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Post-Ulcerative anaemia in *Schizothorax niger* Haeckel, caused by an ectoparasitic protozoan *Chilodonella* spp.

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Abstract
Effect of a ciliate protozoan *Chilodonella* spp. (ectoparasite), on some haematological parameters of *Schizothorax niger* were studied. Analyses of blood parameters (cell counts, haemoglobin content and haematocrit) were carried out on parasitized and unparasitized *Schizothorax niger* from a fish farm in Anantnag, Jammu & Kashmir, India. Parasitized fish had significantly lowered erythrocyte counts, haematocrit and haemoglobin values and significantly increased leucocyte counts. Desquamation, necrosis of epithelial cells, ulceration and haemorrhages by this parasite thus produces a post-haemorrhagic anaemia and the fish appear to mount an immune response to the presence of these parasites.

Keywords: Anaemia, ectoparasite, *Chilodonella*, blood, feeding

Introduction
Chilodonella spp. are ectoparasitic ciliated protozoans of fresh water teleost fish. *Chilodonella* spp. parasitise numerous families and species of fish, including many of commercial importance, particularly in tropical, subtropical and temperate waters (Brusca 1981). Generally, they have been considered free living but two species viz. as *Chilodonella hexasticha* and *Chilodonella piscicola* are reportedly ectoparasitic in nature feeding on skin tissue, Gills and Leaked Blood out of ulcers. (Brusca 1981). There are also reports of various pathologies such as tissue damage, host behavioural changes, growth defects, decreases in mean weight and size, and mortalities. Parasitic disease can seriously compromise the sustainability of fish industry as they cause mortality, slow fish growth, lower food conversion rates and decreased marketability (see Nowak 2007; Buchmann 2013 and Shinn *et al*. 2015 for reviews) [5]. Ciliates are considered some of the most harmful parasites of cultured fish (Lom & Dykova 1992) and can facilitate secondary infections (e.g., bacterial infections) in farmed fishes (Lom & Dykova 1992; Hossain *et al*. 2013; Padua *et al*. 2013). Ciliates of the genus *Chilodonella* (Phyllopharyngea: Chilodonellidae) are primarily free-living, although at least two species, *Chilodonella hexasticha* (Kiernik 1909) and *C. piscicola* (Zacharias 1894; syn. *C. cyprini* Moroff 1902), can infect animal hosts (Lom & Dykova 1992) and cause severe epizootic outbreaks in wild and farmed freshwater fishes.

Materials and methods
In the present study 20 specimens from same population (10 parasitised by feeding on *Chilodonella* spp. and 10 unparasitized) of *Schizothorax niger* were studied in separate fish ponds of a farm here at Anantnag, Jammu & Kashmir, India. Sex, weight, length and confidence intervals of 10 parasitised fish with (mean length ± 95%, confidence intervals= 31 ± 3.5 mm and mean weight ± 95%, confidence intervals = 16.5 ± 26.6 g) and 10 unparasitized with (mean length ± 95% confidence intervals= 20 ± 5.3 mm, and mean weight ±95% confidence intervals =32 ± 5.8 g) were measured. Our sampling scheme thus ensured that all fish were effectively from the same population. Blood samples were taken at harvest by Heart punctures, severing of caudal peduncle (Blaxhall and Daisley, 1973; Hatting 1975). Blow was given on the head of the fish and the needle was inserted at an angle of 45° in to the heart and one ml of blood was collected using 1 mL disposable plastic syringes with 13 mm needles in to the glass tubes already containing anticoagulant EDTA (Ethylene diamine tetra acetic acid) at a concentration of 5 mg mL. Blood analysis was carried out very quickly after sampling within one hour.

For differential counts, a blood smear was prepared and the slides were immediately fixed in 95% methanol for 5 min before staining. The slides were first rinsed in pure Leishman's stain for 4 min, then placed in a working Giemsa solution (1 mL Giemsa, 2 mL phosphate buffer, 47 mL distilled water) for 30 minutes. Stained slides were rinsed with distilled water, dried and mounted using DPX mounting medium. The first 100 leucocytes observed in a blood smear were categorized into thrombocytes, lymphocytes, neutrophils and other granulocytes. Roberts (1978), Groman (1982), Takashima & Hibiya (1995). (Blaxhall and Daisley, 1973). Leishman staining was used to determine the percent leukocyte (Goel *et al*, 1981).

Haemoglobin was determined according to the method of Haldane and was carried out using the haemoglobin assay kit from Sigma-Aldrich Diagnostics as it is recommended for use with fish blood (Handy & Depledge 1999). Twenty microlitres of whole blood were added to 5 mL of Drabkin reagent, mixed and allowed to stand for 15 min. The absorbance of the samples was read at 540 nm using a CCS 175 spectrophotometer. Absorbance values were converted into blood haemoglobin values in g dL^{-1} using a calibration curve.

