Indian J. Fish., **46(4)** : 375-381, Oct.-Dec., 1999

Identification of female specific protein in seabass, *Lates calcarifer* (Bloch)

KULDEEP KUMAR LAL[1], A. GOPALAKRISHNAN[1], VINDHYA MOHINDRA[1] AND P.S.B.R. JAMES

Central Marine Fisheries Research Institute, Cochin-682 014, India

ABSTRACT

Electrophoretic serum protein profile of female *Lates calcarifer* reveals appearance of female specific protein during gonadotropic dependent stages of ovarian growth. The protein is conspicuously absent in the serum of maturing males. The protein stains positively for carbohydrate, lipids and calcium indicating it to be vitellogenin. The vitellogenin band first appears in fish with maturing ovaries (stage 3) and stain intense and sharp till stage 4. At stage 5 (ripe) the band appears diffused. The protein is lacking in the serum profile of immature (stage 1), resting-preparatory (stage 2) and spent (stage 6) as well as in maturing male fish. The correlative pattern of vitellogenin band with ovarian maturation stages provides evidence of single spawning in *L. calcarifer*.

Introduction

Lates calcarifer, a large sized centropomid teleost inhabiting coastal, brackish and freshwaters of the Indo-Pacific region, is one among the valuable resources for aquaculture for commercial as well as sport fisheries in the region. The fish occupies the list of threatened Indian fishes (Mahanta *et al.*, 1994) and does not contribute much to the total fish landings except in certain areas like Chilka Lake and Hooghly-Matlah estuary (Kasim and James, 1987). The necessity to develop commercial calture of *L. calcarifer* has been emphasised by aquacultutists due to high consumer demand and price coupled with fast growth and euryhaline nature of the fish (Bensam and Nammalwar, 1991). The available information and issues related to aquaculture of *L. calcarifer* in India have been reviewed by James and Marichamy (1987).

The *L. calcarifer* exhibits a complex life history and is a protandric hermaphrodite. (Moore 1979; Davis, 1987; Lal, 1992; Guaigven *et al.*, 1994). Despite the fish being of commercial significance and an interesting specimen for academic studies as well, limited studies exist on aspects of physiological changes associated with reproductive cycle and gametogenesis (Lal, 1992; Lal and Pandey, 1998). The present study attempts to analyse the eletrophoretetic profile of serum proteins at different stages of maturation and attempts to trace the presence of female specific protein or vitellogenin.

* Present address : *National Bureau of Fish Genetic Resources, Canal Ring Road, P.O. Dilkhusha, Telibagh, Lucknow - 226 002, U.P., India.*

Vitellogenin, principal yolk precursor protein has been demonstrated in the serum of several teleosts (Hara *et al.*, 1987, Tyler *et al*, 1988, 1990, Rinchard *et al.*, 1997), even in estradiol - 17β treated immature fish. (Fujii *et al.*, 1987; Hara *et al.*, 1987).

Vitellogenin is synthesised by hepatocytes in non-mammallian vertebrates, induced by estradiol-17β under gonadotropin influence. This protein is directly released and transported via blood from liver for incorporation in developing oocytes (Wallace, 1985). Changes in vitellogenin patterns, though not absolute values, can be indicated conveniently and reliably through electrophoretic separation and estimation of total calcium, alkali labile protein bound phosphorous (Wallace and Jared, 1968, Rinchard *et. al.*, 1997). Vitellogenin is sequestered into developing oocytes and uptake has been demonstrated to be selective (Tyler *et al.*, 1988, 1990). In the oocyte, the precursor protein undergoes structural conversion and maturatin to form yolk globules and is responsible for majority of oocyte growth. (Wallace, 1985). The relationship between vitellogenin and egg yolk protein has been confirmed immunologically (Fujii *et al.*, 1987; Hara *et al.*, 1987). Yolk is essential for sustanace of newly hatched fish larvae.

