STUDIES ON MONOGENEAN PARASITE(S) IN *PANGASIANODON HYPOPHTHALMUS*: CHARACTERIZATION, PATHOLOGY AND IMMUNE RESPONSES

Thesis submitted in partial fulfillment

of the requirements

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Ph.D. (Aquatic Animal Health Management)

By

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Dedicated to

My parents, wife & CIFE



Date: 27 September 2017

CERTIFICATE

Certified that the thesis entitled **"STUDIES ON MONOGENEAN PARASITE(S) IN *PANGASIANODON HYPOPHTHALMUS*: CHARACTERIZATION, PATHOLOGY AND IMMUNE RESPONSES"** is a record of independent bonafide research work carried out by **Mr. Saurav Kumar** during the period of study from September 2012 to August 2017 under our supervision and guidance for the degree of **Doctor of Philosophy (Aquatic Animal Health Management)** and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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सारांश

वर्तमान अध्ययन का उद्देश्य *Pangasianodon hypophthalmus* में मोनोजेनीयन परजीवी का संक्रमण, मेजबान एवं सूक्ष्मनिवास के प्रति ग्राह्यता, रोग अभिव्यक्ति और मेजबान के प्रतिरक्षा प्रतिक्रिया की जाँच करने के लिए क्रियान्वित किया गया। महाराष्ट्र स्थित मुरबाड से कुल ६० पिंजरे संवर्धित मछली को मोनोजेनीयन परजीवी के अध्ययन हेतु एकत्रित किए गए । अध्ययन के उपरांत गिल मोनोजेनीयन का व्यापकता, बहुतायत और औसत संक्रमण दर क्रमश: ७०%, १९.५ और ३९ परजीवी प्रति संक्रमित मछली पाई गयी। गिल मोनोजेनीयन की पहचान पहले के नमूनों के लग्नक एवं मैथुन अंगों के गुणों की समानता के आधार पर *Thaparocleidus siamensis* एवं *T. caecus* के रूप में गई । इसके अलावा *T. siamensis* का पहचान २८S rDNA जीन अनुक्रमण का उपयोग कर किया गया तथा वंशावली विश्लेषण द्वारा इसकी मलेशियन *T. siamensis* के साथ सतप्रतिशत समानता की पुष्टि की गयी। इस परजीवी (*Thaparocleidus* spp.) के बहुतायता का स्वीकृतिय सम्पर्क एवं संबंध मेजबान के मानक लम्बाई के साथ प्राप्त हुआ अपितु शरीर भार के साथ कोई संबंध नहीं पाया गया । परजीवी के स्थानिक वितरण क्रमश: १ से ४ गिल एवं उनके ६ विशिष्ट क्षेत्रों के विश्लेषण से पता चला कि *Thaparocleidus* spp. का अधिक समग्र गिल आर्क १ एवं २ (कुल ६९.३१%), गिल के पीछे-मध्य (८३.५०%) और पृष्ठीय-औसत (८०.७०%) क्षेत्रों पर होता है। *P. hypophthalmus* के संक्रमित गिल का ऊतकीय विश्लेषण घाव का केंद्रीय एवं बहुकेंद्रीय होने, गंभीर अवसाद और द्वितीयक गिल पटलिका के संलयन होने का पुष्टि करता है। गिल के उपकला कोशिकाओं में रक्त संचय एवं अतिवर्द्धन के साथ इसका विमोचन भी देखा गया। असंक्रमित, कम और उच्च *Thaparocleidus*-ग्रसित समूहों में नैदानिक रोग मापदंडो का विश्लेषण एवं तुलना किया गया। असंक्रमित मछलियों के समूह के अपेछा परजीवी से संक्रमित मछलियों के समूहों का रुधिर मापदंडो जैसे टीईसी, टीएलसी, एचबी, पीसीवी, एवं सेरम जैवचिह्नों जैसे प्रोटीन, एसओडी, सीएटी, एसजीओटी, एसजीपीटी, एलडीएच एवं लैक्टेट के स्तरों में महत्वपूर्ण (पी < 0.05) वृद्धि व् एमसीएच, एमसीवी, एमसीएचसी तथा लिम्फोसाइट के स्तर में महत्वपूर्ण (पी < 0.05) कमी पाई गई। असंक्रमित मछलियों के समूह के तुलना में *Thaparocleidus*-संक्रमित मछलियों के एएलपी और बिलीरुबिन के स्तर में महत्वपूर्ण (पी > 0.05) अतंर नहीं पाई गयी। *Thaparocleidus* spp. के साथ प्रयोगात्मक संक्रमण के माध्यम से *P. hypophthalmus* के प्रतिरक्षा गतिविधियो में अधिमिश्रण व् ऑक्सीडेटिव तनाव प्रतिक्रिया का अध्ययन किया गया था। स्वस्थ प्रयोगात्मक मछलियों को संक्रमित करने के लिए मानक सहवास विधि का उपयोग किया गया। १४ दिनों के उपरांत, पीड़ित मछलियों के नमूनों को तीन अलग समूहों टी१ (निम्न), टी२ (मध्यम) और टी३ (उच्च) क्रमशः <10, 10-49 और ≥50 परजीवी प्रति गिल प्रति मछली में वर्गीकृत किया गया तथा एक नियंत्रण समूह टी0 (असंक्रमित मछली) भी रखा गया। नमूनों के जन्मजात प्रतिरक्षा, रक्त और सीरम में तनाव के जैवचिह्नों के विश्लेषण से पता चला कि मध्यम एवं उच्च *Thaparocleidus*-ग्रसित समूहों की टीईसी, टीएलसी, एचबी, इओबी, एसजीओटी, एसजीपीटी, एलडीएच, लैक्टेट, बिलीरुबिन एवं क्रिएटिनिन के स्तरों में महत्वपूर्ण (पी < 0.05) वृद्धि पाई गयी थी। इसके अलावा, मध्यम एवं उच्च *Thaparocleidus*-ग्रसित मछलियों के समूहों के आरबीए, एमपीओ, सीरम लीसोज़ाइम, अल्फा-२ एम और सीरम की कुल इम्मुनोग्लोबुलीन के स्तरों में महत्वपूर्ण (पी < 0.05) वृद्धि पाई गयी थी। इसी तरह, असंक्रमित मछलियों के समूह के तुलना में उच्च *Thaparocleidus*-ग्रसित मछलियों के कोशिकीय ऑक्सीडेटिव जैवचिह्नों में एसओडी, सीएटी, जीएसटी और सोडियम-पोटाशियम-एटीपेस के स्तरों में महत्वपूर्ण (पी < 0.05) वृद्धि पाई गयी। हालांकि निम्न *Thaparocleidus*-ग्रसित मछलियों के समूहों के सीरम एल्ब्यूमिन, ए:जी, कुल सीरम एंटीप्रोटीएस और सेलोरोप्लास्मीन के स्तर में कमी परन्तु मध्यम एवं असंक्रमित समूहों के साथ कोई भी अंतर की पुष्टि नहीं की गयी I परिणामों से संकेत मिलता है कि विभिन्न स्तरों के गिल मोनोजेनियन (*Thaparocleidus* spp.) संक्रमण से मेजवान के जन्मजात प्रतिरक्षा प्रतिक्रिया के साथ ऑक्सीडेटिव तनाव जैवचिह्नों में भी परिवर्तन होता है I वर्तमान अध्ययन में प्राप्त हुए मूलभूत तथ्यों के माध्यम से इस परजीवी का *P. hypophthalmus* के स्वास्थ्य पर महत्वपूर्ण प्रभाव तथा मेजबान के साथ इसका संबंध समझ कर प्रभावी प्रबंधन रणनीति तैयार करने में सहायता प्राप्त होगी I

ABSTRACT

The present study aimed to investigate the monogenean parasite infection in *Pangasianodon hypophthalmus*, its host and microhabitat preference, pathological manifestation and immune responses of the host. A total of 60 fish were collected from cage farms located at Murbad, Maharashtra, India and screened the presence of monogeneans. The study revealed prevalence, abundance and a mean intensity of 70%, 19.5 and 39 gill monogenean per infected fish, respectively. The isolated gill monogeneans were identified as *Thaparocleidus siamensis* and *T. caecus* based on morphological similarities of haptor and copulatory organs of previously reported specimens. Further *T. siamensis* was identified using *28S rDNA* gene sequencing and phylogenetic analyses revealed 100% identity with *T. siamensis* reported from Malaysia. Though a good interaction and relationship were observed between host standard length (R^2 quadratic= 0.699) and intensity of *Thaparocleidus* spp. but not with host body mass (R^2 quadratic= 0.466). Analysis of spatial distribution of parasite between 4 gill arches and 6 regions (anterior-posterior and dorsal-ventral) of gills revealed that *Thaparocleidus* spp. prefered to aggregate more on gill arch 1 and 2 (summation of 69.31%) and posterior-central (83.50%) and dorsal-medial (80.70%) regions of the gills. Histological analysis of infected gills of *P. hypophthalmus* showed a focal to multifocal lesion, severe depression and fusion of secondary gill lamellae. Congestion and marked hyperplasia of the epithelial cells and sloughing of gill epithelium were also noticed. Clinical pathological parameters were analysed and compared between uninfested, low and high *Thaparocleidus*-infested groups. A significant ($p<0.05$) increase in TEC, TLC, Hb, PCV, serum protein, SOD, CAT, sGOT, sGPT, LDH and lactate level and a decrease in MCH, MCV, MCHC and lymphocyte were detected in the infected fish. However, no significant ($p>0.05$) difference in ALP and bilirubin level in *Thaparocleidus*-infested fish were observed when compared with uninfested group. The modulation of innate immune activity and oxidative stress response of *P. hypophthalmus* through experimental infection with *Thaparocleidus* spp. was also studied. Standard cohabitation method was used to infect healthy experimental fish. After 14 days, experimental fish were sampled and categorised into four different groups as T1(low), T2 (moderate) and T3 (high) corresponding to <10, 10-49 and ≥50 mean parasite per gill per fish respectively, along with a control group T0 (uninfested fish). Analysis of the markers of innate immune and stress in blood and serum showed significant ($p<0.05$) increase in TEC, Hb, TLC, EOB, SGOT, SGPT, LDH, lactate, bilirubin, and creatinine in moderate to high monogenean-infested group. Further, levels of RBA, MPO, serum lysozyme, α-2 macroglobulin (α-2M) and total serum immunoglobulin were significantly high ($p<0.05$) in moderate and high level of parasite-infested group compared to the control. Similarly, cellular oxidative biomarkers SOD, CAT, GST and Na^+-K^+-ATPase in gills and liver were significantly high ($p<0.05$) in *Thaparocleidus*-infested fish in comparison to the control. However, decreased levels of albumin, A:G ratio, total serum antiprotease and ceruloplasmin level were observed in fish infested with low degree of *Thaparocleidus*, while no significant ($p>0.05$) difference was observed between low to moderately infested, and the control group. The results indicate that varying degree of gill monogenean dactylogyrid infestation not only modulates the innate immune response but also the oxidative stress biomarkers in *P. hypophthalmus*. The baseline data obtained in the present study will be valuable in understanding the host-parasite relationship and thereby, in formulating effective management strategies for the parasite which has significant impact on the health of farmed *P. hypophthalmus*.

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1. INTRODUCTION

Aquaculture is recognised as one of the potential food producing sector which provides high-quality animal protein to growing population. The sector also improves the nutritional level and contributes to the livelihood by generating income and employment for rural communities. India is blessed with huge productive aquatic resources that reflect 10 percent of the global fish diversity. Further, India produces about 10.79 million tonnes of fish annually and there is a tremendous potential to increase fish production (DAHD, 2017). Diversification of species in aquaculture is an important step towards enhancing fish production in order to meet the increasing domestic and foreign demand. Further, this also provides scope to bridge the gap of low productivity existing in the various culture systems. The introduction and popularization of catfish pangasius (*Pangasianodon hypophthalmus*) in various parts of India is an example of successful diversification of aquaculture species (Singh and Lakra, 2012). Owing to the strong market demand, fast growth and standardized culture practices, pangasius has quickly become the 3rd most cultured freshwater fish in India. Currently, pangasius is also being cultured in cages in reservoirs including ponds and tanks. However, few studies have been conducted to assess the risk and benefits of the pangasius culture in India (Singh and Lakra, 2012). In recent years, farming practices have intensified to increase production and maximize the profit. It is widely recognized that this intensification is generally accompanied by an increase in occurrence of diseases and problems caused by various infectious agents.

Disease is the primary constraint in all types of culture systems including pangasius farming. Few reports of bacterial diseases caused by *Enterobacter clocae, Aeromonas hydrophila, A. jandaei, Pseudomonas sp.* and *Edwardsiella tarda* have been reported in grow out ponds of *P. hypophthalmus* in India (Kumar *et al.,* 2013, 2015; Kumar and Ramulu, 2013; Shetty *et al.,* 2014). In addition, severe parasitic infestation can occur in early larval stages and hamper the production (Singh and Lakra, 2012). Gill fluke infection is commonly seen in *P. hypophthalmus* farms with prevalence ranging from 60% to 90% of fish (Lakra and Singh, 2010). High mortalities due

to gill fluke are generally noticed during the first week after stocking. It has been observed that the prevalence and intensity of the parasites on fish host correlates positively with high stocking density, water temperature and organic load in culture ponds. Despite the fact that gill flukes contribute to significant losses due to the secondary bacterial infections that follow the initial infections with the parasite, this receives little attention compared to the direct economic losses due to disease outbreaks. Monogenean is not yet considered the great hurdle in aquaculture, the growth reduction due to biological stress, severely affects the economies of aquaculture.

Among the bewildering array of fish parasites, monogeneans (Platyhelminths) are flat flukes characterized by a posterior attachment organ 'the haptor' (opisthaptor) which facilitates infestation on external surface of fish i.e. gills, skin, and fins with few exception of infecting internal organs. Monogeneans are hermaphrodite and display both oviparous and viviparous, and rarely exhibit self-fertilization. Monogeneans exhibit a direct life cycle, and thus transmission of monogenean to the host is primarily by means of direct contact and it spreads horizontally by a ciliated, free swimming larva the oncomiracidium. The Class monogenea is traditionally divided into 2 Subclasses, the monopisthocotylea, which possess a haptor comprising a single attachment unit, and the polyopisthocotylea, members of which have a complex haptor with multiple attachment units, including hooks, suckers, clamps, or both (Buchmann and Bresciani, 2006).

Monopisthocotyleans are tissue grazers that generally inhabit the external surfaces and gills of their hosts. Heavy infestations may elicit epidermal hyperplasia due to disruption by parasite attachment and feeding; increased mucus production can also occur. Secondary infections by protozoa, bacteria and fungi often ensue and can cause dermal erosion and haemorrhage. In several infestations, death occurs, usually due to loss of osmotic regulation which impairs the normal physiological function of the organs (Paperna, 1963; Buchmann, 1997; Buchmann and Bresciani, 2006). Polyopisthocotyleans usually inhabit the gills and are sanguinivorous, causing tissue compression and sometimes hyperplasia of the gill tissues. Pathogenesis results from feeding and blood loss, which is reflected in

decreased level in haematocrit, haemoglobin and mean corpuscular indices in heavily infected fish host. The anaemia caused in such cases characterized as a microcytic hypochromic type. Further heavy infections can cause interruption of gill function by physical disruption through compromised oxygen uptake associated with lethargy, anorexia, dark skin colour and paleness of muscle, gills, kidney and liver of the infected fish (Paperna *et al.,* 1984; Buchmann and Bresciani, 2006). Recently in India, taxonomic identification and molecular characterization of *Thaparocleidus siamensis* and *T. caecus* (dactylogyrids) from *P. hypophthalmus* has been carried out (Tripathi *et al.,* 2014; Chaudhary *et al.,* 2014; Rajvanshi and Agrawal, 2014). The pathological implications of these monogeneans and host responses are yet not studied.

Fish has a strong immune system to protect it from various infectious agents. However, the information of fish innate and adaptive immune responses to monogenean parasite is meagre. Cellular and humoral components of blood play a central role during stress to maintain the homeostasis in the host, and fight against pathogens during infection. Additionally, haematological and serum biochemical profiles provide an array of valuable information, related to fish health status and act as a monitoring tools. In general, fishes greatly rely on non-specific immune responses to monogenean infestation. The host responds to biological stressors by mobilizing energy, active phagocytosis, and agglutinins (the secreted protein). Further, synthesize the active protein against antigens to maintain the homeostasis. Red blood cell (RBC) count, haemoglobin (Hb), packed cell volume (PCV), total leucocytes count (TLC) and haematological indices (MCH, MCV and MCHC), including erythrocyte osmotic brittleness (EOB) are suitable parameters of the physiological responses and damage to the organs in humans as well as in fish (Bain *et al.,* 2011; Kumar *et al.,* 2013). Serum glutamate oxaloacetate transaminase (sGOT), serum glutamate pyruvate transaminase (sGPT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) are considered as important biomarkers of cellular physiological stress (Winkaler *et al.,* 2007; Kumar *et al.,* 2013). At the same time, serum biomarker like blood glucose, lactate and creatinine can be viewed as part of a stress response triggered by the presence of biological

stressors in aquatic system (Grutter and Pankhurst, 2000; Mansell *et al.,* 2005; Wells *et al.,* 2006).

In general, fish largely rely on innate immune system as first defence barrier to monogenean infestation in the form of localized inflammation which appears as hyperplasia and severe mucus secretion on the fish gills and skin (Mansell *et al.,* 2005; Del Rio-Zaragoza *et al.,* 2010). Reports of gill monogenean infestation on *P. hypophthalmus* and associated health risk are available. However, no systematic studies have been conducted on host immune responses against this parasite. Moreover, the knowledge of nonspecific immune response of *P. hypophthalamus* is scarce. Estimation of humoral component of innate immunity like serum lysozyme, α-2 macroglobulin (α-2M), ceruloplasmin level, antiprotease activity and cellular component performance via respiratory burst activity (nitroblue tetrazolium (NBT) assay) and myeloperoxidase levels significantly reflect the immunological status and leucocyte performance. Consequently, it is important to recognize the change in the immune system of host occuring during monogenean infestation. Moreover the host responds to biological stressors by an antistress or oxidative response mechanism such as catalase, superoxide dismutase (SOD), peroxidase and glutathione-S-transferase (GST), and levels of total prtotein, globulin, and albumin (Wu *et al.,* 2007; Kumar *et al.,* 2013). Catalase and SOD are considered as important biomarkers of oxidative stress. Fish are endowed with defensive mechanisms to counteract the impacts of reactive oxygen species (ROS) resulting from the catabolism of various antioxidant defense enzymes such as SOD, Catalase and GST family (Tjalkens *et al.,* 1998). Although, alteration in haemato-serum biochemical parameters and immunological parameters of fish infested with parasite are reported (Ghiraldelli *et al.,* 2006; Del Rio-Zaragoza *et al.,* 2010; Ferreira and Oldewage, 2013; Yin *et al.,* 2015; Rastiannasab *et al.,* 2016; Rana and Das, 2016), there is no systematic information available either in online or printed on immunomodulatory and haemato-serum biochemical parameters of pangasius infested with *Thaparocleidus* sp. (dactylogyrids), a gill monogeanean.

Therefore, it is important to recognize the host-parasite interaction in terms of changes in the haematological and serum biochemical parameters, and immunological responses during monogenean infestation. The present study aims to investigate and identify the systematic position of monogenean parasite and a better insight into pathology associated with infection of the parasite in *P. hypophthalmus*. Further, the study aimed to evaluate the effects of varied degree of monogenean infestation on innate immune response and oxidative responses of *P. hypophthalmus* by artificial challenge experiments. Keeping the above in view, the present study was focused to achieve following objectives:

1. To isolate and characterize the monogenean parasite(s) infecting *Pangasianodon hypophthalmus*
2. To study the pathology associated with monogenean(s) infecting the host
3. To study the immune responses of *Pangasianodon hypophthalmus* to monogenean parasite(s)

2. REVIEW OF LITERATURE

2.1. Status of aquaculture in India

The Fishery and aquaculture sector of India is immensely contributing to the economy of the country and emerge as a flourishing sector with varied resources and potentials. Aquaculture is a significant socio-economic activity especially for rural communities, contributing to livelihoods, food security and poverty alleviation through income generation, employment, services, use of local resources, diversified farming practices, domestic and international trade and other economic investments (Edwards *et al.,* 2002; FAO, 2016). Only after the Indian Independence, fisheries together with agriculture have been recognized as an important sector. The vibrancy of the sector can be visualized by the 11–fold increase that India achieved in fish production in just six decades, *i.e.* from 0.75 million tonnes in 1950-51 to 9.6 million tonnes during 2013–14 which is now the par of 10.5 million tonnes (DAHD, 2017). This resulted in an unparalleled average annual growth rate of 4.5% over the years which has placed the country on the forefront of global fish production, only after China. Besides meeting the domestic needs, the dependence of over 14.5 million people on fisheries activities for their livelihood and foreign exchange earnings to the tune of US$ 4.69 billion (2015–16) from fish and fisheries products, amply justifies the importance of the sector on the country's economy and in livelihood security (DAHD, 2017).

India is home to more than 10 percent of the global fish diversity. Further, India is the second largest producer of freshwater fish in the world. Fish production has increased from 41.57 lakh tonnes (24.47 lakh tonnes for marine and 17.10 lakh tonnes for inland fisheries) in 1991-92 to 107.90 lakh tonnes (35.8 lakh tonnes for marine and 72.10 lakh tonnes for inland fisheries) in 2015-16 (DAHD, 2017). Remarkably, freshwater aquaculture contributes to over 80 percent of the total aquaculture production (DAHD, 2017). This comprises the culture of carp fishes, catfishes (air breathing and non-air breathing), freshwater prawns, pangasius, and tilapia. In addition, in brackishwater sector, the aquaculture includes culture of shrimp varieties mainly, the native giant tiger prawn (*Penaeus monodon*) and exotic white legshrimp (*P. vannamei*).

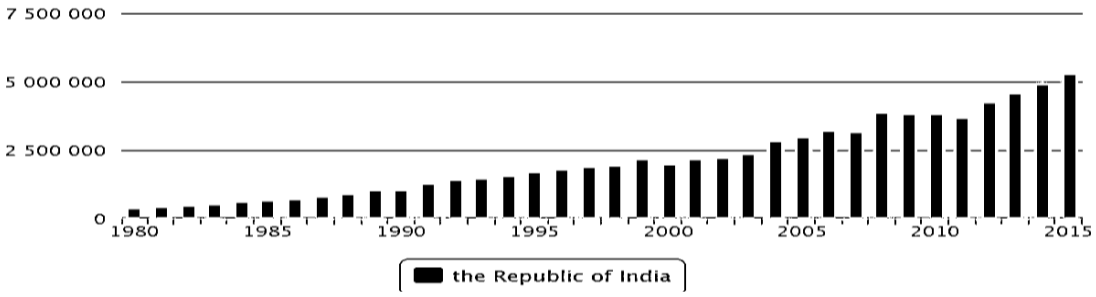


Figure 1: Total aquaculture production of India (tonnes) during 1980-2015.
Source: FAO, 2016

The production of carp in freshwater and shrimps in brackishwater form the bulk of major areas of aquaculture activity. The three Indian major carps, namely catla (*Catla catla*), rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*) contribute the bulk of production to the extent of 70 to 75 percent of the total freshwater fish production, followed by silver carp, grass carp, common carp, catfishes forming a second important group contributing the balance of 25 to 30 percent. It is estimated that only about 40 percent of the available area of 2.36 million hectares of ponds and tanks has been brought under use and an immense scope for expansion of area exists under freshwater aquaculture (Ayyappan *et al.,* 2013). The national mean production levels from still-water ponds has gone up from about 600 kg/hectare/year in 1974 to over 2,900 kg/hectare/annum at present and several farmers are even demonstrating higher production levels of 8–12 tonnes/hectare/year (Ayyappan *et al.,* 2013). Aquaculture in India has evolved as a viable commercial farming practice from the level of traditionally backyard activity over last three decades with considerable diversification in terms of species and systems, and has been showing an impressive annual growth rate of 6-7 percent. While the carp-based freshwater aquaculture, mainly constituted by the Indian major carps, such as, catla, rohu and mrigal, has been contributing over 90 percent of the aquaculture production satisfying the domestic need, the shrimp-based coastal aquaculture contributes to only about 5% of the export earnings.

2.2. Need of diversification in aquaculture and candidate species in India

While sustainability is being addressed, the present concern is with regard to species diversification, in spite of the fact that the country possesses several other endemic potentials and cultivable medium and minor carp species having regional demand, such as, *Labeo calbasu*, *L. fimbriatus*, *L. gonius*, *L. dussumieri*, *L. bata*, *Cirrihinus cirrhosa*, *C. reba*, *Puntius sarana*, *P. jerdoni*. Efforts are being made to standardize the technology of mass-scale seed production of these species and their inclusion as a component of conventional carp polyculture, based on their regional importance. In addition, there is contribution from cold water fisheries, although insufficient for fish basket, is of high value and low–volume category with a projected volume of 1 percent. Important food fishes of cold waters are mahaseer and schizothorax belonging to the indigenous species and trout among the exotic varieties (FAO, 2017). *Pangasianodon hypophthalmus* is a native of Mekong River in Vietnam introduced to many Asian countries such as Singapore, Philippines, Taiwan, Malaysia, China, Myanmar, Bangladesh, Nepal and India. In view of its escalating trade, some of these countries have been culturing this fish to boost up aquaculture production (De Silva *et al.,* 2009). Based on the information provided by FAO in the Database and Introduction of Aquatic Species (DIAS) and Fishbase, this fish has established itself in the natural ecosystems of many countries where it was introduced. The data shows that this fish has a wide range of adaptability to water quality and eco-physiology and hence suitable for various types of aquaculture practices.

2.3. *Pangasianodon hypophthalmus*

2.3.1. Scientific classification *Pangasianodon hypophthalmus*

Kingdom Animalia

Phylum Chordata

Class Actinopterygii

Order Siluriformes

Family Pangasiidae

Genus *Pangasianodon*

Species *P. hypophthalmus*

2.3.2. Biological features of *Pangasianodon hypophthalmus*

P. hypophthalmus is a catfish native to Vietnam which has long body, flattened laterally with no scales on the body. It's head is relatively small with large eyes, broad mouth with small sharp teeth on jaw, vornerine and palatal bones. It has two pairs of barbels, upper one being shorter than the lower and fins dark grey or black. Six branched dorsal-fin rays and four pair of gill rakers on both side of gill operculum are normally developed. Young fish have a black stripe along the lateral line and another long black stripe below lateral line; large adults are uniformly grey, sometimes with greenish tint, and sides are silvery. A dark stripe is present on the middle of the anal fin and in each caudal lobe, and small gill rakers are regularly interspersed with larger ones. *P. hypophthalmus,* when young, are carnivorous and cannibalistic feeding at the bottom on snail, gastropods, insects, etc. and turn to omnivorous feeding habit at a later stage, readily accepting artificial feeds under confined culture conditions. The species reaches sexual maturity by 3 years of age and can grow up to a weight of 4-6 kg. Females are larger than males. In nature, they are seasonal spawners and breed during warmer months. The fish can be artificially bred in hatcheries by hormone injection (Bui *et al.,* 2010).

2.3.3. Aquaculture of *P. hypophthalmus* in India

P. hypophthalmus, one of the swift growing catfishes was first introduced into India in the year 1995–1996 in the state of West Bengal from Thailand through Bangladesh. Initially farming was carried out in limited area in the states of West Bengal and Andhra Pradesh. This fish grows to 1–1.5 kg during one year and in monoculture system a minimum of 10–15 tonnes/hectare/year is produced in India. According to National Fisheries Development Board, Hyderabad estimates, since 2004 its farming has increased due to the commercial importance and by 2008 it is estimated that *P. hypophthalmus* is being farmed in an area of about 40,000 ha with an expected production of 1.80 to 2.20 lakhs tonnes. There is a growing interest among the farming community in other states as well to take up *P. hypophthalmus* culture in a larger extent, thus paving way for demand for its seed and for establishment of commercial scale hatcheries. Culture of *P. hypophthalmus* is mainly restricted to land-based pond aquaculture systems in India. However, it is also being adopted for cage aquaculture because of its good response to commercial diets. It is tolerant to high stocking densities and easily

breeds artificially. Thus, *P. hypophthalmus* is being farmed under monoculture or polyculture with carps. The seed of *P. hypophthalmus* is not only being used in aquaculture but also as ornamental fish. Different varieties (striped and albino) of *P. hypophthalmus* are produced for aquaculture and aquarium trade (Singh and Lakra, 2012).

P. hypophthalmus culture in India was legalized recently by the Government of India. The Government of India has stipulated strict guidelines for regulating introduction of *P. hypophthalmus* in the country and the guidelines clearly suggested keeping the upper limit of production to a level of 20 tonnes/hectare/year. The outbreak of diseases is a major constraint in aquaculture systems resulting in mortality and reduced yield. Though the information on *P. hypophthalmus* diseases is sparse in India, studies in other Asian countries suggest that a number of bacterial and parasitic diseases affect this species.

2.3.4. Economically important diseases of *P. hypophthalmus* with special emphasis on parasitic diseases

Diseases are major constraint to the sustainable development of aquaculture. It is estimated that about 10 percent of all cultured fish throughout the world die because of infectious diseases; however in some instances this estimate may be conservative (Woo *et al.,* 2011). Contemporary, many diseases affecting aquaculture result from intensification of culture practices without a basic perception of the intricate balance between host, pathogen and environment (Snieszko, 1975). Often, disease outbreaks are closely linked to environmental deterioration and stress associated with intensification of culture practices. Stress factors such as inadequate physico-chemical and biological (microbiological) quality of culture water, inferior nutritional status and high stocking densities can cause infection by opportunistic pathogens, leading to mortality. Unprecedented movement of fish seed and broodstock between regions and localities has created fish populations with inherent pathogens, making this population more prone to serious disease when they are under stress (Woo *et al.,* 2011). Indiscriminate use of chemicals including antibiotics may cause serious environmental problems. Outbreaks of disease caused by virulent primary pathogens can be minimized through careful quarantine, prophylaxis, and therapy. However, the much-emphasized practice of ‘good on-farm management’ will become costly and ineffective if basic culture

requirements such as appropriate site selection, farm design, quality of incoming water, nutritional status of feed and quality of seed are not guaranteed (Subasinghe and Barg, 1998). In this section, a critical overview of the available information regarding the disease of pangasius is presented.

Bacterial, viral and parasitic diseases are already reported in *P. hypophthalmus* from different part of pangasius farm in Vietnam, Malaysia, Thailand, Bangladesh, Java, West Indies and India (Ferguson *et al.,* 2001; Crumlish *et al.,* 2002, 2010; Singh and Lakra, 2012; Shetty *et al.,* 2014; Faruk, 2008; Kumar *et al.,* 2013, 2015; Tripathi *et al.,* 2014; Phillips *et al.,* 2017). Additionally non-infectious diseases like pale liver, yellow fillet and nutritional deficiency diseases are case setback to pangasius farming.

2.3.4.1. Bacterial disease of *P. hypophthalmus*

Bacterial disease outbreaks, resulting in higher rate of mortality in fishes are a significant constraint to the further development of aquaculture industry. Bacterial diseases are among the most important causes of loss among fish stocks. Since last decades several literatures are published that mainly emphasized on bacterial diseases of *P. hypophthalmus.* Although, first case study on bacterial diseases reported from Vietnam, the reports of diseases along with culture of pangasius in South East Asian countries also increased. However, first detail and establish disease bacillary necrosis (BNP) was reported by Ferguson *et al*. (2001), described the pathology in clinically sick animals as severe multifocal necrotizing bacterial infection and named the disease as BNP. Further the causative agent of BNP has been identified as *E. ictaluri* (Crumlish *et al*., 2002). A report from New Zealand on risk assessment of Vietnamese *P. hypophthalmus* has highlighted the possible transfer of *E. ictaluri* in the aquaculture areas in Vietnam which is a concern of World Organization for Animal Health (OIE) listed disease (Reed, 2008).

Two main bacterial diseases in *P. hypophthalmus* industry are BNP and MAS, where the aetiological agents have been identified as *E. ictaluri* and *A. hydrophila* respectively (Crumlish *et al.*, 2010). Previously, infections by *A. hydrophila,* resulting heavy mortality of cultured fish in Vietnamese *P. hypophthalmus* production system, had also been reported (Subagja *et al.,* 1999).

2.3.4.1.1. Bacillary necrosis of *P. hypophthalmus*

BNP in one of the important bacterial disease associated with *P. hypophthalmus. E. ictaluri* has been isolated from *P. hypophthalmus* in Vietnam, which has not been reported from the co-habitant *P. bocourti* (Crumlish *et al.*, 2002). A previous report of bacillary muscle necrosis reported from Vietnam (Ferguson *et al.*, 2001) has now been identified as being attributable to *E. ictaluri* (Crumlish *et al.*, 2002). This bacterium was first identified as a significant threat to the USA channel catfish *Ictalurus panctatus* industry causing enteric septicaemia of catfish (Hawke *et al.,* 2013). BNP exhibits multifocal irregular white lesions of varying sizes on several organs including liver, spleen and kidney. Histopathological, the lesions are acute to sub-acute multifocal areas of necrosis and polygranulomatous inflammation (Crumlish *et al.*, 2002). *E. ictaluri* isolates were identified as Gram-negative, non-motile rod, oxidase-negative fermentative, and positive only for lysine decarboxylase and glucose using API 20E kit. This bacterium gave no haemolysis on sheep blood agar. The optimal growth temperature was determined to be 20 ^{o}C (Crumlish *et al.*, 2002).

2.3.4.1.2. Motile aeromonad septicaemia (MAS)

Aeromonas hydrophila is the aetiological agent of motile aeromonad septicaemia (MAS), which causes disease in a wide range of freshwater fish species (Newman, 1993) and is often associated with stressed or immunocompromised hosts (Roberts, 1993). Septicaemia, abdominal dropsy and blood-tinged peritoneal fluid are typical signs of *A. hydrophila* infections in warm-water fish species (Roberts, 1993). Further, in experimental study, Crumilsh *et al.* (2010) showed that *E. ictaluri* and *A. hydrophila* both are responsible to cause septicaemia in *P. hypophthalmus*. Similarly, Nusbaum and Morrison (2002) described a potentially synergistic infection of MAS from channel catfish exposed experimentally to *E. ictaluri*. Further, it is highlighted that carriers of *A. hydrophila* are the chief source of the latent MAS infection that can be either from an endogenous infection or from bacteria released and shed into the environment from stressed fish (Nusbaum and Morrison, 2002). Subsequently along with these two bacterial diseases, red spot, pop eye, and edwardsillosis in *P. hypophthalmus* were reported from various part of the world. Kumar *et al.* (2013, 2015) isolated pathogenic strains of *Enterobacter cloacae* and *A. jandaei* from infected pond

reared *P. hypophthalmus* from Andhra Pradesh, India. Interestingly, in Thailand, concurrent infection of *Flavobacterium columnare* and *E. tarda* was also found in naturally infected *P. hypophthalmus* (Dong *et al.,* 2015). The list of bacterial diseases in pangasius from various countries is presented in table 1.

2.3.4.2. Viral diseases in *P. hypophthalmus*

There are no published reports of viral diseases of pangasid catfish in the usual fisheries literature databases. Of the virus diseases listed by the OIE as being of concern in fish none lists pangasid catfish as susceptible species. There have been no reports of infection of Pangasiidae over a considerable period of time (Ministry of Agriculture and Forestry (MAF) reports, 2008). Further iridoviruses, as a family, will be considered as risk, but uncertainty regarding the host specificity and occurrence of iridovirus. The number of susceptible species being identified is increasing, as are the number of countries infected with a range of iridoviruses. However, the first incidence of channel catfish virus, CCV (herpesvirus) infection in *P. hypophthalmus* was reported in Malaysia (Siti-Zahrah *et al.,* 2014). Although channel catfish virus is known to cause an acute haemorrhagic disease in channel catfish fry and fingerlings (Plumb, 1986) and is believed to be host specific (Davison, 2002). Interestingly, Plumb (1986) revealed that CCV is frequently found concurrent with *A. hydrophila* or *F. columnaris* and suggested that the virus might be latent (Gray *et al.,* 1999) but predisposing fish to secondary bacterial infection.

