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Histopathological alterations and distribution of *Pomphorhynchus kashmirensis* in intestines and their seasonal rate of infestation in three freshwater fishes of Kashmir

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Abstract
In the present study water, fish and parasite samples were collected during different seasons from various sites and processed. Three fish species (*Schizothorax niger, Schizothorax esocinus, Schizothorax curvifrons*) were recovered from two water bodies of Kashmir valley. Acanthocephalan parasite *Pomphorhynchus kashmirensis* (27.47%) were recovered from all the three species of *Schizothorax*. The parasite showed higher prevalence during summer and least during winter. Overall the mean intensity of *Pomphorhynchus kashmirensis* was highest in Dal Lake whereas its abundance was higher in River Jhelum. Parasitic infections were prevalent more in male fishes compared to females. The presence of the parasites had reduced the condition coefficient of the infected fishes in both the water bodies. Histopathologically, the parasite induced various intensities of enteritis coupled with hyperplastic goblet cells with increased acid mucopolysaccharide concentrations.

Keywords: Water; fish; parasite; Dal Lake; River Jhelum

Introduction
The distribution of parasites varies not only in different species of fish but also seasonally and from one water body to other. The pathogenicity of parasitism has been reported to cause extensive damage to the host leading to the lower production of the fish (Rai, 1986) [28]. In certain studies the parasite has been found to be responsible for the death of the host (Bookmer *et al*., 1981) [4]. The relationship of parasitism and pollution is not simple and involves a double edged phenomenon in which parasitism may increase host susceptibility to toxic pollutants or pollutants may result in an increase or decrease in the prevalence of certain parasites. Numerous reports are available on the harmful effects of many acanthocephalan parasites on the digestive tract and associated organs of different fish species Dezfuli, *et al*., 2009) [11], but, no information is available due to *P. kashmiriensis* infections in snow trout, *Schizothorax niger*. The present study was therefore undertaken to determine the pathological changes in the intestine of naturally infected *S. niger* with *P. kashmiriensis* as well as to establish the host's responses against the parasite, which may be helpful in understanding the host parasite relationship.

Materials and Methods
Examination of live helminth parasites of fishes and preliminary treatment of the fish
The fishes were first given serial number and then the total length (cm) and sex of each fish were recorded. After collection, the fish samples were subjected to helminthological examination and then preserved in ice. Fishes were dissected in the laboratory and intestine were removed and examined for gastrointestinal helminth parasites.

Isolation of helminth parasites
Identification of fishes was carried out by morphological examination, as described by (Yamaguti, 1958; Soota, 1983 and Retief *et al.,* 2009) [34, 31, 29]. Acanthocephalans were relaxed in tap water so that specimens with proboscis fully everted were produced.
The regular record of the collection was maintained and the prevalence of acanthocephalans were carried out by the following formulas and such Ecological terms are studied as per Margolis *et al*. (1982) [34] and Gudivada *et al*. (2010) [15].

$$\text{Prevalence} = \frac{\text{Total No. of hosts infected}}{\text{Total No. of hosts examined}} \times 100$$

$$\text{Mean intensity} = \frac{\text{Total No. of parasites}}{\text{Total No. of infected hosts examined}}$$

$$\text{Abundance or relative density} = \frac{\text{Total No. of parasites}}{\text{Total No. of hosts examined}}$$

Pathology

Gross pathology Fishes were systematically subjected to detailed macroscopic examination with special emphasis on liver, intestine and the lesions were recorded.

Histopathology Representative Tissue samples from the liver, intestine affected by parasites were collected in 10% formalin. The tissue samples were processed for routine paraffin embedding technique and 5u thin section were stained with Haris haematoxylin and Eosin (Bernet *et al*. 1999) [2].

Histochemistry Parallel tissue section selected on the basis of histopathological examination was stained for following histochemical observation.

