General Materials and Methods

II. GENERAL MATERIALS AND METHODS

A. The Experimental Animal

The experimental animal for the present study is the fish *Etroplus suratensis*. It originated from South Asia. It belongs to the family Cichlidae. It is also called Green Chromide and Pearlspot. It is euryhaline and inhabits in the coastal states of India. The euryhaline nature tends to dwell Pearlspot and grow better in low saline fresh water conditions. It is a potential fish species for aquaculture because of its big size and high market demand.

It feeds on algae, plant material and insects. It attains an average length of 22 cm and weighing about 250 g. The fish is caught mainly using gillnets. Regarding diet, quality flakes and pellets can provide the staple diet for the fish. Vegetable matter should also be given in the form of spinach, peas, and lettuce. It will also accept blood worms and brine shrimp. Regarding sexing, there are no visible differences between the sexes. Regarding breeding, these fish are classed as open water spawners but may use caves as their spawning sites. Normally the eggs are deposited on a flat rock which has previously been cleaned by the parents. The eggs hatch after 36 + hours and the fry will be free swimming a few days later. The fry can be fed on newly hatched brine shrimp, rotifers or crushed flake. The expected life span for this fish is 5-8 years. This fish can tolerate freshwater for short periods of time but prefers brackish conditions. Uses fine sand for the substrate and make hiding places with caves or wood. It also needs open swimming spaces.

B. Taxonomy of *Etroplus suratensis*

The taxonomic position of *E. suratensis* is described as below:

Kingdom: Animalia

Phylum: Vetebrata

Subphylum: Craniata

Super class: Gnathostomata

Series: Pisces

Class: Teleostei

Sub class: Actinoptergii

Super order: Acanthoptergii

Order: Perciformes

Sub order: Labroidei

Family: Cichlidae

Genus: *Etroplus*

Species: *suratensis*

C. Biology of the Animal

The body of the fish is deep and laterally compressed, mouth small and terminal with a small cleft. Snout is spout-like, eyes large and lateral, lips thin and jaws equal. The teeth of the fish are observed as villiform and are present on both jaws. Palate is edentulous. Dorsal fin is inserted above the pectoral fin base; caudal fin is emarginated while pelvic fin is characterized with one spine. Scales are weakly ctenoid; the lateral line is interrupted and there after continuing as small open pores in each side.

Body is light greenish with eight yellowish oblique bands, the first passing through the occipital part of head and last across base of the caudal. Scales above lateral line have a central pearly spot; and possess some triangular black spots on the abdomen. Fins, except the pectorals are of dark leaden colour while the pectoral fin is yellowish with jet black base. Anal fin possesses 12-13 spines and 11 or 12 soft rays. Specimens from salt waters have a deep purple colour and bands are almost black. Fingerlings possess a conspicuous eye-spot (ocellus) on the dorsal fin.

D. Distribution

The family cichlidae is comprised of over 700 species of fishes found in freshwater as well as brackish water habitats in Africa, South America, North America and parts of Asia. The larger African lakes of Malawi, Victoria and Tanganyika contain about 500 endemic species of Cichlids (Fryer and Ilnes,, 1972). *Etroplus* is the only Cichlid genus endemic to peninsular India and Sri Lanka. The two well known species are *E. suratensis* and *E. maculates.*

In Sri Lanka, *E. suratensis* forms an important proportion of the catches of reservoirs (Amarasinghe and Samarakoon, 1988). In India, it is distributed widely in brackishwater and freshwater enenvironments from Goa on the west coast to Chilka Lake on the east coast. It has also been introduced into the different interior watersheds like dams, reservoirs, freshwater lakes and tanks from early times. As a result, at present, the species is available in coastal water bodies of Maharashtra on the west coast and West Bengal on the east coast.

