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Myxospridean Parasite In Fish Culture Ponds And Histochemistry Due To Physico-Chemical Parameters

Mr. D. Ravi[1], Dr. Ch. Krishnamurty[2], Ch. Hema Prathyusha[3], Dr. V. Sailaja.[4*]

[1]*Research Scholar, Department of Zoology, Vikrama Simhapuri University College, Kavali, Nellore*
[2]*Faculty, Department of Zoology, Andhra University, Visakhapatnam*
[3]*Research Scholar, Department of Zoology, Vikrama Simhapuri University College,Kavali, Nellore*
[4*]*Assistant Professor in Zoology, Vikrama Simhapuri University College, Kavali, Nellore*

[*]***Corresponding Author:*** *Dr. V. Sailaja.*
[*]*Assistant Professor in Zoology, Vikrama Simhapuri University College, Kavali, Nellore,*
vemulurisailaja68@gmail.com

Abstract

Hennaguya sp. is a myxosporean parasite of *Cyprinus carpio* that caused damage to the muscles and gills by dense infestation which resulted in respiratory problems and caused locomotory disturbances. In this study histochemical observation on tissues with Heidenhain's iron hematoxylin, Feulgen's Techniques, PAS and Best's Carmine and were saliva labile indicating that they are probably glycogen granules. The fine ground cytoplasm was positive to PAS was saliva fast, positive to Alcin blue, Toludine blue and dialysed iron showing its nature to be a mucopolysaccharide. Fish was swimming near the surface with distended operculum, trail for jumping outside the water and mortality due to heavy cardiac infection. In the 1980s, it was discovered that *Hennaguya sp.* needs to infect a unified oligochaete to complete its life-cycle. Thus the parameters physical and chemical parameters were influenced on Fish. Which characterized by changes in Water temperature, pH, Dissolved oxygen(DO) and Alkalinity maintenance of water quality parameters within the normal range is one of the most important factors for culturing fish.

Key words:, *Hennaguya sps., Cyprinus carpio*, Heidenhain's iron hematoxylin, Feulgen's Techniques, PAS and Best's Carmine,. Physico-Chemical parameters.

INTRODUCTION:

Freshwater bodies which include reservoirs, tanks, lakes and rivers offer extensive feeding and breeding grounds for a variety of fishes such as carps, murrels, catfishes etc. Owing to the complex nature of environmental conditions in these areas, there are abrupt changes in dissolved oxygen, pH, alkalinity, temperature and other physicochemical factors. As a result fishes and other organisms inhabiting these water bodies exhibit great endurance physically. Consequently, many of these organisms are considered best suited for fish farming and other aquaculture practices being carried out at a global level. The need for such an exercise appears obvious in the context of increasing demand by man for inexpensive protein food from the aquaculture water bodies to feed the growing millions living in the third world countries. Despite excellent logistical and per capita yield noticed, one major constraint against expanding aquaculture practice has been the disease

induced affliction following large scale fish kills which have often been attributed to acute parasitic invasions causing death due to "disease".

The term "disease" may be defined in its broadest sense as any departure and deviation from an animal (Sindermann, 1970). Among aquatic animals fish diseases are mainly two types namely microbial and metazoal; the two forms including virus, bacteria, fungi and protozoa; later myxozoans, cnidarians and many helmenths, copepods and acantho cephalans. Among these myxozoans are undoubtly the most pathogenic to fishes, causing behavioural, physiological and anatomical changes such as the swimming abnormalities (due to *Myxobolus, Myxosoma* infection in salmonids), skeletal deformities(*Myxosoma cartilaginis* in centrarchids), tissue erosion and inflammation (*Myxobolus dogieli* in carps) and metaplasia and necrosis (*Henneguya sp*. in cat fishes).