- Acid and neutral mucin were determined by combined alcian blue Periodic-acid Schiff (PAS) stain as per Bancroft and Gamble, 2002 [1]
- Mast cells were determined by toluidine blue staining protocol as per Gandalfo *et al*. 2006. [4]

Statistical analysis

SPSS for Windows 17.0 (SPSS Inc.) was used for statistical analysis of the data. Post hoc test should be used to interpret the data. One-way analysis of variance (ANOVA) was used to see the significant differences in intensity and abundance within two water bodies. Non-parametric analysis i.e. Mann-Whitney test was used to compare the condition factor of uninfected and infected fishes from the same populations.

$$U = n_1 n_2 + \frac{n_2(n_2+1)}{2} - \sum_{i=n_1+1}^{n_2} R_i$$

Pearson's correlation (r) was used to assess associations between different physico-chemical parameters and parasitic infection. The mathematical formula for computing ***r*** is:

$$r = \frac{n\sum xy - (\sum x)(\sum y)}{\sqrt{n(\sum x^2) - (\sum x)^2}\sqrt{n(\sum y^2) - (\sum y)^2}}$$

Where *n* is the number of pairs of data.

Results

Host-wise prevalence of *Pomphorhynchus kashmirensis* in Dal Lake and River Jhelum (Table 1)

Overall out of 444 fish specimens examined 122 (27.47%) were found to harbour the *Pomphorhynchus kashmirensis.* Out of 224 specimens examined from the Dal Lake only 47 specimens were found infected with *Pomphorhynchus kashmirensis* (20.98%). Host-wise distribution of the parasite was significantly varied ($p<0.01$) as *S. niger*, *S. esocinus* and *S. curvifrons* (27.63, 18.18 and 16.90%), respectively. Out of 220 *Schizothorax* spp. examined from River Jhelum only 75 (34.07%) were infected with *Pomphorhynchus kashmirensis* which include *S. niger* (30.20%), *S. esocinus* (30.13%) and *S. curvifrons* (42.25%).The overall mean intensities of *Pomphorhynchus kashmirensis* in *Schizothorax* spp. of Dal Lake and River Jhelum was 2.04 and 1.66 having an abundance of 0.42 and 0.56 respectively.

Table 1: Overall prevalence of *Pomphorhynchus kashmirensis* in various host species

Host	Dal Lake							River Jhelum						
	No. Examined	No. infected	Prevalence (%)	No. of parasites	Mean Intensity	Abundance	P-value	No. Examined	No. infected	Prevalence (%)	No. of parasites	Mean Intensity	Abundance	P-value
S. niger	76	21	27.63	47	1.76	0.48	< 0.01	76	23	30.2	30	1.30	0.39	< 0.01
S. esocinus	77	14	18.18	28	2.0	0.36	< 0.01	73	22	30.13	46	2.09	0.63	< 0.01
S. curvifrons	71	12	16.9	21	1.75	0.29	< 0.01	71	30	42.25	49	1.63	0.69	> 0.05
Total	224	47	20.98	96	2.04	0.42	< 0.01	220	75	34.09	125	1.66	0.56	< 0.01

Seasonal prevalence (Table 2): *Pomphorhynchus kashmirensis* showed a definite trend as the infection was highest in summer and lowest in winter.

Table 2: Infection dynamics of *Pomphorhynchus kashmirensis* recorded of *Schizothorax* spp. from Dal Lake and River Jhelum

Season	Host	Dal Lake							River Jhelum						
		No. Examined	No. infected	Prevalence (%)	No. of parasites	Mean Intensity	Abundance	P-value	No. Examined	No. infected	Prevalence (%)	No. of parasites	Mean Intensity	Abundance	P-value
Spring	*S. niger*	14	6	42.85	14	2.33	1	>0.05	15	4	26.6	12	3	0.8	>0.05
	S. esocinus	19	3	15.78	3	1	0.05	<0.01	20	5	25	8	1.6	0.4	<0.05
	S. curvifrons	16	2	12.5	6	3	0.18	<0.01	19	9	47.3	6	0.6	0.31	>0.05
Summer	*S. niger*	18	7	38.8	9	1.28	0.5	>0.05	16	8	50	7	0.87	0.43	>0.05
	S. esocinus	17	5	29.4	13	2.6	0.76	>0.05	18	8	44.4	15	1.87	0.83	>0.05
	S. curvifrons	19	4	21.05	6	1.5	0.31	<0.05	15	9	60	18	2	1.2	>0.05
Autumn	*S. niger*	25	6	24	13	2.16	0.52	<0.05	20	8	40	7	0.87	0.35	>0.05
	S. esocinus	19	4	21.05	9	2.25	0.47	<0.05	19	7	36.84	12	1.71	0.63	>0.05