Table 1: Bacterial diseases in *P. hypophthalmus*

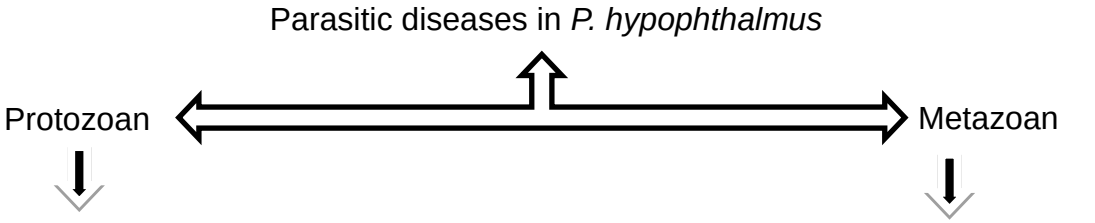
Sl no.	Bacterial disease	Aetiology	Country	References
1	Red spot	*A. hydrophila*	West Java	Supriyadi *et al.,* 1998
2	Red spot	Parasite *and Aeromonas complex, Pseudomonas sp.*	Vietnam	Dung and Ngoc, 1998
3	Bacillary necrosis	*Bacillus* sp. (*B. fumarioli*) associated with Myxobolus and *Sphaerosphora*-like myxosporean	Vietnam	Ferguson *et al.,* 2001
4	Acute septicaemia "Bacillary necrosis"	*Edwardsiella ictaluri*	Vietnam	Crumlish *et al.,* 2002
5	BNP	*E. ictulari*	Indonesia	Yuasa *et al.,* 2003
6	Bacillary necrosis	*E. ictaluri*	Vietnam	Dung *et al.,* 2008
7	Red spot, tail and fin rot, pop eye, dropsy	*A. hydrophila*	Bangladesh	Faruk, 2008
8	Haemorrhage disease	*A. hydrophila, A. sorbia, A. caviae, E. ictaluri, Clostridium* sp.	Vietnam	Ly *et al.,* 2009
9	BNP and MAS	*E. ictulari and A. hydrophila*	Vietnam	Crumlish *et al.,* 2010
10	Bacterial septicaemia	*Enterobacter cloacae*	India	Kumar *et al.,* 2013
11	Erdwardsiellosis	*E. tarda*	India	Shetty *et al.,* 2014
12	Red disease	*Pseudomonas sp.*	India	Kumar and Ramulu, 2013
13	Red spot	*A. jandaei*	India	Kumar *et al.,* 2015
14	Saddleback and Necrosis	*Concurrent infection of Flavobacterium columnare and E. ictaluri*	Thailand	Dong *et al.,* 2015
15	MAS (Reddening of eye and mouth)	*A. hydrophila*	Bangladesh	Sarker and Faruk, 2016
16	MAS (Reddening of eye and mouth)	*A. hydrophila*	Bangladesh	Nahar *et al.,* 2016
17	Bacillary necrosis	*E. ictaluri*	West Indies	Phillips *et al.,* 2017

2.3.4.3. Parasitic diseases in *P. hypophthalmus*

Parasitic diseases are one of the most serious problems faced by the aquaculture industry throughout the world including India (Scholz, 1999; Chanda *et al.,* 2011). Parasitic diseases are often more prevalent in tropical countries than in temperate ones. Parasites are thought to be of not much of concern among the wild stock outbreaks of diseases. Some of the economically important parasites and diseases caused by them in cultures conditions are briefly discussed in this section.

Both protozoan and metazoan groups infect the *P. hypophthalmus* and several authors reported the outbreak of parasitic diseases in wild and cultured pangasius from South East Asian countries including India (table 2&3). Microsporidian parasite causes severe regressive changes in the trunk muscle of *P. hypophthalmus* (Lom *et al.,* 2000). However, gill fluke infection is commonly seen in all *P. hypophthalmus* farms with infection rates varying from 60 to 90% of fish in India. The highest mortality due to gill fluke is manifested during the first week after stocking. The visible infection in spawn and early fry at the hatcheries is low except for gas bubble disease which is probably due to high ammonia level and eutrophic conditions. Farmers pay attention only to the direct economic loss from diseases other than that from gill fluke infection. In fact, gill fluke is understood to contribute significantly to the loss due to secondary bacterial infection which is followed by the initial infections with the parasite (Singh and Lakra, 2012). Dung *et al.* (2008) highlighted the parasitic diseases in *P. hypophthalmus* in Vietnam (table 2).

Table 2: Parasitic group reported in *P. hypophthalmus*



Protozoan	Metazoan
1. Trichodina and Epistylis	1. Monogenean
2. *Ichthyophthirius multifiliis*	2. Digenean
3. Microsporidians	
4. Myxobolus,	
5. Trypanosoma	
6. Balantidium, Ichthyonyctus	

Source: Dung *et al.* (2008)

2.3.4.3.1. Protozoan parasites

2.3.4.3.1.1. *Ichthyophthirius multifiliis*

Ichthyophthirius multifiliis is a common freshwater (protozoan) ciliate that normally infect the gills and skin and cause “Ich” or white spot disease. The life cycle includes the theront (swarmer), a small migratory stage that initially infects the fish; the trophont, a feeding and growing stage in the skin and gills of the fish; the tomont, an encysted stage that develops on a convenient substrate after the trophont within the cyst. The tomites break through the cyst wall to become theronts and infect the host. The parasite invades the skin and gills in heavy infections as a consequence the host loss the osmotic balance. Further, affected fish also show irritation, erratic movement and restlessness with tendency to rub on the sides (Gopalkrishnan, 1964). Dung *et al.* (2008) found a high infection in fry stage of pangasius that swim slowly and mortalities are high within five to seven days when water temperatures are 25-28° C. Clinical signs are white spots less than 1 mm on the skin and gills.

2.3.4.3.1.2. Trichodinids and Epistylis

Mobile peritrichs attach only temporarily to the substrate while feeding, and have been found on (or in) both freshwater and marine fish. Some species are host specific, whereas others are common to many different hosts. Mobile peritrichs may be ectozoic or endozoic. Some ectozoic species occur only on the gills or show a preference for the gills (e.g. *Trichodinella* and *Tripartiella* species), but *Trichodina* spp. occur indiscriminately on the gills and skin (Bruno *et al.,* 2006). Affected fish shows pale and fade gill and there is a creamish coating due to excessive mucus secretion. Heavily infested fish gradually become sluggish and loose the weight and very often there is asphyxia (Pal and Ghosh, 1985; Longshaw *et al.,* 2004). Further Dung *et al.* (2008) highlighted the heavy infections of *Trichodina* and *Epistylis* species in *Pangasius* catfish, responsible for mortalities during the nursing period. Infections could be observed year round but were heaviest when climate conditions were unstable, such as when downpours of rain were followed by periods of sunshine. The parasites are prevalent most in ponds with high stocking densities and poor water quality. The primary symptoms of infestation include breathing difficulties accompanied with anorexia, a dark coloration but with pale gills, fish swimming on the surface, flashing or movement through plants, and in serious cases, fin erosion.

2.3.4.3.1.3. Trypanosoma

Trypanosomatids are transmitted to fish hosts by leech vectors, and can be detected in the peripheral blood of various freshwater including catfish. Infections may be chronic or acute, severe parasitaemia can result in anaemia and death (Bruno *et al.,* 2006). There is no detailed research database on infection of trypanosome in *P. hypophthalmus* till the date. Recently, flagellate blood parasites (*Trypanosoma* species) were noticed in farm reared *P. hypophthalmus* by Dung *et al.* (2008). However they do not reveal any pathological condition associated with flagellate infections in the fish.

2.3.4.3.1.4. *Balantidium*, *Ichthyonyctus*

The vestibuliferan ciliate, *Balantidium* is ellipsoidal or oviform in shape and, although normally commensal, has been observed to cause the disease balantidiosis in a variety of freshwater fish. These are host specific and some species are endocommensals in the intestine but can become histophagous parasites. A report of *Balantidium* infecting *P. hpophtyalmus* was published in the checklist of parasites of fishes in Vietnam (Arthur and Te, 2006). Further, Dung *et al.* (2008) found *Balantidium* and *Ichthyonyctus* species in the large and posterior intestines of juvenile and adult pangasius catfish but pathogenesis was not revealed in such cases.

2.3.4.3.1.5. Microsporeans

The Microsporidia are obligate intracellular parasites found in members of all animal phyla, and are characterized by infectious spores with unique organelles involved in their invasion of a host and causing infections of varying severity. Occasionally, they infiltrate the muscles and viscera of cultured catfish. Cyst forming species may impair organ functions, gill infections may result in respiratory impairment, and gonadal infections clearly have the potential to reduce reproductive capacity and spawning success significantly. Although Lom *et al.* (1990) first reported the infection of microsporidian (*M. arthuri*) in *P. hypophthalmus* from South East Asia but after one decade the second report of microsporidian infection (*Kabatana arthuri*) in *P. hypophthalmus* published by Dykova and Lom (2000). In addition, microsporidians are not regarded as important catfish parasites; however, the presence of certain species can significantly reduce the quality and appeal of fillets (Dung *et al.,* 2008).

2.3.4.3.1.6. Myxozoa/Myxosporeans

Members of this group mostly consists *Henneguya*, *Myxobolus*, *Theohanellus* and *Unicauda*, possess spores that are flattened parallel to the straight sutural line. As a rule, these parasites form large histozoic trophozoited (cysts) with numerous spores, and infect mostly freshwater fish, although some species infect estuarine fish also (Bruno *et al.,* 2006). Dykova and Lom (1997) reported first case of myxozoa (*Sphaerospora ojiroveci*) infection in *P. hypophthalmus* from South East Asia. Further, Molnar *et al.* (2006) recovered

myxozoan from cage cultured infected pangasius fish. Dung *et al.* (2008) highlighted the impacts of myxozoa in pangasius and reported white cysts containing spores could be seen on internal or external organs with notable infections in the kidneys and gills. These infections are widespread, and yet there is no effective treatment available. The disease can be controlled by drying and liming ponds to remove the intermediate oligochaete hosts. Subsequently in Europe, Baska *et al.* (2009) found *P. hypophthalmus* imported from Thailand, infected with *Myxobolus hakyi* and *Hoferellus pulvinatus*.

2.3.4.3.2. Metazoan parasites

2.3.4.3.2.1. Monogeneans

Parasites belonging to the class Monogenea (Platyhelminths) are monogenetic flukes characterised by a posterior attachment organ, the haptor (opisthaptor). Infection of monogenean in *P. hypophthalmus* is reported from various parts of pangasius farm and wild (Komarudin, 1999; Pariselle *et al.,* 2002, 2003; Lerssuthichawal and Lim, 2005; Arthur and Te, 2006; Tripathi *et al.,* 2014). Pariselle *et al.* (2002, 2003) reported infected pangasius fish by a dactylogyrid (*Thaparocleidus* spp.) from South East Asia. Similarly, monogenean infection in pangasius was reported from Thailand (Komarudin, 1999; Lerssuthichawal and Lim, 2005); Bangladesh (Faruk, 2008), and India (Tripathi *et al.,* 2014). Thuy and Buchmann (2008a) reported two gill monogenean species (*Thaparocleidus siamensis* and *T. caesus*) of genera *Thaparocleidus* in *P. hypophthalmus* from Vietnam. The risk associated to gill fluke infestation without referring the classification in juvenile of *P. hypophthalmus* was emphasised by Lakra and Singh (2010). The details of monogenean parasites are written in subsequent section.

2.3.4.3.2.2. Digeneans

Trematodes are commonly found in vertebrates, and the members of the Class Trematodes most frequently encountered in fish are the digenea. Digeneans occur in almost all species of fish, both marine and freshwater. Metacercariae are the digenean larval stages most commonly observed in fish. Cercariae liberated from a mollusc intermediate host actively penetrate and migrate into tissues of the fish (an intermediate host) and encyst as metacercariae (Bruno *et al.,* 2006). Although rare report on digenean infection *Prosorhynchoides ozakii* (an

intestinal trematode) in pangasius (Thuy and Buchmann, 2008b) is documented but concern about zoonotic trematode metacercariae (*Haplorchis pumilio, H. taichui, Centrocestus formosasus and Procerovum* sp.) in pangasius conveyed from Vietnam (Thuy *et al.,* 2010)

2.3.4.3.2.3. Cestodes/ Nematodes/ Molluscs/ Crustaceans

Till the date, we are not perceived any printed or online report related to infection of above parasitic groups in *P. hypophthalmus*.

2.3.4.4. Fungal infection in *P. hypophthalmus*

Literature on fungal infection in pangasius is scarce; however, recently Duc *et al.* (2013, 2015) reported the infection of higher fungi genus *Fusarium* in swim bladder of *P. hypophthalmus* in Vietnam.

2.3.4.4.1. *Fusarium* sp.

Fusarium are distributed in a wide range of environments, including forests, grassland, deserts, littoral zones, agricultural and alpine zones, as well as in aquatic and manmade environments, and these fungi are known to cause various diseases in plants and animals (Marasas *et al.,* 1988; Nelson *et al.,* 1994; Balmas *et al.,* 2010). *Fusarium* species are considered invasive pathogens in species such as red sea bream *Pagrus major* (Hatai *et al.,* 1986), bonnethead shark *Sphyrna tiburo* (Crow *et al.,* 1995), and loggerhead sea turtle *Caretta caretta* (Cabanes *et al.,* 1997). Histological study of swim bladder of *P. hypophthalmus* revealed the *Fusarium* and causing severe swelling (Duc *et al.,* 2015).

2.3.4.4.2. *Aphanomyces invadans*

A. invadans, the causative agent of epizootic ulcerative syndrome (EUS), which is characterised by congested skin lesions and ulceration, affects over 100 freshwater and estuarine fish species (Pathiratne and Jayasinghe, 2001). Whilst *Pangasius* sp. is not listed specifically, some catfish are included as hosts and there are susceptible species in New Zealand.

Table 3: Parasitic disease in *P. hypophthalmus* from various geographical parts

Sl no.	Group of parasite	Species of parasite	Country	References
Protozoan				
1	**Microsporidia**	Microsporidium arthuri	South East Asia	Lom *et al.,* 1990
2		*Kabatana arthuri*	South East Asia	Dykova and Lom, 2000
3	**Ciliates**	Trichodinids, Apiosoma	Bangladesh, India	Haque *et al.,* 2004; Lakra and Singh, 2010
		I. multifiliis	Bangladesh, India	Haque *et al.,* 2004; Lakra and Singh, 2010
	Flagellates	*Trypanosome*	Vietnam	Dung *et al.,* 2008
4	**Myxozoa/myxosporea**	*Sphaerospora ojiroveci*	South East Asia	Dykova and Lom, 1997
		Hennegoides (H. berlandi, H. malayensis, and H. pangasii), Henneguya (H. shariffi) and Myxobolus (M. baskai, and M. pangasii	Malaysia	Molnár *et al.,* 2006
5		*Myxobolus sp. and Henneguya sp.*	Vietnam	Dung *et al.,* 2008
6		*Myxobolus hakyi and Hoferellus pulvinatus*	Europe	Baska *et al.,* 2009
	Metazoan			
8	**Monogenean**	Dactylogyrid (*Thaparocleidus* sp.)	Thailand and Indonesia	Komarudin, 1999
9		Thaparocleidus sp.	South East Asia	Pariselle *et al.,* 2002, 2003
10		*Thaparocleidus sp.*	Thailand	Lerssuthichawal and Lim, 2005
11		*Thaparocleidus spp.*	Vietnam	Arthur and Te, 2006
12		*T. siamensis and T. caecus*	Vietnam	Thuy and Buchmann, 2008a
13		*Gill fluke*	India	Lakra and Singh, 2010
14		*Dactylogyrids*	Bangladesh	Rahman *et al.,* 2006
15		*Thaparocleidus spp. (T. siamensis and T. caecus)*	India	Tripathi *et al.,* 2014; Chaudhary *et al.,* 2014; Rajvanshi and Agrawal, 2014
16	**Digenean**	*Haplorchis pumilio, H. taichui, Centrocestus formosasus and Procerovum sp.*	Vietnam	Thuy *et al.,* 2010
17		*Prosorhynchoides ozakii*	Vietnam	Thuy and Buchmann, 2008b

2.3.4.5. Non-infectious diseases in *P. hypophthalmus*

2.3.4.5.1. Red fillet syndrome

Red fillet syndrome affects adult catfish. It is usually defined by red spots in fillets, which consist of small petechial hemorrhages. Affected fish are unmarketable because they are regarded as poor quality by processors (Dung *et al.,* 2008). The red spots disappear if the feed ration is reduced or stopped for at least one week and the water quality is improved, suggesting an environmental and/or feed related problem. No etiological agent was detected from histology samples.

2.3.4.5.2. Yellow fillet syndrome

Yellow fillet syndrome is typically observed in adult *P. hypophthalmus* during the flooding period. No pathogen has been found in association with the pathology. The syndrome is commonly observed in association with other external parasites and these fish can become vulnerable to bacterial infection. The gross clinical signs are anaemia, weight loss, ascites and a yellowish discoloration of the fillets (Dung *et al.,* 2008).

2.4. Monogenean parasites

2.4.1 Diversity of monogenean parasite

Monogeneans are flatworms (Platyhelminthes) with representatives in freshwater, brackish and marine habitats. Monogenean is one of the largest classes that include more than 5000 known species; all of them are reported as parasitic (Morsy *et al.,* 2012; De Meeus and Renaud, 2002). Among the known species, 95% of the genera have been reported from fishes and remaining 5% have been reported from amphibians. The potential for expansion of our knowledge about platyhelminths was further stressed by Lim (1998), who stated that only 8% of the South-east Asian monogenean species are known. Furthermore, it was suggested that there are about 20,000 species in the genus *Gyrodactylus* alone (Bakke *et al.,* 2002). Approximately including freshwater and marine 112 genera belonging to 28 families on monogenoidea have been abstracted from India (Pandey and Agrawal, 2008). Further Chaudhary *et al.* (2013) critically reviewed the biodiversity of monogenean from the five major Indian riverine systems and found 14 families which belong to 44 genera of freshwater monogeneans. The highest diversity was

found in the Ganges river system followed by East coast river system, the Indus river system, the Brahmaputra and at last west coast river system. Interestingly, if we stick to one host one parasite rule, a very big gap exists in the state of our knowledge as far as biodiversity of freshwater monogeneans are concerned and only limited work has been done in this area in India (Chaudhary and Singh, 2012).

2.4.2. Forms of monogenean parasitism

Monogeneans are an evolutionarily old animal group that is believed to have evolved from ancestral ecto-commensal turbellarians several hundred millions of years ago (Llewellyn, 1965). These platyhelminths have demonstrated an impressive adaptability to changing environmental and host conditions. Thus, the high number of species is probably not only the result of co-evolution between host and parasite but may also result from host switching (Bakke *et al.,* 2002). Monogenean are mainly ectoparasites infect skin, fins, gills, mouth cavity and nostrils with some exception which are endoparasitic (*Acolpenteron ureterocetes*) infects urinary bladder and ducts, *Enterogyrus* sp. infects foregut and stomach and *Calicotyle kroyeri* infects cloaca (Fayton and Kritsky, 2013). They all have a direct life cycle without intermediate hosts.

2.4.3. Morphology of monogenean parasites

The monogenean diversity is huge and identification is always challenging. The most common identification procedure is morphometric and presence and absence of key organ. Morphometric of monogenean are great varied and range from 300 µm long (*Dactylogyrus* and *Gyrodactylus*) to >3 cm long for Capsalidae. The primary characters are the shape and size of hard sclerotinized parts, such as hamuli, clamps and marginal hooks, and their arrangement on the opisthaptor. Additionally, hard reproductive organs, such as the vagina, cirrus and accessory cirrus, are important characters. Studies on live worms are used for description of the excretory systems and distribution of flame cells. However, body length and width, arrangements of testes, ovary, vitellaria, pharynx and intestinal caeca and gland cell distribution may also supplement to the description. It should be noted that the water temperature at sampling sites may influence the size of sclerotized structures (Mo, 1991, 1993). Following treatment with silver nitrate and ultraviolet (UV) exposure, various sensillae on the parasites may be visible. The

arrangement and distribution of these structures represent important taxonomic characters (Shinn *et al.,* 1997).

2.4.4. Biology of monogenean

Two quite different dominant lineages have been suggested for the monogeneans, and they are the monopisthocotyleans and polyopisthocotyleans. The two main groups differ markedly in morphology, anatomy, nutrition, physiology and reproductive. Buchmann and Lindenstrøm, (2002) reviewed the interactions between monogenean parasites and host, and revealed the differences in feeding activity between these two groups have important implications for their pathogenicity. The monopisthocotylea are tissue grazers, which possess a haptor comprising a single attachment unit and generally inhabit the external surfaces mainly gills of their hosts. In other hand the polyopisthocotylea, members of which have a complex haptor with multiple attachment units, including hooks, suckers, clamps, or both usually inhabit the gills and are sanguinivorous. The most important distinguishing characteristics include the presence or absence of eyespots among different families of monogenean (Buchmann and Lindenstrøm, 2002).

2.4.5. Epizootiology and transmission of monogeneans

Monogeneans are exhibited a direct life cycle, and no intermediate host is obligatory for the parasite to reproduce. Thus transmission of monogeneans is primarily by means of direct contact with host. Adults monogeneans are hermaphroditic (each organism has both male and female reproductive structures) and display both oviparous and viviparous, and rarely exhibit self-fertilization (Buchmann and Bresciani, 2006). The viviparous gyrodactylids produce young ones (oncomiracidium) that are capable to free swim or they may be carried by mercy of the water and instantly attach to the host (Buchmann and Bresciani, 2006). Monogeneans possess diverse mode of reproduction and thus developed the various types of host-seeking strategies. Some oncomiracidia hatching from eggs show vivid and elegant movements when swimming through the water (Buchmann, 1997). These free-swimming larvae are able to seek the host actively over short distances. The abilities to reproduce several embryos in short life span makes this ectoparasite successfully survive in aquaculture system.

In contrast to the gyrodactylids (viviparous), hatchlings of oviparous monogeneans often have appendages that have limited or slow movement in the water column which allow them to be easily trapped in mucus or other organic material. The most critical stage of oncomiracidia, need to locate the host and for this either rely on mercy of water currents as well as by their own movement. Further, oncomeracidia crawl on the surface of the host's body to reach their preferred site for feeding.

2.4.6. Pathology

Monogeneans are ectoparasites and mainly parasitizing fish gills, skins and fins (Jarkovsky *et al.,* 2004). The impact of monogenean on fish can be seen with their immense intensity during their feeding, attachment and movements. Monopisthocotyleans are tissue grazers that generally inhabit the external surfaces mainly gills of their hosts and heavy infestation may elicit epidermal hyperplasia due to disruption by parasite attachment and feeding; increased mucus production (Buchmann and Bresciani, 2006; Molnar *et al.,* 2016). Polyopisthocotyleans usually inhabit the gills and are sanguinivorous, causing tissue compression and hyperplasia of the gill tissues, fusion of secondary lamellae associated with hyperplasia, aneurysm, edema and purulent bronchitis. Pathogenesis results from feeding and blood loss, which is reflected in decreased haematocrit in heavily infected fish hosts (Montero *et al.,* 2004; Jeronimo *et al.,* 2014).

2.5. Monogenean parasite infection in *P. hypophthalmus*

Thaparocleidus spp. a dactylogyrids is the most common monogenean group found in *P. hypophthalmus*. This group of parasite mostly infect to catfish (Siluridae). Moreover, 43 *Thaparocleidus* species have been described from 18 species of pangasiidae (Pariselle *et al.,* 2006) and 17 species of *Thaparocleidus* is listed in Indian context collected from fish belongs to family siluridae of five major river system of India (Chaudhary *et al.,* 2013). Historically the only known monogenean from *Pangasius* sp. is *Silurodiscoides pangasi* (Tripathi, 1959) Gusev, 1978 described from *P. pangasius* in India. Previously, two species (*Silurodiscoides caecus* and *Ancylodiscoides caecus*) of *Silurodiscoides* Gussev, 1961 were collected from *P. sutchi* in Vietnam. *Thaparocleidus caecus* was originally described as *Ancylodiscoides caecus* from an unidentified aquarium fish collected in United

States (Mizelle and Kritsky, 1969). Subsequently, this species described and transferred to Silurodiscoides as *Silurodiscoides caecus* (Mizelle and Kritsky, 1969) n. comb (Gusev, 1978). Further, *S. caecus* along with a new species, *S. siamensis* was redescribed based on new morphometric traits which were collected from *P. hypophthalmus* in Malaysia (Lim, 1990). Subsequently, based on the law of priority, Lim (1996) designated *Silurodiscoides* as synonym of *Thaparocleidus* Jain, 1952. Accordingly, the scientific names of *S. caecus* and *S. siamensis* were designated to *T. caecus* (Mizelle and Kritsky, 1969) Lim, 1996 and *T. siamensis* Lim, 1990, respectively. The list of subsequent report of *Thaparocleidus* spp. in *P. hypophthalmus* is presented in table 3.

2.5.1. Taxonomy of *Thaparocleidus siamensis* and *T. caecus*

Kingdom: Animalia

Phylum: Platyhelminthes

Class: Monogenea

Order: Monopisthocotylea

Family: Dactylogyridae

Genus: *Thaparocleidus*

Species: *T. siamensis*

Species: *T. caecus*

2.6. Conventional and genomic diagnosis of monogenean

Choosing the most appropriate method to treat fish infected by monogeneans is completely dependent on adequate parasite identification. Therefore, the proper removal, fixation and preparation of the parasites are critical for the diagnostic process. Monogeneans on the skin and gills can be difficult to detect unaided eye either because they are tiny or on hidden sites such as lip folds and branchiostegal membranes (some capsalids) or the parasites are cryptic because they are camouflaged (*Dendromocotyle* sp.) or transparent (capsalids). Diagnosis of monogeneans invariably is consisted screening of parasite specimen in live condition from host and subsequent fixation with alcohol-formalin- acetic acid (AFA) or 4% formalin and wet mount consisting Canada balsam mounting for permanent slide preparation (Malmberg, 1990).

2.6.1. Morphological description

2.6.1.1. Key for identification of *Thaparocleidus* spp. (Lim *et al.,* 2001)

Body elongate, with four eye-spots. Caeca unite posterior to testis. Haptor may or may not be well demarcated from body, sometimes bilobed. Patches on dorsal anchors. Dorsal anchors usually larger than ventral anchors, with roots of variable length; ventral anchors usually smaller, with roots of variable length. Dorsal bar straight to V-shaped; ventral bar usually V-shaped or divided into two. Marginal hooks of variable shapes and sizes. Seminal vesicle single, blind, sac-like. Copulatory organ consists of usually coiled copulatory tube and accessory piece. Vaginal opening normally sinistral.

2.6.2. Genetical description

2.6.2.1. DNA taxonomy and barcoding of monogenean parasites

The concepts of DNA taxonomy and barcoding can facilitate unambiguous species identification, diagnoses of problematic taxa and discovery of 'cryptic' species (Blaxter *et al.,* 2003; Hebert *et al.,* 2003). The main arguments in favour of DNA taxonomy and barcodes is high conservation of morphology, which makes it difficult to distinguish related species and the low throughput of classical taxonomy apply perfectly for monogenean. Nuclear DNA encoding ribosomal RNA from both large and small subunits of monogenean ribosomes has been the subject of extensive studies (Mollaret *et al.,* 2000; Olson and Littlewood, 2002). These genes have been shown to provide excellent tools for phylogenetic studies and for diagnostic purposes.

Further, mitochondrial DNA has recently been shown to provide a high degree of sensitivity in phylogenetic studies of gyrodactylids (Zietara and Lumme, 2002; Hansen *et al.,* 2003). Sequencing of entire PCR products will elucidate differences and variations between species. The genes for 18S, 5.8S and 28S ribosomal RNA and the spacers between them have shown themselves to be particularly interesting because the intraspecific variation is extremely low. Based on slight variations between species in the internal transcribed spacer (ITS) regions 1 and 2, comprising also the 5.8S gene, a reliable diagnostic tool the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) has been developed by Cunningham (1997) to distinguish different gyrodactylid species.

Further, *T. siamensis* and *T. caecus*, is identified by conducting PCR with specific primers for a fragment of 28S rDNA region in the *Thaparocleidus* species on pangasius in Vietnam, Malaysia and India (Simkova *et al.,* 2013; Rajvanshi and Agrawal, 2014; Tripathi *et al.,* 2014; Chaudhary *et al.,* 2014).

The repetitive ribosomal RNA genes are separated by intergenic spacer (IGS) regions. A very sensitive discrimination between populations of gyrodactylids may be obtained from studying the IGS regions of ribosomal DNA (rDNA) (Collins and Cunningham, 2000). *G. salaris* population was differentiated by using random amplified polymorphic DNA (RAPD) methods (Cunningham and Mo, 1997). Knowledge of specific differences between species in certain genes may allow the use of fast methods for species differentiation. Thus, it is possible to conduct hybridization, in which DNA from a worm is immobilized on a membrane and subsequently hybridized with specific probes conjugated with a marker, allowing visualization of positive reactions (Cunningham *et al.,* 2003).

Table 4: Morphometric data of *T. siamensis* and *T. caecus* from *P. hypophthalmus* in India (Tripathi *et al.*, 2014) and Malaysia (Lim, 1990) and USA (Mizelle and Kritsky, 1969)

Parameters measured		***T. siamensis***		***T. caecus***		
		Tripathi *et al.* (2014)	Lim (1990)	Tripathi *et al.* (2014)	Lim (1990)	Mizelle and Kritsky (1969
Body						
	Total body length	822 (695-1139)	-	1038 (680-1230)	833 (333-1000)	904 (768-1055)
	Total body width	140 (90-170)	-	167 (120-200)	149 (124-183)	176 (140-251)
	Pharynx diameter	40 (30-50)	-	48 (36-60)	-	81 (68-95)
	Haptor length	118 (90-175)	-	125 (80-150)	-	115 (96-137)
	Haptor width	93 (70-145)	-	110 (77-150)	-	144 (111-169)
Reproductive organ						
	Testis length	131 (90-180)	-	113 (36-150)	-	-
	Testis width	64 (45-95)	-	63 (30-80)	-	-
	Copulatory tube length	96 (90-97)	97 (90-100)	60 (48-70)	62 (60-64)	66 (61-71)
	Accessory piece length	129 (121-138)	120 (110-134)	43 (34-53)	41 (40-44)	49 (40-54)
	Ovary length	124 (90-188)	-	141 (60-120)	-	-
	Ovary width	65 (50-85)	-	67 (40-100)	-	-
	Egg length	237 (147-385)	-	77 (56-91)	-	-
	Egg width	25 (19-30)	-	41 (33-50)	-	-
Haptoral parts						
	Dorsal anchor legth	60 (58-64)	64 (60-70)	39 (37-41)	43 (40-44)	46 (45-48)
	Dorsal anchor recuved	30 (27-35)	33 (28-38)	11 (10-13)	12 (12-14)	-
	Dorsal anchor patch	30 (24-36)	30 (24-36)	6 (4-7)	8 (7-9)	-
	Ventral anchor legth	19 (15-21)	23 (22-24)	17 (16-19	20 (19-21)	21 (20-22)
	Ventral anchor recuved	12 (9-15)	13 (12-14)	6 (5-7)	8 (8-10)	-
	Dorsal par length	38 (31-40)	40 (36-44)	42 (29-44)	44 (44-52)	32 (29-34)
	Ventral bar length	28 (25-32)	28 (24-30)	25 (21-27)	25 (22-26)	50 (44-59)
	Hooks length	12 (12-13)	14 (13-15)	14 (14-15)	11 (10-12)	12 (11-19)

2.7. Host-parasite interactions

It is important to appreciate that pathogenicity is not usual potency because killing a host does not enhance transmission. In most instances natural selection results in organisms that cause minimal damage to the host. Epizootics are usually the result of a breakdown in the normal host-parasite relationships created by artificial conditions (Buchmann and Lindenstrom, 2002). Monogenean parasite always influence with the specific host releasing factors for their three important biology (1) egg hatching (2) host finding and (3) nutrition and reproduction (Buchmann and Bresciani, 2006).

(1) Egg hatching: both abiotic and biotic factors can affect even the earliest stage of an oviparous monogenean parasite. It has been found that even changes of light intensities may initiate hatching in certain species like *D. sagittata* egg hatching is induced by darkness (Gannicott and Tinsley, 1997). Further, the mucus extract from a fish host has been observed to act as an efficient hatching stimuli ex. *Pseudodactylogyrus* from eel (Chan and Wu, 1984). Hamwood *et al.* (2002) reported an interesting fact that when an embryonated egg is subjected to mucus extracts, the oncomiracidium will initiate active movements and subsequently escape from the operculum.

(2) Host finding: The high host specificity in monogenean-fish systems; it has been speculated that host factors can be recognized by and attract the monogenean transmitted stage. Some fact related to chemoattraction showed by oncomiracidium (Kearn, 1967). Further, *G. salaris* infects salmon, *S. salar* when it has the choice between rainbow trout, carp and salmon shows specific host selectivity governed by some chemical molecule like lectins of host, and carbohydrate molecule in parasite (Buchmann, 2001). In continuation, it was also highlighted that volatile host substances from intact cells and pH of mucus played a vital role and could be involved in host selection (Hirazawa *et al.,* 2003).

(3) Nutrition and reproduction: finding a correct host and attachment is also regulated to induce feeding, maturation, mating and finally production of offspring that is one of the key parts of parasite life-cycle (Buchmann, 2001).

2.7.1. Pathogenicity of monogenean parasite

Pathogenicity of varied monogenean parasites rely on the anatomy of attachment organs, physiology, gland secretions and feeding strategy, as vary considerably among monogeneans. Further, degree of pathogenicity and pathological impact on host can be mainly due to (1) feeding (2) mechanical effects of opisthaptoral hooks, suckers and clamps and (3) effect of gland secretions.

(1) Feeding: *Heteraxine heterocerca* feeds on blood of yellowtail, *Seriola quinqueradiata,* leading to anaemic condition in the host (Eto *et al.,* 1976). The anaemia was characterized as a microcytic, hypochromic type and the condition was associated with lethargy, anorexia, dark skin colour and paleness of the muscle, gills, kidney and liver of the infected fish. Further, the proteins in the serum, enzyme activities of lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and glutamic pyruvic transaminase were found to be lowered in monogenean parasitized fish (Eto *et al.,* 1976). However, urea and creatinine levels were higher in monogenean-infected fish. Likewise, Paperna *et al.* (1984) reported a negative correlation between the infection intensity and haematocrit values in *Siganus luridus* infected by *Allobivagina* sp.

Browsing of epithelia by monopisthocotyleans elicits another type of pathological disturbance, and this is dependent on the size and number of monogeneans. Large capsalids, such as *Benedenia seriolae*, may produce large feeding wounds with heavy erosion of epidermal layers on yellowtail, amberjack and kingfish (Ernst *et al.,* 2002). Extensive hyperplasia and tissue reactions at the site of attachment are also associated with even smaller monogeneans. Such host reactions may impair the normal physiological function of the organ. Gill tissue developed into club-shaped filaments with fused lamellae or extensive outgrowths, partly embedding the invading parasite leading to decrease in the respiratory surface of gills and some time lead to mortality due to hypoxia (Roubal, 1995).

(2) Mechanical effects of attachment organs

The hamuli, marginal hooklets, suckers and clamps of monogeneans, all have direct contact with host tissue and may afflict mechanical damage to attachment site. This has been extensively reported for cyprinids (Bychowsky, 1957; Paperna, 1963; Molnar, 1972; Bauer *et al.,* 1973), anguillids (Chan and Wu, 1984; Buchmann, 1997) and salmonids (Cone and Odense, 1984). Even light infections with *Bothitrema bothi* on *Scophthalmus aquosus* may result in depressions of host tissue caused by the hard structures on the opisthaptor (Hendrix, 2004). In addition, the mechanical effect can lead to a host reaction of epithelia, including hyperplasia. However, the impact varies; hamuli in gyrodactylids do not penetrate the fish skin, whereas the marginal hooklets may penetrate an epithelial cell. These mobile and active monogeneans can easily move to another position on the skin if there is significant reaction at the site of attachment. In contrast, clamps need intact secondary lamellae in order to attach to the gills and the strategy of these worms is to focus on a minimum of damage to the host structure, although prolonged exposure may produce changes in the gill tissues.

(3) Effect of gland secretions: The enzymes, such as proteases, phosphatases and others in the pharynx, oesophagus and intestinal caeca, associated with digestion may affect the host epithelia and provoke reactions (Buchmann, 2001). Secretions from cephalic glands and opisthaptoral glands involved in attachment of the prohaptor and affect the host tissue. Although monogeneans inflict extensive pathogenic effects on the fish, the host reactions to eliminate them are only partly expressed. This indicates that the parasites are able to produce immune-evading or immunosuppressive substances. The nature of such molecules has to be investigated (Buchmann and Bresciani, 2006).

2.7.2. Impact of *Thaparocleidus* infection on *P. hypophthalmus*

The pathological implications of *Thaparocleidus* monogeneans are not well documented. Nevertheless, the sporadic mortality of *P. hypophthalmus* due to with *T. caecus* and *T. siamensis* infestation has been documented in Vietnam (Thuy and Buchmann, 2008a; Dung *et al.,* 2008; Tripathi *et al.,* 2014). Obligatory to attach with *P. hypophthalmus* gill and infection sometimes co-occurred with *T. caecus* and *T. siamensis* (Tripathi *et al.,* 2014; Simkova *et al.,* 2013). Worm attaches and/or

feeds on the gill, tends to induce hyperplasia and epithelial reactions, which can partly embed the worm (Thuy and Buchmann, 2008a) and also increased mucus production. Tripathi *et al.* (2014) reported the prevalence and intensity of *T. siamensis* and *T. caecus* infecting *P. hypophthalmus* and reported intensity of 200 parasites per aquarium reared fish and 250 parasites per pond-cultured fish, which makes it difficult to envisage that such a high parasitic burden and resulted to threat for host life.