Teleosts not only exhibit wide spectrum of spawning strategies but also considerable plasticity (McEvoy and McEvoy, 1992). This is reflected in oocyte recruitment process too, related to the pattern of physiological as well as cytological events. In single spawners, dynamics and regulation of oocyte development are simpler as the vitellogenesis and maturation are sequential in contrast with multiple spawners where the events persist simultaneously. Rinchard *et al.* (1997) studied the alkali labile protein phosphorous, in three cyprinid fishes, a single spawner, *Rutilus rutilus*, two multiple spawners, *Alburnus alburnus* and *Blicca bjoerkna* and found that the PPP profile differed not only between single and multiple spawner but also between the two multiple spawners. The present attempt draws parallel between pattern of FSP with available cytological evidence in relation to overian maturation of *Lates calcarifer* and discuses the significance of such indices as predictive tool.

Materials and methods

Live *Lates calcarifer* were obtained from commercial catches made through gill net, shore seine and hooks and line fishing. The present work was carried out as part of a larger programme on reproduction in *L. calcarifer*, covering different aspects of biology and gametogenesis, based on assessment of 923 specimens. For the study the blood was drawn at collection site and transported to laboratory over crushed ice. The blood was allowed to clot at room temperature and cerntrifuged for 20 min, at 3000 rpm. The separated serum was stored at -20^0C till analysis. The maturity stage of gonad was assessed macroscopically and confirmed through histological examination (Lal, 1992). After confirmation of maturity stage, the samples from eight fishes for each stage (stage 2 to 6 for females and 3, 4 for males) were analysed for electrophoretic profile of serum proteins.

The electrophoretic seperation was carried out through disc polyacrylamide (tube size 75 mm x 5 mm) as described by Subhashini and Ravindranath (1981).

The final concentration of running gel was 7% acrylamide and 2% bisacrylamide. The serum samples were mixed with ice cold double distilled water in the ratio 1:4 Sixty µl sample mixed with forty µl tracking dye (1% bromophenol blue in 10% sucrose). The current supply was 1ma/tube till dye reached upto spacer gel and 3 mA/tube in running gel. The gels were visualised for proteins, lipids and carbohydrates using the staining method described by Subhashini and Ravindranath (1981). To identify calcium binding proteins, gels were treated with Alizarine red-S (pH 6.5) for 10 min (Dahl, 1952, described in Pearse, 1985). After rinsing and destaining in distilled water and acid alcohol (0.1% HCl in 95% ethanol), gels were stored in 7% acetic acid. The relative mobility (Rf) was calculated for each band (distance migrated by protein/distance migrated by tracking dye).

Results

Electrophoretic profile of serum proteins (Fig. 1) of female *L. calcarifer*, reveals total 24 bands, Rf value ranging from 0.009 to 0.969. The bands exhibit varying thickness, staining intensities as well as characteristics (Table 1).

Comparision of the profile for different stages of maturation (Fig. 1) detects a strong protein band (no. 20) in Zone 1 appearing in maturing and mature fish (stages 3 and 4). The band has low Rf value (0.124). The band stain positively for carbohydrates, lipid as well as calcium indicating the association of these moeties with the protein. This band is strikingly absent in the serum profile of maturing and mature males (stage 3 and 4), confirming it to be

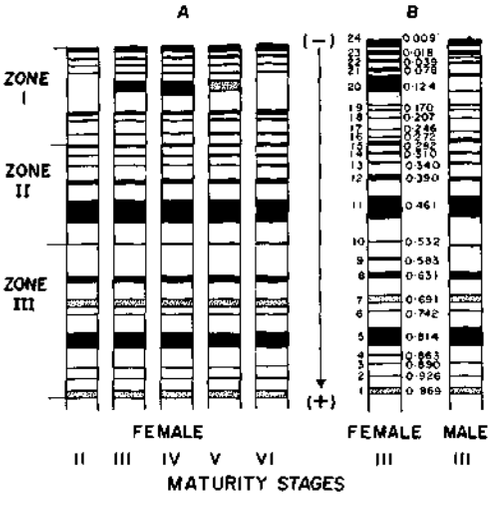

Fig. 1. A. Electrophoretic profile of serum proteins corresponding to different stages of ovarian recrudescence in *Lates calcarifer*. B. Comparison of serum protein of stage III female and male *Lates calcarifer*.

female specific protein (FSP) or vitellogenin. The serum profile of ripe females (stage 5) reveal FSP band to be diffused and of reduced intensity indicating decline of vitellogenin levels. At stage 6 (spent) and 2 (recovering developing) the FSP band is not visualised in the profile.