The two well endemic distribution and abundance of *E. suratensis*, a cichlid endemic to peninsular India and Sri Lanka has been reported by several authors (Munro, 1955; Jhingran and Natrajan, 1969; Fernando and Indrasena, 1969; Ward and Wyman, 1975; De silva and Fernando, 1983; Chandrasoma, 1986; Amarasinghe and Samarakoon, 1988; Sultana *et al.*, 1995; Unnithan *et al.*, 2001; Padmakumar *et al.*, 2002). The biological attributes of *Etroplus* in diverse ecosystems has also been extensively studied by various authors (Menon and Chacko, 1956; Alikunhi, 1957; Hora and Pillai, 1962; Jhingran and Natarajan, 1969; Prasadam, 1971; Devraj *et al.*, 1975; Sundararaj and Krishnamurty, 1975; Jayaprakash, 1980; De Silva *et al.*, 1984; Jayaprakash and Padmanabhan, 1985; Keshava *et al.*, 1988; Ushakurmari and Aravindan, 1992). Although extensive investigations on

various aspects of the breeding biology of pearl spots have also been undertaken (Prasadam, 1971; Thampy, 1980; Jayaprakash and Nair, 1981; Raju *et al.*, 1987; Sumitra Vijayaraghavan *et al.*, 1981; Krishnann and Diwan, 1990) utilization of these information for captive breeding has rarely been accomplished. Studies on the breeding of *H. brachysoma* have been attempted earlier except for some recent studies on reproductive biology (Kurian and Inasu, 2003; Chandran and Prasad, 2006).

E. Feeding

An appreciable rate of growth under a feeding regime, palatability, good non-predaceous habit, highly adaptable feeding habits with ability to grow in compounded feeds, robust and sturdy body and good market price are some of the favourable characters of this fish as a candidate species for brackish water aquaculture.

The food preference of *E. suratensis* has been studied by several authors (Ushakumari and Aravindan, 1992; Sultana *et al.*, 1995). Undoubtedly food and feeding habits indicate the species niche in the ecosystem, their food preference and food spectrum overlaps. As observed in the study, the predominant component of feed of Pearlspot do not differ and there has been little significant seasonal variation as regards major food item. In herbivorous fishes, seasonal fluctuations in dietary component may not be marked, as aquatic vegetation that dominates the diet of such fish is always abundant in the environment. According to Hora and Pillai, 1962 Pearlspot consume blue green algae as food. Jhingran and Natarajan, 1969 reported that in addition to vegetable matter, the fish accept a variety of food items such as copepods, cladocerans, insects and worms. The food preference of *E. suratensis* was reported to vary significantly with reference to size (Jayaprakash, 1980; Jayaprakas and Padmanabhan, 1985), while fingerlings and juveniles prefer an omnivorous diet, the adults and matured fishes are predominantly herbivorous.

Alikunhi, 1957 reported that the young ones of *E. suratensis* feed almost exclusively on zooplanktons while the advanced fry thrive on aquatic insect larvae, filamentous algae and other vegetable matter and the adult fish subsists mainly on filamentous algae, aquatic macro vegetation and planktonic organisms. Apparently, the food of the fish changes as it grows from larva to adult. Devaraj *et al.*, 1975 gave a comparative account on the food of juveniles of *E. suratensis* collected from the estuarine and fresh water habitats and observed that the fish in both the environments feed on a wide variety of items such as filamentous algae, detritus, micro crustaceans, insect larvae and plant materials. Ward and Samarakoon, 1981 reported that *E. suratensis* is a complete herbivore. De Silva *et al.*, 1984 however opined that *E. suratensis* is predominantly a macrophyte feeder and most certainly not a herbivore. The high concentration of detritus in the diet of *E. suratensis* in Vembanad lakes was indicative of selective preference for detritus. Bowen, 1981 has observed that detritus play a significant role in the diet in freshwater systems. Several earlier authors also observed preponderance of aquatic weeds followed by detritus and algae in diet of fish is apparently linked to availability of food in the habitat (Bhatnagar and Karamchandani, 1970).

F. Reproduction

a. *Sexual Dimorphism*

Pearlspot, *E. suratensis* is heterosexual and is gonochoristic exhibiting external fertilisation. Fish is monogamous and identification of sexes possible only during the breeding season. Like other chchlids such as *Oreochromis* spp., *Etroplus suratensis* exhibits some degree of sexual dimorphism, males being larger than females of equivalent age. Just prior to spawning, the males become deeply coloured and the colour bands become strongly marked with a greenish blue iridescence and pearly white spots. The

rayed portions of the dorsal and anal fin also become slightly reddish. Sexual dimorphic features during the breeding season are summarised as follows:

b. Male

In male, body coloration and iridescence become more intense close to spawning. The sexually motivated males often develop black occipital stripes between the eye and opercula spot. The genital papillae are thin and pointed.

