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Implicating serum proteomics: Vitellogenin as biomarker of reproductive state in a Himalayan trout, *Schizothorax plagiostomus*

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Abstract
Accurate knowledge regarding gonadal development and maturity is imperative for predicting the reproductive potential of fish, time and frequency of spawning and size at first maturity. *Schizothorax plagiostomus* plays a significant role in capture fishery in different Himalayan regions and is a demanding food fish. Several studies on reproductive biology of *S. plagiostomus* based on histology of gonads, gonadosomatic index, ova diameter and fecundity group it as asynchronous type, with long breeding period, spawning only once while some claim it to be a batch spawner. The present study attempted to correlate ovarian histology, gonadosomatic index, blood glucose concentration with serum vitellogenin as scorable protein biomarker, revealing that the species spawns at least in two or more batches during the same spawning season. Three isoforms of vitellogenin were identified after 2D gel electrophoresis and MS analysis i.e. Vitellogenin 1, Partial Vitellogenin 2 and 4. No expression was observed in immature phase from January to mid-February, thereafter serum samples showed significant expression of vitellogenin. However, the expression decreased in late-March and increased again in mid-April indicating species is a batch spawner; vitellogenin production seem to cease a little after spawning and increases before next batch of ovum are spawned.

Keywords: Fecundity, vitellogenin, spawning, GSI, 2D-gel electrophoresis, MALDI/MS

1. Introduction
Reproductive success in a species cannot be accurately predicted from simple measurement of morphology or behaviour as it is influenced by complex environmental factors and social interactions. Fish are appealing study model as they share close physiological relationship with their environment. Different species inhabit different set of ecological conditions, exhibit unique reproductive strategies and have peculiar behavioural, physiological or energetic adaptations. An accurate timing of breeding with optimal environment and food availability is required for the successful reproductive outcome. Coordinated interplay of animal's physiology with environmental cycle i.e. endogenous rhythms of physiological processes accounts for seasonal reproduction [1]. Several external stimuli are capable of modifying the excitability of GnRH neurons which in turn control reproductive physiology and behaviour in almost all vertebrates studied to date. Different proximate factors or environmental cues are perceived by the diverse exteroceptors as a signal for approaching favourable reproduction season and which inturn affect the central neurons system, pituitary and ultimately the gonads. Interplay of environmental factors and biological rhythm phase the precise breeding time, accordingly the some fishes are either seasonal breeders or some breed continually while some only once in life time [2].
Study on reproductive biology of fishes is imperative for its rational exploitation towards management of fisheries, development of quality brood stocks, domestication as well as further genetic improvements. Different visual and histological examinations have since long being used to determine the extent of ovarian maturation in fishes. Most widely followed is Bieniarz and Epler [3] method of examining nuclear changes occuring before and during ovulation. Several components as germinal vesicle, cytoplasm, cortical alveoli and follicular wall exhibit distinct variations in morphology at ultra-structural level and biochemical alteration as well during pre-vitellogenic and vitellogenic stages of oocyte maturation.

Even though, histological studies have proved to be among the easiest efficient methods for assessing reproductive phase of fishes, it is often the laborious and time consuming process. Proteomics is a rapidly expanding analytical tool providing means to study the changes occurring at the level entire protein pool in response to both the external environment and ontogenetic events in an organism. The study of changes in the abundance of certain specific protein marker or its modification could be a powerful tool for generating hypotheses regarding how the environmental factors signal and mediate the reproductive biology of organisms.

Vitellogenesis is a seasonal or cyclic phenomenon of deposition of yolk while development of oocytes in most vertebrates. It is the final stage in oocyte maturation prior to ovulation. Yolk is either auto-synthesised in the oocyte or in any other organ (hetero-synthesis) and transported to the egg. A major product of vitellogenesis is a multi-component lipophosphoprotein Vitellogenin (termed by Pan *et al*.) [4]. Vitellogenin is synthesized in liver in fishes under estrogen stimulus produced from the ovary. It is thereafter secreted into circulation and transported to the ovary where it is taken up by the oocytes. Variations in the plasma electrophoretic patterns have been observed in parallel to the progress of vitellogenesis with most conspicuous changes in appearance of vitellogenin. Being specific to the female, it is also known as female specific plasma protein [5] and its presence in the plasma of maturing female coincides with formation of yolk granules underneath vitelline membrane [6]. Several immunological and electrophoretic methods have been used to detect the presence of vitellogenin with varied implications [6-11]. The molecular weight of vitellogenin is species dependent ranging from 350-600 kDa. It is transported to developing oocytes via blood stream where it is cleaved into two daughter yolk proteins, phosvitin and vitelline. Volume of oocytes increase several times during ripening of eggs with incorporation of yolk protein for which vitellogenin serves as the precursor. Phosvitin accounts for nearly all the phosphoprotein and lipovitellin for almost all the lipid associated with the vitellogenin molecule. The high phosphorus content makes it active in Ca^{++} transport while lipophilic moieties are involved in transport of lipophilic components and certain hormones.

