

Environmental Risk, Infections, Fish Protein Biomarkers in Fish Food Safety: A Prospective Study

Environment temperature stress causes physiological stress and infections in fish muscle

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- A hypothesis was proposed that bacterial and fungal diseases, environmental climate risks cause physiological stress that may decrease table fish protein quality. The study puts forth the following evidences and our findings in support.
- Environment temperature, water salinity factors were identified that likely induced physiological stress in fishes to make them vulnerable to infectious diseases.
- Bacterial infectious and fungal diseases harboring in fishes caused poor protein quality of edible fish.
- Edible carp, *Catla catla* and *H. fossilis* fish revealed altered amino acid constituents of fish proteins.
- Histological, histopathological and electron microscopic data of *C. catla* and *H. fossilis* supported the bacterial and fungal infection induced tissue changes and proteins in muscle, liver and serum.
- Poor fish protein quality of flesh may affect human health among fish eaters with possible implications of huge market loss and considerable economic and health concern to fisherman families.

Key Words: fish proteins, Electron Microscopy of fish, Histology and histopathology of fish muscle.

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I. Introduction

Fresh fish flesh was reported as an excellent source of proteins for human diet (Gangwar *et al.*, 2007). Fish protein has relatively high biotransformation and digestibility due to all available ten essential amino acids in recommended dietary values or balanced amino acid quantity for human consumption and cardiac protection (Sharma 2013; Chandrashekhar *et al.*, 2004). Fish proteins are digested in human intestine. After digestion proteins get converted finally into amino acids to meet metabolic requirements and excess protein metabolites are assimilated or incorporated into muscle buildup (Dabhade *et al.*, 2009).

Fish aquaculture practices of poor handling, crowding, transporting, fluctuating temperatures, polluted environment and poor water quality can impose „physiological stress“ on the homeostatic mechanisms in fish. These make fish vulnerable to a wide variety of pathogens such as parasites, water molds, viruses, and bacteria and ultimately fish death (Plumb, 1999). Monitoring such hazards **in turn may reflect the overall health of entire fish population in the ecosystem. In our view, histological alterations in cells and tissues and histopathological changes in fish serve as surrogate biomarkers in aquatic ecosystem. Major experimental histopathological data is available in muscle, liver, ovary, skeleton system and skin changes in contaminated fish as environmental stress biomarkers (Hinton *et al.*, 1985). In following section, infection in fish responsible for fish mortality is highlighted.**

A disease outbreak in fish causes huge losses to fish production, economic loss and socioeconomic revenue of many countries. Two major problems limiting fish production and mortality are: environmental stress reported by Rajkumar *et al.*, (2005) and Acharya and Dutta (2005); bacterial and fungal infections in fishes reported by Das *et al.*, (2005); Hussain *et al.*, (2005); Sharma *et al.*, (2005); Dhar *et al.*, (2006); Baeket *et al.*, (2006); Ariadna *et al.*, (2007); Chalkoo *et al.*, (2007); and by Mastan (2008). **Infection of bacteria and fungi deteriorates the productivity of the fish and muscle protein values.** In following section, fish muscle, protein content and high quality fish protein nutrition value is reviewed.

Fish proteins are rich in lysine and low in tryptophan when compared with red meat. Muscles contribute upto 60% of the total fish mass as its nutritive value based on biochemical composition of fish muscle protein (Chandrashekhar *et al.*, 2004; Kitts *et al.*, 2004; Sahin *et al.*, 2006; Kandemir and Polat, 2007) without any data on deterioration of protein quality induced with infection (Mustafa, 2000; Soliman *et al.*, 2004). In this

direction, efforts are in progress such as protein content, protein analysis, amino acid composition, protein turnover rates etc.:

1. Specific protein contents in fish muscle were reported in number of teleosts such as, *H.fossilis* (Hunge and Baile, 2004); *Schizothorax richardsonii* (Basade *et al.*, 2006); *Babylonia spirata* (Shanmugamet *et al.*, 2006) and *L.rohita* (Gangwar *et al.*, 2007);
2. A more specific Electron Microscopic studies of fish muscle were reported by Evgen'eva *et al.*, (2000); Medina *et al.*, (2000) and Ridgway *et al.*, (2007).

In following section, a feasibility study is proposed on overview of normal and infected (bacterial and fungal) fresh water fishes: *C.catla* a major carp; and *H.fossilis* a common *catfish* of central India. **Major hypothesis was that bacterial and fungal diseases in fish due to environment temperature stress decrease the edible protein quality of *C.catla* and *H.fossilis* fish.** Authors put forth a new concept "Environment temperature stress causes physiological stress in muscles" with following objectives:

1. Prevalence of infection was studied in diseased fish having significant pathological lesions by microbiological investigation.
2. Histological and histopathological changes in muscle tissue in normal and infected fishes.
3. Electron Microscopic studies to illustrate the ultrastructural changes in muscles.

1. Scope of fish farming: In following section, three consecutive five-year fish farming plans of co-operative society - fishermen's multipurpose co-operative society limited, Nagpur is reviewed on aquaculture and tanks for fish culture.

2. Common Fish: Several varieties of fish were farmed *Catla catla*, *Labeo rohita* and *Cirrhinus mrigala*, locally called as Catla, Rohu and Mrigal every year. The catfish *Heteropneustes fossilis* is in high demand so far for monoculture but it can be cultured in combination with *Clarias batrachus*, or *Anabas testudineus*. *C.catla* is the fastest growing common species such as Catla (*C.catla*) Rohu (*L.rohita*) and Mrigal (*C.mrigala*) contributing about 87% of total freshwater production. (ICLARM report (2001). In following section, fish infections are highlighted.

3. Common identified infections:

3.1 Epizootic Ulcerative Syndrome (EUS): Epizootic Ulcerative Syndrome is a seasonal epizootic disease which occurs due to low temperature and heavy rainfall. The first states of India to receive the Epizootic Ulcerative Syndrome (EUS) were Tripura, Assam, Meghalaya, West Bengal and Bangladesh in May 1988. Later reported from Mizoram, Arunachal Pradesh, Manipur, Bihar, Orissa and U.P. in 1989, Sikkim, Nagaland, M.P., Maharashtra, A.P., Tamilnadu in 1990; Kerala, Haryana and Rajasthan in 1991 (Das and Das, 1993).

3.2 Bacterial Infections: Bacterial infections are setback for successful fish farming. Bacteria are the most common pathogens of cultured warm water fish cause major losses to the freshwater aquaculture industry in India (Mohanty and Sahoo, 2007). Epizootics are often associated with rising temperatures in the spring and summer. Chalkoo *et al.*, (2007) reported on bacterial diseases of fishes of Wular Lake. Occurrence of *Listeria sp.* in meat and fish was investigated by Kumar *et al.*, (2005). Hussain *et al.*, (2005) reported occurrence of fin rot disease in common carp (*Cyprinus carpio*) infected by *Aeromonas hydrophilla* in Kashmir. Authors believe that bacterial presence in water is major cause of organ damage mainly muscle. In following section, we explore fungal infections in fish.

3.3 Fungal Infections : Fungal infections are known found to be commonly associated with ulcerative lesions in advance ulcers in diseased fish: a virulent *Aphanomyces laevis* parasite in oomycotic disease of the fresh water fish *Aplocheilichthys panchax* causing cotton-wool disease involving the skin and fins (Mondal and De, 2002); *Aspergillus flavus*, *Aspergillus sp.* and *Penicillium sp.* 33 fungal isolates from fish organs in 191 fish by Dhar *et al.*, (2006); *Aphanomyces* fungal infection induced mycotic granulomas, haemorrhages, cell necrosis, cotton wool like lesions on fresh water fish body surface, ulceration and erosion of skin and muscle reported by Zahura *et al.*, (2004). In next section, we mention mixed infections and fish diseases.