2.7.2.1. Histopathology associated with dactylogyrids infection

Histopathology provides a rapid method to detect effects of irritants and pathogens in different organs and it can be considered as the indicator for abnormal condition for fish. It also shows the qualitative changes of the affected organs and the patterns of recovery. Montero *et al.* (2004) reported severe pathological changes in gills of amberjack (*Seriola dumerili*) infected by a polyopisthocotylean monogenean *Zeuxapta seriolae.* Further, a proliferative gill epithelial response, a high number of mucous cells, infiltrating inflammatory cells, distortion of the lamellar structure, fusions of secondary lamellae, rupture epithelia and depression in gill filaments were observed in histological sections in the gills of gilthead seabream (*Sparus aurata*) artificially infected with gill monogenean, *Sparicotyle chrysophrii* (Sitja-Bobadilla and Alvarezo-Pellitero, 2009), and in spotted rose snapper (*Lutjanus guttatus*) artificially infected with dactylogyrid monogenean (De Rio-Zaragoza *et al.,* 2010). Jerônimo *et al.* (2014) reported severe hyperplasia, sub-epithelial edema, fusion of the secondary lamellae, focal and multifocal necrosis in highly parasitized *Piaractus mesopotamicus* by monogenean. Further, Molnár *et al.* (2016) reported depression in the epithelium of the filaments at the site of monopisthocotylean monogenean gill parasite *Ancyrocephalus paradoxus* attached to the gill of pikeperch (*Sander lucioperca*). They also observed fusion in gill filaments, formation of clubs and composed of epitheloid-type regeneration tissue in heavily infected fish.

2.7.2.2. Haematological and serum biochemical responses

Haematology and biochemical analysis can provide valuable information for monitoring the health condition of host. Its depend on various factors like fish species, sexes, age, cycle of maturity and health condition (Wilhelm *et al.,* 1992). Moreover, haematological tests and analysis of serum constituents have showed useful information in detection and diagnosis of metabolic disturbance and diseases in fishes (Sandnes and Waagbo, 1982). Golovina (1976) reported that intense infections of parasites lead to reduced numbers of lymphocytes and increased numbers of monocytes and granulocytes in circulating blood. Further, some reports are briefed below related to impact of monogenean infection on host haemato-serum biochemical profiles. Sitja-Bobadilla and Alvarez-pellitero (2009) reported the monogenean infection produced hypochromic anaemia, since Hb concentration significantly decreased at 5 to 10 weeks of post infection. Mean cell haemoglobin concentration (MCHC) and mean cell haemoglobin (MCH) were found significantly low in parasitized fish but, immature erythrocytes, granulocytes and plasma cells were found higher in infected fish. Shah *et al.* (2009) studied the impact of helminth parasite on *Schizothorax* spp. and *Cyprinus carpio* in different seasons. They reported the significant reduction in haematological parameters especially haemoglobin and red blood cell (RBC) count in monogenean infected host in summer season whereas reverse trend was reported in case of white blood cell (WBC) counts. Further, a well-marked increase in eosinophils and anaemia was found in infected fishes. Ali and Ansari (2012) reported the significant reduction in RBC, Hb, haematocrit and lymphocytes in monogenean infected common carp *C. carpio*, whereas, total leucocyte counts, neutrophils, eosinophils and monocytes were significantly increased in infected host. Further, analysis of various biochemical parameters in infected fish showed significantly low level of bilirubin content, blood glucose, serum glutamate oxaloacetate transaminase (sGOT) and serum glutamate pyruvate transaminase (sGPT).

2.7.3. Host response to monogenean infection

The parasite infrapopulation under normal circumstances will not increase mortality to the host and it is also applied for monogenean–fish relationship. Several physiological and anatomical factors including immune system may be responsible to counteract the parasitism. Although studies on host responses with reduction or elimination of infrapopulations of monogeneans are limited, it should not be concluded that host responses are uncommon. Most histopathological studies of monogenean infections have demonstrated pronounced cellular reactions at the infection site. Hyperplasia, haemorrhages and infiltration with macrophages, neutrophils and other reactive cells are commonly discerned in histological sections of fish tissue infected with monogeneans (Paperna, 1963; Molnar, 1972; Thuy and Buchmann, 2008a; Simkova *et al.,* 2013).

The mechanisms of host response and the reaction pattern varies from system to system to monogeneans but it seems that both innate and acquired elements are involved (Buchmann, 1999). Both these responses involve humoral elements, such as lectins, complement factors, prostaglandins, leukotrienes and acute phase proteins, in the non-specific path and antibodies in specific reactions. Cytokines in host epithelia may take part in the orchestration of the reactions (Lindenstrøm *et al.,* 2003). Cellular elements, such as neutrophils and macrophages, are involved (Buchmann, 1999). Further, *in vitro* studies have also provided evidence that macrophages may recognize epitopes on monogeneans (Buchmann and Bresciani, 1999). These cells are able to produce reactive oxygen and nitrogen species with adverse effects on monogeneans.

As monogenean–fish host systems are extremely varied and diverse, the mechanisms involved in the host reactions may also vary considerably. However, recent studies *on G. derjavini* have indicated that some of these are influenced by a cytokine network in rainbow trout skin (Lindenstrøm *et al.,* 2003). Post infection, expression of genes encoding interleukin 1-beta (*IL 1β*) and tumour necrosis factor alpha (*TNF-α*) were detected. These genes are involved in the inflammatory response in fish skin, leading to the initial antiparasitic reactions. Further, expression of inducible nitrogen oxide synthetase (iNOS), leading to production of reactive nitrogen (N) species, was subsequently observed (Lindenstrøm *et al.,* 2003). Also cyclo-oxygenase, responsible for prostaglandin

production with regulating effects on the inflammation process, was expressed. However, the expression of genes for transforming growth factor (*TGF*) was found in later stages of the infection. They concluded that cytokine plays an important role in immune regulating and skin repairing (Lindenstrøm *et al.,* 2003). Further, there was no evidence of T-cell receptor (TCR) and major histocompatibility complex (MHC) up-regulation due to monogenean infection and it suggests that the responses are primarily autonomous innate reactions, at least in rainbow trout skin infected with *G. derjavini* (Lindenstrøm *et al.,* 2003).

Adaptive responses comprise antibodies (immunoglobulins) and that is also evident in cases of antibody production in infected fish to gill parasites. The first report indicating immunity in fish against monogenean infections was conveyed from the New York Aquarium, where *Epibdella melleni* had high numbers in *Acanthopteri* naïve fish but only showed limited infection following subsequent exposures (Nigrelli and Breder, 1934). In contrast, gill monogeneans (*Heteraxinoides xanthophilis* on the gills of *Leiostomus xanthurus*) were not recognised to induce antibody production in the host (Thoney and Burreson, 1988).

2.7.3.1. Stress response

Cannon was one of the first researchers, who introduce the concept of stress (Cannon, 1935). He claimed that when the normal homeostasis of an organism was threatened by one or more stimuli then the organism could be considered as stressed. Chrousos and Gold (1992) provided a more comprehensive definition as ‘stress is a condition in which the dynamic equilibrium of an animal or organism called homeostasis is threatened or disturbed as a result of the actions of intrinsic or extrinsic stimuli’ commonly defined as stressors’.

The physiological mechanisms of the teleost integrated stress response have been shown to share many similarities with that of terrestrial vertebrates (Wendelaar *et al.,* 1995). The response exhibited by a fish subjected to a stressor will involve physiological and behavioural response that are induced as mechanism to try and protect homeostasis or maintain the dynamic equilibrium of the stressed organism. These responses have been categorized primary, secondary and tertiary.

The primary response involves dramatic increases in the blood levels of catecholamine and glucocorticoids. These hormones are the dominant hormones involved in the stress response and the primary messengers of the two main routes through which the brain co-ordinates the stress response. These two routes include the hypothalamic-autonomic nervous system and the hypothalamic-pituitary-interrenal-axis (Bonga, 1997). The secondary response comprises metabolic changes (i.e. plasma glucose and lactate levels), hydro-mineral disturbance (fluctuations in plasma chloride and sodium levels), haematological changes (e.g. in haematocrit and haemoglobin content), and changes in the hosts immune system (i.e. immunosuppression). Finally, the tertiary response consists of changes in the organism as a whole including reduced growth, impaired swimming performance, relatively lower reproductive success and reduced disease resistance, all of which can impact negatively upon the survival of the organism.

When examining the effects of a stressors, researchers typically use one or a combination of several parameters. Cortisol is the most widely used hormone involved in the stress response and because it is usually obtained from a fish blood. Many researchers examined blood glucose levels in conjugation with cortisol, and to a lesser extent serum sodium and chloride levels may be measured. Other factors that can be indicative of stress include changes in the skin and gill epithelia. For example; skin from stressed fish typically exhibits abnormally high levels of apoptosis and necrosis, which if not fully compressed by cell proliferation, may result in epithelial disruption. In addition, mucus cell discharge is typically stimulated and infiltration of the epithelia by leucocytes can be observed and is indicative of the immune response being activated. Several effects of cortisol on the skin of rainbow trout have also been showed by Iger *et al.* (1995). Gill lamellar of stressed fish can appear irregular and swollen due to increased blood flow and blood pressure.

2.7.3.2. Inflammatory response

The information on the cellular response to the parasites had been limited to the description of the histopathological lesions produced by the parasites, and changes in leucocytes in blood or infected sites. On many occasions the inflammatory response leads to the formation of granulomas, in which the parasite and their products are encapsulated. This is one of the recognised mechanisms of immune evasion. Thus inflammatory reactions involving cellular responses, either accompanied by the formation of granulomas or not, have been reported for numerous piscine parasites belonging to different groups, such as amoebas, dinoflagellates, diplomonads, kinetoplastids, microsporidians, apicomplexans, ciliates, myxozoans, monogeneans, digeneans, cestodes, nematodes, acanthocephalans and crustaceans (Lom *et al.,* 1999; Buchmann, 2001; Alverez-Pellitero *et al.,* 2004; Alverez-Pellitero *et al.,* 2008). Macrophages and neutrophils were the most abundant leucocytes identified in *Salmo salar* with amoebic gill disease (AGD) (Adam and Nowak, 2001). Cell-mediated responses were demonstrated in salmonids infected with *Cryptobia salmositica* by the presence of macrophages that have engulfed parasites by using delayed-type hypersensitivity and the macrophage inhibition migration test. Inflammatory and cellular reactions are produced in systemic hexamitosis/ spironucleosis of several salmonids (Woo, 2003). By contrast, the slight damage produced in Infection of *Paralichthys olivaceus* by the monogenean *Neoheterobothirum hirame* when a rapid increase of monocyte/macrophages and granulocytes in the blood and infected sites. The blood fluke *Sanguinicola inermis* elicits cellular reactions in carp *C. carpio* (Nakayasu *et al.,* 2005).

Melano-macro phage centres (MMCs) are also frequently associated to inflammation and the retention of parasite spores and antigen (Ag) processing during the immune response has been described among the MMC functions. Myxozoan stages are frequently found in association with MMCs (Lom *et al.,* 1999). Holzer and Schachner (2001) found a closely association of *Myxobolus cyprini* with macrophage aggregation in *Leuciscus cephalus*. Sitja`-Bobadilla *et al.* (2006) reported higher abundance of MMC in *Enteromyxum scophthalmi*-infected turbot (*Psetta maxima*), which also showed melanization of spleen MMC associated to the presence of parasite stages or debris engulfed in macrophages; in addition, the

presence of numerous apoptotic cells suggested an induction of apoptosis by the parasite.

Apoptosis was also induced in turbot head kidney (HK) leucocytes by cysteine proteases of the ciliate *Philasterides dicentrarchi*, and an increase in caspase-3-like activity was observed (Parama *et al.,* 2007). The fish host can also induce apoptosis as a mechanism of pathogen killing. Thus, apoptosis of *I. multifiliis* theronts was detected in the presence of *I. punctatus* skin fluid containing cutaneous antibodies (Abs) (Xu *et al.,* 2005).

2.7.3.3. Self curing mechanism

The importance of mucus in ectoparasitic infections was demonstrated by the correlation of high mucous cell densities with resistance to *Gyrodactylus derjavini* in salmonids (Buchmann*,* 1997). It has been obsevered that the monogenean is highly sensitive to factors in host serum and mucus and parasite death is mediated by host complement acting via the alternate pathway of complement (Harris *et al.,* 1998). Previously, Wunder (1929) and Wilde (1935) showed that cyprinids such as carp and tench respond to the attachment of *D. vastator* and *D. macracanthus*, respectively, by extensive proliferation and hyperplasia of gill epithelia. These reactions were clearly reduces the density of parasites on host gills. Later reports by Paperna (1963) and Molnar (1972) did indicate that cyprinids could clear an infection of dactylogyrids to a certain extent, and Bondad-Reantaso *et al.* (1995) recorded a response in flounders infected with *N. girellae*. One often overlooked aspect of immunity is the interference of the host in communication between individual parasites. Parasites belonging to the same species and present on a particular host fish are probably able to communicate but this particular issue in monogenean is poorly elucidated.

2.7.3.4. Oxidative stress response

Oxidative stress is a key phenomenon in the organisms, and various regulatory enzymes are involved in maintaining redox homeostasis. Alternatively, endogenous and exogenous substances can alter biochemical homeostasis, thus continuously raising reactive oxygen species (ROS) levels much higher than the detoxifying (antioxidant enzymes or non-scavengers) capability of tissue. Oxidative response is often a mechanism to balance between pro-oxidants and antioxidants.

Organisms use both enzymatic superoxide dismutase (SOD), catalase (CAT), and peroxidase (PO) and non-enzymatic (e.g. vitamins and glutathione (Gs)) antioxidants to counteract the abiotic and biotic stressors that cause ROS. Bello´ *et al.* (2000) suggested that the fish response to parasite infection could involve reactive oxygen intermediates and, therefore, induced an oxidative stress, but without inducing significant differences in the superoxide dismutase and catalase activities (antioxidant enzymes) between healthy and parasitized fish.

Antioxidants neutralize free radicals by donating one of their own electrons, thereby ending the electron 'stealing' reaction. SOD eliminated the free radical superoxide (O^{-1}) by converting it to peroxide that can in turn be destroyed by CAT or glutathione peroxidase (GPx) reactions. Glutathione is a low-molecular weight antioxidant in cells and is a major non-protein thiol that plays detoxification, regulation, and immune roles in the cell. Glutathione-S-transferase is a phase II conjugation enzyme during biotransformation. It effectively protect cell against an array of hydrophobic and electrophilic compounds, including peroxidised lipids and xenobiotic, through the –SH group (Dzik, 2006).

2.7.4. Immunological responses of host to monogenean

It is evident that early stages of *P. hypophthalmus* gets susceptible to monogenean in compared to late and adult stage in culture systems. However the mechanism is not known which make a fish susceptible or resistant to the pathogen. Immunological reactions (both innate and acquired) of the fish host are important host factors in monogenean-fish host systems (Buchmann and Lindenstrom, 2002). Indeed, some host molecules can activate benign physiological processes in the parasite whereas others may act adversely on monogenean integrity. It can even be speculated that micro-environmental factors (such as calcium and magnesium) can modulate the activity of host molecules and thereby change their effect on the parasite. The best known of these molecules are lectins (Buchmann, 2001), complement factors (Harris *et al.,* 1998), antibodies (Wang *et al.,* 1997; Mazzanti *et al.,* 1999), acute phase proteins (Secombes *et al.,* 1999; Bayne *et al.,* 2001), lysozyme (Buchmann and Uldal, 1997) and anti-microbial peptides (Cole *et al.,* 1997). Expression of immune genes, particularly proinflammatory cytokines have been shown to increase both locally and systematically during parasitic infection (Sigh and Buchmann, 2004).

2.7.4.1. Myeloperoxidase

Myeloperoxidase is a hemoprotein, an important enzyme secreted by neutrophils and heterophils leukocytes showing a potent antimicrobial activity and stands at the centre of the neutrophil's armamentarium (Beutler, 2004). It utilizes hydrogen peroxide during respiratory burst to produce hypochlorous acid (Dalmo *et al.,* 1997), which are potent oxidants known to have several cytotoxic effects on mammalian and bacterial cells (Munoz *et al.,* 2007). In the various study, an induction in serum peroxidases level was reported in *Enteromyxum leei*-exposed sharpsnout seabream (Munoz *et al.,* 2007) and in gilthead seabream during cohabitation, but a decrease in activity found in subsequent samplings and no differences were detected between parasitized and non-parasitized fish (Cuesta *et al.,* 2006). Similarly, during experimental exposure of *Argulus* in *L. rohita*, no significant difference in MPO activity was observed among different degrees of parasite-infected and uninfected control fish (Saurabh *et al.,* 2010). However, Sitja-Bobadilla *et al.* (2008a) reported depletion of serum peroxidase levels in *Enteromyxum leei*-infected and non-parasitized gilthead sea bream fish.

2.7.4.2. Lysozyme

Lysozyme is a mucolytic enzyme produced by leucocytes, especially monocytes, macrophages and neutrophils and an imperative defence molecule of the innate immune system. It is playing a role in mediating protection against microbial invasion. Fish lysozyme possesses lytic activity against bacteria and can activate complement and phagocytes. It is present in mucus, lymphoid tissue, plasma and other fluids and is also expressed in a wide variety of tissues (Saurabh and Sahoo, 2008). Serum lysozyme levels can change in fish in response to parasite infections. An increase level of lysozyme was detected in rainbow trout following immunisation with live theronts of *I. multifiliis* (Alishahi and Bhuchmann, 2006), in *D. labrax* immunized with *S. dicentrarchi* spores (Munoz *et al.,* 2000), in fish infected by *Ceratomyxa shasta* (Foott *et al.,* 2004*)*, similarly in *C. auratus* infested with *Argulus* sp. (Kumar *et al.,* 2013). In contrast, the low serum lysozyme levels found in *D. puntazzo*, could be involved in the higher susceptibility to *E. leei*. Lysozyme was significantly depleted in *S. aurata* after chronic exposure to *E. leei* (Sitja-Bobadilla *et al.,* 2008a).

2.7.4.3. Ceruloplasmin

The multiple function of acute-phase proteins involve in defence related activities such as limiting the dispersal of infectious agents, repairs of tissue damage, killing of microbes and other potential pathogens, and restoration of the healthy state (Dautremepuits *et al.,* 2004). Ceruloplasmin is one of the vital positive acute-phase proteins, playing important anti-inflammatory roles, as a copper transporter from hepatocytes to other tissues, a regulator for hepatic iron mobilization as well as a scavenger of free radicals and superoxides, triggered and released in response to infectious agents and inflammation (Cousins, 1985; Arnaud *et al.,* 1988). Studnicka and Siwicki (1990) found an elevated level of ceruloplasmin during *Eimeria subepithelialis* infection in *C. carpio*. In certain cases of ectoparasite infection, the decrease level of ceruloplasmin was also observed in *Argulus* infected *L. rohita* (Saurabh *et al.,* 2010).

2.7.4.4. Proteases and Antiproteases

The mucus of fish contains proteases with trypsin-like activity. The trypsin is detected in epidermis of skin, epithelial cell layers of gills and intestine (Braun *et al.,* 1990; Kristjansson, 1991). The protease from trout appears to have destroying power against Gram-negative pathogens, which suggest its role in defense mechanism. Fish plasma contains a number of protease inhibitors, principally α1- antiproteinase and α2-macroglobalin (α2-M). The basic function of proteinase inhibitors is to maintain the homeostasis of the blood and other body fluids, and to regulate the action of mechanism such as the complement and coagulation cascades (Dalmo *et al.,* 1997). Many microorganisms and parasites are carryout extracellular digestion of the host tissue either to facilitate penetration or to obtain nutrients. The proteinase inhibitors can be utilized to counteract some of these activities (Ellis, 1987; Alexander and Ingram, 1992). Furthermore, α-2 mactoglobulin is able to entrap and form covalent linkages with proteins such as transforming growth factor (TGF-β1, IL-Iβ and platelet-derived growth factor (Crookston *et al.,* 1993). The proteinase inhibitors also interact with the specific part of the immune system by modulating the antigen presentation (Chu *et al.,* 1994). The complexes are cleared by receptor mediated mechanism in the reticuloendothelial system through α-2 macroglobulin receptor on mammalian

macrophages and hepatocytes that also recognize other ligands than activated 2-macroglobulin (Kaplan and Keogh, 1983).

2.7.4.5. Chitinases

Chitinase occurs as an extracellular enzyme in fungi and bacteria and also present in gut of certain invertebrates (Alexander and Ingram, 1992). Chitinase can hydrolysis of the 1, 4 glycosanide linkage. It is found in lymphoid tissue of fish except thymes (spleen, plasma and lymph) (Kumari and Sahoo, 2006). It is mostly found in serum, gut mucosa and pancreas of insectivorous fish, and plays protective role against chitin–containing fungi and parasites (Ingram, 1980; Mason and Davis, 1997).

2.7.4.6. α-precipitin

α-precipitin (a group of 13 proteins) isolated from Atlantic salmon serum reacts with carbohydrates and glycoproteins of fungi. It may play role in defence of fish, which shows marked rise during ulcerative dermal necrosis (UDN) (Alexander and Ingram, 1992).

2.7.4.7. Eicosanoids

Eicosanoids are an important group of compounds derived from 20-carbon polyunsaturated fatty acids, include prostaglandins, thromboxanes and leukotriene's. The role of eicosanoids in homeostasis, immune regulation and inflammation is well described in mammals (Smith, 1989). In fish, eicosanoids are leukotriene, lipoxines and prostaglandins are generally produced in organs rich in blood cells and release after ionosphere stimulation (Anderson *et al.,* 1979; Knight *et al.,* 1995).

2.7.4.8. Antimicrobial peptides

Antimicrobial peptides (AMPs) are widespread in nature as defence mechanisms in plant and animals and have been identified from teleost in recent years and some can be activated against bacteria (Cuesta *et al.,* 2008) though rare in parasite. Besides the four previously known different classes of antimicrobial peptides, a fifth class, NK-lysine has been discovered in mammalian NK and T cells. Three distinct transcripts of NK-lysine exist in channel catfish and they seem to be similarly arranged in zebra fish (Wang *et al.,* 2006). Piscidines, α-helical

haemolytic AMPs were detected in eosinophil granular cells (EGCs) and rodlet cells of several teleosts (Noga *et al.,* 2009). Information on the involvement of antimicrobial peptides in response to fish parasitosis is scarce. Some histone-like proteins isolated from fish tissues may be important for defence as they are able to kill the dinoflagellate *Amyloodinium ocellatum* (Noga *et al.,* 2009). In addition, piscidice 2 isolated from mast cells of hybrid striped bass was lethal to the infective stages of *I. multifiliis*, whereas marine parasites (*Amyloodinium ocellatum*, *Cryptocaryon irritans*) were affected to a lesser extent (Colorni *et al.,* 2008).

2.7.4.9. Natural antibodies

Although antibodies (immune globulins) are an acquired immune parameter, natural antibodies can also be classified as component of the innate immune system. Natural antibodies are produced in the absence of gene-rearrangement and without any apparent specific antigen stimulation (Magnadottir, 2006). They provide immediate, early and broad protection against pathogens, making them a crucial non-reluctant component of the innate humoral immune system. Natural antibodies are found in the serum of most healthy vertebrates but are commonly dismissed as background reaction in serological tests. In recent years natural antibodies have received further attention, which stems basically from work carried out on knock–out mice lacking IgM (Casali and Schettino, 1996). In mammals (man and mouse) natural antibodies are produced by long living B1 (CD5+) lymphocytes that are active during embryonic and early developmental stages. Natural antibodies comprise an isotype of IgM, although some IgM and IgA natural antibodies have also been described (Avrameas and Ternyck, 1995). Although the existence of natural antibodies has been known for a long time, their exact role is still unclear and actively debated. The broad reactively of individual natural antibodies, and the broad reactively of the natural antibody population a whole, is important because it provide pre exiting antibody reactively that allows animals to rapidly recognise and protect against pathogens that have not been encountered previously (Baumgarth *et al.,* 2005). Antibody reactively tends to be low affinity suggesting that natural antibodies provides key protection during the period between the onset of infection and the emergence of the adaptive immune response (Baumgarth *et al.,* 2005). Another suggestion is that they are involved in a hypothetical idiotypic-anti-idiotypic network and therefore have some immune-

regulatory function by intensifying or suppressing the specific immune response related to particular idiotype (Countiho, 1989; Ochsenbein and Zinkernagel, 2000).

Natural antibodies are a well-known fact in fish and play a key role in the innate immune response and the link to the adaptive immune response (Vilaion *et al.,* 1984). Natural antibodies of rainbow trout and goldfish take part in both viral and bacterial defence (Castro *et al.,* 2014) and high non-specific antibody activity in the serum of goldfish inhibited the specific antibody response (Sinyakov *et al.,* 2002). Variation in the natural antibody specificity repertoire between different fish species has also been reported (Gonzalez *et al.,* 1988).

2.8. Preventive and control measures

Table 5: List of biological measures, chemicals, drugs and vaccines used for controlling monogeneans

Control measures	Mode of action	Doses
Biological		
Cleaner fish (tilapia, *Labroides dimidiatus*) Turbellarian (*Stenostomum* spp.) Copepods (Cyclopoid)	Feeding on larvae (oncomiracidium) and eggs of monogenean	-
Chemicals		
Aluminium chloride	Toxic to gyrodactylids	Not defined
Zinc chloride	Toxic to gyrodactylids	Not defined
Acetic acid	Dehydration of parasite	5%, 1 min. dip
Ammonium hydroxide/Ammonia		500 ppm
Benzimidazoles (Levamisole/Praziquantel)	Bind to tubulin monomers in the parasite to destroy microtubules in cytoskeletons and cell transport functions.	10 mg praziquantel L^{-1} for 3 h at 22° C
Praziquantel	Interferes with integument integrity	
Formaldehyde (Formalin)	Coagulation of protein and cellular destruction	30 to 100 ppm
Hydrogen peroxide, Sodium percarbonate	Oxidize the organic constituents and destroy the worm surface	
Mebendazole		0.01 ppm
Methylene blue	Dye binds to cellular anions	3 ppm
Potassium permanganate	Oxidizing the cellular component	2.5 ppm
Sodium chloride	Osmotic imbalance of parasites	0.7 to 2.5%
Organophosphate	Inhibit the acetylcholinesterase in the worm nervous system	0.25-0.50 ppm
Trichlorfon	Toxic for parasites directly inhibit neuron system	0.4 to 1.6 ppm
Metrifonate	Neurotoxicants (paralyse the parasoite by inhibiting cholinesterases and neuromuscular transmission)	0.25-0.50 ppm
Dichlorvos	Neuro-inhibitory	0.4 to 1.6 ppm
Vaccine (against monogenean)	**Host fish**	**Authors**
D. vastator	*C. carpio*	Vladimirov, 1971
M. sebastis	Rockfish (*Sebastes schlegeli*)	Kim *et al.,* 2000
D.sagittata	*O. mykiss*	Rubio-Godoy *et al.,* 2003
Neobenedenia girellae	Japanese flounder	Bondad-Reantaso *et al.,* 1995

Source: Buchmann and Bresciani, 2006

2.8.1. Zoosanitary measures

Due to lack of technology to control infections at an acceptable level, it is often necessary to use more strong management measures. Further, the possible application of vaccine or immunostimulants is still not developed for use in commercial fish farming. Thus, it becomes necessary to implement eradication measures to obtain satisfactory control of monogenean infections. This has been applied both in natural systems and in aquacultural enterprises. The pathogenic *G. salaris* has been cleared from more than 16 Norwegian rivers by using rotenone on the entire water system (Mo, 1994). In this way, both fish and parasites were killed and uninfected fish were subsequently stocked in the rivers. Further, aquaculture ponds need to be cleared for infection by emptying, drying and subsequent disinfection.

2.8.2. Integrated measures

This includes primarily a strict monitoring of infection levels concomitant with implementation of control measures aiming at reducing the infection pressure. These actions include:

- Fallowing of infected systems
- Regular movements of facilities to new uninfected sites
- Placement of new production entities in areas with low spreading potential and away from existing facilities
- Mechanical removal of eggs and larvae by filtration or by using replaceable ropes or nets
- Reducing entrapment of eggs and larvae in a stationary production system (e.g. net cages)
- Use of cleaner fish, if available; and
- Strategic treatments using chemicals or anthelmintic.

3. MATERIALS AND METHODS

3.1. Site of the experiment

The experiment was conducted at the Aquatic Environment and Health Management Laboratory of the ICAR-Central Institute of Fisheries Education (ICAR-CIFE), Mumbai.

3.2. Experimental animals

Animals used for experimental purpose were pangasius fish (*Pangasianodon hypophthalmus*). The infested fish with an average weight of 18.0 ± 5.28 g procured from cage culture of Murbad, Thane, Maharashtra. Whereas, healthy fish (an average weight 20.2 ± 2.56 g) were procured from Kurla, Aquarium shop, Maharashtra, India. The fishes were transported in a polythene packing with sufficient oxygen. On reaching the wet laboratory, they were carefully transferred to a circular Fibre Reinforced Plastic (FRP) tank (500 L) and were maintained with adopting best wet laboratory management practices.

3.2.1. Maintenance of animals

The experimental animals were maintained in rectangular FRP tanks (1.52 × 0.65 × 0.62 m, 500 L capacity) covered with perforated lids and the water used for rearing was drawn from bore well. The tanks were initially washed and filled with 4 ppm potassium permanganate solution that was left overnight. They were flushed out the following day and tanks were thoroughly washed with clean water. Chlorine-free borewell water was used as the source of water. The total volume of the water in each tub was maintained at 300 L throughout the experimental period. Aeration was provided and aeration pipe in each tank was fitted with an air-stone and a regulator valve in order to control the air pressure uniformly in all the tubs. The fish were fed at 3% of body weight with a commercial pelleted diet consisting of 35% crude protein.

3.2.2. Cleaning and siphoning

The experimental tanks were cleaned manually and siphoning was done every day in order to remove excess and remaining feed and the faecal matter. An equal volume of clean borewell water at the same temperature was

maintained and used to replace the siphoned water i.e. 25% of the total volume. This was carried out during acclimatization of healthy fish but very little water was exchanged during challenge experimental study.

3.3. Glassware and chemicals

The glassware used throughout the experiment was neutral glass of Borosil (Mumbai). The chemicals used for various analyses are mainly of Sigma (USA), Hi-Media (Mumbai), Qualigen (Mumbai), and Merck (Germany) etc.

Objective 1: To isolate and characterize the monogenean parasite(s) infecting *P. hypophthalmus*

3.4. Sampling of infected *P. hypophthalmus* for isolation of monogenean parasites

A total of 60 numbers of *P. hypophthalmus* (mean weight 18.0 ± 5.28 g and standard length ranges from 7.0 to 20.6 cm) were obtained from cages cultured at Murbad, Thane, Maharashtra. The external body surface (skin and fins) of fish were screened for ectoparasites including monogeneans and then the gills were individually removed and placed in sterilize water in a pertidish for further examination. Randomly selected sub-sample of *P. hypophthalmus* (n=18) was also dissected and vital organs *viz*. viscera, gall bladder, intestine, liver and kidney were visually examined for parasites. Prior to actual sampling for examining the parasite, the morphometric (fish total length, standard length (in cm) and body weight (in g) traits and gross appearance of fish, coloration and clinical signs, if any, were noted for host preference study. Observed monogeneans in gill biopsy were recorded for their prevalence, abundance, and mean intensity as defined by Margolis *et al.* (1982) as follows:

[i] The prevalence of monogenean was estimated as the percentage of infected fishes out of total number of fish examined

$$\boldsymbol{Prevalence}\ (\%) = \frac{\boldsymbol{Total\ no.of\ infected\ fish}}{\boldsymbol{Total\ no.of\ fish\ examined}} \times \mathbf{100}$$

[ii] The abundance was estimated as the ratio between the total number of parasites in a sample and the total number of fish examined

$$\boldsymbol{Abundance} = \frac{\boldsymbol{Total\ no.of\ parasites\ recovered}}{\boldsymbol{Total\ no.of\ fish\ examined}}$$

[iii] The mean intensity was determined as the ratio between the total number of parasites in a sample and the number of infected fish in a sample (Parasites per fish)

$$\boldsymbol{Mean\ intensity} = \frac{\boldsymbol{Total\ no.of\ parasites\ recovered}}{\boldsymbol{Total\ no.of\ infected\ fish}}$$

3.4.1. Collection and identification of parasites using morphometric traits

Parasites were collected carefully from the gills of infected *P. hypophthalmus* obtained from Murbad, Thane, Maharashtra, India and fixed in a mixture of the alcohol-formalin-acetic acid (AFA) for further use. An individual parasite was drawn out from fixative, placed on a glass slide with a drop of water and stained with a mixture of two stain *i.e.,* ammonium picrate glycerine and Mayer's carmalum (as per suggested by Malmberg, 1990) and live specimen exposed to 10% SDS in a ratio of 1:1. SDS makes the parasite transparent which results in a better study of the hard parts of the haptor and copulatory organs of the parasite. The permanently stained and fixed parasites were observed for the morphometric characteristics of their hard parts and copulatory organs. The mounted parasites were photographed with a digital camera (TUCSEN, ISH500, China) attached to phase-contrast microscope (ZEISS, Axioskop40, Germany). Based on these photographs, measurements were obtained with the software ISCapture 4.0.1 (TUCSEN Photonics, China). Parasites were primarily identified to genus level morphologically, according to the existing keys and descriptions made by Kritsky *et al.* (1986) further modified by Lim *et al.* (2001).

3.4.2. Molecular identification of monogenean parasite in *P. hypophthalmus*

3.4.2.1. Isolation of genomic DNA of parasite

Parasites along with gill from infected *P. hypophthalmus*, were fixed in 90% ethanol for genomic DNA isolation. For genomic DNA extraction, monogenean specimen along with minute part of gill was taken in a 2 mL tube from the previous fixed parasites sample. DNA was extracted from the parasite using DNAzol Kit (Invitrogen/Thermo Fisher Scientific, India) according to the manufacturer's

instructions. A region comprising partial 28SrDNA was amplified using the primers, forward (5' TCCCAGTAACGGCGAGTGAACG 3') and the reverse primer (5' CGGACTCGCGCCAGTATTTAGC 3') designed using software Primer3 (Rozen and Skaletsky, 2000).

3.4.2.2. PCR amplification and confirmation

Each amplification reaction was performed in a final volume of 25 µl containing 2 µl of genomic DNA extract, 10X PCR buffer, 0.4 mM dNTP, 10 pM of each primer, 3 U Taq polymerase (Genei, India), 25 mM $MgCl_2$ and milli-Q water in a thermocycler (Takara, India; 96 well Thermal Cycler) under the following conditions: initial denaturation for 4 minutes at 95 °C followed by 35 cycles of 45 seconds at 95 °C, 45 seconds at 53 °C (annealing) and 1 minute at 72 °C (extension) and a final extension at 72 °C for 10 minutes. Amplified PCR products were examined on 1.2% agarose-TAE gels, stained with ethidium bromide and visualized under uv-transilluminator (Gel doc system)

3.4.2.3. Sequencing of amplified product/alignment and phylogenetic tree

The PCR products were purified from the gels using a minielute gel extraction kit (Qiagen, India) according to manufacturer's instructions. The purified gel extracted PCR product was then sequenced using a method based on Sanger *et al.* (1977) (Bioinnovations, Mumbai). Both forward and reverse primers were used for sequencing. Sequence similarity searching for *Thaparocleidus* was performed using the Basic Local Alignment Search Tool (BLAST)-NCBI (National Center for Biotechnology Information) online application (*www.ncbi.nlm.nih.gov*). The 28S rDNA gene sequence generated was submitted to the GenBank. The phylogenetic reconstruction was inferred by Neighbour-Joining (NJ) using MEGA vr. 6 (Tamura *et al.,* 2013).

3.4.3. Preparation of wet mount of gills of *P. hypophthalmus*

Wet mount of gills of *P. hypophthalmus* collected from cages, Murbad and wet laboratory, ICAR-CIFE, Mumbai were prepared following standard protocol and observed for all forms of gill parasites including the monogenean parasites. Although, co-occurrence of monogenean and trematode larvae (metacercariae) was found in few gill samples of fish obtained from cages, separate experiment was not designed to study the interaction of these parasites. However, those gill samples

were carefully segregated and histopathological study was performed without considering the clinical pathology.

3.4.3.1. Microhabitat preference of monogenean in *P. hypophthalmus*

Gill biopsy was prepared and carefully observed for gill monogenean. The experiment for microhabitat preference of monogenean in *P. hypophthalmus* was performed according to Hockley *et al.* (2011). Monogenean parasites were examined and recovered from the gills of 42 infected fish under a stereo-microscope (Leica, Microsystem). The standard length and weight of each fish was measured and marked and relationship was estimated with intensity of parasites. Further, infected fish were grouped into three groups namely. G1, G2 and G3 based on their standard length (<10, 10-15 and >15 cm, respectively), with each group comprising of 10 individuals, excluding the outliers, and the mean intensity of parasite was compared between the groups. To assess microhabitat specificity, the total number of monogenean on each attachment site (left and right gill operculum) of the gills of the fish was recorded. Further attachment sites were classified according to the gill arch number (1 to 4) i.e. GA1 (first gill arch), GA2 (second gill arch), GA3 (third gill arch) and GA4 (fourth gill arch) of both side (Fig. 1) and then gill arch was divided along dorsal-ventral (dorsal, central, and ventral) and anterior-posteriors (anterior, medial and posterior) axis, giving 6 possible attachment sites on each side of the gill arch (figure 2). Further, mean intensity of monogenean parasite aggregated at each region were compared and tested for their statistical difference by one-way analysis of variance (ANOVA) at significance level ($p<0.05$).