Earlier studies though have focussed mainly on histological and serum protein PAGE profiles but have established these parameters as important indices in monitoring the effects of aquatic habitat changes and a consequent adaptive physiological response to achieve a particular reproductive phase. Total serum proteins, pre-albumin, albumin and a-globulins etc have also been studied in relation to age, sex, maturation and season. Albumins being mostly studied exhibit differential level in fish serum depending on fish physiological status, maturation, age, season and geographical location. However, only few studies describe the relationship between other major components of serum proteome and different reproductive phases of the annual cycle. Several investigators have used different strategies to purify vitellogenin from plasma or serum of various teleost species. In the present study we performed 2D gel electrophoresis of blood serum of *S. plagiostomus* pre-reproductive, reproductive and post-reproductive phases. The electrophoretic separation of vitellogenin from few drops of blood of a fish can clearly indicate the reproductive phase and help us determine its exact breeding time. Despite certain limitations, proteomics based on 2D gel electrophoresis and mass spectrometry (MS) can be extremely successful in discovering new insights into complexities of biological systems. Moreover, such analysis would provide a valuable guide to assessing the condition of the fish, as it provides a reliable index of their physiological condition, and will also find importance in fish aquaculture.

2. Materials and methods

In the present study, three phases of female *S. plagiostomus* reproductive cycle were considered i.e. Pre-reproductive phase, Reproductive phase and Post reproductive phase based on the status of ovary (shape, size and colour etc.) observed by studying the samples in local catch by fishermen throughout the year. Accordingly after ascertaining the phases mentioned above, live samples of fishes were caught in the corresponding months with the help of cast net from river Alaknanda (between Sandre to Harkandi) in Srinagar-Garhwal, Uttarakhand located between 30°13'28.83"N and 78°47'55.03"E. Though the species reproduces twice a year (in March-April and in September-October), we collected the samples only during one reproductive period for two years i.e. January to June in the year 2014 and 2015.

2.1 Gonadosomatic Index (GSI) determination

For the determination of GSI, samples were procured from local catch by fishermen and were carried immediately to the laboratory. After thorough wash with tap water the body weight was measured in gram by an electronic balance. Excess water from the fishes was removed with blotting paper before measuring the weight of the fishes. The gonads were dissected out from the fish and weighed and GSI was calculated as the ratio of gonadal and body weight [12].

2.2 Fecundity

Fecundity was defined as the total number of eggs produced by a fish during one season. In this present study fecundity was calculated by the Gravimetric method [13].

2.3 Blood collection

According to the phases defined above, in the corresponding months blood samples were collected in the field from caudal vein using a sterile plastic syringe (2.5ml) without sacrificing the fish. The blood was transported to laboratory under optimum temperature conditions (4°C) to prevent any protein denaturation. Blood glucose concentration was determined using Nelson- Somogyi method [14, 15].

2.4 Sample preparation- Proteomics analysis

Serum was isolated from blood after clotting and subsequent centrifugation at 4000rpm for 5 minutes with the help of cooling centrifuge (MPW- 65R med instruments, Poland). Serum proteomics analyses were performed for all the blood sample of *S. plagiostomus* on the regular basis for the two years in different reproductive phase. The samples were processed for dilution, subsequent protein isolation, acetone precipitation. For protein precipitation acetone was used, three fold volume of acetone was mixed in serum sample and vortex for 1 minutes in 1.5 ml tube. Overnight precipitation was done at -20° C. After precipitation centrifugation was done at 4000rpm for 5minutes at 4°C, protein pellet was dissolve in rehydration buffer (GE health care). SDS PAGE was performed initially following Laemmli [16]. Protein quantification was done using Bradford assay kit (GeNei). Further, before performing 2-D gel electrophoresis, sample

preparation was done using 2D-cleanup kit (BioRad) for the removal of ions and detergents from the protein sample which hinders proper Iso-electric focussing.