3.4 Mixed Infections: Heavy mortality of the Indian major carp, *C.catla* was reported due to *Trichodinidciliophoran* and *Tripartiella species* (Acharya and Dutta, 2005). Dykova and Lom (2000) reported *Kabatana arthuri* (Microspora) infection in sutchi catfish, *Pangasius sutchi* from Thailand. Microsporidian *Kabatana arthuri* induced severe regressive changes in trunk muscles. Necrotic changes were developed in muscle fibers around the developmental stages and on the periphery. Udomkusonsri and Noga (2005) noticed that when fish lost their protective skin barrier, viruses, bacteria (eg. *Pseudomonas Sp.*) Water molds (eg. *Saprolegnia Sp.*), or parasites more readily invaded the skin and caused infection. One of the most classical stress related pathogens were the water molds (oomycetes).

3.5 Electron Microscopic Studies: EM studies suggested sarcolemmal invaginations constituting the „T“ system in fish muscle fibers and myofibrillar structures by electron microscopy. Shindo (1984) examined effect of fixative concentration in freshwater and salt water fish and showed the preparation of electron microscopy specimens of fish muscle. Battaram and Johnston (1991) carried histochemical and electron microscopic study on muscle growth in demersal stages of the Antarctic teleost, *Notothenianeglecta*, Nybelin. Stoiber et al (2002) investigated the possible source of new muscle fibers in teleost fish by transmission electron microscopy. Medina *et al.*, (2000) observed muscle ultrastructural pathology in tropical fish *Colossoma macropomum* treated with herbicide. The muscle fiber changes consist of swelling of sarcotubular elements, mitochondria and sarcomere disorganization as the common myopathic changes in other vertebrates. In following section, we introduce to readers with methods used and fish species reported.

II. Materials and Methods

1. Fish species: The Central Indian major carp *C.catla* and the catfish *H.fossilis* were procured from fresh water lake during regular netting. The collected fishes were transported to the laboratory and acclimatized in the aquarium.

2. Environmental temperature and Rainfall: The data of temperature (air) and rainfall was collected from records available with the Regional Meteorological Centre, Nagpur. The water temperature was taken fortnightly.

3. Screening of fishes: Screening of normal and infected fish was done on the basis of following observations. Normal fishes show good color, gills reddish in color, scales compactly arranged and without any gross lesions.

4. Gross Pathological Observations: Fishes were carefully observed for external gross lesions and specific signs of disease by external and internal examination. The infected fishes were found to be weaker and showed:

- Whirling movement, coming to the surface splashing water (*i.e.* changes in fish behaviour).
- Skin became dull /discoloration (pale color) and descaling.
- Gills pale.
- Slimy and showed small haemorrhages on skin and muscles (Sometimes lead to inflammation), skin ulceration.
- Red ulcers noted on head and other body parts.
- Whitish margins of fins and loss of fins, fins destroyed severely and skeleton was exposed.
- Abnormal swelling of belly (not confused with egg ripening).
- Fungal infection on body.

5. Prevalence of infection:Prevalence of infection can be calculated by following formula:

$$\text{Prevalence of infection (\%)} = \frac{\text{No. of infected fish}}{\text{Total no. of fish examined}} \times 100$$

6. Sample Collection:The moribund fishes with typical sign and symptoms of bacterial and fungal infection; were biopsied for wet mounts and culture, and sampled for histopathological evaluation. The fishes were anaesthetized with Phenyl Cellosolve muscle were excised and the samples were collected for microbiological study taken in broth. For histological, histopathological and electron microscopic studies tissues were fixed in various fixatives.

7. Microbiological Examination: Isolation and Identification of Bacteria and fungus from infected *C.catla* and *H.fossilis* were done as per standard procedures (Bergey's 1984; Carter, 1990).

7. i. Isolation of Bacteria: When specific signs of infection were confirmed by external and internal examination, samples were collected aseptically with the help of a platinum loop and transferred to nutrient broth, incubated at 37°C for bacteriological investigation. Growth of bacteria, if any, was observed after 24- 48 hours by noting turbidity in the broth. Primary cultures were made from the turbid broth by streaking on nutrient agar plates (9 cm) and incubated at 37°C for 24 hours. Nutrient agar is enrichment, non-selective medium for general bacterial culture. The dominant colonies in the nutrient agar were identified as *Gram* positive or *Gram* negative by *Gram* staining.

a. *Gram*'s staining

- Isolated bacterial culture was taken on the slide in the form of smear. The smear was stained with crystal violet for 30 second to 1 min.
- Isolated bacterial culture was taken on the slide in the form of smear. The smear was stained with crystal violet for 30 second to 1 min.
- Rinsed with water (did not squirt water directly on the smear but let water run over smear).
- Added *Gram*'s Iodine solution for 30 second to 1min.

- Again rinsed the slide with water.
- Decolorised with 95% ethyl alcohol by letting alcohol run over smear until excess stain washed out (10-20 second).
- Immediately washed with distilled water.
- Added the secondary stain Carbol fuschin for 30 seconds.
- Washed slide with water.
- Blotted and dried the slide.
- Covered the preparation with immersion oil and observed under microscope.

b. Morphological characteristics: The characters of colonies such as shape, size and color were noted using binocular microscope and selected colonies were picked up for slant culture. The agar slants were inoculated at 37°C for 24-48 hours. Preliminary identification was made on the basis of colony characteristics. In addition to this *Gram* staining was done.

c. Cultural characteristics: For identification upto genus, isolated colonies from slants were restreaked on selective media (Table.1) such as Mac-Conkey agar, Eosin Methylene Blue (EMB) agar, Mannitol salt agar, Brilliant green agar (BGA) along with responses to various biochemical tests for comparing as per the standard procedure ((Bergey's, 1984; Carter, 1990).

d. Isolation of single colonies from mixed culture: Four quadrant streaking method was employed. The mixed cultures were streaked on fresh surface so that isolated colonies were grown.

7. ii. Fungi Isolation: Fungi were cultured on agar plates containing sterilized medium prepared as follows: 3 g of Sabouraud dextrose agar (SDA) and 3 g of agar agar was rehydrated by suspending in 100 ml of deionized water. The medium was sterilized by autoclaving for 15 minutes (15 lbs pressure and 121°C). The sterile medium was allowed to cool to 55°C and then poured into approximately (9 cm) sterile Petri dishes. Covered agar plates were stored at 4°C until ready for use. For fungal isolation it is essential to minimize the length of time the plates are uncovered and exposed to potential contaminants.

For fungi isolation, samples were collected from moderate pale, dermal lesions by removing scales around the periphery of the lesion and underlying skin with a red hot spatula. Then by using a sterile scalpel blade and fine pointed forcep, superficial tissues were exposed, taking precautions that blade and forcep do not make any contact with contaminated external surface thereby contaminating the underlying muscles. Using aseptic techniques, excised pieces of muscles and liver were placed on a petridish containing nutrient agar and Sabouraud Dextrose Agar (SDA). Then plates were sealed; incubated at room temperature and observed daily. After fungal growth was observed on plates the hyphal tips were transferred repeatedly on fresh plates to get isolated culture. The cultures were then stained with Lactophenol Cotton Blue and observed for identification of fungi.

Table1: Details of various isolated media.