In more recent years, increasing attention is being paid to studying ecology of myxosporidians since as parasites of aquatic organisms they are far more dependent on changes in water quality than their counterparts elsewhere. Further, the mobility of water would also enhance the possibility of rapid increase in view of infections are sometimes a flare in chronic invasions through easy spread of the parasites. Often, these effects are compounded when there is a decrease in host resistance due to stress caused by adverse water quality. Under such conditions, an adequate knowledge of the parasites, its "entity" seems therefore essential for an overall assessment of the host parasite environment relationships. A number of workers have carried out work on the ecology of myxosporidian parasites. The most significant of which include those of Noble(1957,1973,1984), Paperna (1975), Paperna and Lahan (1971-75), Paparna and Overstreet (1977,1981), Overstreet and House (1977), Knight *et al.* (1977,80), Pellitero *et al*. (1983), Hartigan (2012) and Beth Okamura, Alexander *et al.* (2015).

Material and Methods:

The present investigation was undertaken both under laboratory in the Department of Zoology, Government Degree College, Ponduru and field condition from selective study areas i.e., Fish ponds Eluru, West godavari and Kakinada East Godavari district, Andhra Pradesh, India.

To collect, isolate and identification of certain Myxosporidian parasites of common carp fish (*Cyprinus carpio*). During the study, Common carp fish were collected at regular monthly intervals for a period of one years during June-2021 to May-2022 at two selected study areas i.e., Fish culture ponds from East and West Godavari districts. Host specimens were randomly sampled from the ponds and brought alive to the laboratory or in moribund condition.

Methods Adopted:

The water samples have been collected from two different stations of Kakinada and Eluru culture ponds Godavari districts. Water sample has been collected from column region of the ponds in 500ml polypropylene bottles. Studies of water quality parameter have been started from January-2021. The following parameters have been taken into account for the determination of dissolved oxygen and alkalinity. Water temperature and pH were measured on field.

In *Cyprinus carpio* clinical signs of infection were evident with certain conformity of diagnostic significance. The fish after collection were immediately examined with the aid of a hand lens to detect any external indications of infection. Fish suspected to have infection were taken to the laboratory and examined under a binocular microscope to detect infection. When infection was not detected externally, the fish were dissected out and the different internal organs such as gills, intestine and muscle were examined to detect infection.

When infection was present, the spores were isolated from the host tissue and smears were prepared from them, fixed; with acetone-free methyl alcohol and stained with Giemsa after an initial hydrolysed in 1N HCl at 60^0 C for 10-15 minutes. Smears were wet-fixed with Schaudinn's, Carnoy's or alcoholic Bouin's fluid and stained with Heidenhain's iron haematoxylin or treated according to Feulgen's or Periodic Acid Schiff (PAS) techniques. Some spores along with the tissue were fixed along with the host tissue in aqueous or alcoholic Bouin's fluid and paraffin sections were cut at 8 µm thickness. They were stained with Heidenhains iron haemotoxylin or Azan technique for histological studies. For the extrusion of polar filament a drop of Hydrogen peroxide (H_20_2) was added to the air-dried smears and 90% of the spores extruded the polar tube, which were clearly observed under dark ground illumination as well as by negative staining with India ink.

Hennaguya sps. parasites in the gill, intestine and muscle of fish were chosen for a detailed study. Histochemical observations were carried out by fixing bits of infected tissues showing spores in different fixing fluids such as aqueous Bouin's fluid, alcoholic Bouin's fluid, Carnoy's fluid, 10% formalin, cold absolute

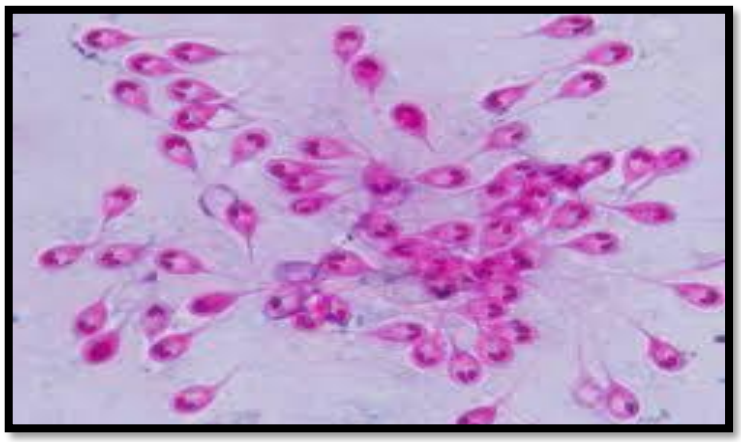
alcohol or formal calcium, sectioned at 8µm thickness and stained them with appropriate stains. All the methods were followed from Pearse (1968) unless otherwise mentioned.
The histochemical techniques employed were Periodic-Acid Schiff (PAS) method for the detection of carbohydrates and various other materials, and followed by PAS after acetylation and deacetylation for control of 1:2 glycol groups. For the detection of mucopolysaccharide, Steedman's Alcian blue and dialysed Iron methods were used. Metachrornatic methods such as Toluidine blue and alcoholic Toluidine blue were also used.