Discussion

The histology of *L. calcarifer* ovary (Lal, 1992) provided the evidence recruitment of oocytes to gonadotropin dependent phase at stage 3 and development continuing through stage 4 upto stage 5. Stage 5 ovaries contain oocytes with completed vitellogenic growth. The occurrence of pinocytotic avtivity near vitelline envelope has been observed through electron microscopy. This cytological evidence and concurrent presence of female specific protein

TABLE 1. *Details of protein fractions, their relative mobility (RF) values and their staining characteristics in the serum of female* Lates calarifer *during different stages of maturity*

Band No.	Rf value	Maturity stages																			
		Stage II				Stage III				Stage IV				Stage V				Stage VI			
		Gen.	Gly.	Lipo.	Cal.	Gen.	Gly.	Lipo.	Cal.	Gen.	Gly.	Lipo.	Cal.	Gen.	Gly.	Lipo.	Cal.	Gen.	Gly.	Lipo.	Cal.
Zone III (6-11 cm)																					
1.	0.969	xx	–	–	–	xx	–	–	–	xx	–	–	–	xx	–	–	–	xx	–	–	–
2.	0.926	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–
3.	0.890	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–
4.	0.863	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–
5.	0.814	xxxx	xx	xx	xx	xxxx	xx	xx	xx	xxxx	xx	xx	xx	xxxx	xx	xx	xx	xxxx	xx	xx	xx
6.	0.742	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–
7.	0.691	xx	–	–	–	xx	–	–	–	xx	–	–	–	xx	–	–	–	xx	–	–	–
8.	0.631	xxxx	xx	xx	xx	xxxx	xx	xx	xx	xxxx	xx	xx	xx	xxxx	xx	xx	xx	xxxx	xx	xx	xx
9.	0.583	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–
Zone II (3-6 cm)																					
10.	0.532	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–
11.	0.461	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx
12.	0.390	xxxx	xxxx	–	–	xxxx	xxxx	–	–	xxxx	xxxx	xxxx	–	xxxx	xxxx	–	–	xxxx	xxxx	–	–
13.	0.340	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–
14.	0.310	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–
Zone I (0-3 cm)																					
15.	0.292	xxxx	–	xx	xx	xxxx	–	xx	xx	xxxx	–	xx	xx	xxxx	–	xx	xx	xxxx	–	xx	xx
16.	0.278	xxxx	xx	–	–	xxxx	xx	–	–	xxxx	xx	–	–	xxxx	xx	–	–	xxxx	xx	–	–
17.	0.246	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–
18.	0.207	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–
19.	0.170	xxxx	–	xx	–	xxxx	–	xx	–	xxxx	–	xx	–	xxxx	–	xx	–	xxxx	–	xx	–
20.	0.124	–	–	–	–	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xx	xx	xx	xx	–	–	–	–
21.	0.078	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–
22.	0.039	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–
23.	0.018	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–	x	–	–	–
24.	0.009	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx

x = narrow & sharp bands; xx = broad & diffuse bands; xxxx = broad & intensely stained bands; – = absence of bands; Gen. = general proteins; Gly. = glycoproteins; Lipo. = lypoproteins; Cal. = calcium bound proteins.

in the serum with affinity for lipid, carbohydrate and calcium stains indicate oxogenous vitellogenesis. Vitellogenin is a glycolipophospho protein (Wallace, 1985) and binding with calcium imparts solubility to the protein during transport via blood (Follett and Redshaw, 1974).

Reduced intensity of the FSP band is serum profile of stage 5 females relative to stage 3 and 4, indicates decline in the level of vitellogenin in the bloood. This observation is also supported by the decline in other indices of serum vitellogenin levels viz. alkali labile protein bound phosphorous and calcium at stage 5, when the developing oocytes have completed vitellogenic growth (Lal, 1992). The low vitellogenin level can be due to its incorporation in the developing oocytes and absence of any succeeding clutch to support. The observation agrees well with the evidence from the histology as well as oocyte frequency profile regarding existance of single synchronously developing group of vitellogenic oocytes, to be shed in the single spawning (Lal, 1992). In multiple spawners, like *Misgrunus anguillicaudatus,* serum vitellogenin has been observed to be at higher levels in ripe females, while a group of oocytes has already completed growth to support the succeeding clutch (Teranishi *et al.,* 1981). Rinchard *et al.* (1997) detected low protein bound phosphorous level before spawning season in single spawner (*Rutilus rutilus*) and the highest level in two multiple spawners (*Alburnus albarnus* and *Blicca bjoerkna*). The two multiple spawners further differed as vitellogenin progressively declined in *Alburnus alburnus* but remained high in *Blicca bjoerkna* throughout the spawning season.