2.5 Two Dimensional Gel Electrophoresis

Isoelectric focusing was performed in PROTEAN i12 IEF Cell (BioRad) using immobilized pH gradient (IPG) strips of 18 cm length, pH 3-10 Linear (BioRad). 450µg protein was loaded with final volume of 315ml with rehydration buffer and 0.6% ampholite 3-10 pH (Sigma), overnight at 20°C for rehydration in the supplied rehydration tray. After rehydration the strips were transferred in focusing tray (with gel side down) following protocol: 100V Rapid for 0:20 hr, 150V Rapid for 0:35 hr, 8000V Gradual for 2:30 hr, 8000V Rapid for 26000V Hr and Hold at 500V. After the completion of IEF, equilibration of IPG strip was done prior to second dimension. The IPG strips were equilibrated twice, first with about 10 ml of equilibration buffer-I (6M Urea, 75mM Tris–HCL, pH 8.8, 2% (w/v) SDS, 30% (v/v) Glycerol, trace amount of Bromophenol blue and 0.1mg DDT) for 15 minutes with continuous shaking and then in fresh equilibration buffer-II containing 0.25mg iodoacetamide (in place of DTT) for another 10 minutes.

For second dimension separation 10% resolving gel (10% v/v Acrylamide/Bis, 0.05% Ammonium persulphate 375 mM Tris–HCl, pH 8.8, 0.1% w/v SDS, 0.033% v/v TEMED) was used. 0.5% w/v agarose with traces amount of bromophenol blue was used for the sealing of IPG strips. The gels were subjected to vertical SDS-PAGE simultaneously for 7 hr at 100V with continuously cooling (PROTEAN II xi cell, BioRad) until bromophenol blue front reached the other end. Broad range molecular weight protein standards (Merck) were run with protein sample. Five 2-D gels were run for each sample so the 2-D gel spots shown here are representative of the five gel images.

2.6 Colloidal Coomassie Brilliant Blue staining

Gels were stained with coomassie brilliant blue (G-250) for visualization and further analysis. Prior to staining, gels were fixed in fixing solution (85% o-Phosphoric acid 1.3% w/v, Methanol 20%) for 60 minutes. Overnight staining was done with staining solution (0.25% w/v coomassie brilliant blue (G-250), 10% w/v o-Phosphoric acid, 10% w/v Ammonium sulphate, 20% v/v Methanol). After the staining gels were transferred into neutralization buffer (Tris-base 0.1 M w/v, titrated with o-Phosphoric acid to pH 6.5) then gels were washed with 25% Methanol for less than 1-3 minutes and finally transferred in stabilizing solution (Ammonium sulphate 20% w/v).

2.7 Gel image analysis

GS-800 Calibrated Densitometer (BioRad) was used for gel image scanning. Image analysis was performed using PD Quest software (BioRad) to quantify pre-ovulatory and ovulatory phase serum protein of *S. plagiostomus*. Spot density was calculated as percentage of total protein density in the same 2D gel image and was used to identify up regulated and down regulated protein spots. Protein spots that were differentially expressed were selected and were manually excised for further analysis.

2.8 In Gel Digestion and MALDI-TOF analysis

Prior to MALDI analysis excised protein gel plugs were subjected to in-gel digestion to extract the protein. Protein gel plugs were washed with miliQ water twice (for 20 minutes). 25mM Ammonium bicarbonate with 50% acetonitrile (Sigma) was used for destining of gel plugs, and further dehydrated with 70% acetonitrile (20 minutes) twice and left overnight. The dried spots were then digested with trypsin (porcine sequencing grade modified trypsin, Promega), after which they were redissolved in 0.1% TFA, followed by desalting and concentration using ZipTipµ-C18 tip containing a small (0.2µl) bed of C18 reverse phase resin (Sigma-Aldrich). The peptides were eluted directly onto the MALDI using 0.5 ml matrix solution (5 mg/ml α-cyano-4-hydroxycinnamic acid in 70% v/v CH_3CN and 0.1% TFA). Mass spectrometric analysis was performed on an Ettan™ MALDI TOF/Pro spectrometer (GE Healthcare). Spectra were internally calibrated using trypsin auto digestion products. The obtained peptide masses were searched on the Mascot™ Peptide Mass Fingerprint database (Matrix Science).