Observation:

Henneguya sp.

The genus *Henneguya thelohan*, 1892 (characterized by bivalvular spores with valves continuing as caudal projections) is the second largest one out of the nine genera composing the *Myxobolidae* family. In the last decade, the number of *Henneguya* species spread throughout the world reached 80 species. Their importance as pathological agents is recognised either in aquaculture or in nature. Myxozoan parasite *Henneguya sp.* was found to cause an important reduction in gills and muscle affects in the *Cyprinus carpio* fish from Kakinada and Eluru culture forms, Godavari districts. Infection was intense with several plasmodia.
The spores are white and measure up to 0.3 – 0.5 mm in length (n=50). Mature spores are ellipsoidal in the frontal view measuring 36.4-36.3 µm in length, 3.8-4.7µm in width and caudal process 28.1-27.5 µm. In the lateral view, the spores are biconvex, measuring 4.3-4.6 µm in thickness. The valves are symmetrical; the polar capsules are elongated and equal in size measuring 4.2-4.8 µm in length and 1.7-2.2µm in width. The polar filaments have five to seven turns and are arranged perpendicularly to the longitudinal axis of the capsules.
Histological analysis revealed that the parasite develops on the gill filaments and lamellae. The development of the plasmodia leads to the stretching and deformation of these structure, thereby reducing the functional area.
The spore have single wall, which is in direct contact with host cells and has extensive, multiple pinocytic canals connecting the wall to the plasmodial ectoplasm (Azevedo and Matos, 2002; El-Mansy and Bashtar, 2002; Adriano *et al*., 2005b). The plasmodium wall is seen as the organelle responsible for feeding the structure of the plasmodium wall is necessary to understanding the stress incurred on the host by the parasite (EL-Mansy and Bashtar, 2002). The hennaguya cysts are frequently seen surrounding the endothelium and the projections in the direction of the muscle suggest an important interaction between the parasite and muscle tissues, possibly characterizing an important source of spore nutrition.
The gill is the major respiratory organ, the primary site of nitrogenous waste excretion and plays an important role in ionic balance (Noga, 2000). Species from the genus *Henneguya* have different manners of interaction with fish gill structures, resulting in different levels of disease (Current and Janovy, 1978; Dykova and Lom, 1978; Bowser and Conroy, 1985; Kalavati and Narasimhamurti, 1985; Duhamel *et al*., 1986; Martins *et al.,* 1997, 1999; Molnar, 1998; Adriano *et al.,* 2005a,b; Molnar *et al*., 2006a,b). No inflammatory response was observed in the gills infected with *H. pseudoplatystoma* n. sp., which is a common finding in infections by *Henneguya sp*. (Barassa *et al*., 2003; Adriano 2005a,b; Eiras *et al*., 2008; Eiras *et al*., 2009), despite the responses induced by some species (Dykova and Lom, 1978; Martins *et al*., 1997, 1999; Molnar, 1998; Work *et al.,* 2008). Regardless of the absence of inflammatory response, the development of the plasmodia of *Henneguya sp*. *n. sp*. Cause stretching and deformation of the filament and lamella structures, thereby substantially reducing the area of functional epithelium, which certainly has a negative effect on the development of infected pintado specimens and may lead to significant losses, especially if a high prevalence and high infection intensity of the parasite are associated with poor environmental and biological conditions, which are common in fish farms. The histopathological analysis revealed a layer in the plasmodial ectoplasm near the plasmodial wall, with intensely eosinophilic areas associated to other, less eosinophilic areas. In the ultrastructural study, this layer corresponded to fibrous material, which, based on the thickness of the microfibrils (7 nm), resembles aggregated actin. Actin is one of the most abundant proteins in eukaryotic cells and has an important role as a major component of the cytoskeleton in the cell (Kabsch and Vandekerckhove, 1992). This fibrous material appears to act as a support to the plasmodium and the projections observed on its surface. Fibrous material resembling aggregated actin has also been reported anchoring the mural cells of the presporogonic cells of *Tetracapsuloides bryosalmonae* (Myxozoa: Malacosporea) and presumably helping stabilize the spore sac (Morris and Adams, 2007). Casal *et al.* (1997) also report the presence of microfilaments occupying the entire space of pericyte cells in *Henneguya striolata*. The authors suggest, however, that these are myosin filaments. Just below of the fibrous material found in the plasmodial ectoplasm of *H. pseudoplatystoma* there were numerous mitochondria, generative cells and the earliest stages of sporogenesis, whereas far from periphery there were different spore developmental stages, which is a similar characteristic