c. Female

Generally females are small as compared to males of the same age with a muddy yellowish colour. During the time closer to breeding season, the genital papillae become larger, broader, reddish, swollen and appear modified into an ovipositor.

d. Stages of Testis in Male *Etroplus suratensis*

There are five stages in the testis development of the male. At immature stage (stage I), the testes are long, thin, elongated, thread- like and not easy to separate from viscera. Light pinkish in hue, translucent and 50 to 84 mm in length. At maturing stage (stage II), the testes are slightly enlarged, flesh coloured and opaque measuring about 55 to 87 mm in length. At maturity (stage III) the testis become creamy white in colour and is opaque. The length of the testis ranges between 60 to 100mm. During ripened stage (stage IV), the testis is dull pinkish in colour and measures 80 to 112 mm in length. The spent (stage V) testis are shrunken and dull reddish in colour and measure 50 to 57 mm.

e. Stages of Ovary in Female *Etroplus suratensis*

The reproductive structure of females shows five stages as like males. At immature stage (stage I), the ovaries are small, thin elongated and somewhat cylindrical and translucent with a reddish yellow hue and occupy less than a third of body cavity. The ova are not

distinct. The length of the ovary ranges form 30 to 45 mm and the largest mature ova falls at l mm.

In the maturing stage (stage II), the ovaries occupy one third of the body cavity. They are slightly larger and pale yellow in colour. Contain spherical and opaque ova owing to the commencement of yolk deposition. Length of the ovary varies from 35 to 52 mm. The diameter of the ova ranges form 0.5mm to 1.75 mm and the mode of the largest maturing ova falls at 1.75 mm.

In mature stage (stage III), the ovaries are yellowish brown in colour and occupy approximately half of the body cavity. The ova are quite distinct, yolk laden, with ovarian wall distended and semi-transparent. Ovary length is in the range of 35 to 65 mm. The largest maturing ova fall at 25 mm.

In ripened stage (stage IV), the ovaries are much broader and distended and occupy more than half of the body cavity; brownish in hue with a ramification of blood vessels. The ovary has an envelope which is distended, thin, delicate and easily rupturable. The ovary measures about 40 to 70 mm. Mode of the largest ova falls at 2.75 mm.

In the spent stage (stage V), the ovaries appear shrunken and flaccid with a few scattered blood patches in fresh condition. They were yellowish brown in colour, contained a few large eggs along with numerous small eggs resembling stage III. The ovary measures 38 to 60mm and the mode of the largest ova falls at 1.5 mm.

f. Gonadal Morphology and Breeding Behaviour

It was observed that the variations in morphology of gonad ware linked to the breeding habit of the fish (Billiard *et al.*, 1982). Large testis and high GSI values are characteristic to species presenting sperm competition where as males of species that invest energy in

parental care will have a small testis (Valdes *et al.*, 2004). The tubular testis in *E. suratensis* also appears to be linked to their unique habit of parental care and monogamy.

g. Breeding Behaviour

The breeding behaviour of Pearlspot is complex involving courtship, pairing, nest-making and parental care. Panikkar, 1920, 1924; Samarakoon, 1985 reported on the breeding behaviour of Pearlspot. The various steps involved in the breeding process are given in detail below:

h. Pair Formation

According to Samarakoon, 1985, before pair formation, the fishes form several groups, the largest group with 20-30 members and the smallest with 5-8 members. During the breeding season, courtship commences between some members of the groups and pairs are formed. The above author also noted that pair formation was independent of the availability of spawning surfaces.

i. Nesting Behaviour

The Pearlspot *E. suratensis* exhibits interesting nesting behaviour. The process involves selection of nesting materials and preparation of the nest for laying the eggs.