	S. curvifrons	18	5	27.7	7	1.4	0.38	>0.05	22	9	40.9	14	1.55	0.63	>0.05
Winter	*S. niger*	19	2	10.5	11	5.5	0.57	<0.01	25	3	12	4	1.33	0.16	<0.01
	S. esocinus	22	2	9.09	3	1.5	0.13	<0.01	16	2	12.5	11	5.5	0.68	<0.01
	S. curvifrons	18	1	5.55	2	2	0.11	<0.01	15	3	20	11	3.6	0.73	<0.05
	Total	224	47	20.98	96	2.04	0.42	<0.01	220	75	34.09	125	1.66	0.56	<0.01

Gender wise prevalence of *Pomphorhynchus kashmirensis* (Table 3)

Pomphorhynchus kashmirensis in S*chizothorax* spp. of Dal Lake revealed that the overall prevalence of the parasite in males was 23.1% whereas it was 17.7% in females. In River Jhelum the overall prevalence in males was 35.8% while as in females it was 32.03%.

Table 3: Genderwise infection dynamics of *Pomphorhynchus kashmirensis* recorded of *Schizothorax* spp. from Dal Lake and River Jehlum

Season	Host	Dal Lake							River Jhelum						
		No. Examined	No. infected	Prevalence (%)	No. of parasites	Mean Intensity	Abundance	P-value	No. Examined	No. infected	Prevalence (%)	No. of parasites	Mean Intensity	Abundance	P-value
S. niger	Male	43	16	37.2	28	1.75	0.65	<0.05	33	12	36.36	22	1.83	0.66	<0.05
	Female	33	5	15.5	19	3.8	0.57		43	11	25.58	8	0.72	0.18	
S. esocinus	Male	39	8	20.5	15	1.8	0.38	>0.05	47	14	29.78	26	1.8	0.55	>0.05
	Female	38	6	15.7	13	2.16	0.34		26	8	30.76	20	2.5	0.76	
S. curvifrons	Male	52	7	13.46	15	2.14	0.28	>0.05	37	16	43.24	32	2	0.86	>0.05
	Female	19	5	26.3	6	1.2	0.31		34	14	41.17	17	1.2	0.5	

Influence of sex and condition factor on the level of infection (Fig. 1)

Insignificant relationship existed between gender and helminth infection. Condition factors was found to be lower in infected fish than in uninfected fish in both water bodies. Mann-Whitney test revealed *S. niger* (U=13, $p<0.01$), *S. esocinus* (U=45, $p>0.05$) and for *S. curvifrons* (U=34, $p>0.05$) of Dal lake While as in River Jhelum it was *S. niger* (U=3, $p<0.01$), *S. esocinus* (U=3, $p<0.01$) and for *S. curvifrons* (U=16, $p<0.05$).

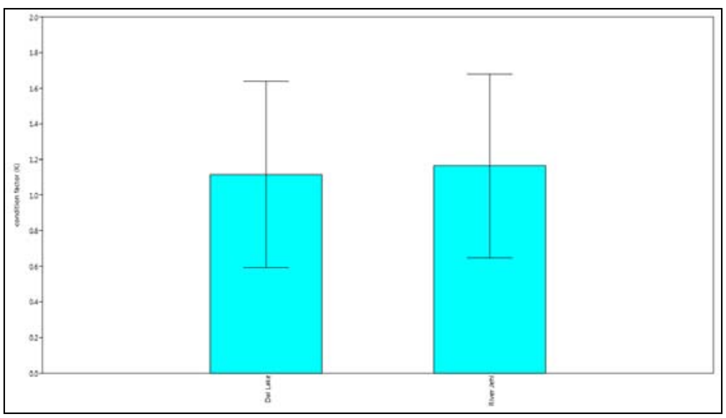

Fig 1: Overall comparative analysis of condition factor of fish in two water bodies with different water quality

Correlation between prevalence and water quality in two water bodies (Table 4)

Temperature was the most important abiotic factor that affected the parasites at all life cycle stages. A positive correlation ($P<0.01$) existed between water temperature and parasitic prevalence in Dal Lake and River Jhelum.

Prevalence of *P. kashmirensis* in the fishes of Dal Lake presented a significant negative correlation ($P<0.01$) with dissolved oxygen whereas it showed insignificant negative correlation ($P>0.05$) in all other cases of patterns of infection under various locations.

pH showed insignificant positive correlation ($P>0.05$) with parasitic infections. Prevalence of infections showed insignificant positive correlation ($P>0.05$) with Carbon dioxide except for *P. kashmirensis* of River Jhelum.