to other parasites from this group (Casal*et al*., 1997; Abdel-Ghaffar*et al.,* 2008; Azevedo and Matos, 2002; Azevedo and Matos, 2003; Casal *et al*., 2003; Adriano *et al*., 2005a,b. The reduction in the area of functional epithelium in the gills demonstrates that this species has considerable pathogenic potential. It leads to respiratory distress and suffocation and leads to mortality of the fish.



Photograph showing the *Hennaguya sp* spores smear stained with Giemsa.

HISTOCHEMICAL OBSERVATIONS

In heavy infections the xenomas occurred in different internal organs of the fish, but the cysts collected from the intestinal tissue and muscle tissue alone were chosen for detailed histochemical studies. The cyst wall was 12 – 15 µm thick and typically showed 3 layers. The outer most layers which was cellular was situated nearer the host tissue and showed rhomboidal cells filled with fine ground cytoplasm and disc-like granules. These granules were positive to PAS and Best's Carmine and were saliva labile indicating that they are probably glycogen granules. The fine ground cytoplasm was positive to PAS was saliva fast, positive to Alcin blue, Toludine blue and dialysed iron showing its nature to be a mucopolysaccharide.

The middle non-cellular layer was positive to PAS, Stedman's Alcian blue, Toluidine blue and dialysed iron indicating its mucoidal nature. It was also positive to Alcian blue at pH 6.0. There were a few sparsely dispersed glycogen granules in this layer.

The inner most layer was fibrous in appearance and stained deep blue with Azo-caramine and Mallory's triple stain. It was strongly positive to aldehyde fuchsin without oxidation; Van Giemson's stain and showed a weak reaction to PAS. It was positive to Alcian blue with 0.6 M $Mgcl_2$, 0.2 M $MgCl_2$, showing the presence of strongly sulphated chondroitin sulphate A and C type of Mucin. These mucins are generally present in connective tissues (Cook, 1977). It stained deep red with Verhoeff's stain indicating its nature to be collagen.

The lumen of the xenoma was divided into a number of compartments by fine filamentous extensions from the inner layer which spread out as they reached the centre of the lumen. These filaments showed similar histochemical reactions like those of the inner layer. The compartments nearer to the periphery showed the developing sporoblasts while the centre of the lumen was filled with fully formed refractile spores.

Owing to the small size and densely packed nature of the spores it was difficult to study their chemical nature in the sectioned material. Hence, all the observations on the histochemical nature of the spores were made on smear preparations only. The spore wall was highly refractive; double layered and was resistant to several chemicals and stains. All the spores were present in a matrix of cytoplasm which was positive to Steadman's Alcian blue, aqueous Toluidine blue, and dialysed iron showing the presence of mucus material.

The spore wall in general was positive to all protein tests and the outer layer was soluble in alkaline sodium sulphide. The spores gave a positive reaction to Chitosan test and Zander method, after they were treated with alkaline sodium sulphide showing that the nature of the exo-spore was proteinaceous and that of the endo-spore was chitinous. The outer exo-spore was thick, refractile, negative to PAS and positive to protein tests. It was also positive to Performic acid – Alcian blue (PFA-AB), paraldehyde fuchsin and Alcian blue after oxidation showing the presence of S-S groups.

