, 1970&1978**). If such is the case, then it can be postulated that the rhythm of gonadal development is generated by a feedback loop within the hypothalamo-pituitary-gonadal axis and that the periodicity of the rhythm is determined by the time taken to develop the ovary, and that the variations observed could be a result of factors such as availability of food and nutritional status of the animal (**Duston & Bromage, 1991**). Evidence against such a hypothesis came from a finding that fish with advanced spawning timing due to photoperiod manipulation exhibit reduced mature oocyte size (**Duston & Bromage, 1988**), suggesting that oocyte or ovary size does not dictate the timing for ovulation and therefore cannot determine the periodicity of the gonadal maturation rhythm. Another point of disagreement was identified when virgin female rainbow trout exposed to LD 18:6 and then LD 6:18 failed to mature in year one but exhibited a phase advance when spawned in the second year, 18 months after the start of treatment (**Duston & Bromage, 1988**). Such results indicate the need for photoperiod history to be "remembered" and would, therefore, be difficult to achieve if the rhythm of gonadal maturation were derived solely from a pituitary-gonadal feedback loop, but perhaps better explained if the basis for such a rhythm is considered to be an endogenous circannual clock analogous in its overt behaviour to a circadian oscillator (**Duston & Bromage, 1991**).

There is only one study on the effect of manipulated photoperiod on the gonadal maturation of the rabbitfish *S. canaliculatus*. In this, January-March spawning fish were subjected to two photoperiod regimes in August for a period of 16 weeks. One group was exposed to a 12L:12D simulated natural photoperiod and the second to a 18L:6D (long day) photoperiod, water temperature was held constant. Loss of most of the experimental fish meant that no statistically significant results could be drawn. However, the authors concluded that long daylengths could be used to delay gonadal development (**Lam & Soh, 1975**).

The manipulation of photoperiod and temperature may induce reproduction in a reliable manner in some species. In others, these agents may be ineffective or too costly. The control of reproduction by endocrine intervention has been reviewed frequently (**Lam, 1982; Billard, 1986a; Crim et al., 1987; Abraham, 1988; Lin & Peter, 1996**). Hormonal induction of reproduction can be achieved by the intervention at several levels in the natural sequence of endocrine events:

1. Intervention at the Level of the Pituitary Gland:

The injection of gonadotropin-releasing hormone (GnRH) agonist analogs and dopamine antagonist drugs, alone and in combination, has been used in fish culture to induce ovulation (**Lin et al., 1986a; Peter et al., 1987a, 1988a&b**). The effectiveness of such applications in ovulation of cultured freshwater fish can now be judged by a set of criteria called the Linpe technique, the criteria used are whether ovulation rate was high, complete or partial, whether ovulation can be repeated from one group to another within each species, whether time from injection to ovulation was short and predictable, the quality of the gamete, and whether this technique affected subsequent reproductive cycles by the same brood fish (**Peter et al., 1988a**). Studies on the structure and activity of a series of agonist analogs of mammalian GnRH (mGnRH) and salmon GnRH (sGnRH) seem to favour the latter for applications in inducing ovulation (**Peter et al., 1985&1987a; Habibi et al., 1987&1989**). sGnRH-A was very active in stimulating GtH-II release *in vivo* in common carp and Chinese loach (**Lin et al., 1988,1991**), and highly resistant to enzymatic degradation by the pituitary, kidney and liver of gilthead seabream (**Goren et al., 1987; Zohar et al., 1989**). However, there are some indications that the superactive analogue of mGnRH-A can also be highly active or at times even more potent than sGnRH-A in inducing GtH-II release and ovulation in some teleosts, such as the Atlantic salmon (*Salmo salar*) and gilthead seabream (**Zohar et al., 1989**).

The inhibitory action of dopamine on GtH-II secretion can vary in potency between species. In goldfish and common carp, dopamine inhibition is strong and use of mGnRH-A and sGnRH-A can only stimulate a modest increase in serum GtH-II levels and are thus

ineffective in inducing ovulation (**Peter et al., 1985, 1986, 1987b; Sokolowska et al., 1985a&b; Lin et al., 1987a**), but the use of dopamine receptor antagonists greatly potentiated their action and induced ovulation (**Lin et al., 1988; Peter et al., 1988a&b**). On the other hand, in the cyprinid *Parabramis pekinensis* and the Chinese loach the inhibitory effect of dopamine is not very strong and injections of high dose of mGnRH-A and sGnRH-A alone can overcome the dopaminergic inhibition. However, the use of dopamine antagonists still shortened the response time from injection to ovulation (**Lin et al., 1985, 1986a&b**). In contrast, there are some marine species, such as the Atlantic croaker (**Copeland and Thomas, 1989**), gilthead seabream, *Sparus auratus* (**Zohar et al., 1987**) and striped bass, *Morone saxatilis* (**Sullivan et al., 1992**), where there is no evidence for a dopaminergic inhibitory regulation of GtH-II secretion or ovulation.

Out of a variety of dopamine antagonist drugs, Domperidone is thought to be the most potent and highly specific D-2 type dopamine receptor antagonist, that does not cross the blood-brain barrier when injected i.p. but is concentrated in the pituitary, at least in the goldfish (**Omeljaniuk et al., 1987**), and is thus considered optimal for applied purposes (**Lin & Peter, 1996**). A commercial formulation of a sGnRH and domperidone has recently been available in the market under the trade name of Ovaprim (Syndel Lab. Inc., Canada).

2. Intervention at the Level of the Ovary:

Induced stimulation of the process of gametogenesis was for many years carried out using injections of crude or purified heterologous or homologous gonadotropins: the so called "Hypophysation Technique". The surge in circulating GtH resembles the normal event occurring just prior to oocyte maturation and ovulation (**Yaron & Zohar, 1993**). Though somewhat improved by the introduction of a GtH-calibrated carp pituitary extract (**Yaron & Levavi-Zermonsky 1986**), the crude preparations containing fish gonadotropin have been replaced in many instances with heterologous human gonadotropin (hCG), and to some extent with semi-purified fish GtH (**Lam, 1982; Donaldson & Hunter, 1983; Peter**

et al., 1988a). The method of introduction of the hormone may be by injection or implants.

Experiments to induce early stages of reproductive development by repeated injection of gonadotropin or GtH+Oestradiol has been less successful, especially in females (**Crim et al., 1987; Crim & Evans, 1983**), but more often hormonal treatment is applied to advance and synchronize later stages of the reproductive cycle.

1.5 Plan of Work:

The following plan of work was undertaken in order to provide a better understanding of the environmental and endocrine control of reproduction in the rabbitfish, *Siganus canaliculatus*, with a view to overcoming the failure of hatchery fish to mature and spawn without intervention.

A detailed description was made of the major stages in the morphological and structural development of the normal ovary (Chapter 2), against which to judge the effectiveness of the later studies on environmental and endocrine manipulation.

An assessment was made of the effects of handling stressors, which are commonly associated with experimental studies and routine hatchery practice, on ovarian development, maturation, and gamete and larval quality (Chapter 3).

An investigation into the role of lunar information in promoting ovarian development and maturation was conducted on hatchery fish which are often deprived of lunar exposure (Chapter 4).

The environmental control of the seasonal timing of reproduction was investigated by manipulation of photoperiod (Chapter 5).

The failure of hatchery fish to mature and spawn unaided was investigated by endocrine intervention with a heterologous gonadotropin and two hypothalamic gonadotropin-releasing hormone analogues (Chapter 6).

Chapter 2

Chapter 2

Morphological and Structural Development of the Ovary in *S. canaliculatus*.

2.1 Introduction:

2.1.1 Normal Follicular Development:

The development of teleost oocytes has been reviewed extensively (**Guraya, 1986; Nagahama, 1983; Tokarz, 1978; Wallace et al., 1987; Wallace & Selman, 1981**).

Teleost gonads are invaded by primordial germ cells at a very early stage in embryonic development, and subsequently differentiate around the time of hatching. Ovaries generally differentiate before the testes. The ovaries take shape as hollow organs consisting of many connective tissue septa called the ovigerous lamellae where the eggs develop (**Scott, 1987**). One of the essential functions of the ovary is follicular development, which depends on the inter-play of several control mechanisms originating partly from outside the ovary, mainly the pituitary, and partly from within the ovary and the follicle itself (**Peters, 1979**).

The process of oocyte development can be categorized into the following six phases (**Scott, 1987**):

(1) Oogonial proliferation- In which premeiotic sex cells, termed oogonia, develop from the primordial germ cells. These are small, rounded, found in nests and have large nuclei each containing a single prominent nucleolus. The oogonia proliferate by mitotic division, following which some are transformed at the first meiotic division into primary oocytes (oogenesis). In the majority of teleosts this process of transformation occurs throughout reproductive life, at intervals in some species and continuously in others.

(2) Primary Growth Phase- The oogonia become invested with a layer of follicle cells. The nuclei increase in size and multiple nucleoli appear. The cytoplasm increases in volume and becomes more chromophilic, complexes known as "Balbiani bodies" appear as less densely stained cytoplasmic material. The function of these bodies is not fully understood. Until this phase, oocyte growth is gonadotropin-independent, and is also called protoplasmic growth (**Hussain, 1984; Butskaya & Faleeva, 1987**).

(3) Secondary Growth Phase- This phase is gonadotropin-dependent, trochoplasmic growth (**Hussain, 1984; Butskaya & Faleeva, 1987**), and primarily involves the accumulation of nutrient reserves, the yolk, within the oocyte, in the process known as vitellogenesis. Vitellogenesis involves the synthesis or uptake of three types of "yolk" by three processes:

i. Endogenous vitellogenesis- involves the synthesis of "intravesicular yolk" which is seen early in this phase in the form of vesicles (vacuoles) in the cytoplasm beginning at the periphery of the oocyte and later filling the cytoplasm. These eventually become the cortical alveoli (**Yamamoto,T. 1955; Osanai, 1956; Yamamoto,K. 1956a&b; Malone & Hisaoka, 1963**), which will release their contents into the perivitelline space at fertilization. Careful study (**Selman et al., 1986, 1988**) of 'yolk vesicles' has shown that they do not contain yolk, but instead contain primarily enzymes that are released at fertilization, and serve to harden the vitelline envelope to prevent polyspermy. Thus, 'yolk vesicles' is a misnomer, and these vesicles should be called cortical alveoli.

ii. "Lipid yolk" uptake- most teleost eggs contain lipid inclusions, usually oil droplets (globules), these inclusions may appear earlier or later than the yolk vesicles.

iii. Exogenous vitellogenesis- This process involves the sequestration and packaging of hepatically derived plasma precursor, a female-specific plasma lipophosphoprotein complex known as vitellogenin, into yolk protein (**Wallace, 1978; Wallace & Selman, 1981**). In teleosts, like other nonmammalian vertebrates, this process is the principal event responsible for the enormous growth of oocytes. Exogenous vitellogenesis follows the endogenous phase very rapidly with a very short period where they can be separated.

(4) Oocyte maturation- Maturation of a fully vitellogenic oocyte starts with the migration of the nucleus (germinal vesicle) from the center to a peripheral position at the animal pole. This is followed by disintegration of the nuclear membrane (germinal vesicle breakdown). In many teleosts, particularly marine species, maturation may also involve a rapid increase in diameter due to the uptake of aqueous medium (hydration).

(5) Ovulation- This process involves the actual release of mature oocytes from the follicle into the body cavity or the lumen of the ovary. The process of ovulation involves the appearance of a hole in the follicle, followed by the detachment of the follicular layer, and active expulsion of the egg through the hole by prostaglandin-induced contraction of smooth muscle fibers in the thecal layer. Maturation and ovulation are rapid events of brief duration which may be classified as a single event, although the physiological control of the two is quite distinct..

(6) Postfertilization- The egg resumes meiosis and embryonic development proceeds.

The above description of oocyte development holds for most oviparous teleosts, although the factors and events which initiate and promote the stages of development differ throughout such a diverse group as the teleosts (**Wallace & Selman, 1981**).

Marza (1938) classified ovaries into three basic types:
i. Synchronous- All oocytes grow and ovulate from the ovary in unison.
ii. Group-synchronous- At least two populations of oocytes can be distinguished at some time.
iii. Asynchronous- Oocytes of all stages are present without dominant populations.

The cytological and histochemical descriptions of the morphological changes occurring during oocyte growth (**Takahashi, 1981; Schaefer, 1987; Rajalakshmi, 1966; Khoo, 1979; Mayer et al., 1988**), have led to classifications based on criteria such as cell size, number and morphology of nucleoli, the abundance and distribution of cell inclusions, and degree of development of the follicular envelope (**Coello, 1989**).

The most commonly used classification divides ovary development into eight stages (**Yamamoto & Yamazaki, 1961; Nagahama, 1983**): chromatin-nucleolus stage, early perinucleolus stage, late perinucleolus stage, yolk vesicle stage, oil droplet stage, yolk granule stage, migratory nucleus stage, and ripe or hydrated stage. Other classifications and modifications are also used (**Kesteven, 1960; Nikolsky, 1963; Moore, 1980**).

2.1.2 Atresia in Teleosts:

The word "atresia" is used to denote the degenerative processes during which the ovarian follicle loses its integrity and the oocytes are resorbed and eliminated from the ovary by means other than ovulation (**Byskov, 1979**). Numerous studies on atresia in teleosts have been published (**Saidapur, 1978; Guraya, 1986**), but the process is more extensively studied in mammals and nonmammalian vertebrates other than teleosts (**Byskov, 1979; Tsafriri & Braw, 1984; Dodd, 1983; Gupta & Maiti, 1986**). In mammals and non-mammalian vertebrates, follicles may become atretic at any age and any stage of development. The large proportion of atretic oocytes in the ovary indicate that atresia and not ovulation is the ultimate fate of the majority of developing follicles (**Mandl & Zuckerman, 1950; Himelstein-Braw et al., 1976**). The cause of atresia, the mechanisms that control it, and its role in regulating oocyte development is not well understood; however, hormonal balance plays an important role (**Wallace & Selman, 1981**). Some of the causes suggested include environmental stress (**Ball, 1960; De Montalembert et al., 1978**), poor nutrition (**Rai,1966**), and factors reducing gonadotropin levels (**Chester-Jones & Ball, 1962; Barr, 1968**).

The follicular layer plays an important role during the process of atresia, which generally involves changes in the granulosa and possibly the theca cells and appearance of phagocytic cells, the resorption of the oocyte by these cells, and degeneration of the phagocytic cells. All follicles do not follow a uniform pattern during atresia, instead, the atretic process varies with the stage of development of the follicle. In early developing oocytes, the process of resorption is mainly by phagocytosis with the granulosa cells remaining intact, while in more advanced oocytes degeneration affects both oocyte and follicular layer (**Byskov, 1979**).

In teleosts the main function of atresia is disposal of oocytes, although other roles have been suggested, such as the involvement in the synthesis of steroids or formation of interstitial cells (**Saidapur, 1978; Saidapur & Nadkarni, 1976**), and in oogonial differentiation (**Khoo, 1975**). However, these have been challenged and are controversial (**Van den Hurk & Peute, 1979**). Atresia is most prominent in teleost ovaries at the end of the breeding season, when the process is triggered by either endogenous or environmental factors or both (**Lam, 1983**). It follows the non-mammalian pattern and can be morphologically classified into four stages: alpha, beta, gamma, and delta (**Bretschneider & Duyvene de Wit, 1947**).

2.1.3 Ovary of Siganids:

There are a few studies describing the development of the siganid ovary (**Lavina & Alcala, 1973; Alcala & Alcazar, 1979; Tseng & Chan, 1982; Avila, 1984; Hara, 1987; Ntiba & Jaccarini, 1990; Budiman et al., 1991; Al-Ghais, 1993**). Most are based on macroscopic appearance and measurement of gonadosomatic index, and very few describe the histological changes involved in the process.

Many systems have been used to describe follicular development in the rabbitfish based on macroscopic appearance, histological changes, and gonadosomatic indices. **Lavina (1975)** identified six stages in *S. canaliculatus* based on macroscopic appearance of the ovary (Table 2.1), ranging from immature (stage I) to spent (stage VI). **Alcala & Alcazar (1979)** identified seven stages based on ovary size and histological appearance (Table 2.2), they termed stage I as chromatin nucleolus and early perinucleolar oocytes, and stage VII as resting or resorbing oocytes. **Al-Ghais (1993)** followed the former scheme with some modifications, dividing the sixth stage into three sub-stages (Table 2.3). The above classifications are based on the general scheme used for goldfish (**Yamamoto & Yamazaki, 1967**).

The time taken to complete the process of oocyte development has been reported for some species. Two gonadal cycles were reported for *Siganus canaliculatus* in South East Asia, each cycle extending over a period of 4.5 months from perinucleolar stage to ripe egg stage

Table 2.1 Stages in gonadal development of *Siganus canaliculatus*, based on macroscopic appearance, after **Lavina (1975)**.

Stage	Descriptive Term
I	Immature
II	Developing virgin or recovering spent
III	Maturing
IV	Mature
V	Ripe
VI	Spent

Table 2.2 Stages in gonadal development of *S. canaliculatus*, after **Alcala & Alcazar (1979)**.

Stage	Ovary Size Range (mm)	Histological Appearance
I	14-70	Chromatin nucleolus and early perinucleolus oocytes.
II	14-238	Late perinucleolus and yolk vesicle oocytes.
III	56-350	Yolk vesicle and primary yolk oocytes.
IV	210-364	Secondary and tertiary yolk oocytes.
V	266-406	Mature oocytes.
VI	336-420	Ripe eggs.
VII	14-70	Resting or resorbing oocytes.

(**Lavina, 1975**). In *S. guttatus*, vitellogenesis is usually completed within 27-28 days (**Soletchnik, 1984**).

The ovary of *Siganus canaliculatus* is perciform, the posterior portions are fused together leading to the oviduct, which terminates immediately behind the anus (**Lavina, 1975**). There is no ovipore, thus the eggs are extruded through a rupture in the connective tissue at the end of the common oviduct (**Duray, 1990**).

The topic of follicular development of siganids in general and *S. canaliculatus* in particular has not been studied in detail. The very few and early reports describe oocyte development in general, without giving detailed description of changes occurring during that process and without clear photographic documentation. A knowledge of the morphology and structure of the ovary is essential for understanding the dynamics and control of ovarian development. The following experiments are designed to provide some of that knowledge.

2.2 Materials and Methods:

2.2.1 Ovary Sampling:

A. Wild fish:

Live adult fish were collected directly from fishermen on board boats, fishing near Fasht Al-Adhom reef on the eastern coast of Bahrain (26° 8' N, 50° 40' E) or from near shore traps in the same area, at monthly intervals from June 1994, approximately one month after the end of the spawning season, until May 1995 in the next spawning season. This covered all stages of ovarian development and maturation based on macroscopic criteria as adopted by **Al-Ghais (1993)** (Table 2.3) for *Siganus canaliculatus*. During the spawning season, sampling was done at weekly intervals . The rabbitfish is not sexually dimorphic, so a larger number of fish than needed had to be collected and the first 10-15 dissected females kept.

Table 2.3 The maturity scale of female *S. canaliculatus* based on macroscopic criteria, after **Al-Ghais (1993)**.

Maturity Stage		Macroscopic Appearance
Stage 0	Immature	No easily visible gonads in body cavity.
Stage I	Maturing	Ovary thread-like, transparent, sexually distinguishable.
Stage II	Early developed	Ovary begin to swell and occupy nearly one fourth of body cavity, but still translucent.
Stage III	Mid-developed	Ovary larger, translucent with small eggs visible microscopically
Stage IV	Ripe	Ovary opaque, solid, eggs fully formed and numerous, not translucent
Stage V	Running	About to spawn, ovary turgid, filling body cavity, eggs expelled from body by slight pressure to abdomen.
Stage VIa	Early spent	Ovary reduced in size, reddish in colour.
Stage VIb	Half spent	Ovary much reduced in size, eggs seen by naked eye.
Stage VIc	Late spent	Ovary flaccid, reddish in colour.

Table 2.4 Neutrally buffered formalin solution, after **Bucke (1972)**.

Formaldehyde (40% W/V)..... 100 ml

Distilled water..... 900 ml

Sodium dihydrogen phosphate (dihydrate)..... 4.5 g

Disodium hydrogen phosphate (12 H2O)..... 6.5 g

Fish were measured, for total weight and length, and dissected on board or transported on ice to the laboratory and dissected within one hour after being caught. The ovary was weighed to the nearest 0.01 g, texture and colour were recorded, and the whole was fixed in 4% formaldehyde in neutrally buffered saline (Table 2.4). Large ovaries were injected with the fixative prior to fixation.

B. Captive fish:

300 spent or virgin fish with total length over 15 cm were stocked in a 40 m^3 outdoor concrete tank one month after the spawning season and maintained under ambient conditions with continuous water change and aeration until after the end of the next natural spawning season. Tank bottom was cleaned each month after sampling using a siphon, and fish were also given a 24 hour bath treatment using 0.5 ppm Prefuran (Argent Chemicals, U.S.A.) as a measure of disease prevention. The fish were fed a high protein diet (46%) formulated by mixing two commercial diets enriched with vitamins and fish oil (Table 2.5), the feed was used in moist form at a ratio of 3% body weight divided over two daily meals given at 08:00 and 16:00 hours Sunday to Thursday. On Saturday the fish were fed an equal amount of chopped cuttle fish. No feeding was done on Friday.

Fish were caught for ovary and blood sampling at monthly intervals at feeding time by using a large rectangular net frame, this method was adopted to reduce stress to the remaining fish. The sampling frequency was increased to weekly intervals during the spawning season. Numbers of fish sampled and procedures were the same as for wild fish.

2.2.2 Histological Procedure:

Transverse slices, about 5 mm thick, taken from the mid-region of the ovary, were prepared and dehydrated in ascending concentrations of ethanol in water and then infiltrated and embedded in glycol methacrylate polymer resin (TAAB Technovit 7100) (Table 2.6). Sections were cut to 4 μm thickness on an LKB 2218 HistoRange microtome. Sections were stained with Polychrome stain (Table 2.7), which gives good differentiation of the various types of

2.5 Formulated maturation diet (crude protein=46%) for *Siganus canaliculatus.*

Ingredient	Percentage
Ingredient I	70
Ingredient II	24
Cod liver oil	5
Vitamin premix	1
	100

Ingredient I - Eel diet, crude protein=48%, Sealion Feeds , Taiwan.
Ingredient II - Seabream diet, crude protein=52%, BioMar , France.

Table 2.6 Histological procedure for Technovit 7100 (TAAB), infiltration medium used for ovary of *S. canaliculatus*.

A. Dehydration:

1. Discard fixative, do two half hour distilled water changes.
2. 30% IMS one hour.
3. 50% IMS one hour.
4. 70% IMS one hour (tissue can be stored at this stage).
5. 90% IMS one hour.
6. IMS, three changes, one hour each.

B. Infiltration:

1. Place tissue in 1:1 mixture of IMS and Historesin, store at 4-6 °C overnight.
2. Place tissue in HIS and store at 4-6°C for 24 hours.
3. Change HIS and leave at 4-6°C for two weeks, with a change of HIS after one week

C. Embedding:

Embedding is done in a 1:15 ratio of HIS and Hardener in suitable moulds at room temperature (keep large moulds at 4-6°C for the initial 2-3 hours). Allow to polymerize overnight. Cut down excess plastic and mount on a block.

D. Sectioning:

Sections were cut at a thickness setting of 4μm on a LKB 2218 Historange microtome. The sections were picked up on a microscope slide, coated with Mayer's albumen, from a temperature controlled water bath (36-40°C). Slides were dried on a hot plate for 24 hours and stained.

Notes:

IMS = Industrial Methylated Spirit
HIS = Historesin Infiltration Solution prepared as per manufacturer's instructions.

Table 2.7 Polychrome stain for fish ovaries, modified after **Coello (1989)**.

Staining Solution	Time (min)	Staining Characteristics
1% Periodic acid	10	
running water	5	stains carbohydrates
Schiff's reagent (in the dark) (a)	10	(yolk vesicles)
running water	5	
Ehlrich's haematoxylin	30	highlights cytoplasm and
running water	quick rinse	nucleus
0.1% Bromophenol blue (b)	15	
0.5% Acetic acid	20	stains proteins (yolk granules)
running water	3	
Dry slides on a hot plate and mount in DPX		

(a) Schiff's reagent according to **Barger & De Lamater (1948)**.
(b) 0.1% Bromophenol blue in 95% alcohol.

Table 2.8 Diameter of oocytes and oocyte inclusions, and thickness of follicular structures during oocyte development in the rabbitfish *Siganus canaliculatus*.

Oocyte stage	Diameter (µm)				Thickness (µm)			Months occurring
	Oocyte	YV	YG	LG	RI	RE	GR	
Oogonia	4-8							Jun-May
Chromatin nucleolus	8-15							Jun-May
Early perinucleolus	18-90							Jun-May
Late perinucleolus	45-145							Dec-May
Yolk vesicle	100-170	1-5		1-10	0.8-1.2		1.2-2.4	Jan-May
Early yolk granule	125-280	3-12	1-3	3-9	1.1-1.9		2-2.8	Mar-May
Mid yolk granule	270-485	5-12	3-6	3-9	1.2-1.8		1.9-2.7	Mar-May
Late yolk granule	370-500	3-9	6-12	5-18	6-16.8	<1	1.3-2.8	Mar-May
Migratory nucleus	460-510	5-9	6-11	9-120	6-14	<1	3-8.4	April
Hydrated oocyte	530-600			48-156	3-8		2.4-4.0	April
Post ovulatory follicle	N/A							Apr-May

Sample number is 20 for diameter measurements and 10 for thickness measurements.
LG=Lipid Globule, RI=Radiata Interna, RE=Radiata Externa, GR=Granulosa.

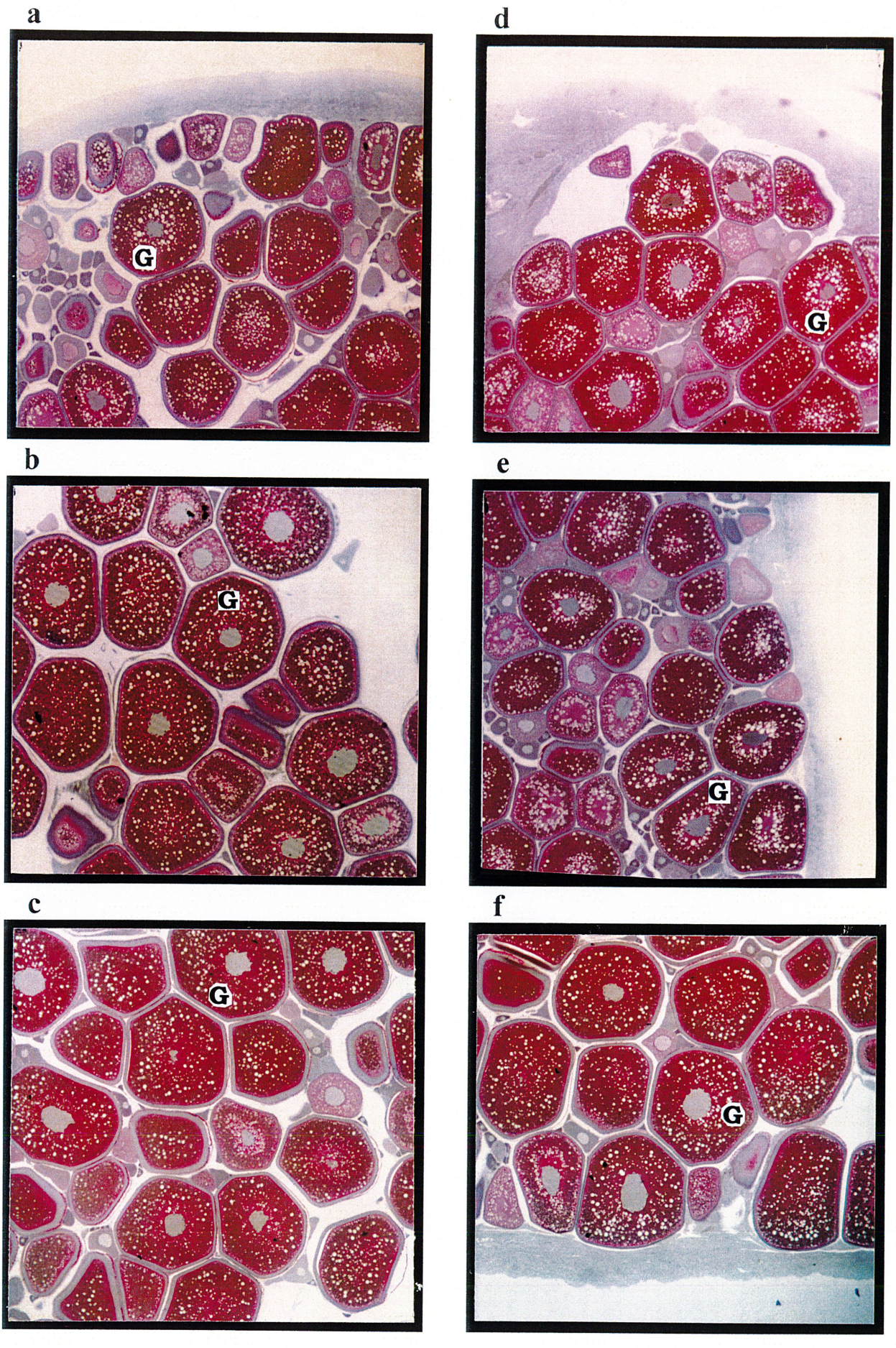
yolk. Sections of ovaries in various stages of development were also stained separately in Ehlrich's hematoxylin, Periodic Acid/Shiff's (PAS) and bromophenol blue to high-light staining characteristics of various oocyte components and facilitate better interpretation of results.

To establish whether there were any variations in the distribution of oocytes throughout the ovary at different stages of development, transverse slices from the anterior, middle, and posterior regions of the ovary covering both lobes were histologically checked. The sampling covered fish with pre-vitellogenic and vitellogenic ovaries. No variation was found in oocyte distribution (Fig.2.1), and hence throughout this work portions cut from the mid-region of the larger left ovary lobe were used in histological preparations.

Oocyte Measurements:

To check for any volume change of oocytes due to preservation in 4% formaldehyde in neutrally buffered saline, diameters of freshly biopsied oocytes were measured using a Nikon profile projector Model V-12, connected to a Nikon readout meter model SC-112. The oocytes were then preserved in the fixative and measurements done again at intervals of 6hrs, 12 hrs, 24 hrs, one week, two weeks and one month. Three fishes were used, the sample number for each was thirty oocytes. Comparison of mean diameters of fresh and preserved oocytes showed that there was a slight shrinkage (1.4%) in oocyte diameter after 6 hours increasing to 2.6% at 12 hours. But at 24 hours the diameter shows an increase with no significant difference compared to initial values ($F_{1,58}$=0.08 P=0.781) (Fig.2.2). Therefore, no adjustments were made to the diameters of preserved oocytes measured.

At advanced stages of development, when biopsy was possible, diameter measurements were also done for individual preserved oocytes, not subjected to histology, using the same technique. The results were used, in conjunction with results of the same oocytes subjected to histology, to check for any effect on oocyte diameter caused by the histology procedure. No significant variation due to histological procedures was detected ($p>0.05$).


a
G
d
G
b
G
e
G
c
G
f
G

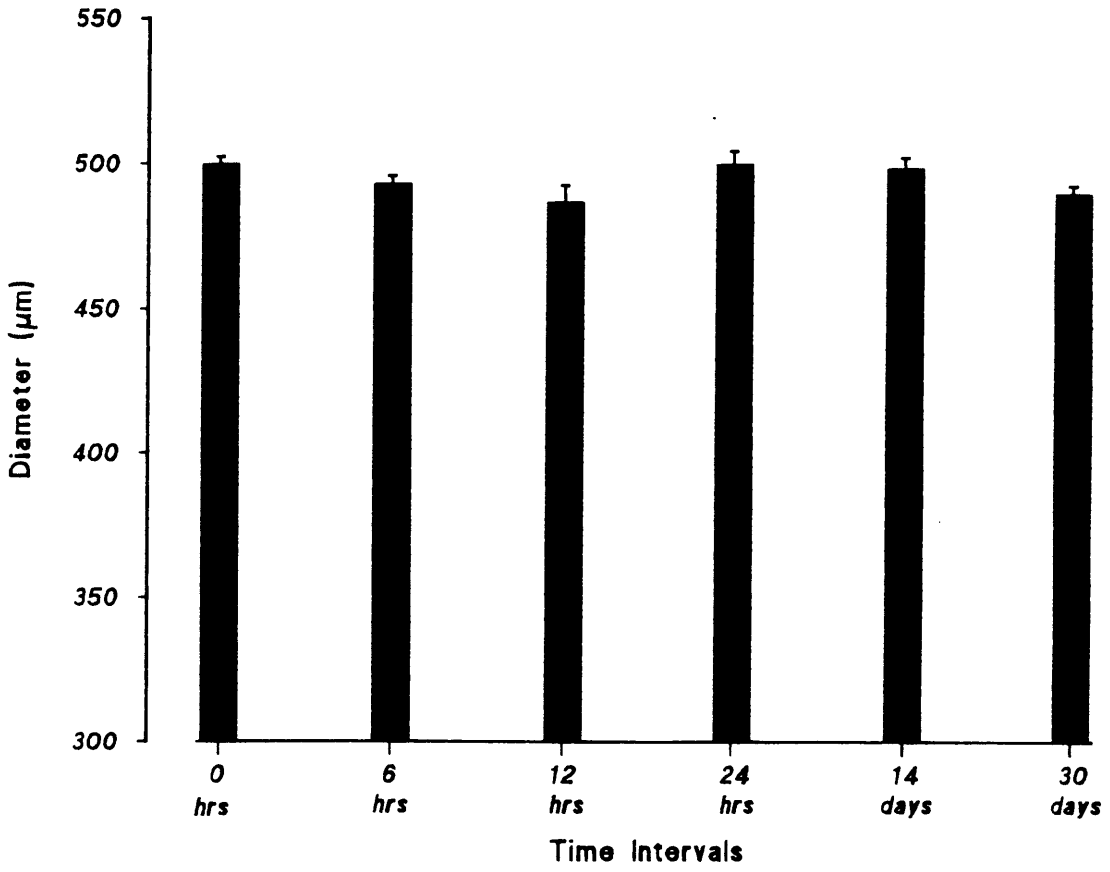


Figure 2.2– Diameter (mean±s.e.m.) of vitellogenic oocytes of *S. canalicualtus* after preservation in 10% neutrally buffered formalin for different intervals of time.The number of samples at each interval was 30.

Oocyte diameter measurements were made only on oocytes sectioned through the nucleus. A random part of the mounted tissue section was projected using a Leitz light microscope fitted with a projection eyepiece onto a mirror which then reflected the image onto a clear sheet of glass covered with tracing paper. The images were drawn on the tracing paper using a fine pencil. These drawings were subsequently digitized into a personal computer by means of a GTCO Corporation DIGI-PAD digitizing tablet with an active area of 50X50 cm and 0.025 mm resolution. Digitizing traced images was found to be more accurate, especially when the profiles of a large number of shapes have to be followed by the five-button digitizer cursor. Special software was used to produce relevant data for the profiles, and this allowed a simultaneous generation of areas and perimeter distances for the measured oocytes. Correction for scale was done using a projected graticule image. Other software was used for calculating the mean diameter from the area of the irregularly shaped oocyte. Also, the new circumference generated for these normalized areas was compared to the original perimeter around the oocyte and the ratio of both indicated the extent of irregularity of the shape. The integrity of the data was validated by using calibrated sizes on graph paper and the results manually checked.

Radiata and granulosa thickness was measured at four points on the perimeter corresponding approximately to the N.,S.,E. and W. sides of the oocyte, using a calibrated eyepiece graticule; measurements were done in 10 oocytes for each of the two follicular layers. Diameters of oocyte inclusions were also measured using a calibrated eye-piece graticule. Three oocytes were randomly taken, inclusions which were run through by a horizontal and vertical line on the eye piece were measured, 20 inclusions of each type were measured in each oocyte. Three ovaries for each development stage was used for the above measurements.

2.2.3 Blood Sampling and Oestradiol Assay:

About one ml of blood was collected from the caudal vessel of live fish tranquilized in 100 ppm 2-phenoxyethanol. Serum oestradiol was assayed from wild and captive fish at monthly intervals except during the maturation phase, when oestradiol was assayed at 24 hours intervals using hormone-injected fish. Full details of sampling and assay procedures are given in Chapter 5.

2.2.5 Gonado-Somatic Index:

A gonadosomatic index (G.S.I), the weight of the ovary as a function of the body weight, was calculated using the equation:

$$G.S.I. = (\text{ovary weight/total body weight}) \times 100$$

2.2.6 Terminology:

The terminology used in this study to describe the macroscopic development of the ovary is a modification of that used by **Lavina (1975)** (Table 2.1), and **Al-Ghais (1993)** (Table 2.3), previously adapted from **Yamamoto and Yamazaki (1967).**

Siganids in the area of the study do not spawn in captivity (see Chapter 1, Section 1.3.2-2). Therefore, all developmental stages after late yolk granule stage (end of vitellogenesis) have been described from fish manipulated into the maturational stage with exogenous hypothalamic or pituitary hormones and observed until spawning.

2.2.7 Statistical Analyses:

All values are given with their mean and standard error of the mean (s.e.m.). Differences between mean oocyte diameters were tested using a One-way analysis of variance (ANOVA) followed by Tukey-Kramer's Multiple Comparison Test. Difference between means of serum E_2 within groups were tested using Kruskal-Wallis Nonparametric ANOVA Test followed by Dunn's Multiple Comparison Test. All tests were performed on a Minitab statistical package V.10 (Minitab, Inc.) and GraphPad Instat2 Statistical Software.

2.3 Results:

2.3.1 The Gonado-Somatic Index (G.S.I):

The seasonal changes in GSI correspond closely to the development stages of the ovary and can be used as a general indication of the gonad condition, especially in late development stages. The GSI is low and consistent at less than 1% from June till January after which it increases to a maximal of over 12% in fully vitellogenic ovaries (Fig.2.3). Values of up to 30% were recorded in hydrated ovaries of fish induced into maturation during the spawning season in April.

2.3.2 The Structure of the Follicle of *S. canaliculatus*:

The structure of the typical follicle of *Siganus canaliculatus* is similar to that of other fish. Oocytes less than 90 microns in diameter constitute the reservoir of oocytes that are present year-round in active, as well as inactive, ovaries. The follicular envelope is composed of three layers, an outer theca forming a layer of epithelial cells, an inner layer of cuboidal cells, the granulosa, and an innermost proteinaceous, acellular layer, the radiata, which at an advanced stage of development can be distinguished into a thin radiata externa and a more prominent radiata interna (Fig.2.4). Within this envelope surrounded by the plasma membrane is the oocyte which ultimately contains three types of yolk material, namely, the yolk vesicles or cortical alveoli, the lipid yolk, and the yolk granules (Fig.2.4).

2.3.3 Oocyte Growth:

Using histological criteria , the development of the oocyte could be divided into eleven stages, four previtellogenic, four vitellogenic, two maturation and one post ovulation. During the spawning season, two populations of oocytes can be seen in a fully vitellogenic ovary, one dominant homogenous population of large oocytes and one composed of less developed oocytes of various sizes and stages (Fig.2.5). In a newly spawned ovary, oocytes in all stages of development can be found, although very few late yolk granule stage can be seen. Their

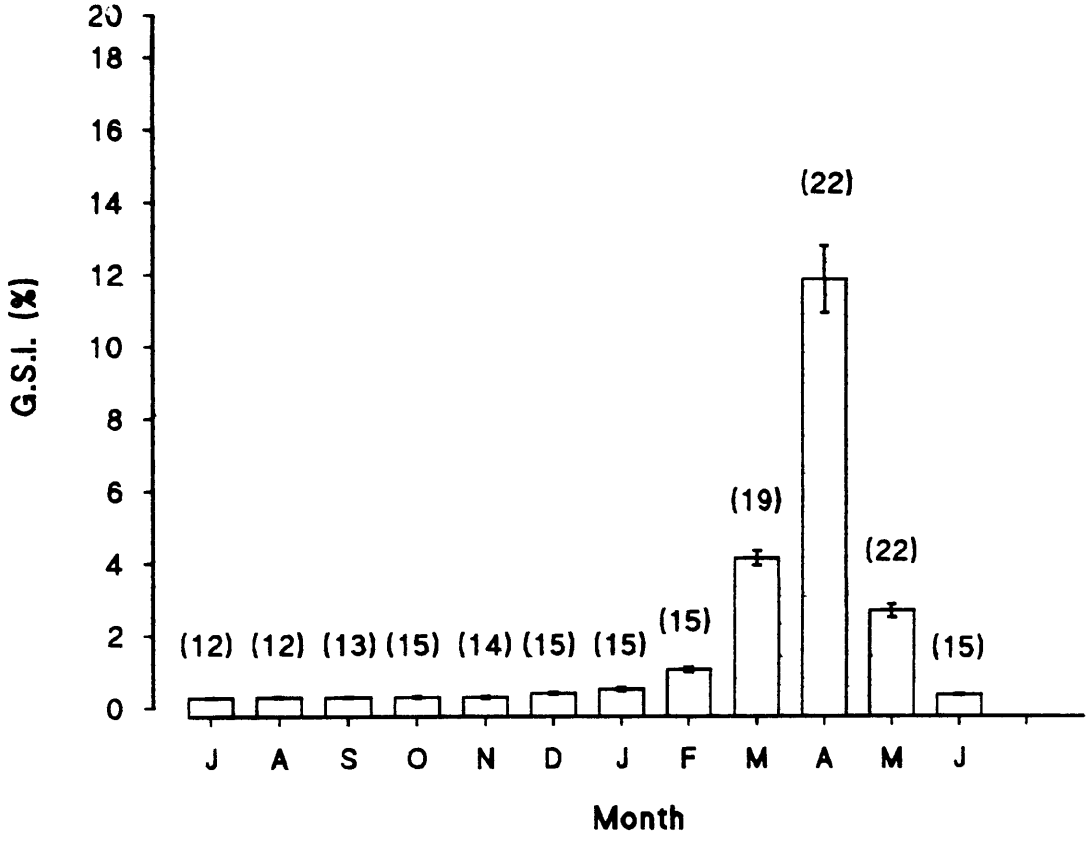


Figure 2.3– Gonadosomatic indices (mean±s.e.m.) in wild *S. canaliculatus* at monthly intervals during one annual cycle of reproduction. Numbers on bars indicate sample number.

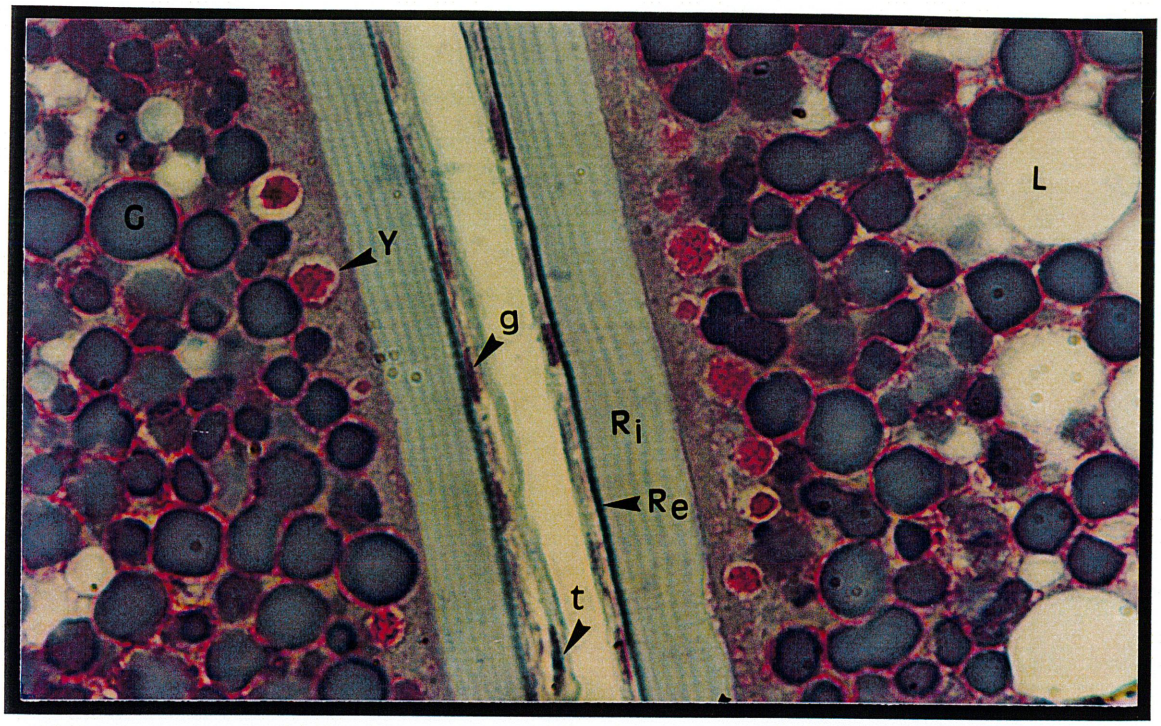


Figure 2.4- Three layers of oocyte follicle of *S. canaliculatus*. t=theca, g=granulosa, R=radiata [composed of radiata externa (Re) and radiata interna (Ri)]. G=yolk granules, Y=yolk vesicles, L=lipid globules. **Scale : 10μ**

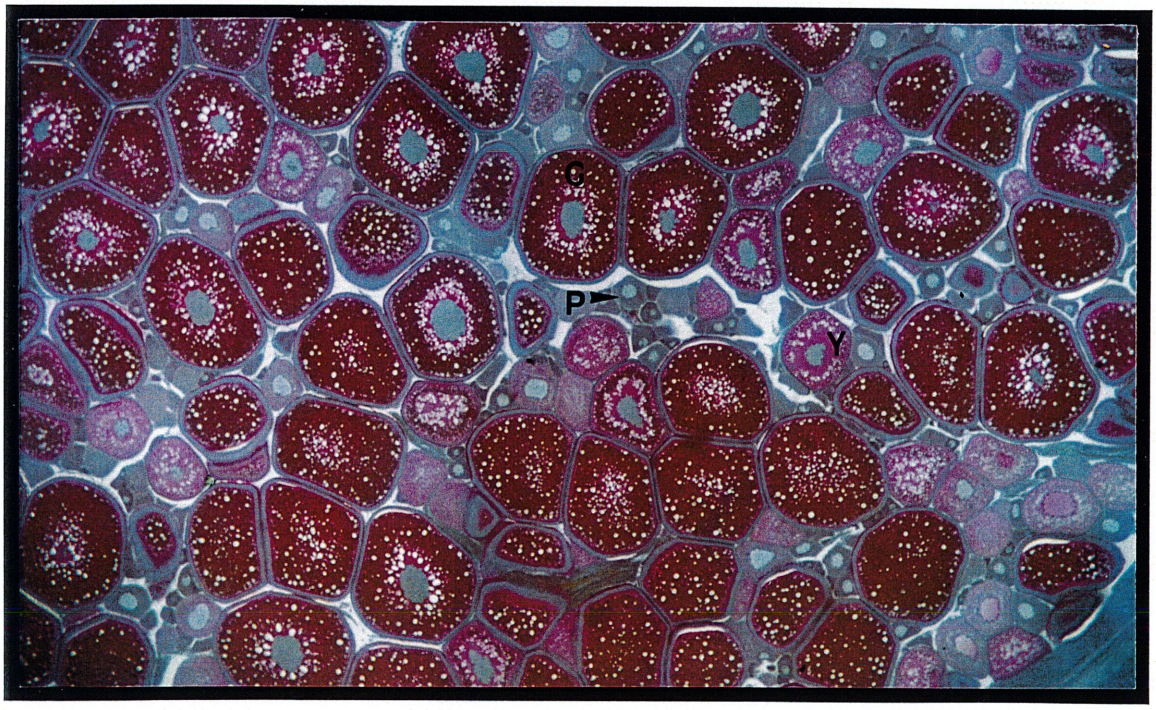


Figure 2.5- Ovary of *S. canaliculatus* showing simultaneous presence of oocytes of various stages of development. P=previtellogenic oocytes, Y=yolk vesicle oocyte, G=yolk granule oocyte. **Scale : 250μ**

scarcity indicates that only oocytes at this stage are capable of responding to maturational stimuli and progressing further, whereas earlier stages are arrested until subsequent resorption.

The granulosa and radiata layers show changes in thickness with the progress of oocyte development (Fig.2.6), the granulosa reaching its thickest in the migratory nucleus stage (mean 5.5±1.3μm), and the radiata interna at the late yolk granule stage (mean=10.3±2.3μm). The radiata externa is first noticeable in the late yolk granule oocyte, but its thickness remains less than one micron (Table 2.8). The three types of yolk inclusions also show consistent changes in diameter as the oocyte grows (Table 2.8), yolk vesicles being largest in early and mid yolk granule oocytes (mean=9.4±0.3μm), and yolk granules in late yolk granule and migratory nucleus stages (mean= 9.9±0.2μm). The lipid globules attain very large sizes by coalescence and reach maximum diameters in hydrated oocytes (mean= 98.0±4.2μm) (Fig.2.7).

1. Oogonium [Fig.2.8(a)]

Oogonia are round cells, with a diameter of 4-8 μm (Table 2.8), with a relatively narrow thin zone of cytoplasm and a single, prominent nucleus. They occurred as solitary cells or in groups (nests) in the stroma of the ovigerous folds. They were mainly noticeable in the post spawning season in recovering spent females.

2. Chromatin Nucleolus Stage [Fig.2.8(b)]

In these primary oocytes the diameter is 8-15 μm (Table 2.8). The oocyte has a weakly basophilic cytoplasm with a single large prominent nucleus with a single more basophilic nucleolus.

3. Early Perinucleolus Stage [Fig.2.8(c)]

The oocyte in this stage measures 18-90 μm in diameter (Table 2.8), and is irregular polygonal in shape, being crowded by adjacent oocytes. The nucleus is large and central containing

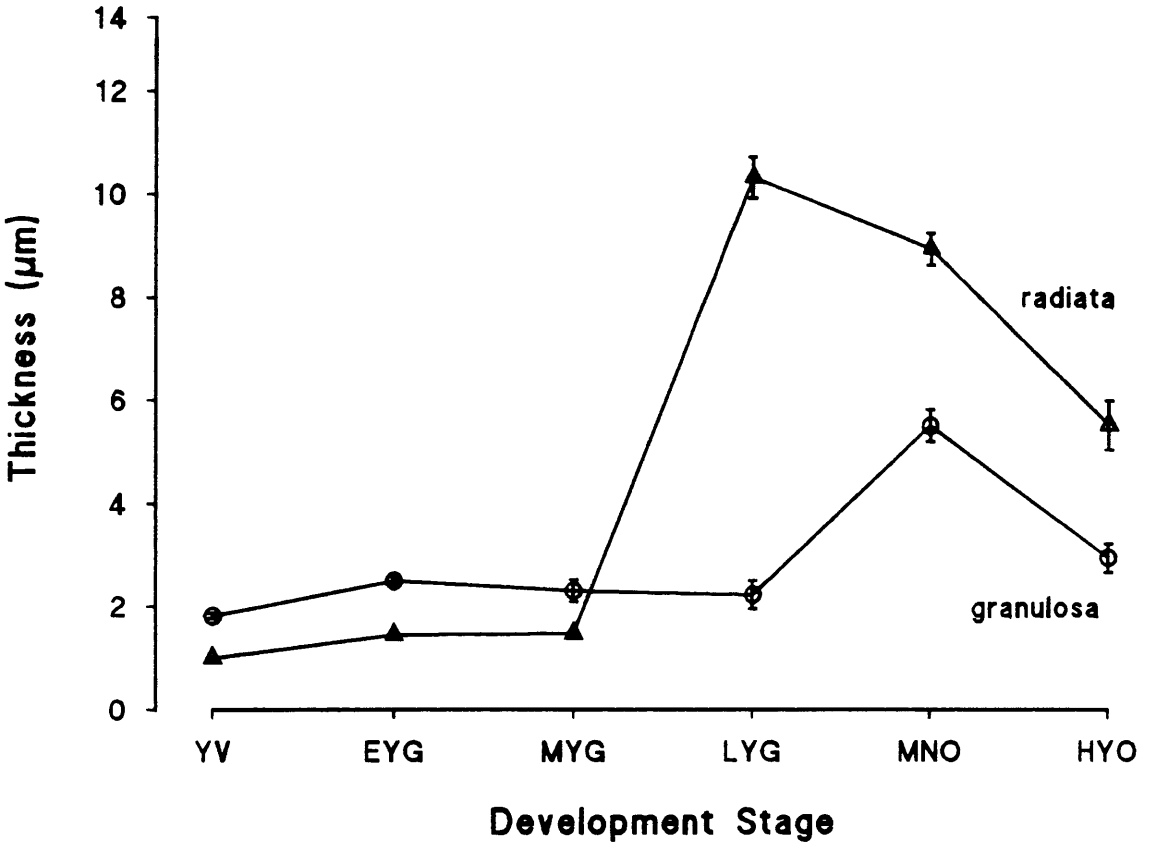


Figure 2.6– Thickness (mean±s.e.m.) of granulosa and radiata layers during different stages of oocyte development in *S. canaliculatus*. YV=yolk vesicle stage, EYG=early yolk granule, MYG=mid yolk granule, LYG=late yolk granule, MNO=migratory nucleus, HYO=hydrated oocyte. Sample number=10.

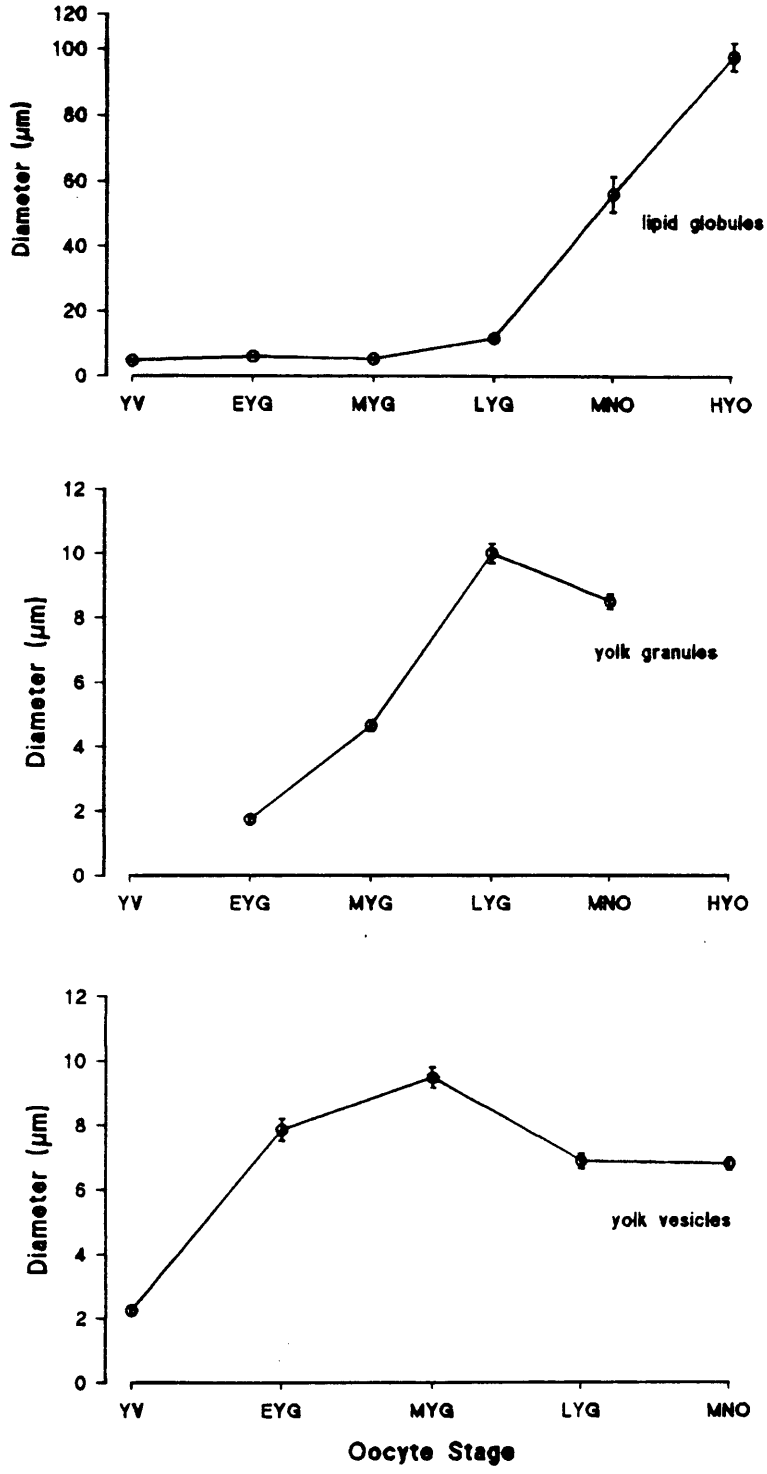


Figure 2.7– Diameter (mean±s.e.m.) of oocyte granules during different stages of vitellogenic oocyte development in *S. canaliculatus*. YV=yolk vesicle stage, EYG=early yolk granule, MYG=mid yolk granule, LYG=late yolk granule, MNO=migratory nucleus stage. Sample number is 20.

many small scattered nucleoli. The cytoplasm and nucleoli are strongly basophilic. During this stage the first appearance of Balbiani's vitelline body was observed, it appeared as a faint less basophilic zone in the cytoplasm near the nucleus.

4. Late Perinucleolus Stage [Fig.2.8(d)]

The oocyte diameter slightly increases to 45-145 μm (Table 2.8). The cytoplasm now appears less basophilic, and the large nucleus contains many small peripheral nucleoli, most of which are flattened against the nuclear membrane.

5. Yolk Vesicle Stage [Fig.2.8(e&f)]

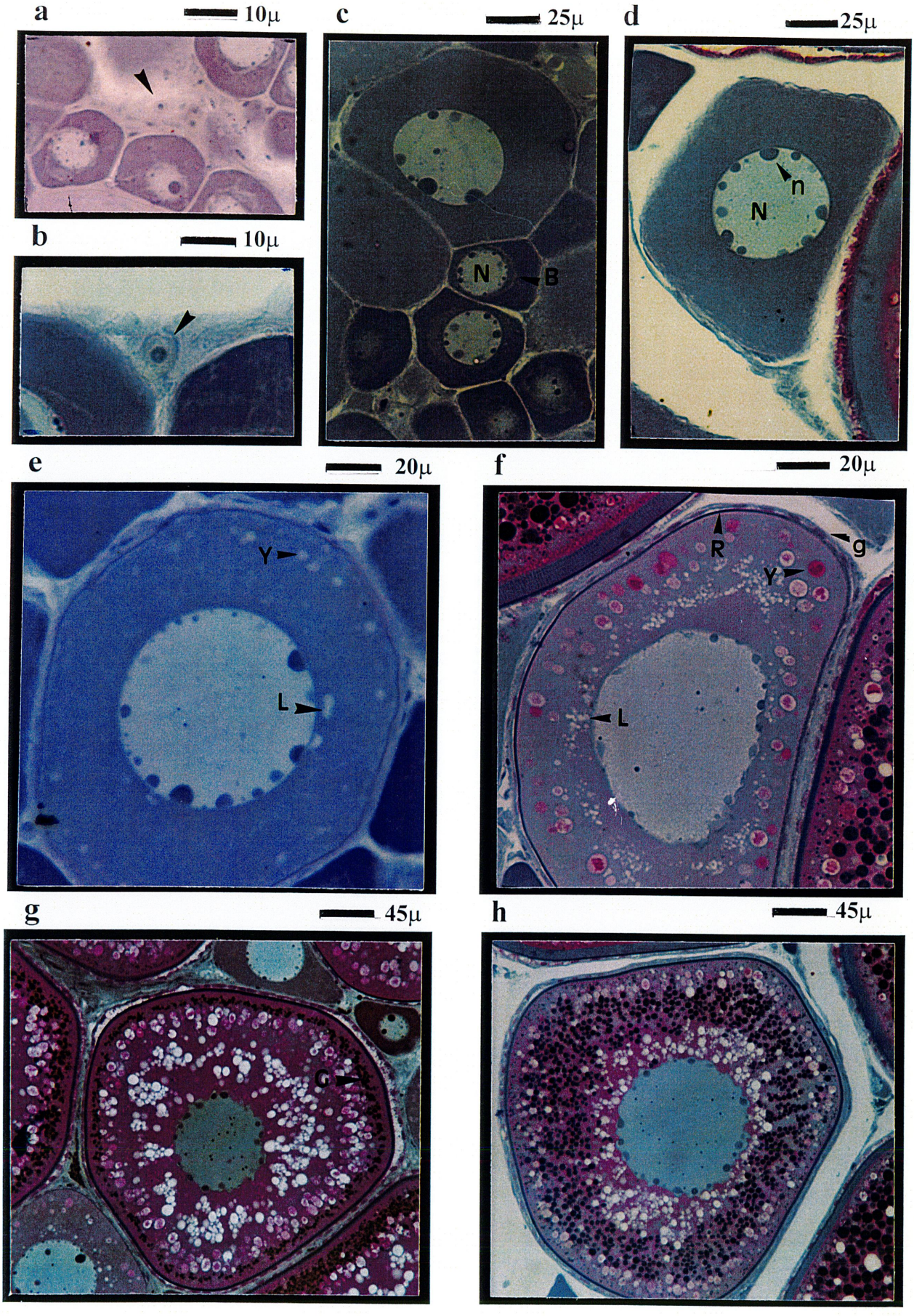
Cell diameter is 100-170 μm (Table 2.8). Transparent colourless cortical alveoli (yolk vesicles) appear at the periphery of the oocyte and increase in number as they move peripherally. Soon after the appearance of the first unstained yolk vesicles, oil droplets appear around the nucleus, later both inclusions start to increase in number in their respective areas, leaving an empty band of cytoplasm in-between. Later in this stage, both yolk vesicles and oil droplets increase in size by coalescence and disperse throughout the cytoplasm, the yolk vesicles reaching a maximum diameter of 10 microns, and becoming strongly PAS positive, staining deep pink. The lipid globules reach diameters of up to 6 microns. A thin granulosa layer is barely visible early in this stage, but becomes evident later with a thickness of 1.2-2.4 μm. The zona radiata is 0.8-1.2 μm thick and is not distinguishable into radiata interna and radiata externa at this stage (Table 2.8).

6. Early Yolk Granule Stage [Fig.2.8(g)]

Exogenous vitellogenesis starts with the deposition of acidophilic yolk granules at the periphery of the oocyte which stain dark green to bluish green with bromophenol blue. The granules enlarge by fusion and with the increase in their number and size, the diameter of the early yolk granule oocyte (EYG) steadily increase (125-280 μm, Table 2.8). The diameter of

Figure 2.8 - Folllicular development in *Siganus canaliculatus*. Polychrome stain.