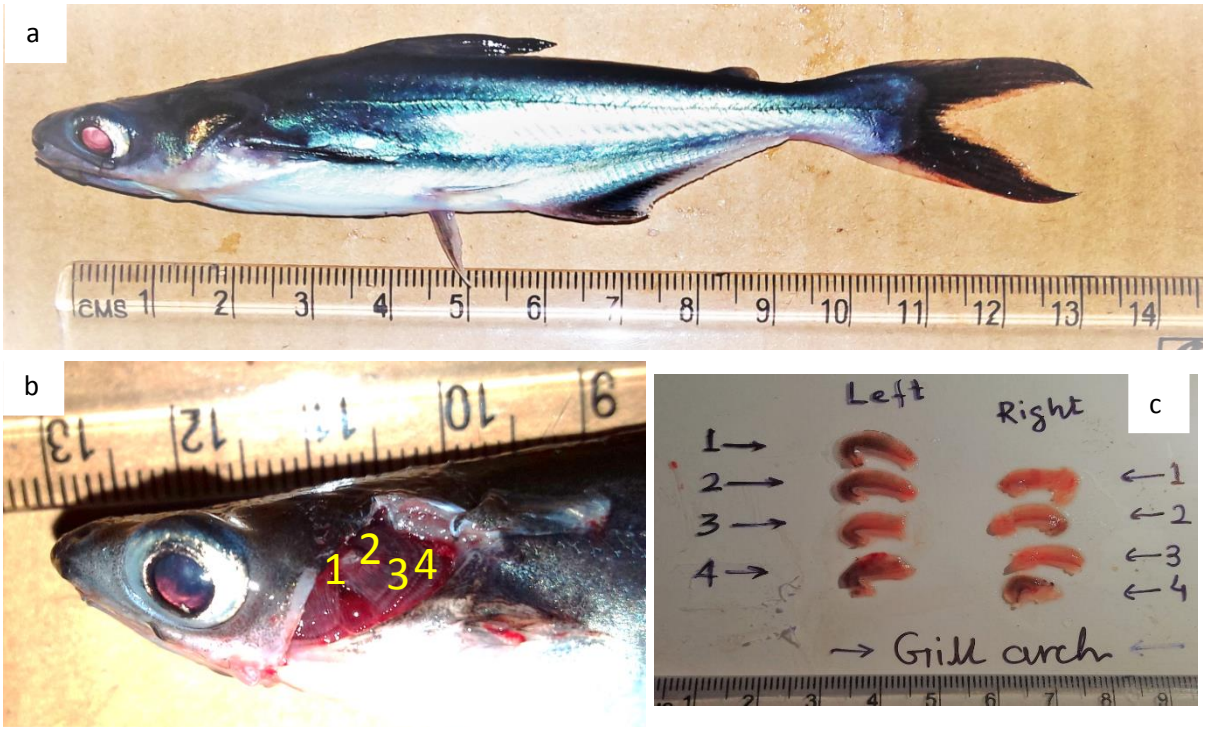


Plate 1: *Pangasianodon hypophthalmus* infected with dactylogyrids. (a) Sampled fish specimen (b) Gill arch numbers assigned to the *P. hypophthalmus*. (c) Gill arch (1-4) from left and right gill operculum of *P. hypophthalmus*.

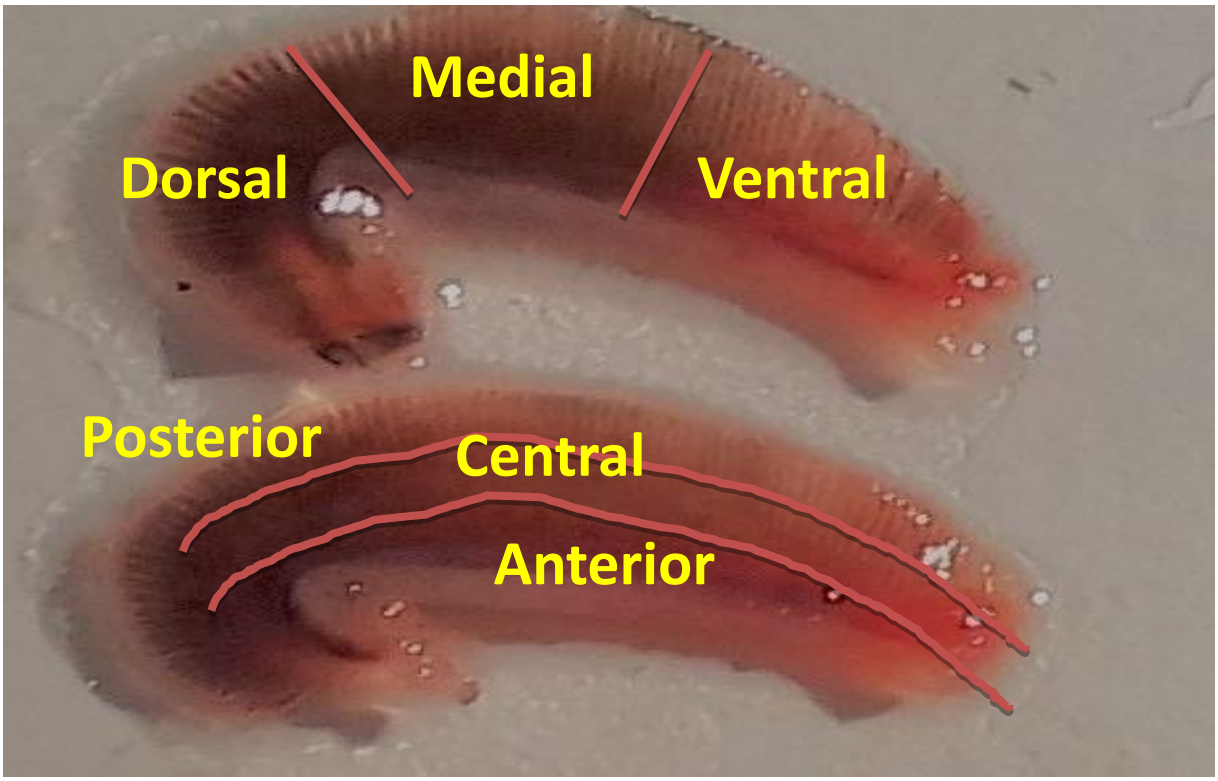


Plate 2: Marking of gill regions of *P. hypophthalmus* (Hockley *et al.,* 2011)

Objective 2: Pathology associated with monogenean infecting *P. hypophthalmus*

3.5. Pathology associated with monogenean infecting *P. hypothalamus*

3.5.1. Histopathological studies

Gills from the parasitized *P. hypophthalmus* were collected after observing and recording the parasite information related to intensity and aggregation along with gill conditions and changes. The collected gills were immediately fixed in 10% neutral buffer formalin (NBF) for histological slide preparation. The preserved gill organ was processed with mild hydration and then dehydrated with a series of ascending concentration of ethanol and cleared in xylene. Further these were embedded into paraffin following the impregnation technique (Leica EG 1140H, Germany). The paraffin-embedded organ was sectioned (microtome) at 5 μm thickness using a microtome (Leica RM 2125RT, Germany) and stained with haematoxylin and eosin (Luna, 1968). Pathological changes manifested in the tissue sections were noted down and microphotographs were taken using a digital camera (TUCSEN ISH500, China) attached to phase-contrast microscope (ZEISS, Axioskop40, Germany). Based on these photographs, scale was depicted with the software ISCapture 4.0.1 (TUCSEN Photonics, China).

3.5.2. Collection of blood and serum for clinical pathology

Prior to biopsy of gills, each fish was marked during parasite screening. The blood was drawn from *Vena caudal* by using 1 mL 26G ½ non-anticoagulant insulin/tuberculin syringes (BD, Singapore), which was previously rinsed with 2.7% ethylenediaminetetraacetic acid (EDTA) solution. The collected blood was immediately transferred to a test tube coated with thin layer of EDTA (as an anticoagulant) and mixed well in order to prevent clotting of blood. Serum was collected without using anticoagulant and serum was separated from blood by keeping the tubes in slanting position for about 2 h. The clotted blood was centrifuged at 1500 *g* for 10 min. at 4 ºC, the serum was collected and stored at -20^0 C for further use. After estimation of parasite load to each fish (median value 34.5, n=60), the sampled fish were categorised into three groups (n=6) based on monogenean parasite load i.e., G0: healthy (uninfested fish), G1: low level (<35 dactylogyrid per gill per fish) and G2: high level (>35 dactylogyrid per gill per fish)

and parameters of clinical pathology were estimated (mean values) (table 6), compared and tested for statistical difference at $p<0.05$ level of significance.

Table 6: The parameters are estimated for evaluating clinical pathology.

Sl no.	Parameters of clinical pathology	Specific estimated parameters	References
1	Haematology	Total erythrocyte count (TEC)	Schaperclaus *et al.,* 1991
2		Total leucocyte count (TLC)	
3		Haemoglobin (Hb) level (g dL^{-1})	
4		Packed cell volume (PCV	
5		Mean cell volume (MCV) (fl)	Haney *et al.,* 1992
6		Mean cell haemoglobin (MCH) (pg)	
7		Mean cell haemoglobin concentration (MCHC) (%)	
8		Differential leucocyte count (DLC)	Schaperclaus *et al.,* 1991
9	Serum protein profiles	Total protein (g dL^{-1})	Reinhold, 1953
10		Albumin (g dL^{-1})	Doumas *et al.,* 1971
11		Globulin (g dL^{-1})	Calculated
12		Albumin to globulin ratio	Calculated
13	Oxidative stress biomarkers	Superoxide dismutase activity (SOD) (U mg $protein^{-1}$)	Misra and Fridovinch, 1972
14		Catalase activity (CAT) (mM H_2O_2 decomposed min^{-1} mg $protein^{-1}$)	Takahara *et al.,* 1960
15	Enzymes related to metabolism	Serum glutamate oxaloacetate transaminase (sGOT) (U L^{-1})	Reitman and Frankel, 1957
16		Serum glutamate pyruvate transaminase (sGPT) (U L^{-1})	
17		Alkaline phosphatase (ALP) activity (U L^{-1})	Ross *et al.,* 2000
18		Lactate dehydrogenease (LDH) activity (U L^{-1})	Wroblewski and La due, 1955
19	Serum marker associated with by-product	Blood glucose (mg dL^{-1})	Nelson and Somogyi, 1945; Oser, 1965
20		Lactate level (nM μL^{-1})	Santulli *et al.,* 1999
21		Bilirubin level (mg dL^{-1})	Pearlman and Lee, 1974

Objective 3: Host immune responses against monogenean parasite

3.6. Host immune responses against monogenean parasite

3.6.1. Experimental Design

Healthy *P. hypophthalmus* (n=120) with mean weights of 20.2 ± 2.56 g and a mean standard length of 9.4±1.02 cm were procured from Kurla Aquarium shop, Mumbai, India. The stock was acclimatized in 500 L water under aerated conditions in 1.52×0.65×0.62 m Fibre Reinforced Plastic (FRP) tank for a period of 14 days, following the standard protocol of best management practices and fed at 3% of body weight with a commercial pelleted diet consisting of 35% crude protein. In order to ameliorate the handling stress, the fish were given a mild salt treatment and ascorbic acid (vitamin C). About one tenth of water was replaced daily in order to remove uneaten feed and faecal matter. The physico-chemical parameters of water were measured weekly with a view to ascertain its optimal condition (temperature 26.8±1.2 °C; dissolved oxygen: 5.80±0.85 mg L^{-1}; pH: 7.5±0.64; ammonia: 0.095±0.055 mg L^{-1}; nitrite: 0.020±0.005 mg L^{-1}) throughout the experiment.

Infected *P. hypophthalmus* (n=40) were obtained from cage culture of Murbagh, Mumbai, India, with a mean weight of 18.0 ± 5.28 g and a mean standard length of 9.2 ± 1.13 cm. Fish were transferred to the Central Wet Laboratory, ICAR-CIFE, Mumbai for acclimatization, and randomly selected 5 fish were biopsied and their gills, skin and fins were examined to ascertain the presence of the monogenean parasite. These fish had a mean intensity (MI) of 30.5 ± 4.0 dactylogyrids, a gill monogenean parasite. Infected fish (n=30) were used as a source of parasitic infection in the cohabitation trial. These fish were naturally infected under prior culture conditions. The presence or absence of adult dactylogyrid (gill monogenea) on the infected fish and uninfected fish during the trials was confirmed by observation of the parasite on gills of fish, using a stereomicroscope (Leica Microsystems, Germany).

3.6.2. Infection by cohabitation

The artificial experimental challenge was performed by cohabitation method with a slight modification (Kumar *et al.,* 2013). Healthy fish (n=120) were given a bath treatment with 2 ‰ saltwater for 15 min, followed by a bath treatment with mebendazole (4.5 mg L^{-1} Sigma, India) for 24 h against helminthic parasites

(Fajer-Avila *et al.,* 2007). Each bath treatment was repeated for 3 consecutive days. The treated healthy fish were used for the experiment after 7 days of acclimatization. Infected fish were marked by making a cut on the tip of the caudal fin to differentiate them and these fish were not sampled. In order to demonstrate the immunomodulation in the host (*P. hypophthalmus*) caused by differential degree of dactylogyrid parasite infestation, experimental infection trials were conducted in 500 L FRP tanks containing 300 L of fully aerated ground water. Healthy fish were challenged by cohabitation mode for artificial infestation for a period of 14 days. The whole experiment was conducted with complete randomized design with triplicates of each treatment. In the control group (T0), 30 healthy fish were kept without introducing any monogenean infested fish. In the artificial infection challenge group, 90 fish were kept in nine tanks with each tank containing 10 healthy fish, cohabited with 2, 4 and 6 infected fish. After 14 days, three grades of infections were defined as (T1) low (<10 mean dactylogyrid per gill arch per fish), (T2) moderate (10-49 mean dactylogyrid per gill arch per fish) and (T3) high (>50 mean dactylogyrid per gill arch per fish) (Del Rio-Zaragoza *et al.,* 2010). The blood, serum, liver tissue, and gill samples were collected from 9 fish with each of the three infection grades in the experimental tanks and from 9 uninfected fish in the control tanks and haemato-immunological parameters were estimated (table 7).

During 14-days experimental period, fish were reared in 500 L tanks supplied with saturated and aerated ground water. Fish were exposed to a natural (12h light : 12h dark) photoperiod, a temperature of 26.6 ± 1.5 °C, pH of 7.6±0.4 and dissolved oxygen of 5.3±0.8 mg L^{-1}, and fed to apparent satiation with a commercial pellets diet (35% protein) twice a day.

3.6.3. Collection of blood, serum and tissue sample

Blood and serum from fish was collected as described previously. Subsequently, fish were dissected under aseptic conditions and the tissues such as gills and liver were collected. Further, the tissues were homogenized (5% w/v) separately in chilled sucrose solution (0.25 M) in a glass tube using Teflon coated mechanical tissue homogenizer (Remi, India). The homogenates were centrifuged at 3500 × *g* for 20 min at 4 °C. The supernatants were collected and the protein contents were quantified following the method of Lowry *et al.* (1951) and stored at -20 °C until further analysis.

Table 7: The following parameters are estimated for evaluating immunological responses of host to different level of dactylogyrid parasite infestation.

Sl no.	Parameters of host responses	Specific estimated parameters	Followed method
1	Haematology	Total erythrocyte count (TEC)	Schaperclaus *et al.,* 1991
2		Total leucocyte count (TLC)	
3		Haemoglobin (Hb) level (g dL^{-1})	
4		Packed cell volume (PCV	
5		Mean cell volume (MCV) (fl)	Haney *et al.,* 1992)
6		Mean cell haemoglobin (MCH) (pg)	
7		Mean cell haemoglobin concentration (MCHC) (%)	
8		Erythrocyte osmotic brittleness	Bain *et al.,* 2011
9	Serum protein profiles	Total protein (g dL^{-1})	Reinhold, 1953
10		Albumin (g dL^{-1})	Doumas *et al.,* 1971
11		Globulin (g dL^{-1})	Calculated
12		Albumin to globulin ratio	Calculated
13	Oxidative stress biomarkers	Superoxide dismutase activity (SOD) (U mg $protein^{-1}$)	Misra and Fridovinch, 1972
14		Catalase activity (CAT) (mM H_2O_2 decomposed min^{-1} mg $protein^{-1}$)	Takahara *et al.,* 1960
15		Glutathione-S-transferase (GST) (U mg $protein^{-1}$)	Habig et al., 1974
16		Na^+-K^+-ATPase activity (µM Pi liberated mg^{-1} protein h^{-1})	Svobaca and Mossinger, 1981
17	Enzymes related to metabolism	Serum glutamate oxaloacetate transaminase (sGOT) (U L^{-1})	Reitman and Frankel, 1957
18		Serum glutamate pyruvate transaminase (sGPT) (U L^{-1})	
19		Alkaline phosphatase (ALP) activity (U L^{-1})	Ross *et al.,* 2000
20		Lactate dehydrogenease (LDH) activity (U L^{-1})	Wroblewski and La due, 1955
21		Blood glucose (mg dL^{-1})	Nelson and Somogi, 1945; Oser, 1965
22		Lactate level (nM μL^{-1})	Santulli *et al.,* 1999
23		Bilirubin level (mg dL^{-1})	Pearlman and Lee, 1974
24		Creatinine (mg L^{-1})	Bower, 1980
25	Innate immune parameters	Respiratory burst activity (RBA)	Anderson and Siwicki, 1995
26		Phagocytic activity	
27		Myeloperoxide content	Quade and Roth, 1997
28		Serum lysozyme activity	Parry *et al.,* 1965
29		α-2 macroglobulin (α-2M) activity	Zuo and Woo, 1997
30		Serum antiproteases activity	
31		Ceruloplasmin level	Pelgron *et al.,* 1995
32		Total immunoglobulin level (g dL^{-1})	Anderson and Siwicki, 1995

3.7. Details of procedure for parameter estimation

3.7.1 Haematological parameters

3.7.1.1 Total erythrocyte count (TEC)

TEC was determined as per the procedure of Schaperclaus *et al.* (1991). Twenty microlitres of the blood sample was taken and mixed with 3980 µl of RBC diluting fluid (Hi-Media, India) in a clean vial. The mixture was shaken well to suspend the cells uniformly in the solution. A small drop of this mixture was charged to Neubauer's counting chamber of haemocytometer (Rohem, India). Care was taken that there were no trapped air bubbles. The numbers of erythrocyte were counted in five groups of squares. All the cells lying inside the five small squares were counted under high power (40X) magnification of a light microscope. The number of erythrocyte per cubic millimetre of the blood sample was calculated using the following formula:

$$\boldsymbol{TEC\ (cells\ mm^{-3}) = \frac{N\ \times dilution\ factor}{Area\ counted\ \times depth}}$$

$$\boldsymbol{TEC\ (cells\ mm^{-3}) = \frac{N \times 200}{0.2 \times 0.1} \quad = N\ x\ 10000}$$

Where, N is the total number of erythrocyte counted in 5 squares of the haemocytometer.

3.7.1.2. Total leukocyte count (TLC)

TLC was determined as per the procedure of Schaperclaus *et al.* (1991). Twenty microlitres of blood sample was taken and mixed with 3980 µl of WBC diluting fluid (Hi-Media, India) in a clean vial. The mixture was shaken well to suspend the cells uniformly in the solution. Care was taken that there were no air bubbles trapped. The cells were counted in Neubauer haemocytometer. The numbers of WBC cells were counted in four big squares under high power (40X) magnification of a light microscope. The number of leucocyte per cubic millimetre of the blood sample was calculated using the following formula:

$$\boldsymbol{TLC\ (cells\ mm^{-3}) = \frac{N\ \times dilution\ factor}{Area\ counted\ \times depth}}$$

$$\boldsymbol{TLC\ (cells\ mm^{-3}) = \frac{N \times 200}{4 \times 0.1} \quad = N\ \times 500}$$

Where, N denotes the total number of white blood cells counted in 4 squares of the haemocytometer.

3.7.1.3. Haemoglobin content

The hemoglobin level of blood was analyzed by the cyanomethaemoglobin method using Drabkin's fluid (Qualigens). Twenty microlitres of blood was mixed with 5 mL of Drabkin's working solution (Hi-Media, India). The absorbance was measured using a spectrophotometer (Thermo Electron, Merck) at wavelength of 540 nm. The final concentration was calculated by comparing with standard cyanomethaemoglobin (Qualigen's Diagnostics). The haemoglobin concentration was then calculated using the following formula:

$$\boldsymbol{Hb}\ (\boldsymbol{g\ dL^{-1}}) = \frac{[\boldsymbol{OD(T)} \times \mathbf{251} \times \mathbf{60}]}{[\boldsymbol{OD(S)} \times \mathbf{1000}]}$$

Where, $\boldsymbol{OD(T) = absorbance\ of\ test}$

$\boldsymbol{OD(S) = absorbance\ of\ standard}$

3.7.1.4. Packed cell volume

PCV was determined by drawing non-dotted blood by capillary action into microhaematocrit tubes. One end of the tube was sealed with synthetic sealant. The sealed tube was centrifuged in a microhaemotocrit centrifuge for five minutes at 10,500 rpm. The PCV measured using microhaemotocrit reader and expressed as percentage (%).

3.7.1.5. Blood indices (MCV, MCH and MCHC)

The Mean Cell Volume (MCV), Mean Cell Haemoglobin (MCH) and Mean Cell Haemoglobin Concentration (MCHC) were determined according to the equation suggested by Haney *et al.* (1992)

$$\boldsymbol{MCH}\ (\boldsymbol{pg}) = \frac{\boldsymbol{PCV}\ (\%) \times \mathbf{10}}{\boldsymbol{RBC}\ (\boldsymbol{million\ mm^{-3}})}$$

$$\boldsymbol{MCV}\ (\boldsymbol{fl}) = \frac{\boldsymbol{Hb}\ (\boldsymbol{g\ dL^{-1}}) \times \mathbf{10}}{\boldsymbol{RBC}\ (\boldsymbol{million\ mm^{-3}})}$$

$$\boldsymbol{MCHC}\ (\%) = \frac{\boldsymbol{Hb}\ (\boldsymbol{g\ dL^{-1}}) \times \mathbf{100}}{\boldsymbol{PCV}\ (\%)}$$

3.7.1.6. Differential leukocyte count

A drop of blood was taken on one end of a grease-free clean glass slide and a uniform smear was prepared by sliding the edge at 45^0 using another slide. After allowing the slide to dry at room temperature, the smear was fixed in methanol. After a few minutes, the slides were removed from methanol, air-dried and subsequently stained with Field stain A and B (Hi-Media, India). The slides were washed in tap water in between and after staining. The slides were allowed to dry and the smear was observed under the oil immersion objective. The percentage of each type of leukocytes in blood, *i.e.*, heterophils/neutrophils, lymphocytes and monocytes, eosinophils were determined depending on the shape of the nucleus.

3.7.2 Erythrocyte osmolarity brittleness (EOB)

The erythrocyte osmotic brittleness assay was estimated following the standard method (Bain *et al.,* 2011). Briefly, 50 µL of anticoagulant blood was added to a series of 5 mL buffered sodium chloride (NaCl) solutions that ranged in concentration from 1.0 to 9.0 g L^{-1} in 1.0 g L^{-1} increments including a tube contained only distilled water. After incubation for 2 h at room temperature, these solutions were mixed again and centrifuged for 5 min. at 1200 g. The supernatants were tested for the amount of lysis in each using a spectrophotometer at a wavelength of 540 nm and supernatant of 9.0 g L^{-1} was taken as blank. The minimum concentration of NaCl solution containing no obvious intact erythrocytes was taken as the value of erythrocyte osmotic brittleness.

3.7.3 Serum protein profiles

3.7.3.1. Serum total protein

Total serum protein was estimated according to Biuret method (Reinhold, 1953) using the kit (Merck, Germany). Protein present in the serum binds with copper ions in an alkaline medium of the biuret reagent and produces a purple-coloured complex, whose absorbance is proportional to the protein concentration. Three test tubes were labelled as Blank (B), Standard (S) and Test (T) were taken. Into all the tubes, 1 mL of biuret reagent and 2 mL of distilled water were added. A volume of 0.05 mL protein standard was taken in the test tube labelled as S and 0.05 ml of serum was added into the test tube labelled as T. It was then mixed well and incubated at 37 ^{0}C for 10 minutes. The absorbance of S and T were measured

against B in a spectrophotometer at 630 nm. The calculation was done as follows:

$$\textbf{Total protein } (g\ dL^{-1}) = \frac{\textbf{Absorbance of test } (T)}{\textbf{Absorbance of standard } (S)} \times 6$$

3.7.3.2 Albumin

Albumin was estimated by bromocresol green (BCG) binding method (Doumas *et al.,* 1971**)** by using kit (Merck, Germany). Albumin in a buffered medium binds with BCG which produces a green colour whose absorbance is proportional to the albumin concentration. Three test tubes labeled as Blank (B), Standard (S) and Test (T) were taken. Into all the tubes, 1 mL of buffered dye reagent and 2 mL of distilled water were added; 0.01 mL of albumin standard was taken in the test tube labeled as S and 0.01 mL of plasma was added into the test tube labelled as T. It was then mixed well and incubated at 37 ^{0}C for 10 minutes. The absorbance of S and T were measured immediately against B in a spectrophotometer at 630 nm. The calculation was done as follows:

$$\textbf{Albumin } (g\ dL^{-1}) = \frac{\textbf{Absorbance of test } (T)}{\textbf{Absorbance of standard } (S)} \times 4$$

3.7.3.3. Globulin

Globulin was calculated by subtracting albumin values from total serum proteins.

$$\textbf{Globulin } (g\ dL^{-1}) = \textbf{Total protein} - \textbf{Albumin } (g\ dL^{-1})$$

3.7.3.4. Albumin to Globulin ratio

A/G ratio was calculated by dividing albumin values by globulin values.

3.8. Serum Biochemical parameters

3.8.1 Blood glucose

Blood glucose was estimated by the method of Nelson and Somogyi (1945) as described by Oser (1965) in blood glucose diagnostic kit (Merck, Germany). Three test tubes labelled as Test (T), Blank (B), and Standard (S) add 1000 µL of reagent in each of tubes, then add 10 µL of distilled water in blank (B) and same volume of reagent to standard solution in Standard (S) and 10 µL of blood sample in each of Test (T). After this all tubes were mix well and incubate for

approximately 15 min. at 37 °C and read the absorbance against reagent blank within 30 min. The absorbance was read at OD_{540} by using spectrophotometer.

$$\boldsymbol{Glucose\left(mg\ dL^{-1}\right) = \frac{Absorbance\ of\ sample\ \times concentration\ of\ standard}{Absorbance\ of\ standard}}$$

$$\boldsymbol{concentration\ of\ standard = 100\left(mg\ dL^{-1}\right)}$$

3.8.2. Serum GOT

For the determination of GOT activity in serum, GOT (AST) diagnostic kit (Merck, Germany) was used. The diagnostic kit was based on Reitman and Frankel (1957) method. GOT is expressed as U/L.

3.8.3 Serum GPT

For the determination of GPT activity in serum GPT (ALT) diagnostic kit (Merck, Germany) was used. The diagnostic kit was based on Reitman and Frankel (1957) method. GPT is expressed as U/L.

3.8.4 ALP activity

Alkaline phosphatase (ALP) activity was determined following the method of Ross *et al.* (2000). In brief, 50 µL of serum sample was mixed with 150 µL of 100 mM ammonium bicarbonate (pH 7.8) containing 1 mM $MgCl_2$ at 30 °C for 15 min. Further 50 µL of 4 mM p-nitrophenol phosphate substrate was added and incubated for 30 min at 30 °C. Post incubation; the absorbance was measured at 405 nm. One unit of activity was defined as the amount of enzyme required to release 1 µM of p-nitrophenol product in 1 min.

3.8.5 LDH activity

LDH activity (U L^{-1}) in serum was determined by using LDH diagnostic kit (Merck, Germany). The kit was based on Wroblewski and La Due (1955) method. One unit of LDH activity is defined as the amount of enzyme that catalyzes the conversion of lactate into pyruvate to generate 1.0 µM of NADH per minute at 37 °C.

3.8.6 Lactate level

Lactate in serum was estimated using lactate colorimetric assay kit (BioVision, India). The assay is based on Santulli *et al.* (1999), in which lactate is oxidized by lactate dehydrogenase to generate a product which interacts with a probe to produce a colour and absorbance is measured at 450 nm. Briefly, 50 µL of reaction mixture (46 µL Lactate assay buffer, 2 µL Lactate substrate mix. and 2 µL Lactate enzyme mix.) add into each well containing the lactate standard or test samples into microtitre plate. Incubate the reaction for 30 min at room temperature and measure the absorbance at OD 450 nm in a microreader. The concentration of lactate C was calculated as follow:

$$\boldsymbol{C\ (nM\ \mu L^{-1}) = \frac{Estimated\ Lactic\ acid\ amount\ (nM) in\ sample}{Sample\ volume\ (\mu L)}}$$

3.8.7 Total bilirubin level

The bilirubin kit was based on modified method of Pearlman and Lee (1974) in which a surfactant is used as a solubilizer. Bilirubin glucuronate reacts directly with sulpho-diazonium salt and forms coloured derivative azobilirubin. Into three test tubes labelled as Test (T), Blank (B) and Standard (S), 500 µL of working reagent was added. Following this, 10 µL of serum sample was added to each test tube labelled T. Further, 10 µL of distilled water was added into blank (B) and same volume of reagent was added into Standard tube (S). After this, all tubes were mixed well and incubated for 5 min. at 37°C. The colour intensity of formed azobilirubin was measured at 550 nm which proportional to direct bilirubin concentration in the sample.

$$\boldsymbol{Total\ bilirubin\ (mg\ dL^{-1}) = \frac{A\ of\ test}{A\ of\ standard} \times Concentration\ of\ std.}$$

Concentration of standard = 0.2 mg dL^{-1}

3.8.8. Creatinine (CRE) level

Creatinine concentration in serum was estimated using creatinine kit (Erba, India). The creatinine kit was based on Jaffe's method (Bowers, 1980) in which creatinine reacts with alkaline picrate to produce an orange-yellow colour. In brief, 100 µL of test sample (diluted serum) was mixed with working reagent (T), and initial absorbance (A_1) was taken after 20 seconds and final absorbance (A_2) on 80 seconds. The absorbance of the orange-yellow colour formed is directly proportional to creatinine concentration measured at 520 nm in spectrophotometer (Thermo Spectronic, USA).

$$\boldsymbol{Creatinine\ (mg\ dL^{-1}) = \frac{\Delta A\ of\ test}{\Delta A\ of\ standard} \times Concentration\ of\ standard}$$

Where, $\boldsymbol{\Delta A = A_2 - A_1}$ **and Concentration of standard= 2 mg dL^{-1}**

3.9. Immunological parameters

3.9.1. Respiratory burst activity (RBA)

Nitroblue tetrazolium (NBT) assay was carried out to estimate RBA using the method of Anderson and Siwiki (1995). One-hundred microliters of blood was placed into the wells of a flat bottomed microtitre plate and incubated at 37 °C for 1 h to facilitate adhesion of cells. The supernatant was removed and the wells were washed three times with phosphate buffered saline (PBS, pH 7.4), followed by the addition of 100 µL of 0.2% NBT. The plate was incubated for further 1 h and washed once with 100% methanol and thrice with 70% methanol. The plates were allowed to air dry. Potassium hydroxide (2N; 120 µL) and dimethyl sulphoxide (DMSO) (140 µL) were added into the wells to form blue formazone precipitate. The absorbance of the wells on was measured in an ELISA reader (Quant, Universal microplate spectrophotometer, USA) at 620 nm.

3.9.2. Phagocytic activity (PA)

Phagocytic activity was detected using *Staphylococcus aureus* as described by Anderson and Siwicki (1995). A sample (0.1 mL) of blood was placed in a microtiter plate well into which 0.1 mL of *Staphylococcus aureus* 1×10^7 (A_{450}= 0.5-0.6) cells suspended in phosphate buffered saline pH 7.2 was added and mixed well. The bacteria–blood solution was incubated for 20 min. at room temperature. Five microliters of this mixture was taken on a clean glass slide and a smear was prepared. The smear was air dried, fixed with ethanol (95%) for 5 min and air dried,

followed by staining with Giemsa (10%) stain for 10 min. Two smears were made from each fish. A total of 100 phagocytic cells (peripheral monocytes and neutrophils) from each smear were observed under the light microscope (Carl Zeiss, Axioskope 40, Germany) and the number of phagocytising cells and the number of bacteria engulfed by the phagocytes were counted. Phagocytic activity (%) was determined by dividing the number of phagocytising cells by the total number of phagocytes counted and multiplying with 100.

3.9.3. Myeloperoxidase content (MPO)

Total myeloperoxidase content present in serum was measured according to Quade and Roth (1997) with some modifications (Sahoo *et al.,* 2005). About 15 µL of the serum was diluted with 135 µL of hank's balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+} in 96 well plates. The wells were added with 25 µL of 20 mM 3,3'-5,5'- tetramethyl benzidine hydrochloride (TMB) (Hi-Media) and 25 µL of 5 mM H_2O_2 (Qualigens) (Both substrates of MPO). The reaction was stopped after 2 min. by adding 50 µL of 4 M sulphuric acid (H_2SO_4). The plate was centrifuged (400×g) for 10 min, and 150 µL of the supernatant from each well was transferred into a new 96-well plate. The absorbance was measured at 450 nm in a micro plate reader (Quant, Universal microplate spectrophotometer, USA).

3.9.4 Serum lysozyme activity

The serum lysozyme activity was measured using colorimetric method (Parry *et al.,* 1965). In a cuvette, 3 mL of *Micrococcus luteus* suspension in phosphate buffer (A_{450}= 0.5-0.7) was taken, to which 50 µL of serum sample was added. The content of cuvette was mixed well for 15 seconds and reading was taken in a spectrophotometer at 450 nm. The lysis of bacteria was recorded immediately at 15, 30 and 270 seconds interval. A unit of lysozyme activity was defined as the amount of sample causing a reduction in absorbance of 0.001 per min. expressed as U min^{-1}.

3.9.5. α-2 macroglobulin (α-2M) activity

The activity of α-2M in fish serum was estimated as per the procedure of Zuo and Woo (1997) with slight modification. Ten microliter serum was mixed with 100 µL of trypsin (bovine pancreas type I (Sigma, India); 100 µg mL^{-1} of 0.1 M Tris-HCl, pH 8.2). Trypsin was replaced with 100 µL of Tris-HCl in blank and in positive control; no serum was added to trypsin. All tubes were made up to 200 µL with Tris-HCl and incubated at 25 °C for 40 minute. After incubation, 2 mL of Sodium-benzoyl-DL-arginine-p-nitroanilide HCl (BAPNA, Sigma) dissolved in Tris-HCl containing 20mM $CaCl_2$ was added to all the tubes and incubated for 20 min. at 25 °C. The reaction was stopped by the addition of 500 µL of 30% (v/v) acetic acid. The optical density (OD) was measured at 410 nm in a UV-Vis spectrophotometer (Thermo Spectronic, USA). The OD of the positive control was taken as 100%. The OD value of the serum blank was deducted from the trypsinized sera and the percentage of trypsin inhibition by the serum was calculated.

3.9.6. Ceruloplasmin level

The level of ceruloplasmin in the fish serum sample was measured as *p*-phenylene diamine (PPD) oxidase activity (Sigma, USA) according to method of Pelgron *et al.* (1995) with slight modifications. Twenty five microliter serum or standard of ceruloplasmin (Sigma, USA) was mixed with 500 µL (1.2 M, pH 5.0) acetate buffer containing 0.1% PPD as the substrate. Further, each sample was incubated in the presence of 0.5 mL of 0.5% NaN_3 (Sodium azide). The reaction was terminated by the addition of 0.5 mL of 0.5% NaN_3. One unit of ceruloplasmin was defined as the amount of oxidase that catalysed a decrease in absorbance of 0.001 min^{-1} at 550 nm in a UV-Vis spectrophotometer (Thermo Spectronic, USA).

3.9.7. Serum antiprotease activity

Total serum antiprotease in fish serum was estimated as per Zuo and Woo (1997) with slight modification. Serum (10 µL) was mixed with 100 µL of trypsin (bovine pancreas type I, 200 µg mL^{-1} of PBS; Sigma, India). In the positive control, serum was replaced by 10 µL of PBS buffer solution. All tubes were incubated at 25 °C for 30 minute. After incubation, 1 mL of casein dissolved in PBS (2.5 mg mL^{-1}) was added to all tubes and incubated further for 15 min at 25 ^{o}C. The reaction was terminated with the addition of 500 µL of 10% trichloroacetic acid. The sample was centrifuged at 10000 *g* for 5 min to remove protein precipitates. The OD

of the supernatant was measured in a UV-Vis spectrophotometer (Thermo Spectronic, USA) at 280 nm and the percentage of trypsin inhibition was calculated.