The endo-spore was thin, brittle, and positive to PAS, chitosan, Zander method and to all general protein tests showing its nature to be chitin. The solubility tests also confirm the above observations. The polar capsules were positive to PAS Steedman's Alcian blue, Toludine blue and dialysed iron showing its mucoidal nature. The polar filament also gave similar reactions showing the presence of a mucus layer. The sporoplasm reacts weakly to PAS, Alcian blue and Toluidine blue as well as to mercuric bromophenol blue, Ninhyddrin Schiff,

azur Schiff and Malachite green showing the presence to little polysaccharides along with proteinaceous material. A comparison of the infected and uninfected tissues was made to
study the effect of the xenomas on the intestinal tissue. Macroscopically the intestinal tissue showing xenomas appeared enlarge, puffy and bloated. It was generally pale and dirty brown in colour or may even be completely discoloured in heavy infections. There was hypertrophy of the host cell and the host cell nucleus. The host tissue nearer the xenoma showed disrupted cells, the cell walls in many cases were broken down and hence appeared like a syncytium. Some of the nuclei in this layer were dumb-bell shaped. A few of the nuclei appeared to be undergoing amitotic divisions with two masses of chromatin material moving in opposite directions and connected by achromatic strands. A large number of such nuclei were seen distributed in this layer. The cytoplasm was highly vacuolated.
The histochemical observations showed that there was a depletion of glycogen in the infected cells showing fewer granules of acid mucopolysaccharide in the vacuolated cell cytoplasm. A few glycogen granules were found aggregated in the host tissue around the xenoma.(Table A).

S. No.	Tests	Infected Tissues		Uninfected Tissue	
		Cell Cytoplasm	Nucleus	Cell Cytoplasm	Nucleus
1	Heidenhain's iron hematoxylin	Cells hypertrophied; 170 – 180 × 75 – 80 µm in size; Cell membrane sometimes destroyed cytoplasm highly vacuolated ; Wandering phagocytes near the xenoma	Nucleus hypertrophied undergoing amitotic divisions which are present in the cells surrounding xenoma; Nuclei sometimes branched with dispersed chromatin	Cells stains deeply; cytoplasm alveolated, filled with numerous disc-like granules; cells 30 – 40 × 60 – 70 µm	Nucleus typical with a nuclear membrane and reticulate chromatin
2	Feulgen's Techniques	Cells hypertrophied	Nuclei undergo amitotic divisions; Nucleus Hypertrophied DNA released into cell cytoplasm	-------	Nuclei clearly spherical; no DNA particles in thecell cytoplasm
3	Azo carmine	Blue	Nuclei stain yellow. Other characteristics same as in Feulgen's technique	Blue	Blue cells with yellow nuclei. Other characteristics same as in Feulgen's technique.
4	PAS	+	-	+++	-
5	PAS after saliva digestion	-	-	-	-
6	Bests carmine	+	-	+++	-
7	Bests carmine after diastase digestion	-	-	-	-
8	Alcian blue	+	-	+	-
9	Dialysed iron technique	+	-	+	-
10	Touldine blue	+	-	+	-

Table-A: HISTOPATHOLOGICAL OBSERVATIONS ON THE INTESTINAL TISSUE

DISCUSSION:

Data obtained by monthly sampling revealed some differences in the seasonal prevalence of the myxosporidian and ciliate parasites in the target fish *Cyprinus carpio sps.* The prevalence and intensity of myxosporidian parasites of the *Hennaguya sp* were outstanding among two selected study locations. These three species of myxosporidians and ciliate were identified in the *Cyprinus carpio* gills, intestine and gall bladder during study period and it was observed that highest prevalence of myxosporidians and ciliate occurred during end of the dry season (June to November) and the levels of infections decreased during December to May, which characterized by changes in temperature, pH, Dissolved Oxygen(DO) and Alkalinity Maintenance of water quality parameters within the normal range is one of the most important factors for culturing fish. During the investigation the water quality parameters were similar at both culturable ponds. DO content and water temperature fluctuated from time to time.
Based on the observations as gathered in the present study, the occurrence of maximum infection is in rainy season and minimum in summer. This can be explained by the increased possibility to find food in the rainy season; during August to September. Due to this reason the movement of the fish was increased and muddy conditions made more polluted environment to infection. The parasitisation showed a significant positive relationship with size of the fish, very small fishes were nearly free of parasites, this exhibit a correlation with feeding habits of the fish.
Kamal *et al.,* recorded DO content varying from 1.80 mg/l to 9.8 mg/l in the seasonal ponds of Nator, Bangladesh. Banerjea (1967) stated that 5-7 ppm of DO of water body is good for biology productivity. According to Hossain *et al*., (2006), pH 6.5 to 9.0 is suitable for pond fish culture and pH more than 9.5 is

