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ENVIRONMENTAL AND HORMONAL CORRELATES OF GONADAL FUNCTION IN THE FISH *Cyprinion watsoni*

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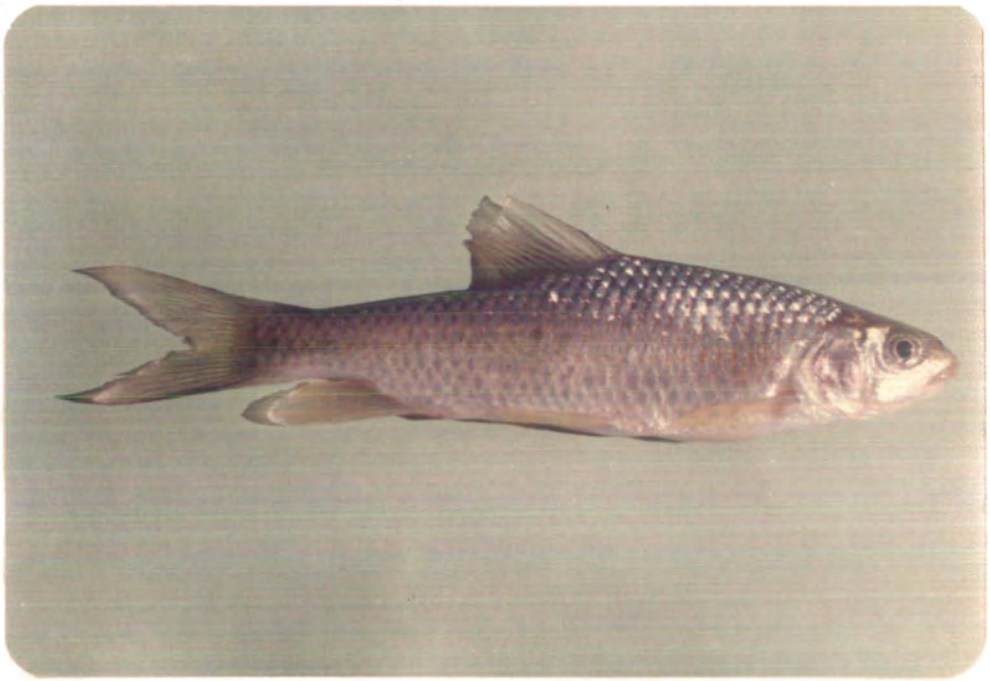

Cyprinion watsoni

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S. A. Shaikh

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ABSTRACT

Studies were conducted to assess role of photoperiod and temperature in regulation of gonadal development and function during annual reproductive cycle in the fish, *Cyprinion watsoni* (Family: Cyprinidae). In addition, to seasonal variations in gonadal steroids and changes following experimental exposure of fish to photoperiod-temperature regimes were studied. Also selected stages of ovarian follicles were examined with the electron microscope in an attempt to relate environmental and hormonal events in the light of morphological features of the follicles.

The perinucleolus stage follicle lacks definitive theca and granulosa. A vitelline envelope makes its appearance first in late perinucleolus stage evidently through deposition of material from the prefollicular cells surrounding the oocyte. Cortical alveoli characterize the cortical alveolar stage follicles which too lack definitive theca and granulosa layers. The prefollicular cells appear active in formation of zona radiata interna and externa. The presence of certain special cells which appear physiologically more active than other cells in prefollicular envelope has been noted in the perinucleolus and cortical alveolar stage follicles. Theca and granulosa and a fully developed vitelline envelope with three distinct layers are first seen in the yolk stage follicles. The organization of follicles

and subcellular features of the theca and the granulosa cells have been discussed in relation to steroidogenesis.

In order to Study effects of photoperiod and temperature the fish were exposed in the laboratory to four photoperiod-temperature regimes, namely, long photoperiod-warm and low temperature (15L/9D:25^{o}C or 15^{o}C), short photoperiod-warm or low temperature (9L/15D:25^{o}C or 15^{o}C) in preparatory, prespawning, early spawning and postspawning seasons. The results show that a long photoperiod-warm temperature combination is essential for causing gonadal recrudescence in the preparatory and prespawning seasons. Short photoperiod and warm temperature cause gonadal regression in the preparatory season whereas short photoperiod and low temperature retard gametogenesis in the prespawning season. In the spawning season, warm temperature alone regardless of photoperiod stimulates spawning. Low temperature in either photoperiod supports vitellogenesis in the females and checks spawning in the male. Low temperature-long photoperiod and low temperature-short photoperiod are important in initiating gonadal recrudescence in the female and the male respectively in postspawning season. The gonadal response, thus, varies with the seasonal history of the fish. Both photoperiod and temperature are important in controlling gonadal function in *Cyprinion watsoni*.

It was possible in this study to measure only three gonadal steroids; estradiol and progesterone in the female and testosterone in the male. Their levels varied seasonally. These steroids in the gonads increased to peak values in

April (early spawning period) before declining to basal levels in the postspawning period. The levels of testosterone paralleled the variations in the testis but plasma progesterone continued to rise in the postspawning season (June) before finally declining to basal level. In the preparatory season, ovarian estradiol and progesterone increased only in the long photoperiod-warm temperature regime. Testosterone in the testis increased in long photoperiod at both warm and low temperature. In the prespawning season, ovarian estradiol increased again in the long photoperiod-warm temperature regime, progesterone increased at both warm and low temperature in long photoperiod. Testicular testosterone increased maximally in short photoperiod. In the postspawning season, highest levels of estradiol were observed in the long photoperiod-warm temperature condition. Progesterone increased but insignificantly in long and short photoperiod at both temperatures. Testosterone increase in all experimental groups with the greatest increase occurring in the long photoperiod-low temperature regime. The observations on hormone profiles have been examined in the light of information available for other species of fish, in relation to gonadal stages, other biologically active gonadal steroids and the morphological details of the ovarian follicles gleaned in the present study.

INTRODUCTION

Reproductive functions and annual maturational cycles in fishes are governed by a variety of external and internal factors. These encompass environmental and hormonal regulatory agencies. The environmental factors trigger hormonal outputs at the hypothalamic, hypophyseal and gonadal levels. In this scheme of relationships, a variety of environmental parameters have been recognized to be of importance, particular hypothalamic hormones and at the pituitary level not only gonadotropins but other trophic hormones are implicated. The gonads respond by producing various steroid hormones which guide local developmental events as well as peripheral physiological and behavioral processes. Earlier literature in the field is exhaustive and has been reviewed by de Vlaming (1974). More recent information has been critically analysed by Liley (1980), Baggerman (1980), Peter (1981, 1983), Lam (1983), Lam and Munro (1987), de Vlaming (1983), Fostier *et al.*, (1983) and Bye (1987). The present program of work was envisaged in the context of the current understanding of reproductive mechanisms known in the literature and the fact that hardly any work has been done in Pakistan to study reproductive adaptions of local species. The attempt here has been made to relate selected environmental factors with accompanying changes in gonadal steroid hormones in a fresh water fish.

Cyprinion watsoni. This species belongs to the family Cyprinidae and its distribution is restricted to the northern areas of Pakistan, parts of Afghanistan, Iran, Syria. and Eastern part of the Arabian peninsula (Jaya Ram, 1981). Shaikh (1986) has studied the annual reproductive cycle of this species.

Several environmental cues, both proximate and ultimate impinge in timing gonadal developmental events and breeding seasonality in fishes (de Vlaming, 1972, 1974, 1975, 1983; Scott, 1979; Baggerman, 1980; Lam, 1983; Lam and Munro, 1987). Their effects vary according to geographical distribution and within given regions on species-specific basis. Of the various proximate factors, photoperiod and temperature are considered to be the most important synchronizers of gonadal development and spawning activities of fishes at least in the temperate regions of the world (de Vlaming, 1974, 1983; Scott and Canario, 1987; Lam, 1983; Lam and Munro, 1987). Since most work in the field of reproductive biology of fishes has been done by workers in this region, these two factors have also been the most extensively studied. Photoperiod and temperature are thought to be of lesser importance for species resident in subtemperate and subtropical areas. In the tropics, photoperiodic variations are minimal and rainfall appears to be a more dominant factor in determining breeding periodicities (Scott, 1979; de Vlaming, 1983; Lam, 1983). Whether both photoperiod and temperature or either one of these act as key factors in timing reproductive events in fishes also varies with

geographical location and species. Thus, it was important in the present program of study on *Cyprinion watsoni* to examine the relative importance of photoperiod and temperature in the control of gonadal development and seasonal breeding rhythm and to determine how the accompanying changes in selected gonadal steroid hormones are related with the gonadal responses.

Gametogenic progress and even regressive events in many temperate, subtemperate and subtropical species have been shown to depend on photoperiod and temperature. At one end of the spectrum photoperiod seems to exert principal influence on the gonads and breeding patterns as has been demonstrated for salmonids (Henderson, 1963, Breton and Billard, 1977; MacQuire *et al.*, 1977) and in the gasterosteids (Schneider, 1969, Baggerman, 1980). Long photoperiod together with warm temperature appears important in those temperate species which breed in spring or early summer (Harrington, 1957; Baggerman, 1980; Kaya and Hasler, 1972; de Vlaming, 1975; Gillet *et al.*, 1978). Short photoperiod triggers gonadal recrudescence in species, especially salmonids, which breed in autumn or winter (Breton and Billard, 1977; Billard *et al.*, 1981) although this has not always been found to be true by other investigators (Bromage *et al.*, 1982; Skarphedinsson *et al.*, 1982). While in earlier studies not much attention was given to temperature, it has now turned out that it interacts with photoperiod and thus has an influence on gonadal function. This appears to be particularly true in the case of cyprinids (de Vlaming,

1975). At the other end of the spectrum, temperature dominates in controlling reproductive periodicities in certain species particularly those belonging to the cyprinodontiform group (de Vlaming, 1975). In the cyprinid, *Couessius plumbeus* (Ahsan, 1966), temperature alone is important with hardly any effect of photoperiod on spermatogenesis. On the other hand, in such subtropical species as *Heteropnuestes fossilis* both photoperiod and temperature interact, the latter seems to have a predominant role (Sundraraj and Vasal, 1976). This also holds true for *Mystus tengara* (Guraya *et al*, 1976), *Cirrhina reba* (Verghese, 1975) and *Cyprinus carpio* (Davies and Hanyu, 1986; Davies *et al.*, 1986). It is also noteworthy that responsiveness of various species to photoperiod and temperature in laboratory conditions may vary depending on their initial gonadal condition i.e. depending on the season in which the fish are exposed to given environmental regimes. With the above background in view, it was proposed in the present work to expose *Cyprinion watsoni* in selected seasons of the year to various combinations of photoperiod and temperature in order to assess the relative importance of these two factors in the control of gonadal function and development.

The gonads are responsive to environmental influences via the hypothalamo-hypophyseal axis although a direct influence of such factors as temperature on ovarian and testicular steroid metabolism has not been ruled out (Ahsan, 1966; de Vlaming, 1972; Harrington, 1959; Hubbs and Strawn, 1957; Weibe, 1968). Under the influence of centrally

mediated environmental effects the gonads pass through a sequence of developmental events, in particular those involving gametogenic progress and final maturation. In the female these culminate in ovulation and in the male in spermiation. Gonadal steroids produced in the ovary and the testis guide progress of specific local events and also are responsible for appearance of secondary sexual features and particular behavioral manifestations. Thus, production of particular steroids their tissue and cellular sources, temporal relationships and cyclic variations are of profound interest to reproductive biologists. Fostier *et al* (1983) have provided a comprehensive review of the gonadal steroids, their biosynthesis and relationships with gonadal development and sexual cycles (see also Kime, 1979, 1980; Fostier *et al.*, 1987; Scott and Canario, 1987; Goetz, 1983). Seasonal variations in biologically active steroids during annual reproductive cycles have been extensively studied (Horvath, 1978; Kime, 1979; Campbell *et al.*, 1980; ven Bohemen, 1980; Godovich *et al.*, 1982; Bromage *et al.*, 1982; Scott *et al.*, 1983; Kime and Haider, 1983; Manning and Kime,1985; Kime and Manning,1986; Zermonsky and Yaron,1986; Fitzpatrick *et al.*, 1986; Liley *et al.*, 1986; Rosenblum *et al.*, 1987; Scott and Canario, 1987 and Scott and Sumpter, 1989). Considerable understanding of the timing of production of given ovarian and testicular steroids and their role in gonadal developmental stages has been achieved but in view of species differences no generalizations have yet been possible. While a vast body of information has become available on the

steroid hormones and their biological significance in various species, neither the relationship of trophic factors with specific ovarian and testicular stages nor the sites of steroid hormone synthesis have yet been fully resolved (van den Hurk and Peute, 1979; Lang, 1981; Kagawa *et al.*, 1981; Nagahama, 1983; Asahina *et al.*, 1985; Pudney and Callard, 1984; Selman and Wallace, 1986; Wallace *et al.*, 1987). Both histochemical and ultrastructure methodologies have been considered fruitful in this context and have been applied in attempts for a better understanding of these aspects (Kagawa *et at.*, 1981; van den Hurk and Peute, 1979 and Lang, 1981). One of the constraints in relating steroid hormones with sites of synthesis and timing of appearance of particular steroids has been lack of resolution of morphological and cellular details of ovarian and testicular stages at the ultrastructural level. In the present work, therefore, it was planned to study structure of selected ovarian follicles with the electron microscope. It was hoped that information thus obtained would be of use in relating the environmental and hormonal events with morphological and cellular features of the ovarian follicles. Although it would have been equally desirable to examine the testes too at the ultrastructural level, both the time constraint and available resources did not permit this. In regard to the endocrine mechanisms controlling reproduction and breeding cycles, no work has so far been done on any of the local species of fishes, fresh water or marine. Such information is essential in order to gain a better understanding of regulation of reproductive

functions in the context of regional environmental variations. The potential value of this basic knowledge in managment of local fish resource and application in fish culture programmes is quite obvious. Therefore, it was decided to determine profiles of estradiol and progesterone in the female and testosterone in the male *Cyprinion watsoni* in selected months of the year as well as following exposure of fish to various photoperiod and temperature regimes.

In this work, the ultrastructural details of selected stages of ovarian follicles have been presented first. This is followed by results of experiments designed to determine the role of photoperiod and temperature in gonadal development. Finally, data are presented on seasonal profiles of estradiol, progesterone and testosterone as well as on changes in their profiles in relation to experimentally imposed photoperiod-temperature regimes.

MATERIALS AND METHODS

Cyprinion watsoni is a small (max. size 12 cm) cyprinid fish which is commonly found in the hill streams of Islamabad (33.3°N, 73.0°E) where surface water temperature ranges during the year between 14°C in the coldest month and 29°C in the warmest months. According to an earlier study (Shaikh, 1986), the reproductive cycle of this species comprises a spawning season between March and May (Spring to early summer) followed by a postspawning period extending between June and August. This is followed by a quiescent period lasting from September to November. Gonadal recrudescence begins in December (preparatory period) when the gonads provide first signs of proliferative activity. The fish enter a prespawning phase during January to March when gametogenic progress becomes pronounced and the fish ultimately reach the final stage of preparedness (testes predominantly contain spermatozoa and ovaries ripe ova) to start spawning in March/April and May.

Fish Collection:

Samples of *Cyprinion watsoni* were collected with cast nets in four different periods of the annual reproductive cycle, namely, preparatory (December), prespawning (January - February), early spawning (April) and postspawning (June). The fish were transported live to the experimental fish laboratory of the Department of Biological Sciences and routinely stocked for acclimation to laboratory conditions in

a large indoor cement tank measuring (400 x 104 x 71 cm). Photoperiod and temperature in the laboratory varied according to ambient conditions. The fish were held in this tank for at least one week before starting the experiments designed to study the effects of controlled photoperiod and temperature regimes on gonadal maturation and gonadal steroid hormones.

Experimental Schedule:

In each of the four above mentioned seasons 20 fish from the stock tank were transferred to individual experimental tanks (75 liter glass aquaria) in a ratio of 10 males and 10 females. Four separate combinations of photoperiod and temperature were used in each season. These were,(1) Long photoperiod and warm temperature (15L/9D:25^{o}C), (2) Long photoperiod and low temperature(15L/9D:15^{o}C), (3) Short photoperiod and warm temperature (9L/15D:25^{o}C) and (4) Short photoperiod and low temperature (9L/15D:15^{o}C). The selected photoperiod and temperature fall within the range of annual variations in this region (Fig. 1). All experimental tanks were isolated from room light by housing them in a combination of environmental chambers (temperature regulated) or in chambers screened with light proof black plastic. Water temperature in the experimental tanks was maintained by means of thermostatically controlled aquarium heaters and/or by adjusting the temperature of the environmental chambers (walk in cold room and a Fisher incubator with a temperature range of 0-50^{o}C). Photoperiod regimes were achieved by means of timer clocks which automatically switched on fluorescent tubes placed about 60 cm above the individual tanks according to a preset

schedule of long and short daylength. Light intensity measured at water surface in individual tanks was 600 Lux. All tanks were provided with aged water (dechlorinated) and were continuously aerated with air pumps. The experimental fish were fed to satiation twice daily. All experiments lasted 30 days.

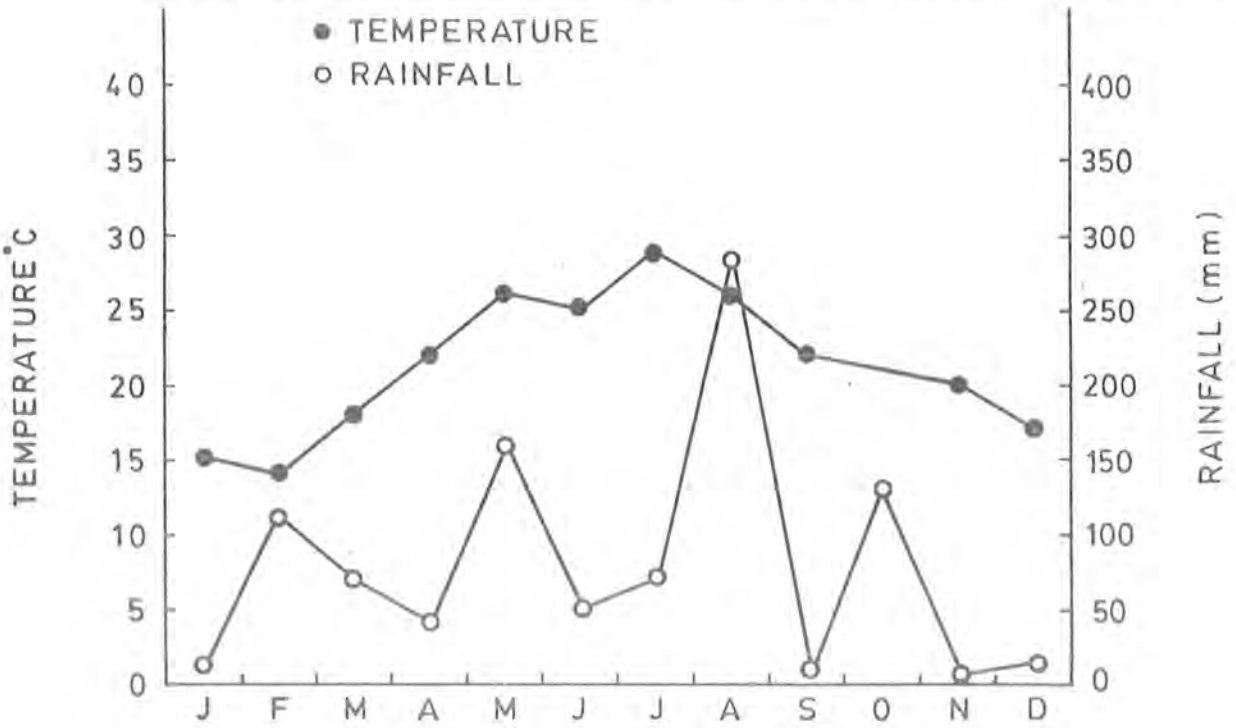


Fig.1: Seasonal variation in average rainfall in the Islamabad area and surface water temperature in Margala streams.