In view of this, *L. calcarifer* can be concluded to be single spawner along the Tuticorin coast in the Gulf of Mannar. Patnaik and Jena (1976) reported single spawning from Chilka lake. Davis (1987) didn't encounter any evidence of multiple spawing in Gulf of Carpentaria and Van Diemen Gulf. However, *L. calcarifer* has been reported to be multiple spawner in Papua New Guinea and Queensland (Moore, 1982). Barlow (1981) reported that smaller specimen (4 kg) released all the eggs in one spawning but bigger fishes are multiple spawners. Such variabilities in reprodu-ctive strategies though are wide spread among teleosts (review McEvoy and McEvoy, 1992) may not be desirable for commercial hatchery and fishery management which need planning based on precise information. Vitellogenin indicator profiles in combination with intraovarian oocytes siphoned through catheter can be predictive tools useful in tracking the course of maturation leading to single or multiple spawning (Lal and Ponniah, 1998). These biopsy oocyte samples have been demonstrated to present representative information of the maturation stage in *L. calcarifer* (Garcia, 1989). The techniques for biopsy as well as vitellogenic indicators including electrophoresis of FSP are simple. Moreover sampling can be done non invasively without losing the precious broodstock.

Acknowledgments

The authors are grateful to Mr. S. Mahadaven and Dr. K.A. Narasimham for providing facilities at the Tuticorin Research Centre of CMFRI and for encouragement extended during the study. The cooperation of the staff of the research centre is acknowledged. Financial assistance provided by the

Indian Council of Agricultural Research in the form of Senior Research Fellowship is gratefully acknowledged.

References

Barlow, C.C. 1981. Breeding and larval rearing of *Lates calcarifer* (Bloch) (Pisces: Centropomidae) in Thailand. New South Wales State Fisheries, Sydney.

Bensam, P. and P. Nammalwar 1991. Seed production and commercial cluture of the seabass, *Lates calcarifer* (Bloch) at Singapore and its lessons for India. *Mar. Fish. Infor. Serv., T&Ser.,* No. 109, p. 5-11.

Davis, T.L.O. 1987. Biology of wild stock *Lates calcarifer* in Northern Australia. *In:* J.W. Copland and D.L. Grey (Eds.). *Management of Wild and Cultured Seabass/ Barammundi (Lates calcarifer).* Proc. of an International Workshop held at Darwin, N.T. Australia, Sept. 1986. ACIR Proc. No. 20.

Follet, B.K. and M.R. Redshaw 1974. The physiology of vitellogenesis. *In:* B. Lofts (Ed.). *Physiology of the Amphibia,* Vol. 2, p. 219-308. Academic Press, London.

Fujii, K., A. Hara, K. Hirose and T. Maruyama 1987. Immunological and electrophoretical studies of specific serum protein induced by estrogen treatment in hybrid sturgeon between female *Huso huso* and male *Acipenser ruthenus* so-called "Bester". *Bull. Natl. Inst. Aquaculture,* **12** : 17-24.

Garcia, L.Ma. B. 1989. Development of an ovarian biopsy technique in the seabass, *Lates calcarifer* (Bloch). *Aquaculture,* **77** : 97-102.

Guiguen, Y., C. Cauty, A. Fostier, J. Fuclis and B. Jalabert 1994. Reproductive cycle and sex inversion of the seabass, *Lates calcarifer,* reared in sea cages in French Polynesia; histological and morphometric description. *Env. Biol. Fish.*, **39** : 231-247.

Hara, A., K. Ouchi, Y. Nagahama and T. Nose 1987. Identification of female specific serum proteins (vitellogenin) and their related egg yolk proteins in red sea bream, *Pagrus Major. Bull. Natl. Res. Inst. Aquaculture,* **12** : 25-35.

James, P.S.B.R. and R. Marichamy 1987. Status of sea bass (*Lates calcarifer*) culture in India. *In:* J.W. Copland and D.L. Grey (Eds.), *Management of Wild and Cultured Seabass/Barramundi (Lates calcarifer).* Proc. of an International Workshop held at Darwin, N.T. Australia, Sept. 1986. ACIR proc. No. 20, p. 74-79.