Table 4: Correlation between environmental variables and prevalence of *Pomphorhynchus Kashmirensis***.**

Environmental variables	Prevalence of *Pomphorynchus kashmirensis*			
	Dal Lake		River Jhelum	
	Pearson's coef.	Spearman's rho	Pearson's coef.	Spearman's rho
Temperature	882**	.853**	.907**	.907**
Oxygen	-.842**	.-.825**	-.451	-.350
pH	.420	.587*	-.227	-.266
Carbon dioxide	.682	.021	.628*	.522

*. Correlation is significant at the 0.05 level (2-tailed); **. Correlation is significant at the 0.01 level (2-tailed).

Histopathology of *Pomphorhynchus kashmirensis* infections of *Schizothorax niger*: Excess secretion of mucous, shedding of host's tissue and yellowish white fibrous nodules was observed macroscopically at the host parasite interface which was more pronounced in heavy infection (Fig 2). The extension of nodules on the external surface of the infected intestine is an indication for the presence of parasite. In heavy infection, the proboscis of *P. kashmiriensis* was found to perforate the intestine and caused damage to the adjoining tissue of liver and pancreas (Fig 3). *P. kashmiriensis* was found to have well developed hooked proboscis and bulb, by which they were firmly attached with the host intestine (Fig 4). They penetrate their pro- boscis and bulb deep into the host tissues and thereby damage the villi, and epithelial layer. In the areas of trunk contact with the host tissue, compression/absence of intestinal folds and loss of columnar appearance of epithelial cells were evident. Intense cellular infiltrations were noticed at the site of attachment which gave a granuloma like appearance and the cells were identified as plasma cells, neutrophils and fibroblasts (Fig 5). During Spring in intestine: Both lamina epithelia and lamina propria showed infiltration of inflammatory cells. Desquamation of epithelial cells was seen. Goblet cells revealed positivity of acid mucopolysaccharides. During Summer Severe enteritis was seen characterized by infiltration of inflammatory cells in lamina propria and desquamated lamina epithelialis. The inflammation had extended the muscular tunic of the intestine (Fig 6). During Autumn Blood capillaries revealed congestion. Goblet cells revealed hyperplasia with presence of acid mucopolysaccharide (Fig 7). During Winter Villi were sometimes completely denuded of epithelial cells. Liver: Revealed cellular swelling, vacuolar degeneration, kupffer cell hyperplasia and nuclear pyknosis almost in all the seasons (Fig 8, 9).

Fig 2: *S. niger* revealing reddish pink discoloration of abdominal viscera.

Fig 3: Intestinal contents showing excessive mucous production. *Pomphorynchus* had penetrated the wall of intestine which was reddish in colour

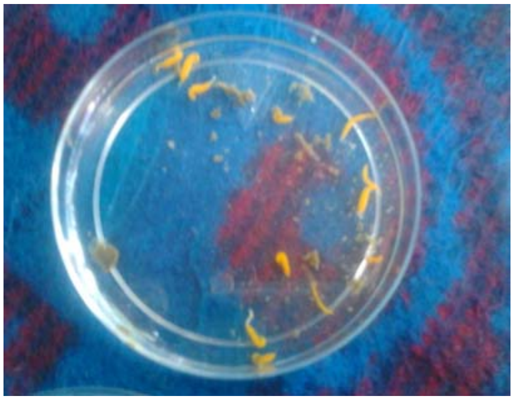

Fig 4: *Pomphorynchus* recovered from the intestine of *S. niger*

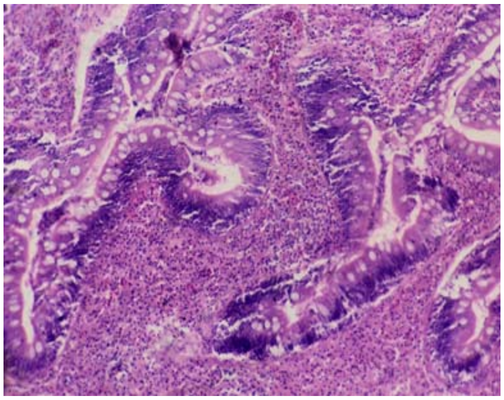

Fig 5: Both lamina epithelialis and lamina propria showed infiltration of inflammatory cells. H & E X 35.