[a] - Oogonia nest (arrow).
[b] - Chromatin-nucloelus stage (arrow).
[c] - Early perinucloelus stage- first appearance of balbiani's vitelline body (B), N=nucleus).
[d] - Late perinucleolus stage (N=nucleus, n=nucleolus).
[e] - Early yolk-vesicle stage- first appearance of lipid globule (L) and yolk vesicle (Y)
[f] - Late yolk-vesicle stage- (L=lipid globule, Y=yolk vesicle, g =granulosa, R=radiata).
[g] - Early yolk-granule stage- first appearance of exogenous yolk granules (G).
[h] - Mid yolk-granule stage.


a
10μ
b
10μ
c
25μ
N
B
d
25μ
n
N
e
20μ
Y
L
f
20μ
R
g
Y
L
g
45μ
C
h
45μ

yolk granules increases up to 3 μm, and lipid droplets to 3-9 μm. The radiata shows a slight increase to 1.1-1.9 μm, and the granulosa thickness is 2-2.8 μm (Table 2.8).

7. Mid Yolk Granule Stage [Fig.2.8(h)]

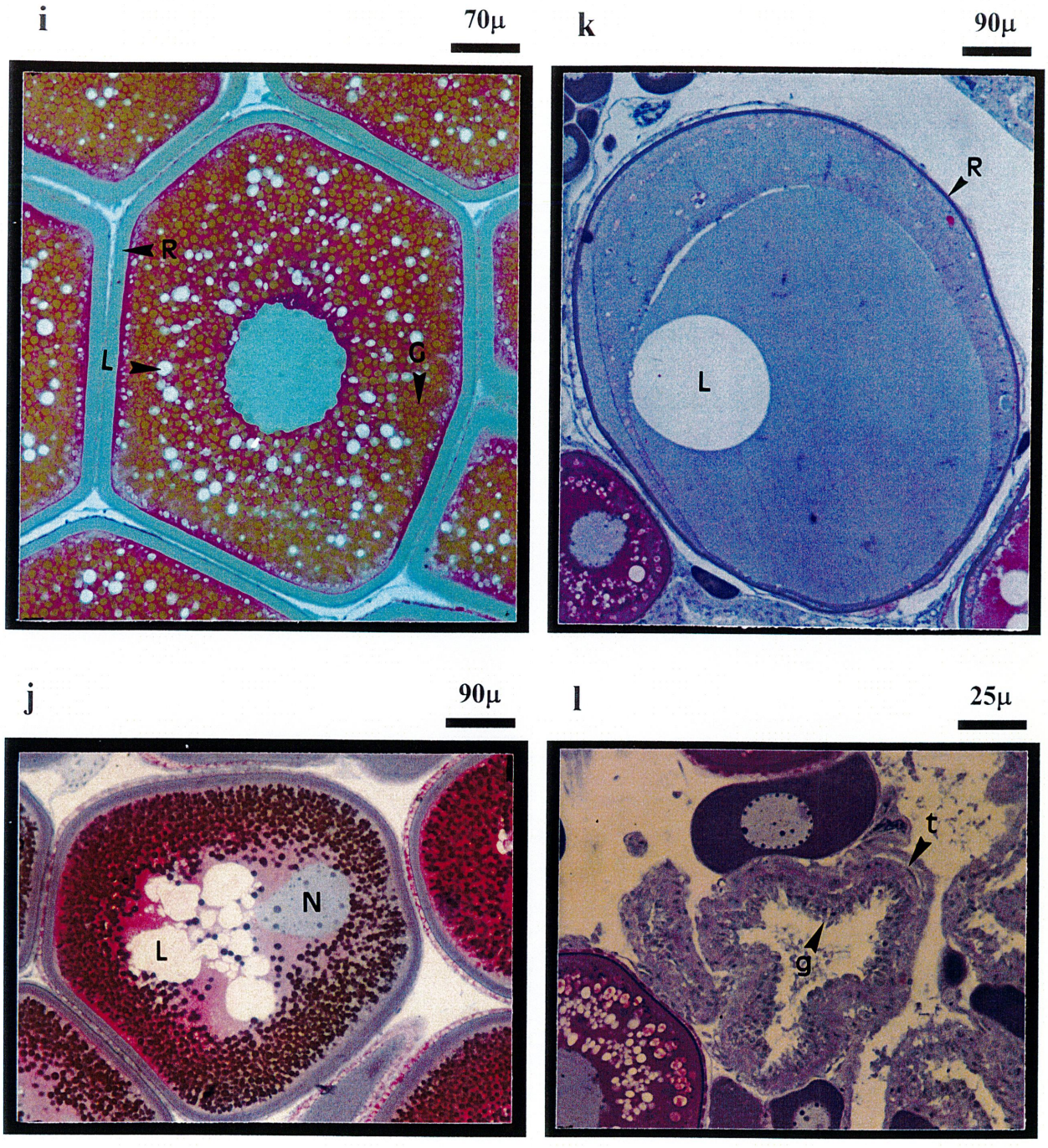
Yolk granules continue to develop at the periphery, while existing larger granules move centripedally and come to fill the middle zone of the cytoplasm. The diameter of the mid yolk granule (MYG) oocyte shows no significant increase (Table 2.8). The yolk granules diameter increase to 6 μm. Yolk vesicles and lipid droplets show no significant change, and neither do the granulosa or radiata (Table 2.8).

8. Late Yolk Granule Stage [Fig.2.8(i)]

This stage marks the completion of vitellogenesis. The oocyte diameter is 370-500 μm (Table 2.8). The yolk granules, now up to 12 μm diameter, almost completely fill the cytoplasm, and indent against the nuclear membrane, giving it a wavy appearance. Yolk vesicles are confined to a thin layer adjacent to the plasma membrane. Lipid globules are conspicuous, and grow by fusion up to diameters of 18 μm. The radiata interna reaches its maximum thickness of 16.8 μm whereas the radiata externa, although visible, remains less than 1 μm in thickness. The granulosa shows no change in thickness (Table 2.8).

9. Migratory Nucleus Stage [Fig.2.8(j)]

The remaining oocyte development stages are based on observations from fish induced into maturation by injection of maturation-inducing doses of hCG (Human Chorionic Gonadotropin). Wild fish in this stage are rarely caught, only two were encountered in wild samples and showed no histological differences from the ones described. 24 hours after the injection of hCG, the nucleus begins moving towards the animal pole. At this stage, oocyte diameters show no significant increase (460-510 μm, Table 2.8). Yolk granules still show some fusion, as evidenced from the dumbbell-shaped granules. Lipid droplets coalesce into a smaller number of large globules, some reaching diameters of 120 μm. The radiata interna


i
70µ
R
L
G
k
90µ
R
L
j
90µ
N
L
l
25µ
t
g

and externa show no significant increase in thickness, but the granulosa increases in thickness (3-8.4 μm, Table 2.8). This process may take up to 36 hours, at the end of which the nuclear envelope disintegrates (germinal vesicle breakdown, GVBD). This latter change, which may take less than 24 hours, was not observed.

10. Oocyte Hydration [Fig.2.8(k)]

The process of fluid uptake into the oocyte causes a significant increase in the diameter of the oocyte, up to 530-600 μm, and consequent thinning of the follicular envelope (Table 2.8). Coalescence of yolk granules continues until there is a single homogenous mass of featureless yolk, and a peripheral zone of cytoplasm with yolk vesicles and small numbers of large lipid globules. The hydration-induced increase in oocyte diameter may cause the observed thinning of the granulosa and radiata interna (Table 2.8). The hydrated oocyte (HYO), which can now be called an egg, is now ready for ovulation.

11. Post-Ovulatory Follicle [Fig.2.8(l)]

After ovulation, the collapsed follicular wall can be seen as convoluted bilayer of theca and granulosa (Post-Ovulatory Follicle, POF). The fate of the POF is considered later. Macroscopic appearance of the POF ovary shows an increased vascular supply. POFs were observed in post-spawning females only and disappeared in less than one month.

2.3.4 Follicular Atresia:

In healthy fish, follicular atresia in previtellogenic oocytes was observed throughout the year. In oocytes at the later stages of development, atresia was less frequently observed except in post-spawning ovaries (Fig.2.9) and in ovaries of laboratory-stressed fish (Chapter 3), and was not restricted to one particular stage of development. Resorption of previtellogenic and hydrated oocytes is less complicated than that of other stages. In fully vitellogenic ovaries, atresia does not affect all stages of oocytes simultaneously, instead, oocytes in the late yolk granule stage usually being the first to undergo atresia.

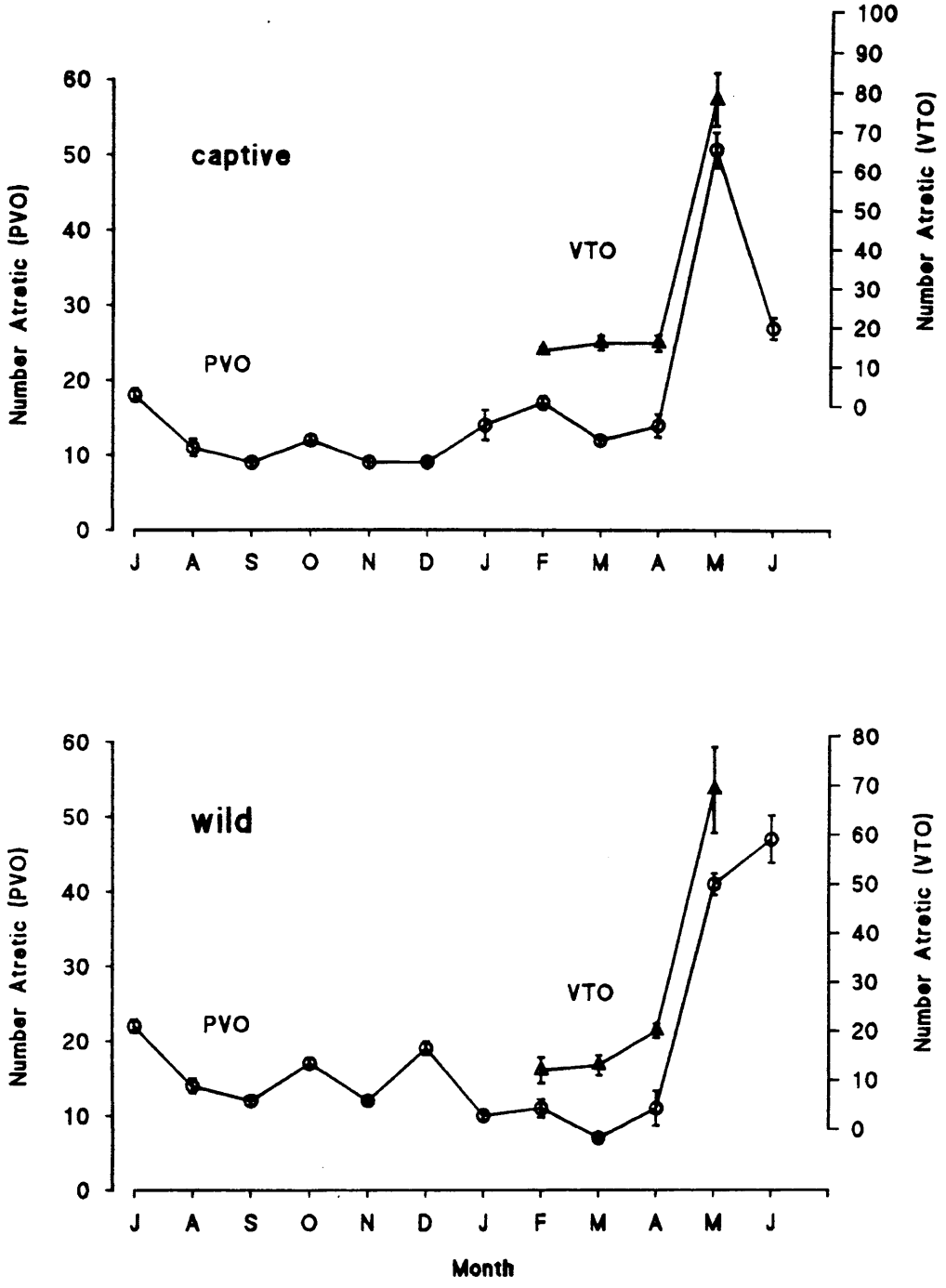


Figure 2.9– Number (mean±s.e.m.) of atretic previtellogenic oocytes (PVO) and vitellogenic oocytes (VTO) in the ovary of wild and captive *S. canaliculatus*. Measurements were done on replicate samples of 100 oocytes from three ovaries from each group.

1. Pre-Vitellogenic Oocytes (PVO):

Early signs of atresia in PVO is the textured appearance of the cytoplasm with hair-line cracks, possibly due to shrinkage of the now less strongly basophilic cytoplasm [Fig.2.10(a)]. The cracks widen and the cytoplasm now appears in the from of chunks, although the nucleus is still intact [Fig.2.10(b)]. Later the nucleus also shrinks and disintegrates, the theca, if present, holds together the pieces of cytoplasm [Fig.2.10(c)].

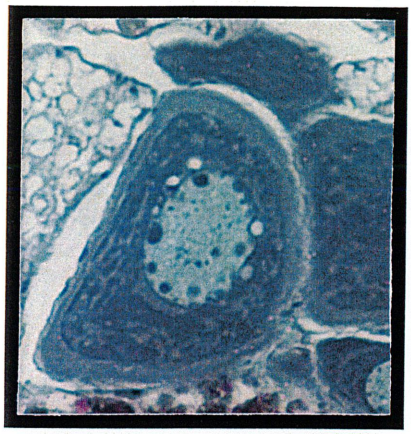
2. Yolk Vesicle Oocytes (YVO):

In early yolk vesicle oocytes, early indication of atresia is the enlargement of the granulosa layer followed by texturing of the cytoplasm [Fig.2.11(a)]. In the last stages, the entire contents of the oocyte, including the nucleus, become a single amorphous mass [Fig.2.11(b)]. In late yolk vesicle oocytes, early atresia may also start with the enlargement of the granulosa. Later, [Fig.2.11(c)] the cytoplasm becomes divided into two zones, the inner one around the nucleus is clear and almost devoid of any yolk inclusions except for a few lipid droplets, whereas the outer zone is dense, containing yolk vesicles and lipid droplets. The nucleus disintegrates, the yolk vesicles begin to breakdown and the lipid droplets coalesce into disorganized lumps. The whole mass of yolk vesicles and lipid droplets are later incorporated into a single featureless mass, whereas the granulosa and the radiata remain intact [Fig.2.11(d)]. In the last stage, the radiata separates from the granulosa and the whole thing collapses into the remains of the cell [Fig.2.11(e)].

3. Yolk Granule Oocytes (YGO):

Early and mid yolk granule oocytes quickly advance into late yolk granule stage, so atresia was not fully observed. In these two stages, the radiata is not as prominent as in later stages. The first observed signs of atresia were the shrinking of the oocyte and disintegration of the nucleus and fading and breakdown of cytoplasm. However, in late yolk granule oocytes, atresia is very distinct. The first sign of atresia being the corrugation of the radiata interna, which may expand, on its inner side where it is in contact with the oocyte (Fig.2.12(a)]. The

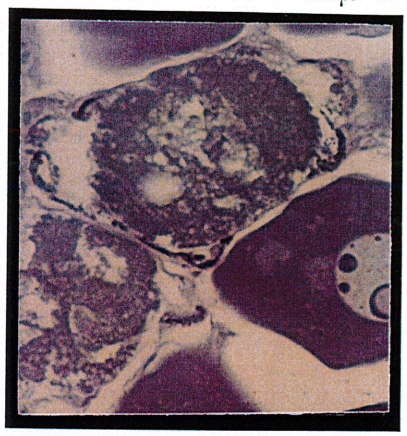
10a 25μ



10b 25μ



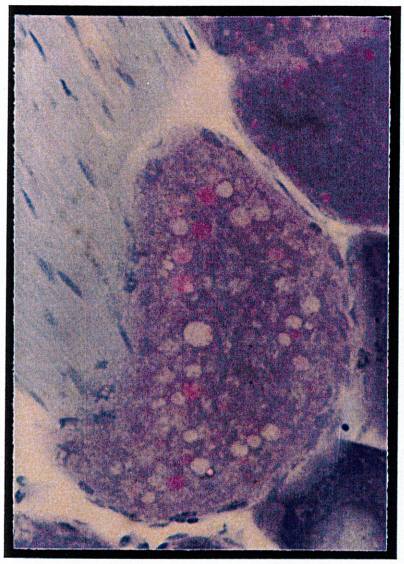
10c 25μ



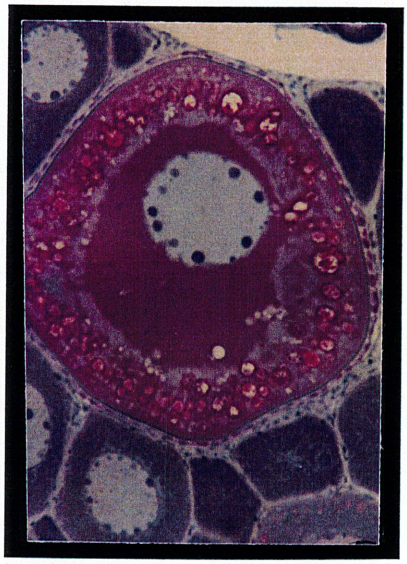
11a 25μ



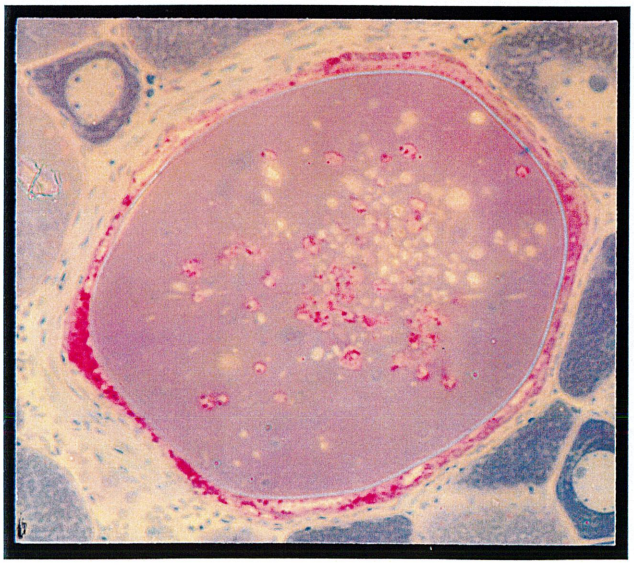
11b 25μ



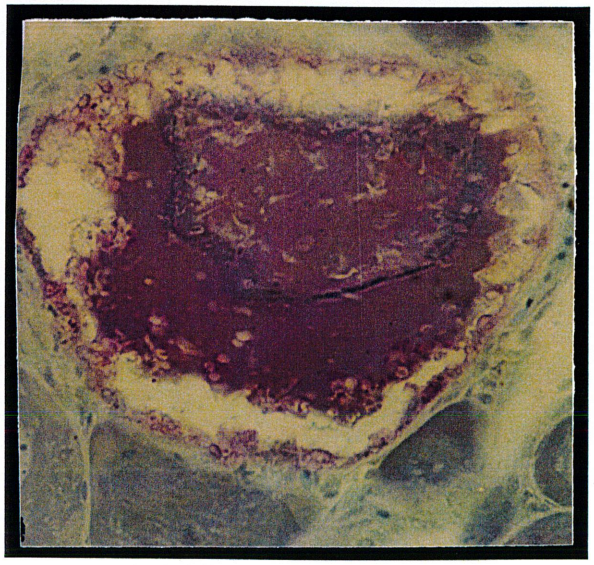
11c 40μ



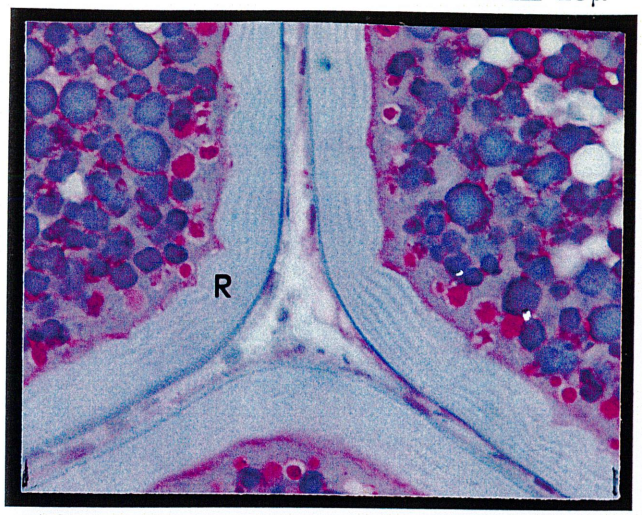
11d 30μ



11e 20μ



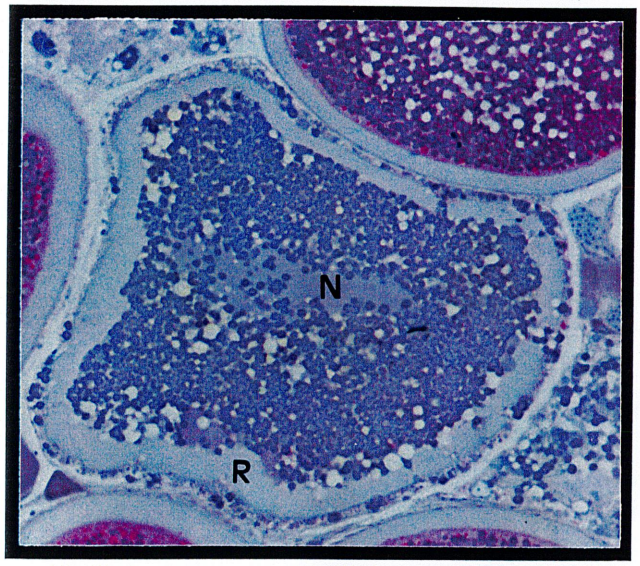
12a



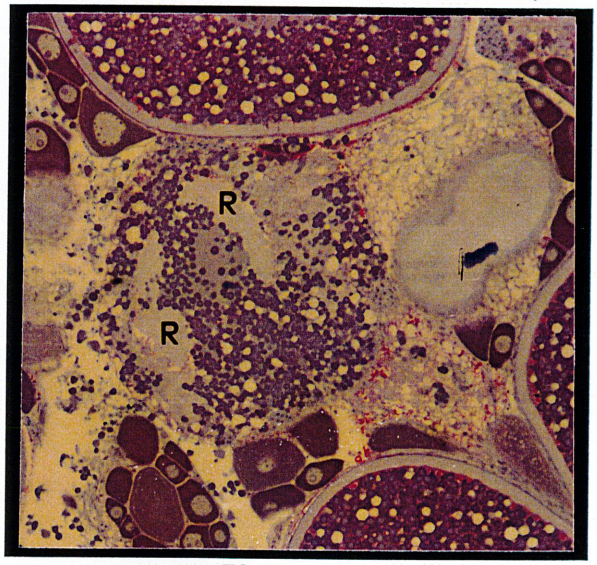
12b



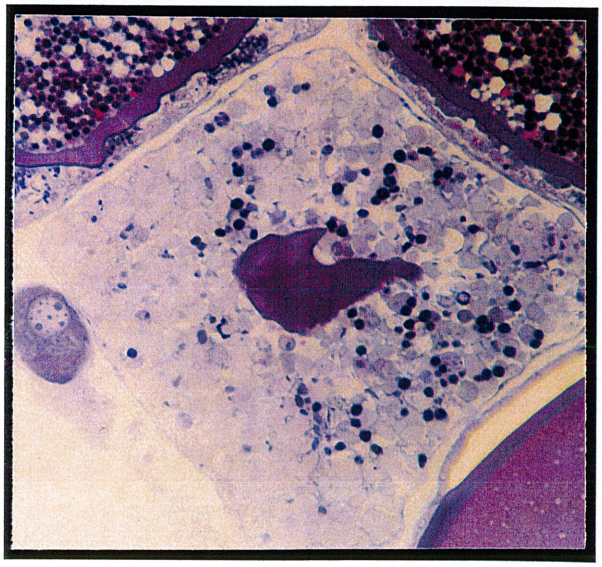
12c



12d



12e



radiata interna develops fissures at several points, and the oocyte may shrink, causing the zona radiata to separate from the granulosa layer, the radiata externa remaining intact [Fig.2.12(b)]. Shrinking of the oocyte may start earlier. The entire zona radiata fractures at several points and the contents of the oocyte leak into the follicular envelope, although the theca remains intact and contains the leakage [Fig.2.12(c)]. The nuclear membrane soon begins to disintegrate and the nucleus slowly disappears, by this time the zona radiata has broken into pieces and collapsed into the oocyte, granulosa cells separate and fall into the mixture of pieces of radiata and yolk granules, although the theca is still intact [Fig.2.12(d)]. Following this the yolk granules and yolk vesicles begin to break down and coalesce into lumps forming an amorphous mass [Fig.2.12(e)].

4. Migratory Nucleus Oocyte (MNO):

Early in this stage the nuclear migration may be arrested. Shrinkage of the oocyte may cause the separation of radiata from the granulosa [Fig.2.13(a)]. The oocyte continues to shrink, leaving large lacunae of PAS positive material between oocyte and radiata [Fig.2.13(b)]. Lipid globules stay intact for longer, but eventually break down and mix with the amorphous mass of other cell contents [Fig.2.13(c)], very similar to what happens to yolk granule oocytes.

5. Hydrated Oocyte (HYO):

The atresia of hydrated oocytes is seldom observed. The process is quick and distinct atretic stages are difficult to distinguish. The early sign of an atretic hydrated oocyte is the shrinking of the cytoplasm, causing it to separate from the radiata interna [Fig.2.14(a)]. The radiata remains evident until later, but eventually begins to disintegrate also, lipid droplets break down and disappear leaving a collapsed homogenous mass held together by the remaining follicular envelope [Fig.2.14(b)]. Later stages have not been observed and it is assumed that since the oocyte contents are already broken down and dispersed in the cytoplasm, the removal of cell remains is considerably faster than in vitellogenic oocytes.

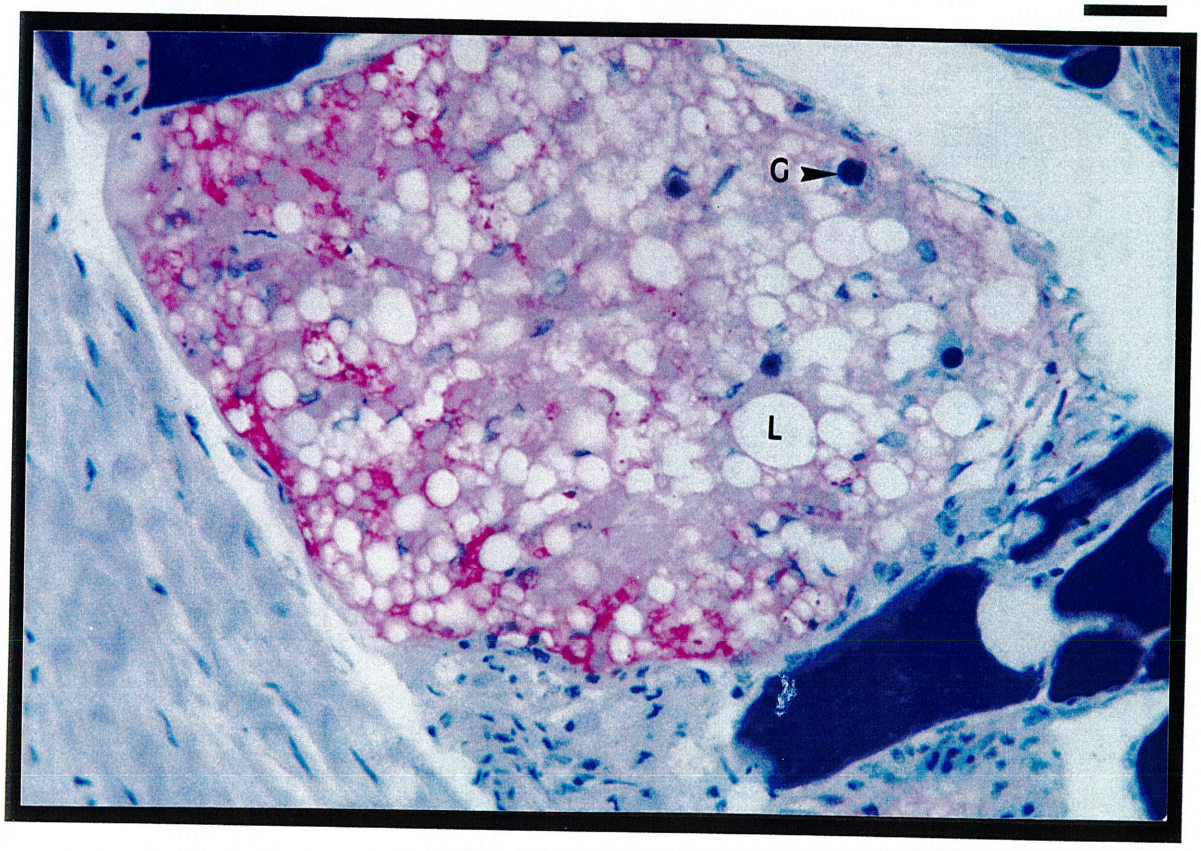
13a



13b



13c



14a

90µ



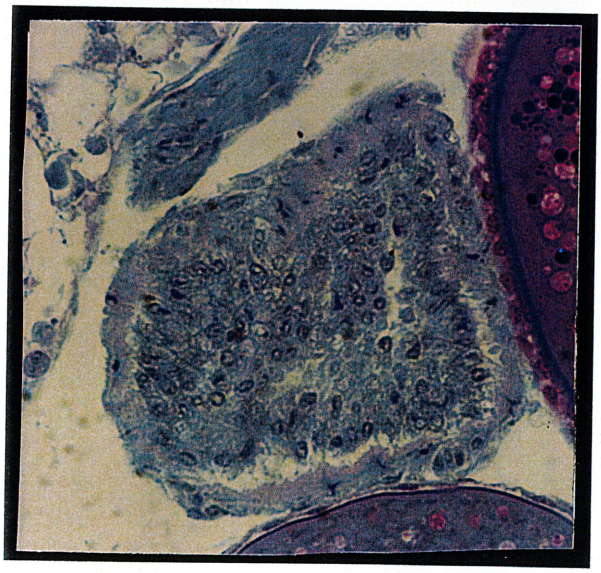
14b

90µ



2.15

25µ



6. Post-Ovulatory Follicle (POF):

Breakdown and resorption of the post-ovulatory follicles was not very distinct. The granulosa layer disintegrates and the cells separate, "falling" into the cavity of the follicle. The theca and granulosa become mixed into an amorphous mass [Fig.2.15]. Later stages were not observed.

2.3.5 The Ovarian Cycle in *S. canaliculatus*:

A similar ovarian cycle of growth and maturation is observed both in virgin females in their first spawning season and in adult females in their second or later seasons. Virgin and immature resting gonads were found predominantly from early summer in late May, about one month after the spawning season, until late winter in January. Primary oocytes were present in all months, their frequency declining from January to a minimum in maturing ovaries in the breeding season in April (Table 2.9a&b). Partial spawning was observed many times in captive induced fish, but only in one instance in wild fish.

Vitellogenic ovaries can be identified by the ability to biopsy the fish. Biopsy could only be carried out in fishes that have started the process of vitellogenesis, insertion of the cannula is not possible in previtellogenic ovaries but in advanced vitellogenic ovaries this process is much easier. The reason may be because of the small size of the early developing ovary or it may possibly indicate that the ovary lobes do not have ovipores opening into the common oviduct. If release of eggs occurs by rupture of the connective tissue, such rupture is only possible when vitellogenesis starts, which involves the increase in the size of the oocytes and thus the thinning of the ovary wall and other related tissues. Females that have completed vitellogenesis or advanced through maturation also show an enlarged genital papilla, which swells outwards with gentle pressure on the abdomen. The annual cycle of development of the ovary can be divided into eight stages based on histological criteria, macroscopic appearance of the ovary, gonadosomatic indices (GSI) and abdominal condition. These stages correspond approximately to particular months of the year (Table 2.10). The color of the ovary and the condition of the abdomen change with the stage of ovary development. From June to January the ovary is in a resting stage, barely visible and opaque in color. The lower part of the

Table 2.9a Percentage occurrence of oocytes at previtellogenic and vitellogenic development stages during one annual reproductive cycle of wild *Siganus canaliculatus*.

Month	Percentage Occurrence						N
	EPN	LPN	EYV	EYG	MYG	LYG	
Jul	100						100
Aug	100						100
Sep	100						125
Oct	100						120
Nov	100						129
Dec	87	13					168
Jan	58	38	4				157
Feb	50	10	40				138
Mar	31	13	35	15	6		155
Apr	24	16	8	11	3	38	101
May	70	17		3	3	7	220
Jun	100						100

N = Total number of oocytes counted.

Table 2.9b Percentage occurrence of oocytes in various stages of development in a mature ovary of *Siganus canaliculatus,* observed at hourly intervals after hormone injection. The fish is spent at 72 hours.

Hours	Percentage Occurrence									N
	EPN	LPN	EYV	EYG	MYG	LYG	MNO	HYO	POF	
0	37	18	3	3	5	34				206
16	38	23		3	1	35				216
28	44	15		7	2	28	4			279
40	41	17		1		22	19			82
60	22	10				2	4	62		110
72	60	21			1	2		1	15	138

EPN=Early Perinucleolus, LPN=Late Perinucleolus, EYV=Early yolk Vesicle, EYG=Early Yolk Granule, MYG=Mid Yolk Granule, LYG=Late Yolk Granule. MNO=Migratory Nucleus, HYO=Hydrated Oocyte, POF=Post-Ovulatory Follicle. N = Total number of oocytes counted.

Table 2.10 Description of gonad development stages for female *Siganus analiculatus* in Bahrain. Macroscopic appearance refers to fresh ovaries. Gonad stages VI, VII, and VIII are in fish induced to spawn.

Stage	General Terminolgy	Histological Stage	Histological Description	Macroscopic Description	Month
I	Virgin or resting	Early perinucleolus oocytes (EPN)	Mainly early perinucleolus oocytes. Well spaced ovigerous folds orientated towards ovary center, containing both oogonia and chromatin nucleolus oocytes.	Small thread-like ovary, translucent and smooth, GSI less than 0.5%. Hard abdomen	Jun-Jan.
II	Early developing	Late perinucleolus (LPN) and early yolk vesicle (EYV) oocytes	Late perinucleolus oocytes develop, secondary growth phase started in some of the larger ones with the appearance of peripheral cortical alveoli. Small lipid droplets also appear in early yolk vesicle oocytes near the nucleus. Zona radiata visible. Spaces still present between the ovigerous folds.	Ovary starting to take a tubular shape, reddish opaque in colour. GSI 0.7-1.6%. Hard abdomen.	Feb.
III	Mid-developing	Late yolk vesicle (LYV) and early yolk granule (EYG) oocytes	Yolk vesicles larger and fully dispersed. Small protein yolk granules appear first at the periphery. Granulosa evident. Ovigerous folds start filling the cavity.	Ovary pinkish white in colour beginning to show rapid increase in weight. GSI 2.9-6.3%. Thin abdomen.	Early March
IV	Late developing	Mid yolk granule (MYG) oocytes	Yolk granules increase in number and size moving towards the center reaching half-way at his stage.	The reddish tinge decreases and the ovary becomes whitish in colour but with visible superficial blood vessels. Ovary surface feels textured. Abdomen still thin.	Late March to April
V	Running ripe	Late yolk granule (LYG) oocytes	Oocyte fully vitellogenic. Yolk granules densely fill the cytoplasm, with a granule diameter reaching 18 microns. Lipid droplets well dispersed. Zona radiata distinguishable into interna and externa. Interna at its thickest reaching 15 microns.	Ovary colour is creamy white, surface is highly textured. GSI 6.2-24.3%. Abdomen becoming softer to the touch.	April
VI	Maturing	Migratory nucleus oocytes (MNO)	Nucleus (germinal vesicle) appears off center with varying positions as it moves towards the periphery. Lipid droplets coalesce into fewer but larger globules, some measuring 120 microns in diameter At end of this stage germinal vesicle breaks down (GVBD).	No significant change in appearance or weight of ovary. Abdomen soft and becoming fuller.	April
VII	Ready to spawn	Hydrated oocyte (HYO)	Oocyte contents appear as a homogenous mass clear of any inclusions, except yolk globules which at this stage are few but large. Sometimes with some inclusions in the process of liquefaction.	Ovary very swollen, opaque with a golden yellow tinge, surface is stretched thin to a smooth finish. Larger hyaline eggs visible and ooze out with gentle pressure. GSI can reach up to 30%. Distended Abdomen.	April to early May
VIII	Spent or resorbing	Post-ovulatory follicle (POF)	Irregular convoluted ovigerous folds containing oocytes in all stages,, but very few stage V oocytes . Many empty follicles consisting of residual follicle layers. Some signs of early atresia.	Ovary slack, reddish opaque and vascular. Thick ovary wall. GSI drops sharply to 1.3-4.5 %. Abdomen feels soft and empty.	April to mid May

abdomen, where the ovary is located, is thin with both sides of the abdomen pressed against each other, making it hard to the touch. It is only in February that a change is seen, the ovary becomes reddish opaque due to increased supply of nutrient-carrying blood to the ovary. At this stage the abdomen begins to soften, due to increased size of the ovary. As vitellogenesis progresses and the ovary increases in size, the color changes to yellowish white and then to creamy white at the end of vitellogenesis, when the ovary surface acquires a textured appearance with blood vessels running across it. At this time the abdomen is soft and full. During maturation and ovulation, the only significant change in ovary appearance is after hydration, when there is a distinct increase in size. The ovary wall becomes thinner as it is stretched, with the increase in size giving the ovary a translucent appearance, also indicating the increased aqueous content. The abdomen at his stage is distended and the slightest pressure make eggs ooze out from the genital aperture. After spawning the ovary size decreases considerably and takes on a reddish colour, due to the abundant blood supply now used in the mobilization and removal of ovarian debris, prior to the return to resting state.

2.3.6 Seasonal Changes in Levels of Oestradiol (E_2) :

In wild fish, post-spawning serum E_2 levels from July to November remain at levels below 0.15 ng ml^{-1} with no significant differences ($p>0.05$). In December there is a significant elevation of E_2 levels to a mean of 0.38 ± 0.65 ng ml^{-1} ($p<0.05$). From December there is a steady increase to a peak level in April (mean=1.09 ± 0.357 ng ml^{-1}). Between May and June, E_2 levels decrease to the post-spawning levels seen from July to November (Fig.2.16). A similar E_2 profile was observed in captive fish (Fig.2.17).

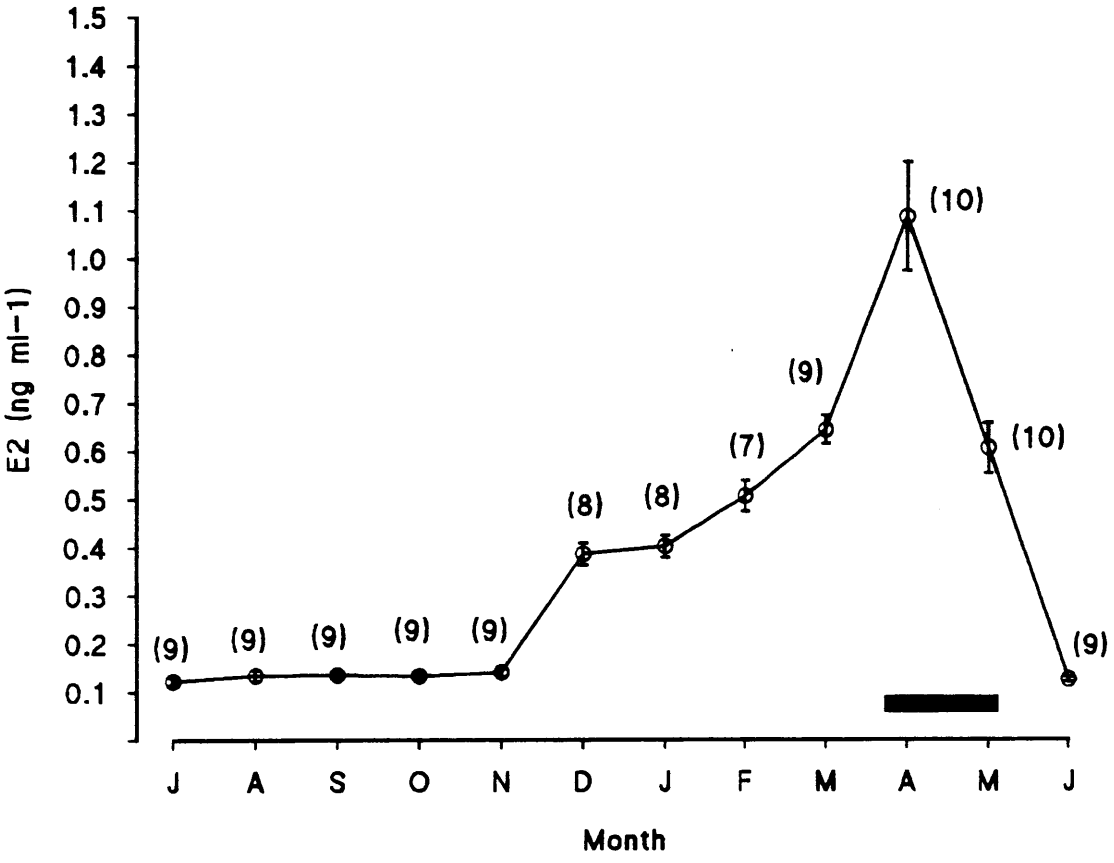
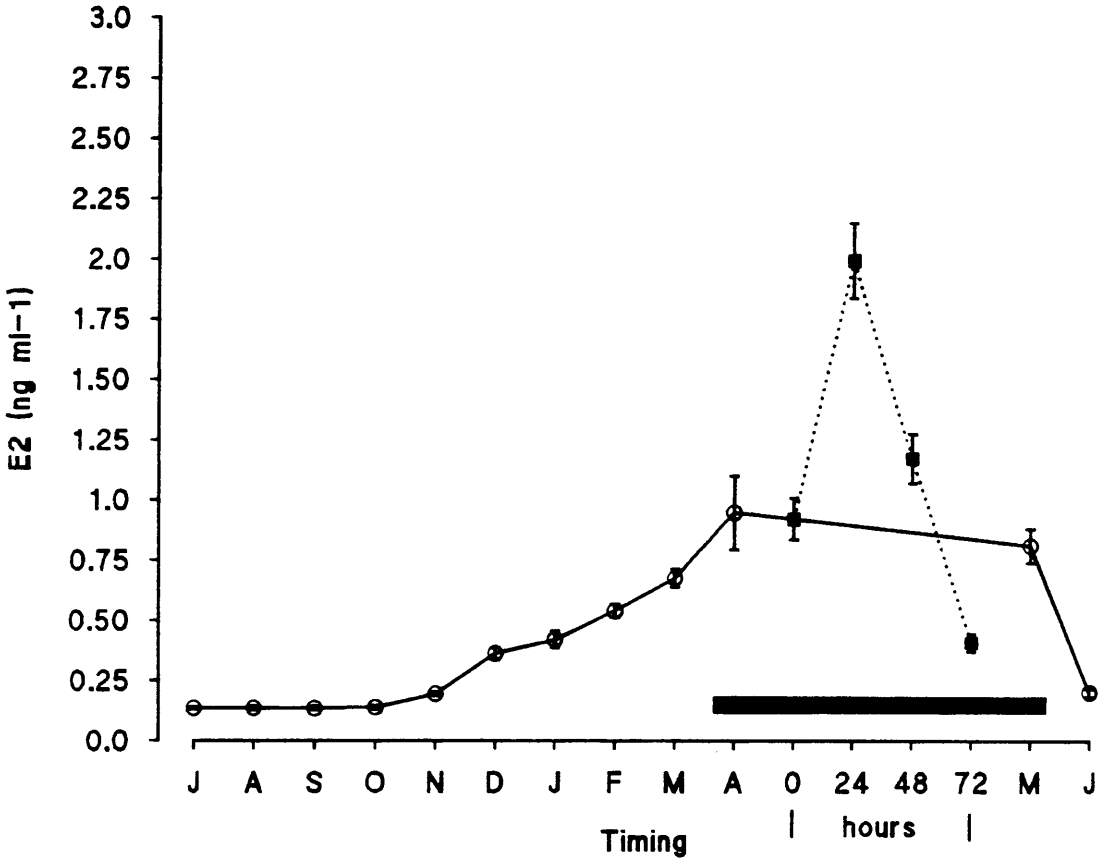


Figure 2.16 Monthly serum oestradiol levels (mean±s.e.m.) in wild female *S. canaliculatus*. Numbers on data points indicate sample size. Bar on the X axis indicates the natural spawning season.



Figure– 2.17 Monthly serum oestradiol levels (mean±s.e.m.) in captive *S. canaliculatus.* Solid square data points indicate levels in fish induced into maturation by HCG injection during the normal season of maturation and spawning (indicated by the bar on the X axis). Sample number is nine.

2.4 Discussion:

2.4.1 Pattern of Oocyte Growth in *Siganus canaliculatus*:

The pattern and progress of oocyte growth in *S. canaliculatus* is broadly similar to that seen in other group synchronous spawners (**Yamamoto, 1956**). They could be placed into one of eight development stages corresponding to previtellogenesis, vitellogenesis, and maturation phases (Table 2.10). The first stage of oocyte development (early perinucleolus) was present throughout the year, oogonia and chromatin nucleolus oocytes were noticeable just after spawning. The number of oocytes in chromatin nucleolus stage was relatively low, possibly indicating a short life span of such oocytes before advancing into early perinucleolus stage. Such cyclic accelerated transformation from secondary oogonia to primary oocytes (oogenesis) soon after spawning is common in most teleosts (**Bromage & Cumaranatunga, 1988**), but cases of occurrence of oogonia and oocytes in later stages of development throughout all phases of ovarian development has also been reported, as in the flounder *Pleuronectes flesus* (**Janssen et al., 1995**). The development of oocytes is arrested for several months in early perinucleolus stage, the initiation of a new generation of developing oocytes begins with the transformation from early into late perinucleolus stage oocytes in early February.

As reported for the majority of teleost species studied (**Khoo, 1979; Wallace and Selman, 1981; de Vlaming, 1983; Selman and Wallace, 1989**), the first type of yolk inclusions to accumulate in developing oocytes are the PAS positive yolk vesicles, the appearance of which can be considered to mark the start of endogenous vitellogenesis. Yolk vesicles develop at sites in the outer cortex. Next to appear is the lipid yolk in the form of distinct colourless lipid droplets first appearing in the perinuclear cytoplasm. The two processes occur with so little variation in timing that they may be mistaken as simultaneous. After the appearance and spread of endogenous yolk inclusions, the incorporation of exogenous yolk in the form of yolk granules takes place. The zona radiata, which becomes prominent in late secondary oocytes, exhibits a bipartite structure, the zona radiata interna and zona radiata externa. Both the

exogenous yolk granules and zona radiata stain strongly for proteins. The start of the process of vitellogenesis coincides with a significant rise in serum oestradiol levels (Fig.2.16).

Resting ovaries of *S. canaliculatus* show the presence of only oocytes in the previtellogenic stage of development. Therefore, the presence of oocytes in early yolk vesicle stage can be a definitive stage for distinguishing a developing ovary from a resting one. Few oocytes in the mid-yolk granule stage can be seen, indicating that this stage is short and that oocytes advance rapidly to the late yolk granule stage by rapid uptake of vitellogenin.

As a whole, the development of oocytes in the rabbitfish is a group synchronous process. In a developing ovary two populations of oocytes can be distinguished, one 'clutch' of fairly synchronous and dominant larger oocytes and a population of smaller oocytes from which recruitment is done, a situation that is most common among teleosts **(Wallace & Selman, 1981)** and also reported in another rabbitfish species, *S. sutor* **(Ntiba & Jaccarini, 1992)**. The occurrence of post-ovulatory follicles only in spent females and none in maturing ovaries indicate that release of eggs is done in one batch (complete spawning) in a single or multiple very closely timed actions, a feature of group synchronous development **(Howell, 1983)**.

Histological examination of samples from the wild indicated the presence of vitellogenic oocytes only in the period from February till post spawning season in May. This proves histologically that the fish is an annual spawner, with one spawning season starting in late March and ending in early May with slight variations depending on water temperature and phase of the moon. A similar conclusion, but based on more general criteria such as gonadosomatic indices, was reported for the same species in earlier reports from the same geographical area (**Aradi & Shams, 1993**), and also in the adjacent region (**Al-Ghais, 1993**). The duration of endogenous and exogenous vitellogenesis is generally similar, each is of short duration, lasting less than one month. This may be explained by the short spawning season (1-1.5 month).

The process of maturation and spawning was studied in fish induced to spawn with exogenous Human chorionic gonadotropin (hCG). The whole process is short and can be completed in

about 72 hours. It is marked by the migration of the nucleus (germinal vesicle), which starts about 24-28 hours after the surge of hCG. Hydration of the oocyte starts about 60 hours after injection. No female S. canaliculatus accomplished maturation and ovulation in captivity. Trials to trigger spawning by water change and provision of substrate were not successful. Natural spawning of captive *S. canaliculatus* has been reported and it has been suggested that tidal level is the most important factor involved **(McVey, 1972)**. The failure of fish to reach the final stages of gonad maturation has also been reported for cultured seabream *Diplodus sargus* (**Micale & Perdichizzi, 1994**), and is not uncommon in captive populations, which very often need hormonal stimulation to spawn (**Gordin & Zohar, 1978; Lam, 1983; Goodall et al., 1987**).

2.4.2 Follicular Atresia:

In *Siganus canaliculatus,* follicular atresia in previtellogenic oocytes was observed throughout the year with low frequencies (<20%) in healthy ovaries during all stages of ovarian development before spawning, after which natural atresia takes place and the frequency of atretic follicles begins to increase (Fig.2.9&2.10), and was not restricted to one particular stage of development, thus suggesting it may represent a normal part of the reproductive biology of this fish. Resorption of previtellogenic and hydrated oocytes is less complicated than that of other stages. In fully vitellogenic ovaries, atresia does not affect all stages of oocytes simultaneously, oocytes in the late yolk granule stage usually being the first to undergo atresia.

The early indications of atresia differ between previtellogenic (PVO) and vitellogenic oocytes (VO). In PVO, signs of atresia begin within the oocyte itself, with breakdown of the cytoplasm, whereas in stages from yolk granule onwards, the earliest indications are observed in the follicular envelope, with the disintegration of the granulosa and radiata followed by the breakdown of oocyte inclusions. The findings of this study indicate that in *S. canaliculatus* atresia does not necessarily imply the end of spawning. Fish with relatively high levels of atresia may proceed into maturation and spawning, but with possible deleterious effects on egg and gamete quality (see Chapter 3). High levels of atresia typically have been used as

indicators to identify regressing ovaries, and for many teleosts have been described as representing a key histological marker for the cessation of spawning (**Hunter and Macewicz, 1985a&b; Hunter et al., 1986; Dickerson et al., 1992**). The function of resorbing of residual oocytes after spawning is to remove unwanted material, but the reason for pre-ovulatory degradation is not well understood (**Macer, 1974**). The triggering factors and mechanisms controlling atresia in individual oocytes are not clear. In some fish it has been related to environmental and dietary factors (**Hunter and Macewicz, 1985a**), while in mammals genetic and metabolic defects and shortage of nutrients have considered as causes for atresia (**Baker, 1982**). This may also apply to atresia of PVO, in fish where absence of pituitary hormones by itself was found not to cause atresia (**Dodd, 1955**), but appropriate hormonal stimuli was found to be necessary to sustain healthy vitellogenic oocytes (**Guraya, 1986**). In such case atresia in PVO could be a mechanism for the removal of defective cells.

It is generally considered that post-ovulatory follicles (POF) have a lifetime of a few days or weeks. This is true in *S. canaliculatus* where POF are rapidly reabsorbed and are found only for a period of 2-3 weeks only after spawning.

Chapter 3

Chapter 3

Effect of Handling Stressors on Ovarian Development and Maturation in *S. canaliculatus*

3.1 Introduction:

The definition of stress in biological systems has been the subject of considerable debate (**Pickering, 1993**). It has been defined as 'a state produced by any environmental factor which extends the normal adaptive responses of an animal or which disturbs the normal functioning to such an extent that the chances of survival are significantly reduced' (**Brett, 1958**). Such a strict physiological view of stress may not be holistic, as there are other behavioral, psychological or toxicological components to stress that apply to the manner by which the organism responds (**Schreck et al., 1997**). The term 'stress' refers to the response made by the animal to the environmental change which is known as the 'stressor', i.e. stressors cause stress. It is unfortunate that some biologists in the past have applied the term 'stress' to the environmental change and the term 'stress response' to the reaction of the animal.

Exposure to one or more stressors can provoke stress, which in the short term may manifest itself as changes in the secretory patterns of the hypothalamic-pituitary-adrenal axis (**Pottinger et al., 1991**). Over the longer term, stress can result in a range of damaging consequences to the fish, including stunted growth (**Pickering, 1990**) and immunosuppression (**Wiik et al., 1989**). Of particular relevance to the present study are the deleterious effects of stress on the function of the reproductive system (**Pickering et al., 1987a**).

In mammals the primary response to stressors involves the activation of two components of the neuroendocrine system, the adrenergic system and the pituitary-adrenal axis (**Selye, 1956**). These two pathways switch the animal's metabolism from an anabolic state of growth and

energy storage, to a catabolic state, in which protein and energy mobilization predominate. Such a shift in energy utilization is presumed to be of survival value in the natural environment (**Pickering, 1993**).

A similar response to most of environmental stressors can be seen in teleost fish in the form of rapid increases in the concentrations of plasma catecholamines (**Mazeaud & Mazeaud, 1981**), and corticosteroids (**Donaldson, 1981**). The former group of hormones are both protein-catabolic, hyperglycemic and lipolytic (**Larsson, 1973; Sheridan, 1987**), enhance branchial lamellar perfusion (**Booth, 1979**) and increase the oxygen affinity of the erythrocytes (**Nikinmaa, 1982**). The corticosteroids play an important role in enabling the fish to regain osmotic equilibrium with the environment during recovery from stress by promoting ion-transport (**Balm, 1986**). They also stimulate lipolysis (**Sheridan, 1986**) and protein catabolism. Neuroendocrine control of these two hormonal axes is likely to be interrelated (**Axelrod & Reisine, 1984**). Although the details of the response to different stressors may vary with the status of the fish, there is nevertheless a remarkable uniformity in the general pattern of the primary catecholamine and corticosteroid response (**Sumpter, 1997**).

Although plasma cortisol and catecholamines are often considered as primary stress indicators, other hormones, including growth hormone, prolactin, gonadotropins, and somatolactin, also respond to stressors (**Sumpter, 1997**). These primary hormonal indicators may induce major and widespread secondary responses, including changes in liver and muscle glycogen, and in blood parameters such as cell counts, haemoglobin level, plasma chloride and protein. Chronic long term stress may in time lead to gross tissue changes (**Barton, 1997**).

In general, stressors have an inhibitory effect on reproduction in fishes, such effects being detectable at various levels of the reproductive endocrine axis, on egg quality and survival, and on larval survival and development (**Pankhurst & Van Der Kraak, 1997**). A specific inhibitory effect on hypothalamic release of gonadotropin-releasing hormone (GnRH) and dopamine (DA), and on pituitary gonadotroph cells has still to be established. However, plasma GtH levels have been shown to respond to stressors in male brown trout (**Pickering et al., 1987a**) and in the white sucker (**Stacey et al., 1984**), by a mechanism still undescribed. At

the gonadal level, the effects are less ambiguous, with acute or chronic stressors resulting usually in depression of plasma levels of androgens and/or estrogens in most studied species. For example, acute handling and confinement of male brown trout for periods ranging from hours to one month caused depressed plasma levels of testosterone (T) and 11-ketotestosterone (11KT) (**Pickering et al., 1987a; Sumpter et al., 1987**). Female brown and male rainbow trout, *Oncorhynchus mykiss,* showed similar changes after a 2 weeks confinement, whereas female rainbows showed no changes in plasma gonadal steroids (**Pickering et al., 1987a**). In contrast, **Pankhurst & Dedual (1994)** found that female rainbow trout did respond to handling and confinement with reduced levels of plasma testosterone and oestradiol. Depression of plasma E_2 and T levels in response to stress has also been reported in non-salmonid fish like *Pagrus auratus* (**Carragher & Pankhurst, 1991**) and wild spotted sea trout, *Cynoscion nebulosus* (**Safford & Thomas, 1987**).

Not all gonadal steroid levels are depressed by stressors. Physical and chemical stressors which depress T and E_2 may either elevate the levels of maturational steroid 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP), as in female snapper, *Pagrus auratus* (**Carragher & Pankhurst, 1991**), and white sucker (**Van Der Kraak et al., 1992b; McMaster et al., 1994**), or have no effect as in wild rainbow trout (**Pankhurst & Dedual, 1994**). Such findings suggest that some steroid-converting enzymes, like $P450_{c17}$, which mediate the conversion of progestins to androgens, are more sensitive to stressors than others (**Van Der Kraak, 1992b**). Such a concept has been shown in the rat, where acute immobilization stress inhibits both 17α-hydroxylase and 17,20-hydroxylase activities which are catalyzed by the P450 enzyme (**Orr et al., 1994**).

The stressor-induced depression of plasma levels of gonadal steroids during the vitellogenic phase may be a direct consequence of oocyte atresia, the incidence of which increases in both snapper and red gurnard on confinement of wild fish (**Clearwater, 1992**). After entering the maturation phase the post-vitellogenic oocyte seems to be relatively immune to stressors and stressors may even have a benign effect on the progress of maturation (**Carragher & Pankhurst, 1991; Pankhurst & Carragher, 1992**).

Stressors can affect gamete quality and survival of young. In both rainbow and brown trout, stress caused by repeated emersion and confinement resulted in reduced egg size, lower survival rates, and lower sperm count (**Campbell et al., 1992&1994**). Both of these studies suggest that reduced egg size alone does not account for the lower survival of progeny. This view is supported by studies on the Atlantic cod, *Gadus morhua,* in which stressed broodstock showed higher rates of deformed larvae simultaneous with normal egg production, fertilization and progeny survival (**Short et al., 1995**).

Handling and repeated injections of hormones in *Siganus guttatus* significantly enhanced spawning in captive females. Groups of one female and two males were subjected to either handling stress (transfer to experimental tanks, anaesthetizing and cannulation) or a combination of handling and injections of hCG or saline at 24 hours intervals until spawned. The results indicated that fish stressed by handling and injections (whether hCG or saline) had significantly higher spawning rates than controls. It was concluded that some stress is needed for *S. guttatus* to spawn. It was also suggested that corticosteroids that are secreted in response to stressors were responsible for inducing spawning in handled fishes that were given sham injections. No difference in survival rates of larvae reared for five days was found between the groups (**Ayson, 1989**). Several other suggestions were made linking handling-induced stress with enhanced spawning in other siganid species. It was suggested that transferring spawners from tank to tank and lowering the water levels induced spawning in *S. fuscescens* (**McVey, 1972**), *S. rivulatus* (**Popper & Kissil, 1973**), *S. canaliculatus* (**Bryan et al., 1975**), and *S. argenteus* (**Burgan and Zseleczky, 1979**).

In Bahrain, *Siganus canaliculatus* broodstock are collected from the wild and held in captivity. Ovarian growth proceeds normally until the completion of vitellogenesis but fish do not progress further spontaneously. Induction of maturation and spawning requires intervention in the form of hCG injections. The collection, handling, monitoring by biopsy, and injections to which the broodstock are subjected may act as stressors which promote or inhibit the progress of the reproductive cycle. The aim of the experiments was to investigate the effect on oocyte development and plasma oestradiol of subjecting broodstock, during the

vitellogenic and maturational phases to the routine manipulations necessary to promote and monitor the female reproductive cycle through to spawning.

3.2 Materials and Methods:

3.2.1 Effect of Potential Stressors on Vitellogenesis:

I. Experimental Conditions:

480 spent or virgin fish, with a total length over 15 cm and body weight over 140 g, were taken from a stock of fish collected in early February when the ovary is in a previtellogenic stage. After total length and weight were measured, fish were distributed between four 40 m^3 concrete tanks (for maintenance procedure see Chapter 2, Section 2.2.1 A). 2mx2mx2m cage nets suspended in the tanks were used to hold the three experimental groups to make sampling easier and to make stocking densities (weight of fish per unit volume of water), similar in all cases. The fourth control group had 300 fish and the three experimental groups consisted of 60 fish each. The groups were subjected to potential handling and sampling stressors as follows:

Controls: No intervention except that caused by "fishing" the tank on occasions for the collection of samples for sacrifice.

Biopsy only: Intervention by repeated biopsy procedure.

Blood sampling only: Intervention by repeated blood collection.

Blood and biopsy sampling: Intervention by repeated combination of biopsy and blood collection.

II. Sampling Procedure:

At the beginning, 15 females from each of the four groups were sacrificed for blood and ovarian samples. Ovaries were staged, weighed, and fixed in 4% formaldehyde in neutrally buffered saline solution (see Table 2.4). At the second sampling, when sexes could be distinguished, the first 20 females identified in all groups except the controls were tagged using numbered internal anchor tags, in order that identified fish could be resampled on later occasions. Both the tags and the tagging needle were disinfected using an iodine-based antiseptic solution (Betadine, NAPP Lab.). An antiseptic ointment (Soframycin) was applied to the puncture site. Appropriate samples were collected every two lunar quarters coinciding with either the new or full moon, until one month after the natural spawning season. Ovaries were freshly dissected, staged, weighed and fixed. Oocytes were biopsied using a 0.8 mm (internal diameter) cannula and fixed for further histological treatment. Blood samples were collected from the caudal vessel, processed and stored prior to oestradiol assay (for blood sample collection details see Chapter 2, Section 2.2.3). In the group from which only biopsies were taken, blood was sampled once only at the peak of the spawning season when oocyte size and abdominal condition would normally indicate that vitellogenesis was completed. This sample was used to measure E_2 as an additional indicator of completed vitellogenesis and for comparison with fish of other groups for the same sampling date. A final blood sample was also taken at the end of the experiment.

In "blood" group, where only blood was sampled, information on the status of the oocyte was obtained from spare fish subjected to similar handling conditions.

III. Measurements:

1. Time of Vitellogenesis- The timing of successive vitellogenic stages was assessed by observations on ovarian tissue and biopsied oocytes using the criteria and histological procedures outlined in Chapter 2, Section 2.2.2. Three ovaries from each group were used.

2. Oocyte Diameter- Diameters of vitellogenic oocytes were measured using a digitizing pad (see Chapter 2, Section 2.2.2). Measurements were done on a triplicate sample of 30 oocytes from each of three fish in each group.

3. Incidence of Atresia- The number of atretic oocytes expressed as a percentage of all vitellogenic oocytes was determined from histological sections of ovaries from three fish in each group at each sampling interval. In each ovary, three counts of 100 vitellogenic oocytes were taken. A square etched on a microscope slide was used for counting, with the etched slide placed over the specimen slide. Oocytes that lay within the square or had their nucleus touching the top or right margins were counted.