3.9.8. Total immunoglobulin level

The modified method of Anderson and Siwicki (1995) was followed for the estimation of total immunoglobulin in plasma. The serum (0.1 mL) was placed in a plastic serum vial and 0.1 mL of 12% PEG (Polyethylene glycol) was added and suspended in deionised water. Incubation was done at room temperature for 2 hours under constant mixing, followed by centrifugation at 5000 X *g* for 10 minutes. The protein concentration in the supernatant was determined by Biuret method using a commercial kit (Merck, Germany). Protein reading from supernatant was the amount of protein taken out by absorption into the polyethylene glycol. The total immunoglobulin expressed as unit g dL^{-1} was calculated by the formula given below.

$$\boldsymbol{TI = (TP\ in\ individual\ sample\ of\ serum - TP\ taken\ out\ by\ absorption\ to\ PEG)}$$

3.10. Enzymes of oxidative stress

3.10.1. Superoxide dismutase (SOD)

Superoxide dismutase was assayed as described by Misra and Fridoyich (1972), based on the oxidation of epinepherine–adreno chrome transition by the enzyme. Fifty-microliters of the sample (gills, liver and serum) was taken in the cuvette and 1.5 ml of 0.1M carbonate–bicarbonate buffer containing 57 mg dL^{-1} EDTA (pH-10.2) and 0.5 mL (3 mM) epinephrine was added and mixed well. Change in the optical density at 480 nm was read immediately for 3 min in a UV-Vis spectrophotometer (Thermo Spectronic, USA). One unit of SOD activity was the amount of protein required to give 50% inhibition of epinephrine auto oxidation. SOD was expressed as unit activity (amount of protein required to give 50% inhibition of epinephrine auto oxidation).

3.10.2. Catalase (CAT)

Catalase activity in gills, liver and serum was assayed according to the method described by Takahara *et al.* (1960). The reaction mixture of 3 mL consisted of 2.50 mL phosphate buffer (50 mM, pH 7.0), 50 µL tissue homogenate (enzyme source) and the reaction was started by the addition of 1.0 mL of H_2O_2 solution. The decrease in absorbance was measured at 240 nm at 15 second intervals for 3 minute. An enzyme blank was run simultaneously with 1.0 mL distilled water instead of H_2O_2 solution. Enzyme activity was expressed as nano-moles H_2O_2 decomposed min^{-1} mg^{-1} protein.

$$\boldsymbol{Catalase\ activity} = \frac{\boldsymbol{\Delta OD/\min \times total\ reaction\ volume \times 1000}}{\boldsymbol{equivalent\ weight\ of\ H_2O_2 \times sample\ volume \times protein\ concentration}}$$

3.10.3. Glutathione-S-transferase (GST)

The activity of glutathione-S-transferase (GST) of gills and liver was determined spectrophotometrically (Habig *et al.,* 1974) following the formation of glutathione (GSH) conjugate with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm using extinction coefficient of 9.6 mM^{-1} cm^{-1}. The reaction mixture contained 3 mL volume of 0.3 M sodium phosphate buffer (pH 6.5), 30 mM GSH, and 30 mM CDNB in ethanol, 0.1 mL tissue homogenate and 1.3 mL of distilled water. The mixture was incubated at 37 °C for 5 minutes. The change in absorbance was measured at 340 nm for 3 minutes at 60 seconds intervals. GST activity was expressed as nM CDNB conjugates formed min^{-1} mg of $protein^{-1}$ where ε =9.6 mM^{-1} is extinction coefficient for CDNB conjugate at 340 nm for test in 1 mL cuvette.

$$\boldsymbol{GST\ activity} = \frac{\boldsymbol{\Delta OD_{340}/\min \times (total\ reaction\ volume \times 1000)}}{\boldsymbol{\varepsilon \times sample\ volume \times protein\ concentration}}$$

3.10.4. Na^+-K^+-ATPase activity

Na^+-K^+-ATPase activity of gills and liver was measured by liberating PO_4 (Pi) from a hydrolysis reaction with ATPase, according to Svobaca and Mossinger (1981) with slight modification (Agrahari and Gopal, 2008). The activity in the gill and liver was assayed in reaction mixture of 1 mL of 0.1M Tris-HCl buffer (7.8), 0.1 mL 100 mM NaCl, 0.1 mL 20 mM KCl, 0.1 mL 3 mM $MgCl_2$, 0.5 mL 5mM ATP and 0.1 mL tissue homogenate (test). For control experiments, Trichloroacetic acid (TCA) (Sigma, USA) was used instead of tissue homogenate. The reaction mixture was incubated for 15 minutes and the reaction was terminated by adding 10% TCA. The liberated phosphate was estimated at 660 nm according to Fiske

and Subbarow (1925) and the values were calculated and expressed as µM Pi liberated mg^{-1} protein h^{-1} in the tissues using the following formula.

$$ATPase\ activity = \frac{OD\ at\ 660\ nm(Test-Control)\times concentration\ of\ standard\ \times 0.5}{OD\ of\ standard\times volume\ of\ sample\times protein\ concentration\times time}$$

3.11. Statistical analysis

All statistically analyses were conducted using software Statistical Package for Social Sciences (SPSS) version 16. Differences in parasite intensity with respect to fish length and weight were analysed by correlation and data are tested for linearity and further Pearson and quadratic correlation coefficient were estimated. Parasite intensity of each gill arch number and position are plotted in box-plot and comparison of mean intensity was tested using One-way analysis of variance (ANOVA).

Principal component analysis was used to summarise the spatial distribution of the parasites over the 6 gill regions of 4 gill arches and various morphometric traits. Relationships between the dominant principal component score of various parameters are depicted by biplot of PCA.

Further, the data of haematological and physiological biomarkers and immune parameters were also analyzed using (SPSS) version 16 and their normality tested using Shapiro-Wilk test. The data were subjected to one-way ANOVA, and Duncan's multiple range tests (DMRT) was used to determine the significant differences between the means of the various degree of monogenean infested and control groups. Comparisons were made at 5% probability level in all the cases.

Principal component analysis was used to summarise the haematological and serum biochemical biomarkers of the high level naturally parasitized fish. Relationships between the dominant principal component score of various parameters are depicted by biplot of PCA.

4. RESULTS

4.1 Isolation and identification of monogenean parasite(s) infesting *P. hypophthalmus*

4.1.1. Infestation parameters

Sixty samples of *P. hypophthalmus* were collected from cage farms at Murbad, Thane, Maharashtra. The standard length of samples ranged from 7.0 to 20.6 cm. Forty-two fish were found positive for infestation with gill monogenean. Further, estimations of the infestation parameters showed 72% of monogenean prevalence, with an abundance of approximately 20 parasites per individual and mean intensity of 39 parasites per infected fish (figure 2). The highest intensity observed was 72 dactylogyrids in an individual host belonging to size (standard length) range of 10-15 cm.

Two morphologically distinct dactylogyrids belonging to *Thaparocleidus* spp. (*Thaparocleidus siamensis* Lim, 1990 and *Thaparocleidus caecus* Lim 1996) were collected from the gill filaments of *P. hypophthalmus*. The morphometric traits (Mizelle and Kritsky, 1969) of these two species have been described in the present study.

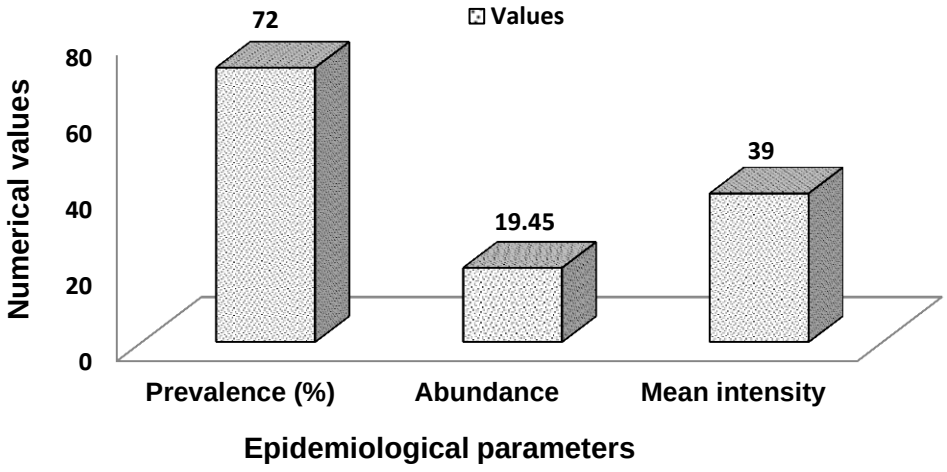


Figure 2: Infestation parameters (prevalence (%), abundance (no. of parasite per individual) and mean intensity (no. of parasite per infested fish)) of dactylogyrid (*Thaparocleidus* spp.) infection in *Pangasianodon hypophthalmus*.

4.2. Description and morphometric traits of dactylogyrids (*Thaparocleidus* spp.)

4.2.1. *Thaparocleidus siamensis*

Description:

The body is elongated, long with rounded anterior and posterior ends. The body shape is cylindrical with distinct prohaptor and opisthohaptor (plate 3 & 4). Parasites measured 1067 μm (968–1198 μm) in length and 167 μm (85–267 μm) in maximum width at the mid-region of the body (table 8). Head organs were not visible clearly at prohaptor region. Above pharynx, 2 pairs of eye spots present, made up of melanistic granules, the anterior pair being smaller than the posterior pair (plate 3). The eye spots are found to be distinct apart (vertical distance: 14 μm; horizontal distance: 34 μm). Pharynx small, muscular, proximate to round in shape, 39×37 μm (29-48) (table 8). Esophagus short, bifurcation of caeca takes place posterior to pharynx. Crura extend throughout the length of the body and unite posteriorly to testis, and anterior to haptor.

Opisthohaptor is slightly elongated, and measures 137 μm (121–156 μm) × 116 μm (106–130 μm) (table 6). Haptoral armature composed of 2 pairs of anchor (dorsal and ventral), dorsal transverse bar, ventral connective bar, and 7 pairs of marginal hooks (figure 3).

Dorsal pair of anchors is wunderoid-type (W) with well-defined base, but inner and outer root absent (plate 3b & 4b,c). Measurements (in μm) include: total length: DTL 145 (125-173), main part: MPD 75 (49-116), recurved point: DRP 59 (41–76) (table 8). Straight and tapering shaft terminates smoothly to bent recurved point. Sleeve sclerite present at the mid-length of shaft and reaches up to the beginning of recurved point (plate 4b). Supporting patches are small and knee in shape, measuring (in μm) 21 (11–27) × 4 (4–5), present at the base of each pair of dorsal anchors (table 8). Dorsal anchors are connected with each other by dorsal transverse bar measuring (in μm) DBL×DBW 65 (54–74) × 17 (14–19), which is slightly curved in the medial part and sharp-rounded at the lateral ends (figure 3b; table 8).

The ventral pairs of anchors are comparatively smaller than dorsal pairs. Ventral anchors are boreal-type in which inner roots are larger than outer roots, and the anchors are almost of equal length (plate 4b & c). Measurements (in μm) include total length: VTL 44 (40-49), main part: MPV 24 (21-29), inner length and outer length of root (not measured), recurved point VRP 20 (18-22). The shaft is cylindrical and straight with smoothly recurved point. The ventral bar is broad shallow V-shaped, consisting of 2 identical pieces; each measuring (in μm) 28 (23–34) × 11 (8-16) (figure 3c). Two pieces joined in such a way that they seem to be a single piece.

Marginal hooks, larval-type are morphologically similar in shape and size except for hook 2. Each hook has a total length of 22 (18–27) μm, consisting of an elongated handle, a flattened loop, and a sickle-shaped blade (plate 4b; table 8).

The male reproductive system comprises of a single testis, vas deferens, and cirrus (plate 3c). Testis is single, elongated, intercecal, postovarian, postequatorial, and saccular structure measuring (in μm) TL×TW 124 (104–142) × 58 (50–63) (table 8). Male copulatory organ is simple and initial-type, consisting of a cirrus tube. Vas deferens leaves anterior part of testis, crosses along dorsal side diagonally to left side, loops round left intestinal tract to ventral side, extends upwards forming thin, long blind seminal vesicle sac, ductus ejaculatorious leaves seminal vesicle to enter initial part of copulatory tube (figure 3c). Copulatory organ consists of slightly undulating tube CTL 178 (142-224) μm with lightly scleotised funnel-like accessory piece (plate 3c; table 8). Prostatic gland is small, sited near initial part of copulatory organ, point of entry into copulatory tube not clear.

Vagina and egg could not be located clearly and hence vaginal apparatus was not observed in detail. However in some cases, small bulbous structure (seminal receptacle) could be seen opening into oocyte. Some lightly sclerotized tubes, probably vitelline ducts and vaginal duct, enter oocyte and opens into uterus. Single ovoid ovary is measure (in μm) (OL×OW) 178 (164-198) × 81 (49-104) anterior to single testis (table 8). Oviduct leaves anterior of ovary to oocyte. Uterus is sac-like, uterine opening ventral, mid-body, below

the copulatory organ. Vitellaria is co-extensive with intestines, confluencing at anterior, posterior and mid region.

4.2.2. *Thaparocleidus caecus*

Description:

The body is cylindrical and elongated, long with rounded anterior and posterior end. Body shape is cylindrical with distinct prohaptor and opisthohaptor (plate 5). It measures (in μm) 487 (330–635) in length and 73 (64–98) in maximum width (in μm) attained at the mid-posterior region of the body (plate 5a). Head organs were not clearly visible at prohaptor. Above pharynx, 2 pairs of granulated eyespots are present, the anterior pair being smaller than the posterior pair (figure 4a). Pharynx is small, muscular, proximate to rounded, 23×22 (18-33) μm (table 8). Esophagus is short, bifurcation of caeca takes place posterior to pharynx. Crura extends throughout the length of the body and unites posteriorly to testis and anterior to haptor.

Opisthohaptor appears slightly spharocylindrical shaped and measures (in μm) 62 (42–79) × 69 (48–83) (table 8). Haptoral armature is composed of 2 pairs of anchor (dorsal and ventral), dorsal transverse bar, ventral connective bar, and 7 pairs of marginal hooks (plate 5b & c).

Dorsal anchors are with length of main part, inner root, stumpy outer root and recurved point. Measurements (in μm) include total length: DTL 105 (76-120), main part: MPD 61 (42-77), recurved point: DRP 23 (18–29) (table 8). Triangular patch ends present in spine at the base of each pair of dorsal anchors have a dimension of 8 μm (6-10, average) x 4 μm (3-6, average). Straight and tapering shaft terminates smoothly to bent recurved point (figure 4b). Dorsal anchors are connected with each other by dorsal transverse bar of broad deep curved in the medial part and rounded at the lateral ends measuring (in μm) DBL×DBW 48 (22–68) × 13 (5–17) (table 8).

The ventral pairs of anchors are comparatively smaller than dorsal pairs. Ventral anchors are boreal-type in which roots are not well developed, and anchors almost equal length. Measurements (in μm) include

total length: VTL 33 (23-39), main part: MPV 23 (13-39), recurved point: VRP 9 (5-18). The shaft is cylindrical and straight with smoothly recurved point. The ventral bar is flattened V-shaped, consisting of 2 identical pieces; each measuring (in μm) VBL × VBW 20 (18–58) × 8 (4-11).

Marginal hooks, larval-type are morphologically similar in shape and size, except for hook 2. Each hook has a total length 20 (17–22), consisting of an elongated handle, a flattened loop, and a sickle-shaped blade (table 8).

The male reproductive system comprises of a single testis, vas deferens, and cirrus. Testis is single, elongated, intercecal, postovarian, post-equatorial, and saccular structure measuring TL×TW 69 (42–105) × 41 (31–54) (table 8). Vas deferens leaves anterior part of testis, crosses diagonally along dorsal to left side, loos round left intestinal tract to ventral side, extends upwards forming thin, long blind seminal vesicle sac, ductus ejaculatorious leaves seminal vesicle to enter initial part of copulatory tube (figure 4c). Copulatory organ consists of tapering sigmoid tube, length CTL 72 (42-115) μm with spine like structure near distal end, sclerotized funnel-shaped initial and grooved accessory piece with expanded distal part (plate 5d). Prostatic gland is bulb-shaped with duct which dilates slightly before, entering initial part of copulatory tube.

Vaginal opening is ventral, mid-body, posterior to copulatory organ, connected by short vaginal tube to large oval-shaped seminal receptacle which open into a common area together with uterus and a trilobed structure. Single ovoid ovary is measured (in μm) (OL×OW) 95 (48-135) × 45 (22-72) (table 8). Oviduct arises from anterior part of ovary to oocyte. Vitellaria co-extensive with intestines, confluencing at anterior, posterior and mid region (figure 4).

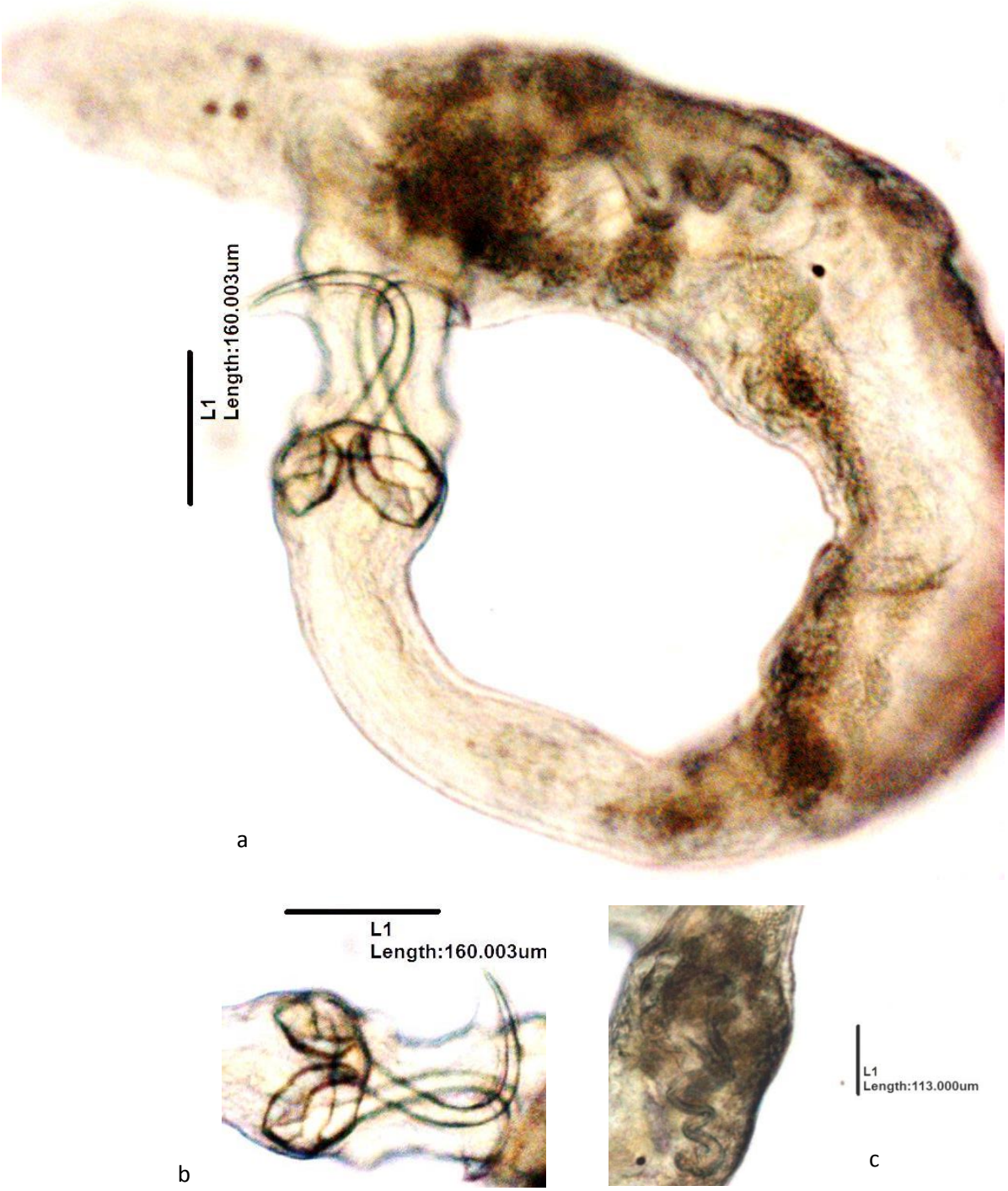


Plate 3: Microphotographs of *Thaparocleidus siamensis*. (a). Whole mount; Scale bars: 160.00 μm (b). Dorsal anchors with dorsal bar; Scale bars: 160.00 μm (c). Copulatory complex; Scale bars: 113.00 μm.

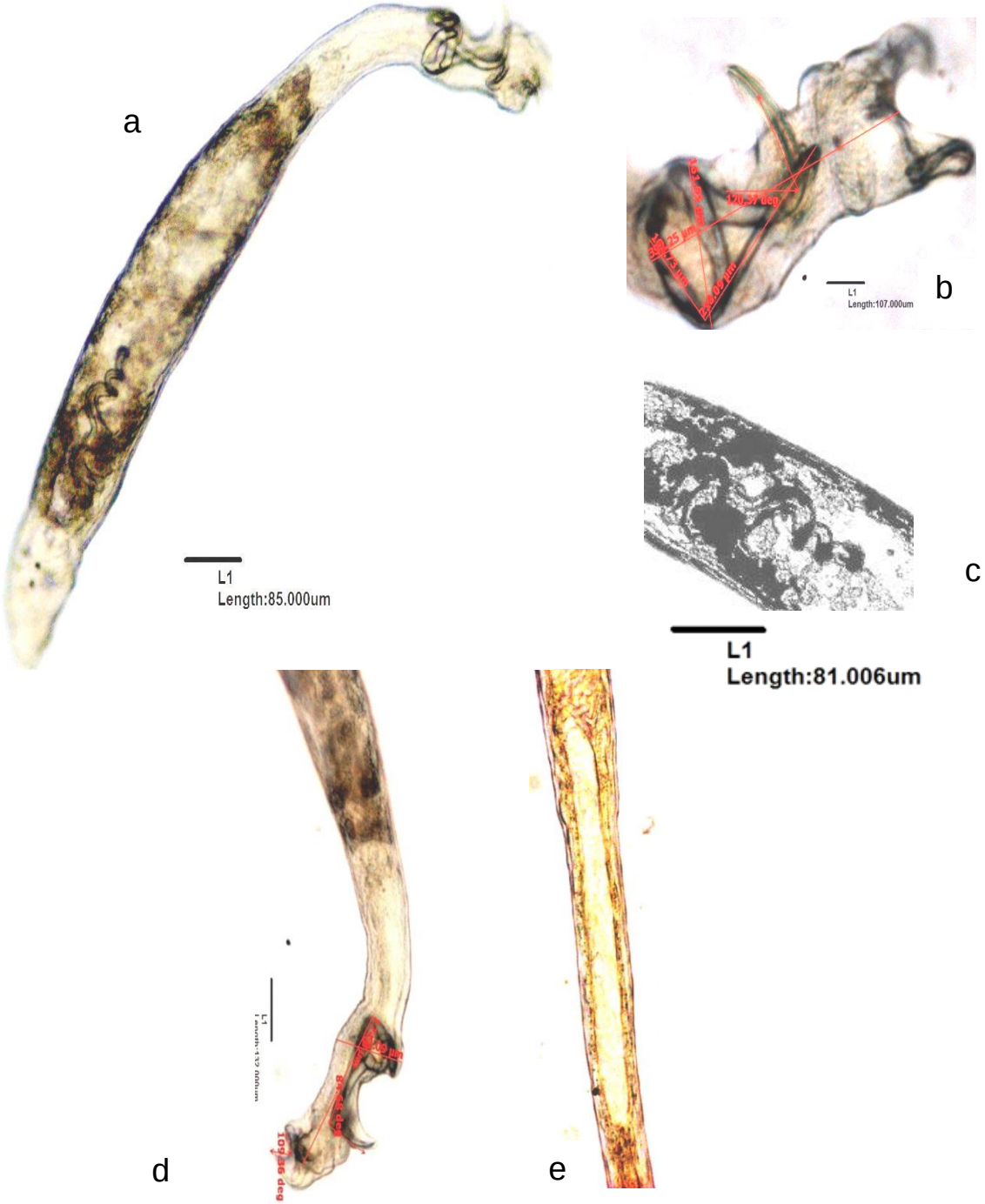


Plate 4: Microphotographs of *Thaparocleidus siamensis*. (a). Whole mount; Scale bars: 85.00 µm (b). Dorsal anchors with dorsal bar and marginal hooks; Scale bars: 107.00 µm (c). Copulatory complex; Scale bars: 81.00 µm. (d). Posterior part (haptor armature); Scale bar: 132.00 µm (e). Medial part (Ovary and testis) Scale bar: 132.00 µm

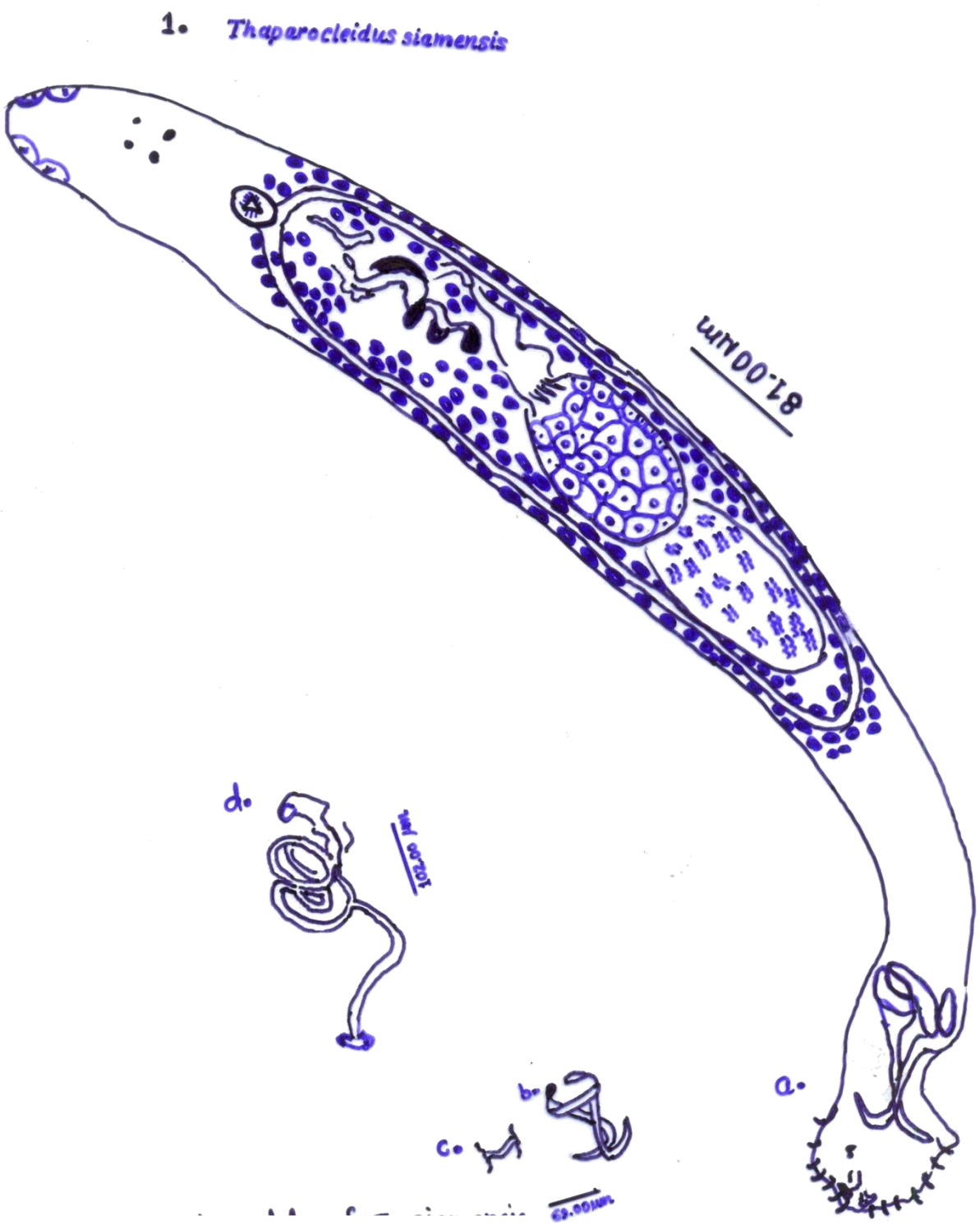


Figure 3: Line diagram of *T. siamensis*

a. Whole mount; Scale bar: 81.00 µm

b. Dorsal anchor and bar; Scale bar: 69.00 µm

c. Ventral anchor and bar; Scale bar: 69.00 µm

d. Copulatory organ; Scale bar: 102.00 µm

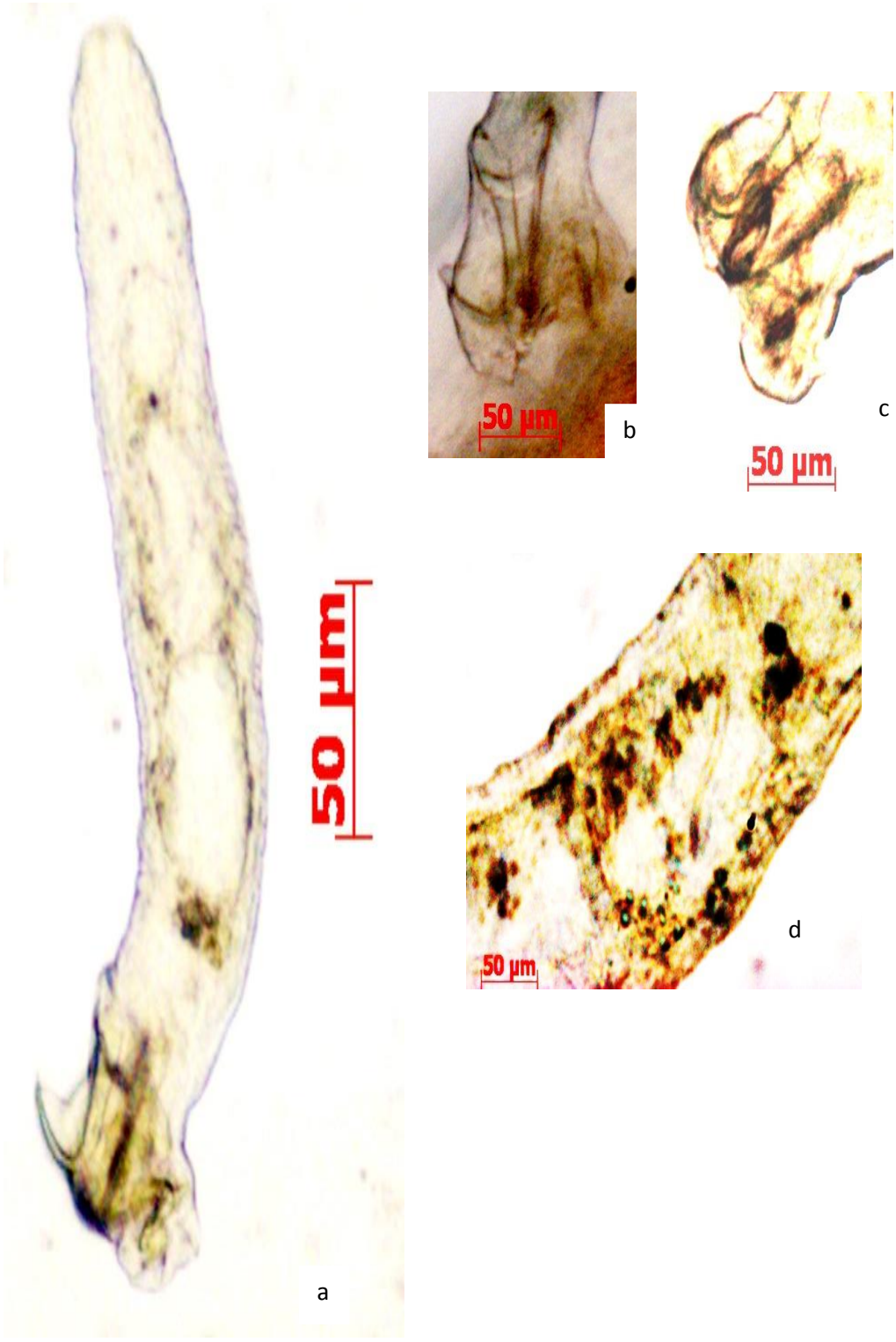


Plate 5: Microphotographs of *Thaparocleidus caecus*. (a). Whole mount; (b). Haptor armature and marginal hooks; Scale bars: 50.00 µm (c). Dorsal and ventral anchors with bars (d). Medial part (copulatory complex).

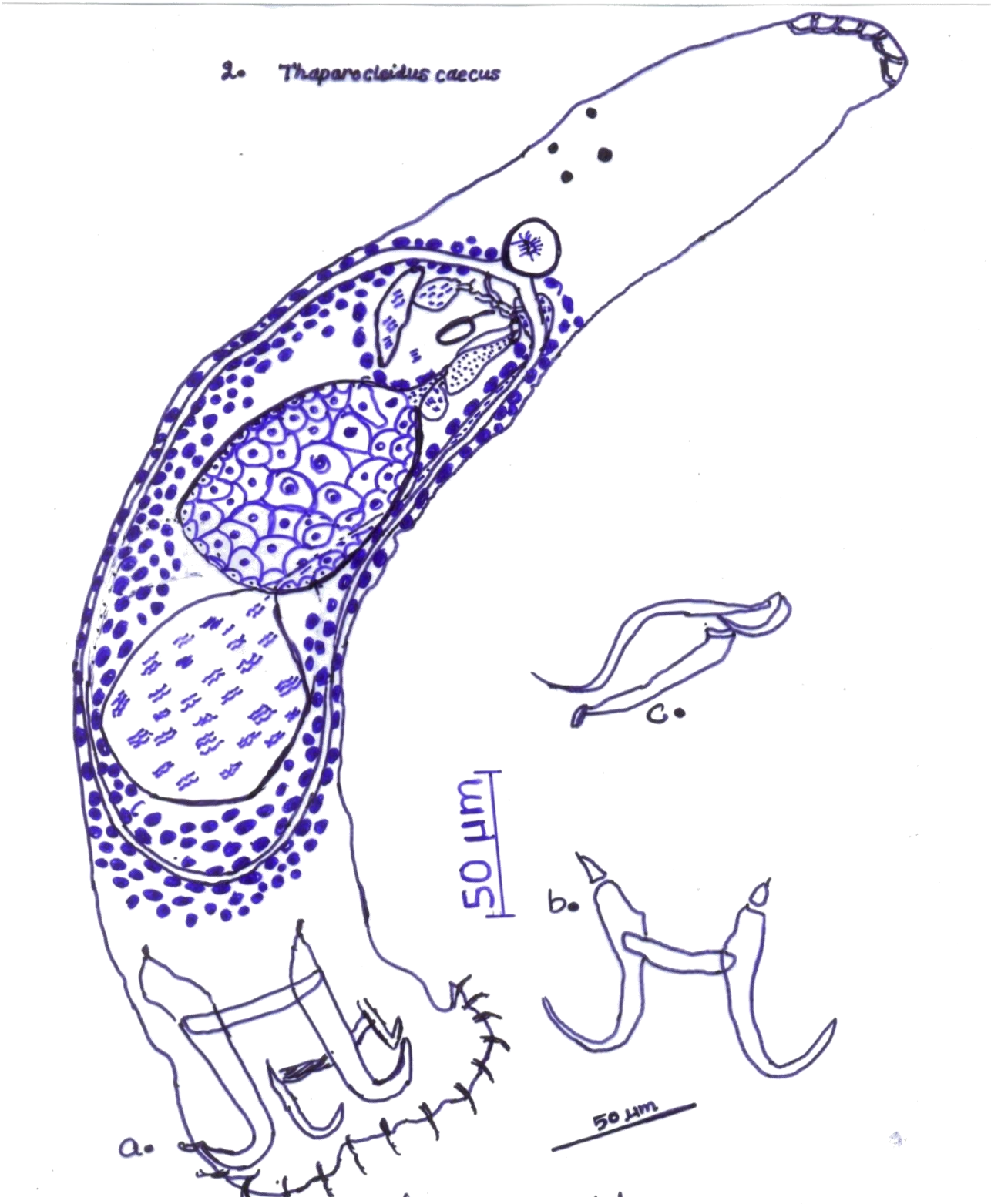


Figure 4: Line diagram of *T. caecus*

a. Whole mount; Scale bar: 50.00 µm

b. Dorsal anchor and bar; Scale bar: 50.00 µm

c. Copulatory organ; Scale bar: 50.00 µm

Table 8: Morphometric (in µm) of *Thaparocleidus siamensis* and *T. caecus*, isolated from *P. hypophthalmus* (host fish). Data is presented in mean (min-max) values; (n=6)

	Body parts	**Morphological variables (length in µm)**	**Symbol**	***Thaparocleidus siamensis***	***Thaparocleidus caecus***
1	**Body**	Body length	(BL)	1067 (968-1198)	487 (330-635)
2		Body width	(BW)	167 (85-267)	73 (64-98)
3		Pharynx length	(PL)	39 (33-46)	23 (18-33)
4		Pharynx width	(PW)	37 (29-45)	22 (18-30)
5		Haptor length	(HL)	137 (121-156)	62 (42-79)
6		Haptor width	(HW)	116 (106-130)	69 (48-83)
7	**Haptoral parts**	Total length of dorsal anchor	(DTL	145 (125-173)	105 (76-120)
8		Main part length of dorsal anchor	(MPD)	75 (49-116)	61 (42-77)
9		Inner root length of dorsal anchor	(DIR)	absent	Present
10		Outer root length of dorsal length	(DOR)	absent	Present
11		Recurved point of dorsal anchor	(DRP)	59 (41-76)	23 (18-29)
12		Dorsal bar length	(DBL)	65 (54-74)	48 (22-68)
13		Dorsal bar width	(DBW)	17 (14-19)	13 (5-17)
14		Total length of ventral anchor	(VTL)	44 (40-49)	33 (23-39)
15		Main part length of ventral anchor	(MPV)	24 (21-29)	23 (13-39)
16		Recurved point of ventral anchor	(VRP)	20 (18-22)	9 (5-18)
17		Ventral bar length	(VBL)	28 (23-34)	20 (18-22)
18		Ventral bar width	(VBW)	11 (8-16)	8 (4-11)
19		Hook Length	(HL)	22 (18-27)	20 (17-22)
20	**Reproductive organs**	Copulatory tube length	(CTL)	178 (142-224)	72 (42-115)
21		Testis length	(TL)	124 (104-142)	69 (42-105)
22		Testis width	(TW)	58 (50-63)	41 (31-54)
23		Ovary Length	(OL)	178 (164-198)	95 (48-135)
24		Ovary width	(OW)	81 (49-104)	45 (22-72)
25		Vaginal length	(VL)	42 (40-45)	20 (6-35)
26		Accessory piece length	(APL)	Not measured	Not measured

4.3. Principal component analysis of morphometrics of *Thaparocleidus* spp.

In the principal component analysis (PCA) of the morphometric traits of *Thaparocleidus* spp., the two principal components (PCs) together constituted 100% of the total variation. PC1 contributed 70.81% of the total variation and PC2 contributed 29.19% for the same (figure 5). The first PC is significantly high and positively correlated with parameters DBW, TW, VL, OW, TL and DRP and negatively correlated with parameters MPD, DTL, MPV, OL, VTL and HL. Further, second PC is significantly high and positively correlated with parameters VRP, DBL, HW, BL, and HOL (figure 5).