Gonadosomatic Index and histology:

At the end of the experimental period, the fish were lightly anesthetized (with MS 222) weighed immediately on a Metller electrobalance. A random sample of fish was taken on the day of collection in the four selected seasons and processed similarly to serve as the seasonal control group (IC). The body weight of fish ranged during the various

seasons between 6 to 22 grams. The gonads of the fish were dissected out following sacrifice, weighed to the nearest mg and immersed immediately in aqueous Bouin's fluid for 24-48 hours for histological examination. Records of body weight and gonadal weight were used to determine gonadosomatic index (GSI) which was calculated as $\frac{\text{gonad weight}}{\text{body weight}} \times 100$. These two ctriteria (GSI and histology) served to assess the gonadal status of the seasonal controls and the experimental fish.

Histological Procedures:

The gonads (ovaries and testes), fixed in aqueous Bouin's, were rinsed briefly in tap water and dehydrated in ascending ethanol series, cleared in Benzene and embedded in paraffin wax. Tissue blocks were sectioned with a Cambridge Microtome at a thickness of 6-8 μ. The sections were affixed to precleaned albuminized glass slides and stained with hematoxylin and eosin. Microscopic examination was carried out under a research microscope (Optihot Research Microscope, Olympus).

Histological details and morphometric data, in combination with macroscopic features of the gonads were used to determine the stages of development of testes and ovaries in the control and the experimental groups. Measurements of oocyte diameters were made by a precalibrated ocular micrometer in order to obtain their mean size. The annual testicular and ovarian rhythms based on these criteria are shown below:

Stages of annual ovarian rhythm in *Cyprinion watsoni*

Stage	Histological features
O	Ovaries regressed, collapsed, containing many atretic follicles.
I	Recovering ovary with oogonial nests, non yolky oocytes lacking distinction between theca and granulosa.
II	Preparatory phase with oocytes in cortical alveolar and early vitellogenic stages,diameter 90-150 μm.
III	Oocytes in advanced stage of vitellogenesis, yolk granules throughout, diameter 150-500 μm.
IV	Ripe ovary, yolk deposition nearly complete,diameter of oocytes greater than 500 μm.
V	Spent ovaries,collapsed,postovulatory follicles are common, internal hemorrhage atretic follicles also present.

Stages of annual testicular rhythm of *Cyprinion watsoni*

Stage	Histological features
0	Regressed,testicular lobules with nests of degenerating cells,nuclei pycnotic.
1	Testis quiescent,germinal epithelium contains mainly primary spermatogonia and residual spermatozoa
2	Testis showing recrudescence with numerous secondary spermatogonia
3	Testis with germinal epithelium containing spermatogonia, primary and secondary serpmatocytes
4	Spermiogenic phase, few spermatocytes dominated by spermatids and spermatozoa
5	Ready to spawn stage, spermatids present but testis distended with spermatozoa
6	Testis spent,postspawning phase with testicular lobules shrunken containing few germ cells and residual spermatozoa

Electron microscopy:

The ovarian follicle and their seasonal developmental progress were studied not only light microscopically but also with the electron microscope. For this purpose small portions of the ovarian tissue were taken from fish on the day of collection (autopsies of seasonal controls described in the earlier section). The tissues were immersed immediately for 2 hours in 2% cold Glutaraldehyde prepared in 0.1M phosphate buffer (pH 7.2). These were rinsed thrice in buffer and postfixed in 1% cold Osmiun tetraoxide for 30 minutes to 1 hour. The osmicated tissues were dehydrated in acetone and embedded in Durcupan according to standard procedures. Semithin and ultrathin sections were made with glass knives on a LKB ultratome V. Semithin sections were stained with 1% Toluidene blue. The ultrathin sections were transferred to 150 or 200 mesh copper grids. These were contrasted using uranyl acetate and Reynold's solution. The sections were examined and photographed on a Joel Sx-100 Transmission Electron Microscope.

Gonadal steroids:

It was proposed to obtain plasma and tissue profiles of estradiol and progesterone in the female and testosterone in the male *Cyprinion watsoni* collected in the four selected phases of the reproductive cycle as well as in fish subjected to the various combinations of photoperiod and temperature regimes described earlier. At the time of sacrifice of the fish (seasonal control, experimental fish), blood from immobilized individual fish was collected from the caudal

vein in heparinized hematocrit capillaries.Blood of several males and females was separately pooled for assays of steroid hormones. Plasma was separated by centrifugation at 5000 RPM for 10 minutes using a clinical centrifuge, transferred to glass vials, sealed and saved at -20^{o}C for Radioimmunoassay (RIA). Samples of ovaries and testes were saved at the time of autopsies of seasonal controls and experimental fish (See earlier section). While one of the pair of ovaries and testes from individual fish was saved for histological work, the other was saved and frozen for hormone analysis by RIA.

Radioimmunoassay procedures:

Weighed portions of ovaries and testes (10 to 30 mg) were homogenized in 1.0 ml of 0.65% saline. A 500 µl aliquot of plasma or tissue homogenate (ovary or testes) was extracted in 5 ml reagent grade diethyl ether and dried in a water bath at 60^{o}C. The dried extracts were reconstituted with 2.0 ml of assay buffer/(0.1M phosphate buffer, 0.9% Nacl, 0.17% gelatin and 0.1% sodium azide; pH 7.2). The extracts were analysed for estradiol, progesterone (Arslan *et al.*, 1978) and testosterone (Nieschlag and Loriaux, 1972) by radioimmunoassay using specific antisera. The antibody was used at a dilution of 1:50,000. A mixture of diluted antibody and 2,4,6,7-^{3}H, estradiol; 1,2,6,7-^{3}H, progesterone and testosterone (New England Nuclear Corporation) which gave 40-48 % binding of the tritiated steroid (200 µl) was incubated with 500 µl of the sample or standard for 18-24 hours at 4^{o}C. After incubation, the tubes were placed in ice and 200 µl of dextran coated charcoal (0.625 % charcoal,

0.0625% dextran in assay buffer) was added and the tubes were kept for 30 minutes at 4°C. The incubation mixture was centrifuged at 3000 rpm for 10 minutes, the supernatant was transferred to scintillation vials containing 5 ml of the scintillation fluid (0.5% permablend-III containing 5.0 g PPO and 0.5 g bis-MSB, Packard International, Zurich, Switzerland) and radioactivity was counted on a Beckman LS 1801 scintillation counter.

The results of radioimmunoassay (RIA) were calculated according to the procedure described by Roadbard and Lewald (1970). All determinations were made in duplicate . In these assays the minimum detectable dose on the standard curve was 10 pg for progesterone and estradiol and 12.5 pg for testosterone. Reovery of labelled steroid standards added to tissue homogenates was 87-91% . The intra and interassay coefficients of variation were, respectively 6% and 11% for estradiol, 7% and 13% for progesterone and 5% and 11% for testosterone.

Statistical Analysis:

The data were subjected to one way analysis of variance (ANOVA) and the level of significance was determined by Duncan's multiple range test (DMRT) (Steel and Torrie, 1960).

RESULTS

Ultrastructure of Ovarian Follicles:

Morphological changes in ovarian follicles of *Cyprinion watsoni* were examined at the ultrastructural level using previtellogenic and vitellogenic stages of the oocytes. The noteworthy details of selected stages are as follows:

Previtellogenic follicle (Perinucleolus stage):

The oocyte in this stage has a central germinal vesicle with several nucleoli and is surrounded by electron dark prefollicular connective tissue cells. Neither theca nor granulosa cells can be distinguished at this stage. The prefollicular cells are arranged in two layers. Both layers contain flattened and elongate cells (Fig.2). A thick basement membrane separates the two layers of cells. The outer layer, thickened in places, is continuous with the interstitial tissue. In these thickened areas are found collagen fibers and fibroblasts but Particularly noteworthy are large active cells with oval to oblong nuclei. These latter cells characteristically appear vacuolated owing to presence of small to large cisternae of endoplasmic reticulum (Fig.3). Mitochondria, occasional granules and lysosomes are also seen in their cytoplasmic compartment. Blood vessels are common in the outer prefollicular connective tissue layer.

The inner cell layer lies next to the oocyte surface. The dark cells in this layer too have oblong to very elongate nuclei and are similar to those in the outer layer. The membranes of these cells are intimately apposed. These features of the cells are best seen in the older oocytes of this stage in Fig.5 where their inner surface is seen to send cytoplasmic extensions towards the oocyte surface. The surface of the young perinucleolar stage oocytes is smooth Fig.2 but develops numerous microvilli as development progresses. These microvilli make contact with the inner layer of the prefollicular cells which are seen in Fig.2 to lie at some distance due to artefactual (shrinkage) changes in the organization of the oocyte.

A vitelline envelope, absent in young oocytes devoid of microvillar extensions (Fig.2), first appears to be laid down in older perinucleolar stage oocyte between the oocyte microvilli (Fig.4) as dense amorphous material. The relationship between the inner layer of prefollicular cells, their processes and the microvilli (Fig.4) suggests that this amorphous material is deposited by the activity of the prefollicular cells. Since the material of the vitelline envelope is deposited around the microvilli, channels or pore canals are formed through which the microvilli of the oocyte communicate with the overlying extracellular space. The ooplasm of the oocyte contains scattered mitochondria. Rough endoplasmic reticulum is prominent but Golgi complexes are not readily discernible.

Early "vitellogenic" (Cortical alveolar stage):

This stage is marked by appearance of cortical alveoli ("Yolk" vesicles) in the ooplasm which demarcate a period of endogenous synthesis of yolk. The prefollicular connective tissue layers persist with little change and their relationships are best seen in Figs.5 and 6. The most notable change pertains to the vitelline envelope which has now increased in thickness and has assumed the features of zona radiata. It has a palisade-like arrangement of dense material alternating with the microvilli of the oocyte (Figs.5,6). The microvilli can be seen to emerge from the pore canals into the extracellular space where they make contact with the inner surface of the prefollicular cells (Figs.5,6). The prefollicular cells are arranged end to end and lateral cytoplasmic extensions of adjacent cells are intricately interlaced.From their inner surface arise cytoplasmic processes which reach out towards the zona radiata and are often seen to enter the pore canals through which also course the microvilli emanating from the oocyte surface (Fig.6). The basement membrane separating the inner from the outer layer of prefollicular cells (Figs,5,6) consists of sheets of finely granulated fibers stacked one on top of the other. Granular material and sheets of collagen fibers from the outer prefollicular zone seem to be continuously added to the basement membrane (Fig.6). Whether there is uptake of this granular material across the basement memberane into the inner prefollicular cells cannot be judged fully. The general impression one gets from the disposition of the inner prefollicular cells is that they are in a state of transformation and are closely associated with the underlying

zona radiata of the vitelline envelope. An examination of vitellogenic follicles tends to support this observation (See below, next section).

The ooplasm of the cortical alveolar stage oocytes is richly supplied with cylindrical mitochondria (Figs.5,7). Rough endoplasmic reticulum is abundant too. Smooth endoplasmic reticulum is not visible. Smooth surfaced vesicles and vacuolar profiles containing a flocculent or finely granular material are frequently seen throughout the ooplasm (Fig.7) including the cortical region. In places, these occur in clusters particularly adjacent to the germinal vesicle and are either derived from endoplasmic reticulum or represent enlargements of saccules of Golgi complexes.

Vitellogenic follicle (Yolk stage oocytes):

Already in early to mid yolk stage follicles, when the exogenous yolk deposition process is well in progress the follicle seems to have assumed a definitive organization (Fig.8) especially in regard to the vitelline envelope and follicular investments. The ooplasm now shows accumulation of electron dense yolk granules which vary in shape from round to angular. The most striking change again concerns organization of the vitelline envelope and differentiation of definitive granulosa and theca layers, evidently from the outer prefollicular cell layer and/or interstitial connective tissue.

The vitelline envelope now consist of three distinct layers, the innermost of which (zona radiata interna) had partially formed in the early vitellogenic follicles but has

further modified in its details in association with new additions on its outer surface (Fig.8). While the zona radiata interna, comprises variously oriented plaques of electron-dark amorphous material, the middle of the three layers is ornamented by nearly concentrically organized sheets of dense ground material (Figs.8,9). The outermost layer of the vitelline envelope forms a relatively thin sheet of dense material next to the granulosa layer. The two outer layers constitute the zona radiata externa (or zona pellucida, Guraya, 1976) of the vitelline envelope. The relationships of the cellular and acellular investments of the cortical alveolar stage follicles and the organization of the definitive vitelline envelope in the yolk stage oocytes suggest that the two components of the zona radiata externa arise by incorporation of the basement membrane demarcating the inner and outer prefollicular cells as well as the latter cells. The middle ornamented layer and part of the zona radiata interna would thus seem to arise from material of the inner prefollicular cells. Figure 10 shows the various layers surrounding a cortical alveolar stage oocyte. The appearance of the cells, lamination in their cytoplasm and their relationship with zona radiata tend to further support the argument. A comparison of investing layers of the oocyte in Figures 8 and 10 adds strength to this assertion. It is noteworthy that the spacing of the pore canals is also wider in the yolk stage (Figs.8,10) and this is likely to have resulted from further incorporation of material from the transforming cells. The pore canals run across the entire vitelline envelope and contain a pair of microvilli (Figs.8,9,11).

Figure 8 shows that granulosa and theca layers are for the first time recognizable in the yolk stage follicles. They are separated by thin basal lamina and are strikingly moreelectron-light than the cells of the prefollicular investment seen in the perinucleolar and cortical alveolar follicles. The granulosa and the theca would seem to have differentiated from the outer prefollicular layer and/or interstitial tissue compartment (See also Fig.10).

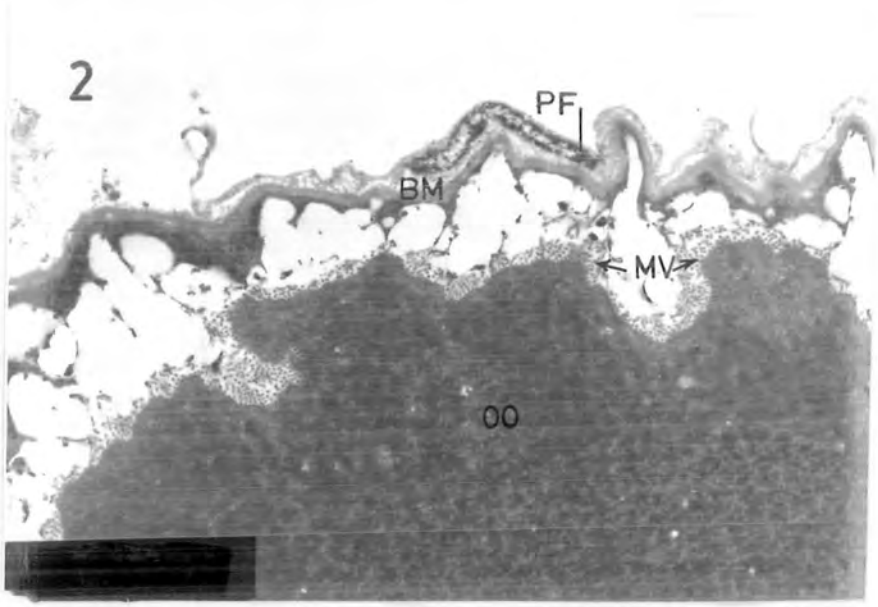
The granulosa cells possess, round, oval or oblong and sometimes indented nuclei with a prominent nucleolus (Fig.8). The chromatin is evenly dispersed. These cells possess abundant cytoplasm. Cylindrical mitochondria (Figs.9,11) are scattered throughout but particularly in the vicinity of the nucleus. Lysosomes are also noted. Rough endoplasmic reticulum is present but is not very abundant (Figs.9,12,13). It is dilated in places and is frequently enlarged into cisternae (Figs.5,8,11) which give a highly vacuolated appearance to the cells and the entire granulosa layer. Smooth endoplasmic reticulum is not discernible. Golgi bodies are common not only in the vicinity of the nucleus but also at variable distances from it and are associated with small empty or dense-core vesicles (Figs.9,13). Such vesicles are also visible adjacent to the plasma membrane facing the extracellular space next to the vitelline envelope as well as subjacent to the basal lamina on the thecal side (Figs.8,9,12). The inner surface of the granulosa cells is folded and interdigitates with the microvilli reaching it from the oocyte surface (Figs.8,9). Although the pore canals often show a pair of microvilli coursing through it and exhibiting

different electron densities (Figs.8,9) suggesting that microvilli of the granulosa cells also exist, no direct evidence for this could be discerned here. The outer surface of the granulosa cells is closely apposed to the basal lamina separating the granulosa from the theca layer. Adjacent granulosa cells make intimate end to end contacts (Figs.5,8,11). Intercellular spaces are rarely seen. The intracellular cisternae are frequently seen to contain profiles with membrane whorls and granular material (Figs.8,9,12).

The theca cells are flatter and more elongated with usually oblong darker nuclei characterized by dense marginal aggregations of chromatin and one or two nucleoli (Figs.8,11). The cytoplasmic compartment of these cells spreads extensively forming interlacing intercellular contacts. Mitochondria and rough endoplasmic reticulum are present (Figs.11,12). A Golgi complex could not be seen readily in these cells. The theca layer is permeated by capillaries and blood vessles (Fig.8), and collagen fibers are abundant in the extracellular spaces especially near the basal lamina (Fig.12).