j. Nesting Materials

Mature Pearlspot spawns on prominent objects which are hard in texture to facilitate attachment of the eggs, if the nesting site is devoid of vegetation. Stones, pieces of wood, coconut husks, water-logged coconuts, petioles and mid-ribs of coconut leaves and palm leaves, tiles, bamboo pieces, asbestos sheets or any other hard objects which are submerged at a depth of not more than 100 cm of water are preferred for egg laying. In ponds, the nesting materials should be kept all along the sides of the pond, about 50 cm from the shore (Samarakoon, 1985)

k. Nest Making

The spawning behaviour of Pearlspot has been described in detail, by Panikkar, 1920. After the completion of pair-formation, the pair-bonded fishes search for a suitable object to lay the eggs. If the nesting object is not sufficiently raised from the ground for convenience of spawning movements, the fish makes a cup-like excavation in the ground just below the selected spawning surface, by digging in the mud out with the mouth. Although both the sexes participate in nest making, the contribution of the male is greater. The whole process of selection of the spawning site and its preparation for the reception of the ova takes about 3 to 5 days.

Pair bonding among the mates was stable and not momentary apparently mate selection was guided by some specific attributes characteristic among members and find choice pairs of appropriate attributes if it was felt necessary to increase the size of the groups. The paired mates clear off algal growth from the nesting substrate, by browsing with their truncated cone like snout. The modified mouth also helped in suction feeding. The female fish was found to probe the artificial nesting substrates with the genital papillae and spontaneously spawn with one or two trial runs, using the modified cup like ovipositor. At this point of time, the male and female positions parallelly one behind the other, lay flat to the spawning surface and then began to attach their adhesive eggs on to the substrate. After the female has completed attaching one or two rows of eggs, the male following close behind spontaneously hover over the egg mass deposited by the female and fertilizes the eggs by sprinkling milt over the egg patches. Duration of sperm motility was found to vary form 3 to 4min. Eggs are never laid on top of other eggs but are concentrated in a single layer so that one egg just touched the other.

When eggs were incubated separately, without parental patronage, hatching of eggs occurred in 70-72 h under a temperature regime of 25-27°C. The hatching was protracted and the whole brood was observed to hatch out in a long interval of 24 to 26 hrs after the first hatching. The hatched out larvae, or the 'wrigglers', were heavily yoked observed to sink to the bottom. They were found to instinctively congregate on the tank floor. In the tank incubation, involving parent fishes, fanning and mouthing of the brood were continued. During parental care, the fish responded even to the slightest disturbance and was found to remove all the eggs away from the substratum when bothered. Eggs were also removed at times when the water became more turbid. Parents continued to maintain a constant current of water over the pits by fanning with fins and were found to clear off any adhering foreign particles on to the larval body.

G. Spawning and Larval Rearing

a. Spawning Season

Pearlspot breeds freely in both brackishwater and freshwater, almost throughout the year with a peak period during December to February (Alikunhi, 1957). It breeds in brackishwater throughout the year and in freshwater, during the dry season (Hora and Pillay, 1962). Several workers reported on the breeding season of Pearlspot from different areas. Raj (1916) reported that the breeding season in Madras extended from

April to May, in Travancore from May to June, and from November to February. Jones, 1946 observed that breeding occurs during July and August in river Adyar, Madras and during May and June, in Travancore. Ganapati *et al.*, 1950 reported that the fish breeds throughout the year in the Madras region, with a maximum from November to February. Menon and Chacko, 1956 also reported that breeding occurs throughout the year in Adyar with a peak immediately after rains.

The fish breeds twice a year along the Malabar coasts once from May to June and again from November to February (Panikkar, 1924). Thampy, 1980 observed two peak breeding seasons in the Kerala backwaters, the main one from February to May and the other from October to November, after the south-west monsoon. The spawning seasons in Veli lakes, Trivandrum, more or less correspond to the two monsoons experienced along the south-west coast, the south west monsoon from June till August and the north-east from October to January (Jayaprakas and Nair, 1981). The peak breeding season in Poothota, near Cochin, occurs in December with a secondary peak in June and July (Krishnan and Diwan, 1990).

Jhingran and Natarajan, 1969 observed the occurrence of maturing and mature specimens throughout the year in Chilka lakes and the breeding shows two peaks, one from December to February and the other from April to May. Kowtal, 1976 also reported that the fish breeds round the year in Chilka lakes with peaks in summer and winter seasons.