4. Serum Oestradiol (E_2) - Blood samples from all groups were processed and serum oestradiol (E_2) levels were measured by direct radioimmunoassay procedures using replicate samples from eight females in each group (for assay details see Chapter 5, Section 5.2.4).

IV. Statistical Analyses:

Where applicable, results are expressed as mean±the standard error of the mean (s.e.m.). Differences between the mean levels of serum E_2 of groups were tested with Friedman's Non-parametric Test followed by Dunn's Multiple Comparisons Test. Differences between mean oocyte diameters and number of atretic vitellogenic oocytes were tested using a one way analysis of variance. All tests were performed on a Minitab statistical package V.10 (Minitab, Inc.) and GraphPad Instat2 Statistical Software.

3.2.2 Effect of Potential Stressors on hCG-Induced Maturation:

I. Experimental Conditions:

A single female/male pair of fish was held in each of 12 separate 60cmx60cmx60cm covered floating net cages suspended in a 7mx2mx1m fibreglass tank (Fig.3.1). Placed on the bottom of each floating cage there was a 15cmX15cm egg collector made from corrugated plastic



Figure 3.1- A partitioned fibreglass tank with floating net cages for holding pairs of *S. canaliculatus* used in the experiments.

sheet, the surface of which was gently scoured to provide a suitably roughened substrate for the attachment of the adhesive eggs. The tank was provided with continuous aeration and running water sufficient to give complete exchange every eight hours. The fish were given a 24 hour preventative bath treatment in 0.5 ppm Prefuran (Argent Chemicals, USA) at the start and halfway through the experiment.

A group of 12 female/male pairs was subjected to one of three treatments shown below. Only females which had completed vitellogenesis were used (stage V, see Chapter 2, Section 2.3.4). The only males used were those from which milt could be expressed by gentle abdominal pressure (running males). All females were given one injection of hCG (Sigma Chemicals), at a dose of 1 IU g^{-1} body weight at the beginning of the experiment, to induce maturation (this dose is most commonly used with siganids and was found to be the optimum, see Chapter 6). Males were given half the dose to ensure spawning readiness. The three groups were subjected to potential handling and sampling stressors as follows:

Controls: Fish were not disturbed after the administration of hCG until spawning or for one week, whichever came first.

Blood sampling only: Fish were subjected to daily blood sampling until spawning or for one week, whichever came first.

Blood and biopsy sampling: Biopsies and blood samples were taken daily until spawning or for one week, whichever came first.

II. Sampling Procedure:

All fish were biopsied at the start of the experiment to confirm by histology that all were in the same stage (V) of development previously established by macroscopic criteria outlined earlier. Blood-only or blood and biopsy samples were taken, at 24 hours intervals until the end of the experiment. Oocytes were fixed in 4% formaldehyde in neutrally buffered saline (see Table 2.4) prior to histology. Blood samples were taken as described in Chapter 2, Section

2.2.3). An antibiotic ointment (Soframycin) was applied to the needle puncture sites at each blood sampling. Egg collectors were checked three times a day for signs of spawning with minimum disturbance to the fish..

III. Measurements:

1. Oocyte Diameter- Diameters were measured using a digitizing pad, and also processed for histological observations (for details see Chapter 2, Section 2.2.2). Measurements were done on a triplicate sample of 30 oocytes from each of three fish in each group.

2. Serum Oestradiol (E_2) - Blood samples were assayed for oestradiol-β (E_2) levels (for assay details see Chapter 5, Section 5.2.4).

3. Spawning Numbers- The number of spawned fish out of 12 was recorded for each treatment group.

4. Latency Period- This was measured as the number of hours between injection and spawning. Since the exact moment of spawning was rarely observed, the time of spawning was back calculated from the embryo stage first observed using known rates of development. The experiment ran for a maximum of 168 hours or until such earlier time when all fish in a group had spawned.

5. Fertilization Rate- The percentage of fertilized eggs were determined by microscopic observation of three samples of 100 eggs each from half the number of the spawned females.

6. Hatching and Larval Viability Rates- Three sub-samples of 100 fertilized eggs from three spawned females in each group were hatched and maintained for 24 hours after hatching in one liter beakers placed in a constant temperature water bath (25°C). The number of hatched larvae, and the number of normal larvae surviving at the end of the 24 hours were used to determine hatching and larval viability rates respectively.

7. Incidence of Atresia- The number of atretic oocytes expressed as a percentage of all vitellogenic oocytes was determined from histological sections of ovaries from three fish in each group at each sampling interval. In each ovary, three counts of 100 vitellogenic oocytes was taken. A square etched on a microscope slide was used for counting with the etched slide placed over the specimen slide. Oocytes that lay within the square or had their nucleus touching the top or right margins were counted. Sampling was done at the start of the experiment only, at the subsequent intervals only blood&biopsy group was sampled. This sampling was done to confirm that all ovaries were in a normal condition as found in healthy wild fish.

IV. Statistical Analyses:

Results are expressed as mean±the standard error of the mean (s.e.m.). The differences in the parameters measured, within and between groups, were tested using a One-way Analysis of Variance followed by Bartlett's test for homogeneity of variances and Tukey-Kramer Multiple Comparison Test. All tests were performed on a Minitab statistical package V.10 (Minitab, Inc.) and GraphPad Instat2 Statistical Software.

3.3 Results:

3.3.1 Effect of Potential Stressors on Vitellogenesis:

1. Time of Vitellogenesis- There was no difference in the timing of the process of vitellogenesis in all groups (Fig.3.2). Ovaries in fishes in the three groups exposed to sampling stress advanced into vitellogenesis phase and completed vitellogenesis in unison with the control group. The first sign of yolk granule oocytes (stage V) which marks the final stage of vitellogenesis, was observed in all groups, except the biopsied group, by 16th March. In the biopsied group, stage V oocytes were first observed on 28th of March. However, the range of rates of vitellogenic progress observed is within that found in a group of natural spawners (Fig.3.2).

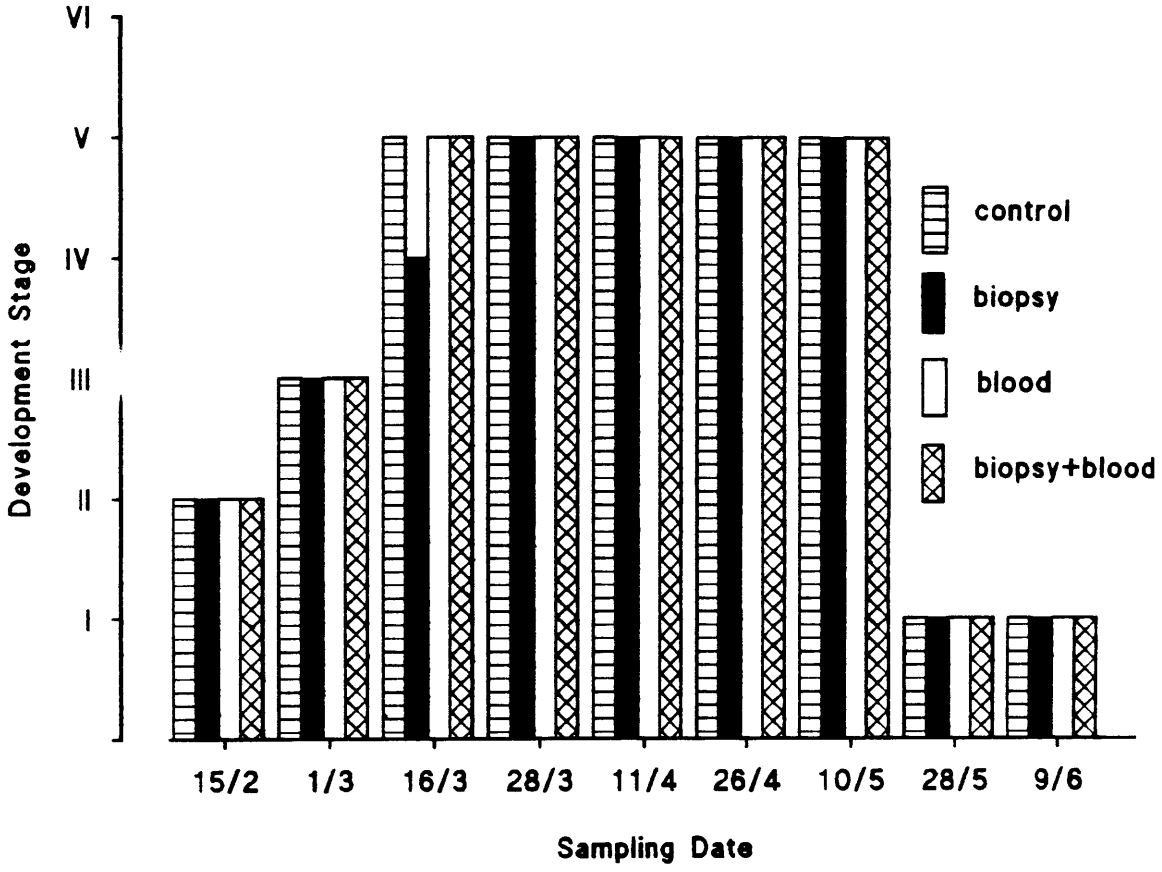


Figure 3.2– Effect of potential stressors on the progress of vitellogenesis in *S. canaliculatus.* Ovarian stages of development by specified sampling dates. I=virgin ovary, II=early developing, III=mid developing, IV=late developing, V=running ripe, VI=maturing. Observations were taken from three fish out of a total of 12 in each group.

2. Oocyte Diameter- Mean oocyte diameters in ovaries which had completed vitellogenesis did not differ significantly between any of the groups ($p=0.48$) (Table 3.1).

3. Incidence of Atresia- There were varying degrees of atresia in all ovaries in all groups, with an increasing trend as the ovary completes vitellogenesis in all except the controls (Fig.3.3). After that stage, the percentage of atretic oocytes in the ovaries show a significant increase. Comparison of all groups at consecutive sampling intervals, shows that one month after the start of the stress treatments, the group exposed to the twin stressors of blood&biopsy shows a significantly higher incidence of atresia than the other groups ($p<0.001$ in all three cases). There was no difference between control and the two groups subjected to stress by biopsy or blood sampling ($p>0.05$). The incidence of atresia in the two treatment groups subjected to a single stressor (biopsy or blood), shows a rise on 28/3. At the time, when all groups had completed vitellogenesis, on 28/3 (1 1/2 months into the experiment), the degree of atresia in all three experimental groups was significantly higher than in the control group ($p<0.05$ or greater). On 11/4, two weeks later, all stressed groups showed significant higher levels of atresia than controls ($p<0.001$). Thereafter, there was a dramatic increase in atresia even in the controls, so that by 10/5 the incidence of atresia exceeded 90% in all groups.

4. Serum E_2 profile- Oestradiol levels in all groups showed a similar pattern throughout the experiment, rising from low levels at the start of the experiment to a maximum during the natural spawning season in April and decreasing to lowest levels in May (Fig.3.4). There was no significant difference between the E_2 profiles of the four treatment groups ($p>0.05$).

3.3.2 Effect of Potential Stressors on Maturation:

1. Spawning- All 12 fish in each of the three groups progressed through the maturation phase and spawned in response of hCG (Table 3.2).

2. Latency Period- The time interval from hCG injection to spawning was similar in all groups. All fish spawned on day three after induction with exogenous hCG (Table 3.3). No

Table 3.1 - Effect of potential stressors on oocyte diameters (mean±s.e.m.) in fully vitellogenic ovaries (stage V) of *Siganus canaliculatus*.

Group	Diameter (µm±s.e.m.)	N
Control	405±2.80	3
Biopsy	410±3.46	3
Blood	408±7.34	3
Biopsy+Blood	405±2.38	3

N= the number of fish out of a total of 12 used for measurements in each group.

Table 3.2 - Effect of potential stressors on spawning numbers in *Siganus canaliculatus*.

Group	Number Spawned	Total Number of Fish
Control	12	12
Blood	12	12
Biopsy&Blood	12	12

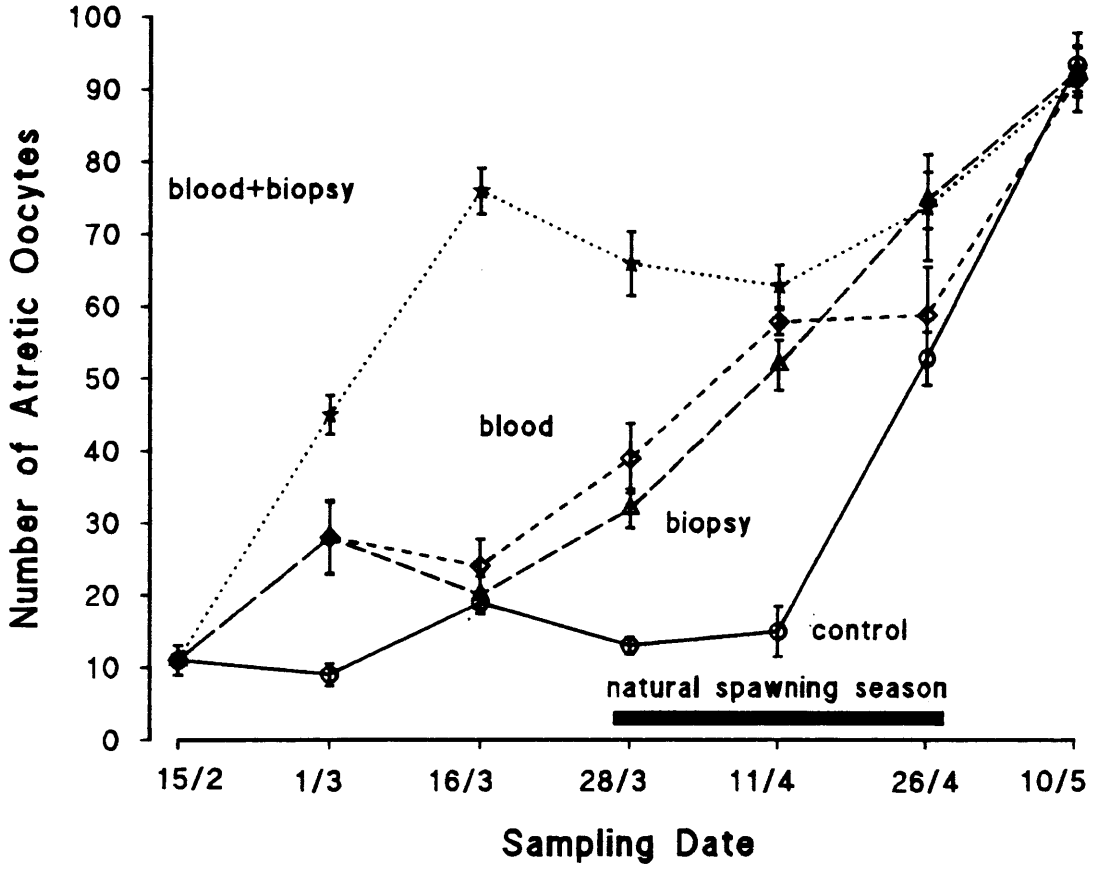


Figure 3.3– Effect of potential stressors on the number of atretic oocytes expressed as a percentage of all vitellogenic oocytes (mean±s.e.m.) in the ovary of *S. canaliculatus* subjected to repeated potentially stressful sampling proced–ures. Obseravtions were taken from three fish in each group.

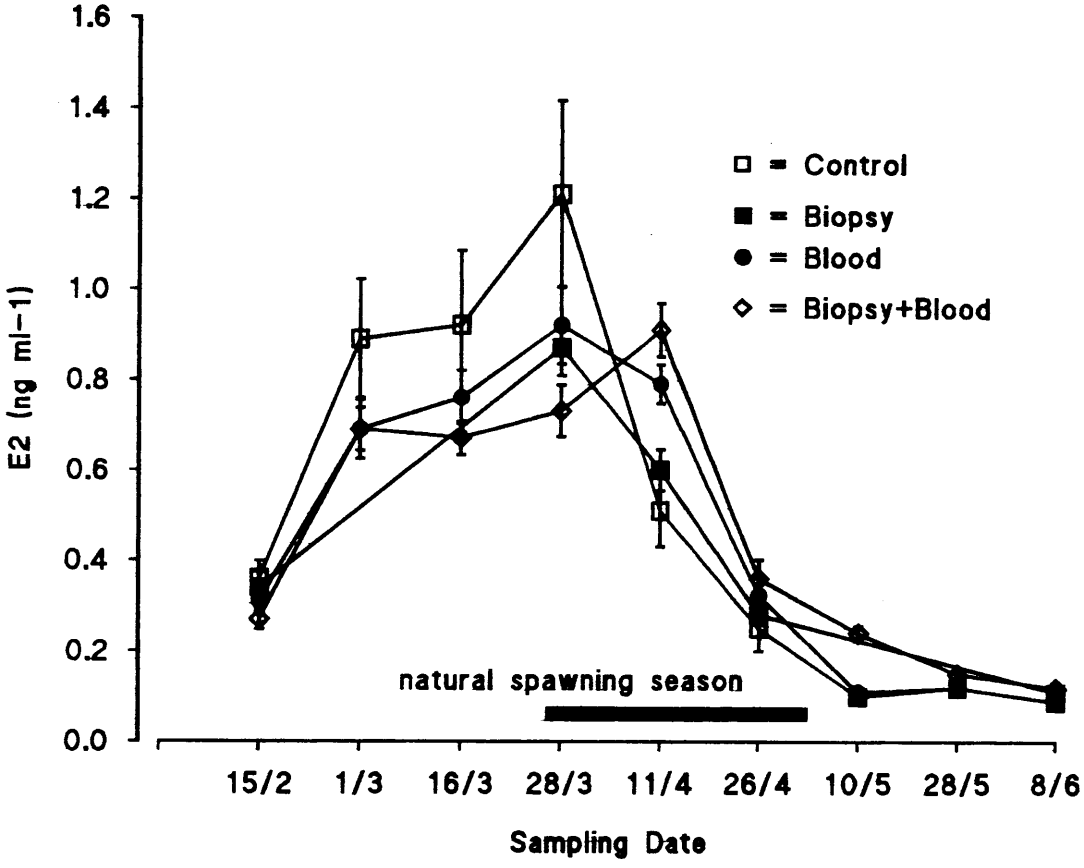


Figure 3.4– Effect of potential stressors on serum oestradiol (E2) levels during vitellogenesis in groups of *S. canaliculatus* subjected to several potentially stressful procedures. Sample number=8.

Table 3.3 - Effect of potential stressors on latency period (mean±s.e.m.) between hCG injection and spawning in *Siganus canaliculatus*.

Group	Latency period (hours)	N
Control	69±1.09	12
Blood	66±1.42	12
Biopsy&Blood	59±1.41	12

N= the number of fish out of a total of 12 used for measurements in each group.

Table 3.4 - Effect of potential stressors on egg fertilization in *Siganus canaliculatus*.

Group	Number Fertilized (mean±s.e.m.)	N
Control	99 ±0.401	6
Blood	99±0.342	6
Biopsy&Blood	89±0.730	6

N= the number of fish out of a total of 12 used for measurements in each group.

statistical difference was detected between the latency periods between control and blood groups ($p>0.05$). However, the biopsy+blood group spawned significantly earlier than the other groups ($p<0.001$ or greater).

3. Fertilization Rate- The blood+biopsy group had a significantly lower rate of oocyte fertilization than the other two groups ($p<0.001$) (Table 3.4).

4. Hatching Rate- As seen in Table 3.5, the number of hatched eggs decreases with the increase in the magnitude of stress. The hatching rate of controls was significantly higher than for the blood sampled group ($p<0.001$), which was significantly higher than for the blood+biopsy group ($p<0.001$).

5. Larval Viability- A shown in Table 3.6, the blood+biopsy group show a significantly lower viability than the other groups ($p<0.001$ in both cases).

6. Incidence of Atresia- Table 3.7 show the number of atretic maturing oocytes in the ovary for individual groups at different sampling intervals. As per the experiment design, there was no sampling of the control and blood sampled group at 24 and 48 hours, so no conclusions can be drawn between the three treatment groups at intervals other than that all ovaries of fish in all groups were in a healthy condition at the start of the experiment. At the start of the experiment, atresia was 8-12% in all groups with no significant difference ($p>0.05$), this degree of atresia in vitellogenic oocytes was found to be normal in captive fish (see Fig. 2.9). However, the group subjected to stress by a combination of biopsy and blood sampling showed a significant increase in the degree of atresia just 24 hours later, and such rate of atresia is only found in fully atretic ovaries. At 72 hours, all ovaries in all groups were spent and natural atresia was underway, so all groups showed similar degree of atresia ($p>0.05$), as such no comparison can be done between this and earlier sampling intervals.

7. Serum E_2 Profile- Fig.3.5 shows oestradiol levels in the two treatment groups in which blood was sampled, and as such no attempt is made to compare results. However, it is worth stating that in both these groups, the serum oestradiol shows a continuing rise into the

Table 3.5 - Effect of potential stressors on hatching in *Siganus canaliculatus*.

Group	Number Hatched (mean±s.e.m.)	N
Control	96 ±0.882	6
Blood	88±0.615	6
Biopsy+Blood	82±0.792	6

N= the number of fish out of a total of 12 used for measurements in each group.

Table 3.6 - Effect of potential stressors on larval viability in *Siganus canaliculatus*.

Group	Number of Viable Larvae (mean±s.e.m.)	N
Control	99 ±0.803	6
Blood	98±0.671	6
Biopsy&Blood	84±1.25	6

N= the number of fish out of a total of 12 used for measurements in each group.

Table 3.7 - Effect of potential handling stressors on the incidence of atretic maturing oocytes in the ovary of *Siganus canaliculatus*. At 72 hours fish are spent.

Group	Number Atretic (mean±s.e.m.)				N
	0 hrs	24 hrs	48 hrs	72 hrs *	
Control	8 1.86	ND	ND	96 2.08	3
Blood	12 1.76	ND	ND	95 2.91	3
Biopsy+Blood	10 0.882	64 4.06	79 4.36	99 0.667	3

N= the number of fish out of a total of 12 used for measurements in each group.

* - Figures at 72 hours are not comparable with samples taken earlier.

ND= No Data.

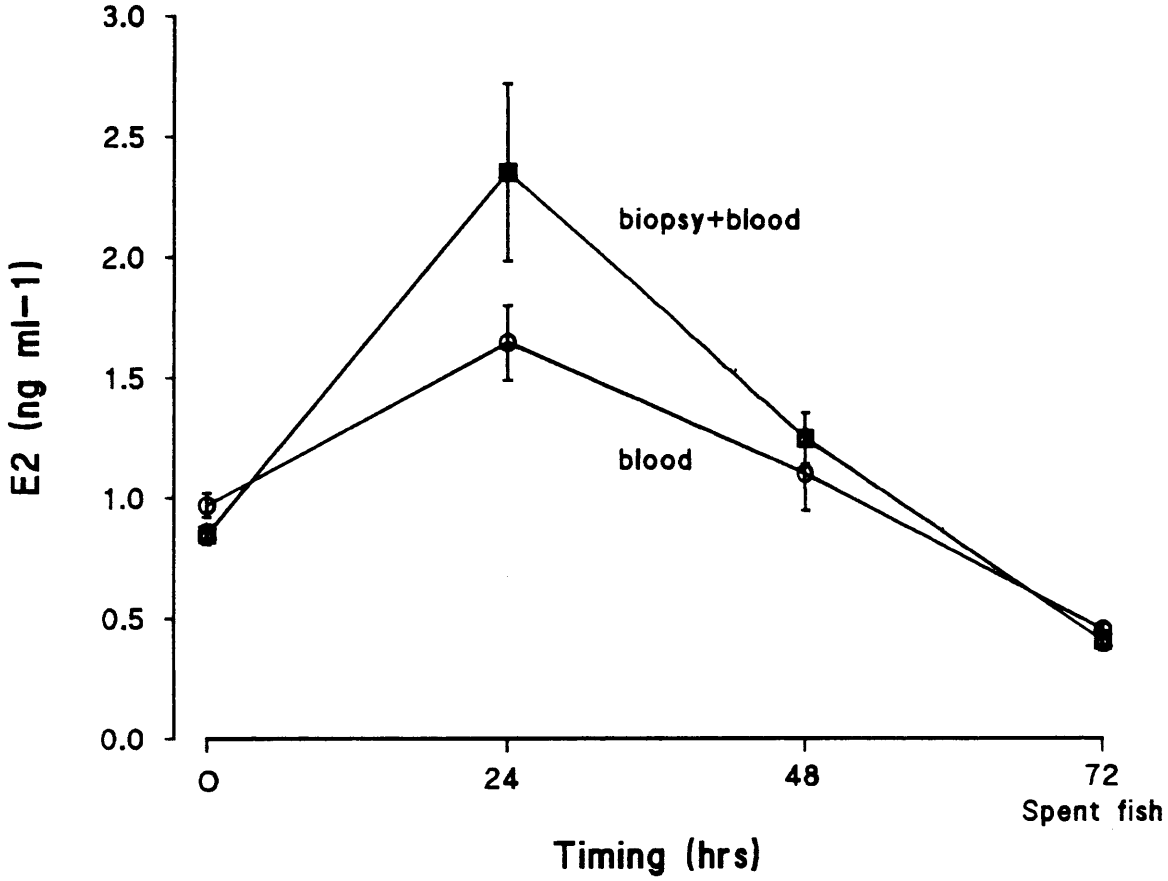


Figure 3.5– Serum oestradiol (E2) levels during maturation in groups of *S. canaliculatus* subjected to several potentially stressful procedures. Sample number=8.

maturation phase until about 24 hours after injection of hCG ($p<0.001$ and $p<0.01$ respectively), after which the levels decrease significantly ($p<0.001$) in both groups.

3.4 Discussion:

This work has shown that reproductive success of female rabbitfish, *Siganus canaliculatus*, is adversely affected by stress caused by sampling during both vitellogenesis and maturation phases. There are some early studies on the effect of stress caused by environmental factors and handling on adult health and spawning rates in siganids (**reviewed by Lam, 1974; Duray, 1990**), but none investigates in depth the effect on the process of ovary development and gamete quality. It is shown here that although siganids are known to be tolerant to environmental changes like salinity (**Lam, 1974; Tobias, 1976; Alcala, 1979**), temperature (**Pillai, 1962**), reduced oxygen concentrations (**Carumbana & Luchavez, 1979a**), and handling and overcrowding (**Ben-Tuvia & Kissil, 1973**), such effects may manifest themselves only in the later stages of reproduction, or when more sensitive indicators are measured.

3.4.1 Vitellogenesis:

The effect of the stressors on vitellogenesis varied. They did not stop the overall process of vitellogenesis, with stressed fish in all treatment groups completing vitellogenesis at the same time. Oestradiol levels in all stressed groups showed no depression but followed a normal pattern, showing a peak during the natural spawning season. Similarly, there was no significant difference in oocyte diameters in ovaries that completed vitellogenesis. There were, however, clear effects and differences in effects in the response of the ovaries in the four groups with regard to number of atretic vitellogenic oocytes. The more intense the stressor, the faster the atretic response. The eventual rise in the incidence of atresia observed in the controls is to be expected in any species in which oocyte development is arrested and does not proceed beyond vitellogenesis.

Deleterious effects of stress on reproductive and other functions in fish have been the subject of numerous reviews (**Pickering, 1981; Wedemeyer et al., 1990; Barton & Iwama, 1991; Fagerlund et al., 1995; Barton, 1997**). A key component of the response to stress is the activation of the pituitary-interrenal axis, resulting in the elevation of plasma cortisol levels (**Donaldson, 1981**). Artificial elevation of circulating cortisol levels in mature female rainbow trout results in a depression of plasma oestradiol and vitellogenin levels. Furthermore, in similarly treated immature female rainbow trout, vitellogenin levels were also suppressed although no effect on oestradiol was apparent (**Carragher et al., 1989**). Such findings have also been reported in mammals, where corticosteroids were implicated in stress-induced gonadal dysfunction (**Sapolsky, 1985**). It was proposed that the mechanism by which cortisol may cause such results is that it has a direct effect on oestradiol secretion and/or it may interfere with the ability of the liver to detect and transduce the oestrogenic signal promoting vitellogenesis (**Pottinger et al., 1991**). The reason that such a depression of E_2 has not be seen in the siganid under study may be due to the lesser intensity of stress induced on the fish.

The fish in this experiment were subjected to normal handling stress used in culture methods; in such procedures care is taken not to subject the fish to unnecessary stress. The experiment lasted for more than three months and the fish could have become "domesticated", or in a way less responsive to that magnitude of stress. In the fish where plasma steroids depression was reported, the fish were caught from the wild and sampled in a few hours after capture, examples are the snapper, *Pagrus auratus* (**Carragher & Pankhurst, 1991**), the red gurnard, *Chelidonichthys kumu* (**Clearwater, 1992**), and the female spotted sea trout, *Cynoscion nebulosis* (**Safford & Thomas, 1987**).

The absence of any effect of stressors on oocyte diameter in *S. canaliculatus* is in contrast with rainbow trout, where repeated exposure to acute stress resulted in delayed ovulation and reduced egg size (**Campbell et al., 1991&1992**).

The observed increases in atresia in this experiment were not accompanied by a depression in serum E_2 levels. The fact that stress does not affect the overall progress of vitellogenesis as measured by ovarian stages, and that it can not be detected by macroscopic measurements of

oocyte diameter, emphasizes the importance of the incidence of atresia as an indicator of the healthy status of the ovary in *Siganus canaliculatus*. This aspect was not considered in earlier studies on the effect of stress on siganids (**Ayson, 1989;** see below). The lack of correlation in the timing of the changes seen in two intimately related processes such as atresia and oestradiol in *S.canaliculatus* is of interest, and in contrast to the response of snapper and red gurnard, where there is a close association between atresia and plasma levels of gonadal steroids (**Carragher & Pankhurst, 1991; Clearwater, 1992**).

It is perhaps plausible to argue that in *S. canaliculatus* more intense stress, whether acute or chronic, would have caused deleterious effects on ovary development through depression of circulating oestradiol and vitellogenin levels and would have lead even to reduced egg size. In the present study the fish were exposed to stress at biweekly intervals. Such a method was chosen because it conforms with the common practice used to obtain and prepare fish for the other experiments done and with the common culture practice used at present for this fish, which is an important consideration in the planning of this work.

3.4.2 Maturation:

The effects of handling stress during the process of maturation, spawning and subsequent larval development were pronounced, with the major adverse effects occurring at and after fertilization. The controls and two groups exposed to handling and sampling stress caused by blood sampling or a combination of biopsy+ blood sampling advanced through and completed the process of maturation, ovulation and spawning in response to exogenous hCG. Fertilization rates in fish exposed to blood sampling only did not differ from the controls, but were significantly lower in fish subjected to the more major blood and biopsy sampling. Compared with controls, hatching rates in blood-only sampled fish were significantly lower, and were even lower in the group subjected to the greater stress induced by both blood and biopsy sampling. Overall, the more intense the stressor, the greater the reduction in hatching and larval viability rates. Impairment of larval viability has also been reported for rainbow and brown trout (**Campbell et al., 1994**), and Atlantic cod, *Gadus morhua*, where stress induced by capture resulted in deformed larvae (**Short et al., 1995**).

Comparison of the number of atretic vitellogenic oocytes in the control and two treatment groups at the start of the experiment showed no significant difference, but the third group exposed to the most acute stress caused by combination of biopsy and blood sampling showed a sharp increase in the number of atretic vitellogenic oocytes just 24 hours after the start of the experiment, reaching over 50%, and the rate of atresia kept on increasing at subsequent samplings. Such high degrees of atresia have not been encountered in normal ovaries, as established in Chapter 2, Section 2.3.4.

In the only traceable published report on the effect of stress on spawning of rabbitfish, it was reported that in *S. guttatus* exposed to stress caused by handling and hormone injections, a larger number of fish spawned than in unstressed controls, and that the stressors had no negative effect on larval survival rates (**Ayson, 1989**). Even though most of the parameters measured here were not studied in that report, the reported stress-induced increase in spawning numbers and the null effect on larval viability do not agree with the findings in this study. No enhancement of spawning was found, in fact stress had no effect on spawning, other than the reduction of the latency period in the biopsy+blood group. The fact that survival of larvae from fish exposed to milder stress did not differ significantly from those of unstressed fish indicates that this species may have reasonable tolerance to potential stressors common in broodstock handling. However, comparison of the present results with the former study may be of limited value because of low number of observations and low and variable survival rates of the progeny (0.5-37.2%) in all groups in the former study, and the difference in species and geographical locations. The siganid under study, *S. canaliculatus,* lives in a harsher environment of salinity and temperature. Stress has varying effect on gamete quality in salmonids, where confinement stress of rainbow and brown trout resulted in production of smaller eggs and lower survival of progeny upto 28 days post-hatch, but the study suggested that reduced egg size alone could not account for the lower survival of progeny (**Campbell et al., 1994**). In non-salmonids, like the Atlantic cod, *Gadus morhua*, chronic stress by netting three times a week resulted in a higher incidence of deformed larvae, even though there was no differences in egg production, fertilization and survival of progeny between stressed and unstressed fish (**Short et al., 1995**).

It can be concluded that although stress may cause adverse effects on gonadal function, fish can tolerate some degree of stress, and can successfully propagate viable number of progeny both in the biological and economical sense. Mild handling stress during the process of vitellogenesis does not have a drastic effect on the fish, but the higher incidence of atresia observed does suggest that egg quality may be affected. A matter that needs to be investigated further. Although post-vitellogenic follicles may appear to be unaffected by stress and do indeed continue to undergo final maturation and ovulation in response to exogenous hormone treatment, the reduction in the number of viable oocytes which complete maturation (at least in the more intensely stressed fish of the blood+biopsy group), and the post-fertilization performance, suggests otherwise, which means that quality and production level of progeny can be negatively affected. Similar results were also observed in the marine snapper, *Pagrus auratus* (**Carragher & Pankhurst, 1991; Pankhurst & Carragher, 1992**).

One of the important applications of the findings of this study can be in the field of mariculture. Among the principal constraints on the production of farmed animals, including fish, are the difficulties in adaptation to culture methods in captivity and the resulting stress (**Pickering, 1993**). Stressors are unavoidable in intensive aquaculture, severe stress can lead to increased mortality, but lower levels can also compromise various physiological and behavioral functions that may contribute to undesirable results like suppressed disease resistance and suboptimal growth rates. It is thus a common notion among aquaculturists that the understanding and management of stress is an important consideration (**Barton, 1997**). Under the more crowded conditions typical of intensive aquaculture systems, water chemistry alterations, environmental factors, common culture practices like handling, sorting, grading, transport, and behavioral interactions between fish all add to the normal demands made by the aquatic environment itself, and the result could be stress that evokes a response from the fish (**Wedemeyer, 1997**). Within limits the fish can survive such challenges by expending energy (**Barton & Iwama, 1991**), but the main problem is that chronic stress can eventually manifest as negative effects on health and physiological condition (**Wedemeyer, 1996a**).

3.4.3 Serum E_2 Increase During Maturation:

In the two treatment groups where blood was sampled there was a continuing increase in serum oestradiol levels for a period of 24 hours after maturation started and only then did it show decreasing values. The 24 hour timing coincides with the start of the migratory nucleus stage of oocyte development (see Chapter 2). The continuing increase in E_2 levels after the start of the maturation phase may be a natural occurrence or an artifact of the exogenous hCG. A third probability is that it is a result of the handling stressors, as suggested by the fact that post-maturation E_2 levels in fish subjected to the more intense stress caused by blood+biopsy sampling are higher than in those subjected to biopsy-only, although both received the same dosage of hCG. But with the absence of natural spawning in captivity without the use of hCG, it is difficult to give credible conclusions in this matter.

Chapter 4

Chapter 4

Effect of Lunar Phase on Vitellogenic and Post-Vitellogenic stages in *S. canaliculatus*.

4.1 Introduction:

Lunar-dependent reproductive cycles are common among marine animals (**Robertson et al., 1990**). In addition, particular aspects of reproduction in many intertidal organisms, as well as patterns of movements and feeding, are closely linked to lunar-influenced tidal cycles (**Berry, 1986; Christy, 1986**). Many tropical reef fishes also have lunar related reproduction (**Johannes, 1978**). Most discussion of the adaptive nature of such cycles in fishes has followed explanations developed for intertidal invertebrates and has focused primarily on the role of the tidal regime or moonlight on the survival of planktonic eggs or larvae during or shortly after their release (**Thresher, 1984; Gladstone & Westoby, 1988**). Such adaptations are included under the "hatchling-biology" hypotheses such as the following (1) Dispersal of planktonic propagules is timed with a lunar cycle in which the high tide is more likely to keep the released eggs or larvae away from the reef-based predators and disperse them quickly by the strong tidal currents. Alternatively, the photo-sensitive larvae may be attracted by the moonlight and move to the surface, thus enhancing their off-reef movement. (2) Synchronized spawning oversaturates predators on planktonic larvae. (3) Spawning cycles of fishes are matched with those of other reef invertebrates, whose larvae provide food for the larval fishes. (4) Temporal spacing of reproduction reduces food competition (**Robertson et al., 1990**).

The "settlement-linkage" hypothesis focuses on later stages of larval life when they settle. It is proposed that temporal patterns of larval production are adaptations to maximize settlement of planktonic larvae (**Christy, 1978; Kingsford, 1980**).

A third category of hypothesis, which was developed largely for fishes, concerns factors affecting spawners themselves, the spawning act, and parental activities. This was called the "adult-biology" hypothesis and is of two types. The first deals with the direct influence of lunar related changes in environmental factors on reproductive success. These include the suggestions that moonlight (1) allows adults to spawn near dawn, when diurnal egg predators are absent (**Pressley, 1980**), or (2) facilitates migration to spawning grounds (**Colin et al., 1987**), or (3) enhances the ability of the adults to guard their eggs (**Allen, 1972**). The second type of "adult-biology" hypothesis stresses the importance of synchronization of reproductive activities which can facilitate protection of eggs against predators due to collective activities of aggregated parents (**Tyler, 1988; Foster, 1989**), and give higher chances of survival of the eggs because of the large clutch sizes (**Sargent & Gross, 1986**).

Examples of lunar-driven spawning are found in both fresh and marine water fishes. In the mummichog, *Fundulus heteroclitus,* which spawns cyclically during its reproductive season, maximum gonad size and gamete production of both sexes occur in synchrony at the high spring tides of new or full moon (**Taylor et al., 1979**). In another study on the same fish, measurements of serum oestradiol and gonadosomatic indices from wild fish showed a cyclic pattern with the highest values coinciding with the highest spring tides associated with new or full moon (**Bradford & Taylor, 1987**).

A lunar-synchronized spawning rhythm was also reported in captive sea bass *Lates calcarifer.* However, in this fish spawning was timed with neap tides associated with quarter moon periods, in contrast with the more common high spring tide timed spawning in other fish. Such an adaptation was attributed to the fact that sea bass spawning grounds are near to coastal swamps which act as nurseries for the larva, and it is thus important that larvae are not dispersed far from such grounds (**Garcia, 1992**). In the tropical Western Atlantic groupers (*Epinephelus guttatus* and *E. striatus*), such adaptations were found to serve better the needs of the adults rather than their progeny. Observations of behavior of individuals within spawning aggregations and the timing of spawning in the wild showed that spawning activities are timed with the new moon. Adult fish migrate to spawning grounds from long distances and the moonlight may serve the needs of the adults to coordinate their activities,

but the relatively small tidal amplitude and the reduced currents passing over the reef at this time do not seem to favour egg or larval survival (**Colin et al., 1987**).

The advantages of lunar-synchronized spawning behavior, whether to the benefit of parent or progeny, is well established. The mechanisms that control such adaptations are less clear. No doubt moonlight and other environmental factors are important cues for the entrainment of such activities, but experiments with fish that were held under constant environmental conditions and subjected to simulated constant new or full moon regimes for long periods of time still exhibited lunar synchronized behaviours that persisted for several months before free-running, suggesting that an endogenous mechanism controls such synchronization (**Hsiao & Meier, 1989; Taylor, 1991; Hsiao & Meier, 1992**).

Where known, spawning in siganid species is synchronized with the lunar cycle. The timing of spawning is between the new and full moon (**Duray, 1990**). The spawning of *Siganus guttatus* in captivity was reported to be during the first quarter of the new moon (**Hara et al, 1986b**), 2-4 days after new moon in *S. argenteus* (**Luchavez & Carumbana, 1982**), 3 days before full moon in *S. lineatus* (**Bryan & Madraisau, 1977**). In *S. canaliculatus* spawning is reported to occur in the first quarter of the new moon, from midnight to early morning (**Bryan et al., 1975; Hasse et al., 1977**). No reasons for such adaptations were investigated or suggested.

The aim of the following experiments is to define the role of lunar phases on the process of ovary development. Captive fish are often held in closed areas sheltered from lunar light, and it is important to know if absence of such light has any detrimental effect on the vitellogenic and post-vitellogenic phase of ovary development.

4.2 Materials and Methods:

4.2.1 Experimental Groups:

A total of 500 resting or virgin fish with a total length over 15 cm were collected in early February, when the ovary is still in a previtellogenic stage of development. The fish were equally distributed in four 40 m^3 concrete tanks, and maintained for experimental work until one month after the expected spawning time (for maintenance details see Chapter 2, Section 2.2.1 A). All environmental conditions, other than moonlight, were ambient throughout the experiment. The four groups were given the following treatments:

Controls - The fish were maintained under ambient conditions, including natural moonlight, until the end of the experiment.

Continuous simulated new moon condition - Every day at sunset the tank was covered by a light proof sheet secured properly to avoid any penetration of external light into the tank. The cover was removed at sunrise.

Continuous simulated full moon condition using artificial light - Light (6 lux/m^2) was supplied by lights fitted with diffusers suspended approximately 30 cm above the water surface. The lights were connected to a dimmer and a timer adjusted to switch on after sunset and off by sunrise, it was adjusted weekly against natural timing. The dimmer was used to increase light intensity in four stages to a maximum 30 minutes after sunset. At sunset the tank was covered with a light proof sheet which was removed at sunrise.

Reversed moon phase condition using artificial light - A similar setup was used as in group 3, (the artificial light was used to simulate normal moon light), but the periods of light and darkness was set in a reverse order to the natural moon phase, e.g. at the start of the experiment when the natural moon was in the 2nd quarter, the artificial moon in

the tank was at the simulated 4th quarter. A dimmer was used to simulate increasing or decreasing moon light intensities.

4.2.2 Sampling Procedure:

At each sampling time, 10-12 females were used. An initial sample from the original stock was taken at the start of the experiment. After that, sampling was done at biweekly intervals corresponding to new and full moon. Fish were caught at feeding time using a rectangular net frame, and after being tranquilized in 2-phenoxyethanol (100ppm), a sample of blood was collected from the caudal vessel and stored (for details see Chapter 5, Section 5.2.4). The fish were then measured, dissected and the ovary condition described, weighed, and preserved for histological analysis (for details of procedure see Chapter 2, Section 2.2.2).

4.2.3 Measurements:

1. Time of Vitellogenesis- Ovary samples were processed for histological determination of the development stage (for details of histological procedure see Chapter 2, Section 2.2.2). Three ovaries from each group were used.

2. Oocyte Diameter- Diameters of vitellogenic oocytes were measured using a digitizing pad (see Chapter 2, Section 2.2.2). Measurements were done on a triplicate sample of 30 oocytes from each of three fish in each group.

3. Incidence of Atresia- The number of atretic oocytes expressed as a percentage of all vitellogenic oocytes was determined from histological sections of ovaries from three fish in each group at each sampling interval. In each ovary three counts of 100 vitellogenic oocytes were taken. A square etched on a microscope slide was used for counting, with the etched slide placed over the specimen slide. Oocytes that lay within the square or had their nucleus touching the top or right margins were counted.

4. Gonado-Somatic Indices (G.S.I) - the weight of the ovary as a function of the body weight was calculated using the equation:

G.S.I = ovary weight/total body weightX100

4.2.4 Statistical Analyses:

All mean values were expressed with their standard error of the mean (s.e.m.). Oocyte diameters and Gonadosomatic indices were tested using a one-way analysis of variance followed by Bartlett's test for homogeneity of variances and Tukey-Kramer Multiple Comparison Test for difference between groups. Difference in number of atretic vitellogenic oocytes between the control and the treatment groups was tested using one way ANOVA followed by standard t-tests. All tests were performed on a Minitab statistical package V.10 (Minitab, Inc.) and GraphPad Instat2 Statistical Software.

4.3 Results:

1. Time of Vitellogenesis- There was no difference in the timing of the process of vitellogenesis between any of the groups. Ovaries from all groups progressed through and completed vitellogenesis at the same rate (Fig.4.1).

2. Oocyte Diameter- There was no significant difference between the mean oocyte diameters of the four groups ($p>0.05$, Fig.4.2). Mean oocyte diameters were indistinguishable from those of wild fish at similar dates, as detailed in Chapter 2.

3. Incidence of Atresia- Atresia occurs in normal ovaries, and occurred in all four groups under investigation, with a trend towards higher incidence as the ovaries complete vitellogenesis, after which the percentage of atretic oocytes rose steeply (Fig.4.3). Comparison of all groups at consecutive sampling intervals, showed that one month into the experiment, there was a higher rate of atresia in the two groups subjected to continuous new moon ($p<0.01$) and continuous full moon ($p<0.05$), as compared to the control. The former two

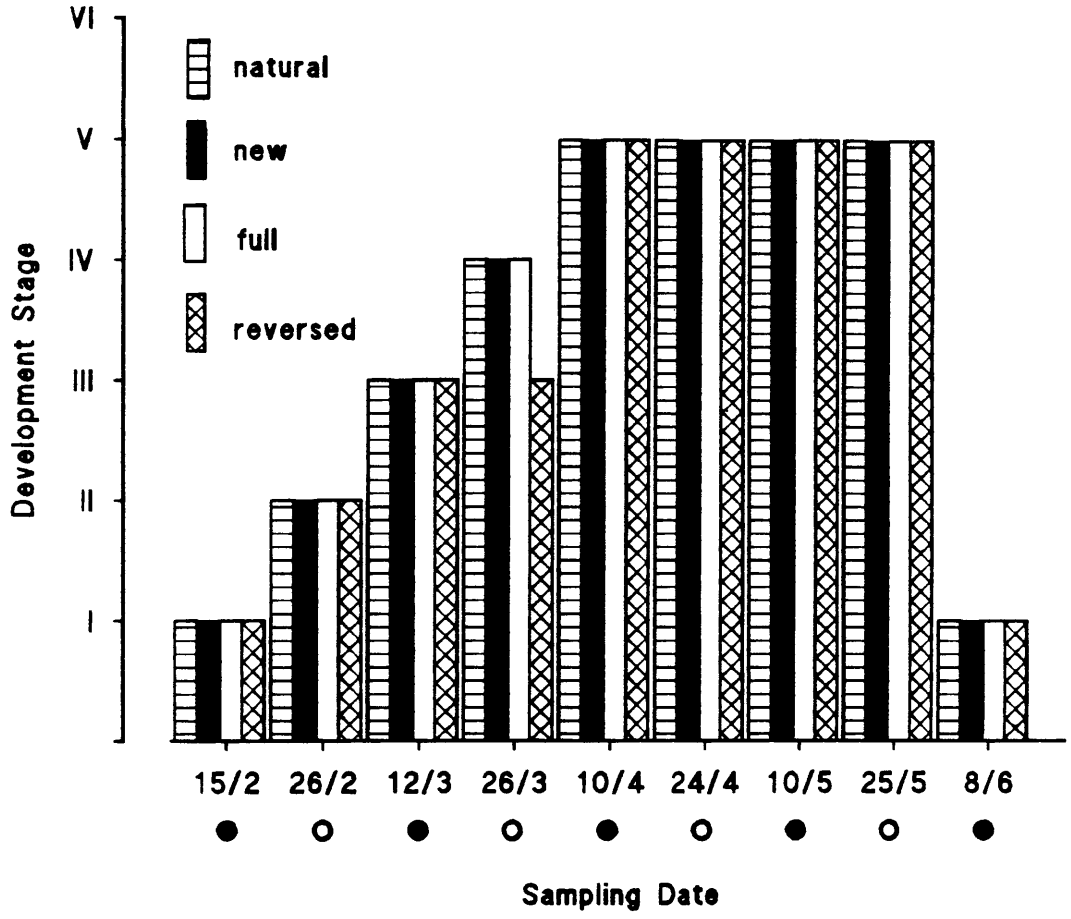


Figure 4.1– Development stages in the ovary of *S. canaliculatus* subjected to natural and different simulated moon phases. Circles on the "X" axis indicate natural new (solid) and full (open) moon phases. I=virgin ovary, II=early developing, III=mid developing, IV=late developing, V=ripe ovary, VI=maturing. Sample number=3.

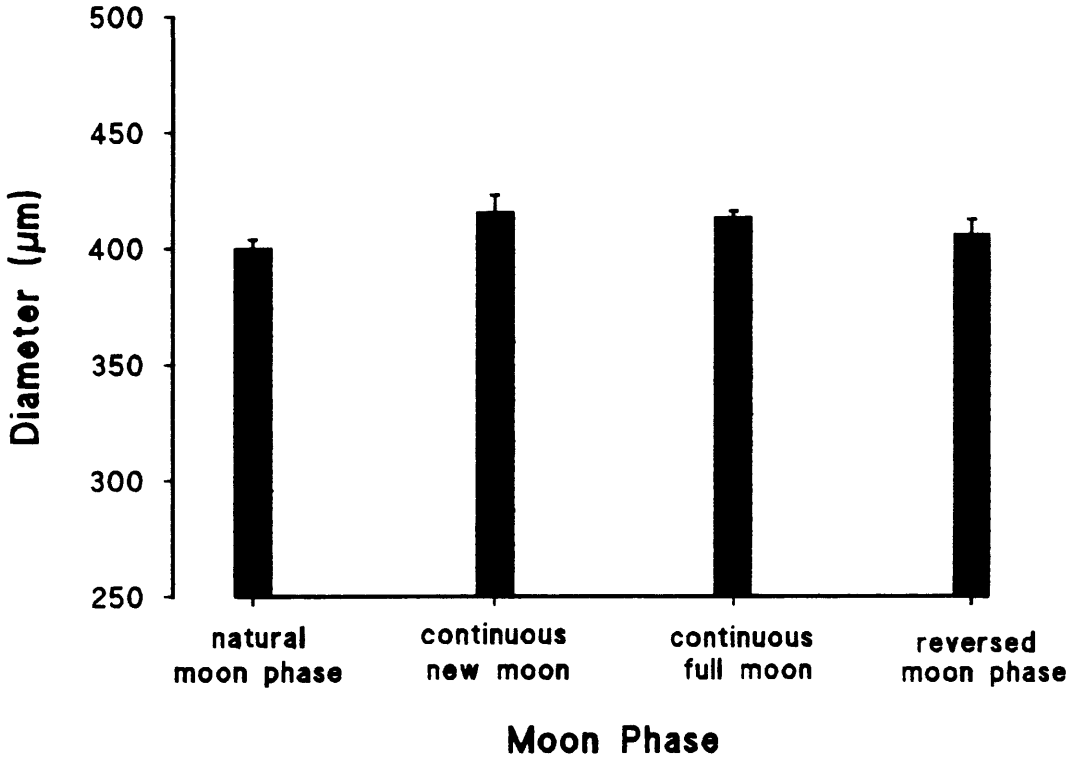


Figure 4.2– Oocyte diameters (mean±s.e.m.) in fully vitellogenic ovaries of *S. canaliculatus* subjected to natural and simulated moon phases. Measurements were done on triplicate samples of 30 oocytes from each of 3 fish in each group.

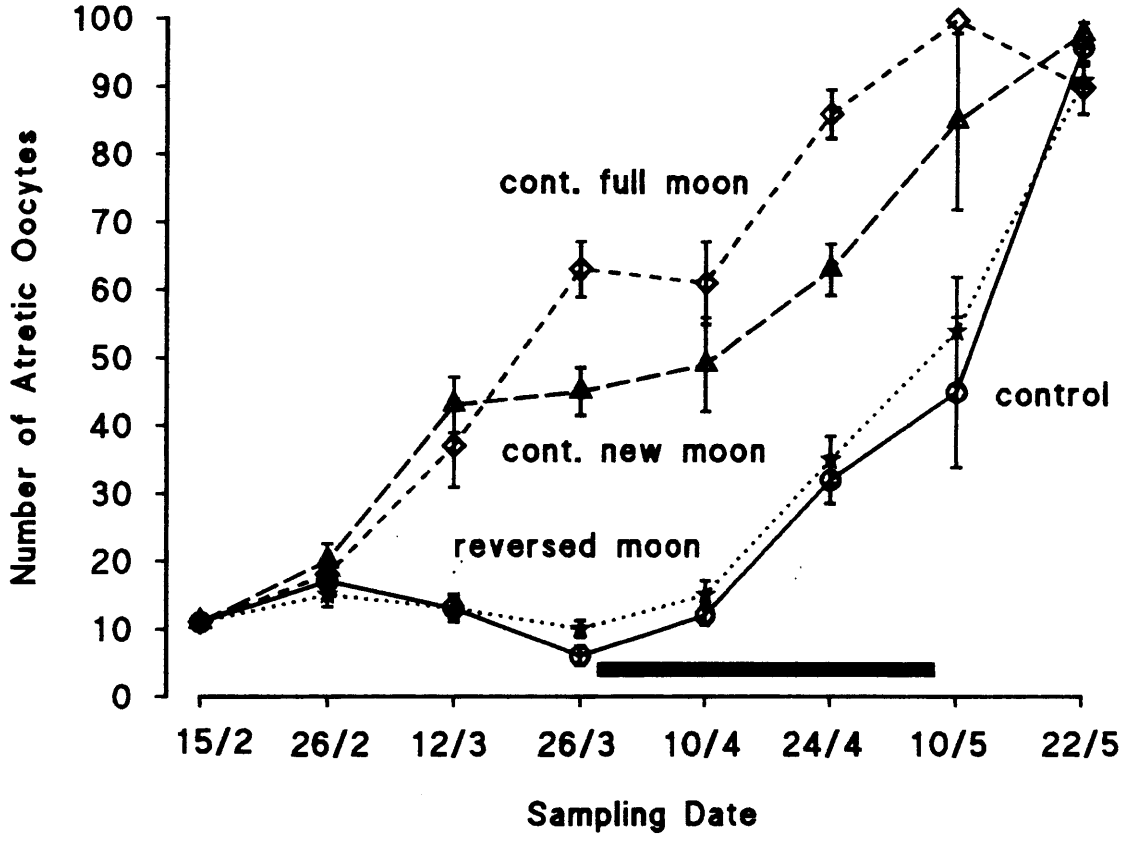


Figure 4.3– Number of atretic vitellogenic oocytes in ovaries of *S. canaliculatus* subjected to natural and different simulated moon phases. Solid line on "X" axis indicates natural spawning season. Sample number is three.

groups had a significantly higher level of atresia than the reversed moon phase group ($p<0.01$ and $p<0.05$, respectively), and these differences persisted until late in the spawning season on 24th of April, after which the normal high levels of atresia found in wild fish (Chapter 2) were observed in all groups.

4. Gonado-Somatic Indices - the gonadosomatic indices (GSI) in all groups followed a similar pattern, starting at their lowest level at the start of the experiment and increasing as the ovary progresses further into vitellogenesis, reaching a maximum during the spawning period in April and decreasing thereafter until the ovary returns to its resting stage in June (Fig.4.4). Comparisons of the timing when the peak values were reached between the three groups show that the control, new and full moon groups all reached maximum values on 10th of April, which coincided with a new moon phase, and such values were observed till the next sampling on the 24th, with no significant difference between the two values ($p>0.05$). In the reversed moon phase group, there was an earlier rise in GSI value observed on the 26th of March that showed significant difference from the control and the other two treatment groups ($p>0.001$).

Neither the normal nor any of the various artificial lunar regimes implemented promoted ovary development beyond vitellogenesis in any of the groups.

4.4 Discussion:

In *Siganus canaliculatus*, there was no observed effect of lunar regimes on the timing of the process of vitellogenesis. Fish in all groups completed vitellogenesis and reached the yolk granule stage (stage V) on 10th of April, which is well within the natural spawning season (late March to early May). There is also an absence of any lunar effect on oocyte diameter of vitellogenic oocytes. Diameters of fully vitellogenic oocytes were within the size range of oocytes in that stage established for wild fish in Chapter 2.

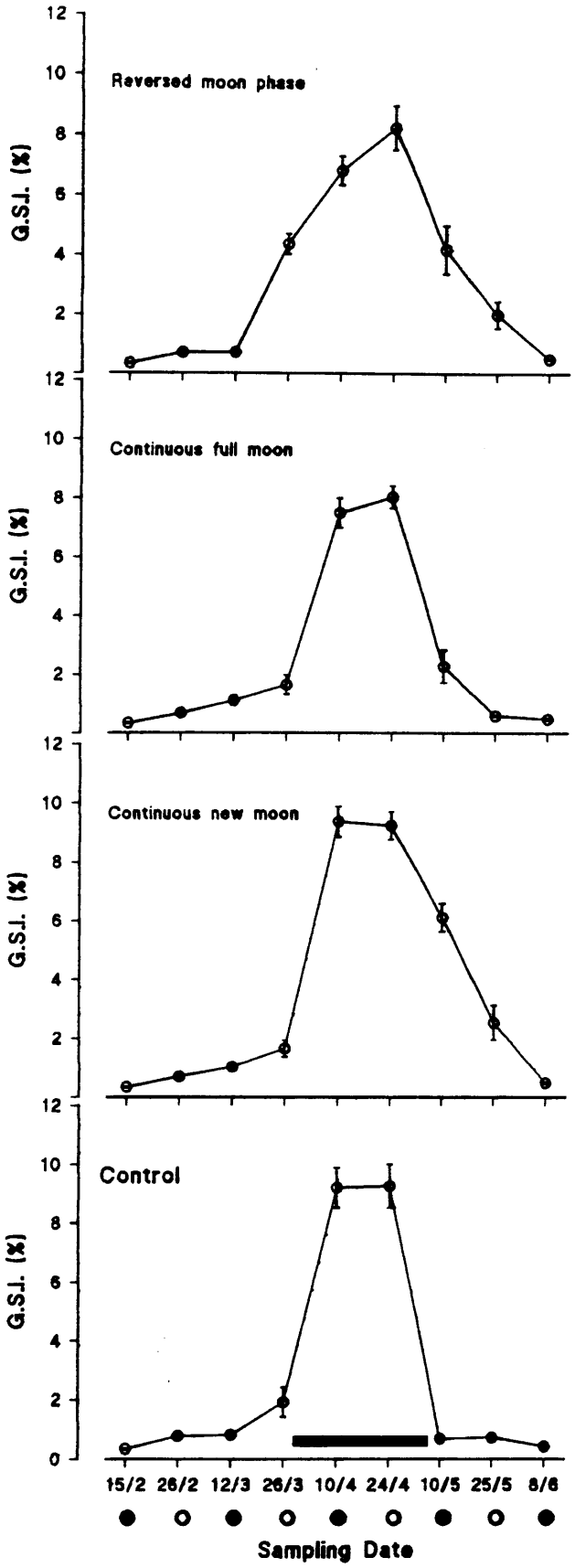


Figure 4.4– Gonadosomatic indices (mean±s.e.m.) in *S. canaliculatus* subjected to natural and different simulated moon phases. Circles on the "X" axis indicate natural new (solid) and full (open) moon phases. Solid bar indicates natural spawning season. Sample number=12.

The gonadosomatic indices also show a normal pattern in all groups as compared to wild and captive fish (Chapter 2), with a peak value reached during the spawning season, the beginning of which coincided with the first new moon phase in that period.

None of the lunar regimes promoted any post-vitellogenic maturation or spawning. It is concluded that some captivity-induced or stress-induced suppression overrides any lunar-induced progression on to the maturation and spawning stages.

It is possible that the spawning stage of the reproductive cycle is controlled by endogenous mechanisms cued to some external factors. Such a mechanism has been reported in *Fundulus heteroclitus*, where a semilunar spawning cycle persisted in constant conditions of light and in the absence of lunar and tidal period cues (**Taylor, 1984; Hsiao & Meier, 1989**). It was expected that, by providing lunar clues (simulated normal and reversed phases), the endogenous mechanism controlling the maturation and spawning would proceed or be re-adjusted accordingly, resulting in spawning within the natural spawning season at the appropriate new moon phase. This did not occur. Such entrainment of the spawning cycle by artificial light was reported for *Fundulus heteroclitus*. Fish held under artificial photoperiod of LD 19:5, in the absence of any lunar light, spawned at intervals that paralleled the lunar cycle in the natural habitat, but when artificial moonlight that was out of phase with the natural lunar cycle was superimposed on it, there was a corresponding phase shift in the spawning cycle with respect to the control cycle (**Taylor, 1991**). Thus if fish are able to use circadian rhythms of day and night to "count" the number of days, as apparently happens in other vertebrates (**Follett, 1984**), environmental cues such as moonlight and tidal pressures are constantly used to entrain their endogenous circadian timing system, which otherwise would either reverse to the endogenous period length or become arrhythmic. And when artificial moonlight conditions are used, the endogenous clock is entrained to it, depending on the exposure time.

Although the lunar regimes had no effects on the progress of vitellogenesis and GSI, there was a clear effect on atresia. Fish subjected to continuous new or continuous full moon conditions showed an earlier and more rapid rise in atresia than fish subjected to reversed and control

lunar regimes. It is apparent that both of these constant night light regimes disrupt ovary development. It is unclear why the two changing lunar cycles (control and reversed) are necessary for the production of healthy oocytes. The process of exogenous vitellogenesis in the studied fish, which accounts for the main increase in size of the oocyte, is short with a duration of less than a month. Such a short time limit means that once the process starts, the fish must reach spawning readiness by the next new moon, and since daylength will change very little during that time, reliance on additional cues to time the final stages of ovary development may be necessary. Possible obvious environmental synchronizers include the lunar cycles of moonlight intensity and the environmental changes that accompany tidal fluctuations. The absence of such synchronizers may explain the high early incidence of atresia in the two groups held under constant lunar regimes.

Lunar periodicity of spawning is a common occurrence among many marine tropical and temperate fish species (**Taylor,1984**), and is generally thought to coincide with lunar-associated tidal cycles like spring and neap tides and also moonlight. It is well established that in all the species of siganids where lunar related spawning rhythms have been studied, spawning is associated mainly with the new moon, and rarely with the full moon (**Duray, 1990**). In *Siganus canaliculatus*, spawning is timed with the new moon, occurring sometimes in the first quarter, 4-7 days after the new moon. Even though natural spawning did not occur in this experiment, the first spawning readiness observed during the spawning season was on the first quarter of a new moon (readiness being assessed as the earliest date at which stage V ovaries and maximum gonadosomatic index was observed, and not by readiness to respond to hCG injection). These results agree with those reported for *S. canaliculatus* by **Manacop (1937)** and **Hasse et al. (1977),** *S. argenteus* (**Luchavez & Carumbana, 1982**), *S. luridus* (**Popper et al., 1976**), and *S. guttatus* (**Hara et al., 1986b**). Similar results have also been reported in the sea bass (**Garcia, 1992**), the damselfish *Abudefduf troschelii* (**Foster, 1987**), and the mummichog *Fundulus heteroclitus* (**Taylor & DiMichele, 1980**).