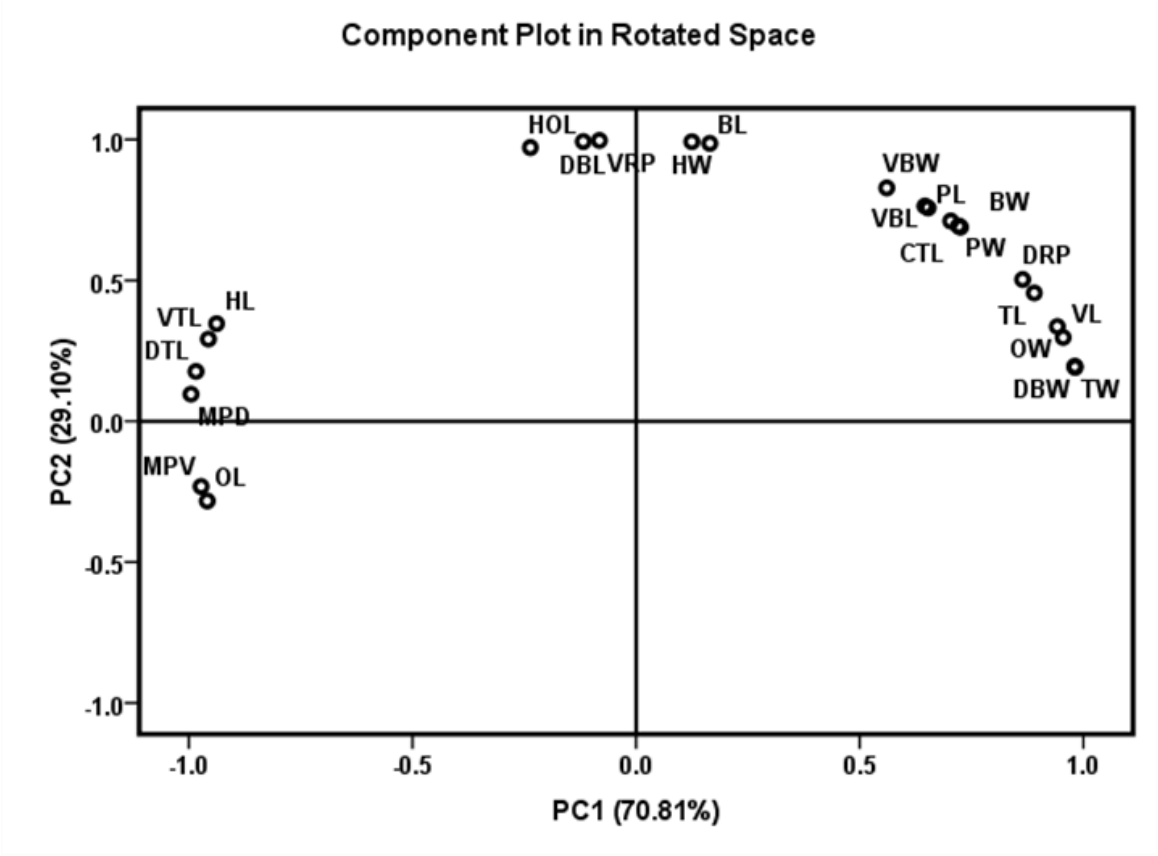


Figure 5: Principal component plot of various traits of morphometric of *Thaparocleidus* spp. isolated from *P. hypophthalmus*, with 100% of total variance.

4.4. Molecular identification of *Thaparocleidus* sp. by 28S rDNA Sequencing

The genomic DNA was extracted from the parasites along with host gills and 28S rDNA gene was amplified using specific set of primers (listed previously). The results showed an expected PCR amplified product of 300 nucleotides in length (figure 6). The PCR products were purified and then sequenced. The 28S rDNA sequences obtained were blast analysed with National Center for Biotechnology Information (NCBI) data base (*www.ncbi.nlm.nih.gov*) and Ribosomal Database Project. Sequencing analysis of the PCR amplified 28S rDNA gene revealed that the monogenean parasite isolates were 100% identical with reported *Thaparocleidus siamensis* AF218123 from Malaysia. Further, the sequence was submitted to GneBank as sequence of *Thaparocleidus siamensis* under the accession number **MF508921**.

4.4.1. Phylogenetic tree construction and relationship

Phylogenetic tree was obtained by comparing the sequence of *T. siamensis* and available 28S rDNA sequences of other monogenean parasite species. Phylogenetic analyses using the methods neighbour-joining (NJ) is depicted in figure 7. The phylogenetic tree constructed in MEGA also revealed a close relationship of *T. siamensis* with other isolates of *Thaparocleidus* spp. and *Silurodiscoides*, as well as phylogenetically distinct monogenean groups such as *Protopolystoma occidentalis* and *Euryhaliotrema johnii* isolated from amphibian and marine fish (figure 7).

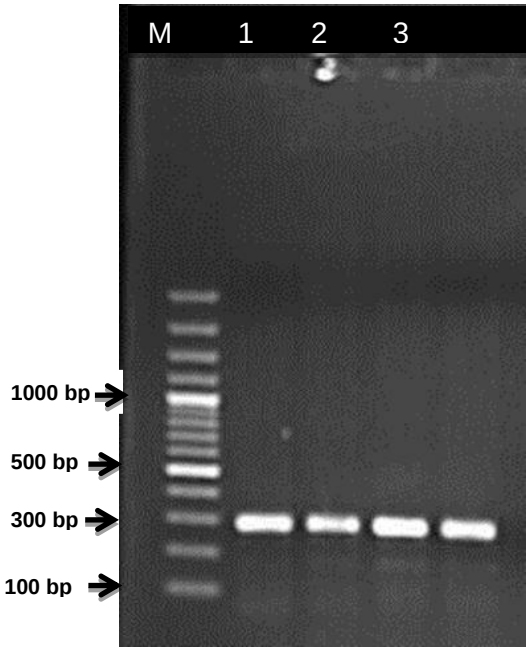


Figure 6: Amplified PCR product of 28S rDNA using *Thaparocleidus* species specific primer.
Lane M: 100 bp DNA ladder
Lane 1-4: Primer set TF&TR (300bp)

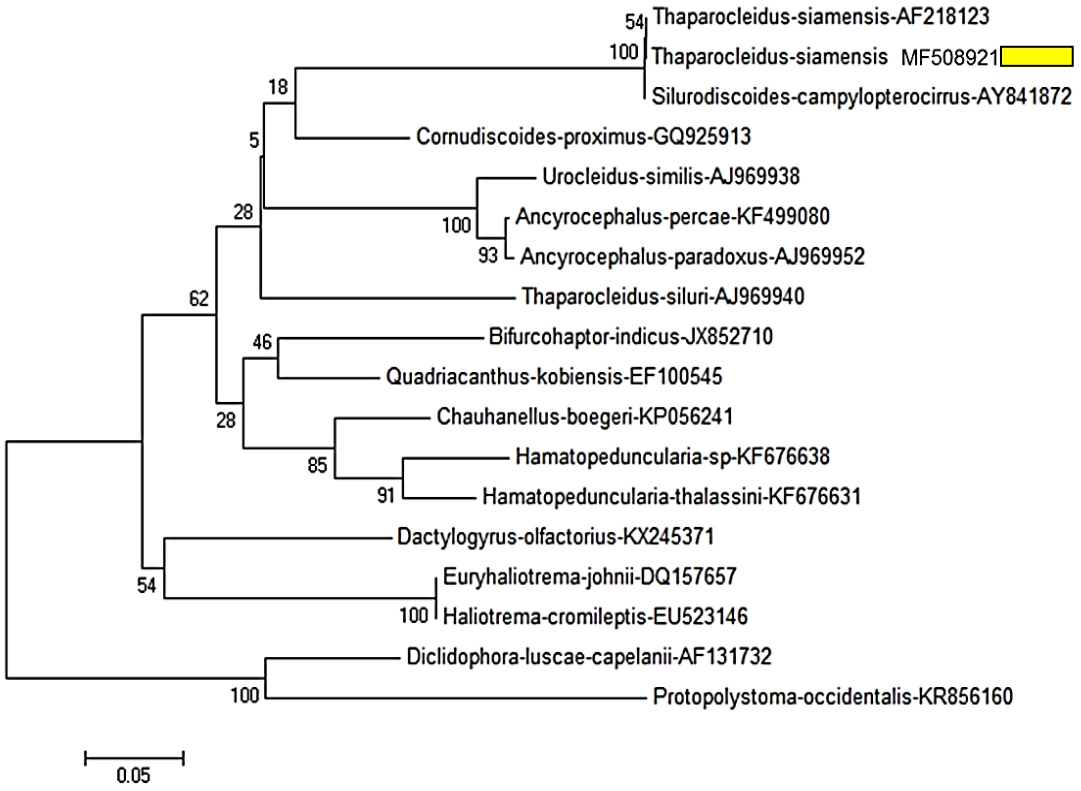


Figure 7: Phylogenetic positioning of *T. siamensis* was obtained based on 28S rDNA sequence using neighbour joining (NJ).

4.5. Aggregation pattern of gill monogenean parasite on gills of *P. hypophthalmus*

Wet mount of infected gills of *P. hypophthalmus* collected from the cages farms at Murbad, Maharashtra showed dactylogyrids, a monogenean attached to gill filaments in differential numbers at various regions (posterior, central and anterior) of gill (Plate 6). Further, in some cases showed co-occurrence of both dactylogyrids and trematode larvae (metacercariae) in gill filaments. Manifestation and attachment of dactylogyrids with gill filaments were observed either singly with one secondary lamellae or interlamellar (Plate 6). However, the metacercariae produced only local changes in the cartilage and appeared to infect the gills non-randomly, occurring both close to the tip and mid-region of the filaments. In the present study, the cysts are attached to the cartilage of the filament on one side only. Consequently, these ellipsoidal cysts become aligned alongside to the gill filaments and were surrounded by a thin layer of cartilaginous cells and a layer of collagenous connective tissue (plate 7).

Although, the interspecific interaction between monogenean and trematode larvae (metacercariae) was not studied in the present study, the parasites' preference of site and host is studied. Moreover, this is the first report of concurrent infection in gills of monogenean (dactylogyrids) and trematode larvae (metacercariae) in *P. hypophthalmus*.

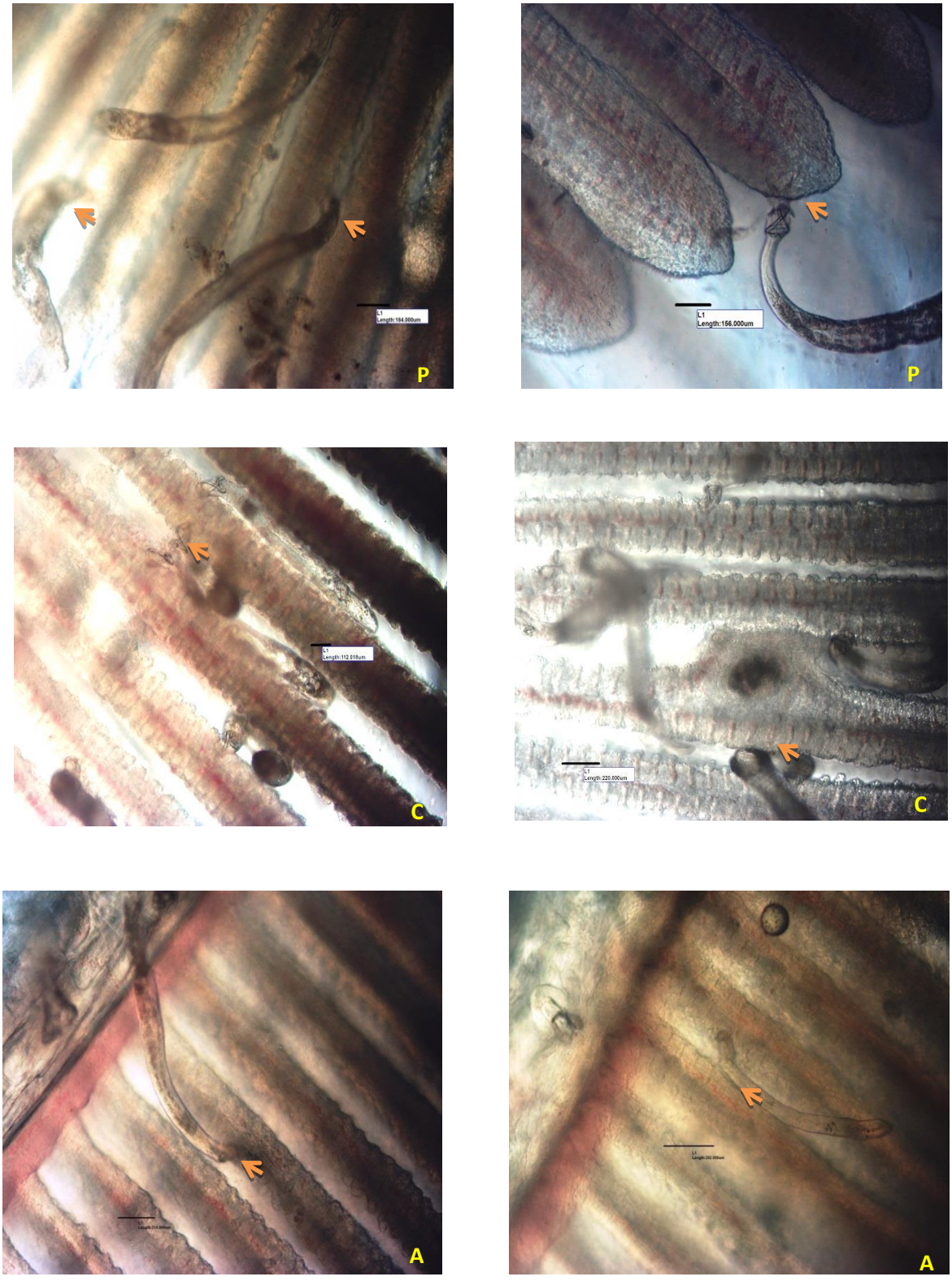


Plate : 6: Micropholographs of Dactylogyrids (*Thaparocleidus* spp.) infection in gills of the *P. hypophthalmus*. Wet mount of Dactylogyrids attached (arrow) to gill filaments at different regions of the gills; (P) Posterior (C) Central and (A) Anterior. Scales bars=200 µm.

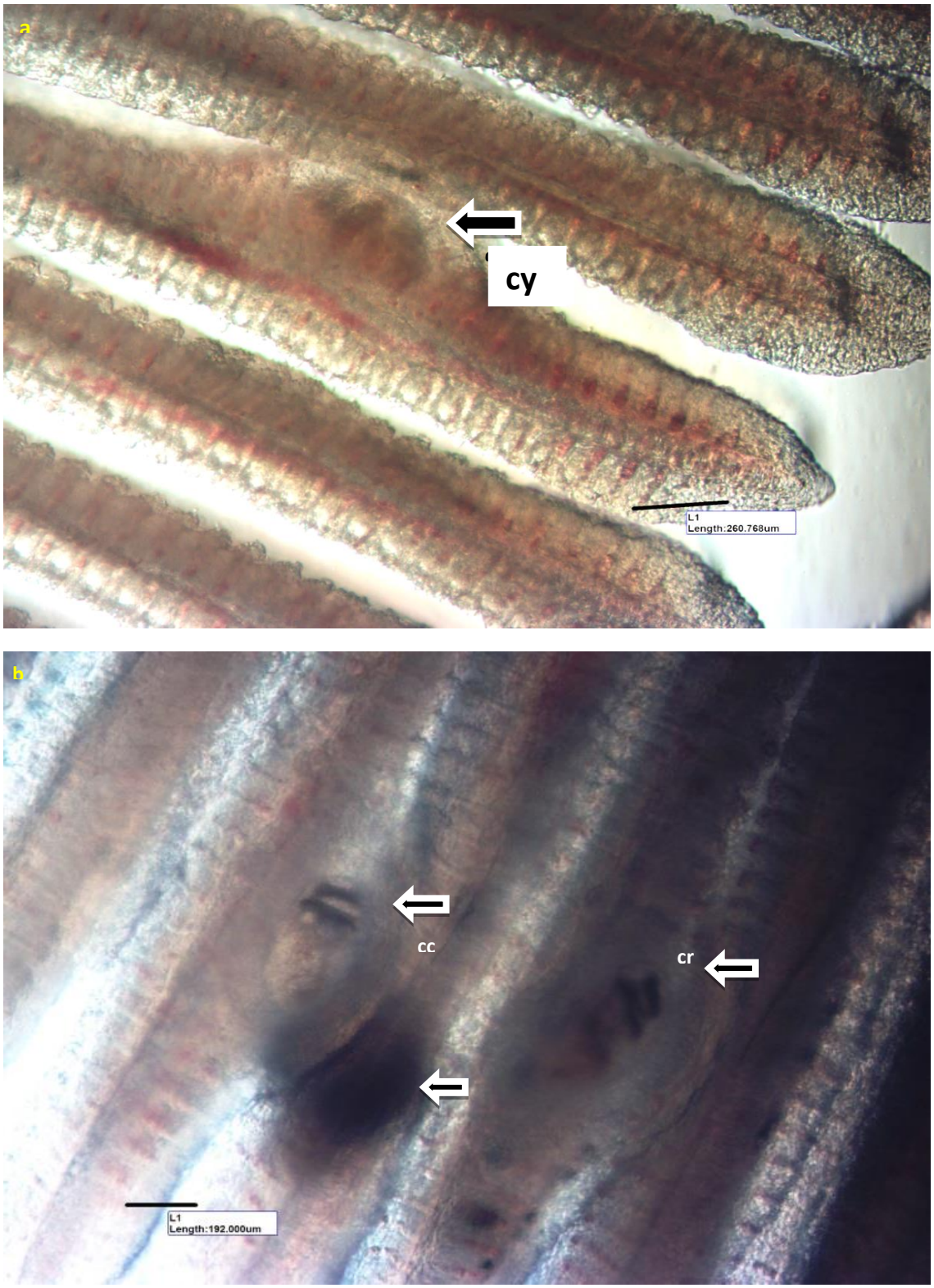


Plate 7: Microphotographs of trematode larvae (Metacercaria) in gills of *P. hypophthalmus*. (a) Wet mount of encysted metacercaria (cy) (arrow) attached on one side and located along to the cartilaginous ray (cr) of a gill filament. (b) Metacercaria (arrow) located in the cartilaginous filament ray and surrounded by cartilaginous cells (cc). (WM, 200X).

4.5.1. Host and tissue specificity of *Thaparocleidus* spp. in *P. hypophthalmus*

Host size in terms of standard length had a significant influence on dactylogyrids (*Thaparocleidus* spp.) intensity in *P. hypophthalmus*. A good quadratic correlation coefficient (r^2=0.699) was found between fish standard length and parasite intensity (figure 8). There was significant ($p<0.01$) difference in mean parasite intensity between <10 cm (25.40) and 10-15 cm (53.30) (t = -7.103, df =18); 10-15 cm and >15 cm (38) (t = 3.265, df =18) and <10 cm and >15 cm (t = -3.565, df = 18) of host standard length (table 9). Importantly, higher parasite density was observed in fish with 10 to 15 cm standard length in comparison to other two groups. However, there were neither significant relationships nor any interaction effects (quadratic correlation coefficient (r^2=0.466) between parasite intensity and host weight (figure 9).

The mean parasite intensity was similar in the gill chamber, both left and the right side. Further, box plot of parasite numbers and each gill showed a decrease in parasite intensity from 1 to 4 (figure 10). Interestingly, the number of parasites was not found to be significantly ($p>0.05$) differed in between gill arch 1 and 2 and between gill arch 3 and 4; but significantly ($p<0.05$) differed when compared gill arch 1 with gill arch 3 and 4 (table 10) of the both sides of gill chambers. The number of parasites decreased with the depth of the gill arch location. Significantly high levels of (69.31%) of total *Thaparocleidus* spp. were recorded on gill arch1 and 2 and subsequently a decreased (30.69%) of total parasite recorded on gill arch 3 and 4 (table 10).

Further, a significant ($p<0.05$) difference in percentage parasite number was recorded among the selected microhabitat of gill arch of *P. hypophthalmus*. Significantly ($p<0.05$) high percentages of parasites, 48.84% and 48.47%, were recorded in the medial and central region of gill arch, respectively. In comparison, other parts such as dorsal (31.84%), posterior (35.04 %), ventral (20.05%) and anterior (17.48%) showed moderate low infestation (table 11). Box plot of microhabitat preference of *Thaparocleidus* spp. (dactylogyrids) on *P. hypophthalmus* showed number of dactylogyrids on

the 3 regions along the dorsal-ventral and the anterior-posterior axis along of the gill arches (figure 11a & b).

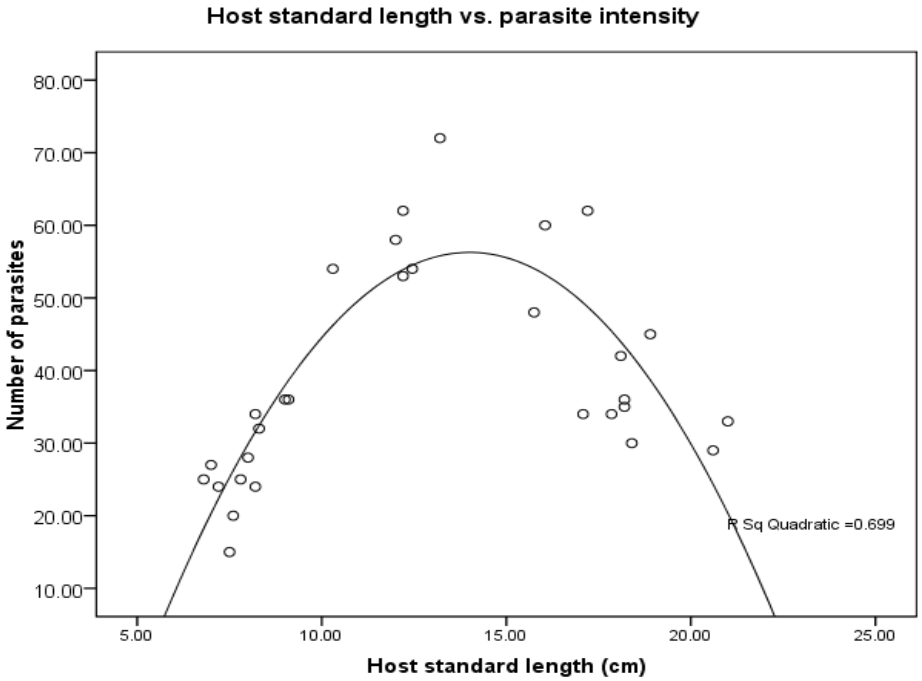


Figure 8: Scatter plot of total number of dactylogyrids verses host (*P. hypophthalmus)* standard length (cm), (n=30). R^2 quadratic= 0.699; Pearson correlation coefficient=0.322

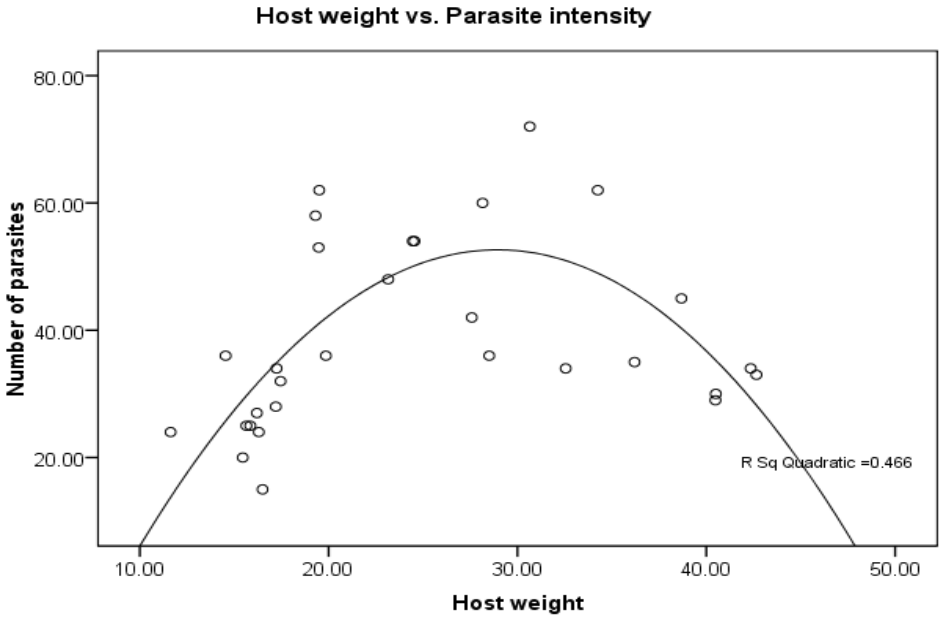


Figure 9: Scatter plot of total number of parasite verses host (*P. hypophthalmus)* weight (g), (n=30). R^2 quadratic= 0.466; Pearson correlation coefficient=0.230

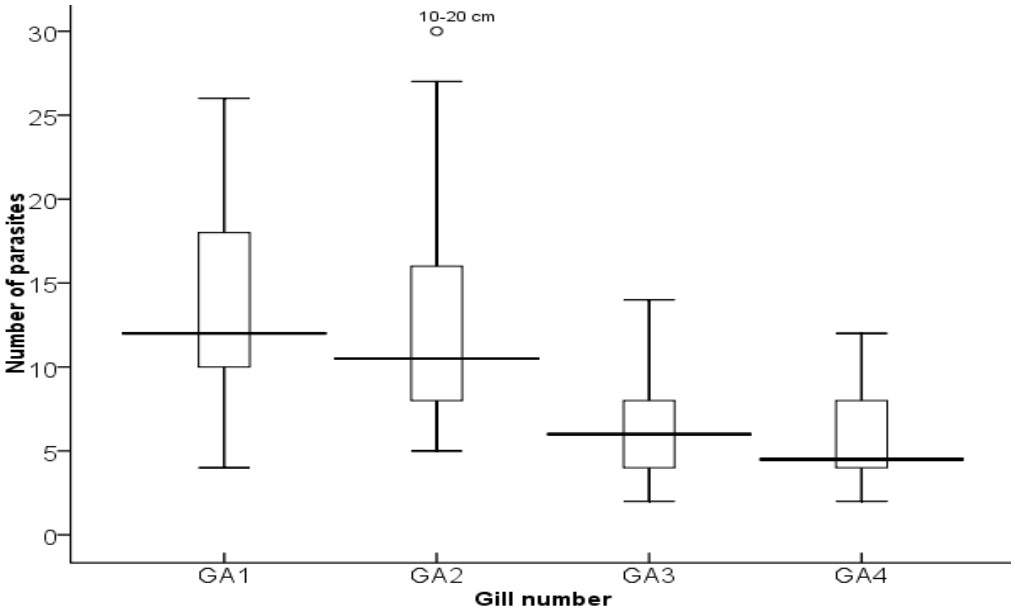


Figure 10: Number of dactylogyrids on the 4 gill arches, 1 to 4, from external to internal of *P. hypophthalmus*.

Table 9: Effect of host size (standard length) on *Thaparocleidus* spp. intensity

Group	Host size (Standard length)	Mean intensity of parasite	t-values	P value
G1	<10 cm	25.40		
G2	10-15 cm	53.30		
G3	>15 cm	38.00		
G1 vs. G2			-7.103	.000
G2 vs. G3			3.265	.004
G1 vs. G3			-3.565	.002

Table 10: Percentage dactylogyrids intensity of four gills of *P. hypophthalmus.* (n=10)

Gill number	% parasite intensity
Gill 1	35.30[b]
Gill 2	34.01[b]
Gill 3	16.62[a]
Gill 4	13.96[a]

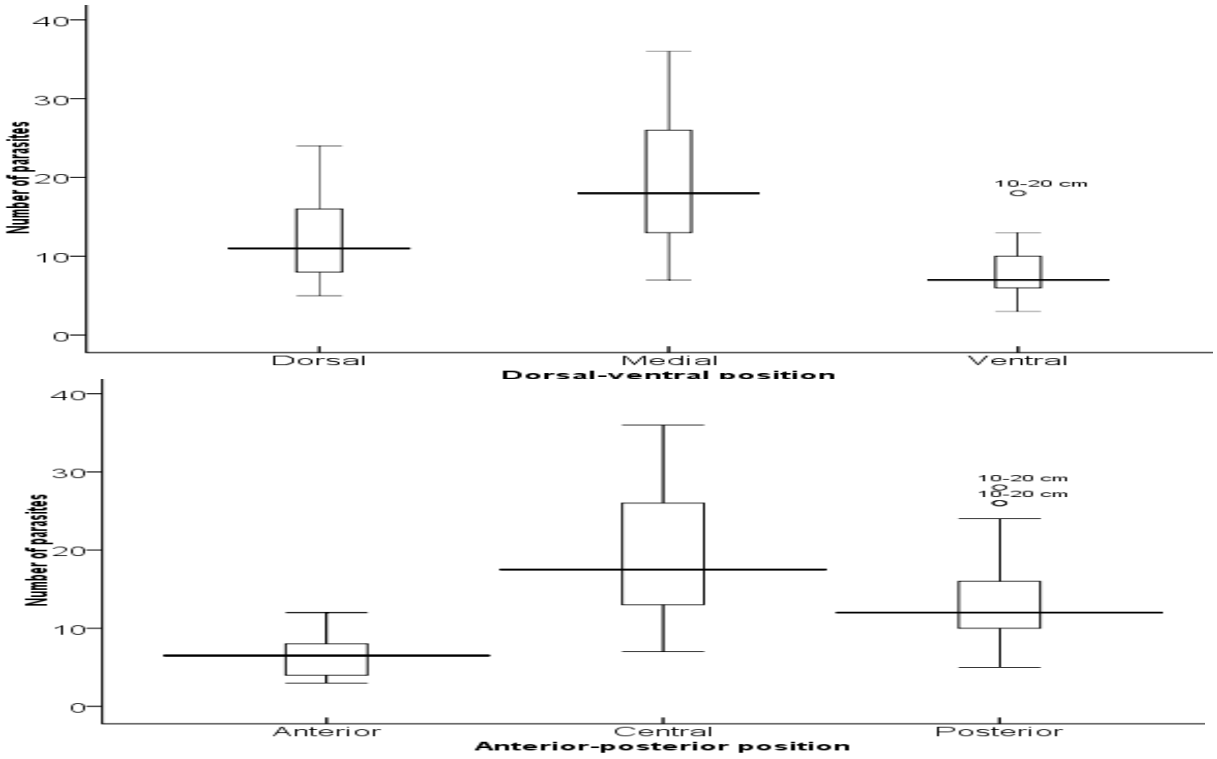


Figure 11: Microhabitat preference of *Thaparocleidus* spp. on *P. hypophthalmus*. (a) Number of parasite on the 3 regions along the dorsal-ventral axis of the gill arches. (b) Number of parasite on the 3 regions along the anterior-posterior axis of the gill arches. Box indicates the interquartile range, centre solid line indicates median number of parasites, wishkers indicate the data range (excluding outliers), and hollow circles indicate outliers.

Table 11: Percentage *Thaparocleidus* spp. intensity at various microhabitats of gills of *P. hypophthalmus.* (n=10)

Regions of gill (microhabitat)	% parasite intensity
Dorsal	31.87[b]
Medial	48.84[a]
Ventral	20.05[c]
Anterior	17.48[c]
Central	48.47[a]
Posterior	35.04[b]

Principal component 1 and 2 constituted 85.18% of the total variability in parasite microhabitat selection over the six different regions including four gill arch (figure 12). X-axis represents extracted component 1 (gill arch 1 and 2, dorsal, posterior, central and medial gill regions) having 72.84% of the variability in distribution of parasites between hosts and Y-axis represents extracted component 2 (gill arch 3 and 4 and anterior gill region) with 12.34% variability. The extracted components of both 1 and 2 were found to be positively related and a significantly high extraction was estimated for GA2 and GA4 respectively.

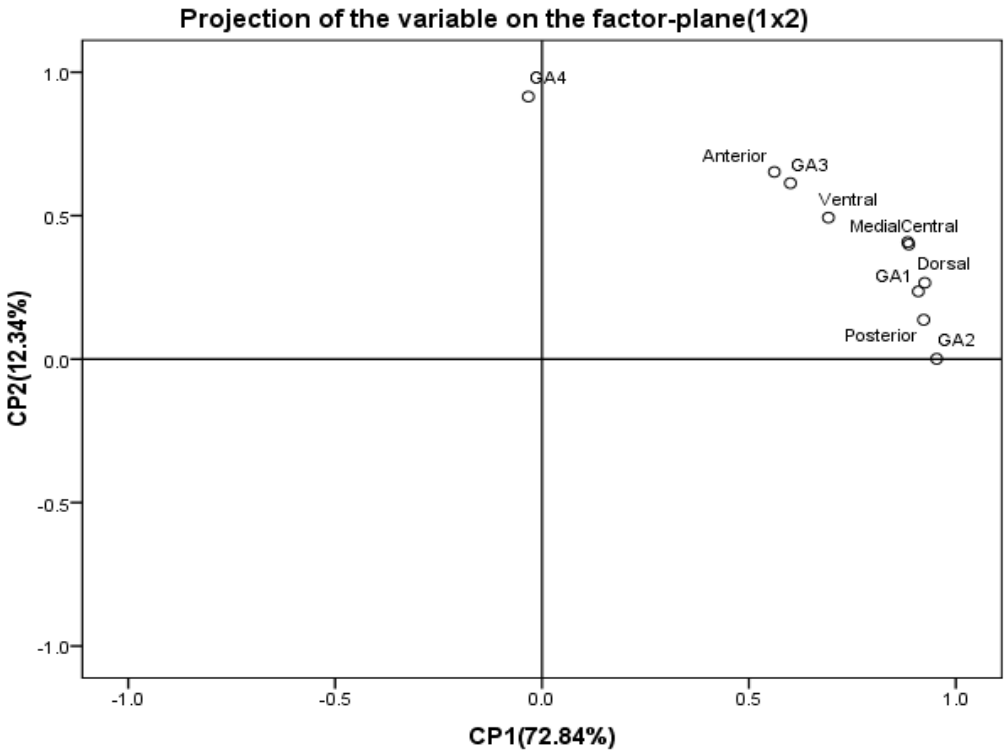


Figure12: Principal component plot of 4 gill arches (1 to 4) and different regions (dorsal, medial, ventral, posterior, central and anterior) of gills of *P. hypophthalmus* infested with *Thaparocleidus* spp. (dactylogyrids), with 85.18% of total variance.

4.6. Pathology associated with monogenean parasite infection in *P. hypophthalmus*

4.6.1 Histo-morphological changes in gills of *P. hypophthalmus* associated with dactylogyrids

Samples of diseased fish were collected from the cages farms at Murbad, Thane. Post observation of gross clinical signs (darkening of gills, excessive mucus secretion and presence of gill monogenean in wet mount of gills); gills were processed for histological studies. Histological analysis of gills indicated interlamellar manifestation and attachment of dactylogyrid (*Thaparocleidus* spp.) monogenean to the gill filaments between the lamellae, particularly secondary gill lamellae of the *P. hypophthalmus* (Plate 8 & 9) by means of their two hamuli pairs, affecting lamellae around (Plate 8b and 8c), and gill tissue reactions ranged from the focal to multifocal. Fish gill with a high level of infection had some low to a mostly elevated severity of reaction and observed distortion of gill filaments associated with parasite at the various regions of attachments (Plate 8a). Further, gill pathology was characterized by depression in the gill lamellae, proliferation of the cells into the interlamellar space, an increase in chloride and epithelial cells, lamellar fusion (Plate 8b), infiltration of inflammatory cells and proliferation of mucous cells (Plate 8c). Occasionally, the attachment and movement across the gills lamellae led to a rupture of distal epithelial and inflammation of vascular structures.