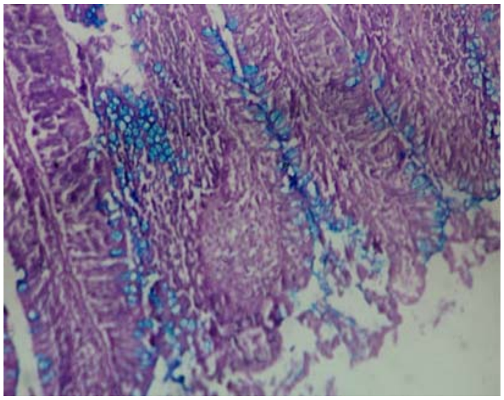

Fig 6: Goblet cell hyperplasia with positivity of acid mucopolysaccharides. Alcian blue PAS staining X 33.

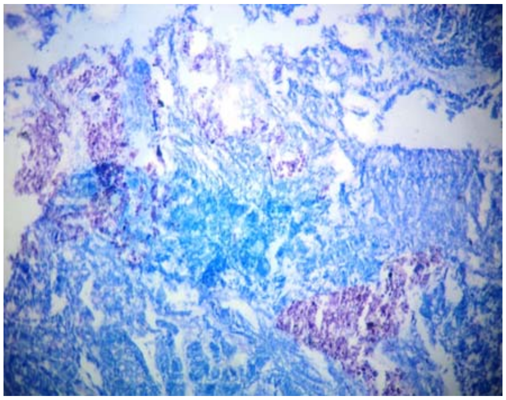

Fig 7: Mast cell infiltration was evident with scattered metachromatic granules. Toluidine blue stain X 25.

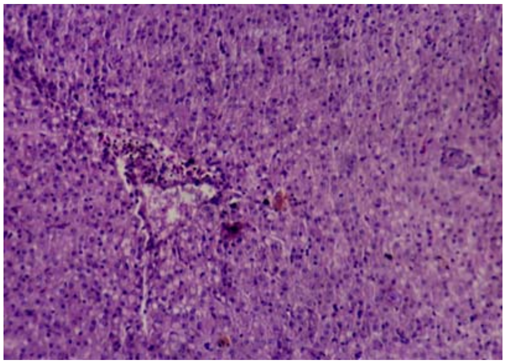

Fig 8: Liver showed cellular swelling, vacuolar degeneration and nuclear pyknosis. H & E X 61.

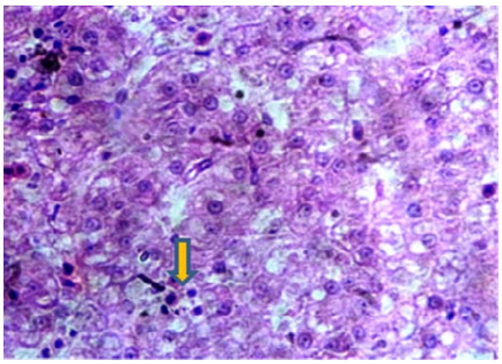

Fig 9: Liver cells revealed swelling, vacuolar degeneration and kupffer cell hyperplasia (arrow). H & E X 160.

Discussion

The present work showed that the infection patterns of *Pomphorhynchus* were greatly influenced by season, fish species and type of water body. It was found that overall prevalence of *Pomphorhynchus* was low, compared to other two helminths, which was in accordance to the studies done by Spall and Summer (1969) [32] and Chishti and Peerzada (1998) [8] who showed 0.7% and 9.3% infection of the acanthocephalan parasite, respectively. Further, the low prevalence might be due to low availability or consumption of intermediate hosts. The present finding also paralleled with Sinderman (1990) [30]; Kuperman (1992) [18] and Dusek *et al.* (1998) [12] who attributed the decrease in the infection rate of fishes lived in highly polluted areas to the fact that, effluents including heavy metals could alter the availability or reduced the number of invertebrate intermediate hosts necessary for life cycle of these parasites. Seasonal variation in incidence of helminth parasitism in fishes was probably influenced by the annual life cycle of the parasites. However, the overall prevalence of 27.47%, observed in the present study, could be attributed to various factors like temperature and availability of food. The host species generally shows a minimum preference for animal food (Chishti and Peerzada, 1998) [8] as they are mostly dependent on plankton (65-70%) which are the intermediate hosts for *P. kashmirensis*, the rest comprises of aquatic invertebrates. In Dal Lake and River Jhelum *Pomphorhynchus kashmirensis* was more prevalent in *S. niger* and *S. curvifrons* during summer, respectively. Further, the increased prevalence of the acanthocephalans in water bodies have been attributed to the contamination of water with municipal and industrial effluents (Billiard and Khan, 2003) [3]. Acanthocephalan infection were prevalent more in male fishes compared to females. Records on higher prevalence in male hosts are supported by the work of Zelmer and Arai (1998) [35] who observed that the slope of relationship between number and abundance of *Bothriocephalus* species and *Proteocephalus* species was greater for male perch than for females. Takemoto and Pavanelli (2000) [21, 33] have reported that male hosts had significantly higher parasite intensity than females. The influence of sex on the susceptibility of animals to infections could be attributed to genetic predisposition and differential susceptibility owing to hormonal control.