In most cases, the proximal factors responsible for initiating and synchronizing these lunar rhythms are unknown. However, among the factors postulated as cues are tidal rhythms in water turbulence and hydrostatic pressures (**Korringa, 1947; Weld & Meier 1982; Ross,**

1983); tidal regimes in the habitat (**Forward et al., 1982**); photoperiod in combination with tidal fluctuations in water temperature (**Miller et al., 1981**), in combination with time of high tide (**Middaugh & Takita, 1983**), or in combination with interrupted current velocity (**Middaugh & Hemmer, 1984**); and moonlight (**Saigusa, 1980**). The advantages of lunar synchronized spawning cycles can be a reflection of adult or hatchling biology. In *Siganus canaliculatus* the synchronization of the spawning cycle with the new moon emphasizes the importance of dim light in the spawning grounds, rather than the tides associated with it. The fact that the eggs are adhesive plays down the importance of spring tides in dispersal of eggs unless they are deposited on floating objects, a matter that is not well substantiated, since though the spawning grounds of this rabbitfish around Bahrain are not well defined, they are commonly thought to be coral reefs and sea weed areas in near shallow waters on which eggs are deposited. Wave action could be advantageous for the mixing of eggs and sperm (**Lam, 1974**), but in captivity it is obvious that body and tail movements are used for that purpose. Spring tides could be advantageous for the dispersal of hatchlings, but again feeding grounds for juveniles are near shore (personal experience), and it is doubtful that a wide dispersal could be practical. Furthermore, spring tides also occur on full moons, but spawning is timed to the new moon which again stresses the importance of dim light. The main advantage of a dim night is protection against predators that may feed on the spawning adults or on the larvae which hatch after a day. It is therefore safe to assume that the synchronization of spawning to new moon may serve the needs of both the adults and progeny in that it provides protection against predation.

The lack of spawning in fish that have been exposed to simulated or natural new moon regimes, in spite of the fact that they were in spawning readiness, indicates that in the wild there may be other factors that stimulate the process of final maturation and spawning in this rabbitfish. Some factors like hydrostatic pressure caused by rising or falling water levels, and substrate for the attachment of eggs, are absent in holding tanks. In other areas, stimulation of natural spawning in captivity, at ambient photoperiod, temperature, and moon light, by reducing water level in the tank has been reported for *S. canaliculatus* (**McVey, 1972**), and *S. rivulatus* (**Popper & Kissil, 1973**). In the latter study, a fresh water change was reported to be the stimulant. However, there are no reports of natural spawning in captivity in the study area.

During the course of this study, trials were done to stimulate natural spawning by using artificial substrates for the attachments of eggs and by lowering the water level in the holding tank, but without success. There may be other physical or chemical factors involved, this needs further investigation and is beyond the scope of this study. Alternatively, failure to mature and spawn in captivity may be due, not necessarily to the lack of one or more of the above factors, but to the presence of some dominant stress-related factor in the hatchery.

Chapter 5:

Photoperiodic Manipulation of Reproduction in the Female Rabbitfish *Siganus canaliculatus.*

5.1 Introduction:

The availability of a year-round supply of gametes has clear commercial advantage in fish farming. Hormones are used extensively to accelerate spawning (**Lin and Peter, 1996**), but generally these methods have been used successfully to advance only later stages of reproduction, namely ovarian maturation and spawning. Manipulation of the earlier stages of ovarian development with hormones has been achieved with varying degrees of success in salmonids (**Fitzpatrick et al., 1984; Crim and Glebe, 1984**). Such limited success is not surprising, bearing in mind the diversity of hormones involved in the control of reproduction and their complexity of interplay. Such limitations and the fact that individual administration of hormones to large numbers of broodstock is an economic burden on fish farmers, make such methods a less effective and convenient option.

Fish are seasonal spawners which synchronize the time of gamete release with the time of optimum conditions for growth of young fish. Such synchronization with annual variation in the external environment is possible only because fish, like other seasonally-breeding organisms, are able to measure and predict both daily and calendar time. All organisms whose reproduction is stimulated by light are cued by the seasonal changes in daylength (**de Vlaming, 1972**). This is true for all studied salmonids, where synchronization is thought to be achieved primarily by using the changes in ambient daylength as the predictive environmental cue (**Bromage et al., 1993**). The relative ease and low cost of using photoperiod to control spawning time has led to the widespread application of this technique. Another advantage of the method is that photoperiodic cues operate at the highest levels of the neuroendocrine pathway which controls reproduction. Provided appropriate photoperiodic signals are

received, then ovarian development proceeds under the direction of natural endocrine mechanisms (**Bromage & Duston, 1986**).

Most of the knowledge on the role of photoperiod in the control of reproduction comes from studies on salmonids, where the necessity for accurate time measurement and developmental coordination with the external environment is emphasized by the long period required for gonadal development and embryogenesis, and the extensive spawning migrations (**Bromage et al., 1993**). Studies on many salmonids have shown that exposure of fish to advanced or compressed seasonal light cycles induces precocious gonadal development, whereas retarded or extended seasonal photoperiodic cycles result in delayed spawnings (**Bromage et al., 1993**). Manipulation of daylength has been shown to advance or delay spawning time depending on the phase of the reproductive cycle at which it is applied. Continuous long days during the earlier part of the reproductive cycle, before the summer solstice, advances maturation and spawning time , with the extent of the advance decreasing as the exposure time nears the summer solstice (**Whitehead & Bromage, 1980; Bromage et al., 1982a, 1984; Bromage & Duston, 1986; Duston & Bromage, 1987, 1988**). The same treatment from the summer solstice onwards delays these processes, with the maximum delay seen when exposure is near to the winter solstice (**Whitehead & Bromage, 1980; Bromage et al., 1982a&1984; Bourlier & Billard, 1984**). Exposure of rainbow trout to long days just after the spawning season advances the next season by 5-7 months (**Bromage et al., 1984; Scott et al., 1984**).

Short days also advance or delay spawning time in salmonids, depending on the season of application. Exposure of fish to short days early in the reproductive cycle, 2-3 months after spawning, leads to a delay in the time of the next spawning (**Whitehead & Bromage, 1980; Bromage et al., 1984; Duston & Bromage, 1987**). In these studies the time of the natural spawning of the treated stock was found to be a contributing factor. A marked change in the effect of short days was observed when fish were exposed to short days 3-4 months into the reproductive cycle until the summer solstice. This treatment caused an advance in spawning time, the maximal advance being at the earlier part of that period. The combination of long days early in the reproductive cycle followed by short days also advances the spawning time

(**Bromage et al., 1993**). Earlier studies have shown that the definition of a long (or short) day is one which is longer (or shorter) than the day to which the fish has been exposed previously- i.e. long and short are relative, not absolute, terms (**Randall et al., 1987; Duston & Bromage, 1987**). This would explain the effectiveness of such treatments in salmonids spread throughout a wide range of latitudes where seasonal photoperiod ranges are different.

The influence of daily cycles of light and dark on the spawning of fish is not restricted to the temperate and polar regions where the annual change in daylength is pronounced and increases with higher latitude (**Scott and Sumpter, 1983; Elliot et al., 1984**). It is also seen in sub-tropical regions, where there is little seasonal change in daylength (**Sundararaj & Vasal, 1976**).

Spawning of sea bass occurs under short and/or increasing daylength (**Carrillo et al., 1993**), in trout and also other salmonids ovulation occurs under a short or decreasing daylength (**MacQuarrie et al., 1978&1979; Whitehead et al., 1978; Bromage et al., 1984; Elliot et al., 1984**), whereas in general flatfish spawn under long or increasing daylengths (**Htun-Han, 1977**). Although the final stages of gonad development in these fish occur under a specific daylength, the initial stages of development would have occurred much earlier under a very different photoperiod. A clear example is the rainbow trout where histological changes in the ovary are apparent about a year before ovulation (**Elliott et al., 1984; Sumpter et al., 1984**), and it has been suggested that a series of cues are involved in the triggering and modulation of the different stages of ovarian growth and recrudescence (**Bromage et al., 1982b, 1984**). This leads to the conclusion that, in the salmonids at least, all stages of ovarian development must be considered when using photoperiodic methods to alter the spawning times.

Photoperiodic manipulation of spawning time has been used successfully for many salmonids and sea breams. It was assumed that rabbitfish would respond to similar environmental cues and that it should be possible to advance or delay the spawning period by using similar techniques. Such alterations would be useful in commercial culture by extending the period of gamete availability and changing harvest time to coincide with favorable market periods. The aim of the following experiments were to test the effect of various photoperiodic regimes on

spawning time with a view to devising practical techniques for extending the season of gamete production.

5.2 Materials and Methods:

5.2.1 Experiment Conditions:

The experiments were conducted on an experimental fish farming station which lacked equipment for maintaining water temperatures below ambient. Therefore, it was not possible to maintain a constant water temperature during investigations on the effect of photoperiod. Cooling the large volumes of water would have been prohibitively expensive. However, heating of water was possible and was used in some experiments to maintain less variable temperatures in winter and early spring.

Fish used in these experiments were from a stock of resting or virgin adult fish with a total length over 15 cm, maintained under ambient photoperiod and temperature (for maintenance procedure see Chapter 2, Section 2.2.1 A).

The tanks used were 6 m^3 (3mx2mx1m) fibreglass reinforced plastic (FRP) with white inner surface (Fig.5.1). The sides of each tank were extended upwards to a height of 90 cm using 6 mm marine plywood, the top was covered with the same material, this formed an inverted box on top of the tank. The inside of the tank was made light proof by sealing all joints and holes with black silicon sealant, the tanks were also checked from the inside for any light leakage coming from outside. A small 60X80 cm opening was left on the side for access. This was provided with a thick black vinyl cover secured at the top and fastened on the other three sides with Velcro strips. Each tank was fitted with two sets of 40 watt twin-tube water-proof fluorescent lights suspended 100 cm above water surface giving an average light intensity of 500 lux/m^2 at the water surface (this figure was based on light intensities at water surface in indoor spawning tanks). Individual light sets were connected to a 24 hour timer switch (Kawamura Electric Ind. Co. Ltd., 30A 1P-ST). Heating, where needed, was provided using 3kW immersion heaters supplied with thermostats with an accuracy of ±1°C.

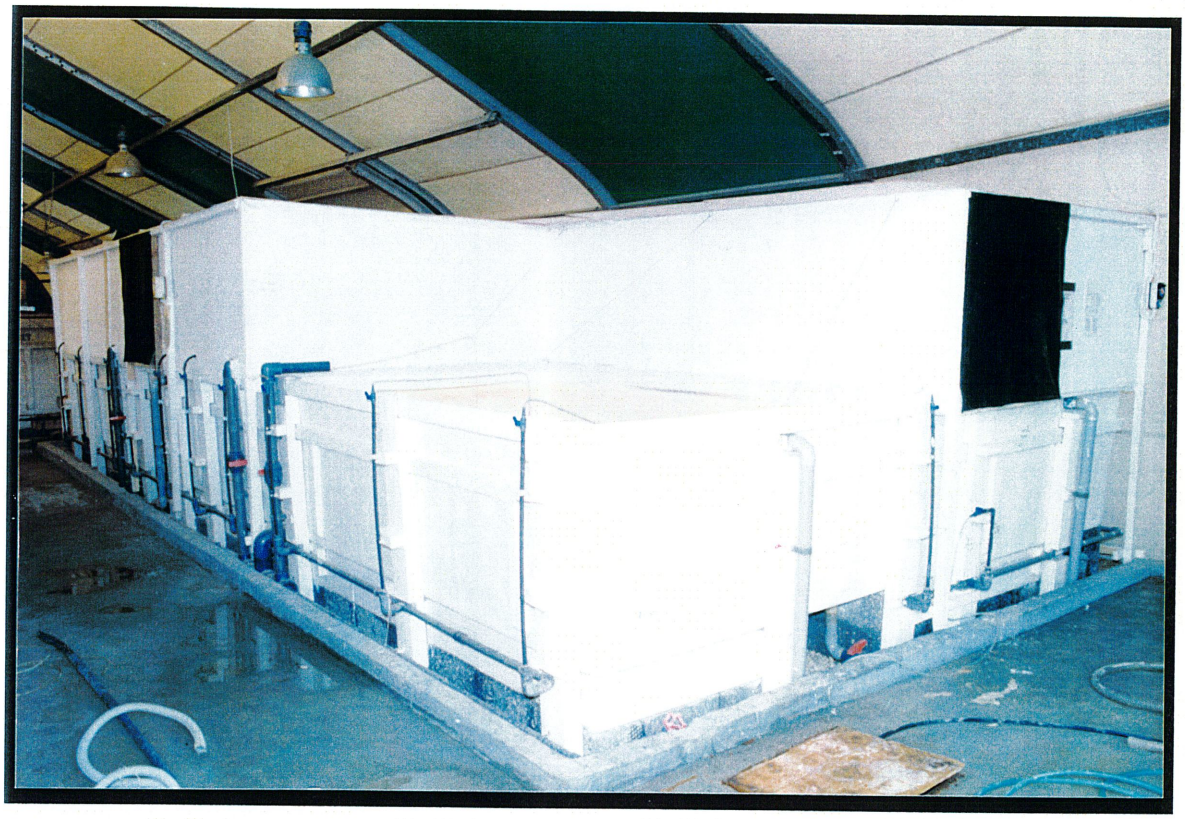


Figure 5.1- Modified tanks used for testing the effect of various photoperiod regimes on the ovary development of *S. canaliculatus*.

All tanks were provided with a continuous supply of aerated sea water. Bottom cleaning of the tanks was done every two weeks using a siphon connected to a pump and the whole tank was cleaned at each sampling time, when the fish were also given a 24 hour bath treatment in 0.5 ppm Prefuran (Argent Chemicals, USA) as a measure of disease prevention. All environmental factors other than photoperiod, and where possible temperature, were ambient.

There is little work on effect of photoperiod on tropical and semi-tropical fish, but it is assumed that they are also governed by the same environmental factors of which the main would be photoperiod and temperature. The specific aims were to examine the effects on the reproductive cycle of various photoperiodic regimes including constant short days, constant long days, combination of short and long days, compressed seasonal light cycles, variations in the seasonal time of application of light regimes, and the effect of constant temperature on the response to selected regimes.

For these purposes, two series of experiments were conducted, the first was under ambient water temperature and the second under constant temperature of 25±1°C. In the first series, the various photoperiods tested were natural, simulated normal, constant short days, constant long days, combinations of 3 months of short and 3 months of long days (i.e. square-wave light regime) and several regimes of ambient to constant long day switches applied at different times in the annual reproductive season. In the second series temperature in the tanks was gradually raised from ambient to 25±1°C before the start of the experiments. Only switches from ambient to long photoperiod were used.

Long and short days in both series refer to 18L:06D and 06L:18D light regimes, respectively. The experiments were ended when fish in their respective groups completed vitellogenesis as indicated by abdomen condition and oocyte diameter, and also confirmed by histological observations, or after the next spawning season, whichever occurred first. The natural spawning season of this fish is April, with about a one week shift earlier or later, depending on water temperature and lunar phase.

The various experimental groups were subjected to the following photoperiodic treatments :

Series 1: Ambient Temperature-

The photoperiodic regimes described below are illustrated in Figs.5.2 and 5.3. All experiments were conducted for one full reproductive cycle starting in July, two months after the natural spawning season, and were ended at the following spawning season or when the fish completed vitellogenesis, whichever came first.

Group A : Natural Normal Photoperiod-

The control group. Fish were exposed to ambient normal photoperiod under natural light.

Group B : Simulated Normal Photoperiod-

Since artificial light was to be used in these experiments, it was decided to compare fish exposed to normal photoperiod using artificial light with Group A. If no difference was found, this group was to be used as a control against which to compare all other groups. Fish in this group were subjected to simulated normal photoperiod under artificial light. The timer was reset weekly to natural daylength.

Group C : Constant Short Photoperiod-

Constant short photoperiod applied shortly after spawning delays spawning in salmonids. It was assumed that this regime would have a similar effect on rabbitfish. Fish were exposed to continuous short days.

Group D : Constant Long Photoperiod-

It was hypothesized that this treatment would accelerate spawning. Fish were subjected to constant long days.

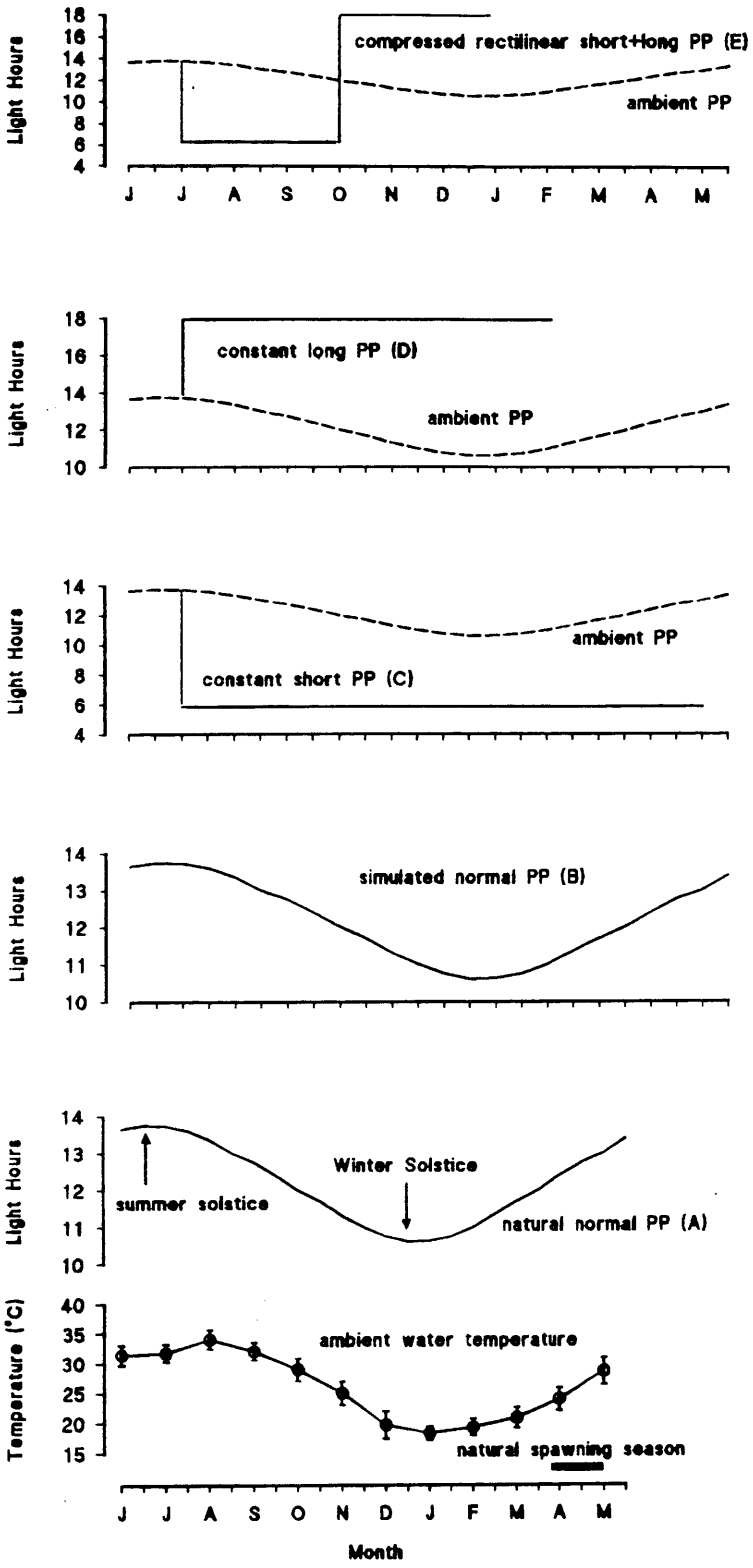


Figure 5.2– Photoperiod regimes used for the manipulation of reproduction in *S. canaliculatus* under conditions of ambient temperature. PP= photoperiod, WS= winter solstice.

Group E : Compressed Rectilinear Short+Long Photoperiod-

A combination of long and short days advances spawning in salmonids. This treatment was designed for the fish under study under the assumption that the aggregate effect of two photoperiodic changes will enhance acceleration of spawning. The difference between the two treatments is that in salmonids the treatment starts with long days followed by short, while the design of this experiment is short followed by long, to ensure that the direction of photoperiodic change matches the direction of change of the natural photoperiod at that time. Fish were exposed to 3 months of short days from July, followed by three months of long days from October.

Group F : Switch from Ambient to Long Photoperiod in October-

The time of application of photoperiodic change along the reproductive cycle has been reported to influence the spawning time for salmonids. The aim of this and the following two experiments was to determine any such influence in *S. canaliculatus*. Fish were subjected to a switch from ambient to long days 10 weeks before winter solstice, which is approximately half way along the annual reproductive cycle.

Group G : Switch from Ambient to Long Photoperiod in November-

Fish were switched from ambient to long days in late November, three weeks before the winter solstice.

Group H : Switch from Ambient to Long Photoperiod in January-

Fish were switched from ambient to long days in January, two weeks after the winter solstice.

Group I : Switch from Ambient to Long Photoperiod in February-

Fish were switched from ambient to long days in February, six weeks after the winter solstice.

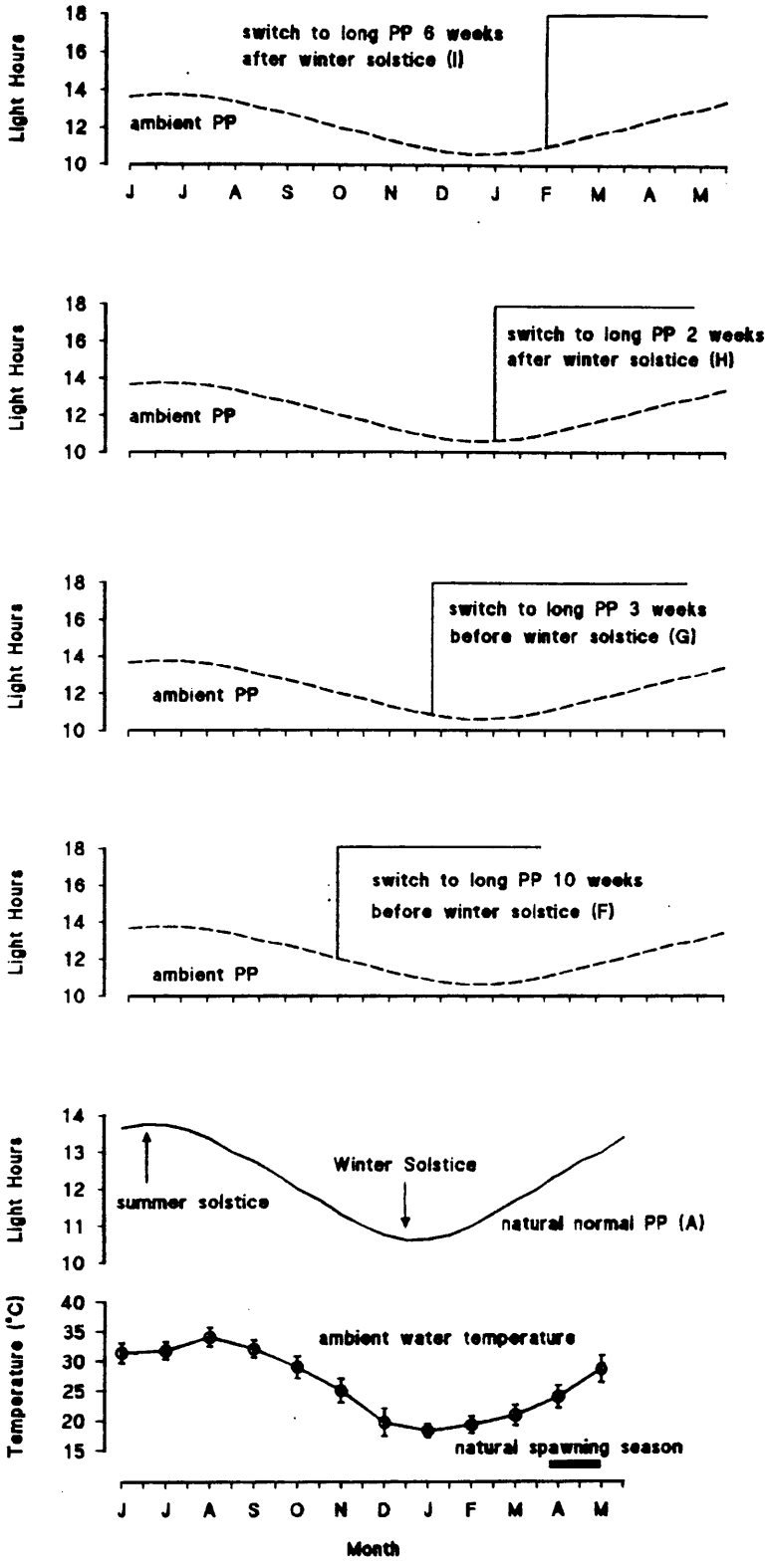


Figure 5.3– Photoperiod regimes used for the manipulation of reproduction in *S. canaliculatus* under conditions of ambient temperature. PP= photoperiod, WS= winter solstice.

Series 2: Constant Temperature-

Four groups of fish were subjected to the photoperiodic regimes described below. Until the winter solstice the fish had been held at ambient temperature. Thereafter they were established in tanks for two weeks, after which water temperature was increased from 20°C to 25 °C over a period of two weeks. One of the four groups was exposed to the ambient photoperiod while the other three were maintained on photoperiods which continued to decrease after the winter solstice for 2, 8 and 14 weeks before switching to a long constant photoperiod of 18L:06D. The reason for the continuing decrease was to deny the fish any information on the winter solstice and the usual increase in photoperiod thereafter. The objective of this series was to determine if the reproductive cycle could be delayed by effectively delaying the winter solstice. The following photoperiodic treatments are illustrated in Figure 5.4:

Group J : Natural Normal Photoperiod-

The control group. Fish were maintained under ambient photoperiod.

Group K : Switch from Ambient to Long Photoperiod in January-

Photoperiod continued to decrease for 2 weeks after the winter solstice before being switched to a constant long day.

Group L : Switch from Ambient to Long Photoperiod in February-

Photoperiod continued to decrease for 8 weeks after the winter solstice before being switched to a constant long day.

Group M : Switch from Ambient to Long Photoperiod in April-

Photoperiod continued to decrease for 14 weeks after the winter solstice before being switched to a constant long day.

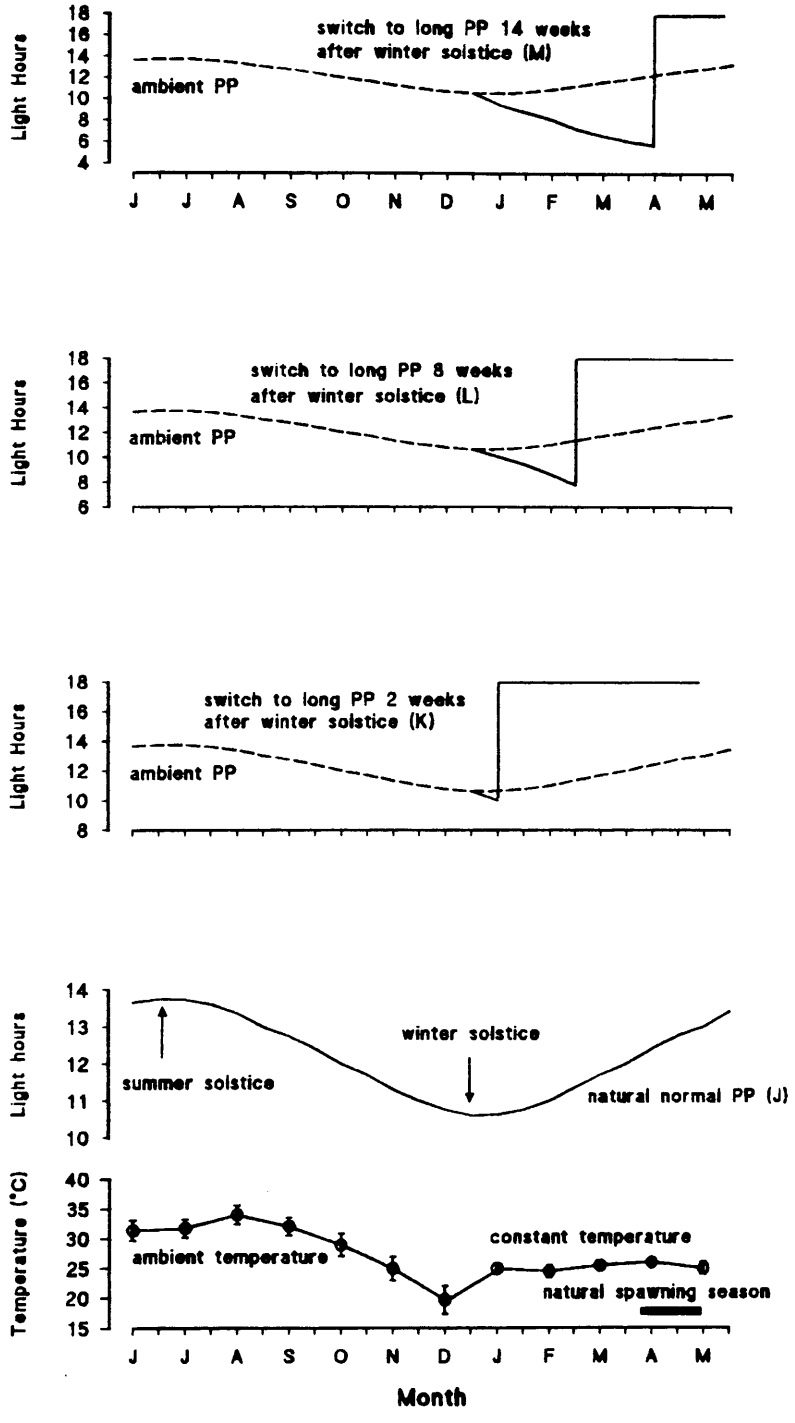


Figure 5.4– Photoperiod regimes used in the manipulation of reproduction in *S. canaliculatus* under conditions of constant temperature (25±1°C). PP= photoperiod, WS= winter solstice.

5.2.2 Sampling Procedure:

S. canaliculatus with undeveloped gonads cannot be sexed using external features. Therefore, 60 fish were stocked in each tank four weeks before the start of the experiments. Fish were tagged using numbered internal anchor tags (Floy Tags, USA). All equipment was disinfected in a commercial iodine solution (Betadine, NAPP LABS.), and an antiseptic ointment (Soframycin) was applied to the puncture points. From the time when sexing was possible using biopsy, the first 15 females were selected and thereafter repeatedly measured until the end of the experiment. Sufficient males were left in the tank for induced spawning trials.

Prior to handling fish, the water level was reduced. Fish were kept anaesthetized with 2-phenoxyethanol (100 ppm) and covered with a damp cloth during sampling. About one ml of blood was collected from the caudal vessel using a one ml syringe fitted with a 25GX5/8" needle and transferred to 1.5 ml capped conical polycarbonate sample vial. The collected blood was kept on ice during sampling and then stored in the refrigerator for a minimum of six hours to allow coagulation. The sample was then centrifuged at 2400 rpm for 10 minutes and the supernatant serum decanted into a 0.5 ml sample vial and stored at -20° C until further treatment.

Oocyte samples were collected using a 0.8 mm internal diameter cannula, and were fixed in 4% formaldehyde in neutrally buffered saline (Chapter 2, Table 2.4). Sub-samples from extra females in the same tanks were used for dissection of ovaries which were also preserved in the same fixative, and also for blood and other sampling at sampling dates after the fish were induced to spawn and the experiment had ended.. In series 'A', sampling was done at monthly intervals in the first week of the month. In series 'B', sampling was done at intervals of 45 days.

5.2.3 Measurements:

The following measurements were made on the same fish in the various treatment groups.

1. Time of Vitellogenesis- Oocyte and ovary samples were processed for histological determination of the development stage (for details see Chapter 2, Section 2.2.2).

2. Serum Oestradiol (E_2) - Blood serum samples were assayed for serum oestradiol-17β (E_2) (see next section).

3. Biopsy Time- The earliest date when the maximum number of fish could be biopsied was determined from the number of fish in which the cannula passed easily into the ovary and from which oocytes were obtained. Biopsy is possible only in fish in which vitellogenesis has started, as determined earlier in Chapter 2, Section 2.3.5.

4. Spawning Readiness- Completion of vitellogenesis and readiness to spawn was tested by attempting to induce spawning using 1.0 IU g^{-1} body weight of human chorionic gonadotropin (hCG).

5. Macroscopic Observations- Abdomen condition and ovary color were described from fresh samples.

6. Oocyte Diameter- Mean oocyte diameters in ovaries which had completed vitellogenesis were measured using a digitizing pad. Measurements were done on triplicate sample of 30 vitellogenic oocytes from each of three fish in each group (for details see Chapter 2, Section 2.2.2).

7. Incidence of Atresia- The number of atretic vitellogenic oocytes was determined from histological slides of ovaries from three fish in each group at each sampling interval. In each ovary, three counts of 100 vitellogenic oocytes were taken. A microscope slide with an etched square was placed over the slide to be counted and oocytes that lay within the square or had their nucleus touching the top or right margins were counted.

5.2.4 Serum Oestradiol (E_2):

Assays for a range of known maturation inducing steroids (MIS), including 17α,20β-P, revealed very low levels of steroid. Instead oestradiol was assayed as a possible, but less appropriate indicator, of the early stages of maturation. The serum concentration of 17β-

oestradiol (E_2) was determined using direct radioimmunoassay (RIA) according to standard procedures. The assay had to be sensitized due to low levels of E_2 by adjusting the concentrations of label and antiserum and varying their ratios. Problems were encountered due to strong interference by serum lipids in vitellogenic fish and strong plasma binding proteins. Steps to denature plasma binding proteins and extract lipids were used based on the procedure described by **Scott et al. (1984)**. Recovery of ^{3}H-labeled E_2 through the extraction procedure was at least 67% from serum of vitellogenic fish and at least 80% from serum of non-vitellogenic fish.

1. Procedure: A flow chart summarizing the procedure is given in Fig.5.4A. Extraction was done using Pyrex borosilicate glass tubes (75 X 10 mm) following trials which showed that recovery of ^{3}H-labeled steroids was only 38% from polyethylene tubes compared with 92% from glass tubes.

Frozen serum samples were thawed to room temperature, centrifuged for two minutes at 1500 rpm and 100 µl aliquots were transferred to assay tubes. After addition of recovery label (25 µl of ^{3}H E_2 of known specific activity), samples were vortexed briefly and allowed to equilibrate for 30 minutes prior to incubation at 70°C for one hour to dissociate steroids from endogenous plasma binding proteins. After returning the samples to room temperature, E_2 was extracted by adding 1 ml of ethyl acetate to 100µl serum samples (10:1 v/v) and allowing the samples to stand for 15 minutes with vortexing at intervals of 4-5 minutes. The samples were then centrifuged for two minutes at 1500 rpm to separate the ethyl acetate phase from the aqueous phase. The upper ethyl acetate phase containing the extracted steroid was transferred to another borosilicate tube and was evaporated in a vacuum centrifuge (GyroVap). The dried extracts were dissolved in a 1:1 mixture of 90% ethanol and heptane, were vortexed for one minute, and centrifuged at 2400 rpm for 15 minutes. The lower 90% ethanol phase was transferred carefully to a polyethylene tube leaving behind the heptane phase containing the extracted lipids. The ethanol phase was evaporated, redissolved in 320 µl of assay buffer (Appendix A) with thorough vortexing and warming to 37-40°C to promote solution. 100µl aliquots were transferred to each of three assay tubes, two of which were used for assay and the third for recovery measurement. To each assay tube, 100µl of tritiated E_2 label

Figure 5.4A - Summary of procedure for radioimmunoassay of serum E_2 for *Siganus canaliculatus*.

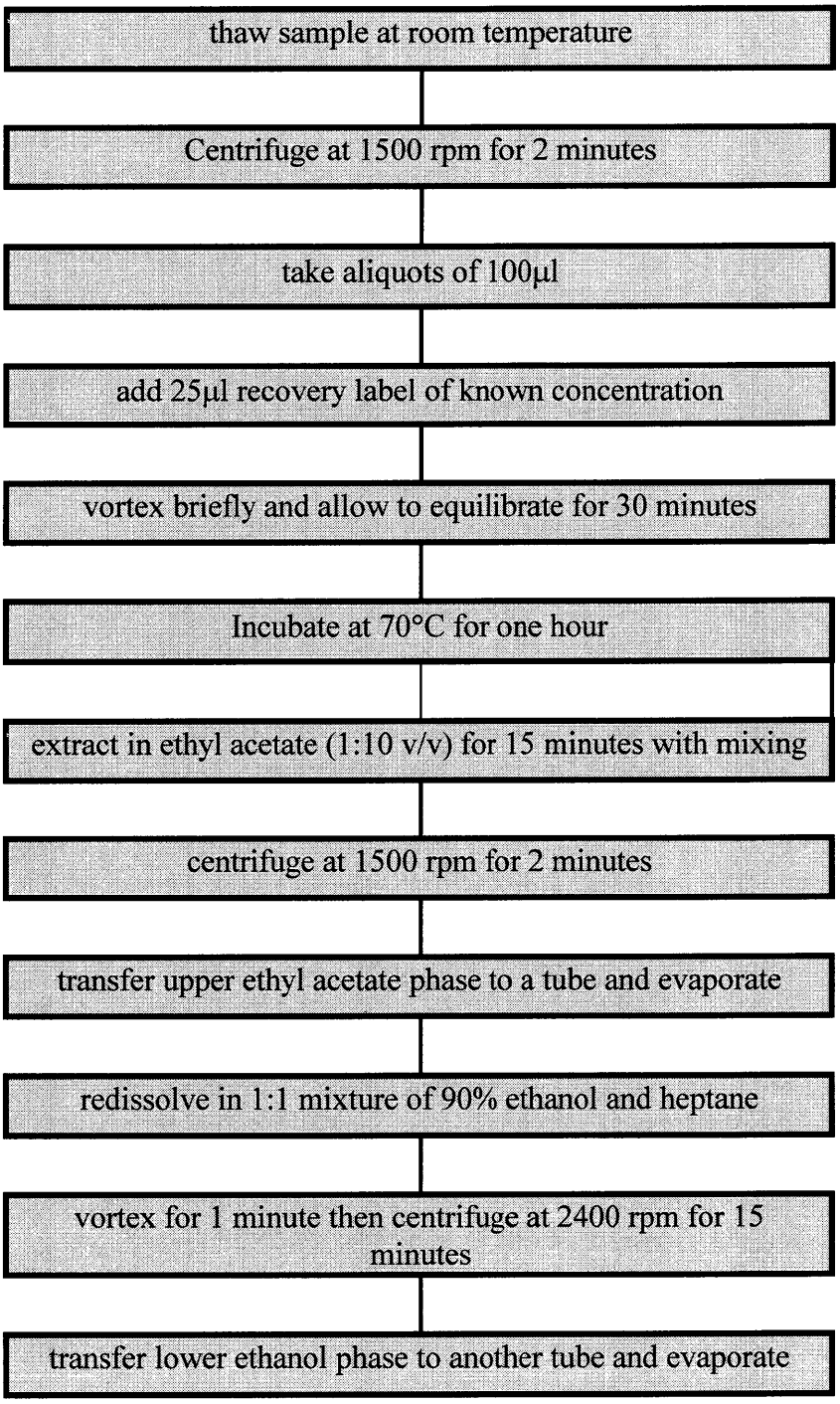
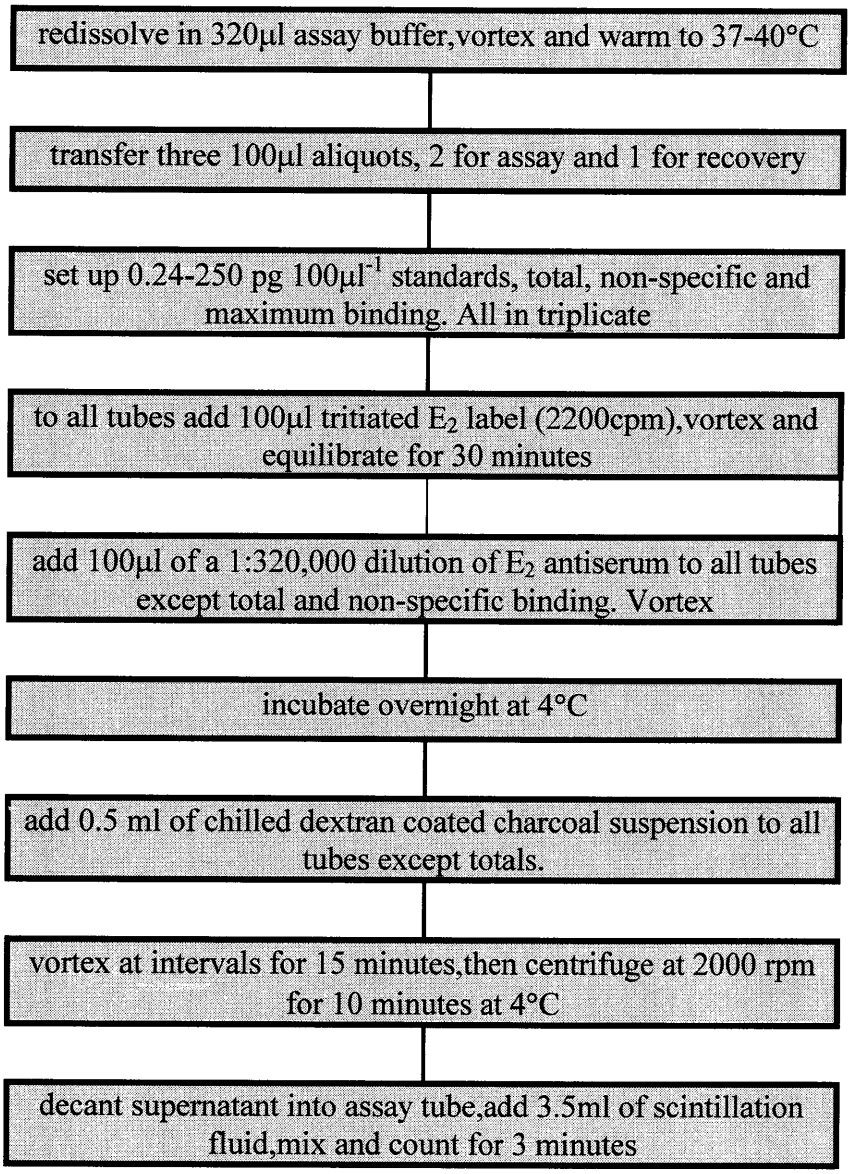


Figure 5.4A - (Continued).



(2200 cpm of 93Ci/mmol 2,4,6,7-^{3}H Oestradiol) were added, mixed and left for 30 minutes. 100µl of a 1:320,000 dilution of E_2 antiserum was added to each assay tube and vortexed. Standards ranging from 0.24 - 250 pg E_2 100µl $^{-1}$, non-specific binding and maximum binding samples were set up in triplicate. After overnight incubation at 4°C to permit the distribution of E_2 label to reach equilibrium, 0.5 ml of chilled dextran-coated charcoal suspension (Steranti Separex, 2 charcoal tablets in 100 ml assay buffer) was added to each tube. After vortexing and standing for 15 minutes at 4°C, the tubes were centrifuged at 2000 rpm for 10 minutes. The supernatant was decanted into a scintillation vial, 3.5 ml of scintillation fluid (OptiPhase Safe, LKB) was added, mixed thoroughly and counted for 3 minutes (Wallac-Win Spectral 1414 Liquid scintillation Counter). The unknown values were calculated using MultiCalc Software.

2. Sensitivity: The sensitivity of the standard curve, taken as the smallest dose of E_2 significantly different from zero at the 95% confidence limit, was 2.2 pg per tube. Serum, stripped of steroids by mixing with activated charcoal (1 ml serum+10mg activated charcoal) for one hour, was used as the blank and as the fluid to which a known mass of standard E_2 was added for sensitivity tests. The stripping efficiency of the charcoal was pre-tested using labeled E_2 and was found to be 89%.

3. Accuracy: Assay accuracy was tested by using built-in recovery checks. A known mass of standard (cold) E_2 was added to 100µl of serum taken from a pool of serum from fish in the resting stage of gonad development with known E_2 levels which are also the lowest values measured. The mean recovery of exogenous E_2 from 12 replicates was 98±2.2% (mean±s.e.m.).

4. Specificity: The E_2 antiserum and data on its cross reactivity with structurally related steroids (Table 5.1) was kindly provided by **Dr. Zvi Yaron** (Department of Zoology, Tel-Aviv University).The percentage cross reaction was less than 0.5% for steroids other than oestradiol.

Table 5.1 Cross reaction of various steroids with standard oestradiol 17β using the rabbit anti-oestradiol-6-BSA serum (Courtesy of Alissa Bogomolanya and Zvi Yaron, Department of Zoology, Tel-Aviv University, 1981-1982).

Steroid	Maximal mass tested (pg)	% Cross reaction
Oestradiol	10^3	100
Oestrone	10^5	0.3444
Oestriol	10^5	0..4246
16α-hydroxyestrone	10^5	0.0674
Testosterone	10^6	0.0031
Androsterone	10^5	<0.0001
Dehydroisoandrosterone	10^5	<0.0001
11β hydroxytestosterone	10^5	<0.0001
11-Ketotestosterone	10^5	<0.0001
Cortisol	10^5	<0.0001
Cortisone	10^5	<0.0001
Corticosterone	10^5	<0.0001
11 deoxycortisol	10^5	<0.0001
Deoxycorticosterone	10^5	<0.0001
Pregnenolone	10^5	<0.0001
Progesterone	10^5	<0.0001

5. Precision: Assay precision was determined by evaluating intra- and interassay variation. The former was determined by 10 replicate measurements from a serum pool in a single assay and the latter by replicate measurements of the same serum pool in all assays. The intra-assay coefficient of variation was 5.6%, the interassay coefficient of variation was 9.1%.

5.2.5 Statistical Analyses:

Oestradiol results are expressed as mean values ± the standard error of the mean (s.e.m.). The serum E_2 profiles within treatment groups were tested using Kruskal-Wallis Nonparametric ANOVA Test. The differences between groups at sampling intervals was also tested using the same test followed by Dunn's Multiple Comparisons Test. Oocyte diameters and atresia numbers were tested using a One-way analysis of variance. Differences between time of completion of vitellogenesis between the groups were tested by One-way analysis of variance at the 95% confidence interval for each month, followed by Tukey-Kramer Multiple Comparisons Test. All tests were performed on a Minitab statistical package V.10 (Minitab, Inc.) and GraphPad Instat2 Statistical Software.

5.3 Results:

The effects of various manipulations of photoperiod on the ovarian cycle of *Siganus canaliculatus* are as follows:

5.3.1 Series 1: Ambient Temperature-

1. Time of Vitellogenesis :

Under ambient photoperoid (natural, normal PP, Fig.5.5), the first histological evidence of vitellogenesis (stage II) was observed in February, 7 weeks after the winter solstice. Full vitellogenesis (Stage V) was observed in early April until early May, which is within the natural spawning season for this fish. The timing of natural spawning in wild fish, and of inducible spawning in captive fish varies slightly from one season to the next, depending on

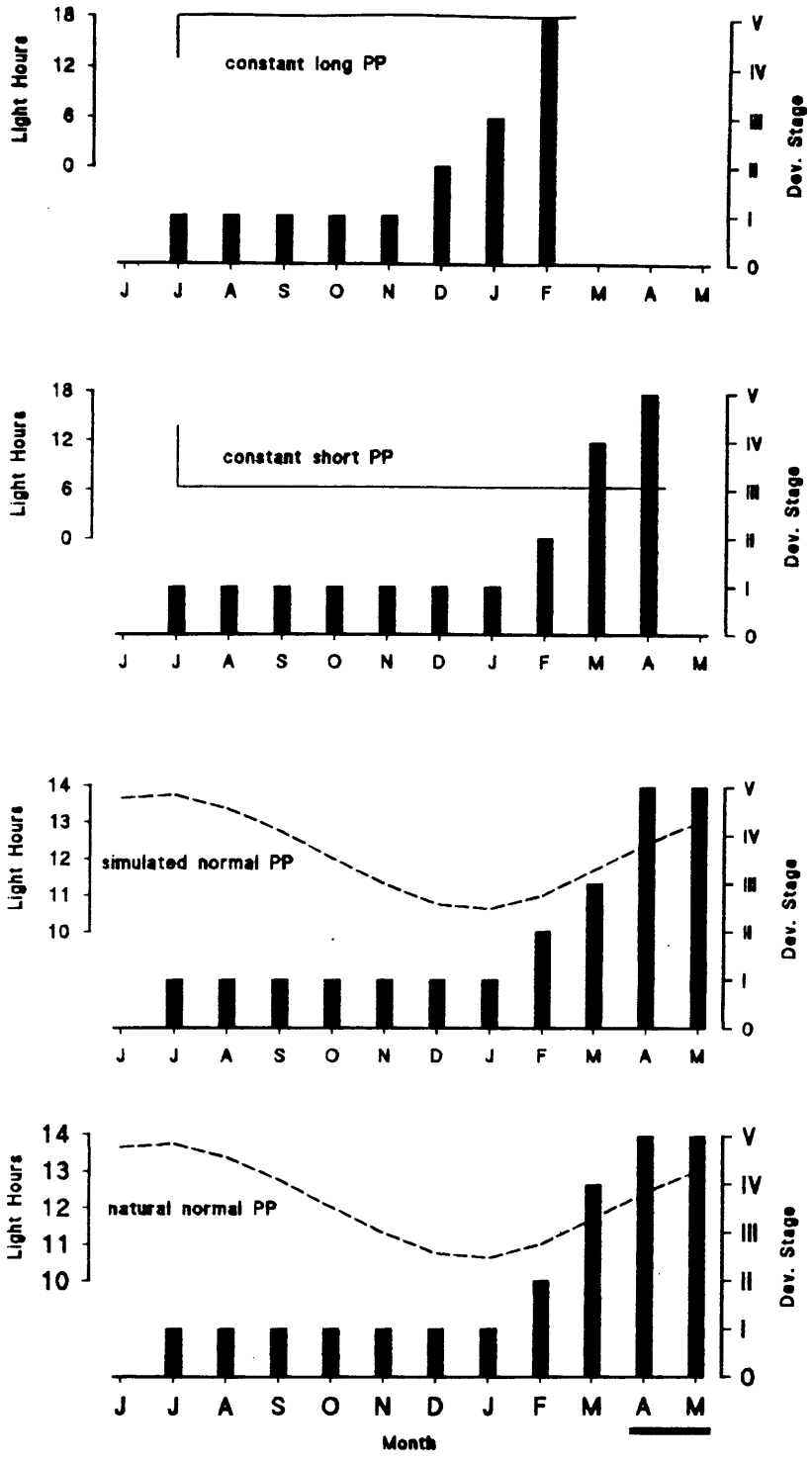


Figure 5.5– Monthly stages of ovary development in *S. canaliculatus* subjected to various photoperiod regimes under ambient temperature. Solid bar under X axis indicates the natural spawning season. I=virgin ovary, II=early developing, III=mid developing, IV=late developing, V=running ripe, VI=maturing. PP=photoperiod. Sample number=3.

the ambient water temperature and the first occurrence of the new moon at the beginning of the spawning period.

Under simulated normal photoperiod using artificial light, histological evidence of vitellogenesis was first observed in February (Fig.5.5). Full vitellogenesis was observed in April until late May, which is similar to the timing seen in the previous group under natural normal photoperiod. The periods and timings mentioned hereafter are related to that of the group held under a simulated normal photoperiod (the control group). The inducible spawning date for the control group was taken as the first of April.

Fish subjected to constant short days from two months after the previous spawning season showed similar timing in the onset (February) and completion (April) of vitellogenesis compared with the control group (Fig.5.5).

Constant long photoperiod had a different effect on ovary development. The onset (December) and completion (February) of vitellogenesis were both advanced by approximately 8 weeks compared with the controls. The timing of completion of vitellogenesis was significantly different from the control group ($p<0.001$).

In fish subjected to a compressed photoperiod of 3 months of short days followed by 3 months of long days, the onset of vitellogenesis was advanced (December), as was the completion of vitellogenesis (January) by approximately 12 weeks (Fig.5.6). The timing of initiation and completion of vitellogenesis were both significantly advanced as compared to the controls ($p<0.001$ in both cases).

The exposure of fish to a long photoperiod from four months after the previous spawning season, i.e. 10 weeks before the winter solstice, also advanced the timing of gonad development. Vitellogenesis was well underway (stage III) by December (Fig.5.6) and was complete by February, some 8 weeks before the controls. The timing of completion was found to be significantly earlier than the control ($p<0.001$).

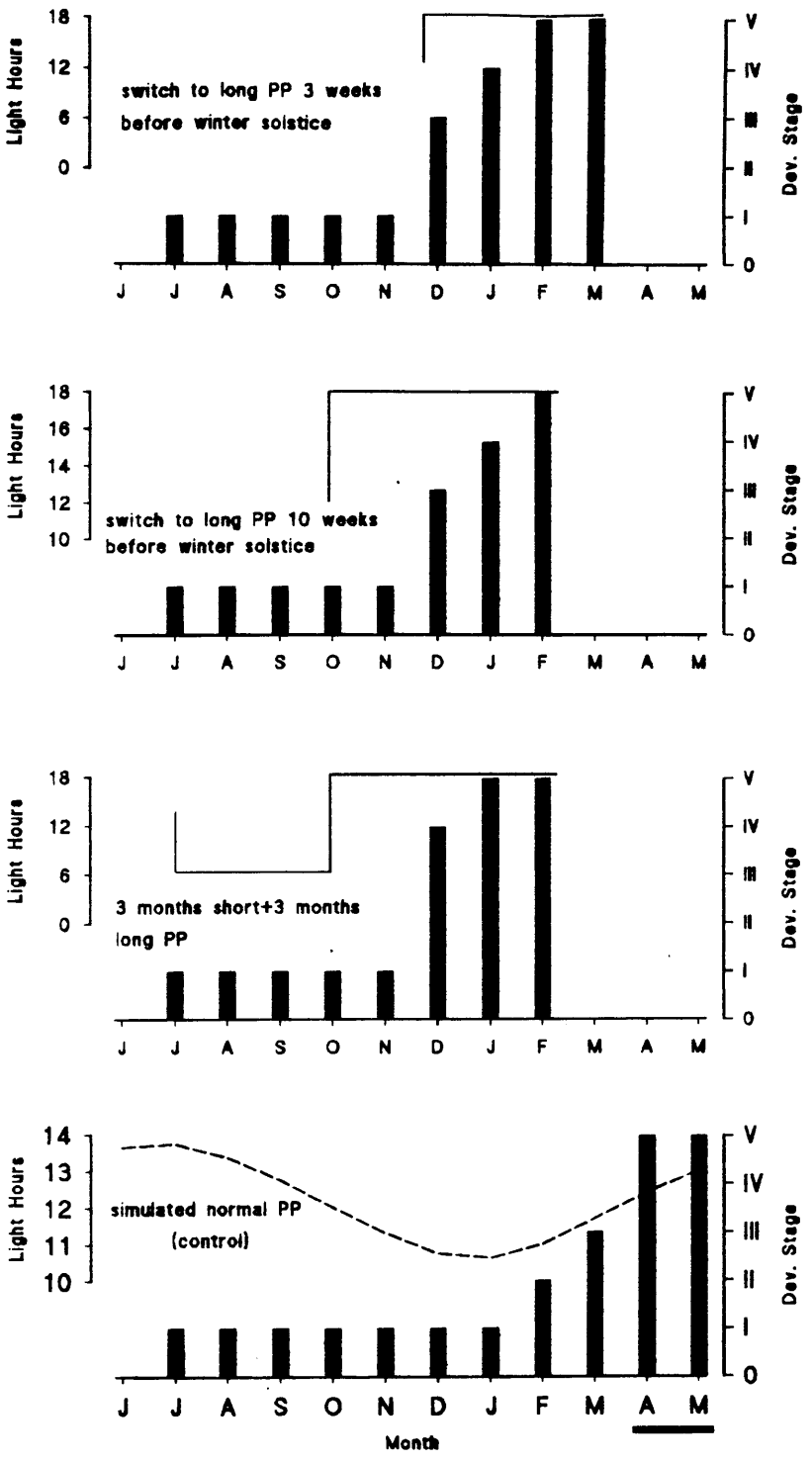


Figure 5.6– Monthly stages of ovary development in *S. canaliculatus* subjected to various photoperiod regimes under ambient temperature. Solid bar under X axis indicates the natural spawning season. I=virgin ovary, II=early developing, III=mid developing, IV=late developing, V=running ripe, VI=maturing. PP=photoperiod. Sample number=3.

In fish switched from ambient to a long photoperiod from 3 weeks before the winter solstice, vitellogenesis was well underway (stage III) by December and complete by February, 8 weeks before the controls (Fig.5.6). The time of completion of vitellogenesis was significantly earlier than in the controls ($p<0.001$).

In fish switched from ambient to a long photoperiod from 2 weeks after the winter solstice, vitellogenesis started in January and was complete by March (Fig.5.7). Completion of vitellogenesis was significantly earlier than in the controls ($p<0.001$).

Switching from ambient to a long photoperiod 6 weeks after the winter solstice had no effect on either the onset (February) or completion (April) of vitellogenesis compared to the controls.

In summary, a switch to long days prior to the winter solstice accelerated the initiation and completion of vitellogenesis. A delay in the switch to long days until after the winter solstice, or the absence of long days, did not delay ovarian development compared with the controls. The one exception was the group in which the switch to long days was made only 2 weeks after the winter solstice. In this group ovarian development was accelerated slightly but not significantly.

2. Serum Oestradiol (E_2):

Serum E_2 levels showed a very similar seasonal pattern in the two groups held on a natural, normal and on simulated, normal photoperiods (Fig.5.8). In both, the serum E_2 rose from <0.2 ng ml^{-1} in July, after the end of the spawning season, to a peak of approximately 1.2 ng ml^{-1} in April at the completion of vitellogenesis.

Serum oestradiol levels in all treatment groups showed a pattern of increase coincident with vitellogenesis. For each of the groups there was a very close correspondence, on the one hand between the first distinct rise in serum E_2 and the initiation of vitellogenesis, and on the other hand between peak serum E_2 and completion of vitellogenesis. i.e. photoperiodic regimes

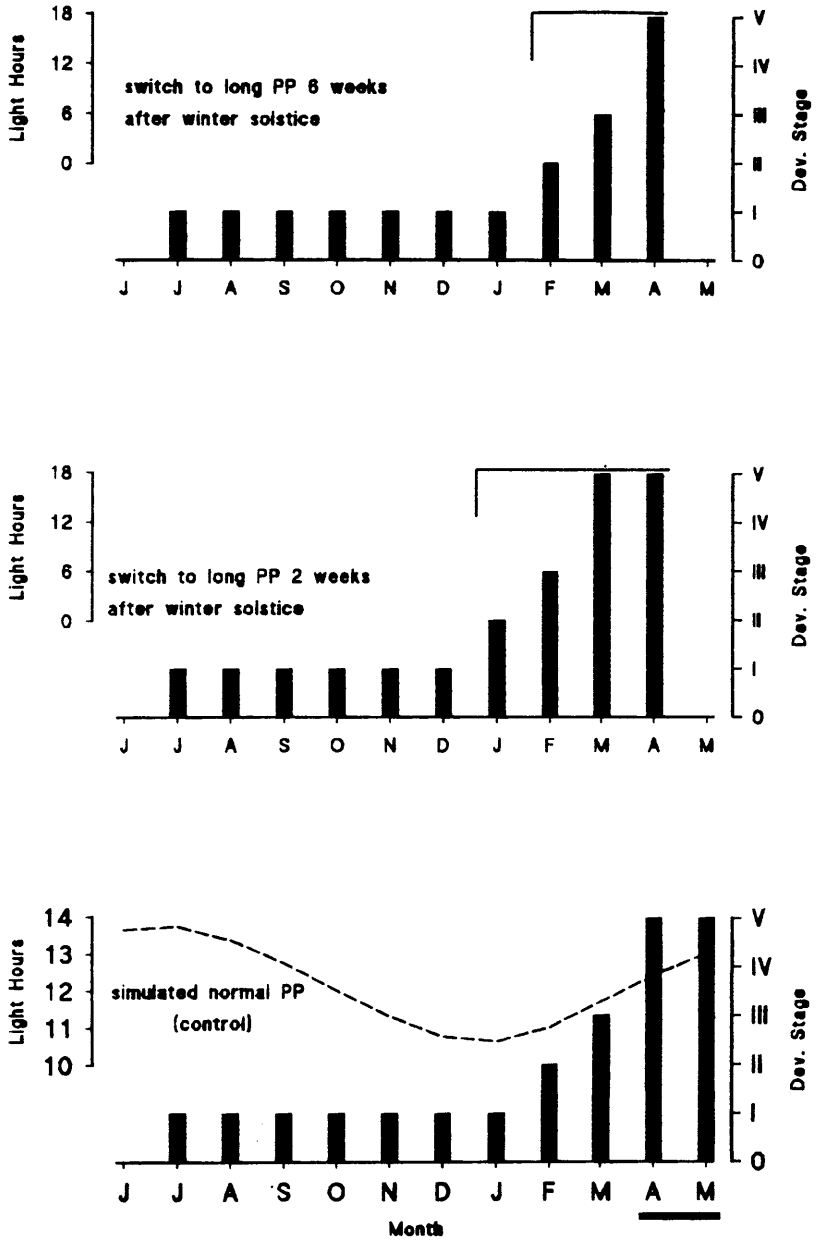


Figure 5.7– Monthly stages of ovary development in *S. canaliculatus* subjected to various photoperiod regimes under ambient temperature. Solid bar under X axis indicates the natural spawning season. I=virgin ovary, II=early developing, III=mid developing, IV=late developing, V=running ripe, VI=maturing. PP=photoperiod. Sample number=3.