3. Results & Discussion

3.1 Reproductive Phases: Stages of ovary maturation

S. plagiostomus reproduce twice in a year (March-April and September-October). In the present study, three phases of female fish reproductive cycle were defined i.e. Pre-reproductive, Reproductive and Post reproductive. The size, shape, colour and texture of ovaries and microscopic structure of the ova were examined and stages identified based on the classification of El-Greisy[17]. In the months, December to January and June to July, the ovaries were observed to be minute, thread like, translucent and pail white in colour localised only in smaller part of the body cavity. This period was considered as pre-reproductive phase defined as resting or immature phase. In the months of February to April and August to October, ovaries gradually matured leading to ovulation. The early maturation starts from ovaries becoming slightly larger and opaque with increase in the weight occupying almost half of the body cavity. With further increase in the weight in the advanced maturation phase and ovary occupies larger volume with deep yellow colour.

On maturation ovaries are further enlarged, occupying almost the entire body cavity with large number of spherical ova visible through the thin ovarian wall. The fishes become gravid with ripe ova and the abdomen round. During spawning the ovarian wall is very thin, almost transparent with eggs also present in the oviduct, with the ovary in running phase. In the months of March to April and September to October, almost all fishes sampled completed their spawning phase. The ovaries became shrinked and sac-like with reduced volume and dull colour. No eggs were released by pressing the abdomen. This period was considered as the post-reproductive period.

3.2 Gonadosomatic index, Fecundity and Nutritional state

Accurate spawning season of any species can be determined by analysing gonadosomatic index and fecundity through its reproductive cycle all year around. During Pre reproductive stage, the mean GSI value was least in early January (3.215±0.02) and gradually increased upon maturing in February (5.675±0.03) and further maturation (6.222±0.02) in late February. A sharp increase in mean GSI was observed during peak reproductive stage in Mid-March (6.858±0.02) and the value decreased during post reproductive phase with lowest value observed in Mid-May (2.176±0.01) (Fig. 1a). Fecundity was also documented for all the samples of *S. plagiostomus* with ripe ovaries. No mature ova were observed

in January. The average number of mature ova ranged from 3587 in early February to 10449 in mid-March, with decrease in late March to further increase in mid to late April (10536) (Fig. 1b).

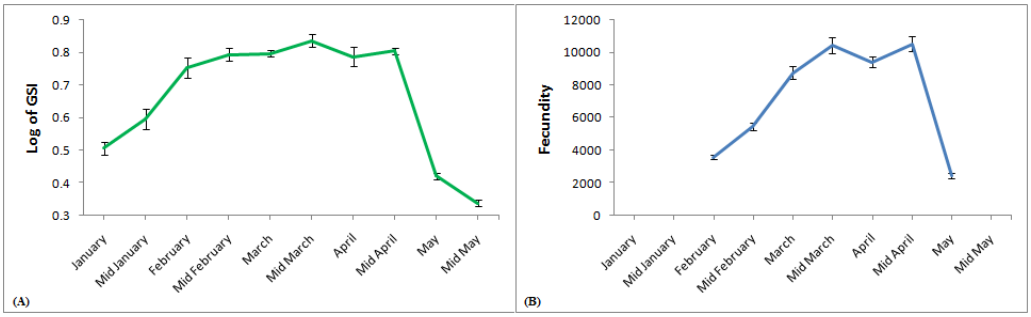

Fig 1: (a) Gonadosomatic index (log_{10}) and **(b)** Fecundity observed for *Schizothorax plagiostomus*. The species reproduces twice a year (in March-April and in September-October). The data plotted is Mean±SD of one reproductive period for two years i.e. January to June in the year 2014 and 2015

The ripened ovary of *S. plagiostomus* exhibited massive pale yellowish oocytes, occupying the entire body cavity with blood vessels ramifying throughout the surface. Additionally, few immature oocytes were also present. Several studies on gonadosomatic index, ova diameter and ovarian histology (as documented by Agarwal) [18] classify this species under category of asynchronism of Marza [19] i.e. several batches of oocytes in different stages of development present at a time indicating a long spawning season with several intermittent spawning acts and under second category of Prabhu [20] of spawning once a year with a long duration. However, on contrary our data suggest the species is a batch spawner, spawning at least two to three times as per peaks observed for GSI and fecundity which we further ascertained via characterization of serum vitellogenin using 2D Gel electrophoresis.