unsuitable because free CO2 is not available in this situation. Fortnightly fluctuations of total alkalinity in the experimental ponds ranged from 128.00 to 188.00 mg/l. Mairs (1966) stated that water bodies having total alkalinity 40 mg/l or more are considered more productive than the water bodies of lower alkalinity.
Hennaguya Sps. Shulman-Albova, 1953 which occurs on the gills of Pleuronectesplatessa (and some other species) is considerably smaller than the present species (cf. Lorn 1970). Another flatfishinhabiting species, T. raabei Lom, 1962 (from the gills of Pleuronectes flesus luscus), is also smaller and possesses a dark-staining adhesive disc centre (see Lorn 1962). Pearse (1972) reported on Trichodina sp. from the skin of captive P. platessa from Port Erin; this species possessed a dark-staining adhesive disc centre and was larger than the present species. From marine fish hosts, several subspecies of the freshwater species Trichodina domerguei (from skin and fins of stickleback) have previously been described (e.g. Lorn 1962). These species all have an adhesive disc with a distinct central circle. T domerguei f. marisnegri Lom, 1962 from the skin of Gaidropsis mediterranaeus L. possess a light central circle and have an adhesive disc and denticle ring of similar size to the present species. However, there are some distinct differences in the denticle morphology.

CONCLUSION:

The present study, carried out for a period of one years during June 2021 - May 2022 at two diverse habitats along the East coast of India namely, Fresh water fish ponds from Kakinada and Eluru, Godavari District, Andhra Pradesh, India was aimed at investigation the biology of the two known myxosporidian parasites of fresh water fish (*Cyprinus carpio*) vis-a-vis environmental conditions. The rational has been that the study, which takes into account of the host-parasite environment relationships, would help to understand some complexities in aquaculture particularly the large scale mortalities of fishes in ponds. Investigations on the ecology of pathogens included an account of their composition, prevalence, distribution, seasonal cycles in relation to abiotic factors. The highest prevalence of myxosporidian occurred during the end of the dry season (June to November) and the levels of infections decreased during December to May, which is characterized by chance water temperature, in conjunction with pH, dissolved oxygen and alkalinity were largely influenced the prevalence of myxosporidian infections. The fish infected tissue were cell cytoplasm and Nucleus when conducted tests with Heidenhain's iron haematoxylin. It shows Cells hypertrophied; 170 – 180 × 75 – 80 µm in size; Cell membrane sometimes destroyed cytoplasm highly vacuolated; Wandering phagocytes near the xenoma and Nucleus hypertrophied undergoing amitotic divisions which are present in the cells surrounding xenoma; Nuclei sometimes branched with dispersed chromatin.
To increase pH add sodium bicarbonate (baking soda), one teaspoon per 10 gallons until the desired level is reached. A higher pH alone is not harmful. Lowering pH can be difficult adding peat or vinegar is somewhat effective. Generally fish formers face several challenges in their operation: Problems related to pest and diseases, Weather conditions, Marketing and prices, Fodder shortage, High feed costs, Lower productivity and profitability, Shortage of good quality fish seeds, Lower probability, Seasonal variations and Water quality management. Environmental impact is the biggest challenges in aquaculture that needs to be addressed if cultivation practices are not carried out properly.
These studies will contribute significantly to predict the disease outbreak, disease surveillance, disease prevention and health management of fry, fingerlings and adults in order to improve the fish health and reduce mortality rate.

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