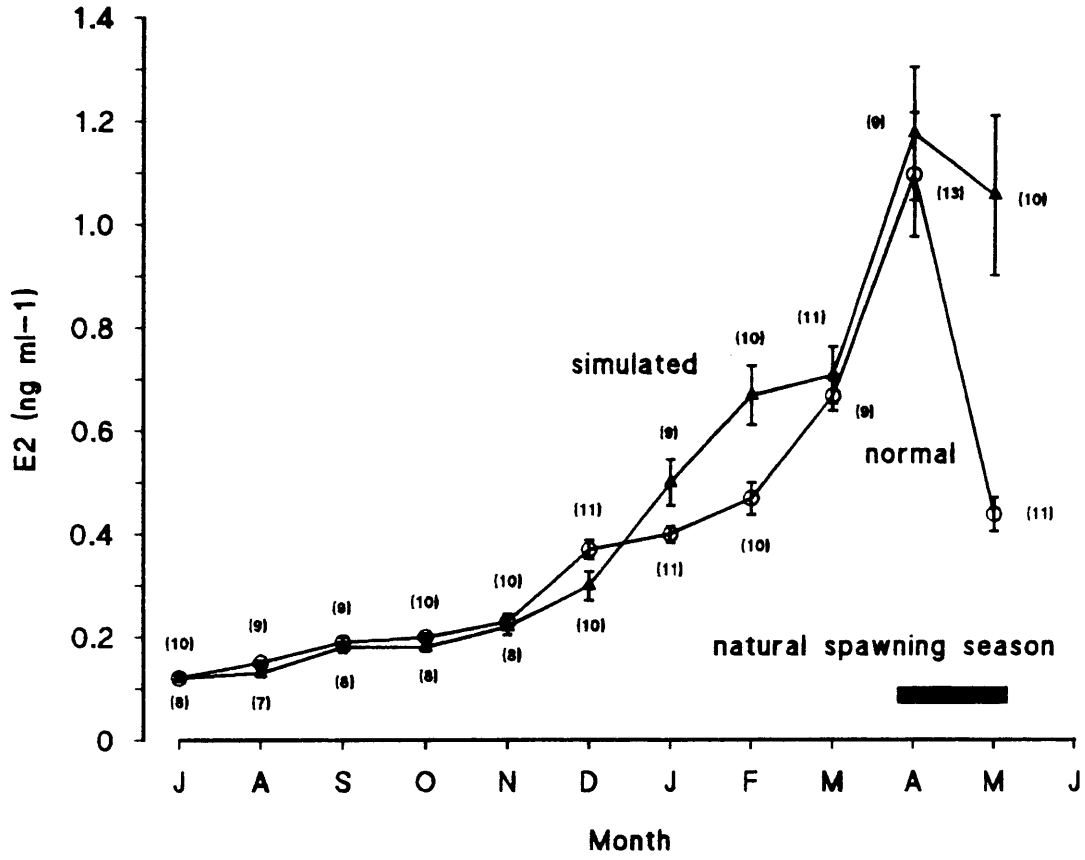


Figure 5.8– Monthly serum oestradiol levels (mean±s.e.m.) in two groups of *S. canaliculatus*, one subjected to natural normal photoperiod and the other to simulated normal photoperiod (using artificial light) under conditions of ambient temperature. Numbers on or below data points indicate sample size.

which advanced the initiation and completion of vitellogenesis, in comparison with the control group, also advanced the timing of the initial increase and peak levels of E_2 (Figs. 5.9, 5.10, and 5.11). Similarly, regimes which did not advance the timing of vitellogenesis also did not advance the seasonal profiles of E_2 (Figs. 5.9, 5.10, and 5.11).

3. Biopsy Time:

For each of the 8 groups, there was an exact match between the time at which it was first possible to insert a biopsy cannula along the oviduct, and the time of completion of vitellogenesis, i.e. regimes which advanced vitellogenesis also advanced the earliest possible biopsy time (cf. Figs. 5.5, 5.6, and 5.7 with Fig.5.12) by a similar interval, e.g. by 3 months in the group exposed to 3 month of short + 3 months of long days.

4. Spawning Readiness:

Readiness to spawn was used as a measure of the normality of oocytes in the groups subjected to various photoperiodic regimes. Fish in all groups responded to the exogenous hormone and spawned. The number of spawned fish out of the total number injected varied in each group (Fig.5.13).

5. Macroscopic Observations:

Changes in the color of the ovary and condition of the abdomen during ovary development are shown in Table 5.2 and 5.3, respectively. During the ovary resting period, the color of the ovary is opaque and the lower part of the abdomen where the ovary is situated is thin and hard to the touch. Ovary color starts to change with the onset of vitellogenesis and becomes reddish opaque, at this stage the abdomen begins to soften due to increased size of ovary. The ovary color changes to whitish and then to creamy white at end of vitellogenesis, at which time the abdomen is soft and full. After spawning the ovary color becomes reddish opaque, before returning to the resting condition. Such changes can be seen in all groups concurrent with the progress of ovary development.

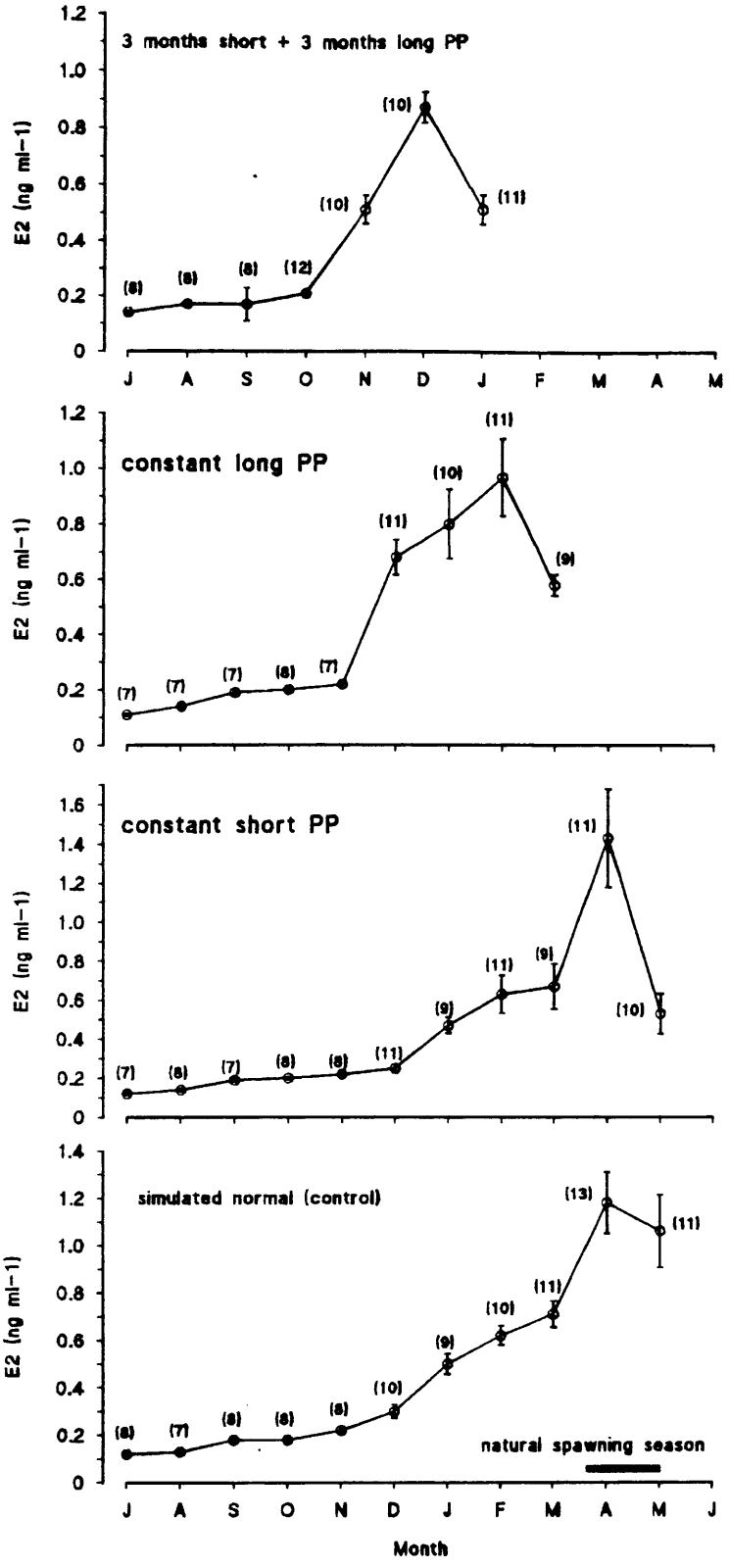


Figure 5.9– Monthly serum oestradiol levels (mean±s.e.m.) in *S. canaliculatus* subjected to various photoperiod regimes under conditions of ambient temperature. PP=photoperiod, WS=winter solstice. Numbers on data points indicate sample size.

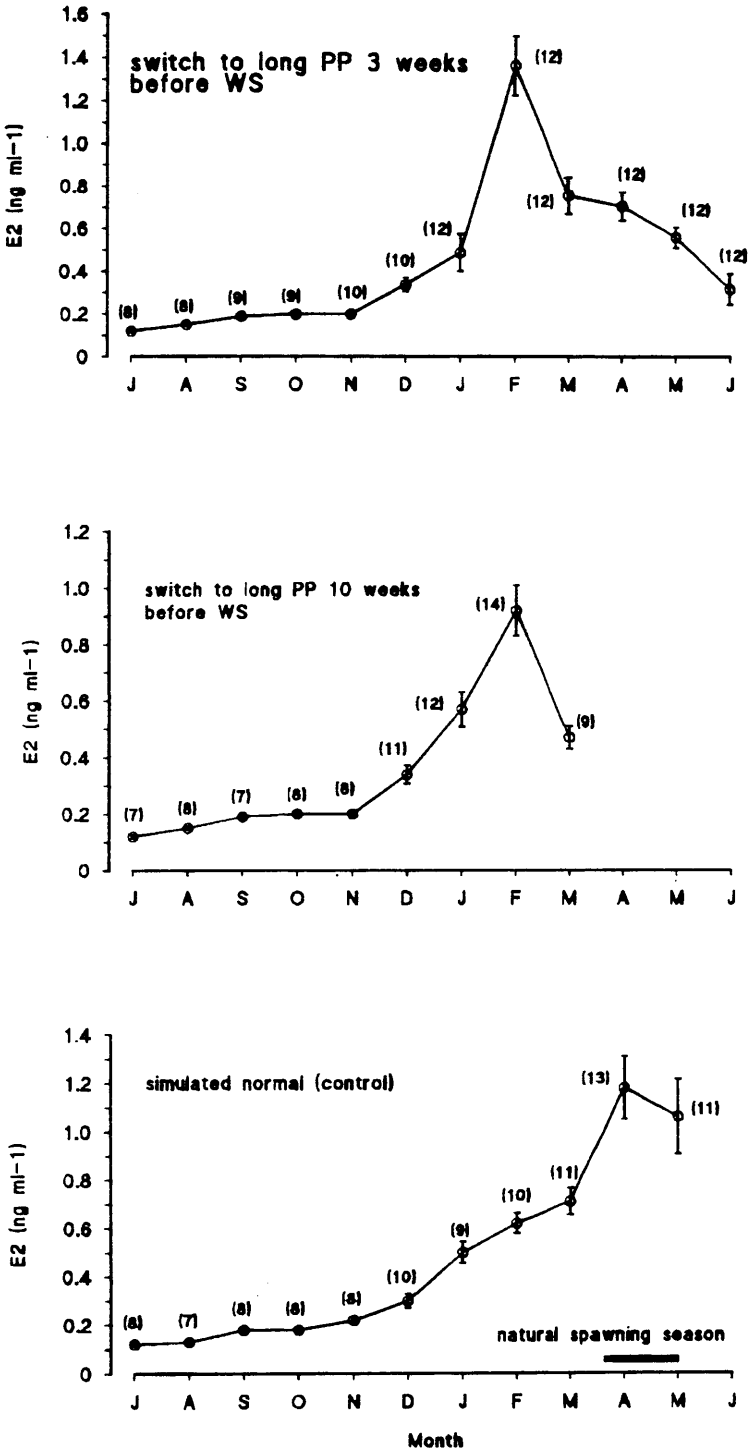


Figure 5.10– Monthly serum oestradiol levels (mean±s.e.m.) in *S. canaliculatus* subjected to various photoperiod regimes under conditions of ambient temperature. PP=photoperiod, WS=winter solstice. Numbers on data points indicate sample size.

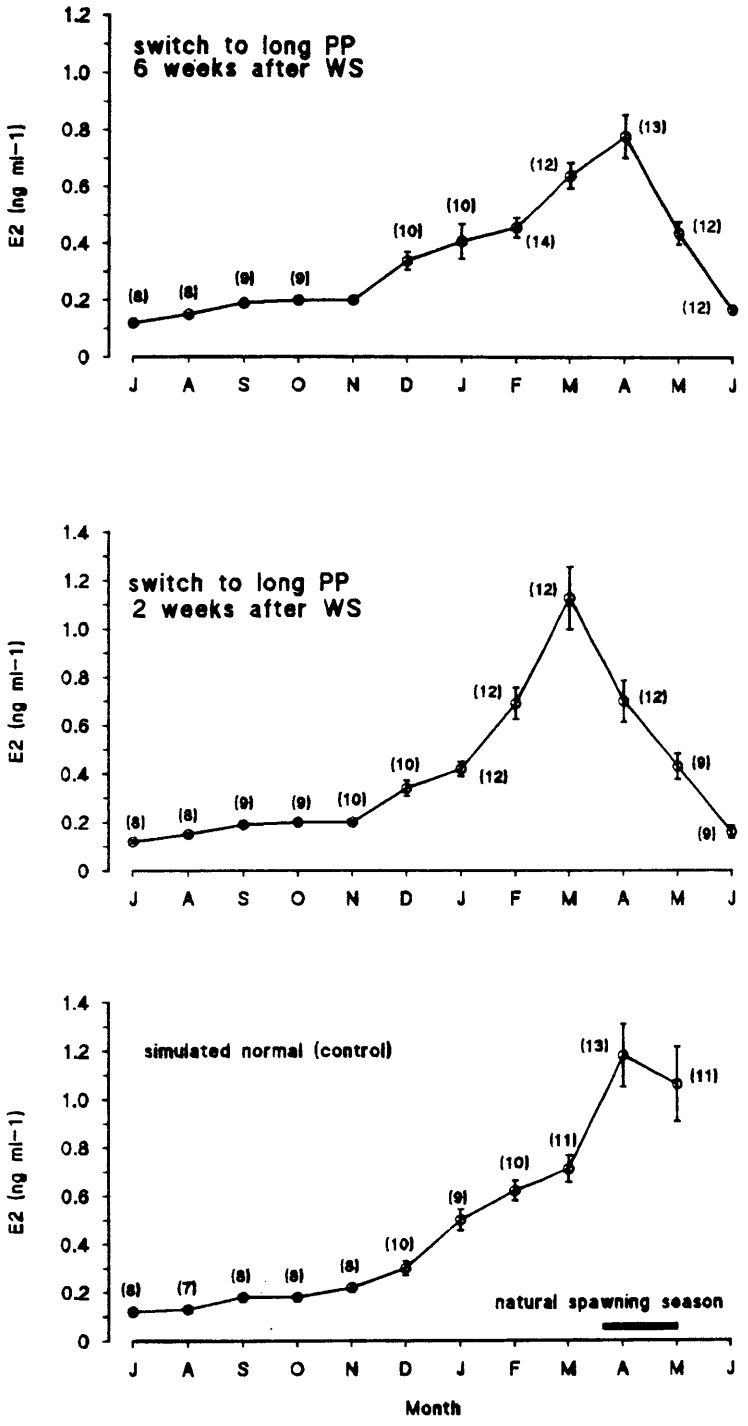


Figure 5.11– Monthly serum oestradiol levels (mean±s.e.m.) in *S. canaliculatus* subjected to various photoperiod regimes under conditions of ambient temperature. PP=photoperiod, WS=winter solstice. Numbers on data points indicate sample size.

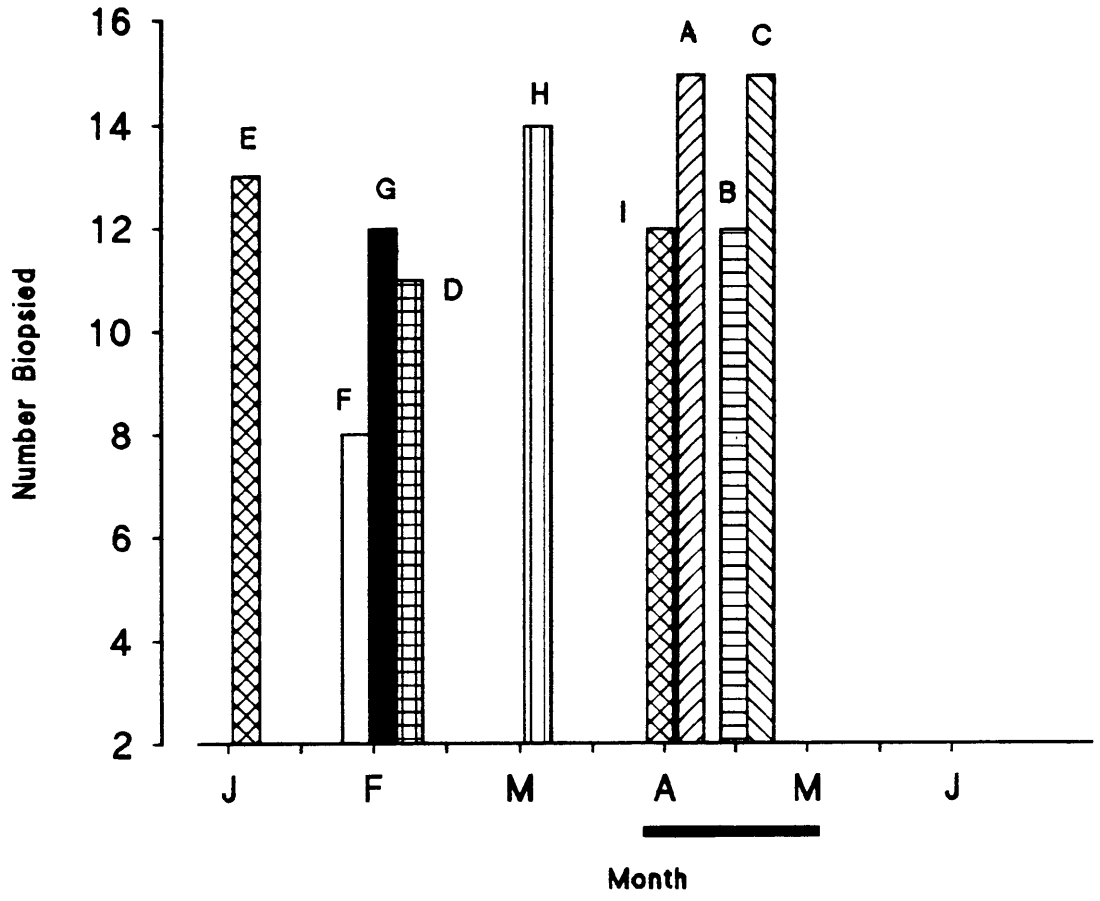


Figure 5.12– Effects of various photoperiod regimes, under conditions of ambient temperature, on the ovary development of *S. canaliculatus.* The earliest date when maximum number of fish could be biopsied. Number of fish in each group=15. Solid bar on the "X" axis indicates the natural spawning season. A=natural normal photoperiod (PP), B=simulated normal PP, C=constant short PP, D=constant long PP, E=3 months short+3 months long PP, F=switch to long PP 10 weeks before winter solstice (WS), G=switch to long PP 3 weeks before WS, H=switch to long PP 2 weeks after WS, I=switch to long PP 6 weeks after WS.

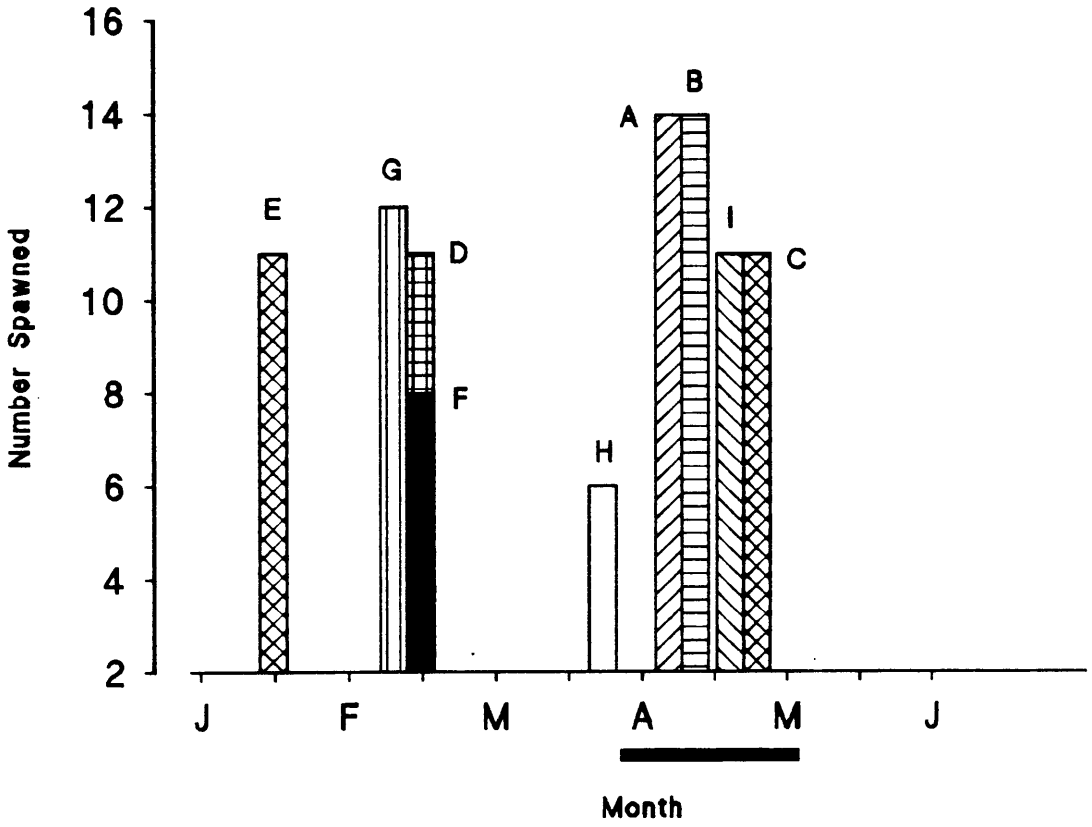


Figure 5.13– Effect of various photoperiod regimes, under conditions of ambient temperature, on the ovary development of *S. canaliculatus*. Number of fish out of a total of 15 which spawned in response to hCG injection. Solid bar under the "X" axis indicates the natural spawning season. A=natural normal photoperiod (PP), B=simulated normal PP, C=constant short PP, D=constant long PP, E= 3 months short+3 months long PP, F=switch to long PP 10 weeks before winter solstice (WS), G= switch to long PP 3 weeks before WS, H= switch to long PP 2 weeks after WS, I=switch to long PP 6 weeks after WS.

Table 5.2 Effect of various photoperiod regimes (Groups A-M) on ovary development of *Siganus canaliculatus*. Monthly changes in ovary colour.

Group	Ovary Colour											
	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun
A	O	O	O	O	O	O	O	RO	WH	CW	RO	O
B	O	O	O	O	O	O	O	RO	WH	CW	RO	O
C	O	O	O	O	O	O	O	RO	WH	CW	RO	O
D	O	O	O	O	O	RO	WH	CW	RO	O	O	O
E	O	O	O	O	RO	WH	CW	RO	O	O	O	O
F	O	O	O	RO	WH	WH	CW	CW	RO	O	O	O
G	O	O	O	RO	WH	WH	CW	CW	RO	RO	O	O
H	O	O	O	O	O	O	O	RO	WH	CW	O	O
I	O	O	O	O	O	O	RO	WH	CW	CW	O	O
J							O	RO	WH	CW	RO	
K							O	CW	RO	O		
L							O	RO	WH	WH	RO	
M							O	RO	WH	WH	RO	

Ovary Color : O=opaque, RO=reddish opaque, WH=whitish, CW=creamy white.

Photoperiodic Regimes Under Ambient (A-I) and Constant (J-M) Temperatures :

A&J = Natural normal photoperiod (PP). B = Simulated normal PP. C =Constant short PP. D = Constant long PP. E = Compressed rectilinear 3 months short followed by 3 months long PP. F = Switch to long PP 10 weeks before winter solstice (WS). G = Switch to long PP 3 weeks before WS. H&K = Switch to long PP 2 weeks after WS. I = Switch to long PP 6 weeks after WS. L = Switch to long PP 8 weeks after WS. M= Switch to long PP 14 weeks after WS.

Table 5.3 Effect of various photoperiod regimes (Groups A-M) on ovary development of *Siganus canaliculatus*. Monthly changes in condition of abdomen.

Group	Monthly Change in Condition of Abdomen											
	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun
A	H	H	H	H	H	H	H	H	E	S	E	H
B	H	H	H	H	H	H	H	H	E	S	E	H
C	H	H	H	H	H	H	H	H	E	S	H	H
D	H	H	H	H	H	H	E	S	E	H	H	H
E	H	H	H	H	H	E	S	E	H	H	H	H
F	H	H	H	H	H	E	E	S	E	H	H	H
G	H	H	H	H	H	E	E	S	E	E	H	H
H	H	H	H	H	H	H	H	E	E	S	H	H
I	H	H	H	H	H	H	H	E	S	S	H	H
J							H	H	E	S	E	
K							H	S	E	H	H	
L							H	H	E	E	H	
M							H	H	E	E	H	

Abdomen Descriptions : (refers to lower part of abdomen behind the anal opening)
H= hard- two sides of abdomen flat against each other and feel hard to the touch,
E= empty- two sides of abdomen are pliable but feels empty.
S=soft and full- The abdomen is distended and gives some resistance to touch.

Photoperiodic Regimes Under Ambient (A-I) and Constant (J-M) Temperatures :
A&J = Natural normal photoperiod (PP). B = Simulated normal PP. C = Constant short PP. D = Constant long PP. E = Compressed rectilinear 3 months short followed by 3 months long PP. F = Switch to long PP 10 weeks before winter solstice (WS). G = Switch to long PP 3 weeks before WS. H&K = Switch to long PP 2 weeks after WS. I = Switch to long PP 6 weeks after WS. L= Switch to long PP 8 weeks after WS. M= Switch to long PP 14 weeks after WS.

6. Oocyte Diameter:

All groups had mean oocyte diameters within the range determined for fully vitellogenic oocytes (Chapter 2, Section 2.3.3) (Fig.5.14). There was no obvious correspondence between oocyte diameter and photoperiodic regimes which accelerated ovarian development.

7. Incidence of Atresia:

The numbers of atretic vitellogenic oocytes sampled from fish in most groups at the end of experiments were well within the normal range determined for healthy, wild caught fish (Chapter 2, Section 2.3.4) (Fig.5.15). A distinctly higher incidence of atresia was obtained in the two groups subjected to the greatest delay in the switch to long photoperiods.

5.3.2 Series 2: Constant Temperature-

The effect of photoperiodic manipulation on the ovarian cycle of *S. canaliculatus* are as follows:

1. Time of Vitellogenesis:

The monthly stages of ovary development in all groups are illustrated in Fig.5.16. In the control group, subjected to natural photoperiod under normal light but at constant temperature, the timing of the start and completion of vitellogenesis was similar to that of the corresponding group at ambient temperature (Group A). In all these experimental groups, vitellogenesis was initiated. However, the completion of vitellogenesis occurred only in the group subjected to the short two week post-solstice delay in long photoperiod (Group K). Longer delays (groups L and M) prevented completion.

2. Serum Oestradiol (E_2):

The E_2 levels are shown in Fig.5.17. Normal peak levels of oestradiol (E_2) were measured in the controls and in the group subjected to a short two week post-solstice delay in long

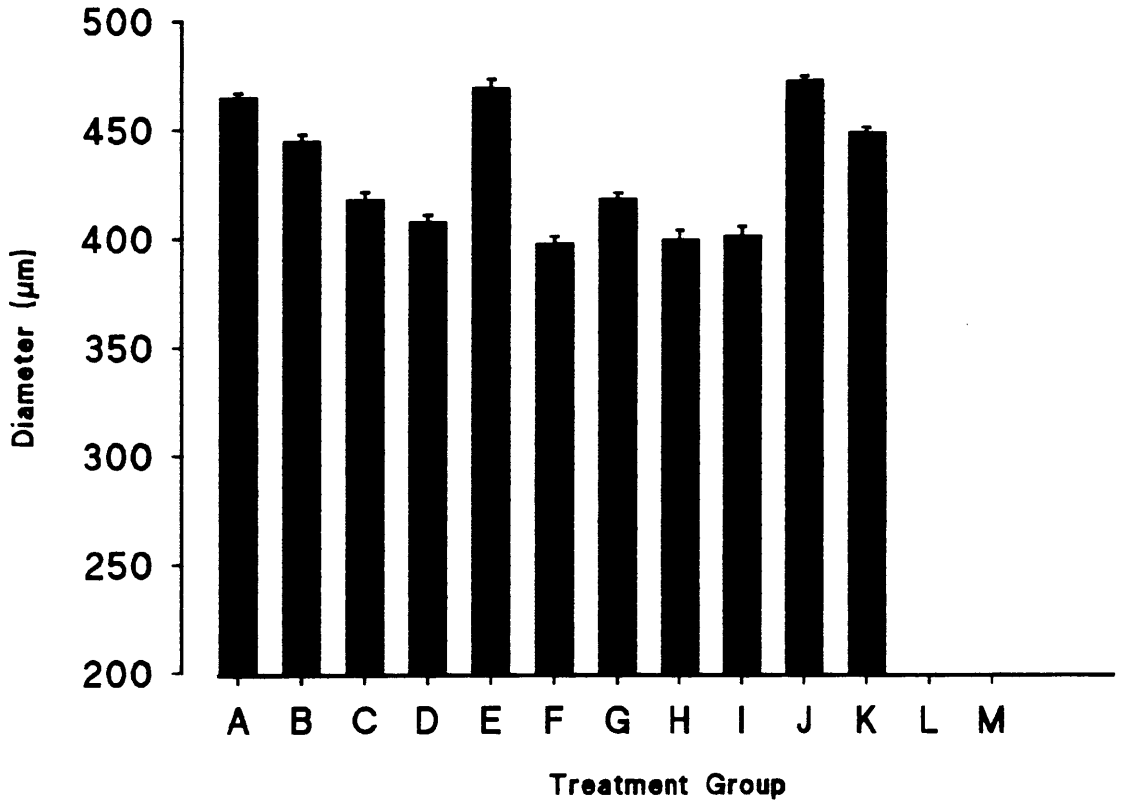


Figure 5.14– Diameters (mean±s.e.m.) of fully vitellogenic oocytes at the end of experiments in *Siganus canaliculatus* subjected to various photoperiod regimes at ambient (A–I) and constant (J–M) temperatures. A&J=natural normal photoperiod PP, B=simulated normal PP, C=constant short PP, D=constant long PP, E= 3 months short + months long PP, F=switch to long PP 10 weeks before winter solstice (WS), G=switch to long PP 3 weeks before WS, H&K=switch to long PP 2 weeks after WS, I=switch to long PP 6 weeks after WS. Sample number=3.

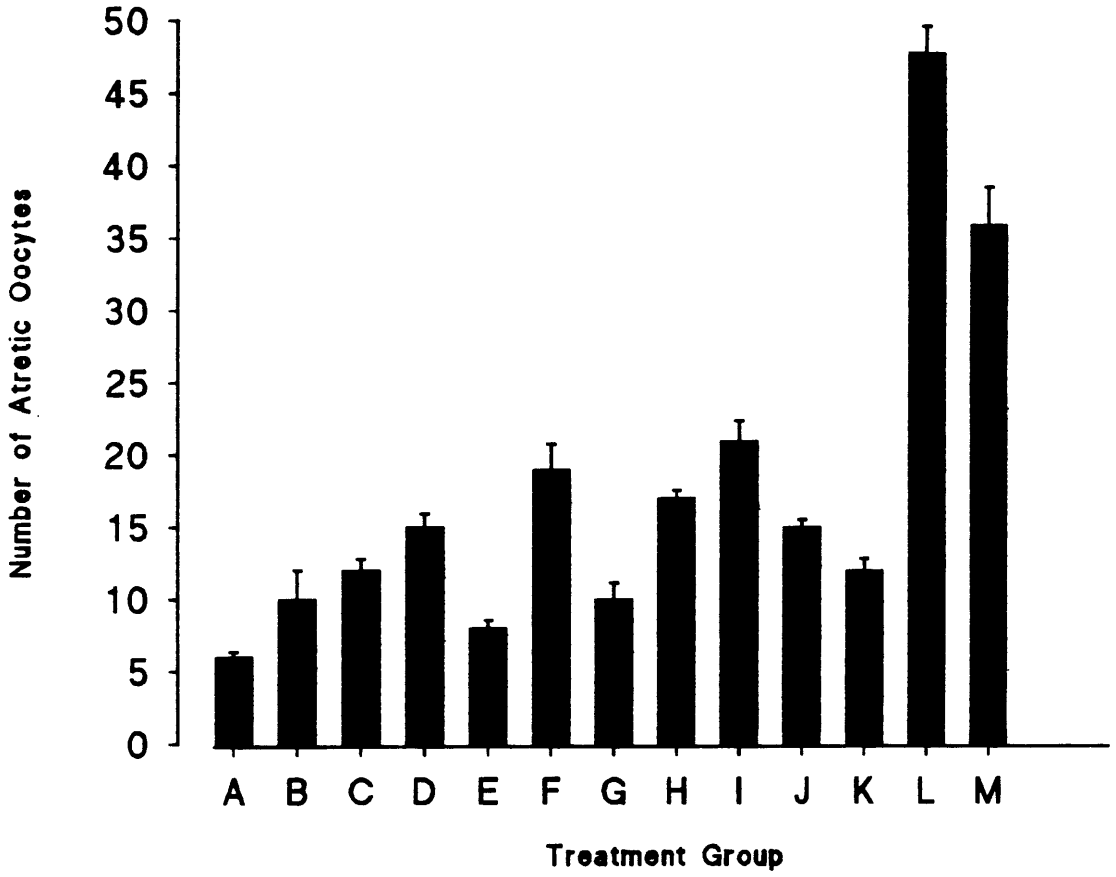


Figure 5.15– Number (mean±s.e.m.) of atretic oocytes in fully vitellogenic ovary of *S. canaliculatus* subjected to various photoperiod regimes in ambient (A–I) and constant (J–M) temperatures. A&J=natural normal PP, B=simulated normal PP, C=constant short PP, D=constant long PP, E=3 months short+3 months long PP, F=switch to long PP 10 weeks before winter solstice (WS), G=switch to long PP 3 weeks before WS, H&K=switch to long PP 2 weeks after WS, I=switch to long PP 6 weeks after WS, L=switch to long PP 8 weeks after WS, M=switch to long PP 14 weeks after WS. Sample number=3.

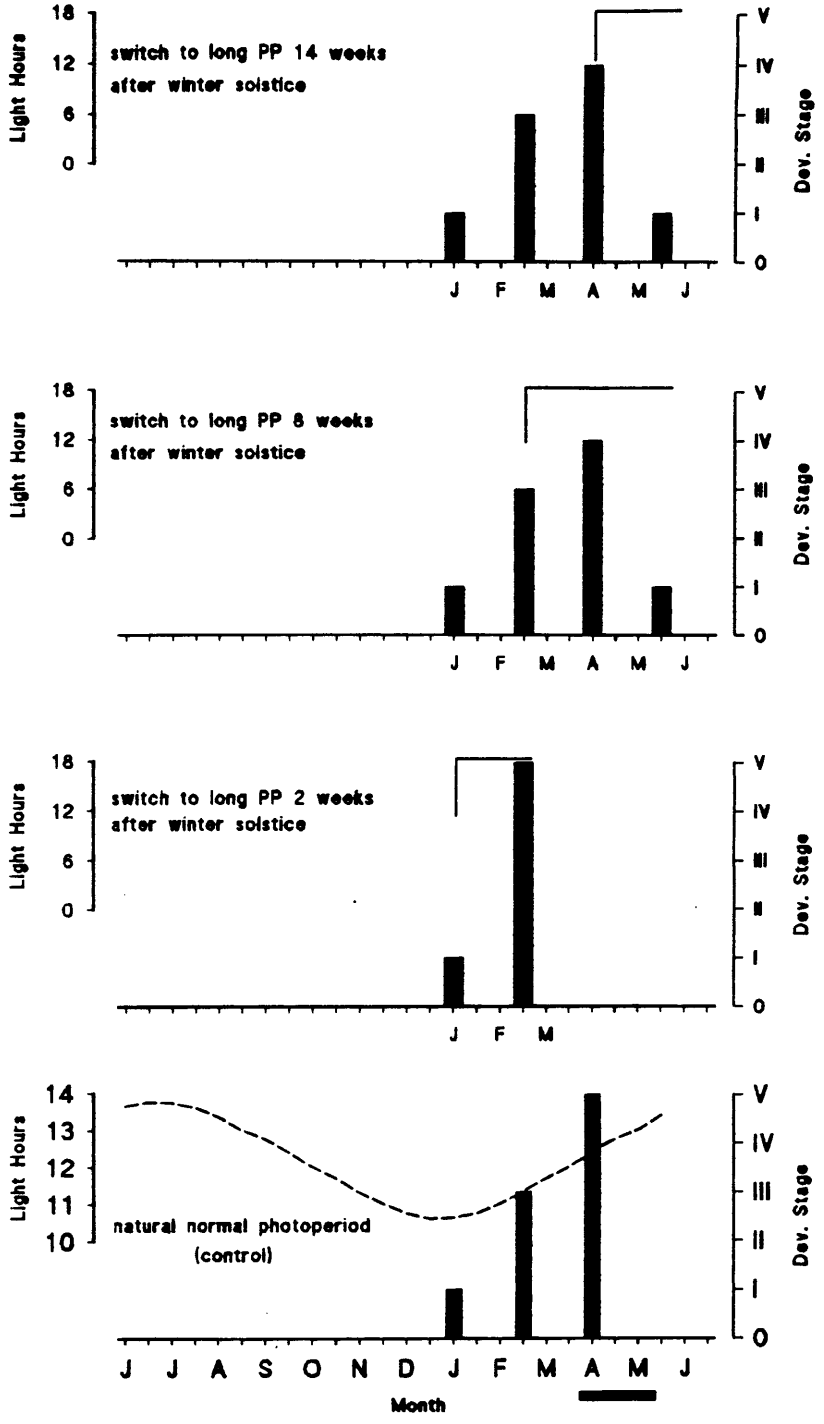


Figure 5.16– Monthly stages of ovary development in *S. canaliculatus* subjected to various photoperiod regimes under constant temperature (25±1°C). Solid bar under X axis indicates the natural spawning season. I=virgin ovary, II=early developing, III=mid developing, IV=late developing, V=running ripe, VI=maturing. P=photoperiod. Sample number=3.

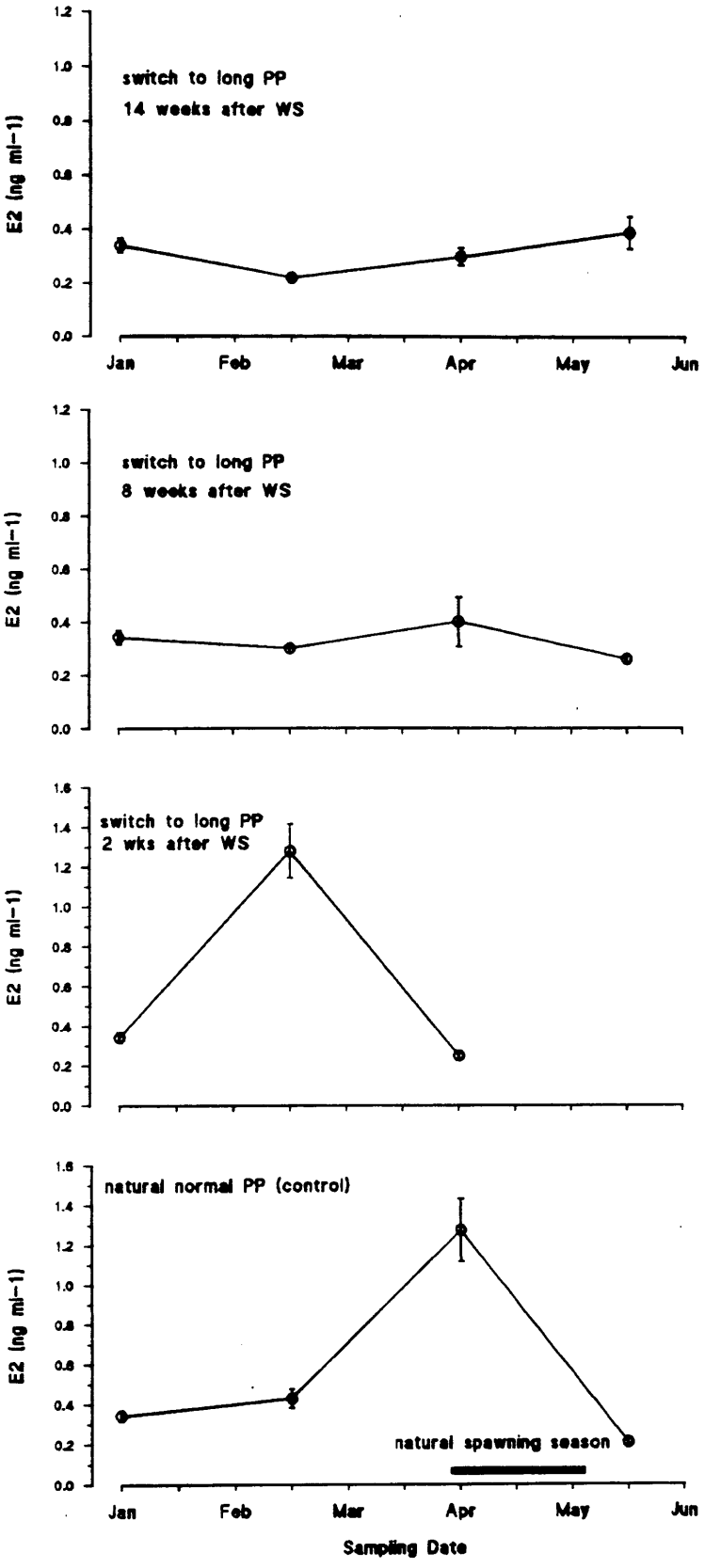


Figure 5.17– Monthly serum oestradiol levels (mean±s.e.m.) in *S. canaliculatus* subjected to various photoperiod regimes under conditions of constant temperature (25±1°C). PP= photoperiod, WS= winter solstice. Sample number=4.

photoperiod. Further delays in the long photoperiod completely inhibited the usual rise in oestradiol.

3. Biopsy Time:

In the control group and in the group subjected to the shortest 2 week post-solstice delay in long photoperiod (J and K), all of the fish were biopsiable before or during the normal spawning season. The numbers of biopsiable fish in the groups subjected to greater delays in long photoperiod (L and M) were fewer (Fig.5.18).

4. Spawning Readiness:

Only the control group and the group subjected to the short 2 week post-solstice delay in photoperiod responded to hCG injection (Fig.5.19).

5. Macroscopic Observations:

Changes in the color of the ovary and condition of the abdomen during ovary development are shown in Table 5.2 and 5.3, respectively. In the control group (Group J), the creamy white ovary color, characteristic of fully vitellogenic ovaries, and soft abdomen were seen in April around the normal spawning time. In the group subjected to a switch to long photoperiod two weeks after the winter solstice, such changes were observed in February, at which time the ovary completed vitellogenesis. Neither feature was observed in the two groups subjected to the long delays in photoperiod.

6. Oocyte Diameter:

Mean oocyte diameter in the groups which completed vitellogenesis (J and K) were within the normal range (see Chapter 2, Section 2.3.3) (Fig.5.14).

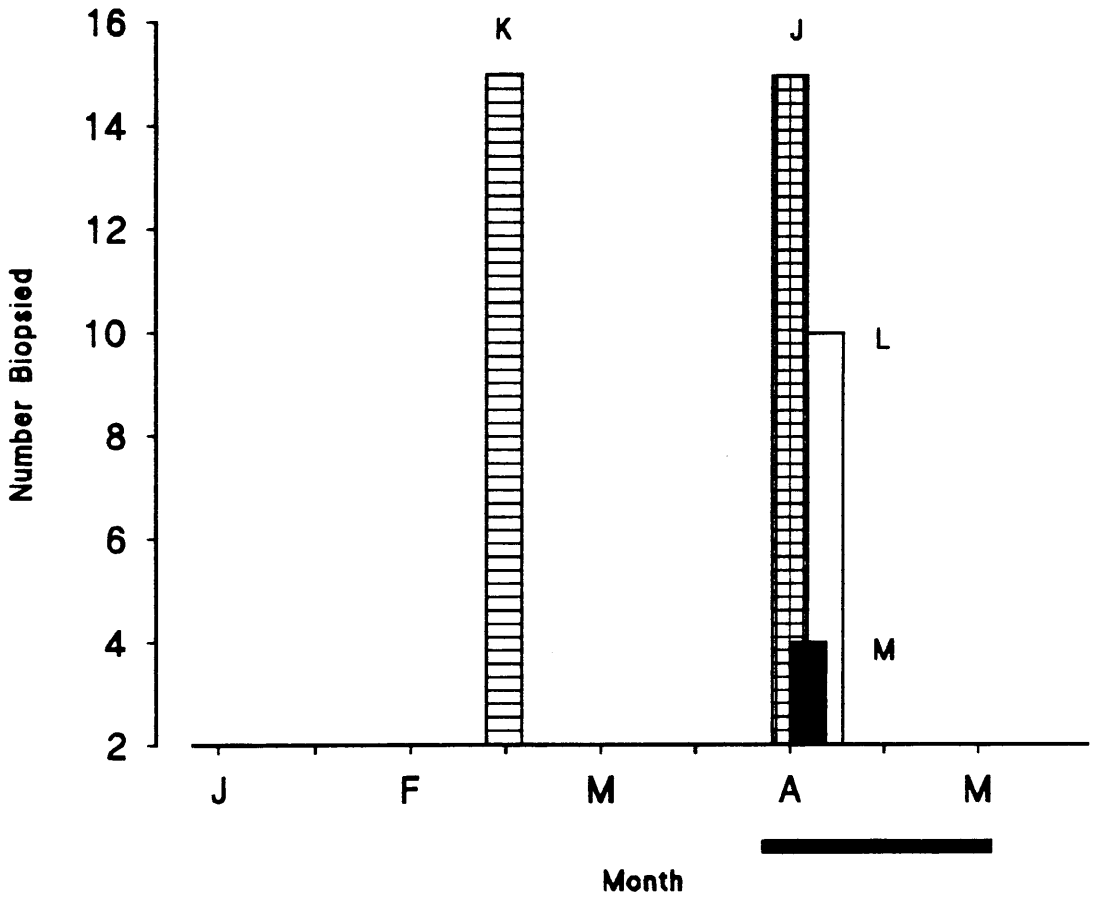


Figure 5.18– Effects of various photoperiod regimes, under conditions of constant temperature, on the ovary development of *S. canaliculatus.* The earliest date when maximum number of fish out of a total of 15 could be biopsied. Solid bar under the "X" axis indicates the natural spawning season. J=natural normal photoperiod (PP), K=switch to long PP 2 weeks after winter solstice (WS), L=switch to long PP 8 weeks after WS, M=switch to long PP 14 weeks after WS.

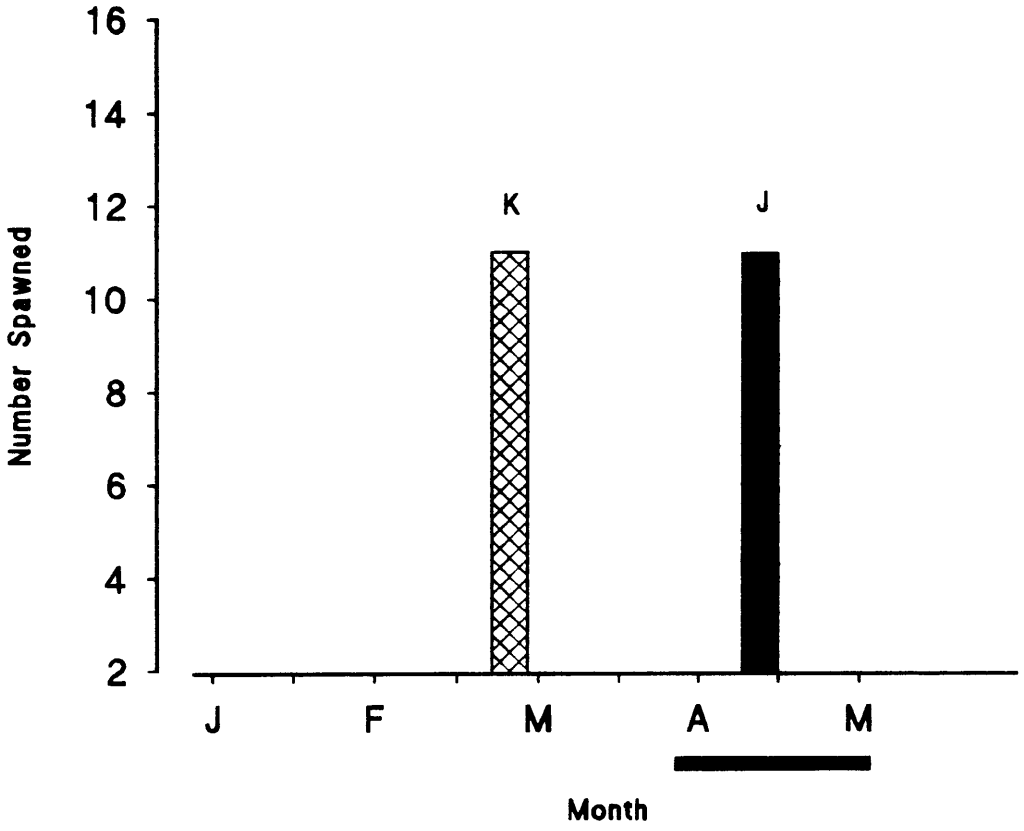


Figure 5.19– Effect of various photoperiod regimes, under conditions of constant temperature (25±1°C), on the ovary development of *S. canaliculatus*. Number of fish out of a total of 15 which spawned in response to hCG injection. Solid bar under the "X" axis indicates the natural spawning season. J=natural normal photoperiod (PP), K=switch to long PP 2 weeks after the winter solstice.

7. Incidence of Atresia:

Numbers of atretic vitellogenic oocytes in the four treatment groups (J-M) are shown in Fig.5.15. The mean number of atretic vitellogenic oocytes in fish subjected to natural normal photoperiod and a switch to long photoperiod 2 weeks after winter solstice was 15±0.51 and 12±0.84 respectively, which is within the range determined for healthy vitellogenic oocytes in stage V fish ready for maturation and before the onset of atresia. In the remaining two groups, where fish were exposed to a switch from ambient to long day photoperiod 8 and 14 weeks after the winter solstice, incidence of atresia was high (48% and 36% respectively). Such high levels of atresia are found in normal ovaries when not induced with hCG (see Chapter 2, Section 2.3.4).

5.4 Discussion:

It is clear that the interval between samples (4 weeks in the ambient series, and 6 weeks in the constant temperature series) sets a limit to the precision of estimating the time of reproductive events. This has to be borne in mind when comparing differences in timing between treatments. For each of the 8 groups maintained at ambient temperature, there was a close coincidence between the timing of the initial rise in serum E_2 and the initiation of vitellogenesis. There was a similar close correspondence between the timing of peak serum E_2 , peak vitellogenesis, first biopsiable date and first inducible spawning date.

In order to aid in the understanding of the discussion, the major findings of the experiments on photoperiodic manipulation have been listed in a quick reference table (Table 5.4).

The duration of vitellogenesis was similar in all groups irrespective of the timing of vitellogenesis. There was one possible exception; in the group exposed to the regime of 3 months of short days followed by 3 months of long days, the duration of vitellogenesis appeared to be shorter (Fig.5.20). More frequent sampling would be required to confirm any such difference.

Table 5.4 Summary of major findings of the experiments on the effect of various photoperiodic regimes on the ovary development of *Siganus canaliculatus*. PP= photoperiod , WS= winter solstice, ND= no data.

Photoperiodic Regime	Start of Vitello-genesis	Earliest Change of Ovary Colour	First Rise in E_2	Peak Vitello-genesis	Peak E_2	First Time Biop-siable	First Time Spawning Inducible	Mean Diameter of Fully vitello-genic Oocytes	Incidence of Atresia (%)
Ambient Temperature:									
3 months short+3 months long PP	Nov-Dec.	November	Nov-Dec.	Dec-Jan	Dec.	January	January	Within Normal Range	8
Constant Long PP	Nov-Dec.	December	Nov-Dec.	January	Jan.	February	February	Within Normal Range	15
Long PP 10 wks before WS.	Nov-Dec.	October	Nov-Dec.	Feb.	Feb.	Late Jan. to February	February	Within Normal Range	19
Long PP 3 wks before WS.	Nov-Dec.	October	Nov-Dec.	Feb.	Feb.	February	February	Within Normal Range	10
Long PP 2 wks after WS.	Dec-Jan.	February	Dec-Jan.	March	Mar.	March	March	Within Normal Range	17
Control Simulated Normal PP.	Jan-Feb.	February	Nov-Dec.	April	April	April	April	Within Normal Range	10
Natural Normal PP.	Jan-Feb.	February	Nov-Dec.	April	April	April	April	Within Normal Range	6
Constant Short PP.	Jan-Feb.	February	Dec-Jan.	April	April	April	April	Within Normal Range	12
Long PP 6 wks after WS.	Jan-Feb.	January	Nov-Dec.	April	April	Late March to April	April	Within Normal Range	21
Constant Temperature:									
Control Natural Normal PP	Feb.	February	Feb.	Mach-April	April	Early April	April	Within Normal Range	15
Long PP 2 wks after WS.	Jan-Feb.	February	Jan.	Jan-Feb.	Mid Feb.	Mid February	Late February	Within Normal Range	12
Long PP 8 wks after WS.	Jan-Feb.	February	No Rise	None	No Rise	Early April	ND	ND	48
Long PP 14 wks after WS.	Jan-Feb.	February	None Rise	None	No Rise	Early April	ND	ND	36

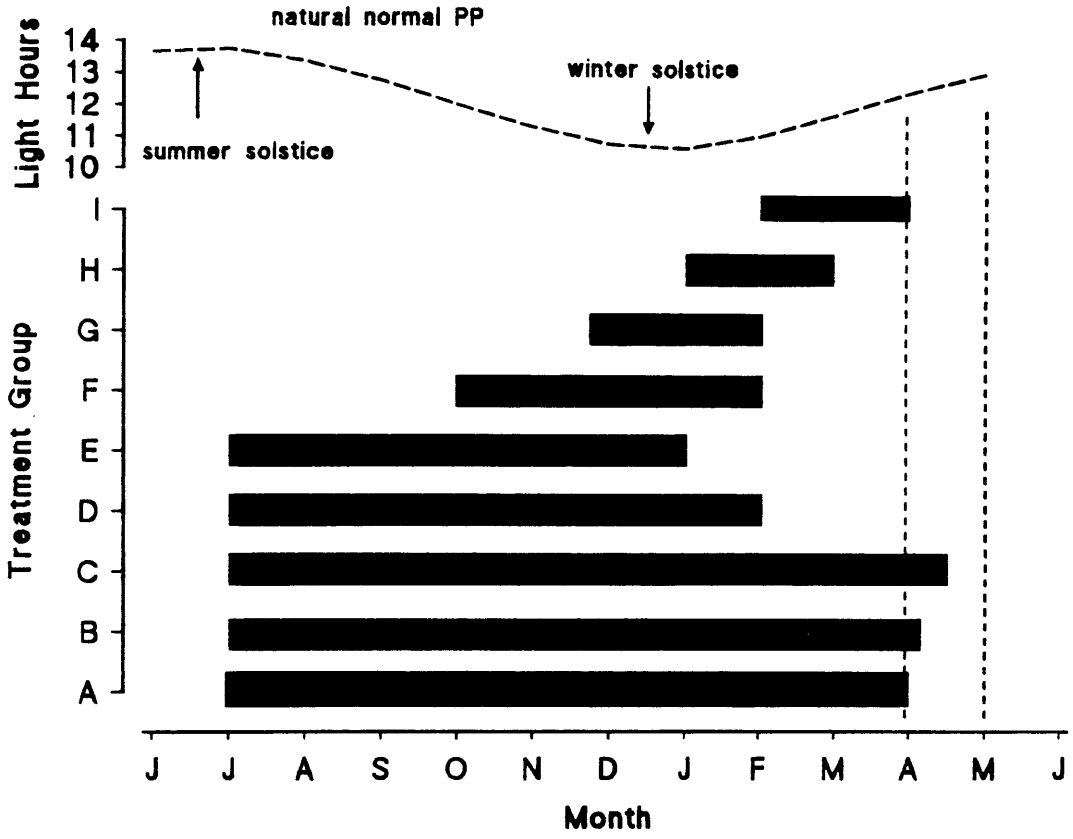


Figure 5.20– Effect of various photoperiod regimes, under conditions of ambient temperature, on the ovary development of *S. canaliculatus*. The period, from the date when the fish were subjected to a modified photoperiod, until completion of vitellogenesis. Dashed vertical lines indicate period of natural spawning. A=natural normal photperiod (PP), B=simulated normal PP, C=constant short PP, D=constant long PP, E=3 months short +3 months long PP, F=switch to long PP 10 weeks before winter solstice (WS), G=switch to long PP 3 weeks before WS, H=switch to long PP 2 weeks after WS, I=switch to long PP 6 weeks after WS.

5.4.1 The Effect of Photoperiodic Regimes on the Timing of Reproduction:

The use of artificial light to simulate the natural ambient photoperiod had no effect on the timing of any of the reproductive events, and had no effect on either mean oocyte diameter or incidence of atresia. Therefore, the group on this simulated normal photoperiod were used as the controls. Compared with the controls, several photoperiodic regimes advanced many of the reproductive stages from initiation of vitellogenesis to spawning inducibility with hCG.

Regimes which advanced reproduction were those involving a switch to long days prior to the winter solstice, and even the regime in which the switch occurred two weeks after the winter solstice. The extent of the advance was greatest (2 1/2 months) in the group on 3 months short days+3 months long days, and least in the group switched to long days 2 weeks after the winter solstice. However, the extent of the advance was not obviously correlated with the date of switching to long days. Groups switched 3, 10 and 20 weeks before the winter solstice all showed a similar advance. Earlier switches did not promote greater advances in the reproductive cycle.

Reproduction was not delayed by any of the photoperiodic regimes, including the regimes in which the long day switch was delayed until 6 weeks after the winter solstice, and the regime of constant short days.

5.4.2 Constant Temperature Series:

Since most of the experiments were at ambient temperature for reasons stated earlier, the role of temperature on the reproductive cycle has to be addressed. A rise in temperature from 20-25°C in December, when the ovary is still in the previtellogenic stage, had no obvious effect on the reproductive cycle. Comparison between the two normal photoperiodic regimes at ambient and at constant temperatures shows no difference in the timing of vitellogenesis or oestrogenic events. Similarly, comparison of the two regimes, in which the long day switch occurred 2 weeks post winter solstice, also shows no evidence of a temperature effect. Based

only on this evidence, it is suggested that temperature acts neither as a cue to trigger reproduction nor does it modify the rate of progress of the reproductive events.

The major finding of the constant temperature series was that a protracted delay in the time of switching to long days (8 and 14 weeks post winter solstice) had a major and deleterious effect on ovarian development soon after vitellogenesis started. Although vitellogenesis was initiated it was never completed, serum oestradiol levels remained low, and levels of atresia were unusually high. Delaying the reproductive cycle was not achieved by any of the regimes involving a simulated delay of the winter solstice in either the constant temperature or ambient temperature series.

A large part of the understanding of the effect of photoperiod manipulation on gonad development in fishes comes from studies on salmonids, and it is only logical to compare the results of this study to those of salmonids, in spite of the difference in climatic conditions prevalent to the different latitudes, salmonids inhabiting temperate higher latitudes and the rabbitfish studied here inhabiting sub-tropical lower latitudes. Salmonids are, however, exposed to lengthening days in the first part and to shortening days in the second part of the reproductive cycle, whereas the entire reproductive development of the *Siganus canaliculatus* studied takes place under lengthening days. Differences in response to the same photoperiodic treatment may thus be expected.

The response of salmonids to constant short days is dependent on the time when it is applied over the annual cycle with relation to the summer solstice. When applied early in the cycle, 2-3 months after the spawning season, but before the summer solstice, it produces a delay in the next spawning, whereas after the summer solstice it advances the process (**Bromage et al., 1984; Duston & Bromage, 1987**). In the rabbitfish, short days applied two months after the spawning season, just two weeks after the summer solstice, did not cause a significant delay in the timing of ovary development. The short photoperiod was held constant for one year until the next spawning season, and the fact that the fish reached vitellogenic phase in time for the natural spawning season, in spite of being under a constant short daylength for about 10, months indicates the presence of an endogenous mechanism that persists in the absence of

external cues. The response of the rabbitfish to constant long days beginning two months after the spawning season, when the fish have also just started on a naturally decreasing photoperiod, advances the timing of next spawning by two months. This conforms with the results for salmonids, where constant long days also advance timing of spawning when applied during the earlier part of the reproductive cycle, but in the case of salmonids they are on a naturally increasing photoperiod (**Bromage & Duston, 1986; Duston & Bromage, 1987,1988**). The difference in the response of the two fish may be attributed to the natural spawning timing of each; salmonids spawn naturally in winter and any application of constant photoperiods soon after spawning will be when fish are on a naturally increasing photoperiod, while in the rabbitfish they would have just started on a naturally decreasing photoperiod, as they spawn naturally in spring. The effect of an early arrival of long days is perhaps perceived by the fish as running 'behind time' and it compensates with a phase advance.

The combination of short followed by long photoperiods for a duration of three months each early in the reproductive cycle produces the most marked advance in the timing of the next spawning. Spawning readiness was observed in fish in January, three months earlier than the natural spawning season. In salmonids, combination of long and short days also produced advancement in spawning time, which was attributed to the aggregate effects of exposure to long days early and short days later (**Bromage et al., 1993**). A similar explanation may also apply to rabbitfish, even though the direction of natural photoperiod in the two fishes when the photoperiodic changes were applied were opposite. The abrupt decrease in daylength just after the spawning season, when the natural photoperiod has just started to decrease, may have the effect of compressing the period from summer to winter solstice, the following increase in daylength can do the same to the natural rise in photoperiod from winter solstice to the next spawning season. In effect, the combination of short followed by long days results in a compressed annual light cycle.