Wallace and Selman [21] also suggested the relationship between oocyte maturation to the nutritional state of the animal. We quantified large amounts of glucose in the circulation before ovarian vitellogenic growth begins in February to mid-February (1019.82±4.36 µg/ml) and the concentrations decline thereafter during the early phase of vitellogenesis to mid-March (867.24±42.56 µg/ml) and then increased significantly around spawning period in early April and early May (906.35±9.54 µg/ml) after which the levels dropped again as the spent phase ensued (Fig. 2). Hepatic glycogen and lipid perhaps represent energy sources to meet the metabolic demands during ovarian growth and spawning [22]. We did not quantified phospholipid in the present study; however it is required for biosynthesis of yolk proteins and oocyte membranes. Hepatic glycogen concentration is net result of the activity of glycogen synthetase and glycogen phosphorylase; thus plasma glucose levels represent a balance between nutrition, hepatic supply and tissue uptake.

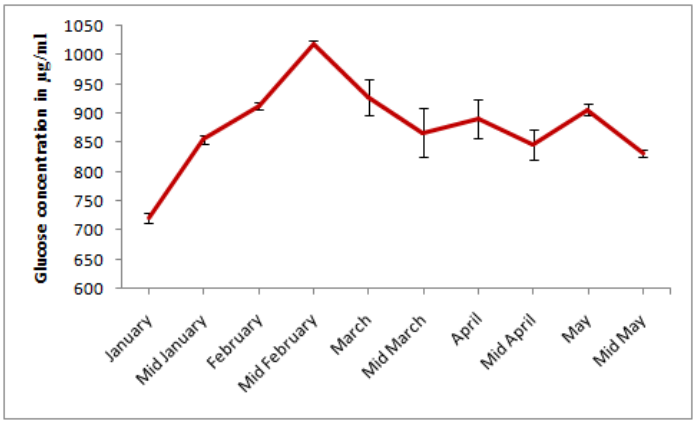

Fig 2: Serum glucose concentration quantified using Nelson-Somogyi method for *Schizothorax plagiostomus*. The data plotted is Mean±SD of one reproductive period for two years i.e. January to June in the year 2014 and 2015

3.3 Serum Proteomics- *Vitellogenin* as a biomarker

A remarkable increase in the ovary weight of up to 20% or more is documented for many fish species, prior to spawning mainly due to the accumulation of yolk and other nutrient reserves. During final stages of oocyte maturation, egg yolk coalesces and mixes with other egg contents [23]. Though diverse opinions have been expressed in terms of origin and nature of these yolk bodies in past literature, several recent studies have tried to elucidate the mechanism of vitellogenesis and its implications on fish reproductive biology [24, 25]. 2D gel profiles of *S. plagiostomus* serum proteins was generated for all pre, post and reproductive period by superimposing gel profile for distinct set (five of each group). Protein spots of interest were manually excised from each gel of set and subjected to in-gel digestion to extract proteins prior to MALDI analysis. The obtained peptide masses were then used to search the Mascot ™ Peptide Mass Fingerprint database (Matrix Science). In particular, we also aimed to assess the rate and reliability of protein identification in a non-model species *S. plagiostomus* without a sequenced genome as a step toward developing this species for future studies of environmental proteomics. It was also aimed to

annotate molecular functions for the proteins using web-based tools.

Three isoforms of vitellogenin were identified after MALDI analysis i.e. vitellogenin 1, Vitellogenin 2 Partial, Vitellogenin 4 Partial, represented most conspicuously in reproductive phase gels. Three spots characterized as vitellogenin I had approximately same molecular weight with slight difference in their isoelectric point, while two other partial isoforms were also identified of lower molecular weight (Table 1). These protein identifications were homologous to the proteins of Zebrafish (*Danio rerio*) than any other species; however this was expected result due to availability of large number of genome and proteome annotations of Zebrafish in the public databases after sequencing of its genome.