Kasim, H.M. and P.S.B.R. James 1987. Distribution and fishery of *Lates calcarifer* in India. *In:* J.W. Copland and D.L. Grey (Eds.), Management of Wild and Cultured Seabass/Barramundi (*Lates calcarifer*). Proc. of an international Workshop held at Darwin, N.T. Australia, Sept. 1986. ACIR Proc. No. 20, p. 101-114.

Lal, K.K. 1992. Studies on the reproductive physiology of *Lates calcarifer* (Bloch). *Ph.D. Thesis,* Cochin University of Science and Technology, Cochin.

Lal, K.K. and Ponniah, A.G. 1998. Reproductive biology estimators for conservation and culture of fish. In: *Proc. Workshop on Germplasm Inventorisation and Gene Banking of Freshwater Fishes,* 12-13 October, 1998, Cochin, p. 4-6.

Lal, K.K. and A.K. Pandey 1998. Hypothalamo-neurosecretory system of the female seabass, *Lates calcarifer* (Bloch), with special reference to gonadal muturation. *Indian J. Fish.,* **45**(1) : 51-60.

Mahanta, P.C., D. Kapoor, R. Dayal and A.G. Ponniah 1994. Prioritization of the Indian fish species for conservation. *In:* P.V. Dehadrai, P. Das and S.R. Verma (Eds). *Threatened Fishes of India.* Natcon Publication 4, p. 379-385.

McEvoy, L.A. and J. McEvoy 1992. Multiple spawning in several commercial fish species and its consequences for fisheries management, cultivation and experimentation. *J. Fish Biol.,* **41** : 125-136.

Moore, R. 1979. Notional sex inversion in giant perch *Lates calcarifer. Aust. J. Mar. Freshwat. Res.,* **30** : 803-813.

Moore, R. 1982. Spawning and early life history of barramundi *Lates calcarifer* (Bloch), in Papua, New Guinea. *Aust. J. Mar. Freshwat. Res.,* **33** : 663-670.

Patnaik, S and S. Jena 1976. Some aspects of biology of *Lates calcarifer* (Bloch) from Chilka lake. *Indian. J. Fish.*, **23**(1&2) : 65-77.

Pearse, A.G.E. 1985. *Histochemistry - Theoretical and Applied.* Vol. 2 Churchill Livingstone, London.

Rinchard, J., P. Kestemont and R. Heine 1997. Comparative study of reproductive biology in single and multiple-spawner cyprinid fish. II. Sex steroid and plasma protein phosphorus concentrations. *J. Fish Biol.*, **50** : 169-180.

Subhashini, M.H. and M.H. Ravindranath 1981. Electrophorectic separation of proteins. *In:* M.H. Ravindranath (Ed.). *Manual of Research Methods for Crustacean Biochemistry and Physiology. CMFRI Spl. Publ.* No.7, p. 103-114.

Teranishi, T., A. Hara and H. Takahashi, 1981 Changes of serum vitellogenin levels during the course of annual reproductive cycle of the loach, *Misgurunus anguillicaudatus. Bull. Fac. Fish. Hokkaido Univ.*, **32** : 281-292.

Tyler, C.R., J.P. Sumpter and N.R. Bromage 1988. Selectivity of protein sequestration by vitellogenic oocytes of the rainbow trout, *Salmo gairdneri. J. Exp. Zool.*, **248** : 199-206.

Tyler, C.R., J.P. Sumpter and N.R. Bromage 1990. An *in vicro* culture system for studying vitellogenin uptake into ovarian follicles of the rainbow trout *Salmo gairdneri. J. Exp. Zool.*, **255** : 216-231.

Wallace, R.A. and D.W. Jared 1968. Studies on amphibian yolk. VII. Serum phosphoprotein synthesis by vitellogenic females and estrogen-treated males of *Xenopus laevis. Can. J. Biochem.*, **46** : 953-959.

Wallace, R.A. 1985. Vitellogensis and oocyte growth in nonmammalian vertebrates. *In:* L.W. Browder (Ed.). Developmental Biology, Vol. 1., 127-175. Plenum Publishing Corporation, New York.