The occurrence of a large number of mature and ripe specimens from January to April and from September to early November in the Kali estuary, Karwar was reported by Raju *et al.*, 1987. Keshava *et al.*, 1988 observed that the spawning season in the Nethravati-Gurpur estuary extended from August to November and again from January to February. Pearlspot inhabiting Colombo lakes, Sri Lanka was a multiple spawner (Pathiratne and Costa, 1984).

H. Larval Development

The larval development of the Pearlspot *E. suratensis* was studied by Raj, 1916; Jones, 1937 and Varghese, 1976. The description based on Panikkar, 1920 is given below.

I. Eggs

The eggs are oblong in shape, about 1 to 2 mm in diameter, attached at one end by means of a short stalk to the nesting object. The newly laid eggs are yellowish in colour and as the embryo develops, the colour becomes brownish and the yolk sac becomes pigmented.

J. Hatching

The incubation period lasts from 82 to 100 h. During hatching, the egg membrane bursts first over the head of the larvae which is at the free end and this continues along the upper side by the waving of the tail. For some moments, the newly hatched larvae remain attached to the egg membrane by means of their cement organs.

K. Early Larval Stage

The early larval stage lasts for 7 days from the day of hatching and is described below.

a. First Day

The newly hatched larva measures about 5 mm in length. It has neither mouth nor gills or fins. The yolk is unpigmented, but the yolk sac is richly pigmented. The auditory organ is distinctly developed. The larvae group together in the centre of the pit and vibrate their tail continuously. This phenomenon lasts till the yolk sac is fully absorbed.

b. Second Day

On the second day, the eyes become pigmented.

c. Third Day

The larvae measure about 6 mm in length. The mouth opens and yolk sac is reduced to half of its size. The cardiac region of the yolk sac is dilated. Heart beats more vigorously and respiratory movements begin.

d. Fourth Day

Pectoral fins appear. Pigmentation appears on the back of the body in two centres.

e. Fifth Day

The yolk sac is almost absorbs, dilation in front of the yolk sac disappears. When the larvae move, the yolk sac lies on one side.

f. Seventh Day

The larvae measure about 7 mm. in length Yolk sac is completely absorbed. Tail fin is continuous with dorsal and ventral fins. The larvae move out of the pit, guided by the mother. Cement organs disappear.

L. Late Larval Stage

The larvae, though free swimming, are quite different from the adult. The tail remains long, the caudal fin continuous with dorsal and anal. After a fortnight, the primary chromatophores on the back disappear and permanent colour bands begin to appear. The larvae assume adult form within a month after hatching and measure about 18 mm. in length.

M. Ayurvedic Herbals

a. Selection of Suitable Immunostimulant and Aphrodisac Herbals

Eighteen herbs having the antibacterial characteristics such as *Aegle marmelos, Andrographis paniculata, Adhathoda vasica, Azadirachta indiaca, Cyanodon dactylon, Lecus aspera, Moringa oleracea, Mucuna pruriens*, *Murraya koeingii, Ocimum sanctum, Ocimum basilicum, Psoralea corylifolia, Quercus infectoria, Solanum surrattense, Solanum trilobatum, Terminalia bellirica*, *Myristica fragrans*, and *Withania somnifera* were selected following Nadkarni, 1995.

b. Collection of Herbs

The herbs such as *Withania somnifera*, *Mucuna pruriens* were collected from Keeriparai forest, Kanyakumari District. The other herbs were purchased from M.S.S. Assan herbal market and Sigma herbal remedies, Nagercoil, Kanyakumari District.

c. Processing

Collected plant materials were shade dried with in a temperature range of 28 – 35°C, the drying process was continued to reduce moisture level less than 14%. After drying, the plant materials were minced with wooden knife before feeding into a grinder; minced materials were made into powder using teeth mills and sieved. Then the powder was stored in airtight container and kept at room temperature until further use.

d. Organic Extraction – Percolation Method

The dried powder of plant material was extracted with ethyl acetate three times. Then methanol was added to the residue and extracted three times and hexane was added to the residue and extracted three times. Then Dichloromethane (DCM) was added to the residue and extracted three times. Finally aqueous extraction was done using sterile water. Soaking the material for overnight carried out each extraction. Each of these solvent extracts was concentrated in rotatory evaporator under reduced pressure at the temperature of 45°C to 50°C in order to avoid the evaporation of plant materials. Aqueous extract was concentrated using lyophilizer and was stored at 4° C.

e. Selection of Best Two Active Extracts for Further Studies

In this present study the above said fifteen plants in the section 2.1, were screened initially against the five pathogens. As per the best activity of the initial screening of five plant extracts, two were selected for further analysis. They are *Mucuna pruriens* (Methanol), and *Withania somnifera* (Ethyl acetate) for further analysis.