Table 1: Mascot based identification of vitellogenin isoforms in reproductive phases of *S. plagiostomus*

Protein name	Mw, PI (Theoretical)	Mw, PI (Observed)	Mascot Score	Query Cover (%)	Organism	Accession No.
Vitellogenin 2 Partial	69.03, 7.84	72.74, 8.10	89	23.4	*Danio rerio*	AAK94945
Vitellogenin 1	149.54, 8.68	146.50, 8.56	114	34.7	*Danio rerio*	AAW56966
Vitellogenin 1	149.54, 8.68	154.67, 8.92	97	29.8	*Danio rerio*	AAW56966
Vitellogenin 1	149.54, 8.68	150.86, 8.23	102	31.2	*Danio rerio*	AAW56966
Vitellogenin 4 Partial	31.2, 9.48	36.79, 9.45	76	18.0	*Danio rerio*	AAW56968

Our findings are in congruence with earliest landmark studies on synthesis and deposition of vitellogenin in fishes and its absence in serum of male fish, immature and post reproductive stages of female fish [5, 6, 26]. Different isoforms of vitellogenin were identified in blood serum of female fish during maturation and early spawning stages as well. Vitellogenin shows affinity for several ions as calcium, iron and magnesium. Carbohydrate content of vitellogenin binds calcium ions and increases the solubility of this protein. Several metals and minerals bound to vitellogenin thus serving as a vital reservoir of ions during embryogenesis. Also, in teleosts usual iron transport is via transferrin, however during yolk synthesis, iron bound transferrin is up taken by liver and incorporated in to vitellogenin, thereafter secreted into the plasma and transported [26]. After being secreted into the blood-stream it is taken up by the developing oocytes and cleaved into yolk proteins [27] chiefly documented as lipovitellin I and II, phosvitin and β'-component[28]. Vitellogenin also undergoes post-translational modification in oviparous vertebrates, like lipidation, glycosylation and phosphorylation before its secretion in the plasma leading to the generation of various isoforms; often not characterized in previous studies.

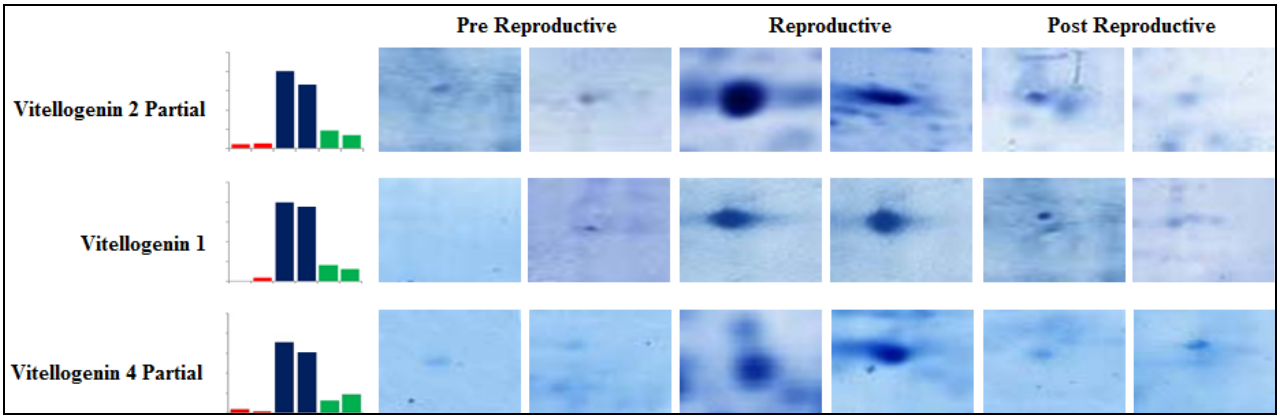

Fig 3: Three isoforms of vitellogenin characterized in this study. Bars presented here denote the variation in expression level of these isoforms along different phases

In the current study, we characterized three isoforms of vitellogenin and variation in their expression level along different reproductive phases (Fig. 3). From January to mid-February no significant expression was observed, with only very faint protein spot for partial vitellogenin 2 in the serum of *S. plagiostomus*. This can be attributed to the fact that ovary is in immature state during this period, with no vitellogenesis whatsoever and thus no yolk transportation or deposition in these months. On progression towards maturity, vitellogenesis ensues and subsequently the yolk deposition starts in oocytes starting after mid-February up to mid to late April resulting in enlargement of the ovary. The first set of serum samples collected from the fishes during initial stage of this phase i.e. between mid-February to mid-March showed the significant expression of all vitellogenin 1, partial vitellogenin 2 as well as 4. The considerable amount of vitellogenin in serum samples clearly indicate that vitellogenin formed in the liver was being transported to ovary. Though the vitellogenin representing spots were broad and intensely stained, the expression decreased to an extent in the late March and increased again in mid-April. It may be due to the fact that this species is a batch spawner; spawning more than once in a single reproductive season. Though the yolk is synthesised exogenously and transported from the liver to ovary through the blood stream for mid-February to late April, the production seem to cease a little after spawning and increases thereafter before next batch of ovum are spawned (Fig. 4). Vitellogenesis is characterized by a significant increase in number of mature ova, growth of the oocyte, subsequent changes in GSI and metabolites as glucose etc. Our data on these parameters too (as discussed earlier, Fig. 1 and 2) affirm the batch spawning nature of this species.