Effect of photoperiod and temperature on gonadal maturation:

The annual reproductive rhythm of *Cyprinion watsoni* has been briefly summarised in the section on methods. Figure 14 shows the seasonal pattern of change in mean GSI based on collections made in selected months of the year (1988-89).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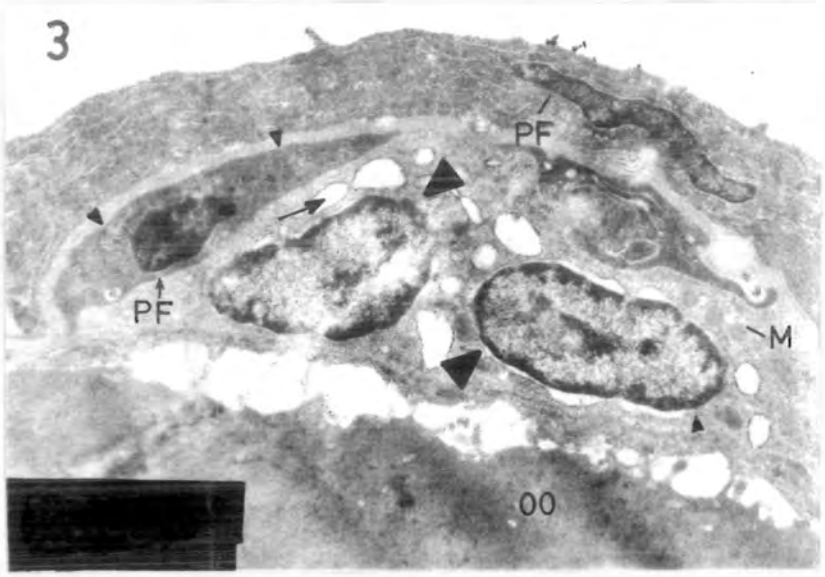
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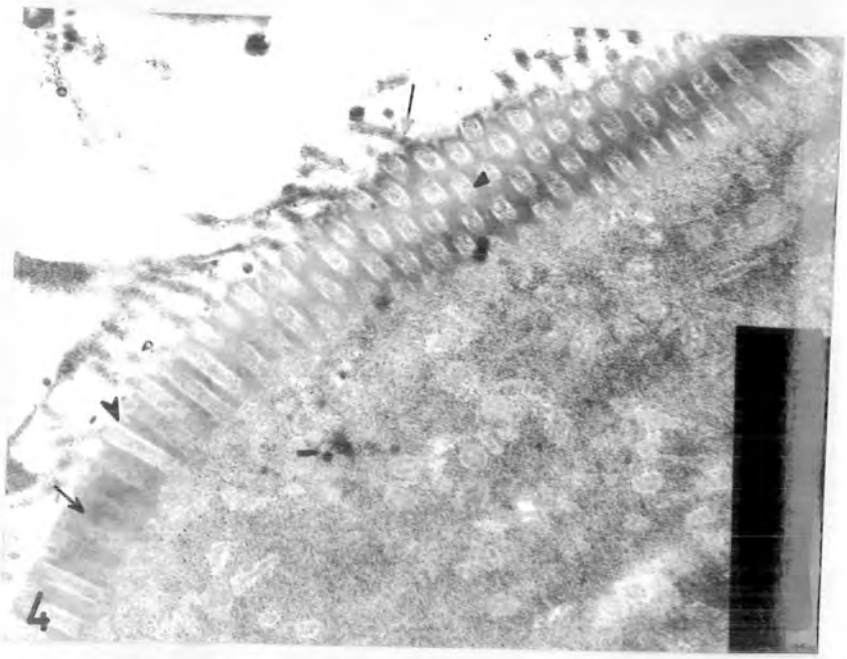
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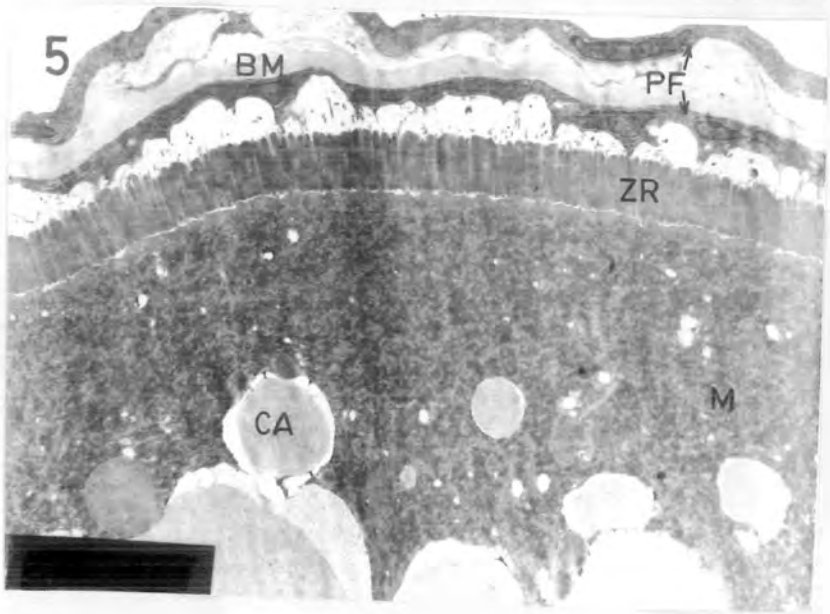
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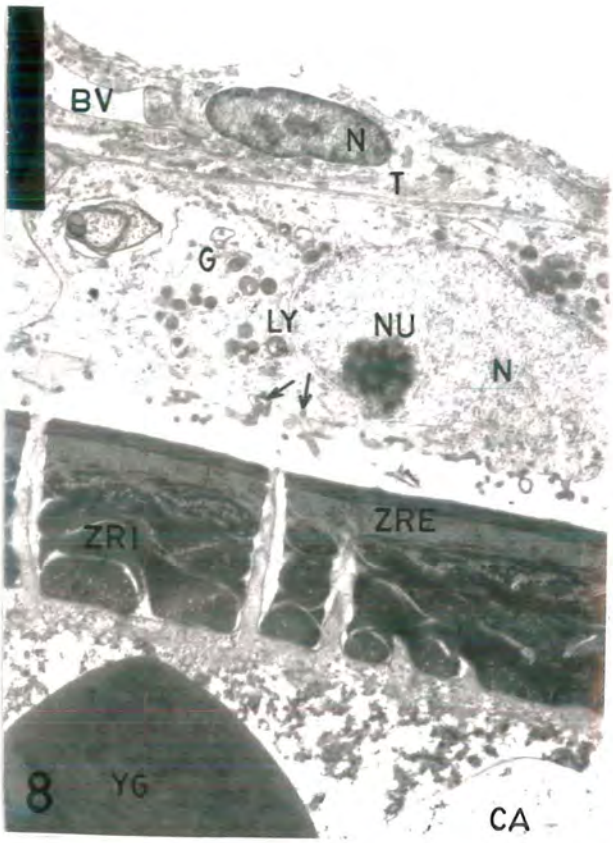
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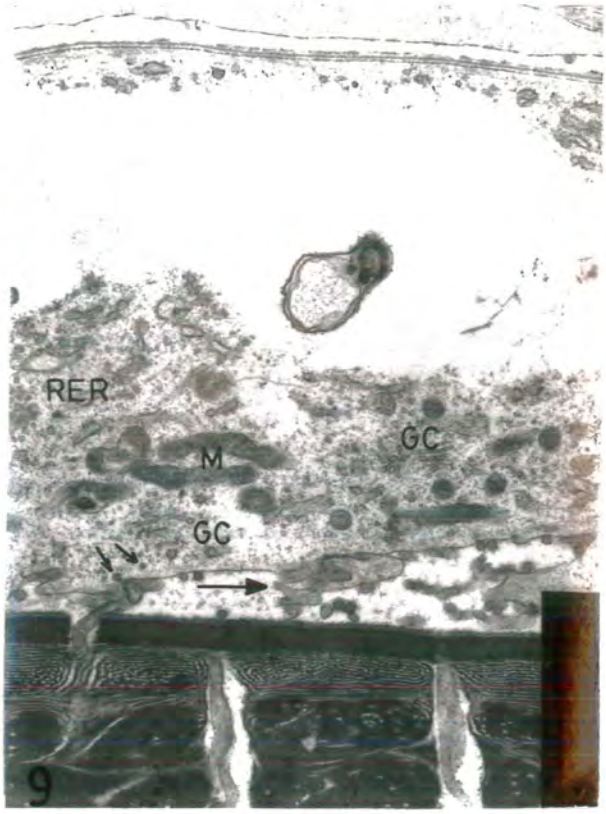
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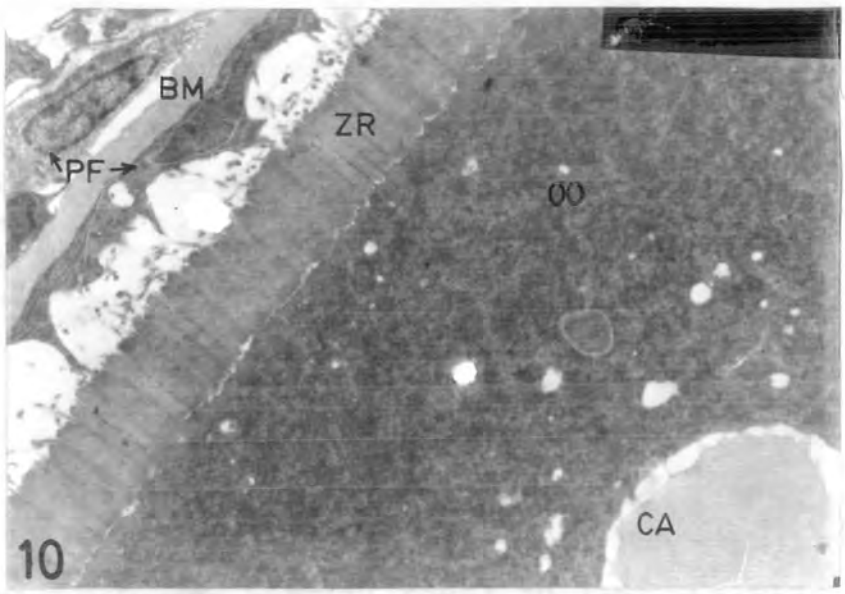
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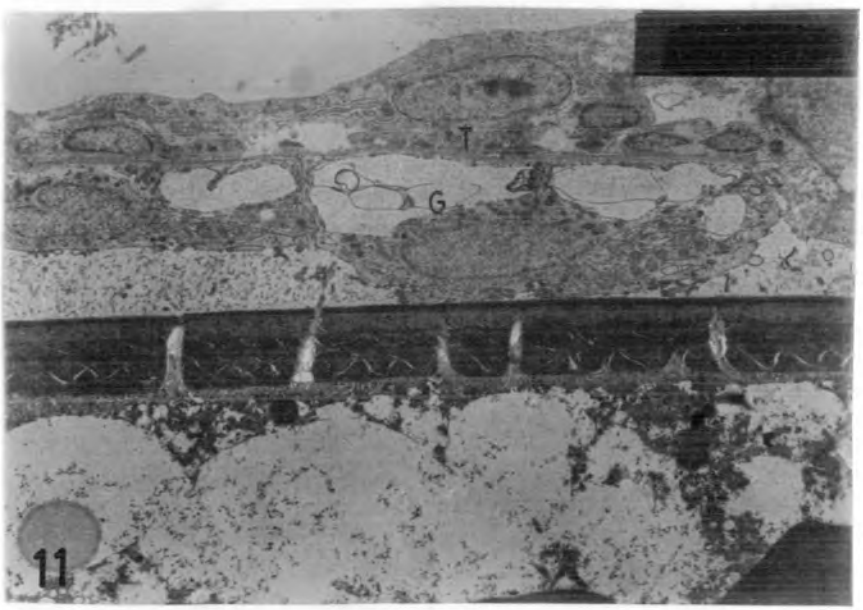
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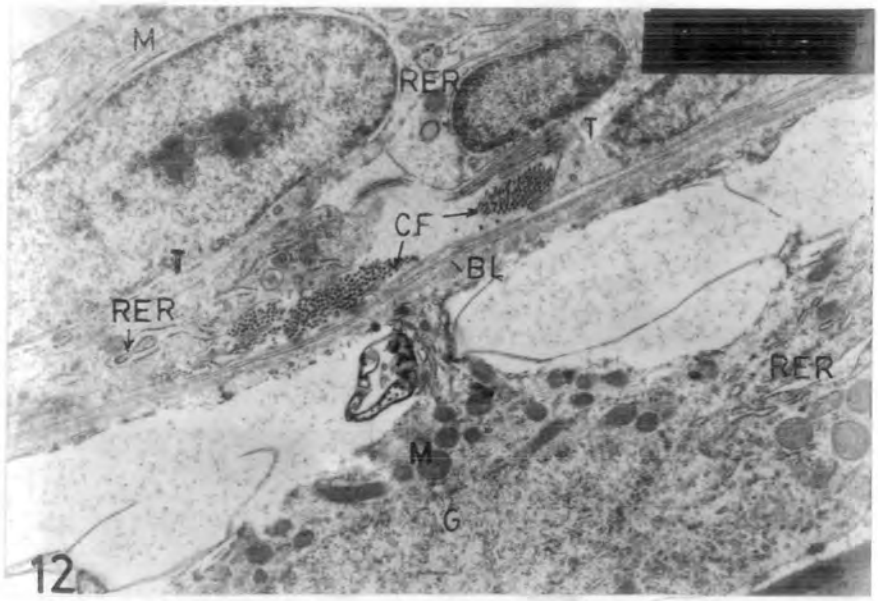

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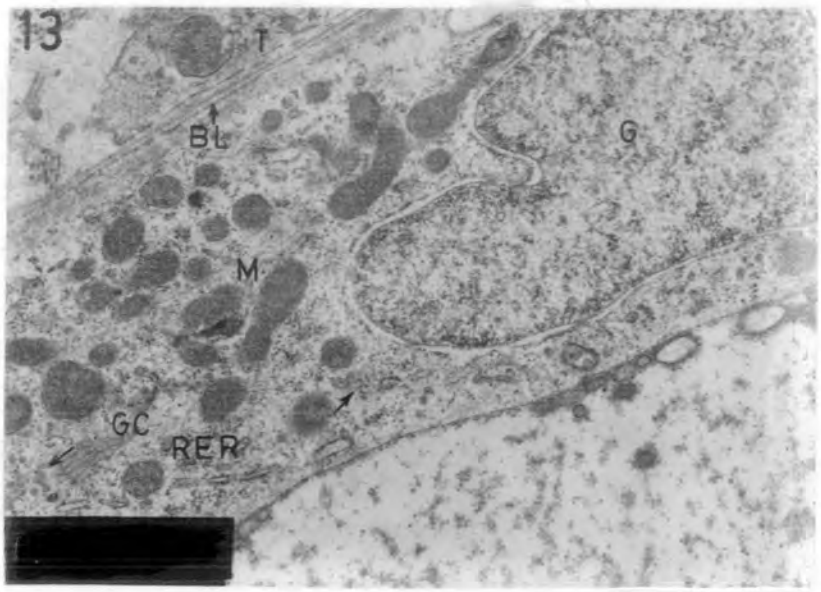

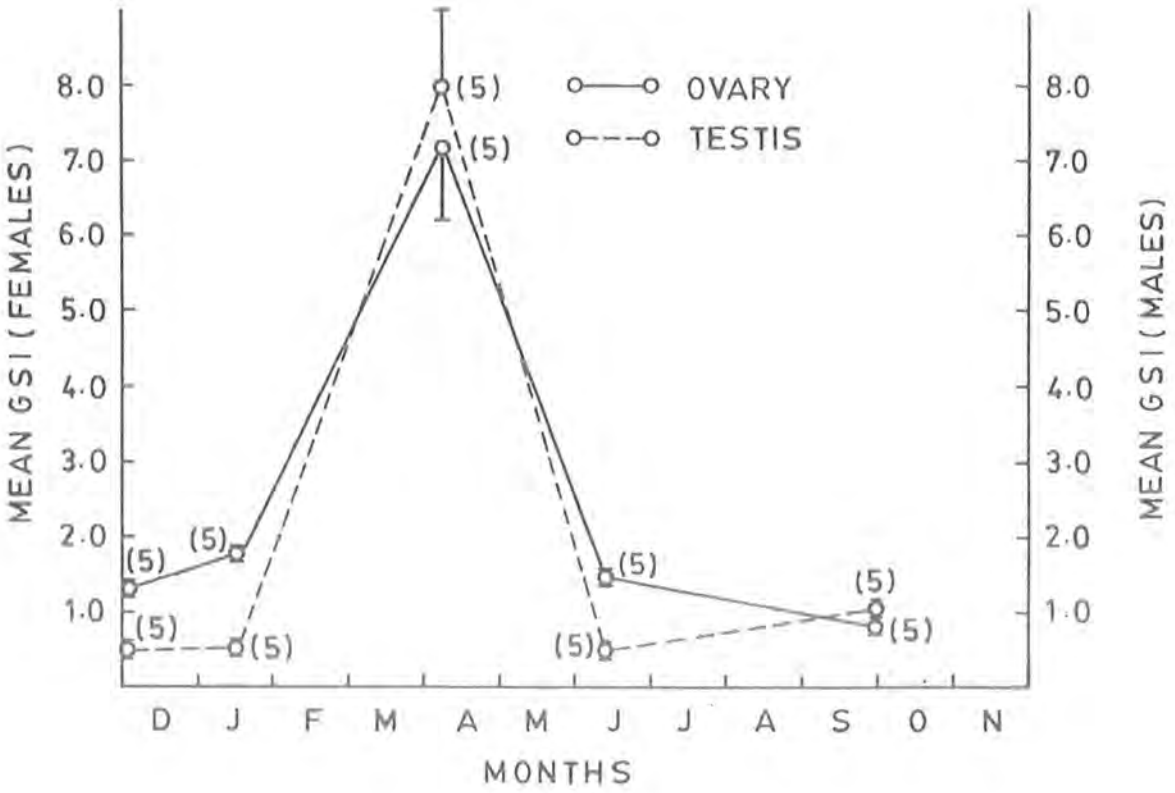

Fig.14: Mean GSI of female and male *Cyprinion watsoni* in selected months during the annual reproductive cycle (1988-89). Vertical lines indicate one standard error of the mean.

As stated earlier, the experiments to check the effect of various combinations of photoperiod and temperature regimes were conducted in preparatory, prespawning, early spawning and postspawning periods. The results of these studies are described below.

Preparatory period:

Long photoperiod-warm temperature (15L/9D:25°C):

Exposure of fish to unseasonally long photoperiod and warm temperature (see Fig.1) caused a significant increase in both ovarian and testicular GSI (Fig.15) over and above the GSI of the seasonal control group. The ovaries of all control fish were in stage II and early stage III (Table 1). In contrast, ovaries in 2 of 5 fish in the experimental group advanced to stage IV. Also the size of the stage III oocytes increased significantly as compared to the stage III oocytes in the initial controls (Table 1). The testes of all fish in the initial control group were in stage 2 whereas they advanced to stage 4 in the experimental fish (Fig.15, Table 1).

Long photoperiod-low temperature (15L/9D:15°C):

Lowering the temperature in long photoperiod did not have a significant effect on the GSI of either the female or the male fish (Fig.15) as compared to the the controls. The ovarian and testicular GSI values of the experimental and initial control groups were nearly similar. Thus, long photoperiod and low temperature, comparable to that prevailing in December, have no modifying influence on the GSI.