Sr. No.	Name of Media	Quantity (ml)	Composition (g)	Company
1	Nutrient agar	100	Peptone(bacteriological) - 1g Meat extract powder - 1g NaCl - 0.5 g Agar agar (Type I) - 3 g	Himedia
2	Nutrient broth	100	Peptone (bacteriological) - 1g Meat extract powder - 1g NaCl - 0.5 g	Himedia
3	MacConkey Agar	100	MacConkey Agar - 5.507g Agar agar (Type I) - 1.5 g	Himedia
4	Eosin Methylene Blue agar (EMB)	100	EMB agar - 3.75 g Agar agar (Type I) - 1.5 g	Himedia
5	Mannitol salt agar	100	Mannitol salt agar - 11.1g Agar agar (Type I) - 1.5 g	Himedia
6	Sabouraud Dextrose Agar (SDA)	100	Sabouraud Dextrose broth - 3 g Agar agar (Type I) - 3 g	Himedia
7	Brilliant Green Agar (BGA)	100	BGA - 5.8 g Agar agar (Type I) - 1 g	Himedia
8	Fungal Stain		Lactophenol Cotton Blue	

8. Histological Methods: The normal and infected fishes (fishes even showing no obvious external gross lesions) were separated and photographed. Then anaesthetized and dissected in normal saline solution. The samples of muscle and liver of about 1cm³ were excised and fixed in aqueous *Bouin's* fixative (18 – 24 hours) and 10% neutral buffered formalin for histolopahtological studies,

The fixed tissue was then dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin wax, sectioned at 4-5 μm thickness and mounted on albuminized slides. The sections were stained with the following staining technique (Humason, 1979).

Haematoxyline Eosin Staining: The sections were deparaffinized in xylene and passed through a descending grades of alcohol and brought to the water. Then stained in haematoxyline (for 2- 5 minutes) and kept in running water (for 10-15 minutes). The sections were partially dehydrated through series of alcohol upto 90% alcohol and counterstained in eosin (for 2 minutes) differentiated in 90% alcohol, dehydrated in absolute alcohol, cleared in xylene and mount in DPX. The slides were then examined under microscope and photographed.

9. Electron Microscopy: To study ultra-structural changes of normal and infected muscles, electron microscopy work was carried out at EM facility centre of Department of Anatomy, All India Institute of Medical Sciences (AIIMS), New Delhi. The samples were sent at AIIMS after primary fixation. The brief procedure was given by the EM centre.

a. Scanning Electron Microscopy (SEM) Protocol for Specimen processing: (specimen Size 2-3 mm)

After primary fixation in 2.5% glutaraldehyde, samples were washed in 0.1M phosphate buffer (3 changes), postfixed in 1% OsO_4 , washed in 0.1M phosphate buffer (3 changes), dehydrated in graded series of acetone (30%, 50%, 70%, 90%, 95%, 100%) dried at critical point drying, mounted on aluminium stubs, gold coated with 20-30 nm thick film and observed under the scanning electron microscope.

b. Transmission Electron Microscopy (TEM) Protocol for Specimen processing: (specimen Size 1 mm^2)

After primary fixation in 2.5% glutaraldehyde, samples were washed in 0.1M phosphate buffer (3 changes), postfixed in 1% OsO_4 , washed in 0.1M phosphate buffer (3 changes), dehydrated in graded series of acetone (30%, 50%, 70%, 90%, 95%, 100%), cleared in toluene, infiltrated, embedded medium (resin) using mould. Embedded blocks were kept in an oven polymerization. After polymerization, blocks could be removed by bending the mould sideways.

c. Sectioning and staining: Both semi thin and ultra-thin sections were cut with glass knife on a *Leica* ultra-cut *UCT-GA-D/E-1/00* ultra-microtome. Semi thin sections of 0.5 – 1.0 μm thickness were mounted on the glass slides, stained with toluidine blue and ultra-thin sections of 60-90 nm thickness were mounted on carbon coated grids, stained with uranyl acetate and counter stained with 4% lead citrate (Bozzola and Russell, 1999) and observed at various magnifications under a transmission electron microscope.

III. Observations and Major Findings

1. Determination of causative factor: Temperature as physiological stress

1.1 Annual temperature variation and fish health: The living environment of fish plays an important role for fish health and growth. The changes in the temperature create stress to fish and favour multiplication of pathogens. Stress response from the environment leads to fish diseases and mortality in extreme cases. Stress is a condition in which organism's equilibrium is disturbed. The fish on crossing tolerance limit shows changes in fish behavior i.e. coming to the surface splashing water and finally exhausted sinking to the bottom.

1.2 Climate of Nagpur: The climate of Nagpur follows a typical seasonal monsoon weather pattern. Being located far away from any major water body at the centre of the Indian peninsula, climate of Nagpur city is dry or mildly humid for most of the year except for the rainy season, extreme variations in temperature, hot summer and chilly winter. Three main seasons are: Summer is from February to May, Winter from October to January and Monsoon from June to September. Summer in Nagpur is extremely hot, with daytime temperatures exceeding 40°C mostly.

1.3 Temperature variation and rainfall: The data pertaining to the temperature (both air and water) and rainfall is presented in Table no.2, Graphs 1, 2 & 3. The water temperature taken during the study period was maximum at 31.2°C to 31°C in May while minimum at 18°C to 19°C during January. The highest air temperature recorded was 43.6°C in May while the lowest temperature recorded in December and January was 30.2°C. The onset of monsoon during the year 2007 was from June and the season extended up to September, with highest recorded rainfall was 362.8 mms in July. The variations in air temperature changes temperature of water and physicochemical characteristics of water thereby induce stress among fishes and makes them susceptible to various diseases. Indian Major Carp *C. catla* die at 39°C and air breathing catfish *H. fossilis* gets exhausted at 42°C.

Table 2: Mean monthly maximum and minimum temperatures and rainfall.

S.No.	Month	Air Temperature ⁰ C		Rainfall in mm	Temp of water ⁰ C	
		Maximum	Minimum		Pond A	Pond B
1.	Jan-07	30.9	13.2	0.0	19	18
2.	Feb-07	32.5	16.3	2.4	21	21
3.	Mar-07	36.4	20.0	8.0	24	23.5
4.	Apr-07	41.4	24.7	3.5	30	29
5.	May-07	43.6	27.9	10.2	31.2	31
6.	June-07	38.3	26.3	226.4	30.2	30.5
7.	July-07	32.0	24.4	362.8	28	27.8
8.	Aug-07	31.5	23.9	187.0	27.5	27
9.	Sep-07	33.2	23.6	207.4	26	27.5
10.	Oct-07	34.1	18.4	2.9	26.5	25.5
11.	Nov-07	31.8	15.7	27.1	24	25
12.	Dec-07	30.2	14.0	0.0	22	21

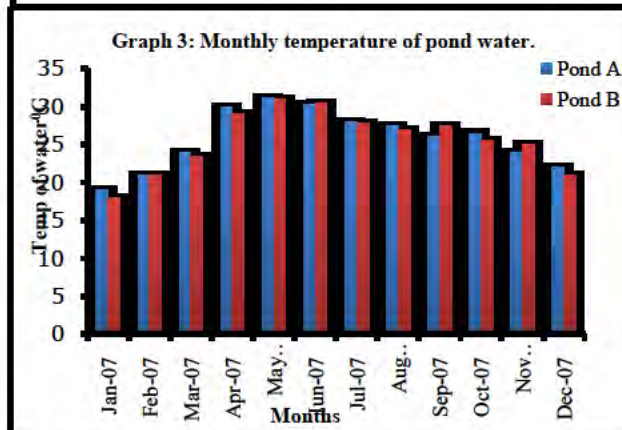
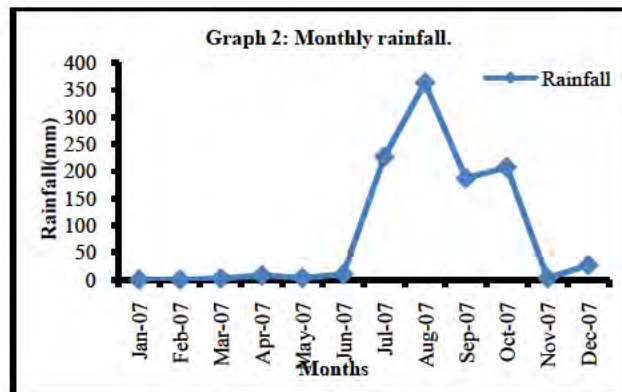
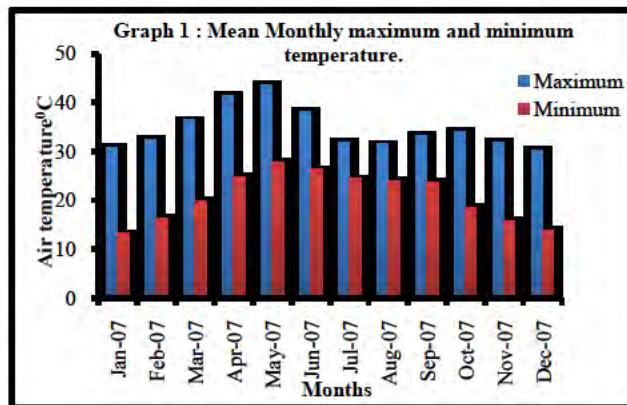




Fig 1. Infected *C. catla* Showing haemorrhage at base of fin and abdominal dropsy. **Fig 2.** Infection present around the eye and mouth in *C. catla*. **Fig 3.** *C. catla* showing skin ulcer deeply invading muscle. **Fig 4.** *C. catla* showing haemorrhages and necrotic patches on body. **Fig 5.** *H. fossilis* showing black patches over skin. **Fig 6.** Infected *C. catla* showing fungus growth.