The presence of the parasites had reduced the condition coefficient of the infected fishes in both the water bodies. However; the overall condition factor of fish in Jhelum River was higher. Condition coefficient was found to be lower in infected fish than in uninfected fish in both Dal Lake and River Jhelum. It might be due to the fact that parasites decrease the immune system of the hosts, which may lead to decreased growth of fish. Decreased growth may lead to decrease in condition coefficient (Khan and Thulin, 1991; Poulin, 1992) [17, 27].

The present study showed that some of the physico-chemical features showed a significant positive correlation with the prevalence. A positive correlation existed between the water temperature and pH with the prevalence of *Pomphorhynchus kashmirensis*. Modu *et al.* (2011) [25] have stated that there existed a significant correlation between Helminth infection and water quality parameters in a pond. A number of workers (Marcogliese, 2001; Lafferty and Kuris, 2005) [23, 19] have suggested that natural abiotic factors such as temperature, oxygen, salinity, hydrogen ion concentration and eutrophication have a positive influence on the occurrence of parasite populations. Evidence from the present study suggested that the water temperature played an important role in the progression of all the three parasitic infection. Dissolved O_2 had been found to be the predictor of habitat selection in monogeneans (Ernst *et al*., 2005) [13]. Our results, however, showed insignificant relationship between O_2 level and acanthocephalan parasites.

Generally acanthocephalans cause more damage to the intestinal tissues and induce more complex host response, mainly, due to deeper penetration into the gut mucosa and worm burdens (Bullock, 1963) [6]. The pathogenicity caused by acanthocephalans also depends on the parasite/host species and site of localization (Hamers *et al.,* 1992) [16]. Over secretion of mucous at the host-parasite interface may be a consequence of host reaction for defence, as mucous layer on intestinal mucosa acts as a physical barrier for microorganisms, parasites and their toxins (Lamont, 1992 and Bosi *et al.,* 2005) [20, 5] and therefore prevents secondary infection. The proboscis and bulb of *P. kashmiriensis* were found to be deeply penetrated till lamina propria and thereby causing erosion of villi and epithelial layer. Similarly, complete penetration of proboscis and bulb and thereby fibrosis, hemorrhage, inflammation and cell necrosis have been reported in infected fishes with several acanthocephalan species (Bullock, 1963 and Dezfuli *et al.,* 2002b, 2008) [6, 8, 9]. The deeper penetration of proboscis and bulb may be assisted by proteolytic enzyme as suggestedby Polzar and Taraschewski (1994) [26] for deeper penetration of *P. laevis.* The compression / erosion of epithelial cells in the areas of trunk contact with the host tissues is similar to those reported by Bullock (1963) [6] and Chaicharn and Bullock, (1967) [7] for *Acanthocephalus jacksoni* and *P. Bulbicoli* infections respectively. Such damages may adversely affect the digestive and absorptive efficiency of fish intestine by which general health and growth of fish was affected. Chronic inflammation leading to an increased amount of connective tissue, thickening of lamina propria and infiltration of leucocytes has been reported at the site of *A. Jacksoni* attachment (Bullock, 1963) [6]. Hamers *et al.* (1992) [16] have reported interspecific differences in the response of leucocytes in infected fish with *P. Ambiguous* and suggested that the cellular defence may be involved in determining the host specificity as unsuitable host expel the parasite within a few days. The cellular degeneration in the liver might also be due to oxygen deficiency as a result of gill degeneration and/or to the vascular dilation and intravascular haemolysis observed in the blood vessels with subsequent stasis of blood (Mohamed, 2001).

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