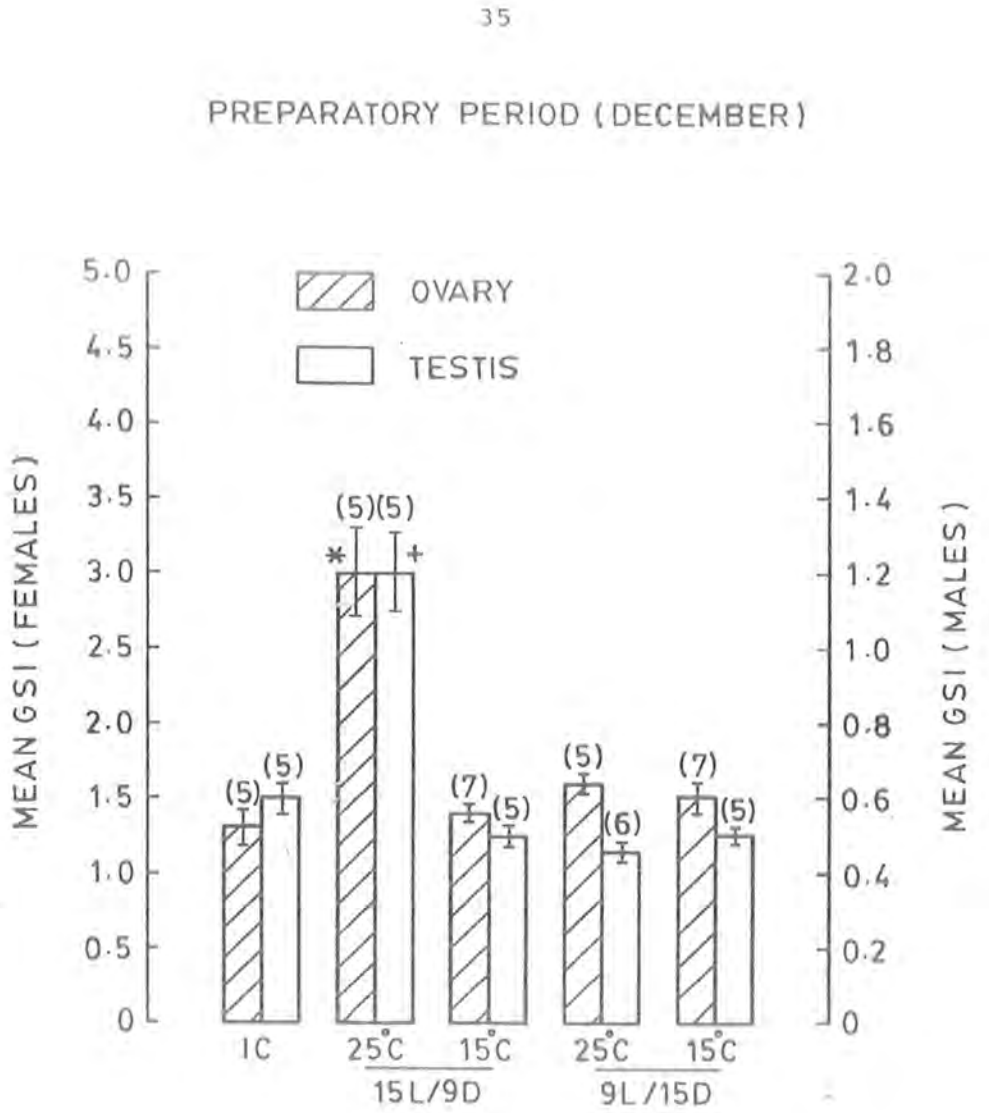

Fig.15: Mean GSI of female and male *Cyprinion watsoni* in preparatory season exposed to various photoperiod-temperature regimes. Figures in parenthesis represent number of aniamls. Vertical lines are one standard error of the mean.

* significantly different ($P<0.01$) from all.

\+ Significantly different ($P<0.01$) from all.

Table-1:Effect of photoperiod and temperature regimes on gonadal activity in Cyprinion watsoni during the preparatory period (December). 30 days treatment. (Figures represent number of fish in particular maturational stage).

Treatment	Maturation stage of testes						
	0	1	2	3	4	5	6
Initial controls	0	0	5	0	0	0	0
Long day 25°C	0	0	0	0	5	0	0
Long day 15°C	0	0	4	1	0	0	0
Short day 25°C	6	0	0	0	0	0	0
Short day 15°C	0	0	3	2	0	0	0

Treatment	Maturation stage of ovaries					
	0	I	II	III	IV	V
Initial controls	0	0	1	4(229.0±6.9)	0	0
Long day 25°C	0	0	1	2(464.0±19.3)	2	0
Long day 15°C	0	0	3	4(264.5±13.0)	0	0
Short day 25°C	4	1	0	0	0	0
Short day 15°C	0	0	3	4(243.6±11.9)	0	0

The ovaries of 3 out of 7 fish kept in the long photoperiod-low temperature regime were in stage II and the remaining ovaries were in stage III (Table 1). The diameter of the oocytes in stage III was significantly lower (264.5±13.0 μm) than that of the corresponding stage oocytes in the long photoperiod-warm temperature regime (464.0±19.3 μm).

The results of the long photoperiod experiments show that it promotes gonadal maturation in the preparatory period only when combined with a warm termperature regime.

Short photoperiod-warm temperature (9L/15D:25°C):

The ovarian and testicular GSI of fish maintained in short photoperiod-warm temperature regime did not differ significantly from the GSI for the initial controls (Fig.15). Histological examination revealed that the ovaries of these fish were regressing; 4 out of 5 ovaries were regressed (Stage 0) containing many atretic follicles and 1 was in stage I (Table 1). The testes of all 6 fish were also regressed (Stage 0).

Short photoperiod-low temperature (9L/15D:15 C):

Neither the ovarian nor the testicular GSI of fish maintained in this photoperiod-temperature regime differed significantly from the GSI of the initial controls or from that of the other experimental groups excepting the long photoperiod-warm temperature fish (Fig.15). The histological details revealed that the oocytes in the ovaries of the 9L/15D:15°C group resembled those in the ovaries of the initial controls and hardly showed any atresia. The testes of the experimental group (short photoperiod-low temperature) were in stages 2 and 3 showing a slight progress over the

initial controls where all testes were in stage 2 only (Table 1).

The results of the short photoperiod tests indicate that short day with warm and low temperature has different effects on the gonads. At warm temperature (unseasonal) the gonads tend to regress during the preparatory period whereas at low temperature, they are maintained in a state nearly comparable to that of the initial controls (ovaries) or cause slight stimulation (testes). The various experiments carried out in the preparatory period reveal that a long photoperiod-warm temperature combination alone can promote gonadal maturation.

Prespawning Period:

Long photoperiod-warm temperature (15L/9D:25^{o}C):

When the fish were exposed to long photoperiod-warm temperature in January the ovarian GSI did not change significantly as compared to the GSI of the initial control group (Fig.16). Histological results indicate that the ovaries of the experimental fish were in stage III and V showing a defninite advancement beyond the condition of the initial control, 3 out of 5 were spent (Table 2). The testicular GSI increased significantly over and above the GSI of the control group (Fig.16), the change being very marked. The testes of all fish were in stage 5 whereas most testes in the control group were in stage 2 (Table 2). Thus long photoperiod and warm temperature accelerated vitellogenesis as well as caused spawning in 3 females and promoted spermatogenesis to the spermiogenic stage.

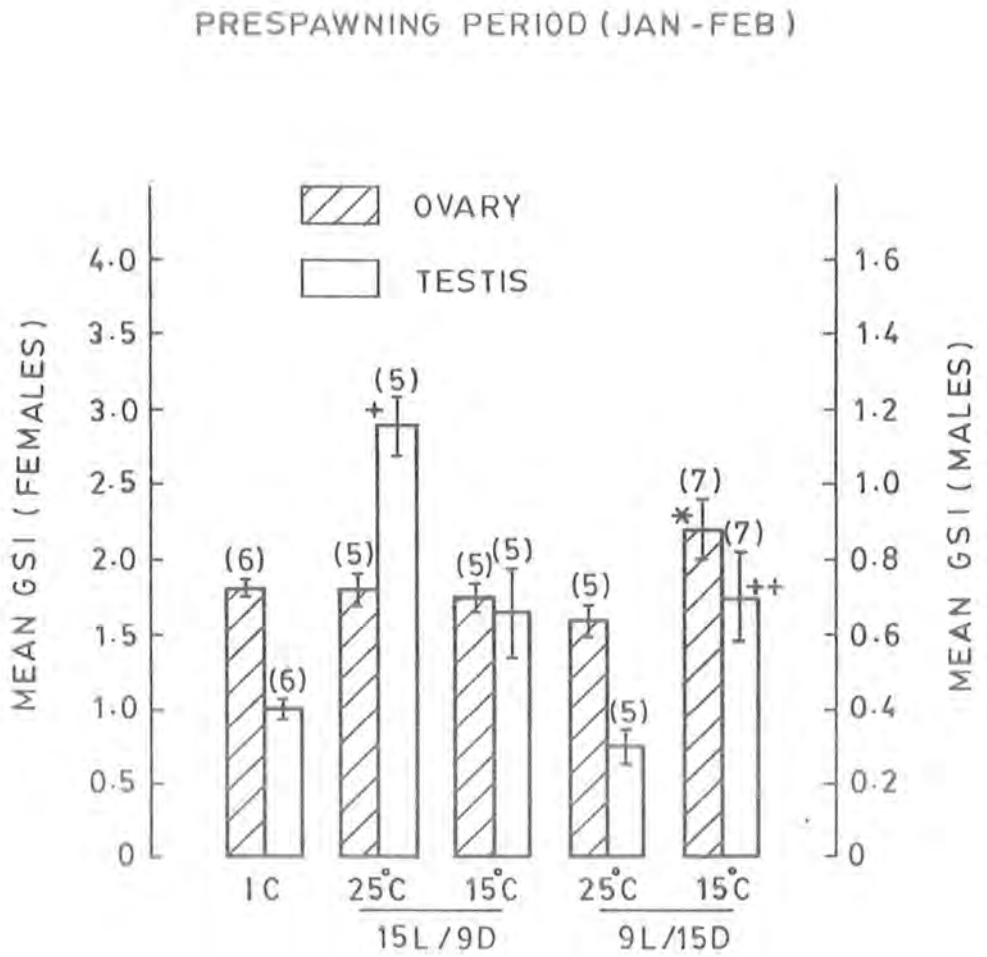

Fig.16: Mean GSI of female and male *Cyprinion watsoni* in prespawning season exposed to various photoperiod-temperature regimes. Figures in parenthesis represent number of animals. Vertical lines are one standard error of the mean.

* significantly different ($P<0.05$) from 9L/15D:25°C.

\+ significantly different ($P<0.01$) from all.

++ significantly different ($P<0.01$) from 15L/9D:25°C.

Table-2 : Effect of photoperiod and temperature regimes on gonadal activity in Cyprinion watsoni during the prespawning period (Jan./Feb.). 30 days treatment.

Treatment	Maturation stage of testes						
	0	1	2	3	4	5	6
Initial controls	0	0	5	1	0	0	0
Long day (25 °C	0	0	0	0	0	5	0
Long day (15 °C	0	0	0	5	0	0	0
Short day (25 °C	0	0	5	0	0	0	0
Short day (15 °C	0	0	0	7	0	0	0

Treatment	Maturation stage of ovries					
	0	I	II	III	IV	V
Initial controls	0	0	2	4(234.3±10.0)	0	0
Long day (25 °C	0	0	0	2(203.0±6.3)	0	3
Long day (15 °C	0	0	1	4(300.3±8.6)	0	0
short day (25 °C	0	0	1	4(188.5±7.2)	0	0
short day (15 °C	0	0	1	6(303.0±14.4)	0	0

Long photoperiod-low temperature (15L/9D:15°C):

Maintaining long photoperiod but lowering the temperature (15°C) had no effect on the ovarian GSI (Fig.16) compared with the initial control. Histologically, the ovaries of the experimental fish were in stage II and stage III. Compared with the seasonal controls, the size of stage III oocytes increased significantly in the experimental fish (Table 2) suggesting some acceleration of vitellogenesis. The GSI of the testis increased in the long photoperiod-low temperature regime (Fig.16) although the increase remained statistically insignificant compared with the seasonal control. Gametogenic progress is however evident in this environmental condition since all fish advanced to stage 3 in contrast to the controls where the majority were in stage 2 (Table 2). The results thus indicate that long day-warm and low temperature regimes stimulate gonadal development; the former has a strong influence, the latter has a marginal influence.

Short photoperiod-warm temperature (9L/15D:25°C):

When the fish were kept in a warm temperature regime in short photoperiod, the ovarian and testicular GSI remained unchanged relative to the seasonal controls (Fig.16). The ovaries of the experimental fish remained in stages II and III as in the controls but the diameter of the oocytes was significantly lower (188.5±7.2, stage III, Table 2). This indicates retardation in vitellogenesis. The testes too stayed in stage 5. There is, thus, evidence of a check on gametogenic progress (compare with the controls, Table 2). A retardation of the process rather than regression is evident

in the warm temperature and short photoperiod regime in both sexes.

Short photoperiod-low temperature (9L/15D:15°C):

Lowering the temperature in short photoperiod led to elevation of both the ovarian and testicular GSI (Fig.16); difference in ovarian GSI being significant only in comparison with the warm temperature-short photoperiod group and that too only marginally ($P<0.05$). A comparison of the histological details for the experimental and control gonads (Table 2) revealed that not only a greater proportion of the ovaries in the former advanced to stage III but the size of the oocytes also increased significantly (303.0±14.4). The testicular GSI of the experimental fish also increased significantly relative to the control GSI and in particular when compared with the warm temperature-short photoperiod group (Fig.16). All male fish advanced to stage 3 whereas most control and all warm temperature fish remained in stage 2 (Table 2). Thus, in the prespawning season, low temperature and short photoperiod combination supported gametogenesis whereas warm temperature and short photoperiod checked the gonadal progress.

Early spawning season:

Long photoperiod-warm temperature (15L/9D:25°C):

Ovarian and testicular GSI of the fish exposed to 15L/9D at 25°C were significantly lower than those for the initial controls (Fig.17). Histologically, the ovaries of the seasonal controls were in stage II, III and IV (Table 3). On the other hand, the ovaries of all fish in the experimental

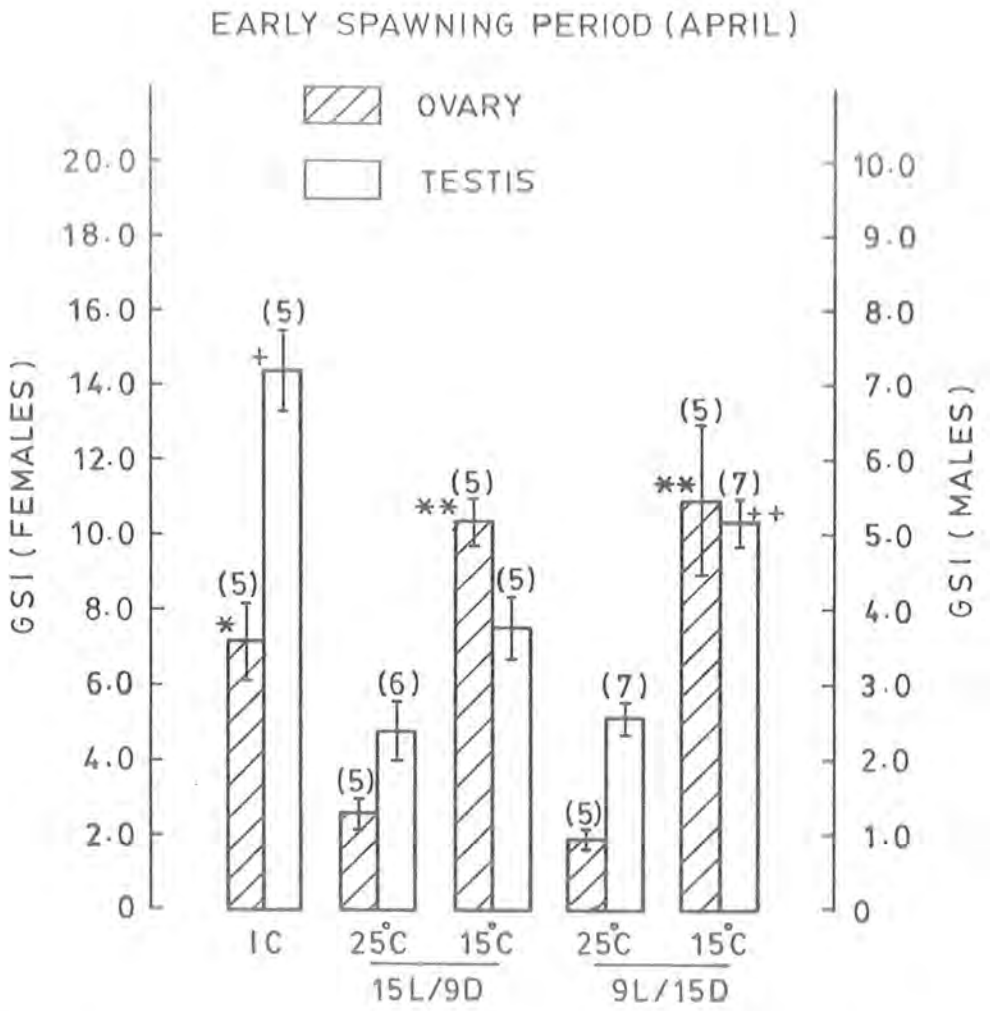

Fig.17: Mean GSI of female and male *Cyprinion watsoni* in early spawning season exposed to various photoperiod-temperature regimes. Figures in parenthesis represent number of animals. Vertical lines are one standarad error of the mean.

* significantly different ($P < 0.01$) from 15L/9D:25°C, 9L/15D:25°C.

** significantly different ($P < 0.01$) from 15L/9D:25°C, 9L/15D:25°C.

\+ significantly different ($P < 0.01$) from all groups.

++ significantly different ($P < 0.01$) from 15L/9D:25°C, 9L/15D:25°C.

Table-3: Effect of photoperiod and temperature regimes on gonadal activity in Cyprinion watsoni during the early spawning period (April). 30 days treatment.

Treatment	Maturation stage of testes						
	0	1	2	3	4	5	6
Initial controls	0	0	0	0	1	4	0
Long day 25°C	0	0	0	0	0	0	6
Long day 15°C	0	0	0	0	1	3	1
Short day 25°C	0	0	0	0	0	0	7
Short day 15°C	0	0	0	0	0	5	2

Treatment	Maturation stage of ovaries					
	0	I	II	III	IV	V
Initial controls	0	0	1	2(249.8±14.3)	2	0
Long day 25°C	0	0	0	0	0	5
Long day 15°C	0	0	0	2(373.7±14.0)	3	0
Short day 25°C	0	0	0	0	0	5
Short day 15°C	0	0	0	2(400.7±20.0)	3	0

group were in stage V (Spent, Table 3). Similarly, the testicular GSI was also significantly lower as compared with the initial control group (Fig.17). Histologically, the testes of the experimental group appeared spent in contrast to the testes of the control group which were in stage 5 (Table 3). Thus, long photoperiod-warm temperature regime stimulated the fish to spawn.

Long Photoperiod-low temperature (15L/9D:15^{o}C):

When the fish were subjected to low temperature and long photoperiod regime the ovarian and testicular GSI achieved a higher mean value as compared to the warm temperature group (Fig.17). The ovarian GSI for this group increased over and above the GSI for the control group as well but the difference remained statistically insignificant (Fig.17). Table 3 shows that 2 of the 5 ovaries of the low temperature fish were in stage III and 3 were in stage IV showing some advance over the control fish. Accelerated vitellogeneic progress is suggested by the larger size of the stage III oocytes as well (Table 3). The testicular GSI of the long photoperiod-low temperature fish decreased significantly relative to the initial controls but remained higher than the GSI for the long photoperiod-warm temperature group (Fig.17). The histological details revealed that the majority of males in the control group were in stage 5 (Spermatozoa) whereas 1 of 5 males had spawned, 3 were still in stage 5 and 1 was in stage 4 in the long photoperiod-low temperature (Table 3). This accounts for the lower GSI of the experimental group compared with the initial control fish. The testes of the long photoperiod-warm temperature group

were in stage 6 (Spent, Table 3). This indicates that whereas warm temperature in long photoperiod stimulated both the male and female fish to spawn, low temperature favoured a retarded vitellogenic progress. In the male, low temperature with long photoperiod checked spawning.