2. Signs and Physical Symptoms of infection in fish: The moribund fishes kept in aquarium showed irregular whirling movements and showed following clinical signs and symptoms given in Table 3.

Table 3: Physical signs and symptoms due to infection.

Fish	Clinical signs and gross lesions.	Fig
<i>C. catla</i>	Haemorrhage at base of fin and abdominal dropsy	Fig 1.
	Infection around eye and mouth.	Fig 2.
	Skin ulcer deeply invading muscle.	Fig 3.
	Haemorrhages and necrotic patches on body.	Fig 4.
	<i>C. catla</i> showing fungus growth.	Fig 6.
<i>H. fossilis</i>	Ulcer in anal and caudal region.	Fig 7.
	Tail and fin rot.	Fig 9.
	Fins destroyed severely, skeleton exposed.	Fig 10.
	Whitish margins of fin.	Fig 8.
	Black patches over skin.	Fig 5.

The distribution of lesions is quite variable and includes mouth back trunk /body wall and caudal peduncle (tail base). The diseased fishes were subjected to microbiological investigations for isolation of bacteria and fungus.



Fig 7. *H. fossilis* having ulcer in anal and caudal region.

Fig 8. *H. fossilis* showing whitish margins of fin.

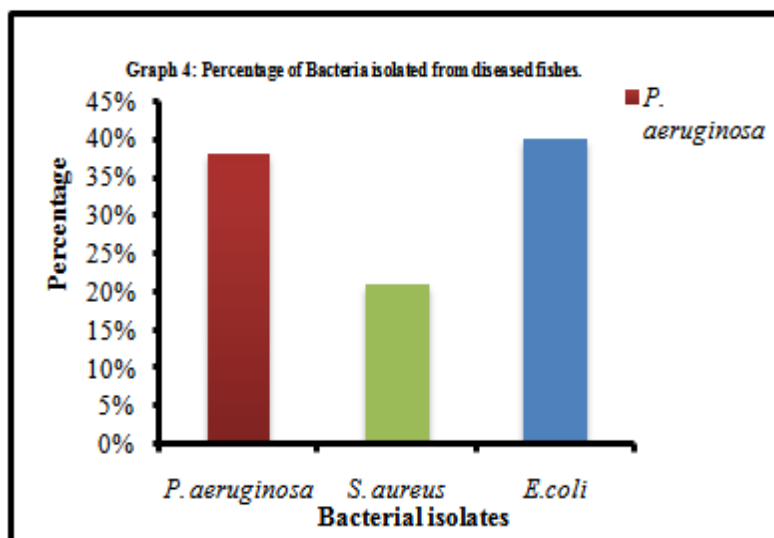
Fig 9. Tail and fin rot in infected *H. fossilis*.

Fig 10. Fins destroyed severely, exposing skeleton in *H. fossilis*.

3. Identification of causative microbial factors to cause Physiological stress: 28 fishes were found to be infected by bacterial pathogens (Table. 4; Graph. 4).

Table 4: Details of bacterial isolates from diseased fishes.

Fish	Site of infection	Bacterial pathogen		
		PBI-1	PBI-2	PBI-3
<i>C. catla</i>	Skin (ulcer)	3	2	3
	Muscle	0	1	3
	Abdominal fluid	6	2	5
	Liver	2	2	0
<i>H. fossilis</i>	Skin (ulcer)	3	1	2
	Muscle	1	0	1
	Abdominal fluid	0	1	2
	Liver	1	0	1
Total	42	16	09	17



- i. **A criteria for identification of bacteria:** Bacteria examined were *Gram* negative rods and *Gram* positive cocci. The bacterial cultures were identified using various criteria such as morphological characteristics, cultural characteristics and biochemical tests. Among 42 isolates from different diseased fishes, three pathogens i.e. PBI-1, PBI-2, PBI-3 (Table 4) were identified and selected for further studies. The morphological characteristics, cultural characteristics and biochemical characteristics of these isolated pathogens are shown in the Tables 5, 6 and 7.

Table 5: Morphological characteristics of isolated pathogens.

Characters	Codes of isolated pathogens		
	PBI-1	PBI-2	PBI-3
<i>Gram</i> 's reaction	<i>Gram</i> -ve	<i>Gram</i> +ve	<i>Gram</i> -ve
Motility	Motile	Non-motile	Sluggishly motile
Colony size(µm)	0.5-1 X 1.5-5	0.5-1.2 X 2-3.5	1.5-2.5X0.8-1.2
Shape	Rods	Cocci	Rods
Flagella	+ve	-ve	+ve
Spore	-ve	-ve	-ve
Flagella	+ve	-ve	+ve
Capsule	-ve	D	-ve
Margins	Erose	Entire	Entire
Elevation	Flat	Convex	Slightly convex
Optimum temperature	37°C	37°C	37°C

Key: +ve: Positive; -ve: Negative;D: Differential

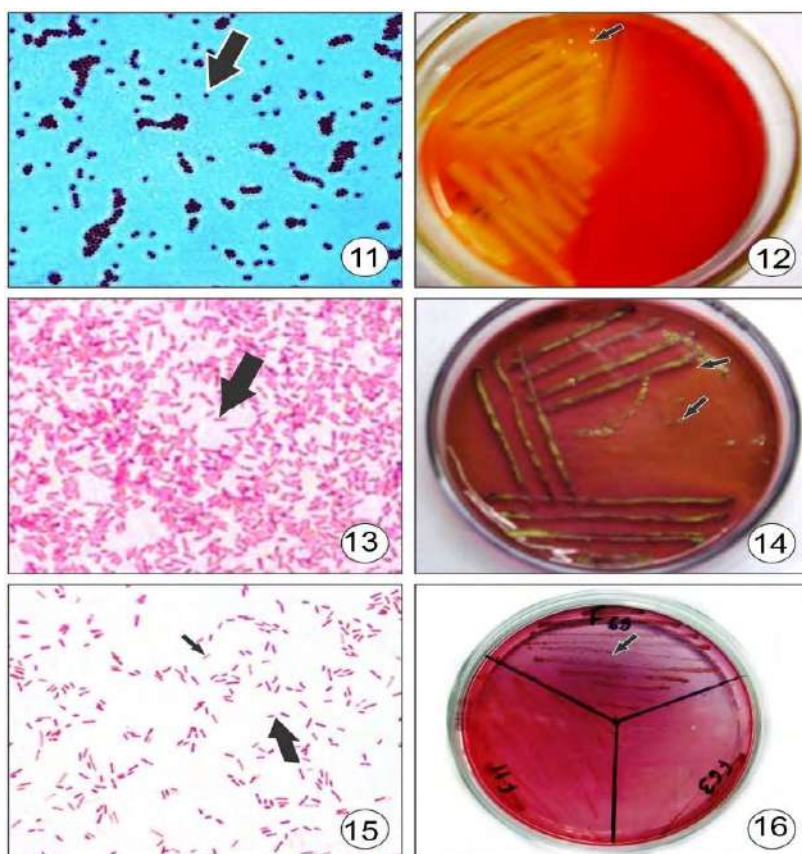


Fig 11.Bacterial smear stained with *Gram*'s stain showing *Gram* positive cocci.**Fig 12.** Isolated pathogens on selective medium *S.aureus* on mannitol salt agar.**Fig 13.**Bacterial smear stained with *Gram*'s stain showing *Gram* negative bacteria.**Fig 14.***E.Coli* on Eosin Methylene Blue agar.**Fig 15.** Bacterial smear stained with *Gram*'s stain showing *Gram* negative, rods.**Fig 16.** *P.aeruginosa* on Brilliant Green Agar

Table 6: Cultural characteristics of isolated pathogens on selective and differential media.