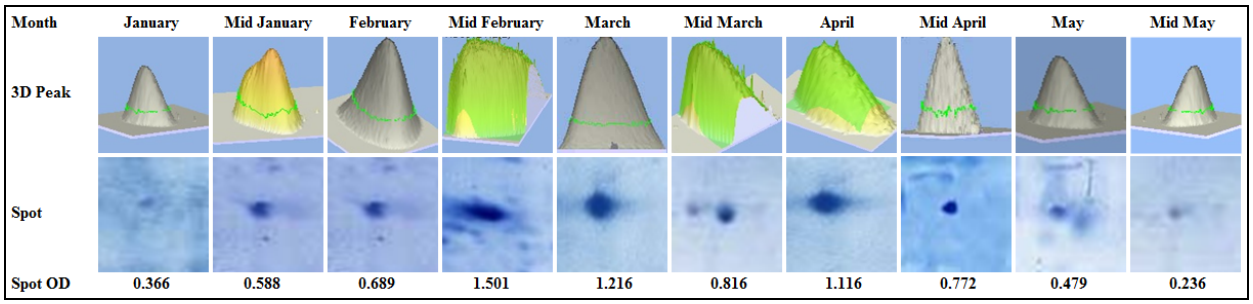

Fig 4: Variation in expression of vitellogenin spots in terms of spot density and comparative 3-D peaks generated using PDQuest analysis software (Bio-Rad)

In the post reproductive phases after complete spawning, the blood samples collected in mid-May showed little expression of vitellogenin than the earlier phases, as the active transportation of vitellogenin from liver is ceases completely and its concentration in the blood is very low. After the spawning, the species enters the spent stage towards the end of May extending up to late June. The superficial evaluation of ovary seemed empty after expulsion of ovum, significant reduction in ovary size and seemed like a flabby structures. As both ovary and serum showed no presence of yolk and thus no vitellogenin spot was observed.

4. Conclusion

The reproductive cycle of teleosts is a seasonal phenomenon which is greatly influenced by environmental factors and the physiological changes associated to the reproductive activity are manifested in the morphological, histological and biochemical variations in organs and tissues. The serum plays an important role in transport of various endogenous and exogenous metabolites, to mediate such responses. The study of changes in the abundance of serum proteins and their modifications could be a powerful tool for generating hypotheses regarding how the environmental factors signal and mediate the reproductive biology of organisms. However, only few studies describe the relationship between other components of serum proteome and different reproductive phases of the annual cycle. Three isoforms of vitellogenin were identified using 2-D gel electrophoresis and MS analysis i.e. Vitellogenin 1, Partial Vitellogenin 2 and Partial Vitellogenin 4. No significant vitellogenin expression was observed in immature phase from January to mid-February. Thereafter the serum samples showed significant expression as vitellogenin formed in liver is transported to ovary. However, the expression decreased in the late March and increased again in mid-April indicating the species is a batch spawner; the vitellogenin production seem to cease a little after spawning and increases before next batch of ovum are spawned. Finally, the species enters the spent stage towards the end of May extending up to late June. Thus, the technique enabled us to identify and characterize the female specific protein, vitellogenin of *S. plagiostomus,* from a few drops of the blood clearly indicating the maturity stages and allowing determining the exact time of its breeding. These proteins represent the molecular phenotype of cells and as such have a direct effect on organismal physiology and thus reproductive fitness. Studying co-expression pattern of different serum proteins along reproductive phases or along seasons could be a more comprehensive step toward understanding of the systems dynamics of cells in response to perturbations in the environment or other external cues.

4.1 Conflict of Interest

Authors declare that there is no conflict of interest.

5. Acknowledgement

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