Short Photoperiod-warm temperature (9L/15D:25^{o}C):

The ovarian GSI of the fish exposed to this combination of day length and temperature remained at nearly the same level as that of the long day and warm temperature fish (Fig.17). The mean GSI was significantly lower than the GSI of the initial control and the long day-low temperature groups (Fig.17). The ovaries of this group were in stage V (Spent, Table 3). The testicular GSI of short day-warm temperature fish was not different from the GSI of the fish exposed to long day-warm temperature (Fig.17) but was significantly lower than that of the initial control group. All the fish in the short day-warm temperature regime were in postspawning condition and, histologically, the testes resembled those of the fish kept in long day-warm temperature environment (Table 3). The above results show that the short day-warm temperature environment also stimulates spawning in fish in the month of April (early spawning period).

Short Photoperiod-low temperature (9L/15D:15^{o}C):

Ovarian GSI of the fish exposed to 9L/15D:15^{o}C increased over and above the control GSI (Fig.17) but the difference remained statistically insignificant. The ovaries in the two groups were in stages III and IV (Table 3) but the stage III oocytes in the experimental fish had a

significantly greater diameter and also more ovaries were in stage IV here than in the control group. This indicates that low temperature in a short day situation maintains or slightly accelerates vitellogenic progress of the ovary as is also true for the low temperature and long day group. Although the ovarian GSI of the low temperature fish is significantly higher than the GSI of the warm temperature fish in short or long photoperiod (Fig.17), this is merely because the warm temperature fish had spawned. In contrast to the ovaries, the testicular GSI of the low temperature-short day fish was significantly lower than the GSI of the initial control group (Fig.17). The histological details of the testes of the two groups showed that in the former 2 of 7 males had spawned (Table 3).

The data for various environmental combinations used in the early spawning period collectively suggest that in this season warm temperature regardless of photoperiod can stimulate the fish to spawn. Low temperature in combination with long or short photoperiod slightly accelerates vitellogenic activity in the ovary and checks spawning in the male.

Postspawning Season:

Long photoperiod-warm temperature (15L/9D:25^{o}C):

Ovarian and testicular GSI of the fish collected in June and exposed to 15L/9D at 25^{o}C did not differ significantly from the initial controls (Fig.18). Nevertheless, some vitellogenic progress did occur in the ovaries as revealed by histological details. Whereas the

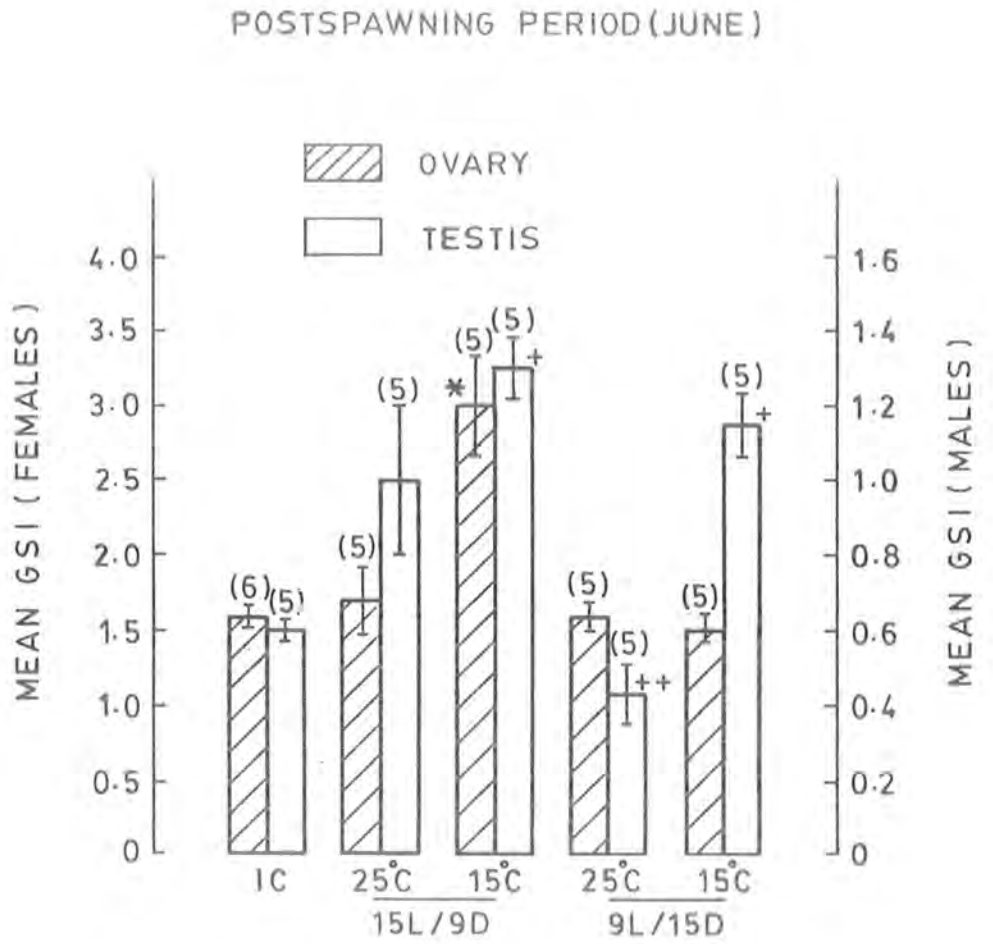

Fig.18: Mean GSI of female and male *Cyprinion watsoni* in postspawning season exposed to various photoperiod-temperature regimes. Figures in parenthesis represent number of animals. Vertical lines are one standard error of the mean.

* significantly different ($P < 0.01$) from all groups.

\+ significantly different ($P < 0.01$) from IC and 9L/15D:25°C.

++ significantly different ($P < 0.01$) from 15L/9D:25°C.

Table-4: Effect of photoperiod and temperature regimes on gonadal activity in Cyprinion watsoni during the postspawning (June). 30 days treatment.

Treatment	Maturation stage of testes						
	0	1	2	3	4	5	6
Initial controls	0	0	0	0	0	0	5
Long day 25 °C	0	0	0	0	0	0	5
Long day 15 °C	0	0	0	5	0	0	0
Short day 25 °C	5	0	0	0	0	0	0
Short day 15 °C	0	0	0	5	0	0	0

Treatment	Maturation stage of ovaries					
	0	I	II	III	IV	V
Initial controls	0	0	3	3(290.0±14.6)	0	0
Long day 25 °C	0	0	4	1(379.6±20.9)	0	0
Long day 15 °C	0	0	2	1(241.0±9.6)	2	0
Short day 25 °C	5	0	0	0	0	0
Short day 15 °C	5	0	0	0	0	0

ovaries in the experimental and the control groups were in stages II and III (Table 4), the mean diameter of the stage III oocytes in the experimental group was greater (Table 4). The testes of all individuals in this regime were in stage 1 in contrast to those of the initial controls which were in the spent stage (Table 4). Histologically, thus, the testes had recovered from the spent conditions but no further gametogenic progress occurred.

Long Photoperiod low-temperature: (15L/9D:15^{o}C):

Ovarian GSI of the fish exposed to long photoperiod but low temperature was significantly higher than the GSI of the controls as well as the warm temperature group (Fig.18). Some of the ovaries in this group had advanced to stage IV (Table 4). This also contrasts with the situation in the warm temperature group where the largest oocytes were still in stage III. These data suggest that unseasonally low temperature in long day is more effective than warm temperature in initiating ovarian recrudescence. The testicular GSI, on the other hand, did not differ significantly from the warm temperature group although it increased significantly over and above the initial control GSI (Fig.18). The testes of all fish in this regime were in stage 3 (Table 4). This indicates that low temperature under long day condition initiates testicular recrudescence as well. This contrasts with the testes of both the seasonal controls and the long day-warm temperature fish where the testes stayed in spent state (Table 4). Evidently, the testis in the postspawning season (June) remains unaffected by warm temperature in a long photoperiod regime but responds to a low temperature environment by initiating

recrudescence.

Short Photoperiod-warm temperature (9L/15D:25°C):

Warm temperature does not bring about a significant change in the ovarian GSI of the postspawning fish in short photoperiod as well (compare with initial controls (Fig.18). The ovarian GSI of the long photoperiod-warm temperature fish and short photoperiod-warm temperature group remained nearly comparable. All ovaries of the fish exposed to short day-warm temperature were regressed containing many atretic follicles. Although the testicular GSI of the short photoperiod-warm temperature fish did not differ significantly from the GSI of the initial control, it was significantly lower than the GSI of the long day fish maintained at 15°C (Fig.18). The testes of all fish maintained in 9L/15D at 25°C were regressed (Table 4) showing shrunken lobules and pycnotic cells. Thus at warm temperature, long photoperiod exerts little influence on the testis, but a short photoperiod causes regressive changes.

Short Photoperiod-low temperature (9L/15D:15°C):

In this regime the ovarian GSI of the postspawning (spent) fish remained unchanged compared with the seasonal group, short photoperiod-warm temperature as well as the long photoperiod-warm temperature fish (Fig.18). Histologically, the ovaries were regressed (Table 4) characterized by many atretic follicles. In contrast, the testicular GSI increased markedly achieveing a significantly higher GSI compared with the initial control and short day-warm temperature groups. However, it did not differ significantly from the GSI of the long day fish at either 25° or 15°C. The testes of all fish

in this regime were in stage 3. Low temperature in a short day situation, thus, has a stimulatory influence on testicular development.

Gonadal Steroids: Seasonal changes and effects of photoperiod and temperature regimes.

Seasonal change:

Estradiol and progesterone were measured in the ovarian tissue and testosterone in the testicular tissue in selected months of the year. Measurements of plasma steroids were possible only for progesterone and testosterone since the volumes of blood collected in the various months turned out to be insufficient for determination of all of the three hormones. Hence estradiol in the plasma could not be measured. The results are shown in Table 5 and Figures 19 and 20.

Estradiol levels in the ovary ranged between the 0.16 to 1.36 ng/gonad. The lowest level was encountered in the preparatory period (December). In the prespawning period it increased to 0.49 ng/gonad, peaked in the spawning period (April) and then dropped again in the postspawning season (June). The level increased again in October.

Ovarian progesterone followed a similar pattern as for estradiol. From its low value in December it increased to a peak value of 2.08 ng/gonad in April and subsequently fell through June to the lowest value of 0.2 ng/gonad in October. Plasma progesterone levels in the female were substantially lower than in the ovarian tissue. The lowest value occurred

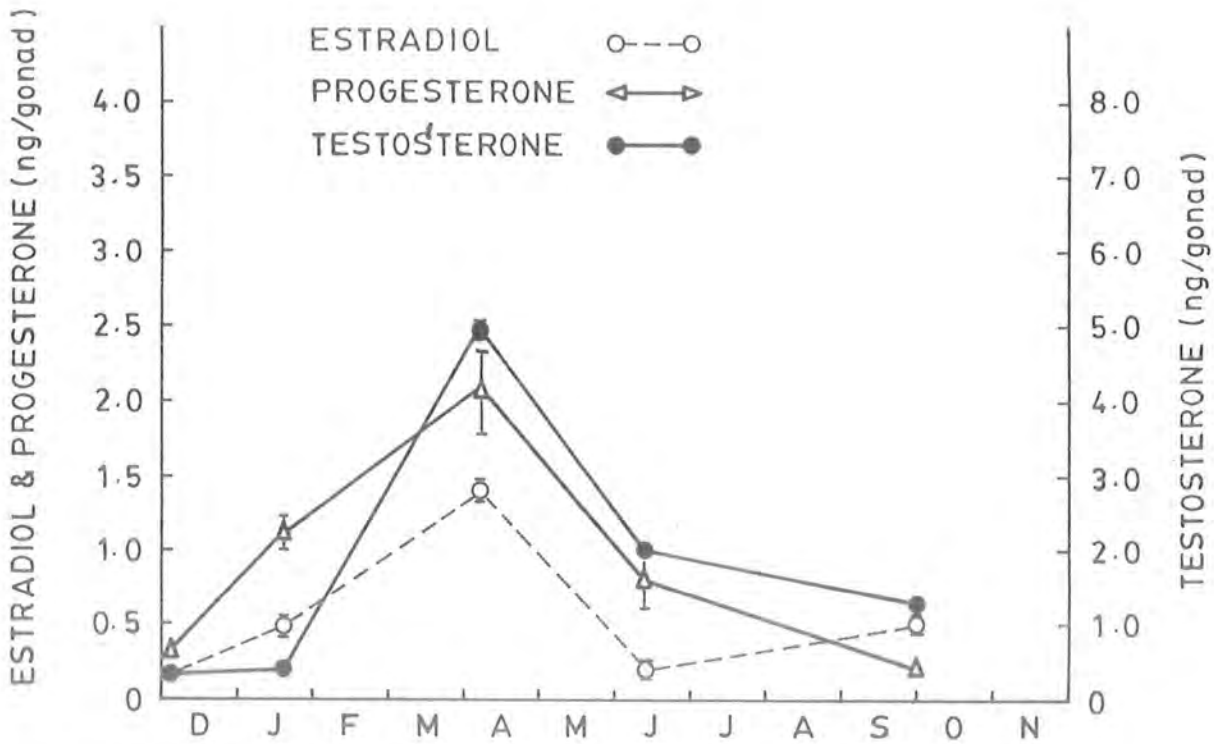

Fig.19: Seasonal variation in estradiol and progesterone in the ovaries and testosterone in the testes based on determinations in selected months of the year.

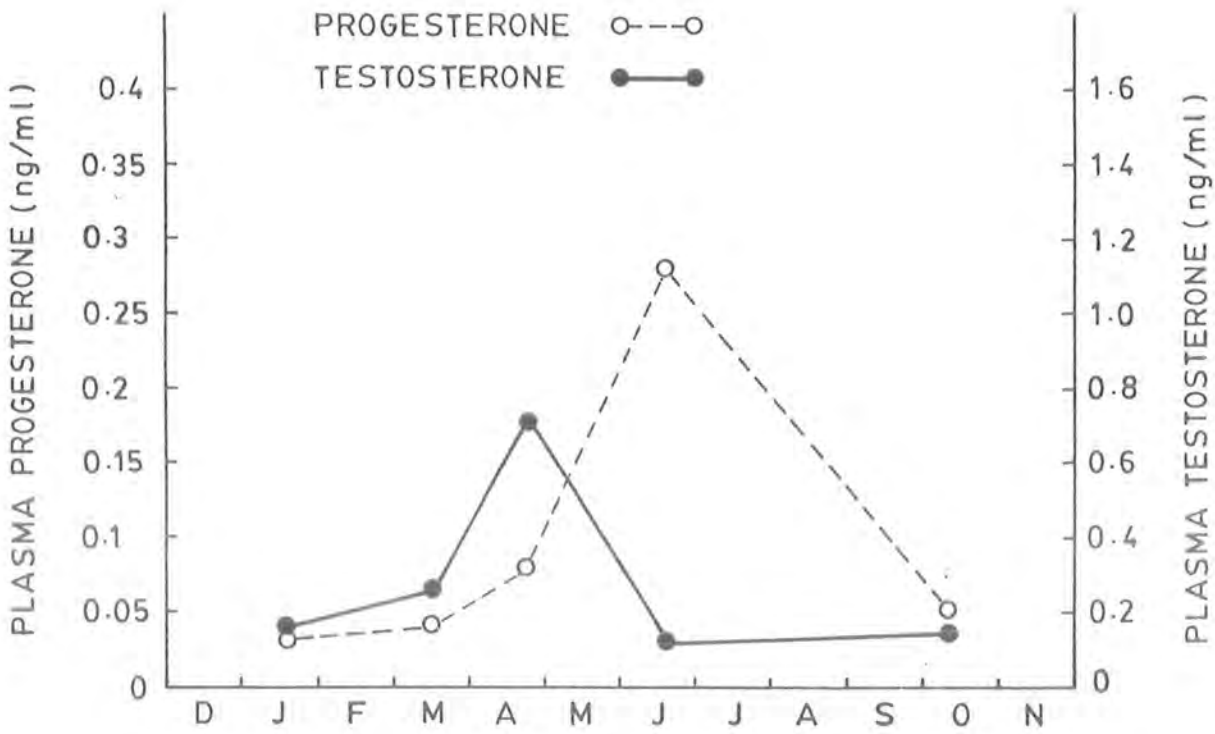

Fig.20: Seasonal variation in plasma progesterone and testosterone in the female and male *Cyprinion watsoni* respectively in selected months of the year. The values are based on single determination of pooled plasma.

Table-5: Seasonal levels Mean ± SEM of estradiol, Progesterone and testosterone in *Cyprinion watsoni*. Means for tissue levels are based on measurements in triplicate. Values for plasma are based on single measurements of pooled samples.

	Estradiol	Progesterone		Testosterone	
	Ovary	Ovary	Plasma	Testis	Plasma
	ng/gonad	ng/gonad	ng/ml	ng/gonad	ng/ml
December	0.16±0.04	0.29±0.03		0.26±0.07	
January	0.49±0.02	1.13±0.08	0.03	0.42±0.05	0.16
March			0.04		0.25
April	1.36±0.06	2.08±0.28	0.08	4.93±0.04	0.72
June	0.22±0.002	0.8±0.20	0.28	1.97±0.01	0.12
October	0.54±0.05	0.2±0.005	0.05	1.34±0.05	0.13

in January (Prespawning period). No measurements could be made in December due to accidental loss of blood samples. The concentration increased in March/April but the peak concentration occurred in June the postspawning season.

Testosterone in the testis was lowest in December. It rose to a peak value of 4.93 ng/gonad in April. In June and October its concentration dropped but remained higher than the December value. Plasma testosterone profile followed the same pattern but the levels were considerably lower. The peak value in April was 0.27 ng/ml plasma.

Effect of photoperiod and temperature on steroid hormones:

The RIA analysis were made on gonadal samples taken from fish subjected in various (four) reproductive phases of the annual cycle to long photoperiod in warm or low temperature (15L/9D:25^{o}C or 15^{o}C) and short photoperiod in warm or low temperature regimes (9L/15D: 25^{o}C or 15^{o}C).

Preparatory season (December).

The results (Table 6) show that the level of estradiol was highest in the long photoperiod-warm temperature regime (15L/9D:25^{o}C) and significantly different from all other groups. This paralleled the significant increase in the ovarian GSI and stimulation of vitellogenesis relative to the seasonal controls (Table 1, also Fig. 15). Estradiol level in the ovary also increased slightly over and above the control group in long photoperiod-low temperature (15L/9D:15^{o}C). It should be noted that the ovarian GSI and histology of the ovary in the control and low temperature

Table-6: Female and male GSI, ovarian estsradiol and progesterone and testicular testosterone (ng/gonad) in *Cyprinion watsoni* in various combinations of photoperiod-temperature in the preparatory season (December). The GSI values are repeated from the previous data (Fig.14). Mean ± SEM based on determination from 3 ovaries and 3 testes.