Name of Media	Codes of isolated pathogens		
	PBI-1	PBI-2	PBI-3
Nutrient agar	Creamy white, irregular , Large, opaque	Creamy white, opaque, moist	Grayish white, large, thick, moist , smooth, opaque
Nutrient broth	Luxuriant growth	Luxuriant growth	Luxuriant growth
MC Agar	Colorless	NA	Button like pinkish coloration
EMB agar	Inhibition of growth	Inhibition of growth	Small dark centered colonies with greenish metallic sheen
Mannitol salt agar	NA	Crimson yellow colonies	NA
BGA	Metallic sheen	NA	NA

Table 7: Biochemical characteristics of isolated pathogens.

Tests	Codes of isolated pathogens		
	PBI-1	PBI-2	PBI-3
Indole	-	-	+
Methyl red	-	+	+
Voges-Prausker	-	+	-
Citrate	+	-	-
H ₂ S production	-	-	-
Oxidase	+	-	-
Catalase	+	+	+
Urease	-	D	-

Gelatin liquefaction	+	+	-
Nitrate reduction	D	+	+
Coagulase	-	-	-
Glucose fermentation	+	+	+
Lactose fermentation	-	+	+
Sucrose fermentation	D	-	+

Key: + ve: Positive; -ve: Negative; D: Differential

The morphological, cultural characteristics and biochemical characters were compared and identification of these bacteria was done by method of Bergey's *et al.*, (1984) and Carter (1990) for isolated pathogens i.e. PBI-1, PBI-2, PBI-3 *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *E.coli* respectively.

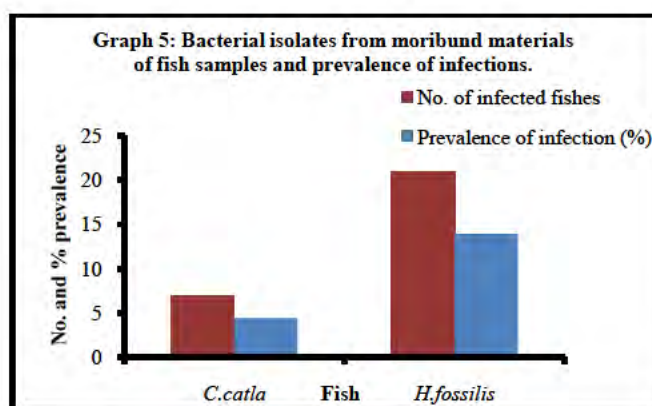
Table 8: Percentage of bacteria isolated from diseased fishes.

Name of the isolates	Pure cultures	Percentage%
<i>Pseudomonas aeruginosa</i>	16	38%
<i>Staphylococcus aureus</i>	9	21%
<i>E.coli</i>	17	40%
Total	42	100%

ii. **Fish Samples for bacterial identification:** (Table. 8). Total 42 bacterial isolates from infected fishes belonging to genera *Pseudomonas aeruginosa* (16); *Staphylococcus aureus* (9) and *E.coli* (17) were recovered (Table. 8, Graph. 4). The average prevalence of infection was 9.03%. In *C.catla* prevalence of infection was found to be 4.4% while in *H.fossilis* prevalence of infection was 13.9% (Table. 9; Graph. 5).

Table 9: Bacterial isolates from moribund materials of fish samples and prevalence of infections.

Fish Samples	No. of samples examined	No. of infected fishes	Prevalence of infection (%)	Bacteria identified
<i>C.catla</i>	159	07	4.4%	<i>P.aeruginosa</i> <i>E.coli</i> <i>S. aureus</i>
<i>H.fossilis</i>	151	21	13.9%	
Total	310	28	9.03%	



iii. **Identification of Fungus**

The diseased *H. fossilis* showing whitish margin of fin, fungus growth on body, grayish fungus over fish, black patches over skin, fluffy white growth like cotton wool disease, haemorrhagic ulceration over the body surface and fins, large red or gray shallow ulcers, were subjected to isolation of fungus. The fungus isolated from samples during present study was identified as *Mucor*, *Penicillium* and *Aspergillus*. It was confirmed that the isolated pathogens i.e. PFI-1, PFI-2, PFI-3 were *Mucor*, *Aspergillus* and *Penicillium* respectively. Out of 310 fishes analysed, 14 were found to be affected by fungal infection (Table. 11). The average prevalence of infection was 6.6%. In *C.catla*, prevalence of infection was found to be 4.4% while in *H.fossilis* prevalence of infection was 4.6% (Tables. 10 & 11; Graph. 6).

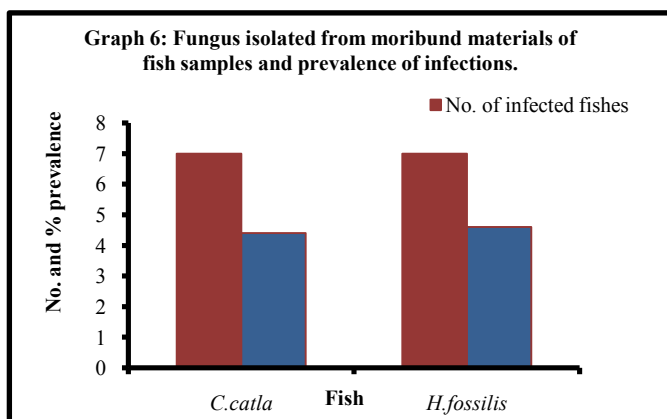
Table 10: Fungus isolated and identified from diseased fishes.

Fish Code no.	Source	Fungus		
		PFI-1	PFI-2	PFI-3
1.Feb-C♂7	Liver	-	-	+
2.Feb-C♂8	Skin & Muscle	-	+	-
3.Feb-C♀7	Skin	-	-	+
4.Feb-C♀8	Skin	+	-	-
5.Feb-C♀9	Skin	-	+	-
6.Oct-C♂7	Skin	+	-	-
7.Oct-C♂8	Skin	-	+	-
8.Apr-H♂7	Liver	-	-	+
9.Apr-H♀7	Muscle	-	+	-
10.Jul-H♂7	Skin	-	-	+
11.Jul-H♂8	Liver	-	+	-
12.Jul-H♀7	Muscle	-	+	-
13.Jul-H♀8	Skin	-	-	+
14.Jul-H♀9	Skin	+	-	-

Key: + ve: Positive; -ve: Negative

Table 11: Fungus isolated from moribund materials of fish samples and prevalence of infections.

Fish Samples	No. of samples examined	No. of fungal affected fishes	Prevalence of infection (%)	Fungus Identified
<i>C.catla</i>	159	07	4.4%	<i>Mucor</i> <i>Aspergillus</i> <i>Penicillium</i>
<i>H.fossilis</i>	151	07	4.6%	
Total	310	14	6.6%	



4. Histological Observations

4.1 Muscles (Normal fish): The muscles of the body consist of a double series of muscle segments, the myotomes in the region of the trunk and tail. The trunk musculature consists of successive segments-the myomeres, running along each flank. The muscle fibers are oriented in anteroposterior position in each myotome and are separated from the adjacent ones by stout sheets of connective tissue; the myocommata. The myotomes are bent forward and backward and fit with the adjacent ones by the cone within the cone arrangement. In the surface view, each myomeres is generally in the form of a „W“ with upper edge turned forward. Prominent blocks of lateral trunk muscle (myotomes) are visible as the meat of a fish when it is skinned. Histologically, muscle fibers have peculiar ribbon like myofibrillar bundles and round edges of the fiber. Muscle fibers are arranged like spokes from a small central sarcoplasmic hub.