Switches from ambient to constant long photoperiods also advance the timing of ovary development. There was no difference in the degree of advancement in relation to the time when the switch is applied over the reproductive cycle. A switch to long photoperiod applied in the period from 10 weeks before the winter solstice and until the winter solstice advances

the completion of vitellogenesis by the same period (2 months). Delay of the exposure time to two weeks after the winter solstice (Group H) reduces the advancement by about one month. Exposure to such photoperiodic changes 6 weeks after the winter solstice, when the fish are already on an increasing natural photoperiod and natural onset of vitellogenesis has started (Group I), has no effect on the timing of ovary development. This appears to be opposite to the effect reported for salmonids, where exposure to long days early in the reproductive cycle, just after the winter solstice, advance maturation (**Bromage et al., 1982a&1984; Bromage & Duston, 1986; Duston & Bromage, 1987,1988**). But later in the cycle, after the summer solstice, causes a phase delay in spawning (**Whitehead & Bromage, 1980; Bromage et al., 1982a&1984; Johnson, 1984; Bourlier & Billard, 1984**). As in the case with constant long days, switches to long days before winter solstice when the natural photoperiod is still decreasing can cause the fish to compensate for the delay by a phase advance. But similar switches more than two weeks after the winter solstice will not trigger the same response, as the fish is already on an increasing photoperiod.

Even though constant long days, when applied at the appropriate time, can advance the timing of ovary development in rabbitfish, it appears that the duration of the change (based on published works, perhaps also the magnitude) is not critical. In group 'F', where the fish were subjected to just one month long photoperiod (square-wave) of a difference of just two hours, four months after the spawning season, timing of vitellogenesis was advanced by two months. An equal advance to that achieved in group 'G', where exposure of fish to a switch to constant long daylength 5 1/2 months after the spawning season, had to be held constant for about two months before effects of advancement were detected. This is in agreement with the opinion that daylengths are considered by the fish as long not because of their absolute length, but when they are preceded by daylengths which are shorter (**Bromage et al., 1993**).

The low spawning percentage response to exogenous injection of hCG in some groups and the high rates of atretic vitellogenic oocytes emphasize the importance of the correct time of application of the photoperiodic treatment. Exposure of fish to changes very late in the reproductive cycle, more than six months after the spawning season, over two weeks after the winter solstice, may have a deteriorating effect on ovary development, and even though

spawning is achieved in some earlier cases, there may be an effect on gamete quality, a matter that needs to be investigated, perhaps not only for these application timing, but also for other light regimes which gave good results. There are some unanswered questions that need to be addressed. It is obvious that the reproductive cycle of *Siganus canaliculatus* could be altered by photoperiodic manipulations. But could it be entrained for longer periods of time?. It is perhaps necessary to design experiments where fish are held under a constant photoperiod for more than just one reproductive cycle, and determine whether there is an endogenous mechanism that will override the external factors. There are also gaps in the time of application of a switch to long days along the annual reproductive cycle. The gap is for the period from July to October, application of the photoperiodic change during this period may give different results than what is available at present.

It is perhaps plausible to conclude that it may be possible to use a variety of light regimes to manipulate the reproductive cycle of *Siganus canaliculatus*, including many others not tested in this study, which include seasonally changing light cycles, compressed or extended annual light cycles, skeleton photoperiods or resonance protocols. But the practical nature of this study necessitates the search for the most economical method to achieve the target, which is extending the spawning period in a fish that has a very short annual one, thus allowing the production of out-of-season seeds and synchronization of culture periods with optimum market policies. The use of equipment for the photoperiod control of spawning time are relatively easy and inexpensive, but the use of constant photoperiods, whether constant long photoperiod of long duration or shorter square-wave methods, require less time and effort than periodic adjustments of light control equipment as required for some of the other photoperiod regimes.

The results of this study indicate that the reproduction of rabbitfish *Siganus canaliculatus* is stimulated by light, like many other organisms, and that they are cued to seasonal changes in daylength. And that photoperiod manipulation does indeed provide a means of accelerating ovary development and advancing the spawning season, therefore extending the period of availability of seed for culture purposes. The use of such techniques in the hatchery for commercial use will be limited by economic considerations. Regimes that require frequent

adjustments of timers may be complex, similarly maintaining the fish on constant daylengths may also be expensive. Clearly, it would be easier to use switches to long days on or near the winter solstice, or a combination of short and long days, if more advancement is preferred, especially since they seem to give similar results to constant long days.

Chapter 6

Chapter 6

Endocrine Manipulation of Spawning in *Siganus canaliculatus*

6.1 Introduction:

An understanding and an ability to manipulate reproduction is critical to the development of hatchery technology. The transfer of fish from natural artificial environments for breeding frequently disrupts the normal hormonal regulation of gonad function, particularly the inhibition of gonad maturation and gamete release. A number of techniques are used to overcome this problem. These either mimic or induce an acute increase in endogenous levels of pituitary gonadotropins or gonadal steroids, which leads to maturation and spawning. Of various methods used for the delivery of exogenous hormones, a common and effective one is the administration of one or two intraperitoneal or intramuscular injections. An additional benefit of this method is the synchronization of spawning, and the elimination of unwanted, asynchronous release of eggs (**Yaron and Zohar, 1993**). In some species held in artificial environments even the earlier stages of gonad development may be inhibited (**Lee et al., 1996**). These may be stimulated by repeated administration of lower levels of hypothalamic hormones, often for long periods, either through oral administration in food, or by use of slow-release, hormone-delivery vehicles implanted in the musculature or abdominal cavity (**Crim, 1985**).

The oral administration methods have certain disadvantages, such as the limited number of usable hormones that can survive the enzymatic activities in the digestive tract, the large quantity of hormone required to overcome the solubility in water and to allow for enough absorption across the wall of the intestine, and the dependency of the actual dosage of hormone the fish receives on its feeding rate, which can be quite variable. These disadvantages can be overcome by the use of a variety of implanted pellets and capsules

which have the additional merit of producing near physiological levels of circulating hormones (**Lee et al., 1996**).

Because of its relative ease and efficiency, spawning induction by hormone treatment has become a routine practice in fish farming. One of the main reasons for lack of ovulation and spawning is failure of the pituitary to release gonadotropin (GtH-II), which is essential for promoting maturation and ovulation (**Lin & Peter, 1996**). Traditional methods of inducing spawning are based on the injection of gonadotropins from different sources, including crude extract of carp pituitary gland (CPE), partially purified fish GtH-II and mammalian GtH, and especially human chorionic gonadotropin (hCG) (**Pankhurst et al., 1986; Peter et al., 1988a**). hCG has been used successfully to induce ovulation and spawning in a number of fish species (**Lam, 1982; Donaldson & Hunter, 1983**). One advantage in using hCG over other hormones is that its potency can be standardized in international units, which makes results comparable among users (**Lee et al., 1988**).

In siganid species the use of hCG to induce spawning in captivity is widely used. Unfortunately the differences in injection protocols adopted and differences in the exact reproduction status of the fish used in the different studies makes it difficult to identify any fundamental species differences in the siganid response to hCG. A single injection of 0.25 IU g^{-1} was sufficient to induce spawning within four days in *Siganus argenteus*, *S. luridus* and *S. rivulatus* (**Popper et al., 1979**); whereas in *S. guttatus* it took four injections of 2.0 IU g^{-1} (**Palma, 1978**), and dosages ranging from 0.3-2.0 IU g^{-1} were used for *S. vermiculatus* (**Popper & Gundermann, 1976; Avila, 1984**). In *S. canaliculatus*, 5-6 injections of 0.25-3.0 IU g^{-1} were found to induce spawning (**Soh & Lam, 1973; Westernhagen & Rosenthal, 1976a**) In another report a single injection of 1 IU g^{-1} hCG was sufficient to induce spawning in *S. canaliculatus* 4-5 days after injection (**Akatsu et al., 1984**).

There are a number of problems associated with the use of heterologous and even homologous gonadotropins for the induction of spawning: 1) Fish GtH-II is generally of high species specificity, e.g. neither carp or salmon GtH-II are effective in gilthead seabream (**Zohar et al., 1987**). Human chorionic gonadotropin is effective in some species, but not in some cyprinids

(**Lin et al., 1986a**). 2) The mixture of hormones that may be present in crude pituitary extracts may have side effects on gametogenesis or other functions. 3) Partially purified fish GtH-II and hCG are costly. 4) The use of heterologous GtH-II may trigger an immune response in treated fish and cause subsequent refractoriness to the hormones (**Peter et al., 1988a**). 5) CPE is highly variable in potency and has a short storage life (**Lin & Peter, 1996**).

Problems associated with the above traditional methods have emphasized the need for alternative hormonal treatments to induce ovulation and spawning. Superactive analogues of mammalian luteinizing hormone releasing hormone (LHRH-A) have been found to be effective in a number of species (**Donaldson & Hunter, 1983**). In some cyprinids, analogs of LHRH have been reported to be effective in inducing ovulation **(Crim et al., 1987)**. Many varieties of salmon and trout have also been shown to respond to LHRH treatment (**Crim et al., 1987**). For example, double injections of 20μg kg^{-1} dosage given on days 0 and 3 induced ovulation in the coho salmon, *Onchorhynchus kisutch,* (**Van Der Kraak et al., 1985**). In the walleye, *Stizostedion vitreum*, injection of LHRH-A at a dosage of 100μg kg^{-1} body weight was effective in advancing the time of ovulation (**Pankhurst et al., 1986**). In the sea bass, *Lates calcarifer*, doses ranging from 4.75-75 μg kg^{-1} body weight stimulated a dose-dependent increase in spawning numbers, but higher doses ranging from 150-300 μg kg^{-1} resulted in fewer fish spawning, with the highest dose also resulting in lower egg fertilization rates (**Garcia, 1989**). The use of LHRH-A implants also advanced spawning by several months (**Garcia, 1990**). In the rabbitfish, *Siganus guttatus*, implants of silastic pellets containing 6.7 mg LHRH-A $250g^{-1}$ fish resulted in accelerated (8-9 days after pellet implantation) and more synchronized spawning as compared to controls (**Harvey et al., 1985**).

Neuroendocrine regulation of GtH-II secretion in teleosts is under a dual hypothalamic control, stimulation by gonadotropin-releasing hormone (GnRH) and inhibition by dopamine, which functions as a gonadotropin release-inhibition factor (GRIF) acting directly at the level of the pituitary to modulate the actions of GnRH as well as the spontaneous release of GtH-II, and inhibits release of GnRH (**Peter et al., 1986&1991**). In the goldfish, *Carassius auratus*, other neurohormones have been reported to regulate the secretion of GtH-II, these include norepinephrine (NE) or serotonin (5-HT) (**Chang & Peter, 1984; Somoza et al., 1988**),

Neuropeptide Y (NPY) (**Peng et al., 1990**), and GABA (**Kah et al., 1990**). Studies on the localization of GnRH show the presence of immunoreactive perikarya in several brain locations, including the ventral telencephalon at the border of the olfactory bulbs (**Munz et al., 1982**), in the ventrolateral preoptic region (POA) (**Nozaki & Kobayashi, 1979; Munz et al., 1981; Kah et al., 1984a**), the nucleus lateralis tuberis (NLT) (**Schreibman et al., 1983& 1984; Kah et al., 1984a**), area dorsalis pars medialis of the telencephalon (**Goos & Murathanoglu, 1977**), in the preoptic nucleus (**Borg et al., 1982; Goos et al., 1985**), and periventricular thalamic nuclei and dorsomedian tegmentum (**Borg et al., 1982**).

In vitro and *in vivo* testing of mammalian decapeptide luteinizing hormone-releasing hormone (LH-RH) on teleosts suggested that there is a structural similarity between teleostean GnRH (tGnRH) and mammalian GnRH (mGnRH) (**Peter, 1982&1983**), with a possible difference in tGnRH by amino-acid substitution in positions 7 and 8, as reported in tilapia (**King & Millar, 1980**), which was further confirmed by Barnett et al., 1982. The primary sequence of tGnRH was subsequently determined in the chum salmon, *Oncorhynchus keta*, as (Trp^7-Leu^8)-LH-RH (**Sherwood et al., 1983**). Structures similar to this salmon GnRH (sGnRH) were also reported in *Salmo gairdneri*, *Carassius auratus*, and *Solea solea* (**Breton et al., 1984**), and in *Mugil cephalus*, and *Chanos chanos* (**Sherwood et al., 1984**). The presence of a second decapeptide, differing at the C-terminal region, was reported in these species, suggesting the existence of another form of GnRH in fish, a situation encountered in birds. This finding was further strengthened by results obtained by **Barnett et al., (1982)** and **Idler and Crim (1982)**.

The actions of GnRH on GtH release has been reported for many fish species (**Peter, 1982, 1983**). Considering the wide distribution of GnRH in the brain, it has been suggested that GnRH actions are not limited to GtH release only, but may extend to being a neuromodulator or even a neuromediator, as in other vertebrates. tGnRH was shown to act as a neurotransmitter in bullfrog sympathetic ganglia (**Jones et al., 1984**).

The presence of dopamine inhibition of GtH-II release has been confirmed in a number of teleost species, including African catfish, *Clarias gariepinus*, (**De Leeuw et al., 1986**), coho salmon, *Oncorhynchus kisutch*, (**Van der Kraak et al., 1986**), European eel, *Anguilla*

anguilla, (**Dufour et al., 1984&1988**), Japanese eel, *Anguilla japonica,* (**Lin et al., 1990**), rainbow and brown trout, *Oncorhynchus mykiss,* and *Salmo trutta*, (**Billard et al., 1984**), and tilapia, *Oreochromis niloticus* x *O. aureus*, (**Gissis et al., 1988**).

This knowledge has led to the use of a two component system for induction of ovulation involving the combination of dopamine antagonist and GnRH analogue (**Peter et al., 1988a**). This dual system was first used in the goldfish, where injection of a dopamine receptor antagonist, pimozide (PIM), caused a marked potentiation of the GtH-II release response to (D-Ala^6 , Pro^9-NEt)-mammalian GnRH (mGnRH-A). The combined use of the two agents was highly effective in inducing ovulation (**Chang & Peter, 1983; Chang et al., 1984; Sokolowska et al., 1984**). Similar results were reported for the coho salmon (**Van der Kraak, 1984**), the common carp (**Billard et al., 1983**), the catfish, *Heteropneustes fossilis* (**Tharakan & Joy, 1996**), and a number of species of cultured Chinese carp and loach (**Lin et al., 1986a, 1987a&b; Peter et al., 1986, 1987a, 1988a&b**).

The inhibitory actions of dopamine on GtH-II secretion differs between species. For example, in goldfish and common carp, dopamine inhibition of GtH-II secretion is strong, and mGnRH-A and salmon GnRH (sGnRH-A) alone stimulate only a modest increase in serum GtH-II levels and are ineffective in inducing ovulation (**Peter et al., 1985, 1986, 1987b; Sokolowska et al., 1985a&b; Lin et al., 1987a**). In the walleye, it has been shown that pimozide (1 mg/kg body weight) can accelerate oocyte development when used on its own (**Pankhurst et al., 1986**). Administration of pimozide or domperidone greatly potentiates the action of mGnRH-A and sGnRH-A on GtH-II release, and combined injections of PIM or DOM and mGnRH-A or sGnRH-A are highly effective in inducing ovulation and spawning in these species (**Lin et al., 1988; Peter et al., 1988a&b**). On the other hand, in bream, *Parabramis pekinensis*, and chinese loach, *Paramisgurnus dabryanus*, the dopamine inhibitory action on GtH-II secretion is not so strong, and injection of a high dose of mGnRH-A or sGnRH-A alone overcomes the dopaminergic inhibition, and is highly effective in stimulating GtH-II release and ovulation. However, the use of a combination of PIM with mGnRH-A potentiated the GtH-II response and shortened the response time from injection to ovulation (**Lin et al., 1985, 1986a&b**). In contrast, in some marine species, such as the Atlantic croaker, *Micropogonias undulatus*,

(**Copeland & Thomas, 1989**), guilthead seabream, *Sparus aurata,* (**Zohar et al., 1987**), striped bass, *Morone saxatilis*, (**Sullivan et al., 1992**) and the catfish , *Heteropneustes fossilis,* (**Tharakan & Joy, 1996**), there is no evidence for a dopamine inhibitory regulation of GtH-II secretion or ovulation. In all the above species there was no inhibitory effect of injection of a number of dopamine agonists and antagonists on either GtH-II secretion or ovulation response to mGnRH-A.

A considerable amount of research has been done on the mechanisms of GtH-stimulated oocyte final maturation and ovulation in teleosts. An understanding of these processes is deemed to be important in the sense that it may lead to techniques of induced ovulation without the use of GnRH or GtH. Increase of GtH levels in the circulation triggers two distinct ovarian processes- final maturation and ovulation of the oocyte. Studies on a variety of teleosts indicate that GtH induces final maturation indirectly by stimulating follicular steroidogenesis (**Hirose, 1976; Jalabert, 1976**). Studies on the nature of the steroids mediating this effect of GtH showed that the steroid most potent in inducing final maturation in many teleosts is 17α-hydroxy-20β-dihydroxyprogesterone (17α-20β-OHP), e.g. in rainbow trout, *Oncorhynchus mykiss*, goldfish, *Carassius auratus*, and northern pike, *Esox lucius* (**Jalabert, 1976**), yellow perch, *Perca flavescens* (**Goetz & Theofan, 1979**), brook trout, *Salvelinus fontinalis* (**Duffey & Goetz, 1980**), and amago salmon, *Oncorhynchus rhoduras* (**Nagahama et al., 1980**). In other species like the zebrafish, *Brachydanio rerio*, deoxycorticosterone is more effective than 17α-20β-OHP in inducing maturation (**Van Ree et al., 1977**), and the catfish, *Heteropneustes fossilis*, evidence shows that GtH stimulates synthesis of interrenal corticosteroids which in turn act on the ovary (**Sundararaj and Goswami, 1977; Truscott et al., 1978**). Ovarian steroids may also act at the level of the brain or pituitary to regulate preovulatory GtH release (**Morell et al., 1975; Kim et al., 1978**). As shown for a variety of mammalian species (**reviewed by Espey, 1980**), final stages of teleost ovulation, rupture of the follicle, and expulsion of the mature oocyte is stimulated by prostaglandins (**see Stacey & Goetz, 1982; Goetz, 1983**).

Studies with synthetic steroids in teleosts have indicated that C_{19} and C_{18} steroids are virtually ineffective in inducing oocyte final maturation. C_{21} steroids, however , especially those with a

17β-hydroxyl group, have generally been found to be very effective (**see Goetz, 1983; Scott & Canario, 1987**). The most effective steroids are 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P), which is believed to be the maturation inducing-steroid (MIS) in fish of the orders Salmoniformes, Cypriniformes, and Siluriformes (**Nagahama & Adachi, 1985; Scott & Canario, 1987; Canario & Scott, 1988**), and 17α,20β,21-trihydroxy-4-pregnen-3-one (17,20β,21-P), which plays a similar role in the Atlantic croaker, *Micropogonias undulatus*, (**Trant et al., 1986; Trant & Thomas, 1988**). The presence of critical oocyte sizes for steroid responsiveness have been found in many fishes (**Canario & Scott, 1990**). In the same study a number of steroids were tested on two marine flatfishes and it was found that the "critical" oocyte size was 940 μm. It was also found that oocytes respond to hCG at a smaller size, indicating that oocytes acquire their responsiveness to gonadotropin before that due to steroids. Similar observations have been made in the yellow perch, *Perca flavescens* (**Goetz & Theofan, 1979**), in killifish, *Fundulus heteroclitus* (**Wallace & Selman, 1980**), and *Sillago japonica* (**Kobayashi et al., 1988**).

Stimulation of the entire process of oogenesis or early gonad development, including vitellogenesis, has been attempted in some fish species. In the Japanese eel, gonadotropin treatments stimulated oogenesis (**Ochiai et al., 1972**), while in the mullet implantation of testosterone and LHRH-A stimulated oocyte growth (**Kelley et al., 1987**). Control of oogenesis was also reported for milkfish, *Chanos chanos*, using LH-RH-A (200 μg) and methyltestosterone (MT) (0.25 mg) (**Tamaru et al., 1988**), the Ayu, *Plecoglossis altivelis*, using 500 μg LH-RH (**Hirose and Arai, 1988**), and the medaka, *Oryzias latipes*, using LHRH-A injected twice a week (**Chan, 1977**).

In Bahrain, the ovaries of captive *S. canaliculatus* do not develop beyond the stage of exogenous vitellogenesis. Spawning is routinely induced using hCG. The objectives of the present study were to test the effects and optimize the regimes of hormonal intervention for the induction of maturation and spawning using combinations of a gonadotropin (hCG), analogues of mammalian and piscine hypothalamic releasing hormones, and the dopamine antagonist, domperidone.

6.2 Materials & Methods:

6.2.1 Hormones and Dopamine Antagonist:

Human Chorionic gonadotropin (hCG) was purchased from Sigma Chemicals (1000 IU/vial). Salmon gonadotropin-releasing hormone analogue, [D-Arg^{6} , Trp^{7} , Leu^{8} , Des-Gly^{10}]-LH-RH Ethylamide (sGnRH-A), and mammalian gonadotropin-releasing hormone analogue, [D-Ala^{6} , Des-Gly^{10}]-LH-RH Ethylamide (mGnRH-A) were purchased from Peninsula Labs. Domperidone was a gift from Janssen Pharmaceuticals, Beerse, Belgium. hCG, mGnRH-A and sGnRH-A were dissolved and made to required concentrations in 0.7% saline. Domperidone was either suspended in 0.7% NaCl and 0.1% sodium metabisulphite, or dissolved in dimethyl sulphoxide (DMSO) and diluted with propylene glycol to the final concentration for injection. There was no difference in the outcome of the experiments using the two vehicles. The latter was adopted because of the greater convenience of dispensing the solution rather than the suspension. Injected volume in all experiments did not exceed one ml.

6.2.2 Experimental Enclosures, Fish and Procedures:

The following conditions were used in all experiments. One male and one female rabbitfish were held in a floating net cage 60cmx60cmx60cm, several of which were placed in a covered 7mx2mx1m reinforced glass fibre tank (see Fig.3.1). Placed below each floating cage there was an egg collector made of a 15cmx15cm sheet of corrugated plastic, the surface of which was gently scoured to provide a suitably roughened substrate for the attachment of the adhesive eggs. The tank was provided with continuous running water and aeration, and the bottom was cleaned whenever necessary. The fish were given a 24 hour bath treatment in 0.5 PPM Prefuran (Argent Chemicals, USA), at the start and halfway through the experiment, as a measure of disease prevention.

Fully vitellogenic females with stage V ovaries and the same number of running males (males that give out milt with gentle pressure on the abdomen) were used. All injections were given intramuscularly at the base of dorsal fin in the anterior third of its length with the syringe needle directed towards the head. All males were given one injection of hCG (Sigma

Chemicals) at a dose of 0.5 IU g^{-1} body weight (half of the commonly used dosage for inducing females) at the beginning of each experiment as a precaution to ensure spawning readiness.

A - Experiments Conducted:

Series I - Optimization of the dose of Human Chorionic Gonadotropin (hCG) for the induction of spawning:

Six groups each consisting of 12 replicate female/male pairs were set up. Females were given a single injection at the start of the experiment of either saline vehicle or hCG at a dose of 0.1, 0.5, 1.0, 2.0 and 5.0 IU g^{-1} body weight. As mentioned above, males in all experiments were given a single injection of hCG at the beginning at a dose of 0.5 IU g^{-1} body weight.

Series II - Effect of salmon gonadotropin-releasing hormone analogue (sGnRH-A) on the induction of spawning:

Four groups each consisting of 12 replicate female/male pairs were set up. The females were given a single injection of either saline vehicle or sGnRH-A at doses of 0.001, 0.01 and 0.1 μg g^{-1} body weight.

Series III - Effect of mammalian gonadotropin-releasing hormone analogue (mGnRH-A) on the induction of spawning:

Six groups each consisting of 12 replicate female/male pairs were set up. The females were given a single injection of either saline vehicle or mGnRH-A at doses of 0.001, 0.01, 0.1, 0.5 and 1.0 μg g^{-1} body weight.

Series IV - Effect of the dopamine antagonist domperidone (DOM) on the induction of spawning:

Three groups each consisting of 12 replicate female/male pairs were set up. The females were given a single injection of either saline vehicle or domperidone at doses of 5.0 and 10.0 μg g^{-1} body weight.

Series V - Effect of combining DOM and hCG on the induction of spawning:
Four groups each consisting of 12 replicate female/male pairs were given an injection of either domperidone vehicle (1 group) or 10 μg g^{-1} domperidone (3 groups). The domperidone vehicle only group was given simultaneously an injection of hCG vehicle. The 3 domperidone injected groups were given simultaneously an injection of hCG at a dose of either 0.1, 0.5 or 1.0 IU g^{-1} body weight.

Series VI - Effect of the combination of DOM and sGnRH-A on the induction of spawning:
Four groups each consisting of 12 replicate female/male pairs were given an injection of either domperidone vehicle (1 group) or 10 μg g^{-1} domperidone (3 groups). The domperidone vehicle only group was given simultaneously an injection of sGnRH-A vehicle. The 3 domperidone injected groups were given simultaneously an injection of sGnRH-A at a dose of either 0.001, 0.01 or 0.1 μg g^{-1} body weight.

Series VII - Effect of the combination of DOM and mGnRH-A on the induction of spawning:
Six groups each consisting of 12 replicate female/male pairs were given an injection of either domperidone vehicle (1 group) or 10 μg g^{-1} domperidone (5 groups). The domperidone vehicle only group was given simultaneously an injection of mGnRH-A vehicle. The 5 domperidone injected groups were given simultaneously an injection of mGnRH-A at a dose of either 0.001, 0.01, 0.1, 0.5 or 1.0 μg g^{-1} body weight.

B - Sampling Procedure:

Fish were biopsied at the start of the experiment to confirm histologically the previous macroscopic assessment of stage (V) status made according to the criteria outlined in section 2.3.4 of Chapter 2. Further biopsies were done at 24 hours intervals until the end of the experiment. Biopsied oocytes were fixed in 4% formaldehyde in neutrally buffered saline (Table 2.4, Chapter 2) prior to histological examination. Sections of oocytes stained in polychrome stain (Table 2.7, Chapter 2) were used for measurement of oocyte diameters and assessment of hydration (see below). Egg collectors were checked three times a day for signs of spawning, without causing any visual disturbance to the fish.

C - Measurements:

1. Oocyte Diameter - Diameters were measured using a digitizing pad on oocyte images projected on a screen. Measurements were done on triplicate sample of 30 vitellogenic oocytes from each of three fish in each group (for details see Chapter 2, Section 2.2.2).

2. Spawning Percentage - Observations were made on the percentage of the 12 females in each group which spawned.

3. Latency Period- This was measured as the number of hours between injection and spawning. Since the exact moment of spawning was rarely observed, the time of spawning was back calculated from the embryo stage first observed using known rates of development. The experiment ran for a maximum of 168 hours or until such earlier time when all fish in a group had spawned.

4. Time to Oocyte Hydration - This was measured as the number of hours between injection and the first observation of hydrated eggs. Hydrated eggs can be identified by the naked eye from biopsied samples; they are transparent and adhesive. Observations were recorded from all fish in each group.

5. Fertilization Rate- The percentage of fertilized eggs were determined by microscopic observation of three random samples of 100 eggs each from half the number of the spawned females.

6. Hatching and Larval Viability Rates- Three sub-samples of 100 fertilized eggs from three spawned females in each group were hatched and maintained for 24 hours after hatching in one liter beakers placed in a constant temperature water bath (26°C). The number of hatched larvae, and the number of normal larvae surviving at the end of the 24 hours were used to determine hatching and larval viability rates, respectively.

6.2.3 Statistical Analyses:

All values are given as the mean and the standard error of the mean (s.e.m.). Oocyte diameters, latency period and gamete quality parameters were tested using a One-way analysis of variance followed by Bartlett's test for homogeneity of variances and Tukey-Kramer Multiple Comparison Test for difference between groups. Evaluation of the effect of treatments on spawning numbers was made using the Fisher Exact test ($P < 0.05$). Percentages were changed to numbers for purposes of statistical testing. All tests were performed on a Minitab statistical package V.10 (Minitab, Inc.) and GraphPad Instat2 Statistical Software.

6.3 Results:

The mean oocyte diameter of every biopsied sample collected at the beginning of each of the six series of experiments was greater than 400 μm, which is similar to the mean diameter of fully vitellogenic oocytes established earlier in Section 2.3.4 of Chapter 2. This was further confirmed histologically by sectioning and staining oocyte samples taken randomly from all groups. All groups of females were therefore judged to have developed to a stage at which they had the potential to respond to hormones and compounds which promote maturation, ovulation and spawning.

Series I - Optimization of the dose of human chorionic gonadotropin (hCG) for the induction of spawning:

All dose levels of hCG from 0.1 to 5.0 IU g^{-1} induced spawning in more than half of the 12 members of each group (Fig.6.1). The number of spawners in each hCG treated group was significantly greater ($p=0.004$ or less) than the control group in which no spawning occurred. Spawning numbers in the groups treated with 0.1, 0.5, 1.0 and 2.0 IU g^{-1} did not differ significantly ($p=0.2$-0.6) from each other. The number of spawners in the group treated with the highest dose of hCG was significantly less ($p=0.02$-0.03) than in all other groups except that treated with the lowest dose of hCG. In general, there was a trend towards a lower incidence of spawning at highest doses of hCG.

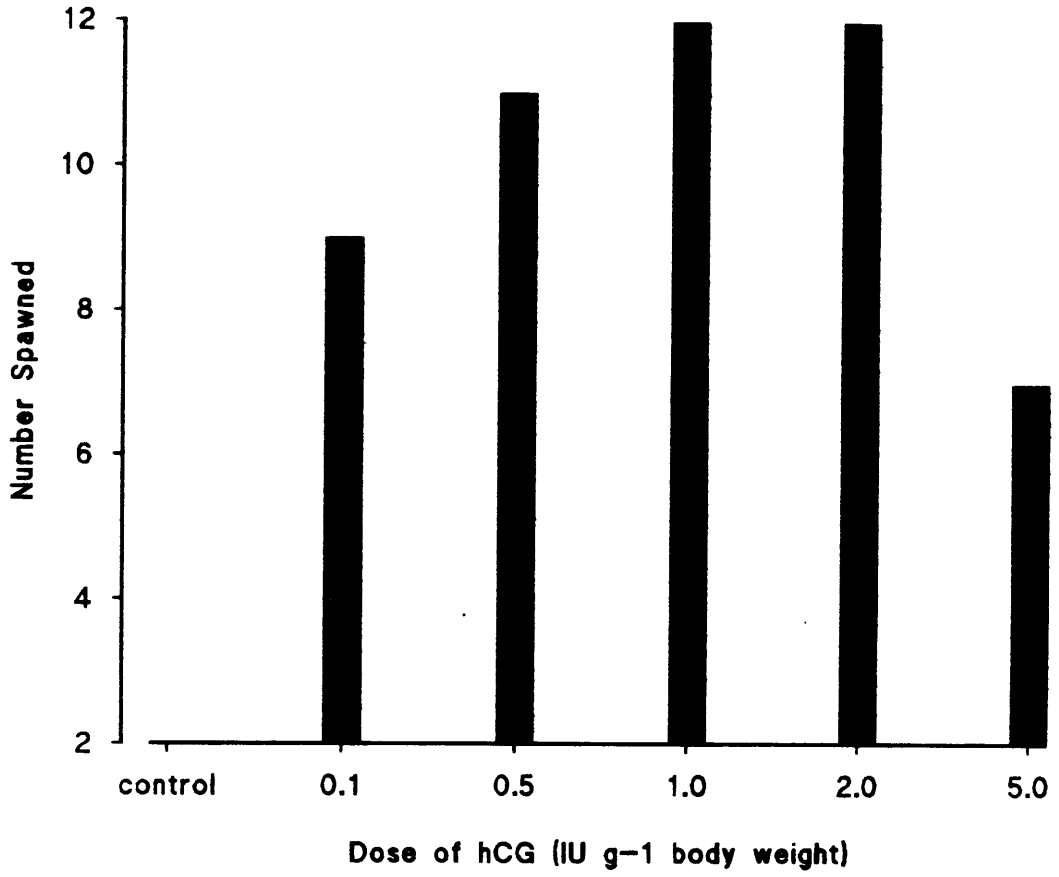


Figure 6.1– Effect of Human Chorionic Gonadotropin (hCG) on induction of spawning in *S. canaliculatus.* Each column shows the number of spawning fish out of a total of 12.

The mean latency periods (Fig.6.2) in the three groups on intermediate doses of 0.5, 1.0 and 2.0 IU g^{-1} did not differ significantly ($p>0.05$), but were significantly shorter than in the groups on the lowest dose ($p<0.01$ or less) and the highest dose ($p<0.001$). Overall there was a trend towards longer latency periods at highest doses of hCG.

The mean fertilization rates (Fig.6.3) for eggs from the three groups on the highest hCG doses (1.0, 2.0 or 5.0 IU g^{-1}) were similar to each other, and were significantly higher ($p<0.001$ in all cases) than the fertilization rates for the two groups on the lower doses, which were similar to one another.

Hatching rates (Fig.6.4) showed a similar pattern of response to hCG as did fertilization rates. Eggs from the groups of fish on the three highest doses of hCG had significantly higher hatching rates ($p<0.001$ in all cases) than for the two groups on lower doses of hCG. The hatching rate of 1.0 IU g^{-1} group was significantly higher ($p<0.001$) than all other groups except the 2.0 IU g^{-1} group.

Larval viability rates (Fig.6.5) showed a distinct peak at an intermediate hCG dose of 1.0 IU^{-1}. This rate was significantly greater than at all higher ($p<0.001$-0.01) and lower ($p<0.01$ or less) hCG dose levels.

Figure 6.6 shows the effect of hCG on oocyte diameter and on the timing of hydration. In controls injected with hCG vehicle the mean oocyte diameter tended, if anything, to decrease, and hydration did not occur. In all hCG-injected groups, mean oocyte diameter increased and hydration occurred with a sharp increase in oocyte diameter to more than 600 μm. At extreme dose levels (0.1 and 5.0 IU hCG g^{-1}), oocyte diameter tended to increase more slowly and hydration occurred later (96h) than in groups given intermediate doses of hCG in which oocyte diameter increased progressively and hydration occurred earlier (72h).

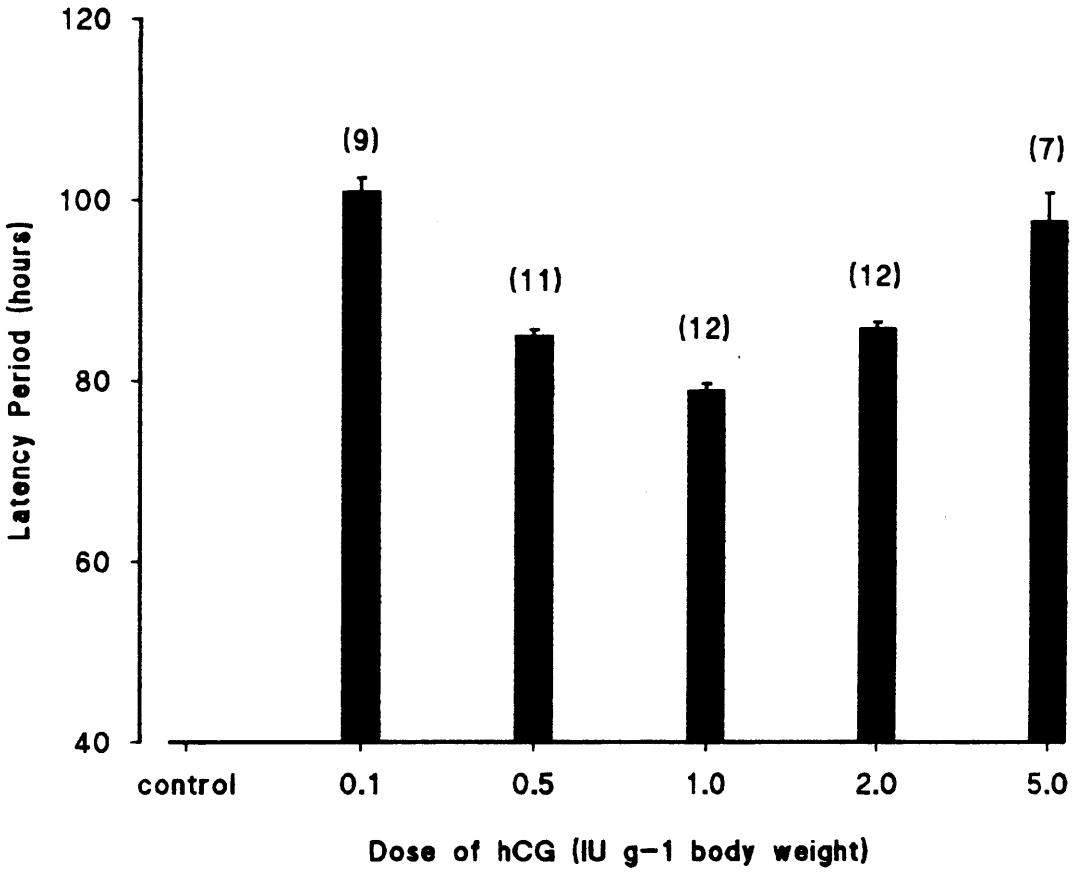


Figure 6.2– Effect of Human Chorionic Gonadotropin (hCG) on the mean latency period (mean±s.e.m.) between injection and spawning in *S. canaliculatus.* Figures in brackets denote the number of fish out of 12 which spawned within a maximum of 168 hours.

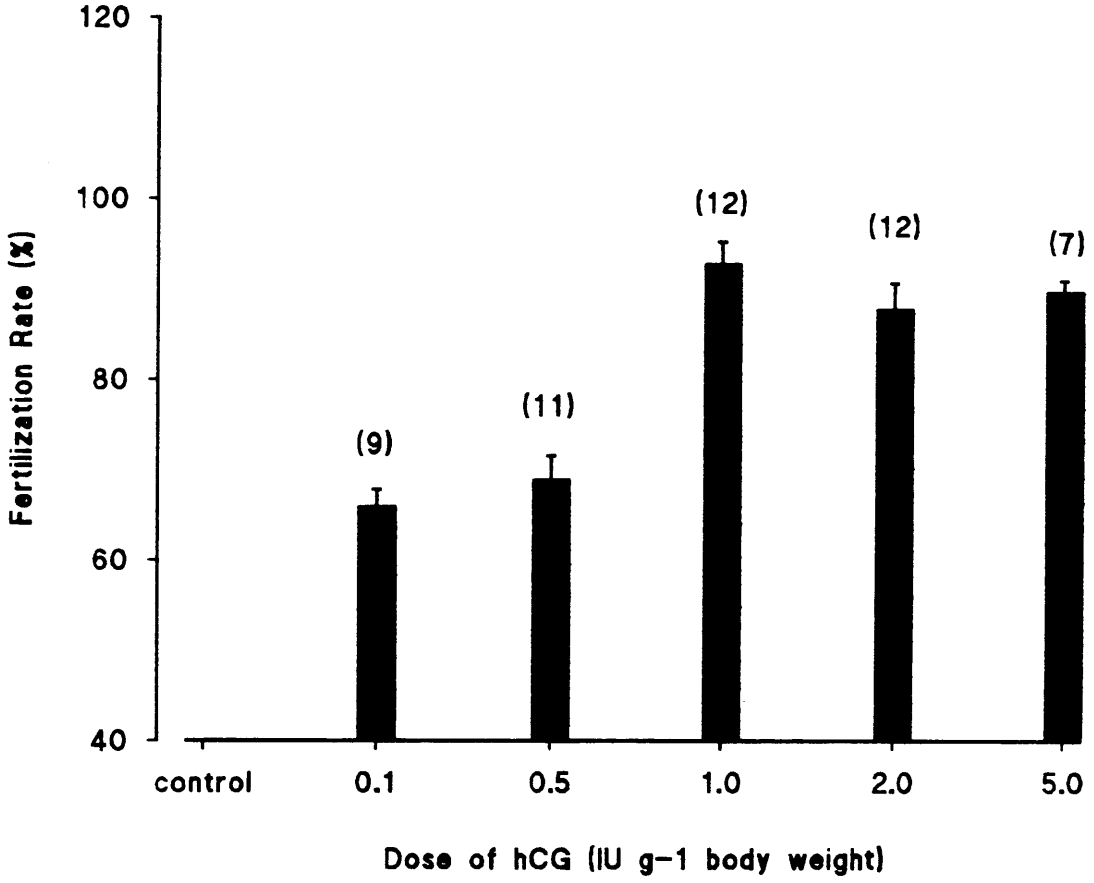


Figure 6.3– Effect of Human Chorionic Gonadotropin (hCG) on fertilization rates of eggs from induced spawnings in *S. canaliculatus.* Fertilization rates (mean±s.e.m.) are expressed as the percentage of fertilized eggs from triplicate samples from half the females. Figures in brackets denote the number of spawning fish out of a total of 12.

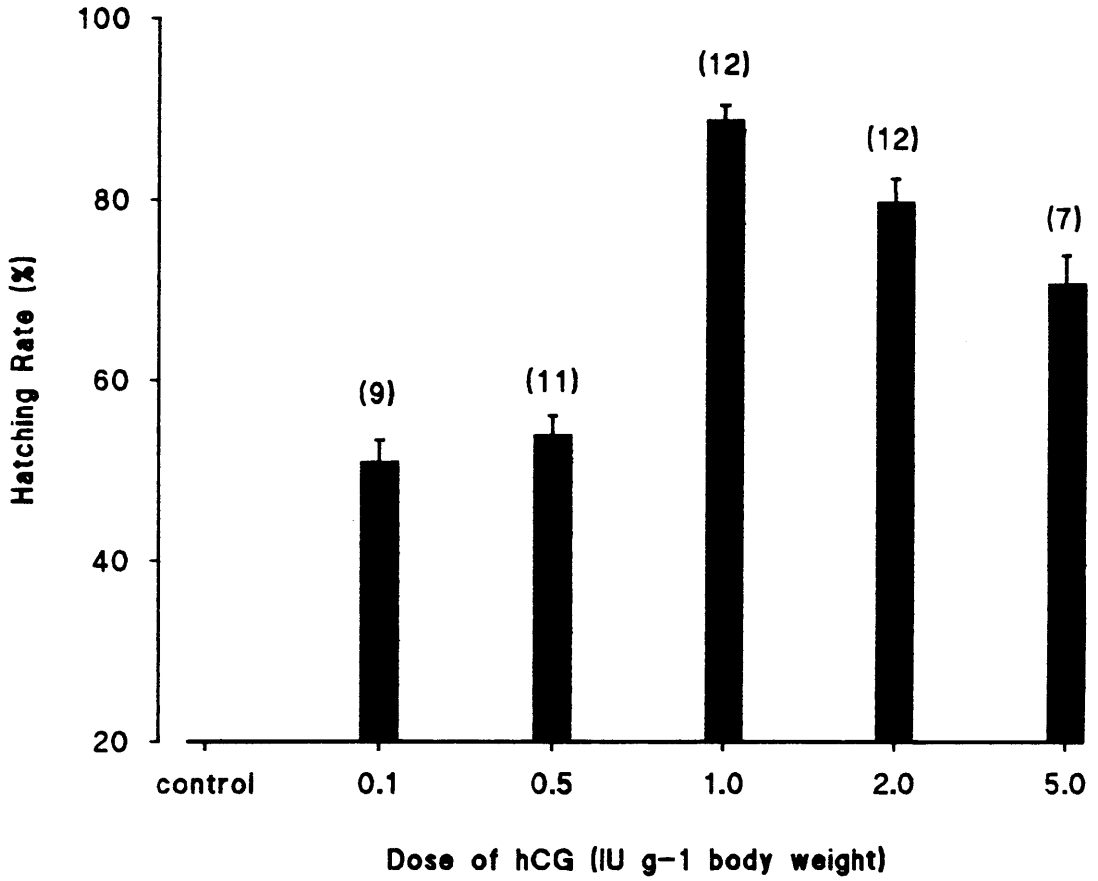


Figure 6.4– Effect of Human Chorionic Gonadotropin (hCG) on hatching rates (mean ±s.e.m.) of eggs from induced spawnings in *S. canaliculatus.* Figures in brackets denote the number of spawning fish out of a total of 12.

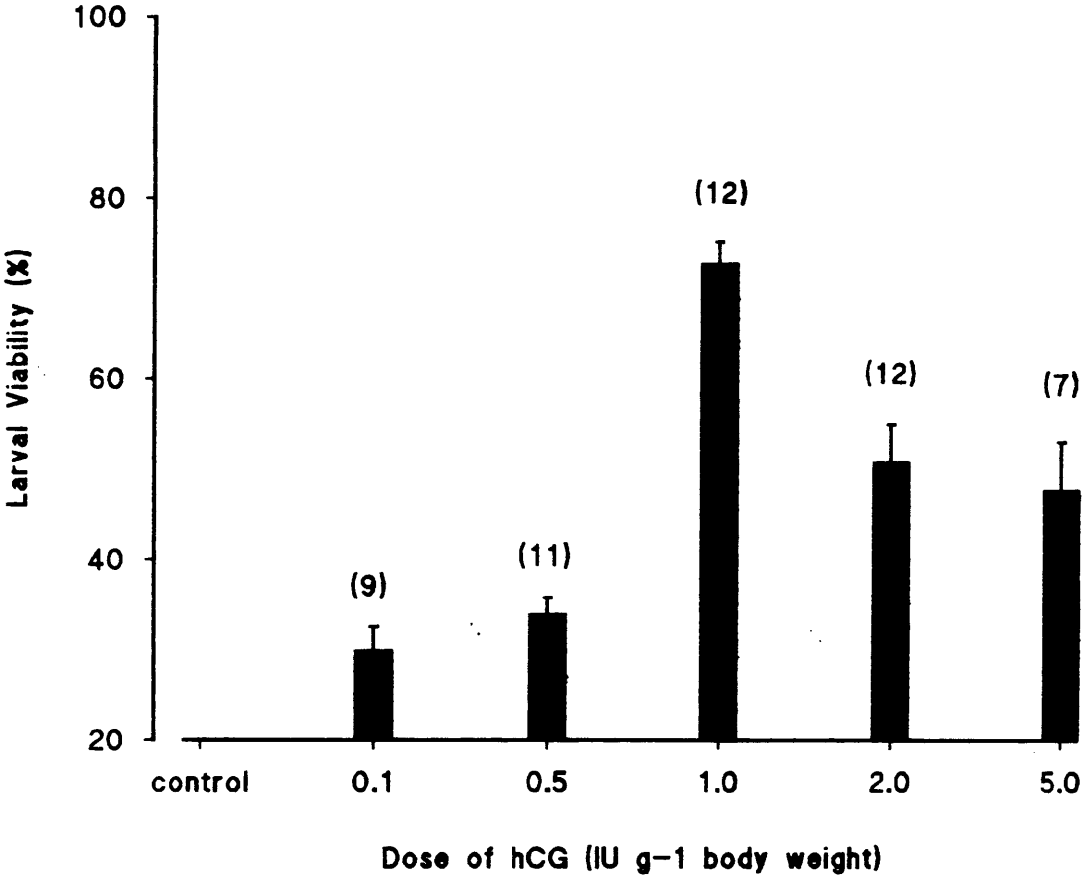


Figure 6.5– Effect of Human Chorionic Gonadotropin (hCG) on viability rate (mean±s.e.m.) of larvae from induced spawnings in *S. canaliculatus*. Figures in brackets denote the number of spawning fish out of a total of 12.

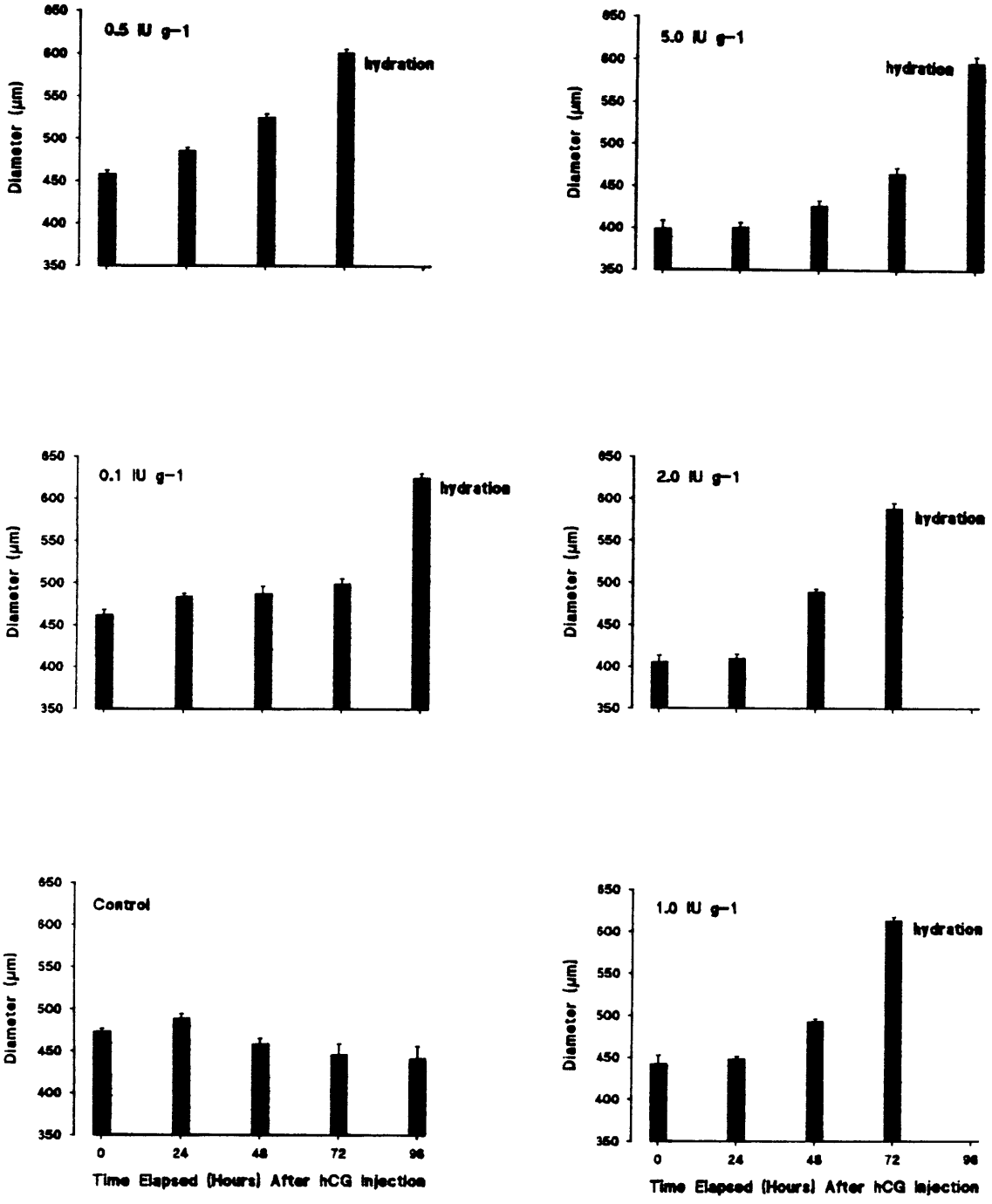


Figure 6.6- Effect of Human Chorionic Gonadotropin (hCG) on oocyte diameter (mean±s.e.m.) and time of completion of oocyte hydration in *S. canaliculatus*. Measurements were done on triplicate samples of 30 vitellogenic oocytes from each of three females in each group. hCG dose is shown at top left of each histogram.

Series II - Effect of salmon gonadotropin-releasing hormone analogue (sGnRH-A) on the induction of spawning:

Non of the females injected with a saline vehicle or the two lower doses of sGnRH-A spawned. In the highest dose of 0.1 $\mu g\ g^{-1}$ body weight, only two fish out of six spawned with no significant difference ($p=0.45$). The mean latency period for the spawned fish was 88±2.0 hours (Table 6.1).

Series III - Effect of mammalian gonadotropin-releasing hormone analogue (mGnRH-A) on the induction of spawning:

Similar results were also observed in this experiment. The only induction of spawning occurred in the groups injected with the higher dose of mGnRH-A (0.1, 1.0 and 5.0 $\mu g\ g^{-1}$ body weight), but no significant difference was found between the groups ($p=0.47$ or more). Latency periods were 190, 120±0.0 and 145 hours respectively (Table 6.1).

Series IV - Effect of the dopamine antagonist domperidone (DOM) on the induction of spawning:

Domperidone given in two dosages of 5 and 10 $\mu g\ g^{-1}$ body weight failed to induce spawning in any fish. There was also no spawning in the control group given a sham injection of vehicle (Fig.6.7).

Series V - Effect of the combination of DOM and hCG on induction of hydration and spawning:

No spawning occurred in control groups injected with DOM, DOM vehicle, and with DOM and hCG vehicles (Fig.6.7). The addition of DOM to hCG at the three lowest doses (0.1-1.0 IU g^{-1}) increased the number of spawners to the maximum (Fig.6.7) and decreased the latency period (Fig.6.8).

The effect on hydration of injecting DOM as well as hCG was to decrease by c.24 hours the time interval between injection and completion of hydration (Fig.6.9).

Table 6.1 - The effect of salmon gonadotropin-releasing hormone analogue (sGnRH-A) and mammalian gonadotropin-releasing hormone analogue (mGnRH-A) on the incidence of spawning and on the latency period.

	sGnRH-A ($\mu g\ g^{-1}$ body weight)			
	control	**0.001**	**0.01**	**0.1**
Number of Spawners out of a Total of 12	0	0	0	2
Latency Period (Hours) from Injection to Spawning	-	-	-	88±2.0

	mGnRH-A ($\mu g\ g^{-1}$ body weight)					
	control	**0.001**	**0.01**	**0.1**	**0.5**	**1.0**
Number of Spawners out of a Total of 12	0	0	0	1	2	1
Latency Period (Hours) from Injection to Spawning	-	-	-	190	120±0.0	145

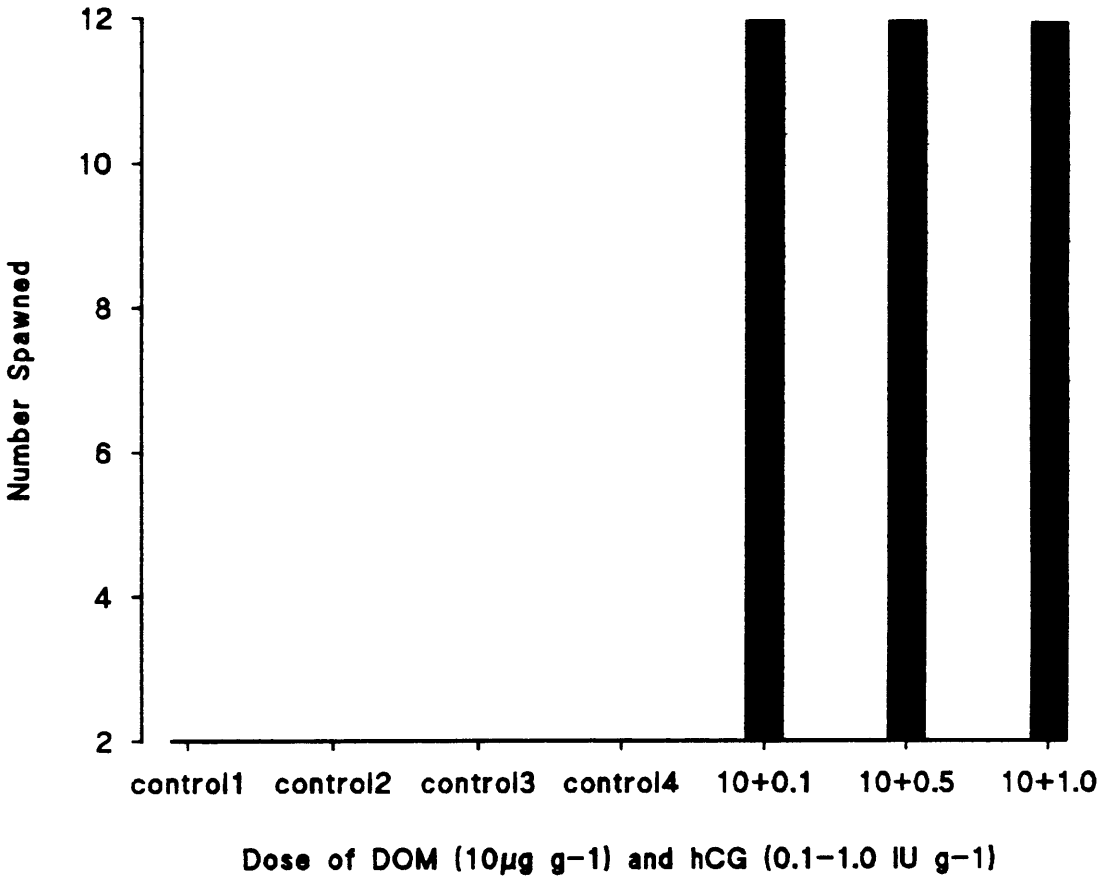


Figure 6.7– Effect of the combination of Domperidone (DOM) and Human Chorionic Gonadotropin (hCG) on induction of spawning in *S. canaliculatus*. Each column shows the number of spawning fish out of a total of 12. Control group1 were injected with DOM and hCG vehicles only, control group 2 with DOM vehicle only, control groups 3 & 4 with DOM only at a dose of 5 and 10 µg g−1 respectively.

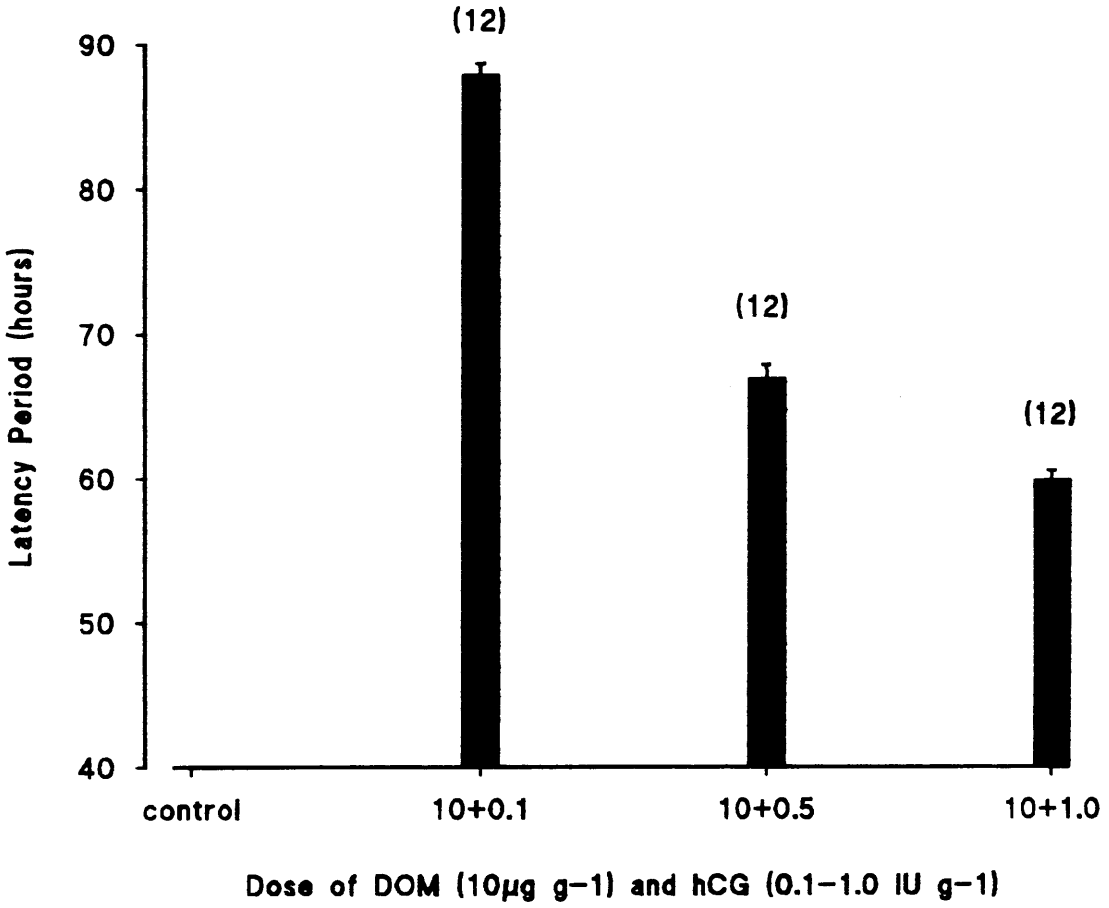


Figure 6.8– Effect of the combination of Domperidone (DOM) and Human Chorionic Gonadotropin (hCG) on the incidence of spawning and latency period (hours). Latency period (mean±s.e.m.) is the interval between injection to spawning. Figures in brackets show the number of spawning fish out of a total of 12.

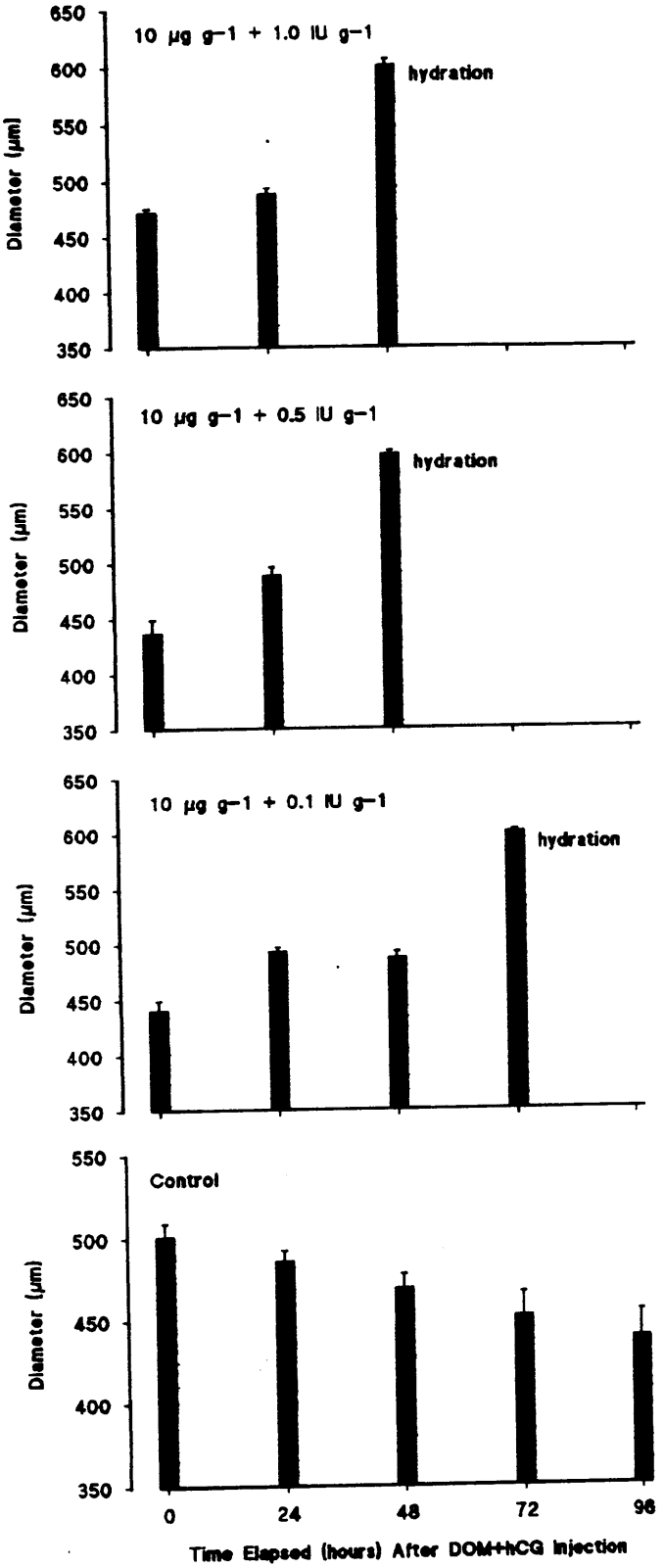


Figure 6.9– Effect of Domperidone (DOM) and Human Chorionic Gonadotropin (hCG) on oocyte diameter (mean±s.e.m.) and on time interval between injection and completion of oocyte hydration. Figures at top left of each histogram are the doses of DOM and hCG respectively. Measurements were done on triplicate samples of 30 oocytes from each of three fish from each group.