Treatment	Female GSI	Estradiol	Proges-terone	Male GSI	Testo-sterone
Initial control	1.3±0.09	0.16±0.04	0.29±0.03	0.4±0.04	0.26±0.07
)25°C LD)	3.0±0.3	1.5±0.14 a	0.85±0.2	1.2±0.34	2.13±0.07 c
)15°C	1.44±0.03	0.39±0.04	6.0±0.8	0.5±0.03	0.86±0.05 cc
)25°C SD)	1.6±0.05	0.43±0.04	20.0±2.74 b	0.45±0.02	0.25±0.005
)15°C	1.48±0.1	0.33±0.04	2.9±0.45	0.5±0.04	0.55±0.05 ccc

a significantly different (P< 0.01) from all groups.
b significantly different (P< 0.01) from all groups.
c significantly different (P< 0.01) from all groups.
cc significantly different (P< 0.01) from IC,9L/15D:25°C 15°C.
ccc significantly different (P< 0.01) from IC,9L/15D:25°C.

groups were comparable (Tables 6,1,Fig. 15) excepting that the size of stage III oocytes in the latter was slightly larger. The estradiol concentration increased slightly in short photoperiod in both the warm and low temperature groups (9L/15D:25^{o} or 15^{o}C) (Table 6). The level in the warm temperature ovaries was slightly higher; the difference being statistically insignificant. The ovarian GSI of the two also did not differ significantly from each other or from the control GSI (Table 6).

The observations in the four environmental situations in the preparatory season reveal that relative to the seasonal control ovarian estradiol increased signficantly only in the long day-warm temperature regime.

The concentration of ovarian progesterone in the preparatory season (December) was low. A significant increase in the progesterone level occurred only in the short photoperiod-warm temperature regime (9L/15D:25^{o}C, Table 6) paralleling the observed regression in the ovary (Table 1). Although the values of progesterone in the other photoperiod-temperature regimes were also higher as compared to the control group, the differences remained statistically insignificant.

Testicular testosterone increased significantly over and above the control level in long photoperiod at both 25^{o} or 15^{o}C (Table 6). Maximum increase, however, occurred in warm temperature regime where in combination with long photoperiod the most significant stimulation of the testes (Spermatogenesis and GSI) was noted (Fig.14, Table 1). The testosterone level in short photoperiod at warm was

comparable to the control group but at low temperature differed significantly from the seasonal control level (Table 6). The lower level at warm temperature matches with the regressed state of the testes (Table 1). At low temperature some of the testes had advanced to stage 3 (Table 1). The apparent increase to 0.55 ng/gonad (Table 5) accompanied this gametogenic progress.

Prespawning season (January-February):

In the prespawning season significant and largest increase in estradiol level (Table 7) occurred in the long photoperiod-warm temperature group where the vitellogenic growth was also accelerated and some fish seemed to have spawned (Table 2). Estradiol level also increased slightly but not significantly relataive to the seasonal control in long photoperiod-low temperature regime (Table 7) where the stage III oocyte diameter increased indicating some stimulation of vitellogenic progress (Table 2). In the short photoperiod group again a significant elevation of estradiol occurred at warm temperature (Table 7) where the GSI dropped and the stage III oocyte diameter was also relatively small (Table 2). At low temperature in this photoperiod, the estradiol level remained near the seasonal control value while vitellogenic progress was better here than in the low temperature-long photoperiod group (Table 2, note oocyte diameter and proportion of ovaries in stage III).

Progesterone increased in long photoperiod at both warm and low temperature (Table 7). This increase was greatest and significant at warm temperature, the group where

Table-7: Female and male GSI, ovarian estradiol and progesterone and testicular testosterone (ng/gonad) in *Cyprinion watsoni* in various combinations of photoperiod-temperature in the prespawning season (January-February). The GSI values are repeated from the previous data (Fig.15). Mean ± SEM based on determinations from 3 ovaries and 3 testes.

Treatment	Female GSI	Estradiol	Proges-terne	Male GSI	Testo-sterone
Initial controls	1.78±0.07	0.49±0.02	1.13±0.08	0.4±0.02	0.42±0.05
)25°C LD)	1.84±0.12	1.26±0.02 a	5.25±0.08 b	1.15±0.08	0.63±0.09
)15°C	1.76±0.12	0.66±0.10	2.0±0.57	0.66±0.13	1.1±0.05
)25°C SD)	1.57±0.14	0.85±0.08 aa	1.16±0.20	0.3±0.04	2.42±0.56 c
)15°C	2.42±0.28	0.55±0.03	1.20±0.13	0.74±0.13	2.66±0.23 cc

a significantly different ($P < 0.01$) from all groups.
aa significantly different ($P < 0.01$) from IC.
b significantly different ($P < 0.01$) from all groups.
c significantly different ($P < 0.01$) from IC.
cc significantly different ($P < 0.01$) from IC, 15L/9D:25 and 15°C.

final maturation and spawning occurred in some fish. In the short photoperiod group the level of progesterone remained similar at low and warm temperatures and also comparable to the level in the seasonal control and other experimental groups excepting the long day warm temperature (Table 7). Relatively lower progesterone levels occurred in groups where the ovaries did not progress beyond stage III (Table 2).

The level of testosterone increased above the seasonal level in all environmental combinations (Table 2). The smallest increase occurred in the long photoperiod warm temperature group where the testes were in the spermatozoa stage (Table 7) and the GSI was highest indicating that spermatogenic and spermiogenic progress had occurred in this regime. Significantly greater elevation over and above the controls occurred in short photoperiod at both warm and low temperature. The highest value characterizes the short photoperiod regime. At low temperature in short photoperiod the testes had advanced to stage 3 compared with the seasonal controls accounting for the observed increase in testosterone (Table 2). At 25^{o}C in short photoperiod where the testes were similar to the control group both in respect to GSI and histology (Table 2), the increase in testosterone was also quite significant. The relationships of the environment, GSI, histology and testosterone show some inconsistency here.

Early Spawning Season (April):

The estradiol levels in the seasonal control and the long day-warm temperature group did not differ significantly

Table-8: Female and male GSI, ovarian estradiol and progesterone and testicular testosterone (ng/gonad) in *Cyprinion watsoni* in various combinations of photoperiod and temperature in the early spawning season (April). The GSI values are repeated from the previous data(Fig.16). Mean ± SEM based on determinations from 3 ovaries and 3 testes.

Treatment	Female GSI	Estradiol	Progs-terone	Male GSI	Testo-sterone
Initial controls	7.24±1.0	1.36±0.06	2.08±0.28	7.20±0.5	4.93±0.04
)25°C LD)	2.6±0.46	1.27±0.39	1.5±0.20	2.4±0.36	4.02±0.03
)15°C	10.44±0.62	0.7±0.07	2.0±0.46	3.76±0.4	3.98±0.08
)25°C SD)	1.92±0.14	0.31±0.04	0.46±0.06	2.65±0.21	2.13±0.45[c]
)15°C	11.02±2.25	2.67±0.65[a]	5.0±1.32[b]	5.17±0.32	18.6±0.27[cc]

a significantly different ($P < 0.01$) from 15L/9D:15°C, 9L/15D:25°C.
b significantly different ($P < 0.01$) from 15L/9D:25°C, 9L/15D:25°C.
c significantly different ($P < 0.01$) from IC.
cc significantly different ($P < 0.01$) from all groups.

(Table 8). The ovaries had completed final maturation and seem to have spawned (Table 3). The most marked and significant increase occurred in the short photoperiod-low temperature group (Table 3) where the ovaries were still in the vitellogenic phase and the oocyte diameter was much larger than in the control group (Table 3). Although the estradiol level dropped markedly below the control value in long photoperiod-low temperature and short photoperiod-warm temperature regimes (Table 8), the difference remained statistically insignificant. In the warm temperature-short photoperiod group the fish had completed final maturation and had spawned (Table 3).

The level of progesterone in the long photoperiod-warm temperature group, where final maturation and spawning had occurred, dropped slightly but remained similar to the control group (Table 8). A significant increase occurred, compared with the seasonal control, in the short photoperiod-low temperature group where most of the ovaries were in stage III with a larger oocyte diameter (Table 3). A noticeable but statistically insignificant decline in progesterone was noted in the short photoperiod-warm temperature (Table 8) fish which had completed final maturation and spawning (Table 3). Progesterone remained unchanged relative to the seasonal control (Table 8) in the long photoperiod-low temperature regime with the fish still in vitellogenic phase (Table 3).

In long photoperiod at both warm and low temperature, the testosterone level dropped only slightly below the seasonal control level (Table 8).The fish, at warm

temperature, had spawned (Table 3). At low temperature, most fish were in the spermatozoa stage, one had spawned and one was in stage 4 (Table 3). In the short photoperiod warm temperature fish which had also spawned, the level dropped significanty below the control value (Table 8). The maximum and significant increase in testosterone occurred in the short photoperiod-low temperature group. The fish were mostly in the spermatozoa stage with 2 fish having spawned (Table 3)

Postspawning Season (June):

In the postspawning season, the highest levels of estradiol were recorded in the long photoperiod-warm temperature and low temperature groups (Table 9). The elevation over and above the seasonal control was significant only in the long photoperiod-warm temperature environemnt. In short photoperiod also at either temperature, no significant change occurred in the estradiol level. The highest rise in long photoperiod-warm temperature was associated with most ovaries being in the cortical alveolar phase (Stage II, Table 4) . More fish in the seasonal control had already advanced to the vitellogenic stage III. The oocytes in the single fish in stage III in long day-warm temperature group were larger in diameter (Table 4). The fish in low temperature-long photoperiod regime had advanced to stage IV. The ovaries in the short photoperiod-warm temperature regime were regressed (Table 4).

The level of progesterone increased over the control value in warm temperature-long photoperiod and short photoperiod at either temperature (Table 9) but none of these

Table:-9 Female and male GSI, ovarian estradiol and progesterone and testicular testosterone (ng/gonad) in *Cyprinion watsoni* invarious combinations of photoperiod and temperature in the postspawning season (June). The GSI values are repeated from the previous data (Fig.17). Mean ± SEM based on determination from 3 ovaries and 3 testes.

Treatment	Female GSI	Estradiol	Proges-	Male GSI	Testo-sterone
Initial control	1.46±0.05	0.22±0.002	0.8±0.20	0.57±0.05	1.97±0.01
)25°C LD)	1.72±0.26	0.76±0.02[a]	1.4±0.17	1.0±0.2	4.81±0.46
)15°C	3.0±0.30	0.6±0.13	0.6±0.28	1.3±0.07	13.2±1.28[c]
)25°C SD)	1.70±0.05	0.36±0.05	1.65±0.37	0.42±0.06	7.2±1.14[cc]
)15°C	1.54±0.17	0.23±0.04	1.85±0.2[b]	1.17±0.07	3.38±0.29

a significanlty different (P< 0.01) from IC,9L/15D:25°C and 15°C.
b significantly different (P< 0.01) from 15L/9D:15°C
c significantly different (P< 0.01) from all groups.
cc significantly different (P< 0.01) from IC,9L/15D:15°C.

values differed significantly. Long photoperiod-warm temperature fish were in stage II/III while the long photoperiod-low temperature fish were in vitellogenic phase stage IV (Table 4). The slight increase in the short photoperiod group matched with the regressed state of the ovaries (Table 4).

The testosterone level increased over and above the control (spent stage) in all groups in the postspawning season (Table 9). However, significant increase occurred in the long photoperiod-low temperature as well in the short photoperiod-warm temperature groups. In the former group, the fish were in spermatogenic phase (Table 4) while in the latter group the testes had regressed. The fish in the low temperature-short photoperiod regime were also in the spermatogenic phase yet the change in testosterone level was insignificant compared with the control (Table 9). At warm temperature in long photoperiod, the insignificant increse in testosterone relative to the control was paralleled by testes which had recovered from the initial spent state and were in stage 1 of gametogenesis (Table 4).

DISCUSSION

Interest in fish reproduction stems not only from a need to understand how a species manages its survival but also from a desire to ultimately utilize new scientific information for exploitation of this fish resource for the benefit of mankind. Gonadal physiology and reproductive behavior of fishes has been known, since long, to be regulated by environmental and hormonal factors. A considerable body of information has become available in this field during the last three decades. Nevertheless, many aspects of hypothalamo-hypophyseal regulation (Peter, 1981; Goos, 1987; Fontaine and Dufour,1987; Fostier *et al.*, 1983) environmental regulation (Scott,1979; de Vlaming,1983; Lam, 1983; Lam and Munro, 1987; Bye, 1984, 1987), follicular development and steroid hormone production (Wallace *et al.*, 1987; Goetz,1983; Scott and Canario, 1987; Fostier *et al.*, 1987; Ng and Idler,1987) in fishes remain to be resolved. Furthermore, the diversity of the teleostean group, regional differences in environmental conditions and the demonstrated species dependent variations in reproductive responses in fishes provide compelling reasons to examine a larger number of teleostean species than has been done so far. It has been possible in the present investigation on *Cyprinion watsoni* to bring out some interesting morphological features of the developing ovarian follicle with relevance to questions regarding production of steroid hormones and environmental control of the ovary. These together with the results of

experimental work on gonadal maturation and steroid hormone profiles discussed below constitute an addition to the currently available information on the reproductive biology of *Cyprinion watsoni*.

Ultrastructure of ovarian follicles:

The developmental progress of gonads of *Cyprinion watsoni* during the annual reproductive cycle has been described in an earlier preliminary study by Shaikh, (1986). In the present study, interest was confined to an examination of follicles in their primary and secondary growth phases at the ultrastructural level. The process of follicular growth and maturation in fishes has received particular attention in recent years especially at the electron microscope level. (Hoar and Nagahama, 1978; Wallace and Selman, 1978; Dumont and Brummett, 1980; Wallace and Selman, 1980, 1981; Selman and Wallace, 1983, 1986; Selman *et al.*, 1986; Wallace *et al.*, 1987). The follicular development in teleost is generally known to progress through several steps. In the earliest stages, the follicle consists of an oocyte invested in a connective tissue sheet of prefollicular cells. These oocytes, arrested in the first meiotic prophase, pass through phases of primary and secondary growth during which their size increases several fold. In the process, definitive thecal, follicular and vitelline envelopes are added externally. In the final phase of the follicular development, events of final maturation occur which are marked by ultimate breakdwon of the germinal vesicle and reinitiation of the remaining

meiotic events. The ovarian follicles in their primary growth phase (upto perinucleolus stage) have been somewhat equivocally (Wallace *et al.*, 1987) considered to be independent of hormonal control (Hoar and Nagahame, 1978; Nagahama, 1983). The secondary growth period begins in the cortical alveolar stage with endogoneous synthesis of "yolk vesicles". Exogenous yolk deposition in the yolk stage oocytes is responsible for most of the growth of the follicle. It seems to continue to some extent in the final maturation period as well at least in some species of teleosts (Selman and Wallace, 1983; *Fundulus heteroclitus*).

Although many of the details of follicular development and maturation in *Cyprinion watsoni* are wanting and require elucidation, principal stress in this electron microscopic study was on aspects of morphological differentiation of theca, follicular and vitelline investments and their relationship with the developing oocyte. Species differences in some of the structural details of these components of the follicle and their interrelationships have been noted in various studies in the past (Nelsen, 1953; Shelton, 1978; van den Hurk and Peute, 1979; Hoar and Nagahama, 1978; Dumont and Brummett, 1980; Kagawa *et al.*, 1981; Selman and Wallace, 1986). Several important questions pertaining to origin of vitelline envelope, organization of the theca, granulosa and presence of special cells as well as their role in steroidogenesis continue to remain unresolved (Selman and Wallace, 1986). This study demonstrates that in *Cyprinion watsoni* young perinucleolar stage oocytes are

surrounded by a double layer of squamous connective tissue cells. This double layer is continuous with the interstitial tissue of the ovary. A noteworthy feature of this prefollicular investment of the oocyte is presence of certain prominent cells which have been designated here "active" cells because they stand out among the fibroblastic cells of the investing connective tissue layer. That these cells are metabolically and physiologically active is suggested by their subcellular details. Ovarian stromal tissue as well as some special thecal cells in advanced follicles have been reported by some investigators to be important in synthesis of steroid hormones (Guraya, 1976; Nagahama *et al.*, 1973, *Salmo gairdneri*; Hoar and Nagahama, 1978, amago salmon; van den Hurk and Peute, 1979, *Salmo gairdneri*; Kagawa *et al.*,1981, *Salvelinus leucomaenis*). Whether the special cells seen in the prefollicular envelope of the perinucleolar stage oocyte in *Cyprinion watsoni* belong to the same line of cells is difficult to judge. Smooth endoplasmic reticulum appears to be absent in these cells, lysosomes exist and granular material is very sparse. The possibility that these cells may be precursors of theca and/or granulosa cells cannot be ignored.

It has been suggested in some of the earlier studies on fishes that the vitelline envelope (Chorion, zona radiata) of the oocyte is formed by activity of the follicle (granulosa) cells (Nelsen, 1953). In more recent works (Tesoriero, 1978; Dumont and Brummett, 1980), it has been argued that its components are formed by material derived

from the oocyte. There is some morphological as well as experimental evidence for this in *Fundulus heteroclitus* (Selman and Wallace, 1986) but limited contribution by the follicle cells has not been ruled out. Evidence is presented in this study on *Cyprinion watsoni* that formation of the vitelline envelope first begins in the late perinucleolus stage follicles. The fully differentiated vitelline envelope appears in the late cortical alveolar or early yolk stage follicles and comprises three layers. The thick inner layer, zona radiata interna, consist of plaques of dense material stacked one on top of the other and lies next to the oocyte. Outside this lies the zona radiata externa comprising an intermediate ornamented and an outer layer of dense homogeneous material. The zona radiata interna is the first to be deposited between the microvilli of the oocyte. The continuity of fibrillar material of the inner prefollicular cells with the vitelline material between the microvilli provides strong support to the argument that these cells are responsible for its synthesis. The images of the inner prefollicular cells seen in the present study and their relationship with the growing vitelline envelope leave little doubt that further increase in the size of the zona radiata interna and development of the zona radiata externa components arise through ultimate transformation of the prefollicular cells into the vitelline envelope material. According to Selman and Wallace (1986) the increase in the thickness of the zona radiata in *Fundulus heteroclitus* occurs by addition of material to its inner surface from the oocyte.

Although a limited contribution by the oocyte cannot be entirely rejected, the events reported to occur in *Cyprinion watsoni* are contrary to the situation in *Fundulus heteroclitus*. Cellular transformation is not unknown in ovarian follicles in fishes. In fact, Shelton (1978) has demonstrated transformation of granulosa cells into egg membrane in *Dorosoma petenense*. The events observed in the present study do not display features which characterize degeneration or follicular atresia (see also Lang, 1981). Furthermore, it would be difficult to visualise how the entirely different relationships in organization of the theca, the granulosa and the vitelline envelope could arise in the yolk stage follicles given the structural details in the perinucleolus stage follicles.