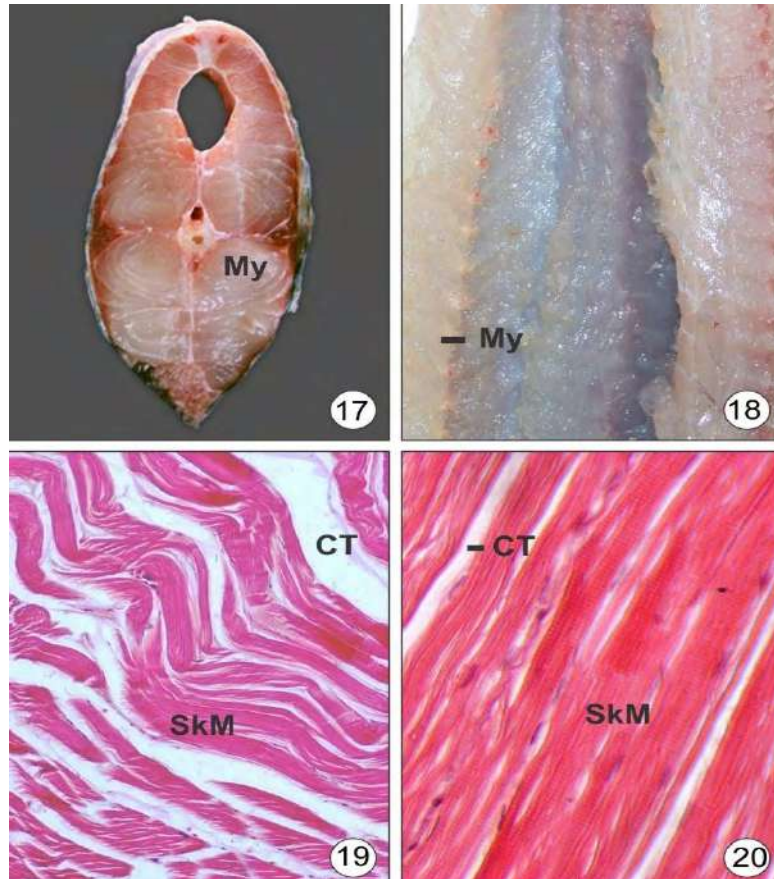


Fig 17.Edible muscle piece of *C.catla*. **Fig 18.** Edible muscle of *H. fossilis*.

Fig 19. L.S.of an intact skeletal muscle(SM) {*C.catla* (H.E.X400)}.

Fig 20. L.S.of an intact skeletal muscle(SM) {*H. fossilis* (H.E.X200)}.

4.2 Skin and Muscles(Gross pathology infected fish): Sloughing off scales with degeneration of epidermal tissue and ulceration on different parts of the body were commonly observed lesions during the present study. Haemorrhage of the fin and abdominal dropsy, haemorrhagic dermatitis of varying sizes mostly in anal fin and at the base of fins were observed during present study.

Microscopically, the changes observed were characterized by muscle haemorrhages and necrosis. The major histopathological alterations in the muscles of *C.catla* and *H.fossilis* included degeneration in muscle bundles, focal areas of necrosis. Also vacuolar degeneration in muscle bundles, atrophy of muscle fibers, oedema between muscle bundles and splitting of muscle fibers were seen. Necrosis in the muscles and epidermis were also observed in some sections (**Table. 12**).

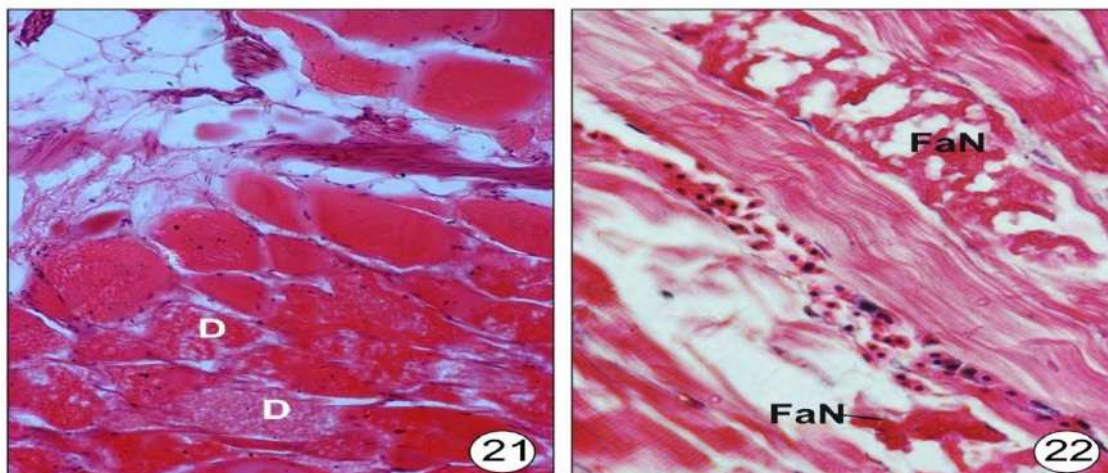


Fig 21.T.S. muscle showing degeneration in muscle fibers {*C.catla* (H.E.X200)}.

Fig 22. T.S. muscle showing focal areas of necrosis {*H. fossilis* (H.E.X400)}.

Table 12: Histopathological observations of fish muscle under study.

Histopathological Observations	Muscle	
	<i>C.catla</i>	<i>H. fossilis</i>
Necrosis	+	+
Degeneration	+	+
Vacuolar degeneration	+	+
Haemorrhage	-	+
Splitting of muscle bundles	+	+
Atrophy	-	+
Oedema	+	+

5. Electron Microscopic Observations

5.1 Scanning Electron Microscopy of Fish Muscle

Scanning Electron Microscopy (SEM) of normal fish muscle show presence of long ribbon like muscle fibers. The muscle fibers are arranged one above the other in clusters. SEM of muscles taken from moribund fishes shows clusters of numerous short, slightly curved rods and cocci along with the myofibrils. A fungus with branched hyphae was also noted on the surface of fish muscles adherent to the muscle fibers. Both bacteria and fungus were observed in the muscle fibers. Due to muscle disintegration, in some areas, cell debris and blood cells were found. Some of the muscle cells clearly shows microridges on the surface of muscle.