Series VI - Effect of the combination of DOM and sGnRH-A on the induction of spawning:
The effect of the addition of DOM on sGnRH-A-induced spawning and latency period were marginal. With domperidone 5 of 24 fish spawned at sGnRH-A doses of 0.01 or 0.1 $\mu g\ g^{-1}$ (Table 6.2) compared with only two spawners without domperidone (Table 6.1). There was also a tendency for latency periods to be shorter in domperidone-treated groups (Tables 6.1 and 6.2).

Series VII - Effect of the combination of DOM and mGnRH-A on the induction of ovulation and spawning:
As with the previous sGnRH-A-treated groups, the effects of the addition of domperidone on mGnRH-A-induced spawning and latency period were marginal. With domperidone 8 of 24 fish spawned at mGnRH-A doses of 0.01 to 1.0 $\mu g\ g^{-1}$ (Table 6.2). There was no clear effect of domperidone on the latency period of mGnRH-A injected groups (cf. Tables 6.1 and 6.2).

6.4 Discussion:

Most of the criteria (spawning and gamete quality) used to judge the effectiveness of the maturation and spawning promoting compounds that were tested are dependent upon the assumption that all the males used are in a state of spawning readiness and therefore sperm quality in addition to availability is good and suitable in all cases. As mentioned earlier in Chapters 3 and 5, precautions were taken to make this assumption as true as possible. Only running males which readily gave out white milt (yellowish white color is low quality sperm as ascertained from previous experience) with gentle pressure on the abdomen were used. In the experiments listed in this chapter, males were also injected with 0.5 IU g^{-1} of hCG to further promote sperm quality; again, such practice has been commonly used for larval production and has given good results.

Another point that also needs to be mentioned is that although captive siganids in the area of study do not mature and spawn naturally without hormonal induction, the judging of optimum values for criteria such as latency period is based on comparisons with data available for fish of the same species that do spawn naturally in other areas (**Duray, 1990**).

Table 6.2 - The effect of the combination of Domperidone (DOM) and salmon gonadotropin-releasing hormone analogue (sGnRH-A) or mammalian gonadotropin-releasing hormone analogue (mGnRH-A) on the incidence of spawning and on the latency period.

	DOM+sGnRH-A (μg g^{-1} body weight)			
	control	**10+0.001**	**10+0.01**	**10+0.1**
Number of Spawners out of a Total of 12	0	0	2	3
Latency Period (Hours) from Injection to Spawning	-	-	87±3.50	76±2.60

	DOM+mGnRH-A (μg g^{-1} body weight)					
	control	**10+0.001**	**10+0.01**	**10+0.1**	**10+0.5**	**10+1.0**
Number of Spawners out of a Total of 12	0	0	2	2	2	2
Latency Period (Hours) from Injection to Spawning	-	-	85±1.50	72±12.5	120±0.0	120±0.0

In the many attempts to control the process of reproduction , whether it is the early part or the late maturation and ovulation phase, quality of the gamete produced was rarely tested. Usually mentioned criteria of success are percentages of spawning and fertilization. The later stages of embryonic development need to be considered more. Larval viability is perhaps the most important factor in deciding the optimum dosage of a spawning inducing agent. The deleterious effects of too high or too low dosages may not be apparent during spawning or early in larval development, as the results of this study clearly show, but may take effect after hatching, when abnormal or weak larvae may be observed. How the strength of the dose can cause such effects is not clear.

The present results demonstrate that injection of human chorionic gonadotropin (hCG) is effective in inducing spawning in the rabbitfish *Siganus canaliculatus*. Results indicate a dome-shaped effect of the dosage, with the peak spawning obtained at the intermediate dose 1.0 IU g^{-1} body weight. However, in deciding the optimum dosage, other considerations must be taken into account; these are the latency period and gamete quality. The latency period in the different dosages may not be a critical issue, since overall the fish takes a maximum of a little over four days to respond to any of the dosages. The deciding factors would therefore have to be gamete quality. There is a tendency for the gamete quality indictors used, egg fertilization and hatching rates and larval viability rates, to decrease with not only lower dosages but also with higher ones (Figs. 6.3-6.5). The negative effect of hCG at doses of 2 IU g^{-1} body weight and more is not well understood, possibly any toxic impurities in the hCG would have increasingly inhibitory effects at higher doses.

The dopamine antagonist Domperidone (DOM) has a marked effect on the response to hCG. The DOM-mediated potentiation of several of the hCG effects (spawning incidence, latency period, and completion of hydration) implies that it is effective in blocking the inhibitory action of dopamine. This, together with the observation that DOM alone has no effect, at least on spawning, in spite of the large dose used, implies that endogenous GnRH secretion is deficient in laboratory held *S. canaliculatus* at the post-vitellogenic stage. There are no reports on the use of dopamine antagonists, whether on its own or in combination with other maturation inducing compounds, on siganids. However, the potentiating effect of DOM in

combination with GnRH analogues has been widely reported in goldfish, carps, Chinese loach, Chinese catfish (**see Lin & Peter, 1996**)

The finding that hCG induces ovulation and spawning in S. canaliculatus is consistent with the results of previous trials at the National Mariculture Center, where this study was carried out, and in the region (**Akatsu et al., 1984**), and also with many previous studies in other parts of the world, where hCG dosages between 0.25-3.0 IU g^{-1} body weight were used in single or multiple injections, resulting in varying spawning percentages (**Soh & Lam, 1973; Westernhagen & Rosenthal, 1976a; Al-Aradi et al., 1985; Akatsu et al., 1984; Ayson, 1991).** hCG has also been effective in inducing final oocyte maturation and spawning in a number of other fish species, including winter flounder, *Pseudopleuronectes americanus* (**Smigielski, 1975**), Japanese flounder, *Limnada yokohamae* (**Hirose et al., 1976**), sole, *Solea solea* (**Ramos, 1986**) and the walleye, *Stizostedion vitreum,* (**Pankhurst et al., 1986**). In vitro studies on intact follicles of the dab (*Limnada limnada*), and the plaice (*Pleuronectes platessa*) have shown that hCG induces oocyte final maturation in a dose-dependent manner (**Canario and Scott, 1990**).

Earlier studies on the use of spawning inducing agents on siganids take into account the incidence of spawning and number of spawned fish as a measure of success, but do not consider the effect on post-spawning stages. The findings of the present study indicate that such considerations are not sufficient to determine the optimum dosage, and that it is necessary to take into account the later effects on gamete quality. Taking into account all the above, it appears to be logical to conclude that the optimum dosage of hCG for inducing final maturation and spawning in *S. canaliculatus* is 1.0 IU g^{-1} body weight. For economic considerations, a dosage between 0.5 and 1.0 IU (possibly 0.75 IU) may be suitable for practical mariculture purposes if the corresponding larval survival rates are economically viable. But this has to be further investigated.

However, it is critical that the maturity of the fish is determined prior to the use of hormones as a management tool as the results depend on that. A practical method, more reliable than observations of external features like palpation or coloration of the fish abdomen, to determine the readiness of the recipient fish for induced maturation and ovulation is essential.

In certain fish (mullets, gilthead seabream, milkfish), it is the diameter and morphological features of biopsied oocytes (**Nash & Shehade, 1980; Zohar & Gordin, 1979**), or calculation of their size distribution (**Tamaru et al., 1980**) that are used as indicators of readiness. In cyprinids it is necessary to determine the extent of germinal vesicle migration (**Brzuska, 1979; Yaron & Levavi-Zermonsky, 1986**). In these experiments, hCG induced maturation and spawning in fish with oocyte diameters of over 400 μm. Histological results showed that these oocytes had completed vitellogenesis, fully vitellogenic oocytes in the species under study are between 370-500 μm (see Chapter 2, Section 2.3.2). The importance of initial oocyte diameter in determining the response to hCG injections has been emphasized in many reports. It was reported that in the rabbitfish, *S. guttatus*, the total dose of hCG required to induce spawning is inversely related to egg diameter (**Ayson, 1988**), and so is the latency period (**Ayson, 1991**). In the species under study, it is safe to assume that the use of hormones to induce spawning would be best directed at fish with oocytes at development stage (V), i.e. those which have completed vitellogenesis, a measure of which could be an oocyte diameter of >400μm.

The effects on spawning of sGnRH-A and mGnRH-A, with and without domperidone, were inconclusive. The comparatively high doses of GnRH given as a single injection induced spawning in only a few of the fish, and the inclusion of domperidone increased only marginally the number of spawners. The modest response to the GnRHs is unlikely to be due to any lack of potential at the level of the pituitary gonadotrophs or ovary, as evidenced by their capacity to respond to domperidone and hCG, respectively. Superactive GnRH analogs are considered to be resistant to enzymatic degradation, and have prolonged biological half-lives in the circulation. However, studies on the gilthead seabream, *Sparus auratus*, show that the mGnRH-A tested disappeared from circulation within about 1-2 hours after injection, and that the GtH-II surge that was caused by a single injection lasted for about 48 hours, which may not be sufficient to induce the whole process of maturation and ovulation of females (**Zohar, 1988**).

The lack of response of *S. canaliculatus* to single injections of each of the two gonadotropin-releasing hormone analogues tested on their own is consistent with studies on the goldfish,

carassius auratus, and common carp, *Cyprinus carpio*, where single injection of LHRH-A and (D-Arg^6 , Pro^9-NEt)-salmon GnRH (sGnRH-A) stimulate only a modest increase in serum GtH-II levels and are ineffective in inducing ovulation (**Peter et al., 1985, 1986, 1987b; Sokolowska et al., 1985a&b; Lin et al., 1987a**). The lack of response in those species was attributed to a strong inhibitory action of dopamine on GtH-II secretion, a concept that was proven by the administration of the dopamine receptor antagonist, pimozide or domperidone, which potentiated the actions of mGnRH-A and sGnRH-A and was found to be highly effective in inducing ovulation (**Lin et al., 1988; Peter et al., 1988a&b**). The use of domperidone in combination with mGnRH-A and sGnRH-A in this study did not significantly potentiate their action in inducing maturation and spawning, a finding that may be explained by the assumption that the inhibitory factor of dopamine may be too strong, or that the dosages of the GnRH analogues were not high enough. But the dosages used exceed the highest used for any other fish species, which is also true for the domperidone dosages. This, combined with the fact that the same dosage of domperidone greatly potentiated the actions of hCG in accelerating maturation and spawning, do not favour that assumption. The use of higher dosages of GnRH analogues than what was used may have more positive effects, but then the practical advantage of their use for mariculture purposes would not be viable. The use of LH-RH analogues for inducing spawning in siganids was reported for *S. vermiculatus,* where 2-3 injections, each at a dosage of 0.002 μg g^{-1} body weight, was given (**Popper at al., 1976**). In *S. guttatus*, implantation of silastic pellets containing 6.7 mg D-Nal(2)^6 LHRH was reported to accelerate and synchronize spawning (**Harvey et al., 1985**). It is perhaps the prolonged presence of GnRH-A in the circulation which is necessary to induce final oocyte maturation (FOM), whether it is by multiple injections or by implanted delivery systems. Such procedures were found to be effective for the striped bass, *Morone saxatilis*, where two GnRH-A injections resulted in 60% ovulated females, and GnRHa-delivery systems resulted in 100% ovulation (**Mylonas et al., 1998**). The use of delivery systems has the added advantage of reducing stress caused by frequent handling for injections, which in turn may have adverse effects on gamete quality.

Chapter 7

Chapter 7

General Discussion and Recommendations.

This thesis provides information that can be useful from both the academic and the practical points of view. Available basic studies on the biology of siganids in general and *Siganus canaliculatus* in particular are not sufficient, considering the importance of commercial farming of this species. We now have a better understanding of the morphological and structural development of the ovary and how that process is affected by certain environmental and endocrinological factors.

The timing of vitellogenesis in the area of the study is histologically defined for both wild and captive fish. It begins in early to mid February and the whole process takes about two months, after which the fish is in spawning readiness waiting for a stimulus to undergo maturation and ovulation. It can remain in this condition for about two weeks, after which the ovary undergoes atresia. The spawning season, or more correctly the season when captive fish can be induced to spawn, may start in late March, depending on water temperature and the time of the first new moon during that period, and may thus extend until early May. The duration of the maturation and ovulation period, as observed in induced fish, is short and can be as little as 72 hours.

The rabbitfish are known to withstand a fair degree of environmental and physical stressors (**Lam, 1974; Duray, 1990**). However, excessive stresses have a detrimental effect on gamete quality, an effect which may not be evident until the later fertilization and post-fertilization stages. This finding may explain the low production numbers in larval rearing trials. This

issue can have great importance, especially since fish are handled during the endocrine manipulation of maturation and spawning. It is therefore important to modify procedures to reduce stress to the minimum. The use of tranquilizers prior to handling and avoiding unnecessary visual disturbance is recommended. The use of slow release delivery systems can be given a consideration as a means to reduce handling stress, but the method of using such systems to induce final oocyte maturation and ovulation needs to be investigated. Another good indicator of good ovaries is the degree of atresia. Healthy vitellogenic ovaries have less than 16% atretic oocytes.

None of the lunar regimes stimulated fish to mature and spawn. Failure to progress beyond the vitellogenic stage may be due to some unknown deficit in the hatchery environment or some stress-inducing factors to which captive fish are exposed, which precludes any normal maturational/spawning response to lunar information. The environmental factors that may be present in the natural spawning grounds but absent in the hatchery may include suitable substrate, hydrostatic pressure, currents...etc.

The timing of the reproductive cycle can be altered by manipulating photoperiod. Constant short days applied directly after the spawning season had no effect, but constant long days applied at the same period advanced the reproductive cycle by two months. Switches from ambient to long photoperiod always advanced the reproductive cycle when applied any time from one month after the spawning season until 2 weeks after the winter solstice. Later applications had no effect. Changes to longer photoperiods are effective probably by simulating the natural increase in photoperiod after the winter solstice. The magnitude of change may not be very critical if one takes into account published works. The amount of variation in natural photoperiod is very small (about 4 hours), the fish may therefore be sensitive to small changes. The period by which the reproductive cycle was advanced was similar in all groups that responded to the treatment, except in the group where fish were subjected to a regime of three months of short days followed by three months of long days. In this group the period was three months, instead of the two in the other groups. Thus it seems that, of the regimes tested, a combination of short and long days is the most effective method for advancing reproduction. Retardation of the reproductive cycle was not achieved in this

study. It is perhaps necessary to try other means for achieving that result, for example, by the use of constant short days but under constant temperature. Although an increase in temperature did not advance the timing of vitellogenesis, it does not mean that it can not be used as an alternative cue by endogenous mechanisms to trigger the natural course of vitellogenesis. Raising temperature, while keeping photoperiod ambient, soon after winter solstice, did not alter the timing of vitellogenesis, perhaps suggesting that it has a more functional role at later stages of ovary development and can act as a limiting factor at extreme ranges.

The use of maturation and spawning inducing compounds is still a necessity for the propagation of *S. canaliculatus* in Bahrain. Using Human Chorionic Gonadotropin (hCG) alone, the optimum dose is approximately 1.0 IU g^{-1} body weight. The simultaneous use of Domperidone (DOM) with hCG reduces the latency period between injection and spawning, and may possibly increase spawning incidence at the lowest doses. However, at the lowest and the highest doses of hCG alone, there are deleterious effects on gamete quality. Whether such effects would persist with combined DOM+hCG treatment needs to be examined. Furthermore, for practical applications, the additional cost of DOM has to be compared with the reduction in the cost of hCG.

Although both GnRH analogues used failed to induce maturation and ovulation when given as a single dose, experiments using multiple doses or implants should be done.

Trials were done during the course of this study, kindly arranged by **Dr. A.P. Scott** at the MAFF Fisheries Laboratory, Lowestoft, Suffolk, to assay other hormones present during induced maturation and spawning that could fit the role of a maturation inducing steroid (MIS), especially 17, 20β-P, but levels were found to be very low (data not shown). The short duration from start of maturation until spawning may indicate the importance of the time of sampling, because the surge of MIS could occur at a specific time, e.g. just prior to germinal vesicle break down, as in the striped bass, *Morone saxatilis* (**King et al., 1994a**) and may be limited both in terms of amplitude and duration, a situation found in many marine fishes

(**Thomas et al., 1987**), and may be cleared rapidly from the circulation by reduction or conjugation.

More information is needed to assess the situation and identify the reason behind this endocrine failure responsible for the absence of final oocyte maturation (FOM) in captivity. This failure could be due to the lack or deficiency of pituitary GtH-II release at the end of vitellogenesis (**Zohar, 1989**), but if this is true, injection of post-vitellogenic females with gonadotropin-releasing hormone agonist (GnRH-A) should induce FOM, but this did not happen with a single injection when used in this study. Increasing the number of injections may have more positive results, but this needs to be investigated. The role of 17β-oestradiol (E_2) in the post-vitellogenic phase needs to be investigated further. The elevated levels of E_2 that persist until nucleus migration could be an artifact of the exogenous hCG, but other possible explanations have to be pursued also. The process of FOM may occur in two phases, as suggested for the striped bass (**Mylonas et al., 1998**), one prior to nucleus migration which may involve lipid globule coalescence and initiation of germinal vesicle migration, and one post nucleus migration, involving coalescence of yolk granules and germinal vesicle breakdown (GVBD). Each phase may be regulated by different hormones. E_2 concentrations may be correlated with the earlier phase during which it is present in noticeable levels. The hormones correlated with the second phase are not known.

References

Ablan, G.L. and Rosario,W.M. (1962). Methods of collecting and transporting live teuthid fry (padas) for stocking. Philipp. Bur. Fish. Gaz., 6, 6-8 and 36.

Abraham, M. (1988). Recent trends in research on induced spawning of fish in aquaculture. J. Appl. Ichthyol., 4:49-64.

Abraham, M, Sagi,G. (1984). Photoperiod regimes and "pineal treatment" as a means of controlling gonadal recrudescence in Liza ramada. Spec. Publ. Eur. Maricult. Soc., (8):105-118.

Abu-Hakima, R. (1984). Some aspects of the reproductive biology of *Acanthopagurus* spp. (Family:Sparidae). J. Fish Biol., 25:515-526.

Akatsu, S., Al-Zahr,C. Al-Aradi J. (1984). Egg and larval development of *Siganus oramin* (Bloch and Schneider) obtained through induced spawning. Kuwait Bull. Mar. Sci., (5):1-10.

Al-Aradi, J., W. Al-Baharna and Z. Al-Alawi. (1985). Feasibility of rearing *Siganus oramin (Siganus canaliculatus)* in closed system in the State of Bahrain. Proc. of Symp. Coastal Aquac., Cochin, India, 12-18 Jan. 1980. Symp. Ser. Mar. Biol. Assoc. India, Dec. 1985, (6):933-940.

Allen, G.R. (1972). Anemonefishes: their classificatoin and biology. T.F.H., Neptune City, New Jersey, USA.

Alcala, A.C. and Alcazar,S.N. (1979). A study on gonad morphology, oocyte development, gonad index, and fecundity in the rabbitfish *Siganus canaliculatus* (Park). Silliman J., 26(2&3): 147-162.

Al-Ghais, S.M. (1993). Some aspects of the biology of *Siganus canaliculatus* in the southern Arabian Gulf. Bull. Mar. Sci., 52(3):886-897.

Annual Meteorology Report (1996). Meteorological Directorate, Civil Aviation Affairs, Ministry of Transportation, Bahrain. pp35.

Aradi, J.S. and Shams,A.J. (1993). The length-weight relationship, condition factor and gonadosomatic index of the Rabbitfish Siganus canaliculatus in Bahrain. Paper presented at the Aqauculture Symp. on Techn. and Investment Opportunities, 11-14 April, 1993, Riyadh, K.S.A.

Avila, E.M. (1984). Hormone induced spawning and embryonic development of the rabbitfish, *Siganus vermiculatus* (Pisces:Siganidae). Philipp. Sci., 21: 75-108.

Axelrod, J. and Reisine,T.D. (1984). Stress hormones: their interaction and regulation. Science, 224:452-459.

Ayson, F.G. (1988). Induced spawning of rabbitfish. SEAFDEC Asian Aquaculture, Vol.X, No. 3, September 1988.

Ayson, F.G.(1989). The effect of stress on spawning of brood fish and survival of larvae of the rabbitfish, *Siganus guttatus* (bloch). Aquaculture, 80(3-4):241-246.

Ayson, F.G. (1991). Induced spawning of rabbitfish, *Siganus guttatus* (Bloch), using human chorionic gonadotropin (HCG). Aquaculture, 95(1-2):135-137.

Baggerman, B. (1980). Photoperiodic and endogenous control of the annual reproductive cycle in teleost fishes. In "Environmental Physiology of Fishes" (M. A. Ali, ed.). pp.533-567. Plenum,New York.

Baggerman, B. (1989). On the relationship between gonadal development and response time to photostimulation of stickle back living under constant short-day conditions for long periods of time. Can. J. Zool., 67:(1):126-135.

Baker, T.G.(1982). Oogenesis and ovulation. In "Reproduction in Mammals"-1. Germ cells and fertilization. (Austin,C.R. & Short,R.V.,eds.), 17-45. Cambridge:Cambrige University Press.

Ball, J.N. (1960). Reproduction in female bony fishes. Symp. Zool. Soc. London, 1: 105-135.

Balm, P. (1986). Osmoregulation in teleosts by cortisol and prolactin. Ph.D. Thesis, Nijmegen University, Holland.

Balon, E.K. (1975). Reproductive guilds of fishes: A proposal and definition. J. Fish. Res. Board Can., 32:821-864.

Barger & Delamatar. (1948). "Volume and bibliographic information is not available".

Barnett, F.H., Sohn,J., Reichlin,S. and Jackson,I.M.D. (1982). Three luteinizing hormone-releasing hormone like substances in a teleost fish brain: None identical with the mammalian LH-RH decapeptide. Biochem. Biophys. Res. Commun., 105: 209-216.

Barr, W. (1968). Patterns of ovarian activity. In "Perspectives in Endocrinology; Hormones in the Lives of Lower Vertebrates (eds. E.J.W. Barrington and C.B. Jorgensen). pp.163-237. Academic Press, New York.

Barton, B.A. & Iwama,G.K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annual Review of Fish Diseases, 1:3-26.

Barton, B.A. (1997). Stress in finfish: past, present and future- a historical perspective. In "Fish Stress and Health in Aquaculture". G.K. Iwama, A.D. Pickering, J.P. Sumpter, and C.B. Schreck (eds.). pp. 1-34. Society for Experimental Biology, Seminar Series 62, Cambridge University Press.

Batten, T.F.C. and Ingleton,P.M. (1987). The hypothalamus and pituitary gland. In "Fundamentals of Comparative Vertebrate Endocrinology". I. Chester-Jones, P.M. Ingleton and J.G. Phillips (eds). Plenum Press. pp. 285-412.

Ben-Tuvia, A. and Kissil,G.Win (1973). Experiments in rearing rabbitfish (*Siganus rivulatus*) in sea water. Aquaculture, 1:359-364.

Berry, A.J. (1986). Semi-lunar and lunar spawning periodicity in some tropical littorinid gastropods. Journal of Molluscan Studies. 52: 144-149.

Beumer, J.P. (1979). Reproductive cycles of two Australian freshwater fishes: the spangled perch, *Therapon unicolor* Gunther, 1859 and the East Queensland rainbowfish, *Nematocentris splendida* Peters, 1866. J. Fish Biol., 15: 111-134.

Bieniarz, K., Epler,P., Breton,B., and Thuy,L.N. (1978). The annual reproductive cycle in adult carp in Poland: ovarian state and serum gonadotropin level. Ann. Biol. Anim. Biochem. Biophys., 18: 917-921.

Billard, R. (1985). Environmental factors in salmonid culture and the control of reproduction. In "Salmonid Reproducton". (Ed. by R. Iwamoto and S. Sower), pp.70-87. Washington Sea Grant Program and university of Washington.

Billard, R. (1986a). The control of fish reproduction. In" Realism in Aquaculture". European Aquaculture Society, Bredene, Belgium.

Billard, R., Alagarswami,K., Peter,R.E. and Breton,B. (1983). Potentialisaton par le pimozide des effets du LHRH-A sur la secretion gonadotrope hypophysaire, l'ovulation et la spermiation chez la Carpe commune (*Cyprinus carpio*). C.R. Acad. Sci., Paris 296: 181-184.

Billard, R., P. Reinaud, M.G. Hollenbecq, and B. Breton. (1984). Advancement and synchronization of spawning in *Salmo gairdneri* and *S. trutta* following administration of LHRH-A combined or not with pimozide. Aquaculture. 43:57-66.

Bogerd, J., Li,K.W., Janssen-Dommerholt,C., Goos,H.J.Th. (1992). Two gonadotropin-releasing hormones from African catfish (*Clarias gariepinus*). Biochem. Biophys. Res. Commun. 187:127-134.

Booth, J.H. (1979). The effects of oxygen supply, epinephrine, and acetylcholine on the distribution of blood flow in trout gills. Journal of Experimental Biology, 83: 31-39.

Borg, B., Goos,H.J.Th. and Terlou,M. (1982). LHRH-immunoreactive cells in the brain of the threespined stickleback, *Gasterosteus aculeatus*. Cell Tiss. Res. 226: 695-699.

Bourlier, A. and Billard,R. (1984). Delayed gametogenesis and spawning in rainbow trout (*Salmo gairdneri*) kept under permanent light during the first and second reproductive cycles. Aquaculture, 43: 259-268.

Bradford, C.S. and Taylor,M.H. (1987). Semilunar changes in estradiol and cortisol coincident with gonadal maturation and spawning in the killifish *Fundulus heteroclitus*. Gen. Comp. Endocrin., 66:71-78.

Breder, C.M., Jr., and Rosen,D.E. (1966). "Modes of Reproduction in Fishes", Natural History Press, New York.

Breton, B. and Billard,R. (1977). Effects of photoperiod and temperature on plasma gonadotropin and spermatogenesis in the rainbow trout, *Salmo gairdneri*. Ann. Biol. Anim. Biochem. Biophys., 17: 331-340.

Breton, B., Weil,C. and Jalabert,B. (1972). Activite reciproque des facteurs hypothalamiques de Belier et de poissons teleosteens sur la secretion *in vitro* des hormones gonadotropes c-HG et o-LH respectivement par des hypophyses de carpe et de belier. C.R. Acad. Sci., Paris 274: 2530-2533.

Breton, B., Motin,A., Kah,O., Le Menn,F., Geoffre,S., Precigoux,G. and Chambolle,P. (1984). Dosage radioimmunoligique homologue d'un facteur hypothalamique de stimulation de la fonction gonadotrope hypophysaire de saumon s-Gn-RH. C.R. Acad Sci., Paris 299: 383-388.

Bretschneider, L.H. & Duyvene de Witt,J.J.(1947). Sexual endocrinology of non-mammalian vertebrates. Monogr. Prog. Res. Holland during War.II. Amsterdam: Elsevier.

Brett, J.R. (1958). Implications and assessments of environmental stress. In "Investigations of Fish-power Problems" (Ed. by P.A. Larkin), pp.69-83. H.R. Macmillan Lectures in Fisheries, University of British Columbia.

Bromage, N.R. (1987). The advancement of puberty or time of first spawning in female rainbow trout (*Salmo gairdneri*) maintained on altered seasonal light cycles. Proc. 3rd Int. Symp. Repr. Physiol. Fish. (Ed. by D.R. Idler, L.W. Crim & J.M. Walsh), p.303. Memorial University of Newfoundland, St. John's.

Bromage, N.R. and Cumaranatunga,R. (1988). Egg production in the rainbow trout. "Recent Advances in Aquaculture", 3, 63-138.

Bromage, N.R. and Duston,J. (1986). The control of spawning in the rainbow trout (*Salmo gairdneri*) using photoperiod techniques. Rept. Instit. Fres. Res. Drottn., 63, 26-35.

Bromage, N.R., Randall,C., Duston,J., Thrush,M., and Jones,J. (1993). Environmental control of reproduction in salmonids. In " Recent Advances in Aquaculture". Edited by J.F. Muir, and R.J. Roberts. Institute of Aquaculture. Blackwell. pp.55-66.

Bromage, N.R., Springate,E.J., & Whitehead,C. (1984). The effects of constant photoperiod on the timing of spawning in the rainbow trout. Aquaculture, 43, 213-23.

Bromage, N.R., Whitehead,C., Elliot,J. & Matty,A. (1982a). Investigations into the importance of daylength in the photoperiodic control of reproduction in the female rainbow trout. Proc. 2nd Int. Symp. Repr. Physiol. Fish. (Ed. by C.J.J. Richter & H.J.Th. Goos), pp.233-35, PUDOC, Wageningen, The Netherlands.

Bruton, M.N. (1979). The breeding biology and early development of *Clarias gariepinus* (Pisces: Clariidae) in Lake Sibaya, South Africa, with a review of breeding in species of the subgenus *Clarias* (Clarias). Trans. Zool. Soc. Lon., 35: 1-45.

Bryan P.G. and Madraisau, B.B. (1977). Larval rearing and development of *Siganus lineatus* (Pisces:Siganidae) from hatching through metamorphosis. Aquaculture, 10(3): 243-252.

Bryan, P.G., Madraisau,B.B. and Mcvey,J.P. (1975). Hormone spawning and natural spawning of captive *Siganus canaliculatus* (Pisces:Siganidae) year round. Micronesica, 11(2):199-204.

Bryan,P.G. and Madraisau,B.B. (1977). Larval rearing and development of *Siganus lineatus* (Pisces:Siganidae) from hatching through metamorphosis. Aquaculture, 10:243-252.

Brzuska, E. (1979) The *in vivo* method of estimating the stages of oocyte maturation in carp (*cyprinus carpio* L.). Acta Hydrobiol., 21: 423-433.

Bucke, D.(1972). Some histological techniques applicable to fish tissues. Symp. Zool. Soc. Lond. 30:153-189.

Budiman, A.A., Hariyanto,H.T., Perbowo,N., Jangkaru,Z. and Karundeng. (Eds). (1990-1991). Notes on ovary development in the rabbitfish (*Siganus guttatus*) of Pari Islands. In" Fish. Newsletter' Direct. Gen. of Fisheries, Jakarta, Indonesia, 2(1-12).

Burgan, B.G. and Zseleczky,K.W. (1979). Induced spawning and early development of the rabbitfish, *Siganus argenteus* (Quoy & Gaimard) in the Philippines. Silliman J., 26(2&3): 163-171.

Burzawa-Gerard, E. (1971). Purification d'une hormone gonadotrope hypophysaire de poisson teleosteen, la carpe (*Cyprinus carpio* L.). Biochemie, 53: 545-552.

Burzawa-Gerard, E. (1982). chemical data on pituitary gonadotropins and their implication to evolution. Can. J. Fish. Aquat. Sci., 39:80-91.

Butskaya, N.A. & Faleeva,T.I. (1987). Seasonal changes in the gonads and fecundity of antartic fishes *Trematomus bernacchii*, *Trematomus hansoni* and *Pagothenia borchgrevinki* (Nototheniidae). J. Ichthyol., 27: 27-36.

Bwathondi, P.O.J. (1981). The culture of rabbitfish *Siganus* spp. in Tanzania. IFS, Stockholm, 35pp.

Bwathondi, P.O.J. (1982). Preliminary investigations on rabbitfish, *Siganus canaliculatus*, cultivation in Tanzania. Aquaculture, 27(3):205-210.

Byskov, A.G .(1979). Atresia. In "Ovarian Follicular Development and Function" (Midgley,A.R. & W.A. Dadler,eds.), pp.41-57. New York: Raven Press.

Campbell, P.M., Pottinger,T.G. and Sumpter,J.P. (1991). Effects of acute stress on time of ovulation fecundity, egg size, egg survival and sperm counts in rainbow trout. Proceedings of the Fourth Int. Symp. on the Reproductive Physiology of Fish, Norwich, U.K., 7-12 July 1991.

Campbell, P.M., Pottinger,T.G. and Sumpter,J.P. (1992). Stress reduces the guality of gametes produced by rainbow trout. Biology of Reproductrion, 47:1140-1150.

Campbell, P.M., Pottinger,T.G. and Sumpter,J.P. (1994). Preliminary evidence that chronic confinement stress reduces the quality of gametes produced by brown and rainbow trout. Aquaculture, 120:159-169.

Canario, A.V.M., and Scott,A.P. (1988). Structure-activity relationships of C21 steroids in an in vitro oocyte maturation bioassay in rainbow trout, Salmo gairdneri. Gen. comp. Endocrin., 71(2):338-348.

Canario, A.V.M., and Scott,A.P. (1990). Effects of steroids and human chorionic gonadotropin on in vitro oocyte final maturation in two marine flatfish, the dab (*Limnada limnada*) and the plaice (*Pleuronectes platessa*). Gen. Comp. Endocrin., 77(2):161-176.

Carragher, J.F., & Pankhurst, N.W. (1991). Stress and reproduction in a commercially important marine fish, *Pagrus auratus* (Sparidae). In Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish. Norwich, U.K., 7-12 July 1991. Scott, A.P., Sumpter,J.P., Kime,D.E. & Rolfe,M.S. (eds). pp. 253-255. FishSymp91, Sheffield.

Carragher, J.F., Sumpter,J.P., Pottinger,T.G., & Pickering,A.D. (1989). The deleterious effects of cortisol implantation on reproductive function in two species of trout, *Salmo trutta* L. and *Salmo gairdneri* Richardson. Gen. and Comp. Endocrinology, 76:310-321.

Carrillo, M., Bromage,N., Zanyu,S., Serrano,R. and Prat,F. (1989). The effect of modifications in photoperiod on spawning times on ovarian development and egg quality in the sea bass (*Dicentrarchus labrax* L.). Aquaculture, 81(3-4):351-365.

Carrillo, M., Bromage,N., Zanuy,S., Serrano,R. and Ramos,J. (1991). Egg quality and fecundity in the sea bass (*Dicentrarchus labrax*) and the effects of photoperiodically-induced advanced and delays of spawning time. Proceeding of 4th Int. Symp. on the Reproductive Physiology of Fish, Norwich, U.K., 7-12 July. 1991.

Carrillo, M., Zanuy,S., Prat,F., Serrano,R. and Bromage,N. (1993). Environmental and hormonal control of reproduction in sea bass. In "Recent Advances in Aquaculture IV". Edited by J.F. Muir and R.J. Roberts. Blackwell Scientific Publications, Oxford. pp.43-54.

Carumbana, E. and Luchavez,J. (1979a). A comparative study of the growth rates of *Siganus canaliculatus*, *S. spinus* and *S. guttatus* reared in laboratory and semi-natural conditions in Southern Negros Oriental, Philippines. Silliman J., 26:187-209.

Chan, K.K.S. (1977). Effect of synthetic luteinizing hormone-releasing hormone (LH-RH) on ovarian development in Japanese medaka, *Oryzias latipes*. Can. J. Zool., 55: 155-160.

Chang, J.P, and Peter,R.E. (1983). Effects of pimozide and des gly^{10},[D-Ala^{6}]luteinizing hormone-releasing hormone ethylamide on serum gonadotropin concentrations, germinal vesicle migration, and ovulation in female goldfish, Carassius auratus. Gen. Comp. Endocrin., 52:30-37.

Chang, J.P., and R.E. Peter. (1984). Influences of norepinephrine and L-adrenergic mechanisms on gonadotropin secretion in female goldfish, *Carassius auratus*. Gen. Comp. endocrinol. 55:89-95.

Chang, J.P., Peter,R.E., Nahorniak,C.S., and Sokolowska,M. (1984). Effects of catecholaminergic agonists and antagonists on serum gonadotropin concentrations and ovulation in goldfish: Evidence for specificity of dopamine inhibition of gonadotropin secretion. Gen. Comp. Endocrin., 55:351-360.

Chang, C.F., Hu,H.J. and Sun,L.T. (1993). Effects of temperature and photoperiod on ovarian development in female ayu, *Plecoglossus altivelis*. J. Thermal Biol., 18(4):197-201.

Chester-Jones, I. and J.N. Ball (1962). Ovarian-pituitary relationships. In "The Ovary", (S. Zuckerman, ed.), Vol.1, pp. 361-434. Academic Press, New York.

Christy, J. (1978). Adaptive significance of reproductive cycles in the fiddler crab, *Uca pugilator*. A hypothesis. Science, 199: 453-455.

Christy, J. (1986). Timing of larval release by intertidal crabs on an exposed shore. Bulletin of Marine Science, 39: 176-191.

Clearwater, S.J. (1992). Reproductive biology and response to capture stress of red gurnard *Chelidonichthys kumu* (Family: Triglidae). MSc Thesis, University of Auckland, Auckland, p.96.

Coche, A.G., Cuzon,G. and Lichatowich,T. (1979). Mariculture Development in Bahrain. Report of a feasibility mission. FAO, Rome, 53P. FAO F1-DP-RAP/71/278/6.

Coello, S. (1989). Reproductive biology of Atlantic mackerel. Ph.D. thesis. University College of North Wales, Bangor, Gwynedd, UK.

Colin, P.L., Shapiro,D.Y. and Weiler,D. (1987). Aspects of the reproduction of two groupers, *Epinephelus guttatus* and *E. Striatus* in the West Indies. Bull. Mar. Sci., 40(2):220-230.

Cook, A.F. and Peter,R.F. (1980). The effect of temperature on the clearance of intraperitoneally-injected gonadotropin in the goldfish *Carassius auratus*. Aquaculture, 19: 275-286.

Copeland, P.A., and P. Thomas. (1989). Control of gonadotropin release in the Atlantic croaker (*Micropogonias undulatus*): evidence for lack of dopaminergic inhibition. Gen. Com. Endocrinol., 74:474-483.

Corson, B. (1955). Four years progress in the use of artificially controlled light to induce early spawning of brook trout. Prog. Fish Cult., 17:99-102.

Crim, L.W. (1982). Environmental modulation of annual and daily rhythms associated with reproduction in teleost fishes. Can. J. Fish. Aquat. Sci., 39:17-21.

Crim, L.W. (1985). Methods for acute and chronic hormone administration in fish. In " Reproduction and Culture of Milkfish", (eds. C.S. Lee and I.C. Liao). pp.1-13. Oceanic Institute, Hawaii.

Crim, L.W., & Evans,D.M. (1983). Influence of testosterone and/or luteinizing hormone releasing hormone analogues on precocious sexual development in the juvenile rainbow trout. Biology of Reproduction, 29:137-142.

Crim, L.W., and B.D. Glebe. (1984). Advancement and synchrony of ovulation in the Atlantic salmon with pelleted LHRH analog. Aquaculture, 43:47-56.

Crim, L.W., Peter, R.E. & Van Der Kraak, G. (1987). The use of LHRH analogs in aquaculture. In" LHRH and its analogs: contraceptive and therapeutic applications", Part 2.(Ed. by B.H. Vickery & J.J. Nestor, Jr), pp.489-98. MTP Press, Lancaster.

Cusick, E.K., & Wilson,F.E. (1972). On the control of spontaneous testicular regression in tree sparrows (*Spizella arborea*). Gen. Comp. Endocrinol., 19:441-456.

Cyrus & Blaber. (1984). "Volume and bibliographic information is not available".

Davies, B., Randall, C. and Bromage, N.R. (1991). Absolute daylength and the entrainment of an endogenous clock controlling reproduction in the female rainbow trout. Proceeding 4th Int. Symp. on the Reproductive Physiology of Fish, Norwich, U.K., 7-12 July 1991.

De Leeuw, R., Goos,H.J.Th. and Van Oordt,P.G.W.J. (1986). The dopminergic inhibition of the gonadotropin-releasing hormone-induced gonadotropin release: an *in vitro* study with fragments and cell suspensions from pituitaries of the African catfish, *Clarias gariepinus* (Burchell). Gen. Comp. Endocrinol., 63: 171-177.

De Montalembert, G., Jalabert,B. & Bry, C. (1978). Precocious induction of maturation and ovulation in northern pike (*Esox lucius*). Annales de Biologie Animale Biochimie Biophysique, 18:969-975.

Devauchelle, N. (1984). Reproduction decalee du bar (*Dicentrarchus labrax*) et de la daurade (*Sparus aurata*). In: G. Barnabe and R. Billard (Editors) "L'Aquaculture du Bar et des Sparides". INRA Publ., Paris. pp. 532-61.

Devauchelle, N. (1987). Four marine spawners in European hatcheries. In: "Production Controlee en Ecloserie. Synthese des papiers presentes dans le cadre du MEDRAP a Roviny-Zadar (Yugoslavia), 18-28 January 1986. Rapport FAO, Rome, 50pp.

de Vlaming, V.L. (1972). Environmental control of teleost reproductive cycles: A brief review. J. Fish Biol. 4, 131-140.

de Vlaming, V.L. (1972b). The effects of temperature and photoperiod on reproductive cycling in the estuarine gobid fish, *Gillichthys mirabilis*. Fish. Bull., 70: 1137-1152.

de Vlaming, V.L. (1975). Effects of photoperiod and temperature on gonadal activity in the cyprinid teleost, *Notemigonus crysoleucas*. Biol. Bull. (Woods Hole, Mass.), 148: 402-415.

de Vlaming, V. (1983). Occyte development patterns and hormonal involvement among teleosts. In "Control Processes in Fish Physiology", J.C.Rankin, T.J.Pitcher and Roger Duggan (Eds). (1983). pp. 176-199. Croom Helm Ltd.

Dickerson, T.L., B.J. Macewicz, and J.R. Hunter. (1992). Spawning frequency and batch fecundity of chub mackerel, *Scomber japonicus*, during 1985. Calif. Coop. Oceanic Fish. Invest. Rep., 33: 130-140.

Directorate of Fisheries. (1996). A summary of water temperature readings from selected sites around Bahrain. Ministry of Works & Agriculture, State of Bahrain. p.24.

Directorate of Statistics. (1996). Statistical Abstracts, 1995. Central Statistics Organization, State of Bahrain. p.681.

Dodd, J.M. (1955). The hormones of sex and reproduction and their effects in fish and lower chordates. Mem. Soc. Endocrin. 4:166-187.

Dodd, J.M. (1983). Reproduction in cartilaginous fishes (Chondrichthyes). In "Fish Physiology" 9A (Hoar,W.S., Randal,D.J. & Donaldson,E.M.,eds.), pp.31-95. New York: Academic Press.

Doherty, P.J. (1983). Diel, lunar and seasonal rhythm in the reproduction of two tropical damselfishes: *Pomacentrus flavicula & P. Wardii*. Marine Biology, 75(2-3): 215-224.

Donaldson, E.M. (1981). The pituitary-interrenal axis as an indicator of stress in fish. In: A.D. Pickering(Editor), "Stress and Fish", Academic Press, London, pp.11-48.

Donaldson, E.M., and G.A. Hunter. (1983). Induced final maturation, ovulation and spermiation in cultured fish. In: Fish physiology, Vol. IXB (eds. W.S. Hoar, D.J. Randall and E.M. Donaldson), pp.351-403. Academic Press, New Tork.

Drew, A.W. (1971). Preliminary report on *klesebuul* and *meyas*, two fish of Palau Islands. Unpublished report, dated January, 1971, 20p.

Dufour, S., N. Delerue-Le Belle and Y.A. Fontaine. (1984). Stimulation de la liberation d'hormone gonadotrope et du pimozide et d'un agoniste de la LHRH chez l'Anguille argentee pretraitee a l'oestradiol. Compte Rendu de l'Academie des Sciences. Paris. Ser. III 299:231-234.

Dufour, S., E. Lopez, F.Le Menn, N. Le Belle, S. Baloche and Y.A. Fontaine. (1988). Stimulation of gonadotropin release and of ovarian development, by the administration of a gonadoliberin agonist and of dopamine antagonists, in female silver eel pretreated with estradiol. Den. Comp. Endocrinol, 70:20-30.

Duray, M.N. (1990). Biology and culture of Siganids. Publication of the Southeast Asian Fisheries Development Center (SEAFDEC), Aquaculture Department, Iloilo, Philippines. pp.47.

Dotan, A. (1990). Reproduction of the slate pencil sea urchin, *Heterocentrotus mammillatus* (L.) in the northern Red Sea. Aust. J. Mar. Freshwat. Res., 41(4):457-465.

Duffey, R.J. and Goetz,F.W. (1980). Gen. Comp. Endocrinol., 41: 563-565.

Duston J. and Bromage, N. (1986). Photoperiodic mechanisms and rhythms of reproduction in the female rainbow trout. Fish Physiol. Biochem., 2:35-51.

Duston J. and Bromage, N. (1987). Constant photoperiod regimes and the entrainment of reproduction in the female rainbow trout (*Salmo gairdneri*). Gen. Comp. Endo., 65:373-84.

Duston J. and Bromage, N. (1988). The entrainment and gating of the endogenous circannual rhythm of reproduction in the female rainbow trout (*Salmo gairdneri*). J. Comp. Physiol. A, 164:259-68.

Duston,J. and Bromage,N. (1991). Circannual rhythms of gonadal maturation in female rainbow trout (*Onchorhynchus mykiss*). J. Biol. Rhythms, 6(1):49-53.

Elliot, J.A.K., Bromage,N.R. and Springate,J.R.C. (1984). Changes in reproductive function of three strains of rainbow trout exposed to constant and seasonally-changing light cycles. Aquaculture, 43(1-3):23-34.

Espey, L.L. (1980). Biol. Reprod., 22: 73-106.

F.A.O. Fisheries Information (1995). A publication of the Food and Agriculture Organization of the United Nations. Rome. pp.154.

Fagerlund, U.H.M., McBride,J.R. & Williams,I.V. (1995). Stress and tolerance. In " Physiological Ecology of Pacific Salmon". Groot,C., Margolis,L. & Clarke,W.C. (eds.). pp. 461-503. University of British Columbia Press, Vancouver.

Fitzpatrick, M.S., Suzumoto,B.K., Schreck,C.B. & Oberbillig,D. (1984). Luteinizing hormone-releasing hormone analogue induces precocious ovulation in adult coho salmon (*Oncorhynchus kisutch*). Aquaculture, 43: 67-73.

Follett, B.K. (1984). Birds. In "Marshall's Physiology of Reproduction", Vol.1- Reproductive Cycles of Vertebrates. Edited by G.E. Lemming. Churchill Livingstone, London, New York. PP.309-337.

Forward, R.B., Jr., K. Lohmann and T.W. Cronin. (1982). Rhythms in larval release by an estuarine crab *Rhithropanopeus harrisii*. Biol. Bull. (Woods Hole), 163: 287-300.

Foster, S.A. (1987). Diel and lunar patterns of reproduction in the caribbean and pacific sergeant major damselfishes *Abudefduf saxatalis* and *A.Troschelli*. Mar. Biol., 95(3):333-343.

Foster, S.A. (1989). The implications of divergence in spatial nesting patterns in the geminate Caribbean and Pacific sergeant major damselfishes. Animal Behaviour, 37: 465-476.

Fujite, S. and Ueno,M. (1954). On the development of the egg and prelarval stages of *Siganus fuscescens* (Houttuyn) by artificial insemination. Jap. J. Icth., 3:129-132.

Garcia, L.Ma.B. (1989). Spawning response of mature female sea bass, *Lates calcarifer* (Bloch), to a single injection of luteinizing hormone-releasing hormone analogue:Effect of dose and initial oocyte Size. J. Appl. Ichthyol., 5:177-184.

Garcia, L.Ma.B. (1992). Lunar synchronization of spawning in sea bass, *Lates calcarifer* (Bloch): Effect of luteinizing hormone releasing hormone analogue (LHRHa) treatment. J. Fish. Biol., 40(3):359-370.

Gissis, A., B. Levavi-Zermonsky, A. Bogomolnaya-Bass and Z. Yaron. (1988). Gonadotropin levels in female tilapia treated with GnRH analog, and reserpine or pimozide. In: Reproduction in Fish: basic and applied aspects in endocrinology and genetics (eds. Y.

Zohar and B. Breton), pp.63-67. INRA, Paris.

Gladstone, W. and M. Westoby. (1988). Growth and reproduction in *Canthigaster valentini* (Pisces, Tetraodontidae): a comparison of a toxic reef fish with other reef fishes. Environmental Biology of Fishes, 21: 207-221.

Goetz, F.W. (1983). Hormonal control of oocyte final maturation and ovulation in fishes. In "Fish Physiology" (W.S. Hoar and D.J. Randall, eds.), Vol. IXB, 117-170. Academic Press, New York.

Goetz, F.W. and Theofan,,G. (1979). Gen. Comp. Endocrinol., 37: 273-285.

Goodall, J.A, Capra,M.F. & Blackshaw,A.W.(1987). Quantitative histology of the seasonal changes in the gonads of summer whiting, *Sillago ciliate*. J. Fish Biol. 30:589-595.

Goos, H.J.Th. and Murathanoglu,O. (1977). Localization of gonadotropin releasing hormone (GRH) in the forebrain and neurohypophysis of the trout (*Salmo gairdneri*). Cell Tiss. Res., 181: 163-168.

Goos, H.J.Th., de Leeuw,R., de Zoeten-Kamp,C., Peute,J. and Blahser,S. (1985). Gonadotropin-releasing hormone-immunoreactive neuronal structures in the brain and pituitary of the African catfish, *Clarias gariepinus* (Burchell). Cell Tiss. Res., 241: 593-596.

Gordin, H. and Zohar,Y. (1978). Induced spawning of *Sparus aurata* (L) by means of hormonal treatments. Ann. Biol. Anim. Biochem. Biophys., 18 (4): 985-990.

Goren, A., Y. Zohar., Y. Koch and M. Fredkin. (1987). Degradation of GnRH and its analogs in the gilthead seabream *Sparus aurata*: an *in vitro* study. Proceedings of the Third International Symposium on Reproductive Physiology of Fish: 96.

Goryezko, K. (1972). A change in spawning season in rainbow trout (*Salmo gairdneri*). Roczn. Rolnic., 94: 57-68.

Grau, E.G., Dickhoff,W.W., Nishioka,R.S., Bern,H.A. and Folmar,L.E. (1981). Lunar phasing of the thyroxine surge preparatory to seaward migration of salmonid fish. Science, 211: 607-609.

Greeley, M.S.Jr. (1984). Spawning by *Fundulus pulvereus* and *Adinia xenica* (Cyprinodontidae) along the Alabama Gulf Coast is associated with the semilunar tidal cycles. Copeia, (3):797-800.

Gundermann, N., Popper,D.M. and Lichatowich,T. (1983). Biology and life cycle of *Siganus vermiculatus* (Siganidae,Pisces). Pacific Science, 37(2):165-180.

Gupta, S.K. & Maiti,B.R. (1986). Study of atresia in the ovary during the annual reproductive cycle and nesting cycle of the Pied Myna. J. Morph. 190:285-296.

Guraya, S.S. (1986). The cell and molecular biology of fish oogenesis. Monographs in Developmental Biology 18. Basel: Karger.

Gwinner, E. (1981). Circannual systems. In"Handbook of Behavioural Neurobiology". Vol.4. Biological rhythms, pp. 391-410. Ed.: J. Ascoff. Plenum Press, New York & London.

Gwinner, E. (1986). Circannual Rhythms. Springer, Berlin.

Habibi, **H.R.**, Marchant,T.A., Nahorniak,C.S., Loo,H. van der, Peter,R.E., Rivier,J.E. & Vale,W.W. (1989). Fuctional relationship between receptor binding and biological activity for analogs of mammalian and salmon gonadotropin-releasing hormones in the

pituitary of the goldfish (*Carassius auratus*). Biol. Reprod., 40, 1152-61.

Habib, H.R., Peter,R.E., Sokolowska,M., Rivier,J.E., Vale,W.W. (1987). Characterization of gonadotropin-releasing hormone (GnRH) binding to pituitary receptors in goldfish (*Carassius auratus*). Biol. Reprod. 36:844-853.

Halpern-Sebold, L.R. and Schreibman,M.P. (1983). Ontogeny of centers containing luteinizing hormone-releasing hormone in the brain of platyfish (*Xiphophorus maculatus*) as determined by immunocytochemistry. Cell Tiss. Res., 229: 75-84.

Hamner, W.H. (1968). The photorefractory period of the house finch. Ecology, 49: 212-227.

Hara, S., Duray,M.N., Parazo,M. and Taki,Y. (1986a). Year round spawning and seed production of the rabbitfish, *Siganus guttatus*. Aquaculture, 59(3-4):259-272.

Hara, S., Kohno,H. and Taki,Y. (1986b). Spawning behaviour and early life-history of the rabbitfish, *Siganus guttatus*, in the laboratory. Aquaculture, 59(3-4):273-285.

Harvey, B., Nacario,J., Crim,L.W., Juario,J.V. and Marte,C.L. (1985). Induced spawning of the sea bass, *Lates calcarifer*, and rabbitfish, *Siganus guttatus*, after implanatation of pelleted LHRH analog. Aquaculture, 47(1):53-59.

Hasse, J.J., Madraisau,B.B. Mcvey,J.P. (1977). Some aspects of the life history of *Siganus canaliculatus* (park)(Pisces:Siganidae) palau. Micronesica, 13(2):297-312.

Henderson-Arzapalo,A. and Colura,R.L. (1987). Laboratory maturation and induced spawning of striped Bass. Prog.Fish Cult., 49(1):60-63.

Himelstein-Braw, R., Byskov,A.G., Peters,H. & Faber,H. (1976). Follicular atresia in the infant human ovary. J. Reprod. Fert., 46: 55-59.

Hirose, K. (1976). Endocrine control of ovulation in medaka (*Oryzias latipes*) and Ayu (*Plecoglossus altivelis*). J. Fish. Res. Bd. Can. 33: 989-994.

Hirose, K. and Arai,S. (1988). Effect of LHRH Cholesterol pellet on ovarian maturation in ayu (*Plecoglossis altivelis*). Bull. Natl. Res. Inst. Aquaculture, 13: 11-16.

Hirose, K., Machida,Y., and Donaldson,E.M. (1976). Induction of ovulation in the Japanese flounder (*Limnada yokohamae*) with human chorionic gonadotropin and salmon gonadotropin. Bull. Jap. Soc. Sci. Fish., 42: 13-20.

Hoar, W.S. (1969). Reproduction, In "Fish Physiology" (W.S. Hoar and D.J. Randall, eds.), Vol.3, pp. 1-72. Academic Press, New York.

Hontela, A. and Peter,R.E. (1978). Daily cycle in serum gonadotropin levels in the goldfish: *Carassius auratus*, effects of photoperiod, temperature, and sexual condition. Can. J. Zool., 56: 2430-2442.

Hontela, A. and Stacey, N.E. (1990). Cyprinidae. In "Reproductive Seasonality in Teleosts: Environmental Influences". A.D. Munro, A.P. Scott & T.J. Lam (Eds.). CRC Press, Inc., Boca Raton, Florida. p.53-77.

Hoover, E. and Hubbard,M. (1937). Modifications of the sexual cycle in trout by control of light. Copeia, 1937, 4: 206-210.

Horstmann, U. (1975). Some aspects of the mariculture of different siganid species in the philippines. Philipp. Sci., 12: 5-20.

Howell, W.H. (1983). Seasonal changes in the ovary of the adult yellowtail flounder, *Limnada ferruginea*. Fishery Bulletin, 81: 341-355.

Hsiao, Shyh-Min and Meier,A.H. (1989). Comparison of semilunar cycles of spawning activity in *Fundulus grandis* and *F. Heteroclitus* held under constant laboratory condition. J. Exp. Zool., 252(3):213-218.

Hsiao, Shyh-Min and Meier,A.H. (1992). Freerunning circasemilunar spawning rhythm of *Fundulus grandis* and its temperature compensation. Fish, Physiol. Biochem., 10(3):259-265.

Htun-Han, M. (1977). The effect of photoperiod on reproduction in fishes: an annotated bibliography. Libr. Inf. Leaf. Dir. Fish. Res. (G.B.), (No.6)-lowestoft.

Hunter, J.R. & Macewicz,B.J. (1985a). Measurement of spawning frequency in multiple spawning fishes. In "An Egg Production Method for Estimating Spawning Biomass of Pelagic Fish: Application to Northern Anchovy, *Engraulis mordax.* (R. Lasker, ed.). NOAA Tehnical Report NMFS 36, pp. 79-99.

Hunter, J.R. & Macewicz,B.J. (1985b). Rates of atresia in the ovary of captive and wild Northern anchovy, *Engraulis mordax*. Fishery Bulletin, 83: 119-136.

Hunter, J.R., Macewicz,B.J. & Sibert,J.R. (1986). The spawning frequency of skipjack tuna, *Katsuwonus pelamis*, from the south Pacific. Fishery Bulletin, 84: 895-903.

Hussein, K.A.(1984). Gonad maturation and fecundity of a fish with partial spawning (Tilapia nilotica L.). Arab Gulf J. Science Res. 2:683-699.

Idler, D.R.. (1982). Some perspectives on fish gonadotropins, In: C.J.J. Richter and H.J.Th. Goos (Editors), Proceedings of the International Symposium on the Reproductive Physiology of Fish. Wageningen, The Netherlands, 2-6 August 1982, Pudoc, Wageningen, pp.4-13.

Idler, D.R. and Crim,L.W. (1982). Gonadotropin releasing factor(s) (GtH-RF) from hypothalamus of winter flounder. In: Proceedings of the Ninth International Symposium on Comparative Endocrinology. Edited by B. Lofts, University of HongKong Press, HongKong.

Idler, D.R. and Everard,B.A. (1987). Mammalian, salmon and chicken-like LHRH's from hypothalami of winter flounder (*Pseudopleuronectes americanus*) as evidenced by chromatographic mobility and immunoreactivity. In "Proceedings of the 3rd International Symposium on the Reproductive Biology of Fish. Newfoundland, Canada". (D.R. Idler, L.W. Crim, and J.M. Walsh, Eds.). p.30.

Jalabert, B. (1976). *In vitro* oocyte maturation and ovulation in rainbow trout (*Salmo gairdneri*), northern pike (*Esox lucius*), and goldfish (*Carassius auratus*). J. Fish Res. Bd. Can., 33: 974-988.

Jalabert, B., Breton,b., Brzuska,E., Fostier,A., and Wieniawski,J. (1977). A new tool for induced spawning: The use of 17a-hydroxy-20B-dihydroxy-progesterone to spawn carp at low temperature. Aquaculture,10:353-364.

Janssen, P.A.H., Lambert,J.G.D., and Goos,H.J.TH. (1995). The annual ovarian cycle and the influence of pollution on vitellogenesis in the flounder, *Pleuronectes flesus*. J. Fish Biol., 47:509-523.

Johannes, R.E. (1978). Reproductive strategies of coastal marine fishes in the tropics. Environ. Biol. Fish., 3(1): 65-84.

Johnson, W. (1984). Photoperiod induced delayed maturation of freshwater reared chinook salmon. Aquacult., 43: 279-287.

Jolles.J., Burzawa-Gerard,E., Fontaine,Y.-A. and Jolles,P. (1977). The evolution of gonadotropins: some molecular data concerning a non-mammalian pituitary gonadotropin, the hormone from a teleost fish (*Cyprinus carpio* L.). Biochimie., 59: 893-897.

Jones, S.W., Adams,P.R., Brownstein,M.J. and Rivier,J.E. (1984). Teleost luteinizing hormone-releasing hormone action on bullfrog sympathetic ganglia is consistent with role as neurotransmitter. Neurosci., 4: 420-429.

Juario, J.V., Duray,M.N., Duray,V.M., Nacario,J.F. and Almendras,J.M.E. (1984). Breeding and larval rearing of the rabbitfish, *Siganus guttatus* (Bloch). Aquaculture, 44(2):91-101.

Juario, J.V. Duray,M.N. Duray,V.M., Nacario,J.F., Almendras,J.M.E. (1985). Breeding and larval rearing of the rabbitfish *Siganus guttatus* (Bloch). Aquaculture, 44:91-101.

Kagawa, H., Young,G., Adachi,S. and Nagahama,Y. (1982). Estradiol-17β production in amago salmon (*Oncorhynchus rhodurus*) ovarian follicles: role of the thecal and granulosa cells. Gen. Comp. Endocrinol., 47: 440-448.

Kah, O. (1986). Central regulation of reproduction in teleosts. Fish Physiology and Biochemistry. Volume 2, nos 1-4, pp.25-34.

Kah, O., Breton,B., Dulka,J.G., Nunez-Rodriguez,J., Peter,R.E., Corrigan,A., Rivier,J.E. and Vale,W.W. (1986a). A reinvestigation of the Gn-RH (gonadotropin-releasing hormone) systems in the goldfish brain using antibodies to salmon Gn-RH. Cell Tiss. Res.

Kah, O., Chambolle,P., Dubourg,P and Dubois,M.P. (1984a). Immunocytochemical localization of luteinizing hormone-releasing hormone in the brain of the goldfish *Carassius auratus*. Gen. Comp. Endocrinol. 53: 107-115.

Kah. O., B.D. Sloley, V.L. Trudeau, K.L. Yu, J.P. Chang and R.E. Peter. (1990). GABA and the neuroendocrine regulation of gonadotropin release in the goldfish. Neuroendocrinology 52 (Supplement) 93 (Abstract:2.88).

Kawauchi, H., Itoh,H. and Koide, Y. (1991). Additional evidence for duality of fish gonadotropins. In: A.P. Scott, J.P. Sumpter, D.E. Kime and M.S. Rolfe (Editors), Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish. Norwich, U.K., 7-12 July 1991, FishSymp91, Sheffield, pp.19-21.

Kawauchi, H., Suzuki, K., Itoh, H., Swanson, P. and Nagahama, Y. (1987). Duality of salmon pituitary gonadotropins. In Proc. 1st Congr. Asia and Oceania, Soc. Comp. Endocrinol. pp.15-18. Edited by E. Ohnishi, Y. Nagahama and H. Ishizaki. Nagoya University Operation, Nagoya, Japan.

Kawauchi, H., Suzuki, K., Itoh, H., Swanson, P., Naito, N., Nagahama, Y., Nozaki, M., Nakai, Y. and Itoh, S. (1989). The duality of teleost gonadotropins. Fish Physiol. Biochem., Vol. 7, nos 1-4, pp. 29-38.