It has been further shown here that the definitive theca and granulosa layers appear late and seem to differentiate from the outer prefollicular cells. These layers become visible for the first time in the yolk stage follicles. Whether they differentiate in late cortical alveolar stage cannot be ascertained without more work. Already in the cortical alveolar stage follicles, there is some evidence of rather infrequent existence of light cells in the outer prefollicular layer where most cells are elongate and electron dark. It seems likely that these cells are precursors of the theca and granulosa cells. It is not very unlikely that the special active looking cells referred to earlier belong to the same category and represent the beginning of the theca and granulosa cells. Traditionally, in

description of ovarian follicles, the investing connective tissue layers of the oocytes are designated as thecal and flattened (squamous) follicle cells (see Selman and Wallace, 1986) as late as the perinucleolus stage. The appearance and morphology of the theca and granulosa cells in *Cyprinion watsoni* are so strikingly different from the investing cells in the perinucleolus stage follicles that a distinction of these cell layers from what have been designated here as "prefollicular" was unavoidable. The currently available literature hardly contains information pertaining to timing of appearance of definitive granulosa and theca cells from the preexisting connective tissue cells.

Both the theca and the granulosa cells reveal a nearly similar subcellular organization. There is hardly any evidence of smooth endoplasmic reticulum which is a usual feature of cells capable of synthesis of steroids. Although a Golgi complex is not readily visible in the thecal cells, Golgi saccules are found in many places in the granulosa cells. These are usually associated with small clear and/or dense vesicles. These vesicles are often present subjacent the plasma membrane of the granulosa cells. Moderate amount of rough endoplasmic reticulum in the granulosa and a slightly more prominent network of this in the theca cells suggests protein synthetic activity. Whether, these cells undergo reorganization of organelles at a latter time remains to be checked. Kagawa *et al.*, (1981) have also reported extensive rough endoplasmic reticulum, Golgi saccules and

dense core vesicles in the granulosa cells of preovulatory follicles in *Salvelinus leucomaenis* implying lack of steroidogenic activity. van Den Hurk and Peute (1979, *Salmo gairdneri*) describe time dependent changes in subcellular transitions in respect to rough and smooth endoplasmic reticulum as well as organelles which could be associated with lack of or assumption of steroidogenic activity in the granulosa cells. A detailed examination of cortical alveolar and yolk stage follicles of *Cyprinion watsoni* according to season may be more illuminating in this context. It has not been possible to provide in the past an ultrastructural basis of steroidogenesis in the granulosa cells of preovulatory follicles in *Carassius auratus* as well (Nagahama *et al.*, 1976). In *Fundulus heteroclitus* too the ultrastructure of the granulosa cells is similar to that seen in the present work. No morphological evidence of steroidogenic activity in any period of follicular growth could be gleaned in this species by Selman and Wallace (1986). Stromal cells in the interstitial tissue and special thecal cells have been shown to have the requisite enzymatic and subcellular features which are characteristic of steroid producing cells (Hoar and Nagahama, 1978; Nagahama *et al.*, 1982). Whereas, Selman and Wallace (1986) have provided some evidence of transport of a flocculent material between the follicular cells (intercellular spaces), a direct participation of these in vitellogenin sequestration in the oocytes has not yet been evident in *Fundulus heteroclitus*. Intercellular spaces in the thecal layer are seen often in the yolk stage follicles in

Cyprinion watsoni but these are not readily visible in the granulosa layer. Instead, the granulosa cells contain intracellular vacuoles and cisternae which usually appear devoid of stored material. In *Salvelinus leucomaenis* too (Kagawa *et al.*, 1981) intercellular spaces in the granulosa layer are common. To what extent and how the granulosa and the thecal cells in *Cyprinion watsoni* participate in transport of vitellogenin to the oocyte cannot be resolved yet since these cells did not display much granular material or marked pinocytotic activity which could serve as direct evidence of the manner in which they might be involved in this process.

That the granulosa cells and the oocyte have intimate physiological association is revealed by microvillar extensions of the oocyte through the pore canals of the zona radiata into the extracellular space of the granulosa layer. The microvilli and the folded inner surface of granulosa cells come in close apposition. Small dense vesicles exist on the inner surface of the granulosa cell membrane. There is evidence that pore canals contain a pair of microvillar profiles. It would seem that microvilli also emanate from the inner surface of the granulosa cells, although a direct evidence in support of this claim could not be obtained at present. The microvilli of the oocyte and those of granulosa cells are a usual feature of vitellogenic follicles in other species as well (Hoar and Nagahama, 1978; Nagahama *et al.*, 1978; Ghaffar, 1989; Rabbani, 1989; review; Selman and Wallace, 1986). In *Cyprinion watsoni*, the microvilli appear

only in late perinucleolar stage follicles as emanations from the oocyte surface around which the vitelline envelope material condenses.

The ooplasm of perinucleolar oocyte in *Cyprinion watsoni* revealed sparse mitochondria and rough endoplasmic reticulum. These become abundant by the time the cortical alveolar stage is reached. Golgi saccules are not readily distinguishable but dilated profiles which often assume cisternal shapes are frequent in the cortical alveolar stage oocytes. These form clusters, in places, in the ooplasm and accumulate a flocculent material which forms a homogeneous matrix in the cortical alveoli. These profiles either represent dilatations of Golgi saccules or of smooth endoplasmmic reticulum and appear to be the source of cortical alveoli (yolk vesicles). In their advanced state a round nucleoid lies in this matrix. This observation appears to be in confirmity with the observations of Selman *et al* ., (1986) and Selman and Wallace (1986) in *Fundulus heteroclitus*. The presence of yolk globules in the ooplasm becomes evident in the yolk stage follicles. Although dense granules of variable size occur at the base of the microvilli of the oocytes, the exact manner of their uptake or formation could not be discerned from the ultrastructural details seen here. In the material examined no evidence of endocytosis at microvillar surface could be gleaned. Further detailed examination is essential to clarify this and other questions pertaining to yolk deposition in the vitellogenic oocytes in *Cyprinion watsoni*.

Gonadal development and environmental effects:

Gonadal development and sexual functions in fishes are governed by a variety of environmental factors. Several good reviews are available with exhaustive analysis of these factors (Donaldson, 1975; Scott, 1979; Baggerman, 1980; Lam, 1983; Bye,1987; Lam and Munro, 1987). The most widely studied of these factors are photoperiod and temperature. Both are considered to be important in synchronising ovarian and testicular functions. The relative importance of the two seems to vary not only one species basis but also according to their geographical distribution (Lam, 1983; Lam and Munro, 1987; Bye, 1987). de Vlaming (1972,1974) for the first time laid stress on the importance of seasonal history of fish in studies aimed at analysis of gonadal responsiveness to experimentally imposed photoperiod and temperature. The present work on *Cyprinion watsoni* is the only one so far in Pakistan where attempts have been made to experimentally elucidate the interplay of environmental factors in control of reproductive function. The data presented here reveal that like many temperate and subtropical species of fish, gonadal development in *Cyprinion watsoni* is controlled by both photoperiod and temperature. The responses of the fish to given photoperiod and temperature combinations vary according to season and the initial stage of gonadal maturity.

The normal cycle of *Cyprinion watsoni* comprises

gonadal recrudescence in December onward and spawning in spring to early summer followed by gonadal regression and quiescence in the succeeding months. The results reported here show that in the preparatory season (December), gonadal recrudescence can be induced in this species only by a combination of long photoperiod-warm temperature regime. Neither warm temperature alone nor photoperiod alone have any effect on ovarian and testicular development. The gonadal development at this time remains unaffected by other photoperiod and temperature combinations. A long photoperiod and warm temperature combination is generally known to have such an effect at any time of the year in several other cyprinid species as well (*Notemigonus crysoleucus*, de Vlaming,1975; *Carassius auratus*, Kawamura and Otsuka,1950; Hanyu *et al*,1983; *Notropis bifrenatus*, Harrington, 1950, 1957; *Cyprinus carpio*, Bienarz *et al.*, 1978; Davies *et al.*, 1986; Davies and Hanyu,1986). Long photoperiod and warm temperature promote gonadal recrudescence in the preparatory season in the Indian catfish, *Heteropneustes fossilis* as well (Sundaraj and Vasal, 1976). In this species temperature appears to be relatively more important than photoperiod.

In the prespawning season when photoperiod and temperature are naturally increasing, long photoperiod plus warm temperature promote gonadal development. Vitellogenesis is accelerated and even spawning occurs in the female *Cyprinion watsoni*. In the male, spermatogenesis is promoted resulting in spermiogenesis. An unusually warm temperature with short photoperiod imposed in January checks the

gametogenic progress (vitellogenesis, spermatogenesis) in this species. It tends to retard development but regressive changes do not occur. In the prespawning season long photoperiod and warm temperature have been shown to accelerate gametogenesis to an extent that spawning occurs also in the cyprinid fish *Notemigonus crysoleucus* (de Vlaming, 1975). *Cyprinion watsoni* is a spring/early summer spawner (Shaikh, 1986). December onwards, both photoperiod and temperature are increasing. In the prespawning season gametogenic development is well in progress. The present observations on *Cyprinion watsoni* in the prespawning season that the long day/warm temperature condition accelerates gametogenesis is thus not surprising. The stimulatory effect of long photoperiod and warm temperature in the preparatory period and at other times of the year is generally the case for most species of fish which normally spawn in spring and early summer (Harrington, 1950, 1957, *Notropis bifrenatus*; Baggerman, 1957, 1980, *Gasterosteus aculeatus*; Kaya and Hasler, 1972, *Lepomis cyanellus;* Smith, 1970, *Lepomis megalotis*; de Vlaming, 1975, *Notemigonus crysoleucas*; Gillet *et al.*, 1978, *Carassius auratus;* Hanyu *et al.*,1983, *Acheilognathus tabira, Carassius auratus;* Scott, 1979, *Phoxinus laevis;* Borg and van Veen, 1982, *Gasterosteus aculeatus*). Although increasing temperatures induce vitellogenesis in *Tinca tinca* (Breton *et al.*, 1980 a,b; Morowska, 1984), it is not known whether photoperiod too interacts with temperature in this species.

Short photoperiod combined with warm temperature

generally exert an inhibitory or regression effect on the gonads in the prespawning season in some species (de Vlaming, 1975; Borg and van Veen,1982; Hanyu *et al* .,1983). In *Cyprinion watsoni* however, warm temperature and short photoperiod cause retardation of vitellogenesis and spermatogenesis in the prespawning season. A regression effect did not become evident at least during the 30-day experimental period. Perhaps a longer exposure time to warm temperature and short photoperiod is needed for regression to occur in this species.

A combination of low temperature with long or short photoperiod has been reported to promote vitellogenesis and spermatogenesis but not final maturation (de Vlaming, 1975, *Notemigonus crysoleucas;* Borg and van Veen, 1982; *Gasterosteus aculeatus*). In *Cyprinion watsoni*, low temperature together with long or short photoperiod accelerates vitellogenesis and spermatogenesis. The influence is marginal or weak when low temperature is combined with long photoperiod. On the other hand, a low temperature combined with short photoperiod exerts a more marked stimulatory effect. This is very likely due to the fact that although in January and February both photoperiod and temperature are increasing, neither has yet deviated too far from the levels of photoperiod and temperature imposed experimentally. Thus, under the experimental condition of low temperature and short photoperiod the normal direction of gametogenic progress is maintained but at a slightly accelerated rate.

In the spawning season, warm temperature regardless of photoperiod induces *Cyprinion watsoni* to spawn. Since the seasonal fish possess oocytes in various stages of vitellogenesis, warm temperature in this season supports both vitellogenic growth and ovulation. Whether such a profound effect of warm temperature occurs in the male *Cyprinion watsoni* cannot be judged since the males were already in the spermatozoa (gametogenesis was complete) stage at the start of the experiment. In *Notemigonous crysoleucus*, a long day-warm temperature regime is essential to cause spawning in the postspawning season (de Vlaming, 1975). Neither long photoperiod nor warm temperature alone are effective cues. On the other hand, the common carp, *Cyprinus carpio*, is capable of maturing at low temperature in long photoperiod but ovulation and spawning occur when the temperature is raised to 24^{o}C in either 16L:8D or 12L:12D photoperiod (Davies *et al.*, 1986). Thus, temperature seems to be the important factor in stimulating spawning. In April and May, *Cyprinion watsoni* reach a near spawning or ready to spawn stage. Presumably, by this time, the minimum required exposure to photoperiod has already occurred and as soon as temperature rises in April-May (23-25^{o}C), spawning occurs. In the spawning season, thus, temperature seems to be the dominant factor in controlling spawning in *Cyprinion watsoni*. The dominance of temperature in controlling reproduction has is also been emphasized in another temperate cyprinid, *Couesius plumbeus* by Ahsan (1966).

Low temperature regardless of photoperiod tends to

retard vitellogenic progress in the early spawning season. The ovarian condition in this regime is slightly more advanced than in the control fish but less advanced than that in the warm temperature groups. Low temperature therefore seems to retard vitellogenic progress and thus prevents the females from spawning. The situation for the male *Cyprinion watsoni* is also similar. Histologically the testes in most males at low temperature in long photoperiod appeared to have completed spermiogenesis but only one male spawned. At low temperature in short photoperiod only two males spawned. It appears, therefore, that in the spawning season temperature has an important role in the spawning season. Warm temperature stimulates spawning regardless of photoperiod. Low temperature, on the other hand, retards ovarian development and checks spawning in the male. Low temperature in combination with long or short photoperiod is known to retard or merely maintain vitellogenic and spermatogenic activity in the gonads in *Notemigonus crysoleucus* as well (de Vlaming,1975). The development of the gonads does not progress through final maturation to the ovulation and spermiogenic stage in this species. Low temperature also allows gametogenic progress in the carp, *Cyprinus carpio*, also without facilitating spawning (Davies *et al.*,1986). The effect of low temperature in the spawning period as well as at other times of the year for the three-spined stickleback, *Gasterosteus aculeatus* is one of retarded ovarian development (Borg and van Veen, 1982).

In the postspawning season long photoperiod in

combination with warm temperature has little or no effect on gonadal development. A warm temperature and short photoperiod regime causes regression of the gonads, the ovaries show atresia and the testes reveal degenerative changes. In contrast, low temperature combined with long photoperiod has a noticeable stimulatory influence on vitellogenesis and spermatogenesis. The response at low temperature in short photoperiod is opposite in the two sexes. The ovaries show regressive change while the testes become recrudescent. The fact that long photoperiod warm temperature regime is ineffective in influencing gonadal development in *Cyprinion watsoni* is not consistent with the general observation that such an environmental imposition causes recrudescence in many species (de Vlaming, 1975; Borg and van Veen,1982; Lam,1983). This aspect requires further investigation since it is possible that a longer exposure is needed to elicit a recrudescent response. The recovery of the male fish from spent condition in this regime also sugggests such a direction in the future.

There is much support, however, to the present observation on *Cyprinion watsoni* that warm temperature with short photoperiod causes gonadal regression. In his earlier work, de Vlaming (1975) presented equivocal evidence regarding a regression effect of warm temperature and short photoperiod on the gonads in *Notemigonus crysoleucus*. However, de Vlaming and Paquette (1977) showed that warm temperature of 25^{o}C and higher results in gonadal regression in this species. This is also true for *Gillichthys mirabilis*

(de Vlaming, 1972) where high temperature cause inhibition of gonadal recrudesence or regression depending upon time of exposure. Gonadal regression at high temperature regardless of photoperiod or only in short photoperiod has been reported in *Lepomis cyanellus* (Kaya, 1973), goldfish (Gillet *et al.*, 1978; Hanyu *et al.*, 1983), *Couessius plumbues* (Ahsan,1966), and grey mullet (Kuo *et al.*, 1974; Kuo and Nash, 1975). There is also evidence that in the postspawning season, the ovaries in *Gasterosteus aculeatus* experience an inhibitory effect of high temperature and short photoperiod; regression was not noticeable (Borg and van Veen, 1982).

Low temperature and long photoperiod exert a stimulatory effect on early development of the gonads in *Notemigonus* at all times of the year (de Vlaming, 1975). However, final maturation is never achieved in such an environmental condition, at least during the term of the experiments. The observation that in the male *Cyprinion watsoni* low temperature with short photoperiod promotes spermatogenesis in the postspawning season also receives validation from the work of de Vlaming on *Notemigonus* (1975). Borg and van Veen (1982) have also made the general observation that low temperature in either photoperiod promote ovarian development in the stickleback, *Gasterosteus aculeatus* but at a retarded rate. The fish tend to maintain vitellogenic progress and hold mature ova. It is often argued that low temperature with increasing day length is important in stimulating early stages of gonadal progress in some species (Ahsan, 1966, *Couessius plumbeus*; Gillet and Billard,

1977, *Carassius auratus;* Breton *et al.*, 1980, *a,b, Tinca tinca;* Gillet *et al., Carassius auratus*, 1981). The present results of long photoperiod and low temperature receive support from these works. The response of the female *Cyprinion watsoni* to low temperature and short photoperiod in the postspawning season remains enigmatic. While no plausible explanations can be extended for the regressive change observed, sex differences in response to environmental factors, particularly photoperiod and temperature, are not unknown in the literature (Lam,1983). Also, the responses of cyprinids to temperature have been considered to be subtle. According to Borg and van Veen (1982) high temperature in either photoperiod regimes accelerates vitellogenesis in the three-spined stickleback in the spring but some animals show ovarian regression. Sexual differences in the gonadal response to the same photoperiod and temperature have also been noted by de Vlaming and Paquette (1977). Haydock (1971) reports that ovaries of the gulf croaker undergo regression in short photoperiod at low and high temperature but the testes remain unaffected. While these observations on other species are somewhat similar, further work is warranted to assess the response of the female *Cyprinion watsoni* to the short photoperiod-warm temperature regime.

It is evident from the information presented regarding control of gonadal development in *Cyprinion watsoni* that both temperature and photoperiod interact to guide it through its annual reproductive cycle. The response of the gonads to these environmental cues varies according to the stage

of development in which the fish are at particular times of the year. In the preparatory period warm temperature and long photoperiod appear indispensable for gonadal recrudesence. In fact, the fish in the wild leave the quiescent phase as temperature and photoperiod start increasing beyond December. In the prespawning period this effect of increasing photoperiod and temperature accelerates the vitellogenic and spermatogenic processes which culminate in final maturation of the oocyte and in spermiogenesis. Long photoperiod and warm temperature do in fact, result in final maturation of the testes and spawning in the female *Cyprinion watsoni* in this season. Low temperature imposed experimentally holds the gonadal progress in abeyance. The interaction of photoperiod and temperature at this time of the year appears prominent. In the spawning season, it appears that temperature assumes a dominant role. Warm temperature is essential to cause spawning with photoperiod playing no role. If a low temperature is imposed at this time, it tends to contain the fish from spawning.

Although the present work provides an understanding of several of the as yet unknown aspects of gonadal development and interaction with photoperiod and temperature in *Cyprinion watsoni*, some of the puzzling results especially in the postspawning season require further resolution. Additionally, cyprinid fishes in this region seem to rely on rainfall particularly for final consummation of the spawning process (Qasim and Qayyum, 1961; Bhatnagar, 1964; Swee and McCrimmon, 1966; Desai, 1973; Crivelli, 1981; Pathani, 1983;

Subhan, 1986; Lam, 1983; Lam and Munro, 1987). Shaikh (1986) has observed a second small peak in the male and female GSI of *Cyprinion watsoni* in the mansoon season (July-August) in Islamabad. Although it could not be ascertained in the above study whether this results in second spawning episode, the observation does indicate that rainfall too has some influence on the reprodutive activity of this species. For a more complete understanding of regulation of annual reproductive cycle of *Cyprinion watsoni*, thus, one needs to focus on interaction of not only photoperiod and temperature but also on other environmental cues together with hormonal control mechanisms.