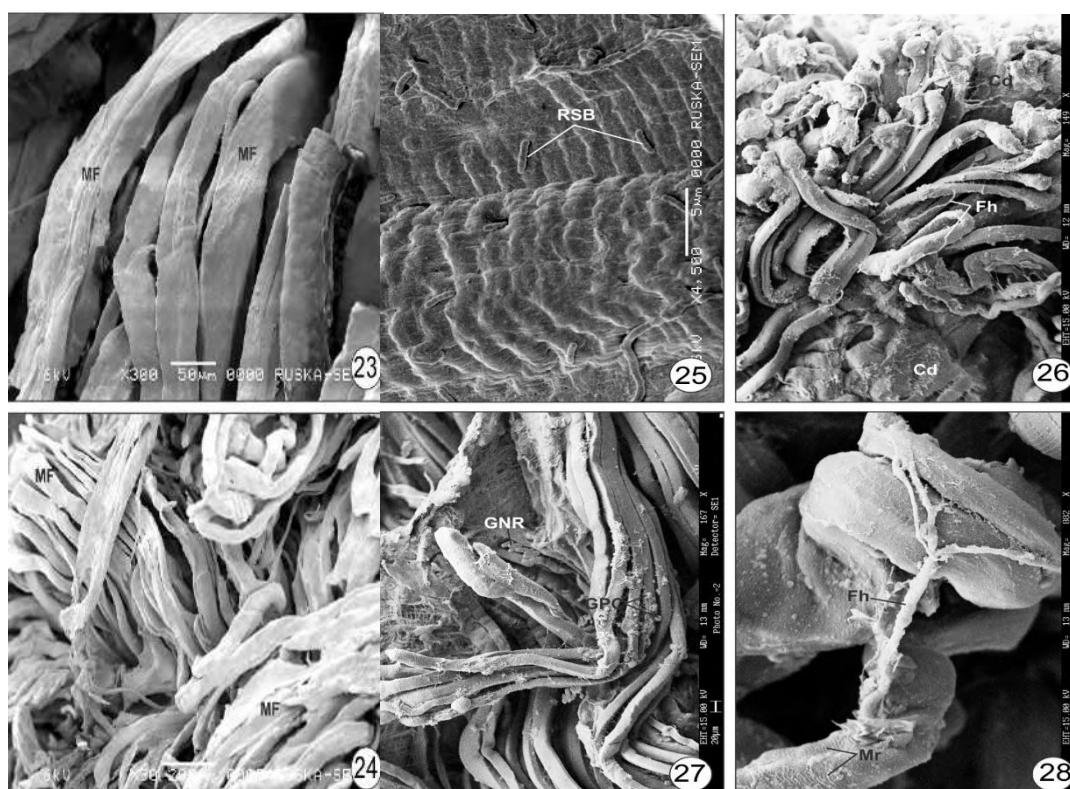


Fig 23. SEM of normal *C.catla* muscles showing long ribbon like muscle fibers (MF). **Fig 24.** SEM of normal *H.fossilis* muscles showing muscle fibers in clusters (MF). **Fig 25.** SEM of infected *C.catla* muscles showing Rod Shaped Bacteria (RSB). **Fig 26.** SEM of infected *C.catla* muscle showing branched Fungal hyphae and Cell debris. **Fig 27.** SEM of infected *H.fossilis* muscles showing clusters of Gram Negative Rods (GNR), Gram positive Cocci (GPC) and Blood Cells. **Fig 28.** SEM of infected *H.fossilis* muscles showing fungal hyphae.

5.2 Transmission Electron Microscopy of Fish Muscle

Examination of ultrathin sections with Transmission Electron Microscope (TEM) of normal muscle showed regular arrangement of sarcomere within the myofibrils giving the muscle striated pattern with normal display of mitochondria. The thick and wavy Z-lines and T-system were noted. The functional units of muscle (sarcomere) were separated from one another by an electron dense Z-line.

Ultrathin section with Transmission Electron Microscope (TEM) shows myofibrils arranged in polygonal shape. Myofibrils were regularly packed at the level of the distinct Z-line and M-line. Among the myofibrils in between intermyofibrillar spaces, sarcoplasmic reticulum was distributed. The sarcoplasmic

reticulum and T -system formed triads alongwith few mitochondria and glycogen granules in between the myofibrils. The mitochondria of muscle fibers were characterized by the possession of numerous cristae. The Transmission Electron Microscopy (TEM) of infected muscle showed following degenerative changes:

- Alteration (increment) in the intermyofibrillar spaces. The formation of dense bodies in the intermyofibrillar spaces and glycogen disappearance. The mitochondria with disintegrated cristae, without cristae and particulate matter were observed. Mitochondria can be heavily damaged changing their normal morphology. A slightly swollen mitochondria surrounded by necrotic areas were seen.
- Destruction of myofibrils at places resulting into formation of vacuoles. The myofibrillar disorganization leading to formation of big vacuoles. Autophagic vacuoles and necrotic areas were noticed.
- Glycogenosomes, loss of glycogen granules with particulate matter were observed. T-system, Z-lines, sarcoplasmic reticulum were disrupted. Total lack of structure among muscle fibers i.e. clear zone at some places in between myofibrils.

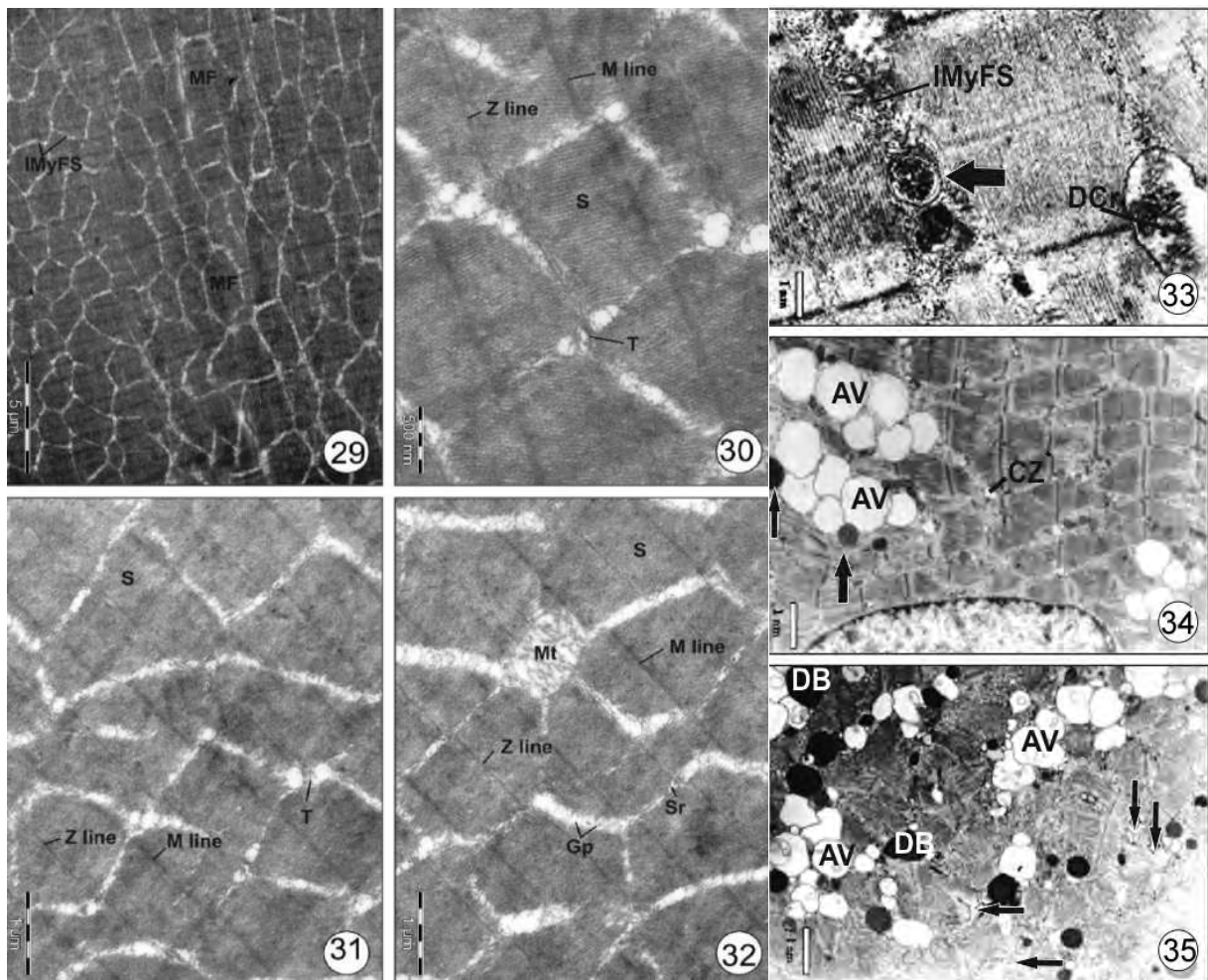


Fig 29.TEM of normal *H.fossilis* muscles showing muscle fibers (MF) arranged in polygonal shape.**Fig 30.**TEM of normal *H.fossilis* muscles showing various cellular components. **Fig 31.**TEM of normal *C.catla* muscles showing regular arrangement of sarcomere (S) within the myofibers.**Fig 32.** TEM of normal *C.catla* muscles showing normal display of mitochondria (MT) and other cellular details.**Fig 33.** TEM of infected *H.fossilis* muscles showing the mitochondria (MT) with Disintegrated Cristae (DCr) and alteration in the Intermyoibrillar Spaces (IMyFS).**Fig 34.** TEM of infected *H.fossilis* muscles showing formation of dense bodies (DB) in intermyofibrillar spaces and glycogen disappearance.**Fig 35.**TEM of infected *C.catla* muscles showing Autophagic Vacuoles (AV), Clear Zone (CZ) in between muscle fibers.