Haematocrit values were determined by the microhaematocrit (Jewet *et al*. 1991). Heparinized 50 µL tubes were used to determine the haematocrit value. Tubes were filled with whole blood then sealed with a commercial sealant and placed in a microhaematocrit centrifuge for 5 min at 5000 g. A micrometre gauge was used to determine the length of the packed cells and the length of the packed cells plus supernatant. The haematocrit % value was then calculated as follows:

Hct = (packed red cells/packed red cells plus supernatant) × 100%

Cell counts were carried out using Neubauer haemocytometer (EMS 68052). Twenty microlitres of fresh whole blood were added to 0.98 mL of fresh filtered Dacie's fluid (1 mL of 40% formaldehyde, 3.13 g trisodium citrate, 0.1 g brilliant cresyl blue, 100 mL distilled water) and the solution was mixed gently to disperse the cells. This provides a 1:50 dilution of the blood sample. The number of cells is expressed as 10^6 cells mm^3. The leucocyte count was carried out using the same sample as that prepared for the erythrocyte count. However, in the case of leucocytes, the count is expressed as 10^3 cells mm^{-3}. Statistical analysis The Kolgomorov–Smirnoff test for normality, and Bartlett's and Levene's tests for equality of variance were used prior to analysis. Parasitized and unparasitized blood sample parameters were compared using either one-way ANOVA or a Mann–Whitney U test. Percentage data were arcsine transformed prior to analysis. Distributions of different types of leucocytes were compared using a chi-square test on a contingency table of pooled data from all samples. Analyses were conducted using Minitab (release 13.1).

Results

Observations on the allow us to confirm that engorged blood vessels could be seen around the site of attachment of the parasite, indicative of blood feeding apart from skin and Gill feeders at the time of observation. ANOVA revealed that parasitized fish had significantly lowered haematocrit values ($F_{1,43}$ = 15.08, P < 0.001) and significantly lowered haemoglobin values ($F_{1,43}$ = 10.67, P= 0.002) than unparasitized fish (see Table 1).

ANOVA revealed that parasitized fish had a significantly lower erythrocyte count than unparasitized fish ($F_{1, 41}$ = 4.66, P = 0.037) (Table 1). A Mann–Whitney U test revealed that the leucocyte counts of parasitized fish were significantly higher than those of unparasitized fish (W = 357.5, P =0.0038) (Table 1). No significant differences were recorded between parasitized and unparasitized fish in the derived values for mean corpuscular volume (MCV = haematocrit×10/no. RBCs), mean corpuscular haemoglobin (MCH = haemoglobin/no. RBCs) and mean corpuscular haemoglobin concentration (MCHC = haemoglobin/ haematocrit) (Table 1).

A chi-square test revealed a significant difference in differential leucocyte cell counts pooled from samples from *Schizothorax niger* parasitized by *Chilodonella* spp. and from unparasitized hosts ($\varkappa^2$ = 8.645, d.f. = 3, P = 0.034). The differences in leucocyte distribution of parasitized compared with unparasitized fish samples are slight and result from an increase in the relative abundance of lymphocytes and granulocytic cells (neutrophils eosinophils and basophils) and a decrease in the relative abundance of thrombocytes in the parasitized *Schizothorax niger*.

Table 1: Blood parameter values for parasitized and unparasitized *Schizothorax niger* (mean 95% confidence intervals), plus derived indices MCV, MCH, MCHC

Blood parameter	Unparasitized (n = 23)	Parasitized (n =21)	ANOVA/Mann–Whitney
Haematocrit (%)	54.93± 2.98	44.465 ± 3.06	$F_{1, 43}$ =14.08, $P < 0.001$
Haemoglobin (g dL^{-1})	7.964 ± 0.45	6.78 ± 0.554	$F_{1, 43}$ = 11.67, P= 0.002
RBC 10^6 (cells mm^{-3})	3.48 ± 0.157	2.70 ± 0.197	$F_{1, 41}$ = 4.76, P =0.037
WBC 10^3 (cells mm^{-3})	77.67 ± 7.17	90.37± 12.35	W =367.5, P = 0.0038
MCV	0.81 ± 0.03	0.65 ± 0.04	$F_{1, 43}$ =1.54, P = 0.246
MCH	0.009±0.06	0.010± 0.04	$F_{1, 43}$ =0.918, P = 0.246
MCHC	0.26 ± 0.06	0.16 ± 0.07	$F_{1, 43}$ = 0.278, P = 0.507

MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration.