Fish with a low level of infection showed a mild tissue reaction and sometimes, showed only a depression in the gill filament caused by the attachment of the parasite (Plate 9). Further, the obvious alteration of the secondary gill lamellae structure with marked hyperplasia in mucous cells and subsequent lamellar fusion; this commonly found at the vicinity of the attachment site of the parasite (Plate 9b).

The histological sections of the gills of infected fish showed manifestation of only dactylogyrid (*Thaparocleidus* spp.) parasite between the lamellae (Plate 10a), attached to the various regions of gill filaments (Plate 10 a & b). The gill section exhibited congestion and fusion of secondary gill

lamellae at various regions of the gill filaments. A pressure on the gill filament and sloughing of the secondary gill lamellar epithelium and marked atrophy were noticed at the site of parasite attached (Plate 10b). Attachment of parasite at the central region of primary lamellae caused depression in gill filament (Plate 11a) and a congestion and hyperplasia of mucus secreting cells of the gill filament was noticed when the parasite attached to the anterior region of gill filaments (Plate 11b).

In few infected gills, both dactylogyrids (*Thaparocleidus* spp.) and trematode larvae (metacercaria) were observed in the wet mount of gill filaments. This was further corroborated by the gill histology sections (Plate 12 & 13). Manifestation of dactylogyrids parasite between primary lamellae caused a loss and sloughing of epithelium of secondary gill lamellae (Plate 12a & b). Further, a distal clubbing (DC) of secondary lamellae and infiltration of inflammatory cells in the distal part were observed in parasite infected gill histology sections of *P. hypophthalmus* (Plate 12c & d). Concurrently, trematode larva (metacercaria) infestation of gill filaments of *P. hypophthalmus* was noticed in gill histology sections (Plate 13). The manifestation of trematode larvae (metacercaria) at the central and distal regions of gill filament was noticed in histological sections of gills of *P. hypophthalmus* (Plate 13a & c). Further, encysted form of metacercaria in the cartilaginous filament ray was observed in histological sections of infected gill of *P. hypophthalmus*. Histological sections showed a local deformation of the cartilaginous filament ray caused by the presence of a metacercaria but did not greatly alter the linear orientation of the ray (Plate 13c). The encysted metacercaria surrounded by young chondrocytes, and chondroblasts were observed in histological sections (Plate 13d).

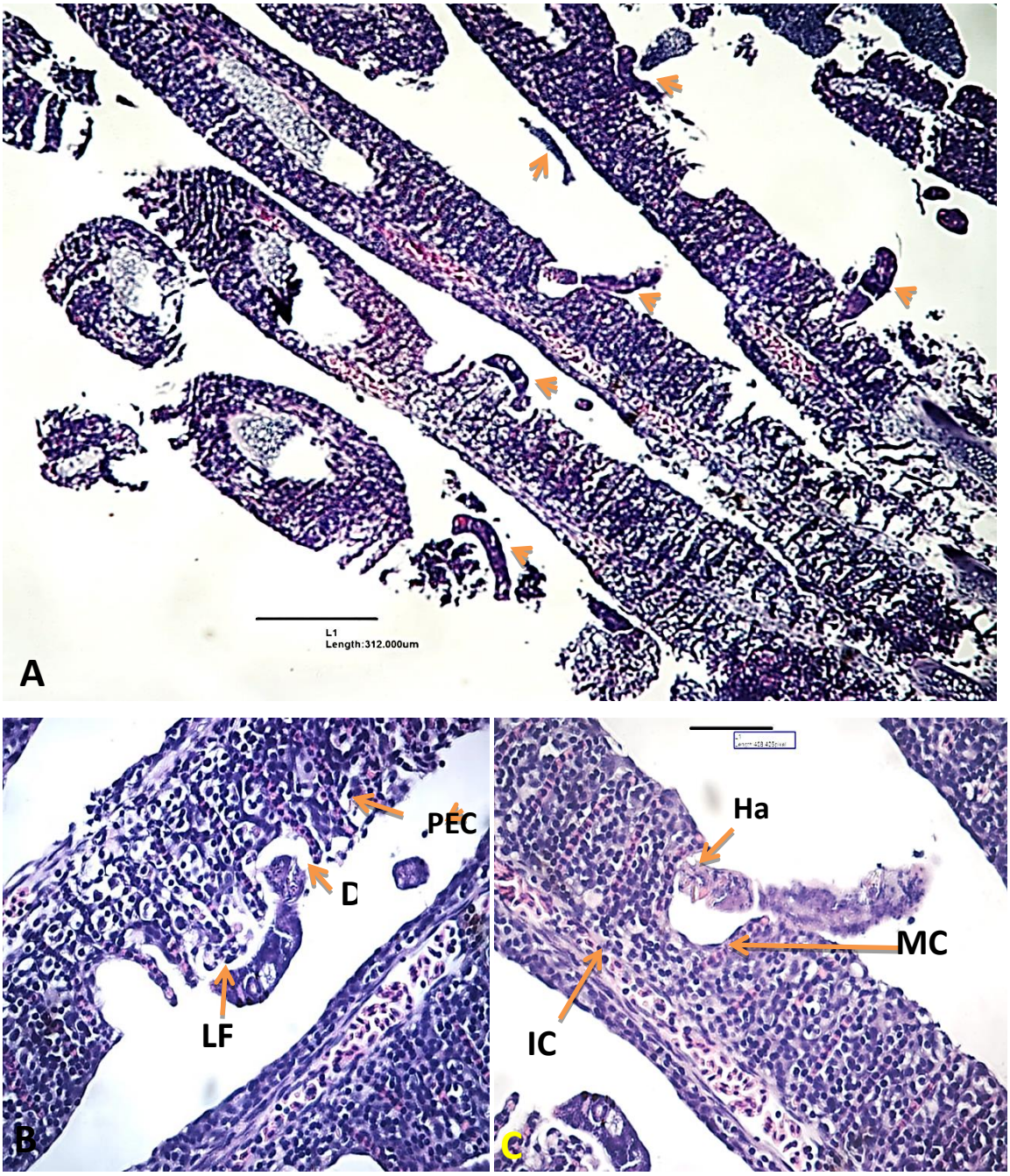


Plate 8: Histological sections of gills showing the Dactylogyrids (*Thaparocleidus* sp.) attached (arrow) to gill filaments at different regions of the gills. Arrows indicate the pathology of interest. (a) High level of infestation showing distortion in gill filaments associating with parasite at the site of attachment; (H&E, 200X) (b) the site of parasite attachment caused a depression in the gill filament with proliferative epithelial cells and causing lamellar fusion; (H&E, 400X). (c) With a severe degree of infiltration of inflammatory cells and a mild to moderate proliferation of mucous cells on the secondary gill lamellae, (H&E, 400X). D, depression; Ha, hamulus; IC, inflammatory cells; LF, Lamellar fusion; MC, mucous cell; PEC, Proliferative epithelial cells.

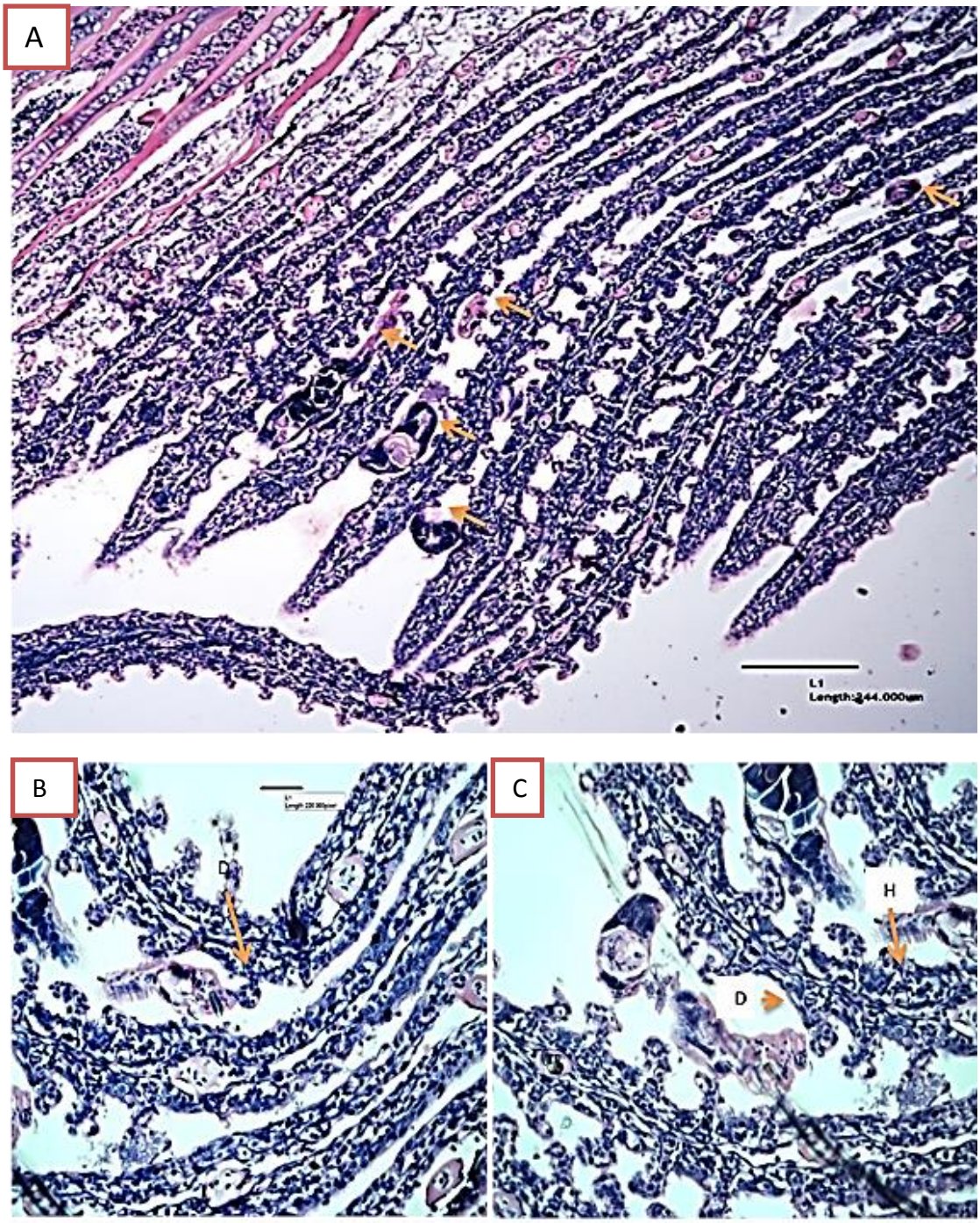


Plate 9: Histological sections of gills showing the Dactylogyrids (*Thaparocleidus* sp.) attached (arrow) to gill filaments at different regions of the gills. Arrows indicate the pathology of interest. (a) Low level infestation, interlamellar presence of parasite at the various site of attachment; (H&E, 200X) (b) the site of parasite attachment caused a pressure on the gill filament; (H&E, 400X). (c) With mild severity on the secondary gill lamellae, depression in gill filament, and marked hyperplasia and fusion of secondary lamellae (H&E, 400X). D, depression; H, hyperplasia.

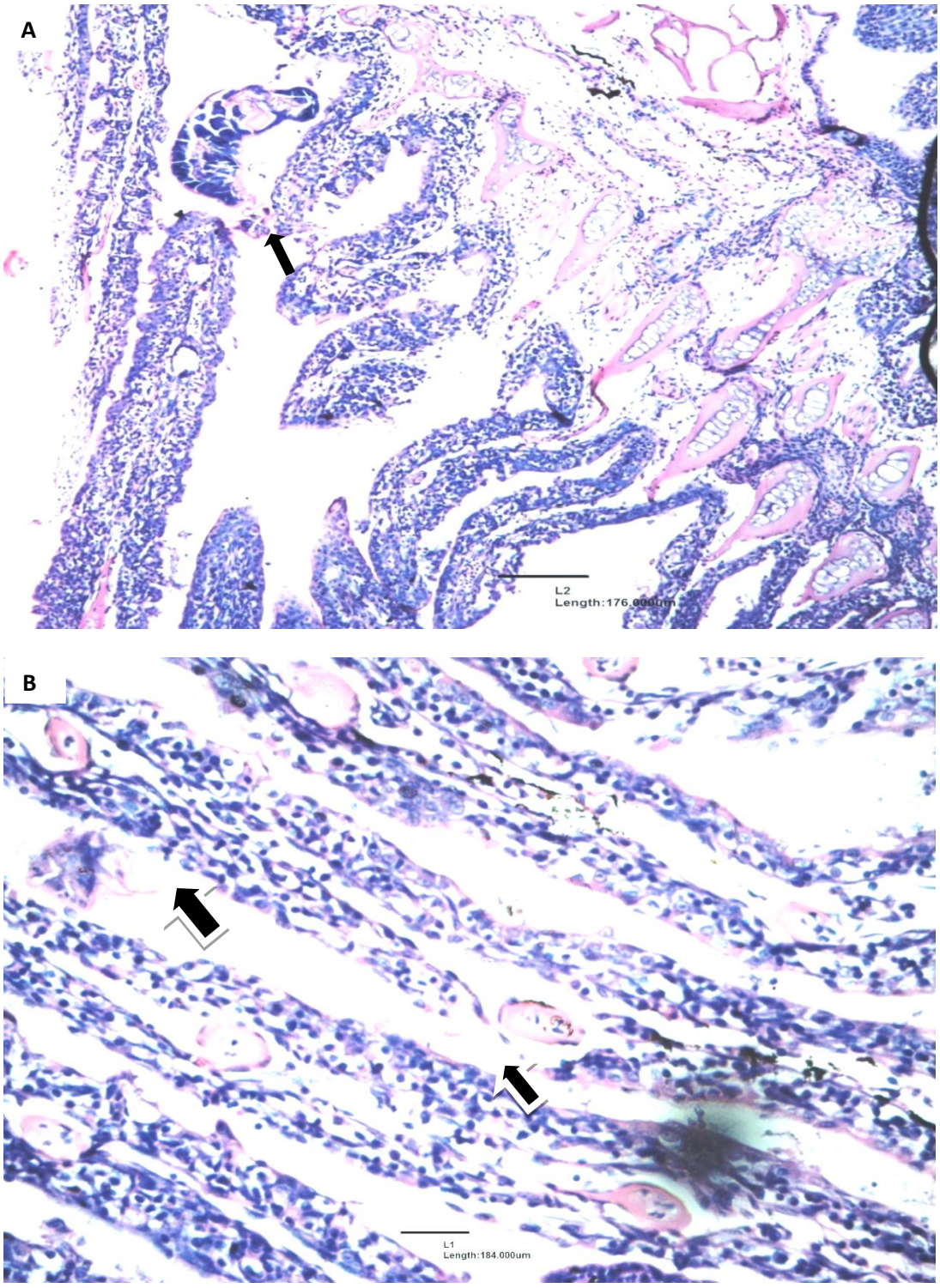


Plate 10: Histological sections of gills showing interlamellar presence of parasite Dactylogyrids (*Thaparocleidus* sp.) and attached (arrow) to gill filaments. Arrows indicate the pathology of interest. (A) Secondary gill lamellae showing congestion and fusion of secondary gill lamellae (arrow) at the many sites of gill; (H&E, 200X) (B) The site of parasite attachment caused a pressure on the gill filament and sloughing of the secondary gill lamellar epithelium and marked atrophy (H&E, 400X).

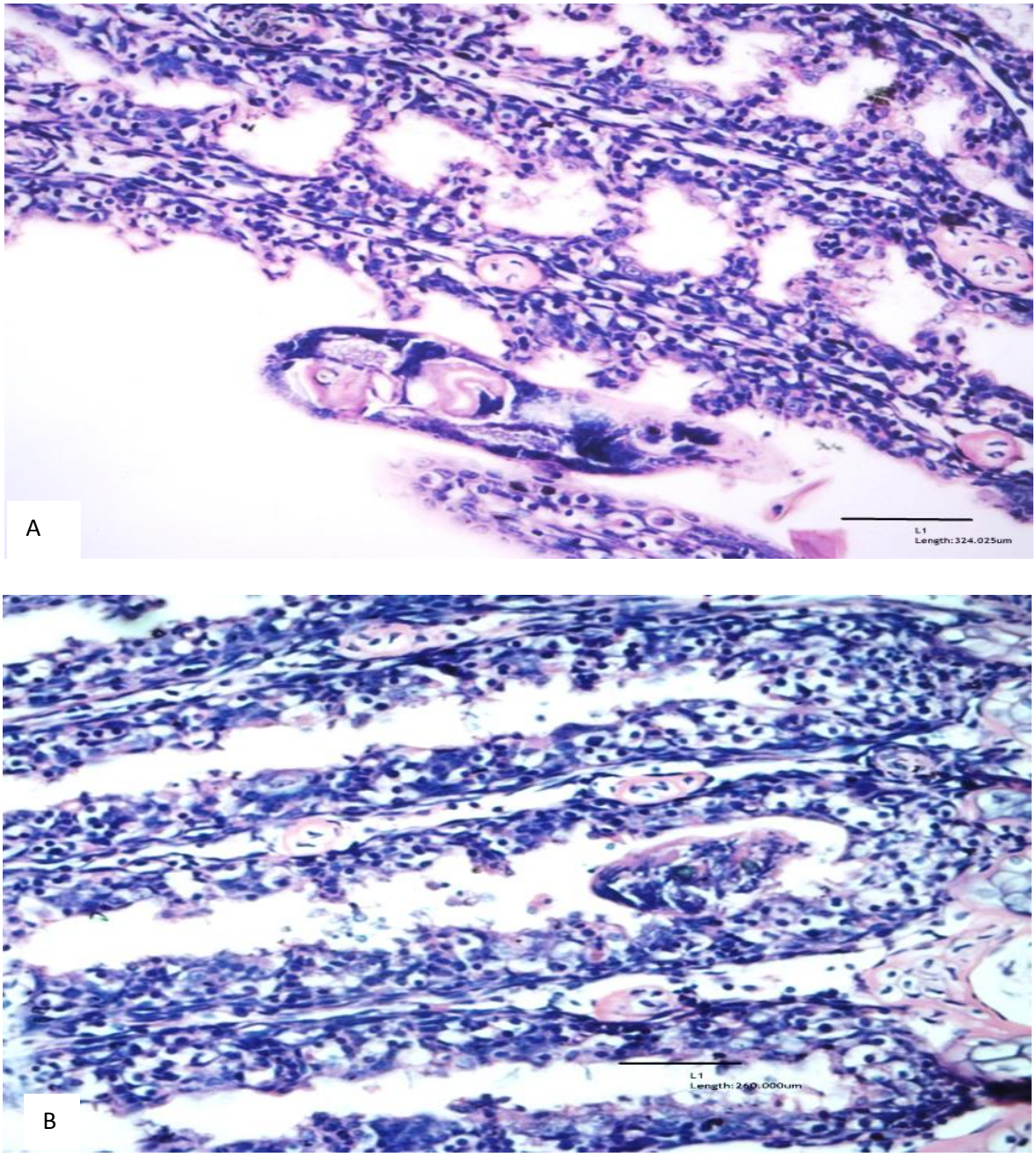


Plate 11: Histological sections of gills of Dactylogyrids-infested *P. hypophthalmus* showing mature monogenean parasite (arrow) attached to the various region of primary gill filament. Arrows indicate the site of attachment and pathology of interest. (A) Presence of parasite at the central region of primary lamellae caused depression in gill filament; (H&E, 400X) (B) Interlamellar presence of parasite at anterior region caused congestion and hyperplasia of mucus secreting cells of the gill filament; (H&E, 400X).

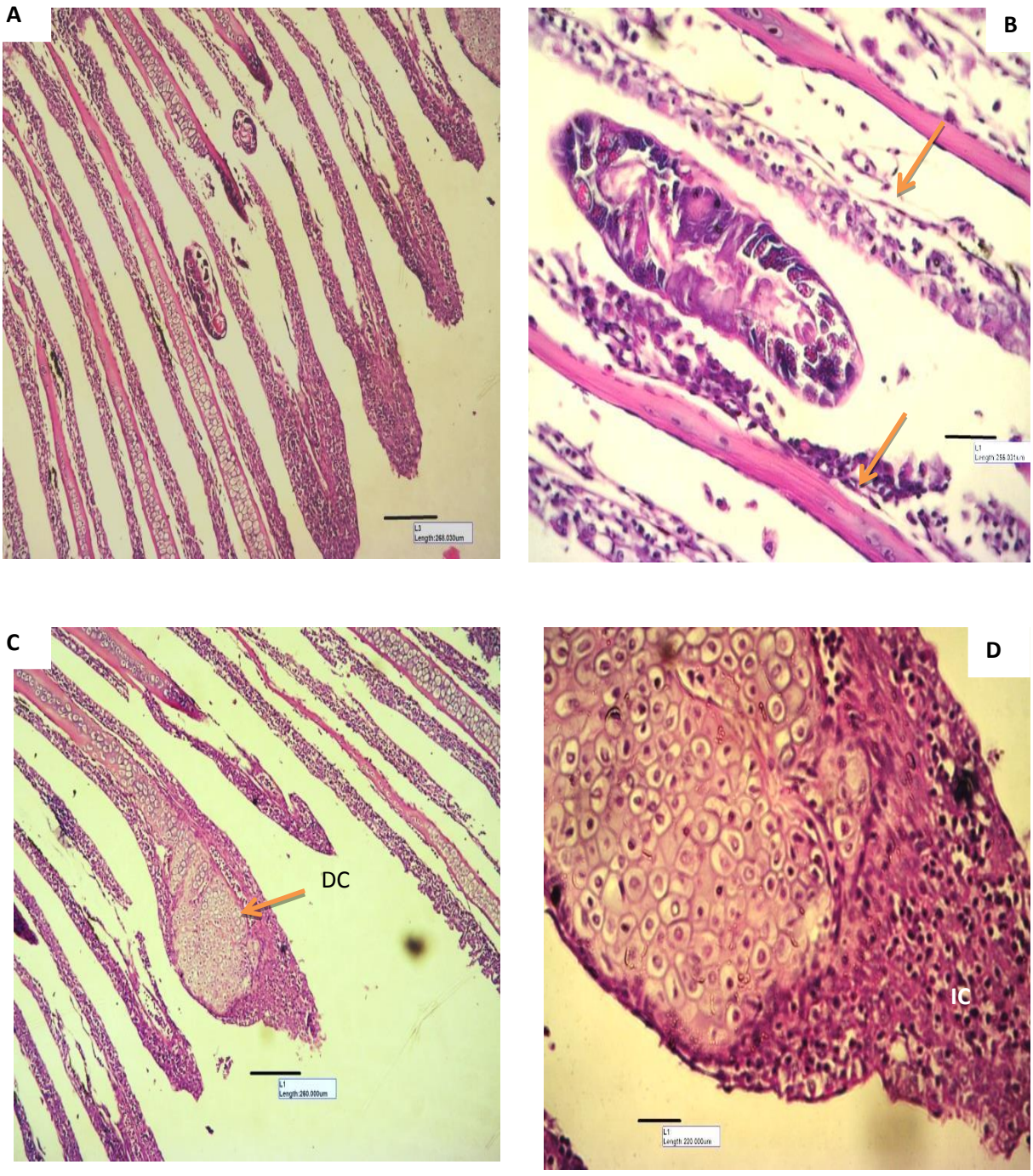


Plate 12: Histological sections of gills of Dactylogyrid-infested *P. hypophthalmus* showing presence of mature monogenean parasite (arrow) at the various regions of primary gill filaments. Arrows indicate the pathology of interest. (A) Interlamellar manifestation of parasite in between primary lamellae; (H&E, 200X) (B) section is showing loss of secondary lamellae, and sloughing of epithelium of secondary gill lamellae; (H&E, 400X). (C) Distal clubbing (DC) of secondary lamellae in the gill of parasite infected *P. hypophthalmus*, (H&E, 200X) (D) Distal clubbing and infiltration of inflammatory cells in the distal part of parasite infected *P. hypophthalmus*, (H&E, 400X).

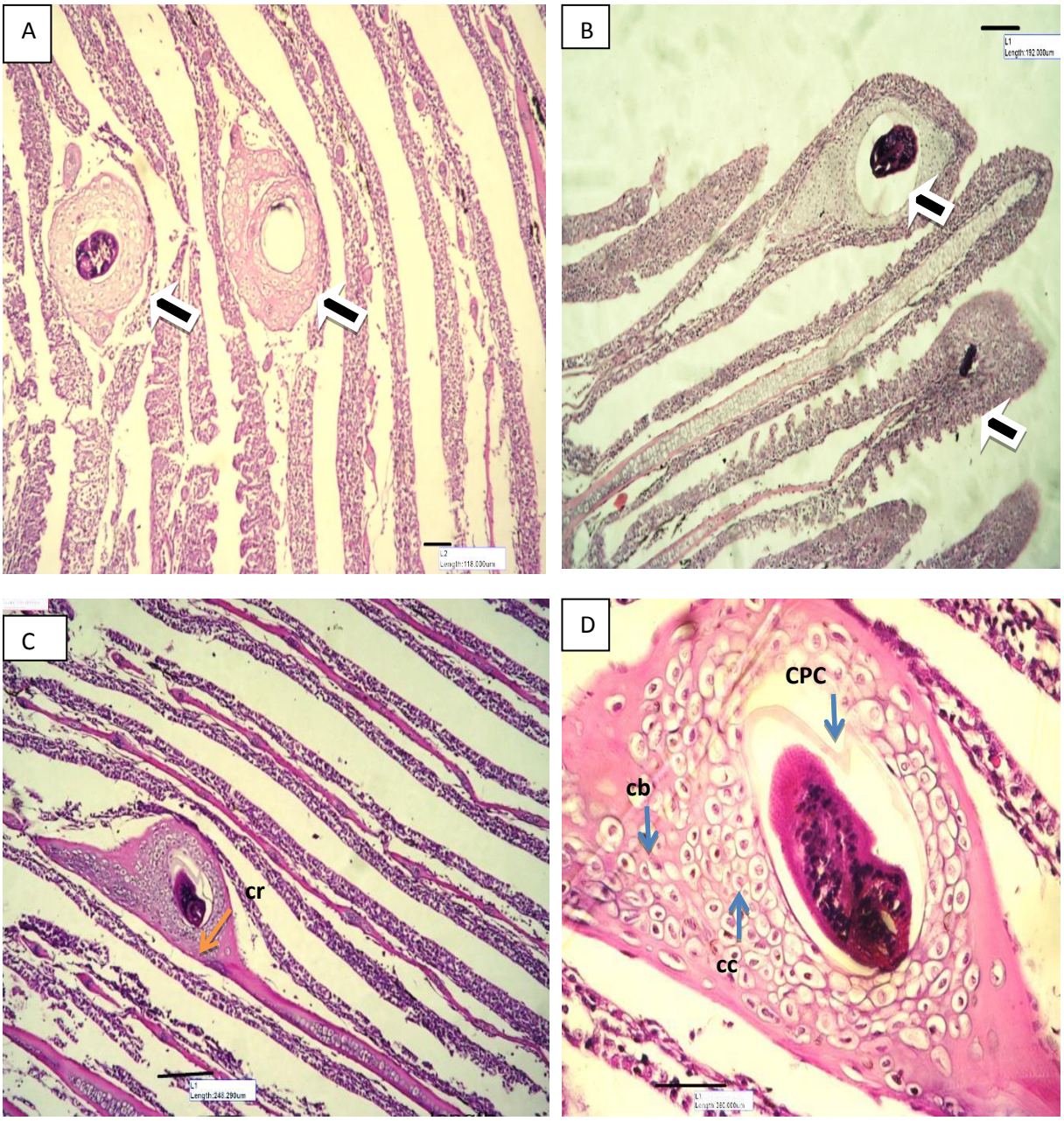


Plate 13: Histological sections of trematode larvae (metacercaria) infested different region of gill filaments of *P. hypophthalmus*. Arrows indicate the site of infection and pathology of interest. (A) Gill of *P. hypophthalmus* is showing trematode (metacercaria) in the central region of gill filament and encysted by chondroblasts and chondrocyte cells, (H&E, 200X) (B) Gill of *P. hypophthalmus* is showing trematode metacercaria at the distal/posterior region of gill filament and encystment by chondroblasts and chondrocyte cells, (H&E, 200X). (C) Histological section showing a local deformation of the cartilaginous filament ray caused by the presence of a metacercaria (arrow) but does not greatly alter the linear orientation of the ray. (H&E, 200X). (d) The metacercaria is surrounded by young chondroblasts and chondrocytes and presence of contentional hyaline parasitic capsule. (H&E, 400X). cb, Chondroblst; cc, Chondrocytes; cr, Cartilaginous filament rays; cy, Cyst; CPC, Contentional hyaline parasitic capsule.

4.6.2. Clinical pathology of *P. hypophthalmus* naturally infested by dactylogyrids (*Thaparocleidus* spp.)

In the present study, a significant effect on haematological and serum biochemical parameters was observed in *P. hypophthalmus* when naturally infested with high level of *Thaparocleidus* spp. (dactylogyrids).

4.6.2.1 Haematological parameters

4.6.2.1.1 Total erythrocyte count (TEC), total leucocyte count (TLC), haemoglobin (Hb), packed cell volume (PCV), blood indices (MCV, MCH and MCHC) and differential leucocyte count (DLC)

Haematological parameters including blood indices of *Thaparocleidus*-infested and uninfested fish are presented in table 12. There was a significant ($p<0.05$) elevation in haematological parameters (TEC, TLC, Hb, PCV) and differential leucocyte count (neutrophil, eosinophil and monocytes) of high level *Thaparocleidus*-infested groups in comparison to low level infested and uninfested fish (table 10). Different leucocyte cells were identified based on forms of nucleus and staining of granules of cells in differential staining (plate 14). The highest (41.66±1.38, 6.33±0.65, and 8.33±0.98) and lowest (23±1.33, 4±0.66 and 2±0.13) percentage of neutrophil, monocyte, and eosinophil in DLC were recorded in high level parasitized and uninfested group, respectively. However in case of lymphocyte, the highest (71±2.25) and lowest (43.66±2.33) percentage counts in DLC was recorded in uninfested and high level parasitized group, respectively. Moreover, significant ($p<0.05$) reduction in MCV, MCH, MCHC and lymphocyte was noticed in high level parasitized fish when compared with uninfested fish (table 12).

4.6.2.1.2. Principal component analysis of blood parameters and indices

The biplot of principal component analysis (PCA) of fig. 13 represented 90.07% of the total variance of haematological parameters of high level *Thaparocleidus*-infested *P. hypophthalmus*. The first component (PC1) depicts approximately 76.71% of the total variance which indicates the separation among the groups through the distribution of the significantly high but negatively related MCV, MCH and positively related TEC, Hb and monocytes of the fish (table 12). The second component (PC2) explains

13.36% of the total variance, showing the separation among the groups with positively high related PCV and negatively with MCHC values of the fish.

4.6.2.2. Serum total protein, A,G AND A:G

The effect of different level of *Thaparocleidus* spp. infestation on serum protein profiles in *P. hypophthalmus* is presented in table 12. A significant ($p<0.05$) elevation in level of total protein, albumin and globulin (U L^{-1}) was noticed with increase in degree of parasite infestation. However, there was no significant ($p>0.05$) difference in A to G ratio between uninfested and low level parasitized group. The highest values 5.06±0.08, 2.43±0.09, and 0.92±0.008 and lowest values 4.73±0.09, 2.19±0.09 and 0.86±0.006 for total protein, A, and A:G were recorded in high level parasitized and uninfested group respectively (table 12).

4.6.2.3. Oxidative stress markers

4.6.2.3.1. Superoxide dismutase (SOD) and catalase (CAT) activity

SOD activity (U mg $protein^{-1}$) and catalase activity (mM H_2O_2 decomposed min^{-1} mg $protein^{-1}$) in naturally infested *P. hypophthalmus* with *Thaparocleidus* spp. and uninfested fish is presented in figure 14 & 15. There was a significant ($p<0.05$) increase in both SOD and catalase activity in *Thaparocleidus*-infested *P. hypophthalmus*. The increase in activity of SOD and catalase was recorded with level of parasite infestation. Significantly ($p<0.05$) high SOD (46.26 U mg $protein^{-1}$) and catalase (5.81 mM H_2O_2 decomposed min^{-1} mg $protein^{-1}$) activities were recorded in the fish group infested with high level *Thaparocleidus*, in comparison to uninfested (28.26±1.85 and 3.5±0.95) groups(figure 14 & 15).

4.6.2.4. Enzymes related to metabolism

4.6.2.4.1 Serum glutamate oxaloacetate transaminase (sGOT), serum glutamate pyruvate transaminase (sGPT) and alkaline phosphatase (ALP) activity

sGOT (U L^{-1}), sGPT (U L^{-1}) and ALP (U L^{-1}) activity in *Thaparocleidus*-infested and uninfested *P. hypophthalmus* is depicted in figure 16. A significant ($p<0.05$) elevation in activity of sGOT and sGPT was noticed

in high level parasitized group in comparison to uninfested group. However, there was no significant ($p>0.05$) increase in ALP activity in parasitized group when compared with uninfested group. The highest sGOT, sGPT and ALP activities recorded were 65.50±3.66, 51.00±3.85 and 5.40±3.05 U L^{-1} respectively, in group infested with high level *Thaparocleidus* spp.. Moreover there was a significant ($p<0.05$) difference in activity of sGPT but not in case of sGOT and ALP in uninfested and low level parasitized group (figure 16).

4.6.2.4.2. Lactate dehydrogenease (LDH) activity

The effect of different levels of *Thaparocleidus* spp. infestation on LDH (U L^{-1}) in *P. hypophthalmus* is depicted in figure 17. Significant ($p<0.05$) increase in LDH (202±5.65) activity was observed in high level parasitized group when compared with uninfested group (176±4.88).

4.6.2.5 Serum markers associated with metabolic by-product

4.6.2.5.1 Blood glucose

Blood glucose level (mg dL^{-1}) in different groups of *P. hypophthalmus*, naturally infested with *Thaparocleidus* spp. and uninfested group is presented in figure 18. A significant ($p<0.05$) elevation in blood glucose level was noticed in high level parasitized group in comparison to uninfested group. Significant high (79.66±3.24) and low (58.50±2.80) level of blood glucose was recorded in high level of parasitized group and uninfested group respectively. However there was no significance difference in blood glucose content between uninfested and low level parasitized group (figure 18).

4.6.2.5.2. Lactate level

Lactate level (nM μL^{-1}) in different group of *P. hypophthalmus*, naturally infested with *Thaparocleidus* spp. and uninfested group is depicted in figure 19. A significant ($p<0.05$) high (0.31±0.001) lactate level was recorded in high level of parasitized group in comparison to (0.20±0.001) uninfested group.

4.6.2.5.3 Bilirubin level

The effect of *Thaparocleidus*-infestation on total biilirubin level (mg dL^{-1}) of *P. hypophthalmus* is presented in figure 20. There was no significant difference in serum bilirubin level in parasitized group when compared with uninfested group. Moreover, a low level of bilirubin was noticed in low parasitized group.

4.6.2.6 Principal component analysis of serum biochemical biomarkers

Biplot of principal component analysis represents 82.78% of the total variance in *P. hypophthalmus* parasitized with *Thaparocleidus* spp. (Fig 21). The first component (PC1) depicts approximately 66.73% of the total variance which indicates the separation among groups through the significantly high and positively related SOD, total protein, CAT, lactate, blood glucose, ALP, albumin, SGPT, LDH and SGOT of fish (figure 21). The second component (PC2) explains 16.05% of the total variance, showing significantly high negative correlation with globulin and positive correlation with A to G ratio and bilirubin level of fish.

Table 12: Haematological and serum protein profiles of different groups of *Thaparocleidus*-infested *P. hypophthalmus*. Values are expressed as mean ± SE (n = 6) in the columns with different superscripts differ significantly ($p<0.05$).