IV. Discussion

Critical Analysis of temperature stress induced physiological stress and fish protein changes.

Diseases in fishes are closely linked to environmental and physiological stress. Diseases develop as a result of complex interactions between pathogen, fish and environmental stress which affect the susceptibility of the host to diseases.

Fish and other aquatic animals are poikilothermic (cold-blooded). Kutty, (1987) has reported the temperature as the master factor among environmental factors affecting aquatic life. The health, nutrient requirements, performance, reproduction and in extreme cases, survival of fish are all dependent on the temperature of the water. In the present chapter, water temperature is shown beyond optimum range (13.8°C – 32°C) during March-June months which directly alters the physicochemical characters of pond waters like pH, DO, BOD, alkalinity etc. Such fluctuations disturb maintenance of homeostasis essential for growth and reproduction and induce stress in fishes making them more susceptible to infections (Bacterial and fungal) during summer months. Das *et al.*, (2005) reported the influence of environmental temperature caused stress on Indian major carp liver and kidneys from the second hour of a heat shock at 37°C. *C.catla* dies at 39°C and *H.fossilis* is exhausted at 42°C as reported by Biswas, (2000).

Bacterial pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherchia coli* infected *C.catla* and *H.fossilis*. Percentage incidence of bacteria infecting the fishes was 40%: *E.coli*, 38%: *P.aeruginosa* and 21%: *S.aureus*. *P.aeruginosa* was reported from skin ulcers of both the fishes along with *S.aureus* and *E.coli*. *S. aureus* was isolated from skin ulcers, muscles, abdominal fluid and liver. Harish *et al.*, (2003) noted high prevalence of opportunistic pathogens such as *Aeromonas* and *Pseudomonas* without any disease outbreaks in Vembanadu Lake of Kerala. Sharma *et al.*, (2006) revealed prevalence of *E.coli* and other enteropathogens of zoonotic significance in fish and fish products from Ludhiana.

The fungi isolated from *H.fossilis* and *C.catla* were *Mucor*, *Aspergillus* and *Penecillium*. Dhar *et al.*, (2006) and Sharma *et al.*, (2005) recorded *Aspergillus* and *Penecillium* from fish mortalities. In present report *Mucor*, *Aspergillus* and *Penecillium* were observed and same were reported by Meshram *et al.*, (1998). Chowdhury *et al.*, (2003) detected *Aphanomyces* and *Saprolegnia sp.* as pathogenic fungi in the ulcer type disease in the fishes. However, no fungal fish pathogens *Saprolegnia*, *Aphanomyces* were observed in present report.

Histopathological studies of muscles of *C.catla* and *H.fossilis* reveal necrosis, degenerative changes, haemorrhage and congestion, cellular infiltration, edema in the diseased fishes. Histopathological biomarkers are closely related to other biomarkers of stress since many pollutants have to undergo metabolic activation in order to provoke numerous cellular changes in the affected organism which manifest as necrosis i.e. histopathological biomarker on a tissue level. The haemorrhagic dermatitis in anal fin or at the base of fins in *C.catla* and *H.fossilis* was in agreement with necrotic ulcers reported by Sahoo *et al.*, (1998) on the posterior part of the body, tail and caudal fin. Most common diseases of fish are those that affect the skin and muscles. Epidermal damage, especially skin ulceration, is a well-recognized biomarker of stressful environment (Udomkusonsri and Noga, 2005). Mohamed, (2009) also reported similar changes in muscles, due to environmental contamination in *Tilapia zillii* and *Solea vulgaris*.

Electron microscopic study to observe the progression of lesion development is state of art. Scanning Electron Microscopy (SEM) of normal *C.catla* and *H.fossilis*, showed presence of long ribbon like muscle fibers arranged one above the other in clusters. Typically, SEM of muscles show clusters of numerous short, slightly curved rods and cocci alongwith the myofibrils. Fungi with branched hyphae were observed on the surface of fish muscles. Due to muscle disintegration, in some areas, cell debris and blood cells were found. Some of the muscle cells show remains of microridges along with fungal hyphae. Strongman *et al.*, (1997) revealed ulcers along with numerous branches of fungal hyphae, Gram-negative bacteria and cellular debris. Fungal hyphae and necrosis on the surface of the skin showed extended damage along the scales through the dermis and musculature. Role of bacteria in the progression of lesion development by Hsu and Smolowitz, (2003) of muscle fibers through SEM and TEM showed fungal hyphae alongwith remains of superficial microridges.

Transmission Electron Microscope (TEM) of normal muscle showed regular arrangement of sarcomere within the myofibrils giving the muscle striated pattern with normal display of mitochondria alongwith cristae and glycogen granules. Muscle fibers were regularly packed at the level of the distinct Z-line and M-line, sarcoplasmic reticulum and T- system were observed. Stoiber *et al.*, (2002) reported TEM of muscle fibers in three species of cyprinid fish, their fine structure, including solid clusters of muscle fibers, thick and wavy Z-lines and T-system.

The fishes infected by bacterial and fungal infection showed peculiar features: loss of balance and poor speed in swimming due to disorganization of muscle fibers, loss of myofibrillar structure, formation of big vacuoles, alteration in the intermyofibrillar spaces, formation of dense bodies in the intermyofibrillar spaces, glycogen disappearance, mitochondria with disintegrated cristae, proteolytic activity, and particulate matter observed in the present investigations, due to infection. In TEM of infected muscle there was no evidence of

bacteria and fungi but clear zone at some places in the muscle fibers suggested the proteolytic activity of bacteria and fungus tissue degrading enzymes.

Histological evaluation of the necrosis and cytoplasmic vacuolation in fish muscles, SEM and TEM studies closely matched the earlier reports: a loss of sarcomeric structure with necrotic lesions, fragments of myofibrils and connective tissue elements, eventual condensation of these elements into separate islands (Ridgway *et al.*, 2007); degenerative ultrastructural changes in the tropical fish *Colossoma macropomum* (Medina, 2000); ultrastructural destruction of the myofibrillar apparatus, with sarcolemma, T-system, and sarcoplasmic reticulum in fish muscle (Evgen'eva *et al.*, 2000).

Bacterial and fungal infection results in economic losses due to the fish mortalities, loss of meat quality and poor external appearance of the fish making it unsuitable for market. A number of causative agents include both *Gram* negative and *Gram* positive species entering via wounds or low-level infections (Cheng and Chen 1998; Evans *et al.*, 1998). Presence of bacteria and fungus in fishes is of special concern to human health (Endtz *et al.*, 1991).

V. Conclusion

- Fluctuations in environmental temperature not only induce physiological stress in fishes but also make them prone to diseases. Bacterial and fungal diseases in fishes affect human health because these are likely to transfer from the food chain to humans.
- Edible carp, *Catla catla* and *H. fossilis* reveal significant changes in the composition of fish proteins. Data from electron microscopic study support the hypothesis that temperature induced bacterial and fungal diseases decrease the table quality of *C. catla* and *H. fossilis*. Loss of fish market value of diseased fish is obvious and may be a cause for considerable economic concern to fisherman families.

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