Gonadal steroid and environmental effects:

Several estrogens, progestins and androgens have been identified and measured in the gonads and blood of both male and female fishes. Earlier literature in this area has been reviewed by de Valming (1974) and more recent information is available in the works of Fostier *et al.*(1983, 1987), Ng and Idler (1983), Horvath *et al.*(1978), Campbell *et al.*(1980); Scott *et al.*(1983), Kime(1979); Scott and Canario (1987), Kime and Hyder (1983) and Scott and Sumpter (1989). Their absolute and relative concentrations in the circulation and in the gonads seem to vary depending on the species and sex. Their differential roles too vary in relation to local developmental events in the gonads, the physiology and behavior of the individual as well as according to seasons (see Fostier *et al.* 1983; Rosenblum *et al.* 1987). The availability of limited resources at present not only prevented estimation of estradiol, progesterone and testosterone in both sexes of *Cyprinion watsoni* but also determinations of profiles of other biologically active steroids. Although the levels of three steroid hormones reported here are low, they do vary seasonally.

Both estradiol and progesterone increased from basal levels in the preparatory period (December) to a peak with onset of the spawning season (April/May) before declining again in the postspawning phase of the cycle in June onwards.

Testosterone in the testis shows a similar trend in seasonal variation. Plasma testosterone in the male also appears in phase with its seasonal rise and fall in the testis. Plasma progesterone too increased through the prespawning period. However, it continue to rise in the postspawning season reaching a peak in June and ultimately dropped to basal level. Such a cyclic change in the steroids is generally consistent with the well known scheme of events involved in environmental and hormonal mediation of seasonal gonadal rhythms in fishes via the hypothelamo-hypophyseal axis (Peter, 1981; Fostier *et al.*, 1983). Some species differences in absolute levels as well as the temporal relationships of the steroidal hormones at specific times during the gonadal cycle are to be expected. This is, indeed, evident from a survey of the available literature. Scott *et al.*(1984) have reported plasma testosterone in the male white sucker, *Catastomus commersoni*, to vary between approximately 2 ng to less than 15 ng/ml and estradiol in the female between nearly 2 ng to less than 10 ng/ml during the reproductive cycle. In *Ictalurus nebulosus* (bullhead catfish), Rosenblum *et al.* (1987) have described that testosterone in the male ranges between 600 pg/ml to nearly 3.0 ng/ml. In the female estradiol varies between 450 pg to 6.5 ng/ml. Plasma levels of estradiol in the female common carp, *Cyprinus carpio*, have been reported to be as low as 700 pg/ml (Down *et al.*,(1987) in spring to early summer. Similar variations in estradiol and testosterone concentrations and the levels of progestins have been described by other workers as well. (Tamaru and Lee,

1987, *Chanos chanos*; Thomas *et al.*, 1987, *Cynoscion nebulosus*; Brighty *et al.*, 1987, *Leuciseus leuciseus*; Harmin *et al.*,1987, *Pseudopleuronectes americanus*; Chapman *et al.*, 1987, sturgeon).

In *Cyprinion watsoni*, the seasonal levels of gonadal estradiol, progesterone and testosterone in the female and the male respectively could not be studied on monthly basis. Therefore a complete resolution of magnitude of change during the annual cycle has not been possible. Basal levels occurred in December when gonadal recrudescence begins and a peak is achieved in April when the GSI was highest and the gonads become ripe or nearly ready to enter the spawning phase. It cannot be said whether further rise would occur in May as well. Both estradiol and testosterone dropped to near basal levels in June (early postspawning phase). Progesterone fell further in October and testosterone in this month stayed near the June level. On the other hand, estradiol in October nearly matched the January (Prespawning) level when vitellogenic growth of the oocytes had already begun. It is difficult to predict how the level of estradiol in the plasma would vary in relation to the ovarian profiles. Plasma testosterone appeared in phase with the gonadal stages, but plasma progesterone continued to rise even in the postspawning period. Species differences in the timing of seasonal rise and fall in steroid levels in relation to the ovarian and testicular development have been recorded in many studies.

In *Ictalurus nebulosus* (Rosenblum *et al.*,1987), basal levels of plasma testosterone in the male and estradiol in the

female occur in the quiescent period, the levels reach a peak prior to spawning followed by declines in the spawning and postspawning periods. However, year to year variations in this relationship have also been noted in the same study especially in the estradiol profiles. Inverse relationships between the stage of gonadal development and hormone levels have also been observed at particular times of the gonadal cycle, the physiological significance of which remains to be fully assessed (Rosenblum *et al.*, 1987). In *Cyprinus carpio*, estradiol achieves a peak in the spawning season (Kime and Dolben,1985) as is also true for the dace (Brighty *et al.*, 1987). A role of estradiol in oocyte maturation has, thus, been suggested. In *Cyprinion watsoni* too ovarian estradiol elevated again in October. At this time the ovaries contain mostly cortical alveolar and younger stages but residual vitellogenic stages are rare. The seasonal oscillations in the estradiol level in the ovary of this species are otherwise generally consistent with its known major physiological commitment in synthesis and uptake of exogenous vitellogenin (Fostier *et al.*, 1983; Wallace *et al.*,1987; Goetz, 1983). The relationship of gonadal and plasma testosterone levels with spermatogenic, spermiogenic and spawning stages as seen in *Cyprinion watsoni* receives support from many studies (See review Fostier *et al.*, 1983). Progesterone, on the other hand, is known to rise during the prespawning period (vitellogenic period) and continues rising beyond ovulation in some species but in others it begins rising in the final stage of oocyte maturation and

stays up for sometime in the postspawning period (Fostier *et al.*,1983). The seasonal pattern of ovarian and plasma progesterone in *Cyprinion watsoni* needs to be investigated in greater detail to fully appreciate its relationship with the process of vitellogenesis and final oocyte maturation in the light of recent *in vivo* and *in vitro* investigations (See reviews, Goetz,1983;Scott,1987). The relatively high concentration of progesterone in the plasma in *Cyprinion watsoni* in the spawning period seem to have significance in respect to a role in final oocyte maturation but furhter rise in the postspawning season cannot be explained fully.

The ultrastructural details presented in a previous section of this work have brought out some interesting features of follicular development and which have relevance to the potential of the ovary in biosynthesis of steroid hormones at particular times during the ovarian cycle. In the preparatory period, at best, a few early vitellogenic stage oocytes exist in the ovary of *Cyprinion watsoni*. The theca and granulosa do not seem to defferentiate until the beginning of vitellogenic or late cortical alveolar stage oocytes (see earlier section). In the late postspawning period once again mainly postovulatory follicles, perinucleolus stages fewcortical alveolar stage oocytes and some residual yolk stage follicles persist. Thus, important questions arise regarding the tissue source of estradiol in the ovary in the preparatory season. The same pertains to the relationship of progesterone and sites of its synthesis particularly since only equivocal views prevail currently about the status of

corpora atretica (postovulatory, preovulatory) and their role in steroidogenesis (Fostier, *et al.*, 1983).

Hormonal correlates and photoperiod-termperature effects:

It was of further interest in the light of seasonal profiles of gonadal steroids reported here to determine the ovarian and testicular hormone levels in fish subjected to the experimental photoperiod and temperature regimes. It was assumed that the levels of estradiol, progesterone and testosterone would show some coincidence with the observed developmental changes in the gonads effected by the imposed environmental regimes. While it has been possible to record the absolute levels, direction and magnitude of change in individual steroids in the male and the female *Cyprinion watsoni*, not all results turned out to be compatible with the responses of the gonadal developmental stages in given photoperiod-temperature regimes especially when the results are viewed against the seasonal pattern of changes for this species. As stated earlier the ovary and the testis in fish are known to synthesize several biologically active estrogens (Fostier *et al.*, 1983), progestogens (Scott and Canario, 1987) and androgens (Kime, 1979; Fostier *et al.*, 1987). The timing of their synthesis and their physiological interrelationships are too complex to enable one to fully comprehend the hormonal basis of the developmental changes observed in this study merely in the light of variations in estradiol, progesterone and testosterone (see also Scott

and Sumpter,1989).

In the present study estradiol levels varied coincidentally with the vitellogenic progress of the ovary in the long photoperiod and warm temperature regime in the preparatory season. This is consistent with the known role of estradiol in vitellogenesis in fishes (Fostier *et al.*, 1983; Goetz, 1983) and provides support to the observation that long photoperiod and warm temperature promote vitellogenesis. Its level also elevated slightly in the other regimes but the increase is insignificant. Neither the GSI nor the histology of the ovary changed significantly relative to the seasonal control. Since the gonadal stage did not change substantially in these latter environmental combinations, it would seem that the magnitude of change is insignificant to promote vitellogenesis. The relationship of estradiol is also compatible with the observed progress of vitellogenesis in long photoperiod-warm temperature regime in the prespawning season. The level of estradiol is known to drop to basal levels prior to ovulation or prior to final maturation depending on the species (Scott *et al.*, 1983, trout; Scott *et al.*, 1984, white sucker; Van Bohemen and Lambert, 1981, trout; Rosenblum *et al.*, 1987, bullhead catfish). Significantly elevated levels of estradiol in the above environmental regime when some of the fish had spawned, raise the possibility that estradiol in *Cyprinion watsoni* may also be involved in the final maturation of the oocytes. Persistence of estradiol well into the spawning period has been observed in several species of fishes during their natural annual

reproductive cycle (Scott *etal.*, 1983, trout; Stacy *et al.*, 1983, goldfish; Kime and Dolben, 1985, *Cyprinus carpio*). The estradiol level increased significantly in short photoperiod-warm temperature when the vitellogenic oocytes were the smallest in size. No significant change occurred at low temperature in short photoperiod in spite of the fact that these oocytes were the largest and the GSI also was significantly higher than at warm temperature. Although from a statistical point of view these results would appear discordant, the magnitude of change in the GSI or histology and in the level of estradiol in the short photoperiod regimes is not too great to cause undue concern. The fish at warm temperature in both long and short photoperiod in the spawning season had spawned. The estradiol level remained statistically comparable to the control group although in short photoperiod the drop was substantial. No significant change in estradiol occurred in long photoperio- low temperature as well as in short photoperiod low temperature where vitellogenic progress was retarded relative to the warm temperature fish in either photoperiod. Since vitellogenesis had already occurred and the ovaries were spent, the estradiol picture appears commensurate with the view that its levels are expected to be low in spawned fish. In fact, the substantial drop at 25^{o}C in short photoperiod is noteworthy in this context. Lack of significant changes at low temperature in either photoperiod relative to the control also appears compatible. There is, however, indication of subtle effects of warm and low temperature in either

photoperiod (see Table 3) on estradiol levels. In the postspawning season, estradiol increased significantly only in long photoperiod-warm temperature. At low temperature in this photoperiod and in other regimes it remained statistically comparable to the situation in the control fish. This is rather interesting. In relation to effects of environment on maturation it has been argued that long photoperiod-low temperature is more favourable for *Cyprinion watsoni* (see earlier section), a contention which does not receive support from the observed estradiol profile. In fact, the estradiol profile favours the argument that a long photoperiod and warm temperature promotes maturation in temperate fishes in the postspawning season as demonstrated by de Vlaming in *Notemigonous crysoleucus* (1975) and Borg and van Veen in *Gasterosteus aculeatus* (1982). This discordance needs to be resolved in future investigations. The estradiol profiles for fish exposed to short photoperiod at either temperature in this season are compatible with the regressed ovarian condition. Progesterone in the preparatory season increased in the ovary in all regimes. However, a significant increase characterized the regressed ovaries in the short photoperiod-warm temperature condition only. This raises the possibility that corpora atretica may be involved in progesterone synthesis at this time (see latter discussion). In the prespawning season a rise in progestrone accompanied the elevation in estradiol in the long day-warm temperature environment where vitellogenesis was accelerated in some fish and spawning had occurred. In other regimes, the GSI, the

ovarian stages and the progesterone levels remained nearly unaltered. In the spawning season a significant increase in progesterone occurred in the short photoperiod-low temperature regime where the oocytes were in early vitellogenic phase and estradiol level also showed some elevation. If progesterone, in *Cyprinion watsoni* too, plays a role in final oocyte maturation or ovulation one expects elevated levels in the warm temperature environment in either photoperiod. The observed relationship of progesterone with ovarian development in these other regimes excepting long day-low temperature in this season appears inconsistent with both the seasonal picture and its known role. In the postspawning season, progesterone elevated though insignificantly in long photoperiod at warm temperature as well as in the two photoperiod-low temperature conditions where vitellogenic progress was evident. This seems consistent with the stage of ovarian development. The marked elevation in fish with regressed ovaries again invites attention regarding its tissue source and physiological role. Progestogens are generally considered to be strongly implicated in the final maturation of oocytes (Nagahama, 1983; Zhao and Wright, 1985; Scott and Canario, 1987; Goetz, 1983; Wallace *et at.*, 1987). Progesterone has been shown *in vitro* to induce germinal vesicle breakdown in several species of fishes (Epler, 1981, *Cyprinus carpio*; Fostier *et al.*, 1978, *Salmo gairdneri*; Zermonsky and Yaron, 1986, *Cyprinus carpio*; Ghaffar, 1989; Ghaffar and Hafeez, 1989, *Bariluis vagra*) although 17 α-20β dihydroxyprogesterone appears to be

the most potent maturation inducing progestogen (Scott, 1987; Goetz, 1983). The observed elevation in progesterone level in parallel with the observed final maturation and spawning in *Cyprinion watsoni* in the prespawning season would appear in agreement with the above understanding. However, progestogens including progesterone have also been reported to show little or no change (Fitzpatrick *et al.*, 1986, coho salmon) or remain elevated throughout the seasonal progression of the ovarian cycle in many species of fish (Scott *et al.*, 1983, 1984, *Salmo garidneri, Catostomus commersoni*; Fitzpatrick *et al.*, 1986, coho salmon; Scott and Sumpter, 1989, trout). Thus, it is difficult to rationalize many of the other observed changesin progesterone level in the various photoperiod-temperature regimes in the different seasons with the stages of ovarian development. To what extent some of the results of this study relate with the unique position of progesterone in the biosynthetic scheme of ovarian steroids cannot be resolved without more detailed work. The coincidence between elevated progesterone levels and ovarian regression raises interest incorpora atretica (preovulatory or postovulatory) as tissue source of this hormone. At present the evidence that corpora atretica may be the sites of its synthesis Browning, 1973; Guraya, 1976; Fostier *et al.*, 1983) remains, at best, equivocal (Lang, 1981). In regressed ovaries where, in addition to atretic follicles, perinucleolus and cortical alveolar stages are present, the interstitial tissue and the special prefollicular cells described in the present study may produce this hormone.

In the preparatory season, testosterone increased in parallel with acceleration of spermatogenesis in long photoperiod and warm temperature. In other photoperiod and temperature combinations too, it showed slight increase which seems to be associated with a progressive shift in spermatogenesis. In regressed testes (short day/25^{o}C) this steroid remained unaltered. This is compatible with the observed effect of short photoperiod at warm temperature and in accordance with the known role of androgens in spermatogenesis (Fostier *et al.*, 1987). In the prespawning season, elevation in testosterone more or less occurred in all combinations of photoperiod and temperature. In the light of the known role of testosterone in spermatogenesis maximum increase is expected to occur in the long photoperiod and warm temperature fish. Although the significant elevation in its level in short photoperiod-low temperature condition is in keeping with the observed spermatogenic progress, it is difficult to justify the situation in the long day-warm temperature or short day-warm temperature fish. In the spawning season, a significant drop in testosterone occurred in spent fish in short photoperiod at warm temperature and a significant elevation at low temperature. Thus a drop in this hormone appears coincident with spent stage. Elevated level of this hormone parallels inhibition of spawning (short day-low temperature). Lack of change in testosterone in long photoperiod appears to result from some subtle effect of long photoperiod and low temperature but the possibility that other hormones may be involved cannot be ruled out.

Testosterone is known to drop to basal levels beyond spermiogenesis in many species and in others to stay up during the active spawning phase or even in the postspawning period pointing to additional roles in more distant physiological or behavioral functions (Godovich *et al.*, 1982, carp; Scott and Baynes, 1982, rainbow trout; Scott *et al.*, 1984, white sucker; Liley *et al.*, 1986, rainbow trout; Pankhurst and Conroy,1988, orange roughy). In the postspawning season, testosterone increased significantly with spermatogenic progress in the long photoperiod and low temperature regime confirming the earlier assertion that such an environmental combinations supports testicular recrudescence in the postspawning season. What hormonal factors are responsible for spermatogenic progress and significant increase in the GSI of short day-low temperature fish cannot be explained in terms of testosterone level alone which remained unchanged in this regime. Other androgens need to be examined. Whereas the insignificant change in testosterone in long day warm temperature is coincident with the histological make up of the testis, the elevation in the regressed testes in the short day warm temperature fish finds no simple explanation. It must be realized, however, that developmentally incompatible steroid hormone levels are not unknown for fish cycling in the natural environment (Scott and Sumpter, 1989).

The observations on seasonal hormonal variations reported in this study in relation to the gonadal rhythm in *Cyprinion watsoni* constitute a useful addition to the existing information on reproductive biology of this species.

However, a more comprehensive understanding of the hormonal basis of seasonal variations in the gonads as well as a critical assessment of results of the experimental study requires focus on other steroids which may be biologically active in *Cyprinicn watsoni* and would vary concomittantly or sequentially under given conditions. Both estradiol and estrone are present in the ovary and the testes although only in low levels in the latter. 17α-hydroxyprogesterone and 17α-20β hydroxyprogesterone exist in both sexes. These progestogens are strong contenders as maturation inducing steroids in the ovary (Goetz, 1983; Scott and Canario, 1987). Of the androgens, testosterone, 11-Ketotestosterone and androstenedione are the most widely studied steroids and exist in both the ovary and the testes. Testosterone has been shown *in vitro* to be one of the most potent oocyte maturation inducing steroids (Nagahama, 1983; Zhao and Wright, 1985; Scott and Canario, 1987). All of these steroids have been described to vary with the gonadal stage of development and control specific local events in the gonads. They are also known to regulate particular peripheral metabolic, physiological and behavioral manifestations. Scott and Sumpter (1989) have described sequential appearance of testosterone, 11-Ketotestosterone and 17α-20β dihydroxy-progesterone during the annual sexual cycle of the male *Salmo gairdneri*. They have further noted that testosterone and 11-Ketotestosterone levels remain unrelated to the testicular developmental stages while 17α-20β dihydroxyprogsterone rises coincidentally with spermiation in the male. The

studies of Thomas *et al.* (1987, *Cynoscion nebulosus*), Tamaru and Lee (1987, *Chanos chanos*), Chapman *et al.* (1987, Sturgeon), Brighty *et al.* (1987, *Leuciscus leuciscus*), Liley *et al.* (1987, *Salmo gairdneri*, behavior), Pankhurst *et al* (1986) and Pankhurst and Conroy (1988, Orange roughy) are particularly illuminating in the context of marked variation in plasma androgens, progestogens and estrogens in relation to the state of development of the gonads of fish in their natural environment. The information avialable in the above studies provides ample justification to examine in greater detail the relationships of the various steroid hormones and their role in regulating the annual reproductive cycle of *Cyprinion watsoni*.

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