Kelley, C.D., Lee,C.S. and Tamaru,C.S. (1987). Acceleration of maturation and double spawning in one season in the grey mullet (*Mugil cephalus*) following chronic horrmone treatment. In "Reproductive Physiology of Fish" (Ed. by D.R. Idler, L.W. Crim & J.M. Walsh), pp.203. Memorial University of Newfoundland Press, St. John's.

Kelley, C.D., Tamaru,C.S., Lee,C.-S., Moriwake,A. and Miyamoto,G. (1991). Effects of photoperiod and temperature on the annual ovarian cycle of the striped mullet, *Mugil cephalus*. Proc. 4th Int. Symp. Repr. Physiol. Fish, Norwich, U.K., 7-12 July 1991.

Kesteven, G.L. (1960). Manual of methods in fisheries biology. F.A.O. Man. Fish. Sci. 1, 152pp. F.A.O. Rome.

Khoo, K.H .(1975). The corpus luteum of goldfish (*Carassius auratus* L.) and its function. Can. J. Zool. 53:1306-1323.

Khoo, K.H .(1979). The histochemistry and endocrine control of vitellogenesis in Goldfish ovaries. Can. J. Zool. 57:617-626.

Kim, Y.S, Stumpf, W.E. and Sar,M. (1978). Topography of estrogen target cells in the forebrain of goldfish, *Carassius auratus*. J. Comp. Neurol. 182: 611-620.

King, J.A., Dufour,S., Fontaine,Y.A., and Millar,R.P. (1990). Chromatographic and immunological evidence for mammalian GnRH and chicken II GnRH in eel (*Anguilla anguilla*) brain and pituitary. Peptides, 11:507-514.

King, J.A. and Millar,R.P. (1980). Comparative aspects of luteinizing hormone-releasing hormone structure and function in vertebrate phylogeny. Endocrinol. 106: 707-717.

King, W.V., Thomas,P., Harrell,R.M., Hodson,R.G., and Sullivan,C.V. (1994a). Plasma levels of gonadal steroids during final oocyte maturation of striped bass, *Morone saxatilis* L. Gen. Comp. Endocrinol. 95:178-191.

Kingsford, M.J. (1980). Interrelationships between spawning and recruitment of *Chromis dispilus* (Pisces, Pomacentridae). Thesis, University of Auckland, Auckland, New Zealand.

Kobayashi, M., Aida,K. and Hanyu,I. (1988). Hormone changes during the ovulatory cycle in goldfish. General and Comparative Endocrinology, 69, 301-307.

Korringa, P. (1947). Relations between the moon and periodicity in the breeding of marine mammals. Ecol. Monogr., 17: 349-381.

Lam, T.J. (1974). Siganids: Their biology and mariculture potential. Symp. Papers of Pacific Sci. Assoc. Special Symp. Mar. Sciences. 7-16 Dec. 1973.

Lam, T.J. (1982). Applications of endocrinology to fish culture. Can. J. Fish. Aquat. Sci., 39: 111-17.

Lam, T.J. (1983). Environmental influences on gonadal activity in fish. In "Fish Physiology", Vol.IX,part B, (W.S. Hoar, D.J. Randall and E.M. Donaldson, eds.). Academic Press, New York, London. pp. 65-116.

Lam, T.J. and Soh,C.L. (1975). Effect of photoperiod on gonadal maturation in the rabbitfish, *Siganus canaliculatus* Park 1797. Aquaculture, 5(4):407-410.

Larsson, A.L. (1973). Metabolic effects of epinephrine and norepinephrine in the eel, *Anguilla anguilla* L. Gen. Comp. Endocrinol., 20: 155-167.

Lavina, A.F.D. (1975). A study of the gonadal cycle of the rabbitfish *Siganus canaliculatus* (Park). Horiz. Unltd., 14(3):37-45.

Lavina, E.M. and Alcala,A.C. (1973). Ecological studies on Philippine siganid fishes in Southern Negros, Philiipines. Abstract (No. MSS/ABS/2/1) submitted to the Marine Sciences Special Symposium, HongKong (7th-14th December, 1973).

Lee, C.-S., C.D. Kelley and C.S. Tamaru. (1996). Hormonal induction of maturation in striped mullet, *Mugil cephalus*. Asian Fisheries Science, 9: 9-20.

Lee, C.-S., Tamaru,C.S. and Kelley,C.D. (1988). The cost and effectiveness of CPH, HCG and LHRH-a on the induced spawning of grey mullet, *Mugil cephalus*. Aquaculture, 73: 341-347.

Lee, C.-S., Tamaru,C.S. and Weber,G.M. (1987). Studies on the maturation and spawning of milkfish *Chanos chanos* Forsskal, in a photoperiod-controlled room. J. World Aquacul. Soc., 18,(4) pp.253-259.

Lichatowich, T. and Popper,D. (1975). Report on the growth of rabbitfish in fish ponds in Fiji. Aquaculture(5), pp.211-212.

Lichatowich, T., Al-Thobaity,S., Arada,M. and Bukhari,F. (1984b). The spawning cycle, fry appearance and mass collection techniques for fry of *Siganus rivulatus* in the Red Sea. Aquaculture, 40(3):269-271.

Lin, H.R., C. Peng, L.Z. Liu, S.J. Zhou, G. Van Der Kraak and R.E. Peter. (1985). Induction of ovulation in the loach (*Paramisgurnus dabryanus*) using pimozide and (D-ala^6, Pro^9-N-ethylamide)-LHRH. Aquaculture, 46:333-340.

Lin, H.R., Kraak,G.van der, Liang, J.Y., Peng, C., Li, G.Y., Lu, L.Z., Zhou, Z.J., Chang, M.L. & Peter, R.E. (1986a). The effect of LHRH-analogue and drugs which block the effect ofdopamine on gonadotropin secretion and ovulation in fish cultured in China. In "Aquaculture of Cyprinids" (Ed. by R. Billard & J. Marcel), pp. 139-150. INRA, Paris, France.

Lin, H.R., G. Van Der Kraak, R.E. Peter, and B. Breton. (1986b). Effects of (D-Ala^6, Pro^9-NEt)-LHRH and catecholaminergic drugs on gonadotropin secretion and ovulation in the Chinese loach (*Paramisgurnus dabryanus*). Gen. Comp. Endocrinol., 64:389-395.

Lin, H.R., X.J. Zhou, G. Van Der Kraak and R.E. Peter. (1987a). Comparison of LHRH-A and sGnRH-A, in combination with pimozide or domperidone, in stimulating gonadotropin release and ovulation in the Chinese loach (*Paramisgurnus dabryanus*). Proceedings of the Third International Symposium on Reproductive Physiology of Fish: 33.

Lin, H.R., J.Y. Liang, G. Van Der Kraak and R.E. Peter. (1987b). Stimulation of gonadotropin secretion and ovulation in common carp by an analogue of salmon GnRH and domperidone. Proceedings of the First Congress of the Asia and Oceania Society for Comparative Endocrinolgy: 155-156.

Lin, H.R., Kraak,G. van der, Zhou,X-J, Liang, J-Y., Peter, R.E., Rivier,J.E., and Vale,W..W. (1988). Effect of [D-arg^{6,} Trp^{7}, Leu^{8}, Pro^{9}, NEt]-luteinizing hormone-releasing hormone (sGnRH-A) and D-Ala^{6}, Pro^{9},NEt]-luteinizing hormone-releasing hormone (LHRH-A), in combination with pimozide or domperidone, on gonadotropin release and ovulation in the chinese loach and common carp. Gen. Comp. Endocrin., 69:31-40.

Lin, H.R., M.L. Zhang, G. Van Der Kraak and R.E. Peter. (1990). Effects of sex steroids, (D-Ala^{6}, Pro^{9}-N-ethylamide)-LHRH (LHRH-A) and domperidone (DOM) on gonadotropin secretion in female silver eel, *Anguilla japonica* Temminck & Schlegel. The Second Asian Fisheries Forum: 591-594.

Lin, H.R., and Peter,R.E. (1996). Hormones and spawning in fish. Asian Fisheries Science. 9:21-33.

Lin, H.R., Zhou,X-J., Kraak,G.van der, Peter,R.E. (1991). Effects of gonadotropin-releasing hormone agonists and dopamine antagonists on gonadotropin secretion and ovulation in chinese loach, *Paramisgurnus dabryanus*. Aquaculture, 95(1-2):139-147.

Lin, Y.-W.p., Rupnow,B.A., Price,D.A., Greenberg,R.M. and Wallace,R.A. (1992). *Fundulus heteroclitus* gonadotropins. III. cloning and sequencing of gonadotropic hormone (GTH) I and II β subunits using the polymerase chain reaction. Mol. Cell. Endocrinol., 85: 127-139.

Lobel, B. (1978). Diel, lunar and seasonal periodicity in the reproductive behaviour of the pomacentrid fish, *Centropyge potteri*, and some other reef fishes in Hawaii. Pacific Science, 32:193-207.

Lofts, B., Pickford,G.E. and Atz,J.W. (1968). The effects of low temperature and cortisol, on testicular regression in the hypophysectomized cyprinodont fish, *Fundulus heteroclitus*. Biol. Bull. (Woods Hole, Mass.), 134: 74-86.

Luchavez, J.A. and Carumabana,E. (1982). Observations on the spawning, larval development, and larval rearing of *Siganus argenteus* (Quoy & Gaimard) under laboratory conditions. Silliman J., 29(1-2): 24-34.

Lundqvist, M. (1980). Influence of photoperiod on growth in Baltic salmon parr (*Salmo salar* L.) with special reference to the effect of precocious sexual maturation. Can. J. Zool., 58: 940-944.

Macer, C.T. (1974). The reproduction biology of the horse mackerel *Trachurus trachurus* (L.) in the North Sea and English Channel. J. Fish Biol., 6: 415-438.

MacQuarrie, D., Market,J. and Vanstone,W. (1978). Photoperiod induced off-season spawning of coho salmon (*Oncorhynchus kisutch*). Ann. Biol. Anim. Bioch. Biophys., 18: 1051-1058.

MacQuarrie, D., Vanstone,W. and Market.J. (1979). Photoperiod off-season spawning of pink salmon (*Oncorhynchus gorbuscha*). Aquacult., 18: 289-302.

Magliulo-Cepriano, L. Schreibman,M..P., and Blum,V. (1994). Distribution of variant forms of immunoreactive gonadotropin-releasing hormone and β-gonadotropins I and II in the platyfish, *Xiphophorus maculatus*, from birth to sexual maturity. Gen. Comp. Endocrinol., 94: 135-150.

Malone, T.E. and K.K. Hisaoka. (1963). A histochemical study of the formation of deutoplasmic components in developing oocytes of the zebrafish, *Brachydanio rerio*. J. Morphol., 112: 61-75.

Manacop, P.R. (1937). The artificial fertilization of dangit, *Amphacanthus oramin* (Bloch and Schneider), probably = *S. canaliculatus*. Philippine Journal of Science, 62:229-237.

Mandl, A.M. & Zuckerman,S. (1950). The number of normal and atretic ova in the mature rat. J. Endoc., 6: 426-435.

Marino, By.G., Mandich,A., Massari,A., Andaloro,F., Porello,S., Finola,M.G., and Cevasco,F. (1995). Aspects of reproductive biology of the Mediterranean amberjack (Seriola dumerilii Risso) during the spawning period. J. Appl. Ichthyol., 11(1-2):9-24.

Marza, V.D. (1938). Histophysiologie de l'ovogenese. Hermann, Paris.

May, R.C. (1973). Report on the Siganid Mariculture Implementation Conference held at the Hawaii Institute of Marine Biology, 1-5 November, 1972. South Pacif. Isl. Fish. Newsletter (Noumea, New Caledonia), No.8 (March, 1973), 14-15.

May, R.C., Popper,D. and Mcvey,J.P. (1974). Rearing and larval development of *Siganus canaliculatus* (Park) (Pisces: Siganidae). Micronesica, 10(2):285-298.

Mayer, I., Shackley,S.E., and Ryland,J.S. (1988). Aspects of the reproductive biology of the bass, *Dicentrarchus labrax* L. I. An histological and histochemical study of oocyte development. J. Fish Biol., 33:609-622.

Mazeaud, M.M. and Mazeaud,F. (1981). Adrenergic reponses to stress in fish. In"Stress and Fish" (Ed. by A.D. Pickering), pp.49-75. Academic Press, London.

McMaster, M.E., Munkittrick,K.R., Luxon,P.L. & Van Der Kraak,G.J. (1994). Impact of low level sampling stress on interpretation of physiological responses of white sucker exposed to effluent from a bleached kraft pulp mill. Ecotoxicology and Environmental Safety, 27:251-264.

Merchenthaler, I., Gores,T., Setalo,G., Petrusz,P. and Llerko,B. (1984). Gonadotropin-releasing hormone (G-RH) neurons and pathways in the rat brain. Cell Tiss. Res. 237: 15-29.

Mcvey, J.P. (1972). Observations on the early-stage formation of *Siganus fuscescens* (should be *S. canaliculatus*) at Palau Mariculture Demonstration Center. South Pac. Isl. Fish. Newsl. (Noumea, New caledonia), (6): 1-12.

Micale, V. and Perdichizzi, F. (1994). Further studies on the sexuality of the hermaphroditic teleost *Diplodus sargus* L., with particular reference to protandrous sex inversion. J. Fish Biol., 45: 661-670.

Middaugh, D.P., and T. Takita (1983). Tidal and diurnal spawning cues in the Atlantic silverside, *Menidia menidia*. Environ. Biol. Fishes, 8: 97-104.

Middaugh, D.P., and M.J. Hemmer (1984). Spawning of the tidewater silverside, *Menidia peninsula* (Goode and Bean), in response to tidal and lighting schedules in the laboratory. Estuaries, 7: 139-148.

Mikolajczyk, T., Weil,C. and Breton,B. (1993). Nicotine stimulates maturational gonadotropin (GtH2) release from carp (*Cyprinus carpio* L.) pituitary cells. Comp. Biochem. Physiol C., 105: 83-88.

Miller, C.A., J.M. Wilson, and A.H. Meier (1981). Induction of semilunar rhythms of reproductive indices in Fundulus grandis. [Abstr.]. Am. Zool., 21:995.

Mills, C.A. (1980). Spawning and rearing eggs of the dace *Leuciscus leuciscus* (L). Fish. Manage., 11:67-72.

Moore. R. (1980). Reproduction and migration in *Lates calcarifer* (Bloch). Ph.D. Thesis, University of London.

Moore. R. (1982). Spawning and early life history of barramundi, *Lates calcarifer* (Bloch), in Papua New Guinea. Aust. J. Mar. Freshwater Res., 33:647-661.

Morell, J.I., Kelley,D.B. and Pfaff,D.W. (1975). In "Brain-endocrine Interaction.II. The Ventricular System in Neuroendocrine Mechanisms" (K.M. Knigge, D.E. Scott, H. Kobayashi and S. Ishii, eds.). pp.230-256. Karger, Basel.

Morrison, J.K & Smith, C.E. (1986). Altering the spawning cycle of the rainbow trout by manipulating water temperature. Prog. Fish Cult., 48, 52-54.

Mrosovsky, N. (1970). Mechanism of hibernation cycles in ground squirrels: Circannian rhythm or sequence of stages?. Penn. Acad. Sci., 44: 172-175.

Mrosovsky, N. (1978). Circannual cycles in hibernators. In "Strategies in Cold: Natural Torpidity and Thermogenesis", L. Wang and J.W. Hudson, eds., pp. 21-65. Academic Press, New York.

Munro et al. (1990). "Volume and bibliographic information is not available".

Munz, B., Stumpf,W.E. and Jennes,L. (1981). LHRH systems in the brain of the platyfish. Brain Res. 221: 1-13.

Munz, H., Claas,B., Stumpf,W.E. and Jennes,L. (1982). Centrifugal innervation of the retina by luteinizing hormone-releasing hormone (LHRH) immunoreactive telencephalic neurons in teleostean fishes. Cell Tiss. Res. 222: 313-323.

Mylonas, C.C., L. Curry Woods III, Peter Thomas, and Yonathan Zohar. (1998). Endocrine profiles of female striped bass (*Morone saxatilis*) in captivity, during postvitellogenesis and induction of final oocyte maturation via controlled-release GnRHa-delivery systems. Gen. Comp. Endocrinol. 110:276-289.

Nakari, T., Soivio, A. & Pesonen, S. (1987). Effects of an advanced photoperiod cycle on the gonadal development and spawning time of 2-year-old *Slamo gairdneri* R. reared in earth ponds under extreme annual water temperatures. Aquaculture, 67(3-4):369-384.

Nakari, T., Soivio, A. & Pesonen, S. (1988). The ovarian development and spawning time of *Salmo gairdneri* R. reared in advanced and delayed annual photoperiod cycles at naturally fluctuating water temperature in Finland. Ann. Zool. Fenn., 25, 335-40.

Nagahama, Y.(1983). The functional morphology of teleost gonads, In "Fish Physiology" 9A (Hoar,W.S., Randall,D.J. & Donaldson, E.M., eds),pp. 223-273. London:Academic Press.

Nagahama, Y. (1987a). 17α.20β,21-trihydroxy-4-pregnen-3-one: a teleost maturation-inducing hormoone. Develop. Growth and Differ., 29: 1-12.

Nagahama, Y. (1987b). Gonadotropin action on gametogenesis and steroidogenesis in teleost gonads. Zool. Sci., 4: 209-222.

Nagahama, Y. and Adachi,S. (1985). Identification of a maturation inducing steroid in a teleost, the amago salmon (*Oncorhynchus rhodurus*). Dev. Biol. 109: 428-435.

Nagahama, Y., Kagawa,H. and Tashiro,F. (1980). Bull. Jap. Soc. Scient. Fish., 46: 1097-1102.

Nash, C.E. & Shehadeh, Z.H. (1980). Review of breeding and Propagation techniques for grey mullet, *Mugil cephalus* L. Iclarm Studies and Reviews No.3, p.87. International Center for Living Aquatic Resources Management, Manila, Philippines.

Nelson, S.G., Lock,S.A., & Collins,L.A. (1992). Growth of the rabbitfish *Siganus randalli* Woodland in relation to the feasibility of its culture in Guam. University of Guam Marine Laboratory Technical Report No. 97. p.30.

Ngamvongchom, S., Lovejoy, D.A., Fischer,W.H., Craig,A.G., Nahorniak,C.S., Peter,R.E., Rivier,J.E., Sherwood,N.M. (1992a). Primary structures of two forms of gonadotropin-releasing hormone, one conserved, from catfish brain. Mol. Cell Neurosci., 3:17-22.

Nikinmaa, M. (1982). The effects of adrenaline on the oxygen transport properties of *Salmo gairdneri* blood. Comp. Bioch. Physiol., 71A:353-356.

Nikolosky. (1963). "Volume and bibliographic information is not available".

Nozaki, M. and Kobayashi,H. (1979). Distribution of LHRH-like substances in the vertebrate brain as revealed by immuno-histochemistry. Arch. Histol. Jap. 42: 201-209.

Ntiba, M.J. and Jaccarini,V. (1990). Gonad maturation and spawning times of *Siganus sutor* off the Kenya Coast: evidence for definite spawning seasons in a tropical Fish. J. Fish. Biol., 37(2):315-325.

Ntiba, M.J. and Jaccarini,V. (1992). The effect of oocytic atresia on fecundity estimates of the rabbitfish Siganus sutor (Pisces: Siganidae) of Kenyan marine inshore waters. Hydrobiologia, 247:215-222.

Ochiai, A., Umeda,S. & Ota,H. (1972). On the eggs of Japanese eel and induction of maturation by hormone injection. Jap. J. Ichthyol., 19: 312-316.

Okuzawa, K., Furukawa,K., Aida,K., and Hanyu,I. (1989). Effect of photoperiod and temperature on gonadal maturation, and plasma steroid and gonadotropin levels in a cyprinid fish. The honmoroko *Gnathopogon caerulescens*. General & Comparative Endocrinology, 75: 139-147.

Omeljaniuk ,R.J., S.H. Shih and R.E. Peter. (1987). *In vivo* evaluation of dopaminreceptor-mediated inhibition of gonadotropin secretion from the pituitary of the goldfish, *Carassius auratus*. Journal of Endocrinology, 114: 449-458.

Orr, T.E., Taylor,M., Bhattacharyya,A.K., Collins,D.C., & Mann,D.R. (1994). Acute immobilization stress disrupts testicular steroidogenesis in adult male rats by inhibiting the activities of 17α-hydroxylase and 17,20-lyase without affecting the binding of LH/hCG receptors. Journal of Andrology, 15:302-308.

Osanai, K. (1956). On the ovarian eggs of the loach, *Lefua echigonia*, with special reference to the formation of the cortical alveoli. Sci. Rep. Tohoku Univ. Ser. 4, 22: 181-188.

Palma, A.L. (1978). Induced spawning and larval rearing of rabbitfish *Siganus guttatus*. Philipp. J. Fish., 16(2): 95-104.

Pandey, S., and Hoar,W.S. (1972). Induction of ovulation in goldfish by clomiphene citrate. Can. J. Zool., 50: 1679-1680.

Pandey, S., Lam,T.J., Nagahama,Y., and Hoar,W.S. (1977). Effects of dexamethasone metopirone on ovulation in the goldfish, *carassius auratus*. Can. J. Zool., 55: 1342-1350.

Pankhurst, N.W. & Carragher,J.F. (1992). Oocyte maturation and changes in plasma steroid levels in snapper *Pagrus* (= *Chrysophrys*) *auratus* (Sparidae) following treatment with human chorionic gonadotropin. Aquaculture, 101:337-347.

Pankhurst, N.W. and Dedual,M. (1994). Effects of capture and recovery on plasma levels of cortisol, lactate and gonadal steroids in a natural population of rainbow trout. Journal of Fish Biology, 45:1013-1025.

Pankhurst, N.W., Kraak,G.Van Der, and Peter,R.E. (1986). Effects of human chorionic gonadotropin, Des-gly^{10} (D-Ala^{6}) LHRH-ethylamide and pimozide on oocyte final maturation, ovulation and levels of plasma sex steroids in the walleye (*Stizostedion vitreum*). Fish Physiol. Biochem., 1(1):45-54.

Pankhurst, N.W., Van Der Kraak,G. (1997). Effect of stress on reproduction and growth of fish. In " Fish Stress and Health in Aquaculture". G.k. Iwama, A.D. Pickering, J.P. Sumpter and C.B. Schreck, eds. pp. 73-94. Society for Experimental Biology Series 62, Cambridge University Press. p.278.

Parazo, M.M. (1989). Effect of protein and energy levels on growth of rabbitfish. SEAFDEC Asian Aquaculture, 11(3): 4-5.

Peng, C., Y.P. Huang and R.E. Peter. (1990). Neuroepetide Y stimulated growth hormone and gonadotropin release from the goldfish pituitary *in vitro*. Neuroendocrinology 52: 28-34.

Peter, H. (1979). Some aspects of early follicular development. In "Ovarian Follicular Development and Function" edited by A.R. Midgley and W.A. Sadler. Raven Press, New York. pp. 1-14.

Peter, R.E. (1981). Gonadotropin secretion during reproductive cycles in teleosts: Influences of environmental factors. Gen. Comp. Endocrinol., 45:294-305.

Peter, R.E. (1982). Neuroendocrine control of reproduction in teleosts. Can. J. Fish. Aquat. Sci., 39:48-55.

Peter, R.E. (1983). The brain and neurohormones in teleost reproduction. In: Fish Physiology. pp.97-135. Edited W.S. Hoar, D.J. Randall and E.M. Donaldson. Academic Press, New York.

Peter, R.E., and Hontela,A. (1978). Annual gonadal cycles in teleosts: Environmental factors and gonadotropin levels in blood. In "Environmental Endocrinology" (I. Assenmacher and D.S. Farners, eds.), pp.20-25. Springer-Verlag, Berlin and New York.

Peter, R.E., Crim,L.W., Goos,H.J.Th. and Crim,J.W. (1978). Lesioning studies on the gravid female goldfish: neuroendocrine regulation of ovulation. Gen. Comp. Endocrinol., 35: 391-401.

Peter, R.E., Habibi,H.R., Marchant,T.A. & Nahorniak,C.S. (1987a). Vertebrate gonadotropin-releasing hormone: phylogeny and structure-function relationship. Annals N.Y. Acad. Sci., 519, 299-309.

Peter, R.E., J.P. Chang, C.S. Nahorniak, R.J. Omeljaniuk, M. Sokolowska, S.H. Shih and R. Billard. (1986). Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. Recent Progresses in Hormone Research, 42: 513-548.

Peter, R.E., Lin,H.R. & Kraak,G. van der (1988a). Induced ovulation and spawning of cultured freshwater fish in China: Advances in application of GnRH-analogues and Dopamine antagonists. Aquaculture, 74, 1-10.

Peter, R.E., Lin,H.R. & Kraak,G. vander (1988b). Induced spawning in Chinese carps. In Proceedings of the Aquaculture International Congress and Exposition, pp. 543-47. British Columbia Pavilion Corp., Vancouver, British Columbia.

Peter, R.E., M. Sokolowska, C.S. Nahorniak, J.R. Rivier and W.W. Vale. (1987b). Comparison of sGnRH-A and LHRH-A, in combination with pimozide, in stimulating gonadotropin release and ovulation in the goldfish, *Carassius auratus*. Canadian Journal of Zoology, 65: 987-991.

Peter, R.E., Nahorniak,C.S., Sokolowska,M., Chang,J.P., Rivier, J.E., Vale,W.W., King,J.A. & Millar,R.P. (1985). Structure-activity relationship of mammalian, chicken, and salmon gonadotropin releasing hormones *in-vivo* in goldfish. Gen. Comp. Endocrinol., 58, 231-42.

Peter, R.E. and Paulencu,C.R. (1980). Involvement of the preoptic region in gonadotropin release-inhibition in goldfish, *Carassius auratus*. Neuroendocrinology, 31: 133-141.

Peter R.E., V.L. Trudeau and B.D. Sloley. (1991). Brain regulation of reproduction in teleosts. Bulletin, Institute of Zoology, Academia Sinica, Monograph, 16:89-118.

Pickering, A.D. (1981). Introduction: The concept of biological stress, In "Stress and Fish" (Ed. by A.D. Pickering), pp.1-9. Academic Press. London.

Pickering, A.D. (1990). Stress and suppression of somatic growth in teleost fish. In "Progress in Comparative Endocrinology". Epple,A., Scanes,C.G. & Stetson,M.H. (eds.). pp.473-479. Wiley-Liss, New York.

Pickering, A.D. (1993). Growth and stress in fish production. Aquaculture, 111:51-63.

Pickering, A.D., Pottinger,T.G., Carragher,J. and Sumpter,J.P. (1987a). The effect of acute and chronic stress on the levels of reproductive hormones in the plasma of mature brown trout, *Salmo trutta*. L. Gen. Comp. Endocrinol., 68: 249-259.

Pickford, G.E., Lofts,B., Bara,G., and A-z,J.W. (1972). Testis stimulation in hypophysectomized male killifish, *Fundulus heteroclitus*, treated with mammalian growth hormone and/or luteinizing hormone. Biol. Reprod., 7: 370-386.

Pillai, T.G. (1962). Siganid fish farming. In: Fish Farming Methods in the Philippines, Indonesia and HongKong. Rome, Fisheries Division, Biology Branch, Food and Agriculture Organization of the United Nations, p.51-52. (Technical paper/FAO Fisheries Biology, No.18).

Poncin, P. (1991). Environmental and endocrine control of barbel (*Barbus barbus L.*) reproduction. Proceedings of 4th Int. Symp. on the Reproductive Physiology of Fish, Norwich, U.K., 7-12 July.

Pongsuwana, U., Rahman,A.B.A. and Khamis,R.B. (1984). Survey of wild fish fry and their suitability for aquaculture in Tanjong Adang, Johor, Malaysia. Coastal aquaculture development, selected papers prepared during the activities of the Coastal Aquaculture

Demonstration and Training Project, FAO Rome, Italy,1984, pp.195-214.

Popper, D., Gordin,H and Kissil,G.W. (1973). Fertilization and hatching of rabbitfish *Siganus rivulatus*. Aquaculture, 2:37-44.

Popper, D. and Gundermann,N. (1975). Some ecological and behavioural aspects of siganid populations in the Red Sea and Mediterranean Coast of Israel in relation to their suitability for aquaculture. Aquaculture, 6:127-141.

Popper, D. and Gundermann,N. (1976). A successful spawning and hatching of *Siganus vermiculatus* under field conditions. Aquaculture, 77:291-292.

Popper, D., May,R.C. and Lichatowich,T. (1976). An experiment in rearing larval *Siganus vermiculatus* (Valenciennes) spawning-inducing agent. Spec. Publ. Eur. Mar. Soc., 8:151-168.

Popper, D., Pitt,R. and Zohar,Y. (1979). Experiments on the propagation of red sea Siganids and some note on their reproduction in nature. Aquaculture, 16:177-181.

Pottinger, T.G., Campbell,P.M. & Sumpter, J.P. (1991). Stress-induced disruption of the salmonid liver-gonad axis. In Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish. Norwich, U.K., 7-12 July 1991. Scott, A.P., Sumpter,J.P., Kime,D.E. & Rolfe,M.S. (eds). pp. 114-116. FishSymp91, Sheffield.

Powell, J.F.F., Zohar,Y., Elizur,A., Park,M., Fischer,W.H., Craig,A.G., Rivier,J.E., Lovejoy,D.A. and Sherwood,N.M. (1994). Three forms of gonadotropin-releasing hormone characterized from brains of one species. Proc. Natl. Acad. Sci. USA. 91: 12081-12085.

Prentice, J.A. and Thomas,P. (1987). Successful spawning of orangemouth corvina following injection with des-Gly^{10}[D-Ala^{6}]-luteinizing hormone-releasing hormone (1-9) ethylamide and pimozide. Prog. Fish. Cult., 49: 66-69.

Pressley, P.H. (1980). Lunar periodicity in the spawning of yellowtail damselfish, *Microspathodon chrysurus*. Environmental Biology of Fishes, 5: 153-159.

Pudney, J.A. (1987). Overall view of the organization of the vertebrate gonad and the structure of steroidogenic cells. In "Fundamentals of Comparative Vertebrate Endocrinology". I. Chester-Jones, P.M. Ingleton and J.G. Phillips (eds). Plenum Press. pp. 57-94.

Rai, B.P. (1966). The *corpora atretica* and the so-called corpora lutea in the ovary of *Tor (Barbus) tor* (Ham). Anat. Anz., 119: 459-465.

Rajalakshmi, M.(1966). Atresia of oocytes and ruptured follicles in *Gobius giuris* (Hamilton-Buchanan). Gen. Comp. Endocr. 6:378-385.

Ramos, J. (1986). Induction of spawning in common sole (*Solea solea* L.) with human chorionic gonadotropin (HCG). Aquaculture, 56:239-242.

Randall, C., Duston,J. and Bromage,N. (1987). Photoperiodic history and entrainment of the annual cycle of reproduction in the female rainbow trout (*Salmo gairdneri*). Proc. 3rd Int. Symp. Repr. Physiol. Fish. (Ed. by D.R. Idler, L.W. Crim & J.M. Walsh), p.310. Memorial University of Newfoundland, St. John's.

Roberts, D.E. (1987). Photoperiod/temperature control in the commercial production of red drum (*Sciaenops ocellatus*) eggs. In "Manual on Red Drum Culture", Chapter II, pp.10-26. Texas Agricultual Extension Service, College Station, Texas.

Robertson, D.R., Peterson,C.W. and brawn,J.D. (1990). Lunar reproductive cycles of benthic-brooding reef fishes: reflections of larval biology or adult biology. Ecol. Monogr., 60(3):311-329.

Ross, R.M. (1983). Annual, semilunar, and diel reproductive rhythms in the Hawaiian labrid *Thalassoma duperrey.* Marine Biology, 72, 311-318.

Rossiter, A. (1991). Lunar spawning synchronicity in a freshwater fish. Natur. Wissenschaften, 78:182-184.

Safford, S.E. and Thomas,P. (1987). Effect of capture and handling on circulting levels of gonadal stroids and cortisol in the spotted seatrout, *Cynoscion nebulosus*. Proc. Third Int. Symp. Repr. Physiol. Fish. (Ed. by D.R. Idler, L.W. Crim & J.M. Walsh), p.312. Memorial University of Newfoundland, St. John's.

Saidapur, S.K. (1978). Follicular atresia in the ovaries of nonmammalian vertebrates Int. Rev. Cytol. 54:225-244.

Saidapur, S.K. & Nadkarni,V.B. (1976). Steroid synthesizing cellular sites in the ovary of catfish, *Mystus cavasius*: A histochemical study. Gen. Comp. Endocrinol., 30: 457-461.

Saigusa, M. (1980). Entrainment of a semilunar rhythm by a simulated moonlight cycle in the terrestrial crab, *Sesarma haematocheir*, Oecologia (Berl.), 46: 38-44.

Sapolsky. R.M. (1985). Stress-induced suppression of testicular function in the wild baboon: role of glucocorticoids. Endocrinology, 116: 2273-2278.

Sargent, R.C. and M.R. Gross (1986). William's principle: an explanation of parental care in teleosts. In "The Behaviour of Teleost Fishes". T.J. Pitcher, ed. pp. 275-293. John's Hopkins University Press, Baltimore, Maryland, USA.

Sawara, Y. (1974). Reproduction of the mosquitofish (*Gambusia affinis affinis*), a freshwater fish introduced into Japan. Jpn. J. Ecol., 24: 140-146.

Schaefer, K.M. (1987). Reproductive biology of the skipjack, *Euthynnus lineatus*, an eastern Pacific tuna. Bull. Int. Am. Trop. Tuna Comm., 19: 169-227.

Schally, A.V., Arimura,A. and Kastin,A.J. (1973). Hypothalamic regulatory hormones. Science, 179.

Schreck, C.B., Olla,B.L., & Davis,M.W. (1997). Behavioral responses to stress. In "Fish Stress and Health in Aquaculture". G.K. Iwama, A.D. Pickering, J.P. Sumpter, and C.B. Schreck (eds.). pp. 145-170. Society for Experimental Biology, Seminar Series 62, Cambridge University Press.

Schriebman, M.P., Halpern-Sebold,L., Ferin,M., Margolis-Kazan,H. and Goos,H.J.Th. (1983). The effect of hypophysectomy and gonadotropin administration on the distribution and quantity of LH-RH in the brains of : a combined immunocytochemistry and radioimmunoassay study. Brain Res., 267: 293-300.

Schriebman, M.P., Margolis-Kazan,H., Halpern-Sebold,L., O'Neill,P.A. and Silverman,R.C. (1984). Structural links between olfactory and reproductive systems: puberty-related changes in olfactory epithelium. Brain Res., 302: 180-183.

Scott, A.P. (1987). Reproductive endocrinology of fish. In "Fundamentals of Comparative Vertebrate Endocrinology". I. Chester-Jones, P.M. Ingleton and J.G. Phillips (eds). Plenum Press. pp. 223-256.

Scott, D.B.C. (1979). Environmental timing and the control of reproduction in teleost fish. Symp. Zool. Soc., London. 44: 105-132

Scott, A.P., Baynes,S. Skarphedinsson,O. and Bye,V. (1984). Control of spawning time in rainbow trout (*Salmo gairdneri*) using constant daylengths. Aquaculture, 43:225-233.

Scott, A.P., and Canario,A.V.M. (1987). Status of oocyte maturation-inducing steroids in teleosts. In Proc. IIIrd Int. Symp. Reprod. Physiol. Fish, (D.R. Idler, L.W. Crim, and J.M. Walsh, Eds.), pp.224-234. Marine Sciences Laboratory, St. John's, Newfoundland, Canada.

Selman, K., Wallace,R.A., and Barr,V. (1986). Oogenesis in *Fundulus heteroclitus*. IV. Yolk vesicle formation. J. Exp. Zool., 239:277-288.

Selman, K., Wallace,R.A., and Barr,V. (1988). Oogenesis in *Fundulus heteroclitus*. V. The relationship of yolk vesicles and cortical alveoli. J. Exp. Zool., 246:42-56.

Selman, K. and Wallace,R.A. (1989). Cellular aspects of oocyte growth in teleosts. Zool. Sci., 6: 211-231.

Selye, H. (1956). "The Stress of Life". McGraw-Hill, Toronto.

Sheridan, M. (1986). Effects of thyroxin, cortisol, growth hormone and prolactin on lipid metabolism of coho salmon, *Oncorhynchus kisutch*, during smoltification. Gen. Comp. Endocrinol., 64: 220-238.

Sheridan, M. (1987). The effect of epinephrine and norepinephrine on lipid mobilization from coho salmon liver incubated *in vitro*. Endocrinology, 120: 2234-2239.

Sherwood, N.M., Doroshov,S., and Lance,V. (1991). Gonadotropin-releasing hormone (GnRH) in bony fish that are phylogenetically ancient: Reedfish (*Calamoichthys calabaricus*), sturgeon (*Acipenser transmontanus*), and alligator gar (*Lepisosteus spatula*). Gen. Comp. Endocrinol., 84: 44-57.

Sherwood, N.M., Eiden,L., Brownstein,M., Spiess,J., Rivier,J. and Vale,W. (1983). Characterization of a teleost Gn-RH. Proc. Natl. Acad. Sci. USA, 80:2794-2797.

Sherwood, N.M., Grier,H.J., Warby,C., Peute,J., and Taylor,R.G. (1993b). Gonadotropin-releasing hormones, including a novel form, in snook, *Centropomus undecimalis*, in comparison with forms in black sea bass, *Centropristis striata*. Regul. Peptides, 46: 523-534.

Sherwood, N.M., Harvey,B., Brownstein,M.J. and Eiden,L.E. (1984). Gonadotropin-releasing hormone (Gn-RH) in striped mullet (*Mugil cephalus*), milkfish (*Chanos chanos*), and rainbow trout (*Salmo gairdneri*): Comparison with salmon Gn-RH. Gen. Comp. Endocrinol., 55: 174-181.

Short, C.E., Crim,L.W. & Morgan,M.J. (1995). The effects of stress on spawning performance and larval development in Atlantic cod, *Gadus morhua*. In "Reproductive Physiology of Fish 1995". Goetz,F.W. & Thomas,P. (eds.). University of Texas, Austin.

Smigielski, A.S. (1975). Hormonal-induced ovulation of the winter flounder, *Pseudopleuronectes americanus*. Fish. Bull. U.S. Natl. Mar. Fish. Serv. 73: 431-438.

Smith, C.L. (1975). The evolution of hermaphroditism in fishes. In "Intersexuality in the Animal Kingdom". edited by R. Reinboth. Springer-Verlag, New York. pp. 295-310.

Smith, R.J.F. (1970). Control of prespawning bahaviour of sunfish (*Lepomis gibbosus* and *Lepomis megalotis*). II. Environmental factors. Anim. Behav., 18: 575-587.

Soh, C.L. and Lam,T.J. (1973). Induced breeding and early development of the rabbitfish *Siganus oramin* (Schneider). Malay. Nat. J., pp.49-56.

Sokolowska, M., Peter,R.E., Nahorniak,C.S., Pan,C.H.,, Chang,J.P., Crim,L.W., and Weil,C. (1984). Induction of ovulation in goldfish, *carassius auratus*, by pimozide and analogues of LH-RH. Aquaculture, 36:71-83.

Sokolowska, M., Peter,R.E., and Nahorniak,C.S. (1985a). The effects of different doses of pimozide and [D-Ala^6, Pro^9-N ethylamide]-LHRH (LHRH-A) on gonadotropin release and ovulation in female goldfish. Can. J. Zool., 63:1252-1256.

Sokolowska,, M., Peter,R.E., Nahorniak,C.S., and Chang,J.P. (1985b). Seasonal effects of pimozide and des Gly^{10} [D-Ala^6] LH-RH Ethylamide on gonadotropin secretion in goldfish. Gen. Comp. Endocrin., 57:472-479.

Soletchnik, P. (1984). Aspects of nutrition and reproduction in *Siganus guttatus* with emphasis on application to aquaculture. Iloilo, SEAFDEC\Aquaculture Department. 75p. (Terminal report to the Aquaculture Department, SEAFDEC).

Somoza, G.M., K.L. Yu and R.E. Peter. (1988). Serotonin stimulates gonadotropin release in female and male goldfish, *Carassius auratus* L. Gen. Comp. Endocrinol., 72: 373-382.

Springate, J., Bromage,N. and Cumaranatunga,R. (1985). The effects of different ration on fecundity and egg quality in the rainbow trout (*Salmo gairdneri*). In "Feeding and Nutrition in Fish" (Ed. by C. Cowey, A.Mackie and J. Bell), pp.371-391. Academic Press, London.

Srivastava, S.J. and R, Singh (1991). Effects of constant photoperiod-temperature regimes on ovarian activity during the annual reprodutive cycle in the murrel, *Channa punctatus* (Bloch). Aquaculture, 96: 383-391.

Stacey, N.E. (1983). Hormones and reproductive behaviour in teleosts. In "Control Processes in Fish Physiology", J.C. Rankin, T.J. Pitcher & R. Duggan, eds. pp. 117-129. Croom Helm.

Stacey, N.E. (1984). Control of timing of ovulation by exogenous and endogenous factors. In Fish Reproduction: Strategies and Tactics (Potts,G.W. and Wooton,R.J., eds.), pp.207-222. London: Academic Press.

Stacey, N.E., Cook,A.F. and Peter,R.E. (1979a). Ovulatory surge of gonadotropin in the goldfish, *Carassius auratus*. Gen. Comp. Endocrinol., 37: 246-249.

Stacey, N.E., Cook,A.F., and Peter, R.E. (1979b). Spontaneous and gonadotropin-induced ovulation in the goldfish, *Carassius auratus* L.: Effects of external factors. J. Fish. Biol., 15 349-361.

Stacey, N.E. and Goetz,F.W. (1982). Can. J. Fish. Aquat. Sci., 39; 92-98.

Stacey, N.E., MacKenzie,D.S., Marchant, T.A., Kyle, A.L. & Peter,R.E. (1984). Endocrine changes during natural spawning in the white sucker *Catostomus commersoni*. I. Gonadotropin, growth hormone, and thyroid hormones. Gen. Comp. Endocrinol.,

56:333-348.

Storey, C.R. & Nicholls, T.J. (1976). Some effects of manipulation of daily photoperiod on the rate of onset of a photorefractory state in canaries. Gen. Comp. Endocrinol., 30: 204-208.

Sundararaj, B.I. and Goswami,S.V. (1977). Hormonal regulation of *in vivo* and *in vitro* oocyte maturation in the catfish, *Heteropneustes fossilis* (Bloch). Gen. Comp. Endocrinol., 32: 17-28.

Sundararaj, B., and Vasal, S. (1976). Photoperiod and temperature control in the regulation of reproduction in female catfish, *Heteropneustes fossilis*. J. Fish. Res. Bd. Can., 33: 959-973.

Sullivan, C.V., R.G. Hodson, Y. Tao, W. King, L.F. Jackson, P. Swanson, P. Thomas, L. Helfrich, W. Blythe, R.M. Harrell and L.C. Woods, III. (1992). Reproduction of striped bass, *Morone saxatilis*: basic and applied research on hormones. Abstracts of the Second International Symposium in Fish Endocrinology: 25.

Sumpter, J.P. (1997). The endocrinology of stress. In "Fish Stress and Health in Aquaculture". G.K. Iwama, A.D. Pickering, J.P. Sumpter, and C.B. Schreck (eds.). pp. 95-118. Society for Experimental Biology, Seminar Series 62, Cambridge University Press.

Sumpter, J.P., Carragher,J., Pottinger,T.G. and Pickering,A.D. (1987). The interaction of stress and reproduction in trout. In "Reproductive Physiology of Fish" (Ed. by D.R. Idler, L.W. Crim, and J.M. Walsh), pp.299-302. Memorial University of Newfoundland, St. John's, , Canada.

Sumpter, J., Scott,A., Baynes,S. and Witthames,P. (1984).. Early stages of the reproductive cycle in virgin female rainbow trout (*Salmo gairdneri* Richardson). Aquacult., 43: 235-242.

Sumpter, J.P., Tyler,C.R. and Kawauchi,H. (1991). Actions of GTH I and GTH II on ovarian steroidogenesis in the rainbow trout, *Oncorhynchus mykiss*. In: A.P. Scott, J.P. Sumpter, D.E. Kime and M.S. Rolfe (Editors), Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish. Norwich, U.K., 7-12 July 1991, FishSymp91, Sheffield, p.27.

Suzuki, K., Kawauchi,H. and Nagahama,Y. (1988a). Isolation and characterization of two distinct gonadotropins from chum salmon pituitary glands. Gen. Comp. Endocrinol., 71: 292-301.

Suzuki, K., Kawauchi,H. and Nagahama,Y. (1988b). Isolation and characterization of subunits from two distinct salmon gonadotropins. Gen . Comp. Endocrinol., 71: 302-306.

Suzuki, K., Nagahama,Y. and Kawauchi,H. (1988c). Steroidogenic activities of two distinct salmon gonadotropins. Gen . Comp. Endocrinol., 71: 452-458.

Swanson, P., Dickhoff,W. (1987b). Variation in thyroid response to thyroid-stimulating hormone in juvenile coho salmon (*Oncorhynchus kisutch*). Gen. Comp. Endocrinol., 68:473-485.

Swanson, P., Dickhoff,W., and Gorbman,A. (1987a). Pituitary thyrotropin and gonadotropin of coho salmon (*Oncorhynchus kisutch*): Separation by chromatofocusing. Gen. Comp. Endocrinol., 65:269-287.

Swanson, P., M. Bernard, M. Nozaki, K. Suzuki, H. Kawauchi and W.W. Dickhoff (1989). Gonadotropins I and II in juvenile coho salmon. Fish Physiol. Biochem. 7: 169-176.

Swanson, P., Suzuki,K., Kawauchi,H. and Dickhoff,W.W. (1991). Isolation and characterization of two coho salmon gonadotropins, GTH I and GTH II. Biol. Reprod., 44: 29-38.

Takahashi, S. (1981). Sexual maturity of the Isaza, *Chaenogobius isaza*. II. Gross morphology and histology of the ovary. Zoological Magazine, Tokyo, 90: 54-61.

Takashima, F. & Yamada,Y. (1984). Control of maturation in masu salmon by manipulation of photoperiod. Aquaculture, 43, 243-57.

Tamaru et al. (1980). "Volume and bibliographic information is not available".

Tamaru, C.S., Lee,C.S., Kelley,C.D. & Banno,J.E. (1988). Effectiveness of chronic LHRH-analogue and 17α methyltestosterone therapy, administered at different times prior to the spawning season on the maturation of mikfish (*Chanos chanos*). Aquaculture, 70: 159-167.

Tawada, S. (1991). Rabbitfishes (*Siganus* sp.). In "Aquaculture in Tropical Areas". S. Shokita, K. Kakazu, A. Tomori, and T. Toma (eds.). Midori Shobo Co., Ltd., Tokyo. pp114-126.

Taylor, M.H. (1984). Lunar Synchronization of fish reproduction. Trans. Amer. Fish. Soc., 113: 484-493.

Taylor, M.H. (1986). Dynamics of the *in vitro* response of *Fundulus heteroclitus* oocytes to 17α,20β-dihydroxy-4-pregnen-3-one. Amer. Zool., 26(4), 25A.

Taylor, M.H. (1991). Entrainment of the semilunar cycle of *Fundulus Heteroclitus.* Proceedings of the 4th Int. Symp. on the Reproductive Physiology of fish. Norwich, U.K., 7-12 July 1991.

Taylor, M.H. and DiMichele,L. (1980). Ovarian changes during the lunar spawning cycle of *Fundulus heteroclitus*. Copeia, (1):118-125.

Taylor, M.H., Leach,G.J., Dimichele,L., Levitan,W.M., and Jacob,W.F. (1979). Lunar spawning cycle in the mummichog, *Fundulus heteroclitus* (Pisces:Cyprinodontidae). Copeia, 2:291-297.

Tharakan, B. and K.P. Joy. (1996). Effects of mammalian gonadotropin-releasing hormone analogue, pimozide, and the combination on plasma gonadotropin levels in different seasons and induction of ovulation in female catfish. J. Fish Biol., 48: 623-632.

Thomas, P. and Arnold,C.R. (1993). Environmetal and hormonal control of reproduction in sciaenid fish. In "Recent Advances in Aquaculture IV". Edited by J.F. Muir and R.J. Roberts. Blackwell Scientific Publicatons, Oxford. pp.31-42.

Thomas, P., Brown,N.J. and Trant,J. (1987). Plasma levels of gonadal steroids during the reproductive cycle of female spotted seatrout *Cynoscion nebulosus*. In Third International Symposium on the Reprodutive Physiology of Fish (Idler,D.R., Crim,L.W. and Walsh,J.M., eds.), p. 219. St. John's, Newfoundland, Canada: Marine sciences Research Laboratory.

Thorpe, J., Talbot,C. and Miles,M. (1989). Research suggests a simple answer to grilsing control. Fish Farmer, 12: 10-11.

Thresher, R.E. (1984). Reproduction in reef fishes. T.F.H., Neptune City, New Jersey, USA.

Titarev, Y.F. (1975). Acceleration of maturation in the rainbow trout (*Salmo gairdneri*) under influence of increased water temperature. J. Ichthyol., 15: 507-509.

Tobias, W.J. (1976). Ecology of *Siganus argenteus* (Pisces:Siganidae) in relation to its maricultural potential in Guam. Tech. Rep. Univ. Guam Mar. Lab, (29):58-93.

Tokarz, R.R. (1978). Oogonial proliferation, oogenesis, and folliculogenesis in nonmammalian vertebrates. In: "The Vertebrate Ovary". (R.E. Jones, ed.), pp. 145-179. Plenum Press, New York.

Trant, J.M. and Thomas,P. (1988). Structure-activity relationship of steroids in inducing germinal vesicle breakdown of Atlantic croaker oocytes *in vitro*. Gen. Comp. Endocrinol., 71: 307-317.

Trant, J.M., Thomas,P. and Shackleton,C.H.L. (1986). Identification of 17α,20β,21-trihydroxy-4-pregnen-3-one as the major ovarian steroid produced by the teleost *Micropogonias undulatus* during final oocyte maturation. Steroids, 47: 89-99.

Truscott, B., Idler,D.R., Sundararaj,B.I. and Goswami,S.V. (1978). Gen. Comp. Endocrinol., 34: 149-157.

Tsafriri, A. & Braw,R.H.(1984). Experimental approaches to atresia in mammals. In oxford reviews of reproductive biology (Clarke,J.R.,ed).pp.226-265. Oxford:Clarendon Press.

Tseng, W.Y. and Chan,K.L. (1982). The reproductive biology of the rabbitfish in Hong Kong. World Maricult. Soc., 13, pp.313-321.

Tyler, C., Sumpter,J. and Bromage,N. (1987). Uptake of vitellogenin into cultured ovarian follicles of rainbow trout. Proc. 3rd Int. Symp. Repr. Physiol. Fish. (Ed. by D.R. Idler, L.W. Crim & J.M. Walsh), p.221. Memorial University of Newfoundland, St. John's.

Tyler, C.R., Sumpter,J.P., Kawauchi,H. and Swanson,P. (1991). Involvement of gonadotropins in the uptake of vitellogenin in vitellogenic oocytes of the rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol., 84: 291-299.

Tyler, J.C., G.D. Johnson, I. Nakamura, and B.B. Collete. (1989). Morphology of *Luvarus imperialis* (Luvaridae), with a phylogenetic analysis of the Acanthuroidei (Pisces). Smithson. Contrib. Zool. 485: 1-78.

Tyler, W.A. III. (1988). Spawning patterns in the Hawaiian sergeant, *Abudefduf abdominalis* (Family:Pomacentridae). Pacific Science, 42; 136.

Vale, W., Grant,G. and Guillemin. (1973). Chemistry of the hypothalamic releasing factor. Studies in structure-function relationships. In: "Frontiers in Neuroendocrinology". Ganong,W.F. and Martini,L. (eds.). Oxford University Press, New York. pp.375.

Van den Hurk, R. & Peute,J. (1979). Cyclic changes in the ovary of the rainbow trout. *Salmo gairdneri*, with special reference to sites of steroidogenesis. Cell Tissue Res., 199: 289-306.

Van Der Kraak, G.J. (1984). Gonadotropin involvement in the control of oocyte maturation and ovulation in coho salmon (*Oncorhynchus kisutch*): neuroendocrine control of gonadotropin secretion, effects of steroid production and properties of ovarian gonadotropin binding sites. Ph.D. thesis, University of British Columbia, Vancouver, B.C. pp. 1-178.

Van Der Kraak, G., Dye,H.M., Donaldson,E.M., and Hunter,G.A. (1985). Plasma gonadotropin, 17β-estradiol, and 17α, 20β-dihydroxy-4-pregnen-3-one levels during luteinizing hormone-releasing hormone analogue and gonadotropin induced ovulation in coho salmon (*Oncorhynchus kisutch*). Can. J. Zool., 63(4):824-833.

Van Der Kraak, G., E.M. Donaldson and J.P. Chang. (1986). Dopamine involvement in the regulation of gonadotropin secretion in coho salmon. Canadian Journal of Zoology, 64: 1254-1248.

Van Der Kraak, G., Suzuki,K., Peter,R.E., Itoh,H. and Kawauchi,H. (1992b). Properties of common carp gonadotropin I and gonadotropin II. Gen. Comp. Endocrinol., 85: 217-229.

Van Ree, G.E., Lok,D., and Bosman,G. (1977). *In vitro* induction of nuclear breakdown in oocytes of the zebrafish, *Brachydanio rerio* (Ham. Buch.): Effects of the composition of the medium and of protein and steroid hormones. Proc. K. Ned. Akad. Wetensch. Ser. C 80:353-371.

Wallace, R.A. (1978). Oocyte growth in nonmammalian vertebrates. In "The Vertebrate Ovary". (R.E. Jones, ed.), pp. 469-502. Plenum Press, New York.

Wallace, R.A., and Selman,K. (1980). Oogenesis in *Fundulus heteroclitus*. II.The transition from vitellogenesis into maturation. Gen. Comp. Endocrinol., 42: 345-354.

Wallace, R.A., and Selman,K. (1981). Cellular and dynamic aspects of oocyte growth in teleosts. Amer. Zool., 21:325-343.

Wallace, R.A., Selman,K., Greeley,M.S., Begovac,P.C., Lin,Y.W.P., McPherson,R. & Petrino,T.R.(1987). Current status of oocyte growth. In proceedings 3rd Int. Symp. on Reprod. Physiol. of Fish. (Idler,D.R.,Crim.L.W. & Walsh,J.M.,eds.),pp. 167-177. St John's:Memorial Univ. of Newfoundland.

Wedemeyer, G.A. (1996a). Transportation and handling. In "Principles of Salmonid Culture". Pennell,W. & Barton,B.A. (eds.). pp.727-758. Elsevier Science B.V., Amsterdam.

Wedemeyer, G.A. (1997). Effects of rearing conditions on the health and physiological quality of fish in intensive culture. In "Fish Stress and Health in Aquaculture". G.K. Iwama, A.D. Pickering, J.P. Sumpter, and C.B. Schreck (eds.). pp. 35-72. Society for Experimental Biology, Seminar Series 62, Cambridge University Press.

Wedemeyer, G.A., Barton,B.A., & McLeay,D.J. (1990). Stress and acclimation. In "Methods for Fish Biology". C.B. Schreck and P.B. Moyle, Eds., pp.451-489. American Fisheries Society, Bethesda, Maryland, USA.

Weld, M.M., and A.H. Meier (1982). Circadian gonadal responses to daily disturbances in gulf killifish. [Abstr.]. Am. Zool., 22:866.

Western Hagen, H.Von, and Rosenthal,H. (1976a). Induced multiple spawning of reard *Siganus oramin* Schneider (= *S.Canaliculatus* Park). Aquaculture, 7(2):193-196.

Westernhagen, H.Von, and Rosenthal,H. (1976b). Some aspects of the suitability of various Philippine Siganid species (Siganidae) for mariculture. Aquaculture, 9, pp.297-311.

Wiik, R., Andersen,K., Uglenes,I. and Egidius,E. (1989). Cortisol-induced increase in susceptibility of Atlantic salmon, *Salmo salar*, to *Vibrio salmonicida*, together with effects on the blood cell pattern. Aquaculture, 83: 201-215.

White, S.A., Bond,C.T., Francis,R.C., Kasten,T.L., Fernald,R.D., and Adelman,J.P. (1994). A second gene for gonadotropin-releasing hormone: cDNA and expression pattern in the brain. Proc. Natl. Acad. Sci. USA 91, 1423-1427.

Whitehead, C. & Bromage,N. (1980). Effects of constant long and short-day photoperiods on the reproductive physiology and spawning of the rainbow trout. J. Endocr., 87, 6-7.

Whitehead, C., Bromage,N., Forster,J. & Matty,A. (1978). The effects of alterations in photoperiod on ovarian development and spawning time in the rainbow trout (*Salmo gairdneri*). Ann. Biol. Anim. Biochem. Biophys., 18, 1035-43.

Woodland, D.J. (1990). Indo-pacific Fishes-No. 19: Revision of the fish family Siganidae with descriptions of two new species and comments on distribution and biology. pp.136. Bernice Pauahi Bishop Museum, Honolulu, Hawaii.

Wootton, R.J. (1984). Functional Biology of Sticklebacks. Croom Helm, London. pp.265.

Yamamoto, K. (1956). Studies on the formation of fish eggs. I. Annual cycle in the development of ovarian eggs in the flounder, *Liopsetta obscura*. J. Fac. Sci. Hokkaido Univ., 12: 362-373.

Yamamoto, K. (1956a). Studies on the formation of fish eggs. VII. The fate of the yolk vesicle in the oocyte of the herring, *Clupea pallasii*, during vitellogenesis. Annotnes. Zool. Jap., 29: 91-96.

Yamamoto, K. (1956b). Studies on the formation of fish eggs. VIII. The fate of the yolk vesicle in the oocyte of the smelt, *Hypomesus japonicus*, during vitellogenesis. Embryologia, 3: 131-138.

Yamamoto, K., Nagahama,Y., and Yamazaki,F. (1966). A method to induce artificial spawning of goldfish all through the year. Bull. Jpn. Soc. Sci. Fish., 32: 977.

Yamamoto, K. and Yamazaki,F. (1961). Rhythm of development in the oocyte of the goldfish, *Carassius auratus*. Bull. Fac. Fish. Hokkaido Univ., 12: 93-110.

Yamamoto, K. and Yamazaki,F. (1967). Hormonal control of spermiation and ovulation in goldfish. Gunma Symp. Endocrino., 4: 131-145.

Yamamoto, T.S. (1955). Morphological and cytochemical studies on oogenesis in the fresh-water fish, medaka, *Oryzias latipes*. Jap. J. Ichthyol., 4: 170-181.

Yamazaki, F. (1965). Endocrinological studies on the reproduction of the female goldfish *Carassius auratus* with special reference to the function of the pituitary gland. Mem. Fac. Fish. Hokkaido Univ., 13: 1-64.

Yaron, Z., Bogomolnaya,A. and Levavi,B. (1984). A calibrated carp pituitary extract as a spawning inducing agent. In: H. Rosenthal and S. Sarig (Editors), Research on Aquaculture. Europ. Maricult. Soc. Spec. Publ. No.8, Breden, Belgium, pp.151-168.

Yaron, Z., Cocos,M., and Salzer,H. (1980). Effects of temperature and photoperiod on ovarian recrudescence in the cyprinid fish *Mirogrex terrae-sanctae*. J. Fish Biol., 16: 371-382.

Yaron, Z., and Levavi-Zermonsky,B. (1986). Flactuations in gonadotropin and ovarian steroids during the annual cycle and spawning of the common carp. Fish Physiol. Biochem., 2 (1-4): 25-34.

Yaron, Z. and Zohar,Y. (1993). An Overview: Applications of comparative endocrinology to fish culture. In "Recent Advances in Aquaculture IV". Edited by J.F. Muir and R.J. Roberts. Blackwell Scientific Publicatons, Oxford. pp.3-10.

Young, G., Adachi,S. and Nagahama,Y. (1986). Role of ovarian thecal and granulosa layers in gonadotropin-induced synthesis of a salmonid maturation-inducing substance (17α,20β-dihydroxy-4-pregnen-3-one). Dev. Biol., 118: 1-18.

Zanuy, S., Carrillo,M. & Ruiz,F. (1986). Delayed gametogenesis and spawning of sea bass (*Dicentrarchus labrax* L.) kept under different photoperiod and temperature regimes. Fish Physiol. Biochem., 2:53-63.

Zohar, Y. (1988). Gonadotropin-releasing hormone in spawning induction in teleosts: basic and applied considerations. In: Reproduction in fish: basic and applied aspects in endocrinology and genetics (eds. Y. Zohar and B. Breton), pp. 269-287. INRA. Paris

Zohar, Y. (1989). Fish reproduction: Its physiology and artificial manipulation. In "Fish Culture in Warm Water Systems: Problems and Trends" (M. Shilo and S. Sarig, Eds.), pp.65-119. CRC Press, Boca Raton, FL.

Zohar, Y. and Billard,R. (1984). Annual and daily changes in plasma gonadotropin and sex steroids in relation to teleost gonad cycles. Trans. Am. Fish. Soc., 113: 444-451.

Zohar, Y. and Gordin,H. (1979). Spawning kinetics in the gilthead sea bream, *Sparus aurata* L., after low doses of human chorionic gonadotropin. J. Fish Biol., 15: 665-670.

Zohar, Y., G. Pagelson, M. Tosky and Y. Finkelman. (1987). GnRH control of gonadotropin secretion, ovulation and spawning in the gilthead seabream *Sparus aurata*. Proceedings of the Third International Symposium on Reproductive Physiology of Fish: 106.

Zohar, Y., Goren,A., Tosky,M., Pagelson,G., Leibovitz,O., Koch,Y. (1989). The bioactivity of gonadotropin releasing hormone and its regulation in the gilthead seabream, *Sparus aurata*: *in vivo* and *in vitro* studies. Fish Physiol. Biochem. 7:59-67.

Appendices

Appendix A - Radioimmunoassay Buffer (Phosphate Buffer Saline).

Sodium Dihydrogen Orthophosphate, dihydrate ($NaH_2PO_4.2H_2O$)..... 7.8 g

Disodium Hydrogen Orthophosphate 12-hydrate ($Na_2HPO_4.12H_2O$)..... 17.91 g

Sodium Chloride (NaCl)..... 9.0 g

Distilled Water..... (to a final volume of).....1000 ml

Mix well to dissolve. To 200 ml of buffer, add 1.0 g of gelatin and allow to stand for a few minutes. Heat in a water bath at 70-80° on a hot plate magnetic stirrer until dissolved completely. Add back to the stock solution and allow to cool. Adjust the pH of the buffer to approximately 7.2, using 1M hydrochloric acid or 0.2M NaOH as required. After adjusting the pH add 1.0 gm of Sodium Azide (NaN_3). Dissolve and store at 4°C.