N. Minimum Inhibitory Concentration (MIC)

The MIC was defined as the lowest concentration of a fraction, which was able to completely inhibit the growth of each bacterial strain. Extracted materials were dissolved in 10% Dimethyl sulphoxide (DMSO) and added to Muller Hinton agar broth (pH 7), "medium 228" (75 % seawater) and "Growth medium 53"- (nutrient broth with 3% NaCl). 50 µl of liquid bacterial culture was added into each tube, which was been already added with different concentration of extracts (10 mg – 100 mg). The MIC values were taken as the lowest concentration of extract that inhibited the growth of the organism after 24 h of incubation at 37°C (Jacobs and Demott, 1994).

O. Minimal Bactericidal Concentration (MBC)

The minimum bacterial concentration (MBC) (Jacobs and Demott, 1994) was determined by plating the culture from the tube without the growth of microorganisms. To determine the minimum bactericidal concentration, aliquots of one loopful of the two lowest concentrations, which inhibited bacterial growth, were streaked on petriplates containing Muller Hinton agar. After an overnight incubation at 37°C, the plates were evaluated by comparing them with control plates containing bacteria without test compounds. The lowest concentration that gave no visible growth was taken as MBC.

P. Characterization of the Active Compounds

a. Soxhlet Extraction

A Soxhlet extractor is a laboratory apparatus invented in 1879 by Franz von Soxhlet. Typically, a Soxhlet extraction is required where the desired compound has only a limited solubility in a solvent, and the impurity is insoluble in that solvent. If the desired compound has a high solubility in a solvent then a simple filtration can be used to separate the compound from the insoluble substance.

Sixty gram of micronized herbal powders were weighed and put in to the extractor tube of Soxhlet apparatus. 100 ml of solvent was added in to the distillation flask and turned on the circulating water bath to the condenser and set the temperature. The temperature is varied depending upon the solvent. Run the extraction process continuously up to 8 to 12 h. After finishing the extraction process, the residual filtrate was concentrated by condense in the rotary evaporator at 45° C. Finally the condensed extract was stored at 4° C.

Q. Artificial Feed Preparation and Feeding

Equal proportion of two extracts namely, *Mucuna pruriens* (Methanol), named as MP and *Withania somnifera* (Ethyl acetate), named as WS were incorporated in five experimental diets containing five different concentrations from 0 (dosage 1, named as D1), 200 (dosage 2, named as D2), 400 (dosage 3, named as D3), 600 (dosage 4, named as D4) and 800 (dosage 5, named as D5) mg kg^{-1} of extracts mixture. There were four feeding treatments given to the experimental fishes, as mentioned below:

1. Control (with out herbal extracts)
2. MP (diet prepared from the crude extract of *Mucuna pruriens*)
3. WS (diet prepared from the crude extract of *Withania somnifera*)

4. MP+WS (diet prepared from the crude extract of mixture of *Mucuna pruriens* and *Withania somnifera*)

Diets were prepared following the basal ratio of Boonyaratpalin, 1993. The basal diet contained 45.1 % protein; 7.2 lipids, 14.6% ash, 7.1 % moisture and 3% fibre. To prepare the feeds, ingredients were mixed thoroughly and 4 % gelatine solution containing active principles in appropriate concentration was added along with oil ingredients. Sufficient water was added and pH was adjusted to 7±0.1. After that, this mixture was cold extruded, cut into pellet, air dried and stored at room temperature. The fishes were fed thrice a day at 8.00, 13.00 and 18.00 h at 10% of the body weight. Uneaten food and waste matters were removed before each feeding.

R. Statistical Analysis

The data were subjected to one-way ANOVA, two-way ANOVA, Post-hoc Tukey test and correlation analysis. SPSS statistical package was used for the Tukey test. All other tests were carried out using MS-Excel in a personal computer.