Discussion

Generally, *Chilodonella* species are considered free-living ciliate protozoans. However, it is unknown whether species considered 'free-living' can also occupy a parasitic lifestyle given optimal conditions and opportunity (Noga 2010). Moreover, it is poorly understood how many *Chilodonella* species elicit harmful pathology to their fish hosts in favourable conditions (Lom & Dykova 1992; Noga 2010).

The haematological study of *Schizothorax niger* has determined that the erythrocyte parameters (cell counts, haemoglobin content and haematocrit) were significantly higher in samples from unparasitized fish than in samples

from parasitized fish. The reduction in haemoglobin content and drop in erythrocyte count in host blood are characteristic features of anaemia induced by blood feeding *Chilodonella* parasites (Kabata 1970). Anaemia caused by blood feeding protozoan parasites has been classified as post-haemorrhagic anaemia (Romestand & Trilles 1977; Romestand 1979; Renaud 1980; Bragoni *et al*. 1983; Nair & Nair 1983; Radujkovic, Romestand & Trilles 1983).

Nair & Nair (1983) studied the effect of the aegid isopod parasite, Alitropus typus Milne Edwards, on the snakehead host fish, Channa striatus (Bloch), and recorded significantly reduced levels of haematocrit, haemoglobin and erythrocyte counts in parasitized fish compared with unparasitized fish. Values of the derived indices MCV and MCH, were all reported to significantly increase in parasitized host fish.

Clinical signs associated with *Chilodonella* spp. infections are not unique, which makes initial diagnosis of chilodonellosis challenging (Noga, 2010). Fish infected with *Chilodonella* spp. can exhibit gasping behaviour, anorexia, skin depigmentation, ulceration, scale loss, excessive mucus excretion and gill lesions (Padua *et al*., 2013). The clinical signs most commonly observed are a mottled/grey appearance on the skin (caused by excessive mucus production), lethargy, gill viscous mucus production, swimming slowly near the surface and edges, slim appearance (caused by appetite loss) and sometimes gill lesions and scale loss (Read, 2007; Padua *et al*., 2013; Bradley *et al*., 2014). Examination of moribund fish is necessary to avoid misdiagnosis. In general, intense parasite infections are accompanied by acute gill lesions sufficient to kill affected fish (Langdon *et al*., 1985; Padua *et al*., 2013; Bowater and O'Donoghue, 2014). Fish infected with few cells usually do not exhibit clinical signs of illness (Read, 2007). However, *Chilodonella* cells can rapidly multiply (population numbers can double in only a few hours; pers. obs.; Read, 2007; Padua *et al*., 2013). Infestations of *Chilodonella* spp. cause gill epithelial hypertrophy, hyperplasia and are generally observed with complete or partial lamellar fusion followed by lymphocytic infiltration. Necrosis and some mild oedema can also be observed (Paperna and Van As, 1983; Padua *et al*., 2013; Bowater and O'Donoghue, 2014; Bradley *et al*., 2014). Fish skin usually demonstrates a nonspecific lymphocytic dermatitis (Padua *et al*., 2013; Bowater and O'Donoghue, 2014; Bradley *et al*., 2014).

Parasitized fish were reported to have larger erythrocytes containing less haemoglobin compared with unparasitized fish. The authors postulated that the increase in the volume of the cells compensates, in part, for the loss of erythrocytes ingested by the parasite. However, although a general downward trend was seen in the number of erythrocytes, haematocrit, haemoglobin and derived values MCH and MCHC, and an increase was recorded in MCV, only the haemoglobin value and related derived values, MCHC and MCH showed significant changes (Bragoni *et al*. 1983). In all host species, a decrease in erythrocyte count, haemoglobin and haematocrit was recorded. No change was reported in the MCV, MCHC or MCH values.

In this study, leucocyte counts were significantly higher in parasitized fish than in unparasitized fish. The relative distribution of cells showed significant changes with parasitism, with increases in the relative abundances of lymphocytes and granulocytic cells and a decrease in the relative abundance of thrombocytes.

However, drop in leucocyte count may be a result of the hematophagy of the parasites reducing the whole blood cell count. The relative proportions of plasmacytes, monocytes and neutrophils first decreased and later increased while the lymphocytes and macrophages showed an upward trend. Romestand (1979) reported no change in the leucocyte count between parasitized and unparasitized individuals of three fish host species. Less work has been done on the post ulcerative anaemia caused by this ciliated protozoan which forced us to conduct a detailed survey on its impact on the blood parameters of the fish as it has been proven that it causes ulceration with engorged blood vessels with blood loss.

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