Parameters	Different level of parasite infested groups		
	G0 (uninfested)	G1 (low)	G2 (high)
TEC (10^6 cells mm^{-3})	$0.94^{a} \pm 0.009$	$1.07^{b} \pm 0.015$	$1.17^{c} \pm 0.005$
TLC(10^3 cells mm^{-3})	$97.53^{a} \pm 3.56$	$99.66^{a} \pm 6.23$	$109.30^{b} \pm 6.86$
HB (g dL^{-1})	$8.58^{a} \pm 0.61$	$9.06^{b} \pm 0.76$	$9.56^{c} \pm 0.78$
PCV (%)	$26.83^{a} \pm 1.41$	$27.51^{a} \pm 1.53$	$29.33^{b} \pm 1.49$
MCV (fL)	$283.50^{b} \pm 3.99$	$257.34^{a} \pm 5.17$	$251.22^{a} \pm 6.37$
MCH (pg)	$95.78^{c} \pm 2.85$	$81.72^{b} \pm 2.09$	$73.36^{a} \pm 2.38$
MCHC (%)	$33.83^{b} \pm 0.33$	$34.87^{b} \pm 0.92$	$29.28^{a} \pm 0.34$
Neutrophils/Heterophils	$23^{a} \pm 1.33$	$25^{a} \pm 1.08$	$41.66^{b} \pm 1.38$
Eosinophil	$2^{a} \pm 0.13$	$2.66^{a} \pm 0.68$	$8.33^{b} \pm 0.98$
Monocytes	$4^{a} \pm 0.66$	$4^{a} \pm 0.38$	$6.33^{b} \pm 0.65$
Lymphocytes	$71^{b} \pm 2.25$	$68.33^{ab} \pm 2.38$	$43.66^{a} \pm 2.33$
Total protein (g dL^{-1})	$4.73^{a} \pm 0.032$	$4.92^{b} \pm 0.073$	$5.06^{c} \pm 0.166$
Albumin (g dL^{-1})	$2.19^{a} \pm 0.023$	$2.29^{b} \pm 0.031$	$2.43^{c} \pm 0.026$
Globulin (g dL^{-1})	$2.54^{a} \pm 0.022$	$2.63^{b} \pm 0.024$	$2.61^{b} \pm 0.031$
A:G	$0.86^{a} \pm 0.016$	$0.87^{a} \pm 0.022$	$0.92^{b} \pm 0.024$

TEC: Total erythrocyte count; TLC: Total leucocyte count; Hb: haemoglobin; PCV: Packed cell volume; MCV: Mean cell volume; MCH: Mean cell haemoglobin; MCHC: Mean cell haemoglobin concentration; DLC: Differential leucocyte count; A : Albumin; G: Globulin

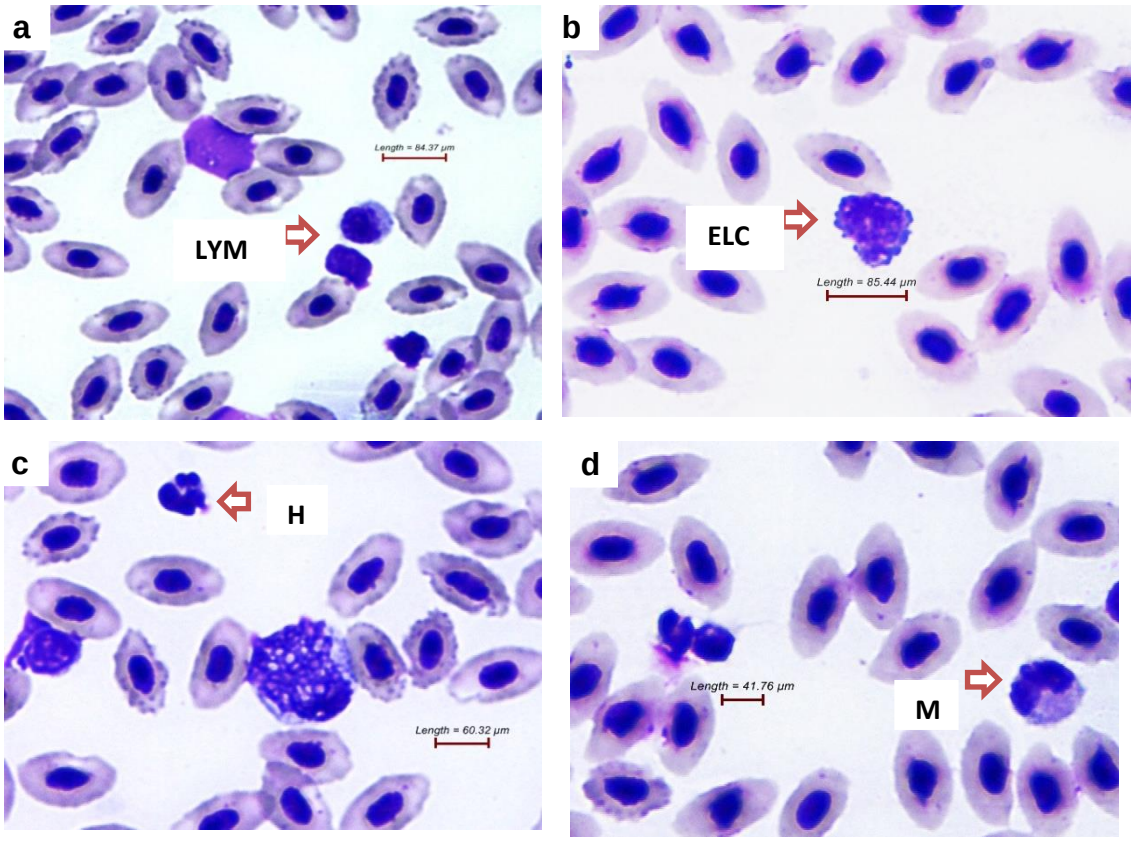


Plate 14: Light micrographs of field-stained blood smear of *P. hypophthalmus* with special reference to different leucocyte cells a. Lymphocytes (LYM) b. Eosionphil like cells (ELC) c. Heterophil (H) d. Monocyte (M).

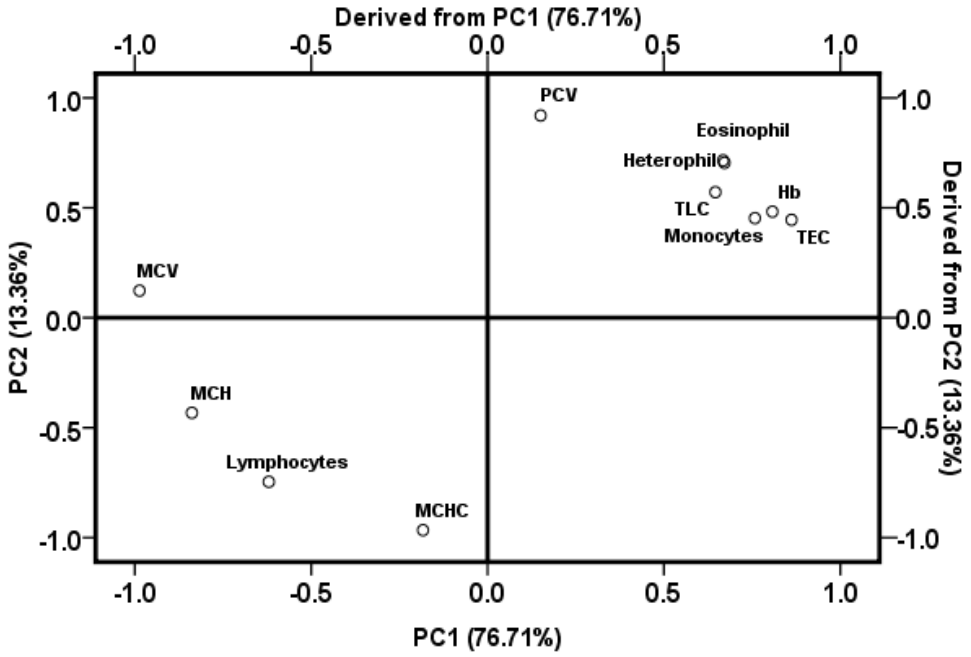


Figure 13: Principal component plot of (a) blood parameters (TEC, TLC, Hb, PCV, MCH, MCV, and MCHC) of high level dactylogrids infested *P. hypophthalmus* with 90.07% of total variance.

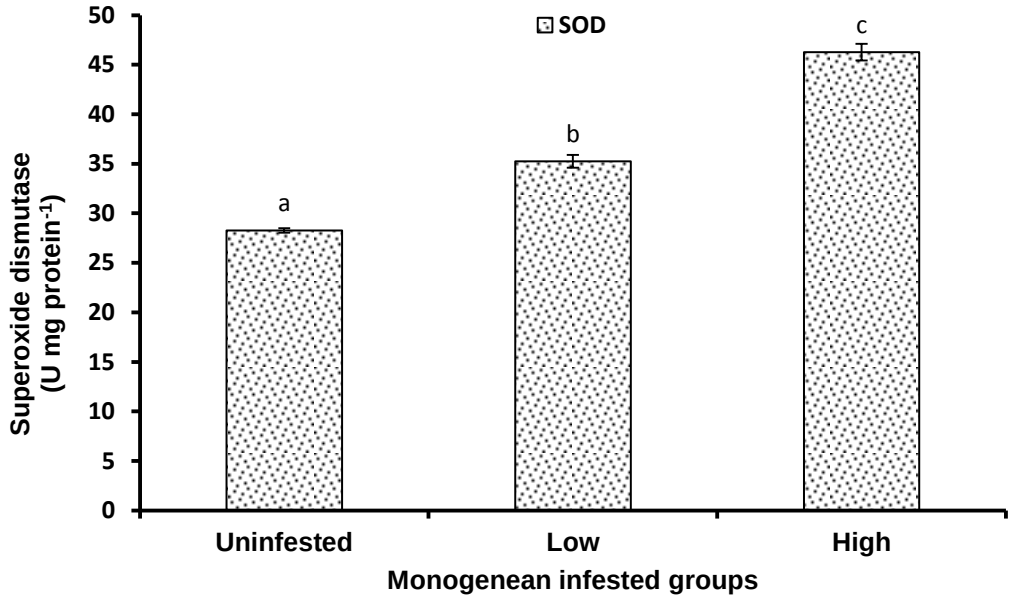


Figure 14: Effect of different level of *Thaparocleidus* sp. (gill monogenean) infestation on superoxide dismutase activity (SOD) in *P. hypophthalmus* (Values with different superscript differ significantly at $p < 0.05$ and expressed as mean ± SE, (n= 6).

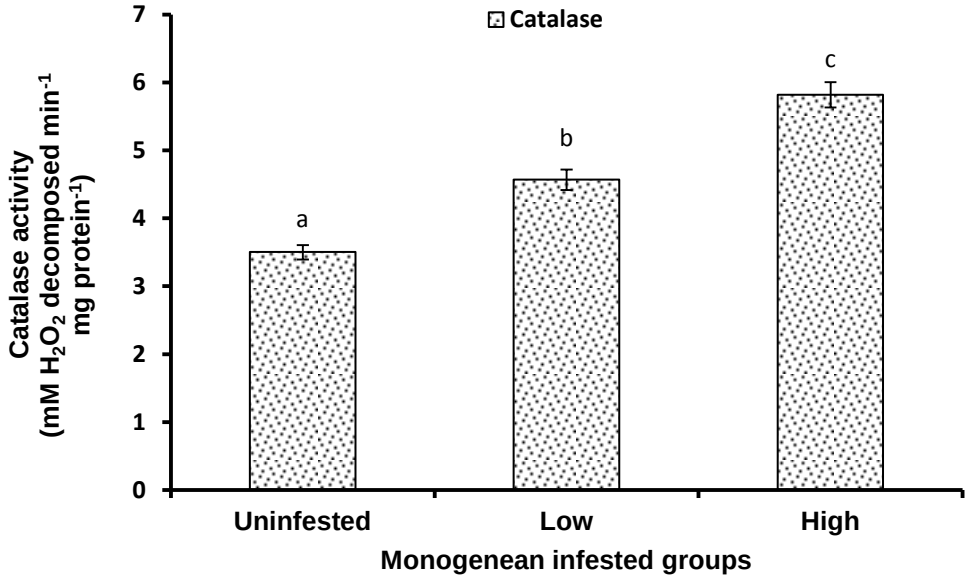


Figure 15: Effect of different level of *Thaparocleidus* sp. (gill monogenean) infestation on catalase activity of *P. hypophthalmus*. (Values with different superscript differ significantly at $p < 0.05$ and expressed as mean ± SE, (n= 6).

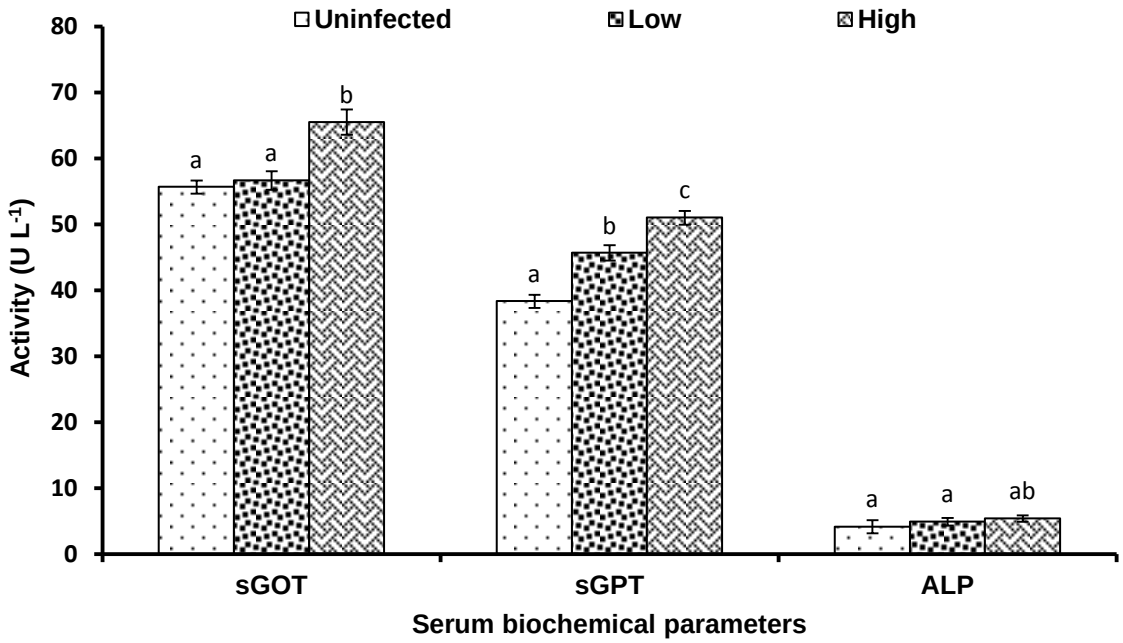


Figure 16: Serum glutamate oxaloacetate transaminase (sGOT), serum glutamate pyruvate transaminase (sGPT) and alkaline phosphatase (ALP) activity in *P. hypophthalmus* infested with different level of *Thaparocleidus* spp. (Values with different superscript differ significantly at $p < 0.05$ and expressed as mean ± SE, (n= 6).

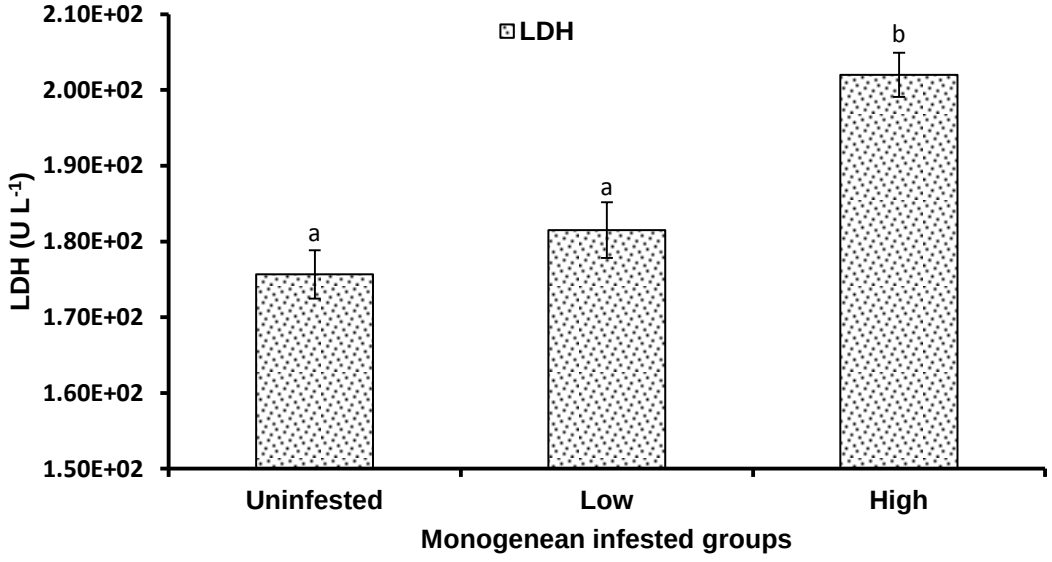


Figure 17: Effect of different level of *Thaparocleidus* spp. infestation on lactate dehydrogenase activity (LDH) of *P. hypophthalmus*. (Values with different superscript differ significantly at $p < 0.05$ and expressed as mean ± SE, (n= 6).

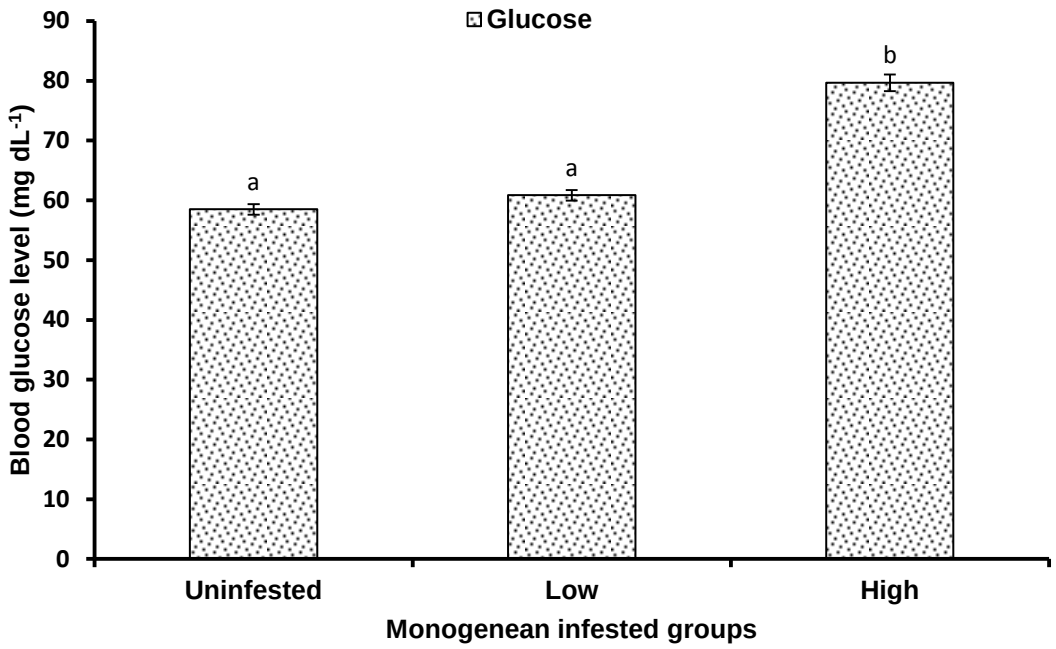


Figure 18: Effect of different level of *Thaparocleidus* spp. infestation on blood glucose level of *P. hypophthalmus*. (Values with different superscript differ significantly at $p < 0.05$ and expressed as mean ± SE, (n= 6).

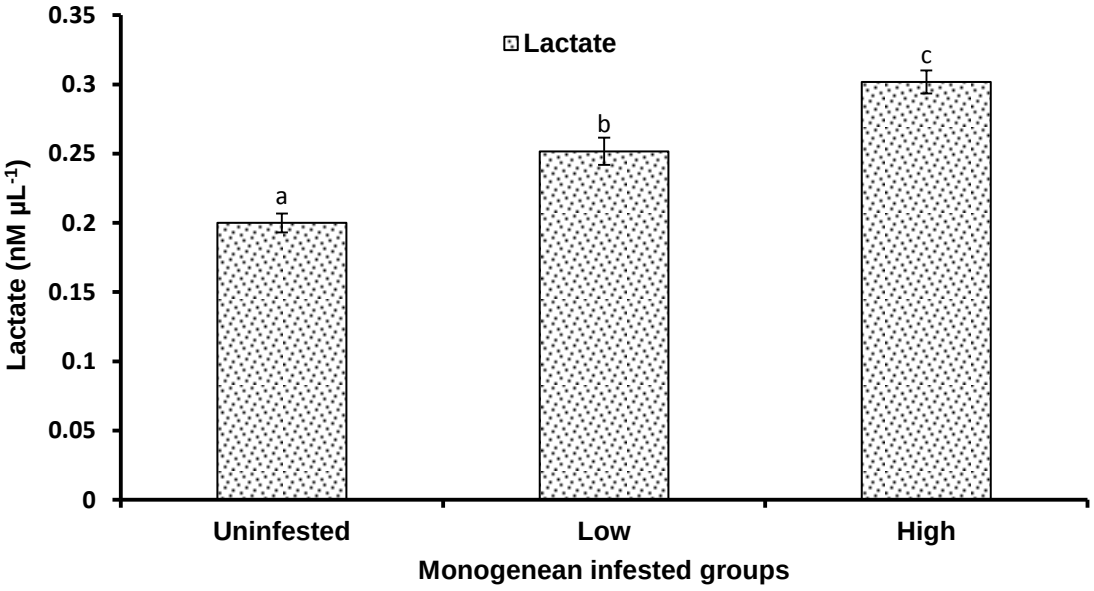


Figure 19: Effect of different level of *Thaparocleidus* sp. (gill monogenean) infestation on lactate level in serum of *P. hypophthalmus*. (Values with different superscript differ significantly at $p < 0.05$ and expressed as mean ± SE, (n= 6).

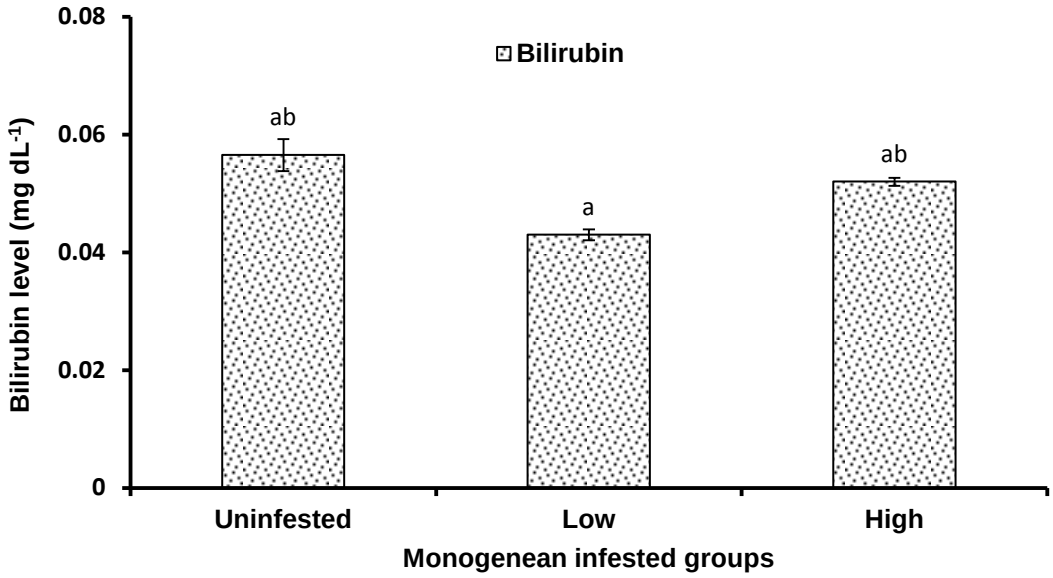


Figure 20: Effect of different level of *Thaparocleidus* sp. (gill monogenean) infestation on bilirubin level of *P. hypophthalmus*. (Values with different superscript differ significantly at $p < 0.05$ and expressed as mean ± SE, (n= 6).

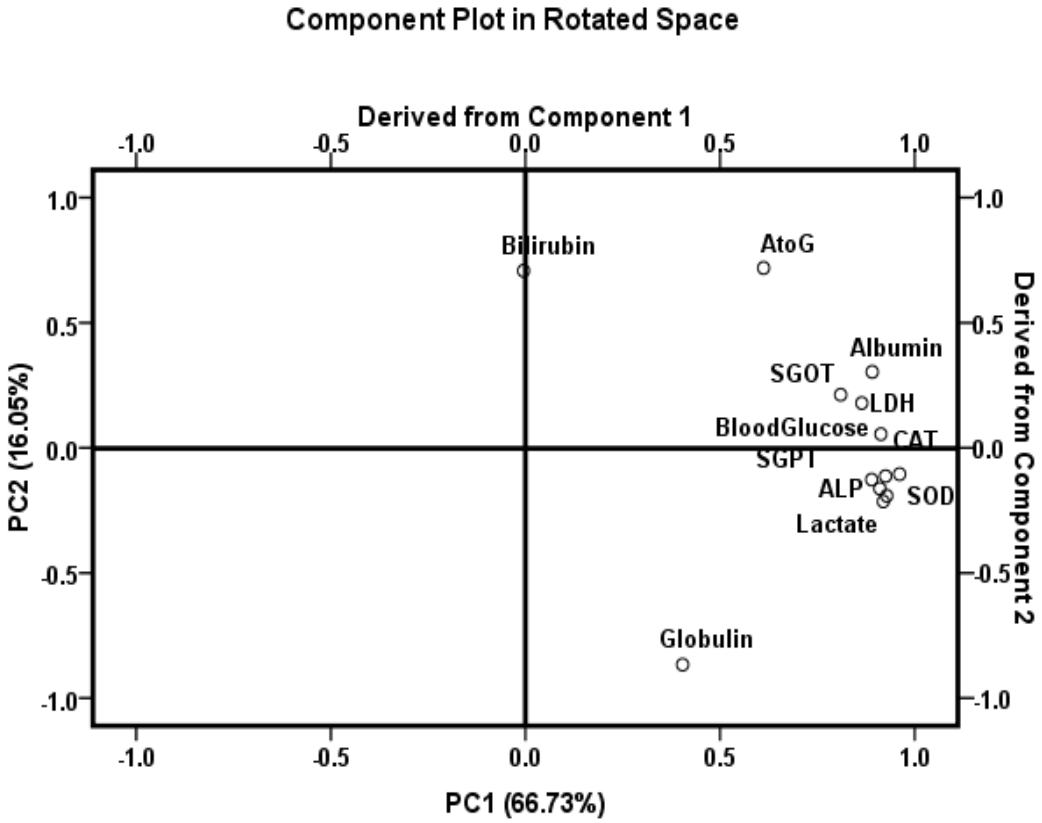


Figure 21: Principal component plot of (b) serum biochemical markers (total protein, albumin, globulin, A:G, sGOT, sGPT, LDH, ALP, lactate, bilirubin, SOD and catalase) of high level *Thaparocleidus* spp. (dactylogrids) infected *P. hypophthalmus*, with 82.78% of total variance.

4.7. Immunological responses of *P. hypophthalmus* artificially infected by dactylogyrids (*Thaparocleidus* spp.)

4.7.1. Haematological responses

Haematological profiles (TEC, Hb, PCV) and indices (MCV, MCH, MCHC, RBD and EOB) of various degrees of *Thaparocleidus*-infested groups differed in comparison to the control (uninfested) group (table 13). Significant ($p<0.05$) increase in TEC and Hb levels were observed in moderate to highly infested groups compared to control and low infested group. However, no significant ($p>0.05$) but increase in PCV values were recorded in infested groups in comparison to control (table 13). Blood indices MCV, MCH and MCHC were recorded significantly ($p<0.05$) decrease in moderate to highly infested groups in comparison to control. The erythrocytes cell integrity index, EOB of high degree of *Thaparocleidus*-infested group was found significantly ($p<0.05$) elevated when compared with all other groups including control. However, significant ($p<0.05$) increase in the count of total leucocyte was observed in all the infested groups when compared with control (table 13).

4.7.2. Serum biochemical parameters

Blood glucose level was found to be significantly ($p<0.05$) elevated in moderate and high level of *Thaparocleidus*-infested groups in comparison to control and low infested group. However, no significant ($p>0.05$) difference was observed in blood glucose level between low degree infested group and the control (figure 22). Significantly ($p<0.05$) elevated activities of SGPT and ALP was recorded in high and moderately infested groups in comparison to the control (figure 23). However, SGOT activity showed significant ($p<0.05$) increase in high degree of parasitized group in comparison to others. No significant ($p>0.05$) difference in SGOT activity was recorded between control and low to moderate infested group (figure 23).

The lactate dehydrogenase activity and lactate leveld were found to be significantly ($p<0.05$) elevated in the high and moderately infested group in comparison to control and low infested group (figure 24). Significantly ($p<0.05$) elevated serum bilirubin was recorded in the high and moderately *Thaparocleidus*-infested groups when compared with the control (figure 25).

Similarly, significantly ($p<0.05$) differed total creatinine level in serum of various degrees of parasitized fish was noticed in comparison to control (figure 25). However, no significant ($p>0.05$) difference was observed between low and control group for bilirubin level, and low and moderately infested group for creatinine level (figure 25).

4.7.3. Immunological parameters

4.7.3.1. Innate immune responses

The respiratory burst activity measured by NBT assay (OD at 620 nm) of the parasitized groups differed significantly ($p<0.05$) in comparison to the control (uninfested fish). Significantly elevated NBT levels were recorded in high and moderately *Thaparocleidus*-infested groups compared to the uninfested control (figure 26a). The myeloperoxidase content of the *Thaparocleidus* spp. dactylogyrid-infested groups measured at 450 nm differed significantly ($p<0.05$) from the control group and elevated MPO values were noticed in the high degree of parasite infested group (figure 26b).

A significant ($p<0.05$) increase in percent of phagocytic activity was found in moderate and high parasitized group. However, no significant ($P>0.05$) difference in phagocytic activity was observed between low degree infected group and the control group (figure 27a). The serum lysozyme activity and α-2 macroglobulin (α-2M) activities were significantly ($p<0.05$) elevated in moderate and high degree *Thaparocleidus*-infested fish in comparison to low and uninfested control fish. However, no significant ($P>0.05$) difference in serum lysozyme and α-2M activity was noticed in low degree parasitized fish when compared with the control (figure 27b & c). No significant ($p>0.05$) difference in total serum antiprotease activity was found between the control group and different degrees of monogenean infested groups except high degree of parasite infested group (figure 27d).

Total protein and globulin contents (g dl^{-1}) were significantly ($p<0.05$) elevated in high and moderate degree of parasite infested group in comparison to low and uninfested control group (figure 28a). However, the albumin content (g dl^{-1}) and albumin to globulin ratio were significantly ($p<0.05$) decreased in all the treatment groups when compared with the

control (figure 28a & b). The level of ceruloplasmin in serum was found to be dependent on the degree of infestation of fish with *Thaparocleidus* spp. However, the level of ceruloplasmin in serum was significantly ($p<0.05$) decreased in low infested fish and increased in high infested fish in comparison to the uninfested control fish (figure 28c). An increased level of total serum immunoglobulin (g dL^{-1}) was observed in all the treatment groups when compared with the control. However, a significantly ($p<0.05$) elevated concentration of serum IgM was noticed in high and moderately infested fish in comparison to low and uninfested fish (figure 28d).

4.7.3.2 Enzymes of oxidative stress (SOD, CAT, GST and Na^+-K^+-ATPase activity)

Significantly ($p<0.05$) elevated activities of SOD, catalase and GST in the liver and gills of various degrees of parasitized fish were recorded in comparison to uninfested control fish. Increased activities of SOD and CAT were observed in gills, liver and serum samples of moderately and highly infested fish when compared with low and uninfested fish (table 14). Similar observation was noticed in case of GST in liver and gill tissues of parasitized fish. However, decrease in the activity of Na^+-K^+-ATPase was estimated in gills and liver of fish with different degrees of parasite infestation when compared with uninfected control fish (table 15).

Table 13: Haematological profiles (total erythrocyte count (cells×10^6), haemoglobin content (g dL^{-1}), packed cell volume (%), mean cell volume (in fl), mean cell haemoglobin (pg), mean cell haemoglobin concentration (%), total leucocyte count (cells×10^3) and erythrocyte osmolality brittleness) of various degree of *Thaparocleidus*-infested *P. hypophthalmus*. Values are expressed as mean ± SE (n = 9) in the columns with different superscripts differ significantly ($p<0.05$).

Parameters	Degree of monogenean infesting			
	Control	**Low**	**Moderate**	**High**
TEC	1.03^{a}±0.01	1.04^{a}±0.00	1.13^{b}±0.00	1.14^{b}±0.01
Hb	8.43^{a}±0.06	8.53^{a}±0.05	8.93^{b}±0.05	8.96^{b}±0.15
PCV	26.33^{a}±0.58	27.37^{bc}±1.15	28.67^{c}±0.83	26.67^{ab}±1.15
MCV	254.92^{bc}±8.20	261.11^{c}±9.62	250.01^{ab}±5.83	232.67^{a}±3.12
MCH	81.62^{b}±1.41	81.52^{b}±0.50	78.82^{a}±0.89	78.21^{a}±2.05
MCHC	32.03^{ab}±0.82	31.25^{a}±1.25	31.53^{a}±0.56	33.66^{b}±1.43
TLC	83.66^{a}±1.15	87.37^{b}±1.57	95.00^{c}±1.73	101.67^{d}±1.52
EOB	0.434^{a}±0.00	0.467^{a}±0.00	0.467^{a}±0.00	0.633^{b}±0.02

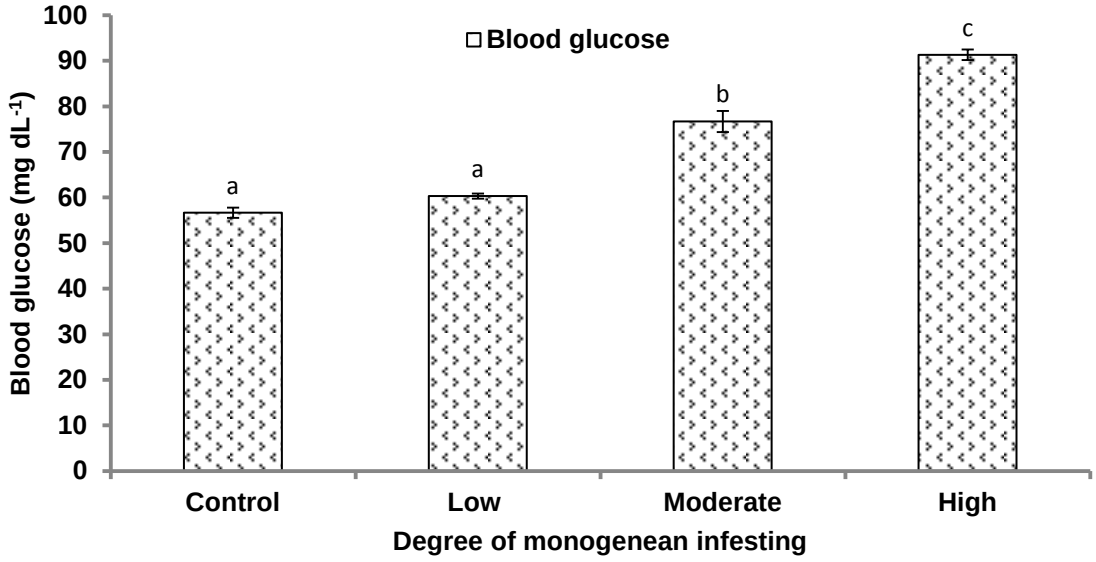


Figure 22: Effect of experimental infection of *Thaparocleidus* sp. (gill monogenean) on blood glucose level in *P. hypophthalmus*. (Values with different superscript differ significantly ($p < 0.05$) and are expressssed as mean ± SE) (n= 9).

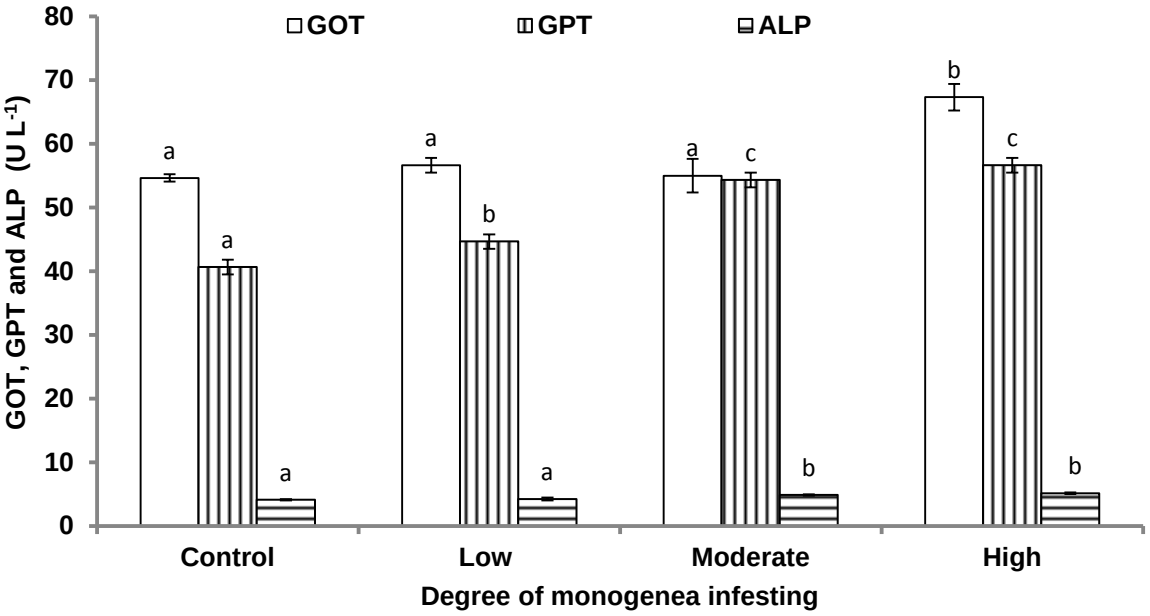


Figure 23: Effect of experimental infection of *Thaparocleidus* spp. on serum glutamate oxaloacetate transaminase (sGOT) and serum glutamate pyruvate transaminase (sGPT) and alkaline phospatase in *P. hypophthalmus*. (Values with different superscript differed significantly ($p < 0.05$) and expressed as mean ± SE) (n = 9).

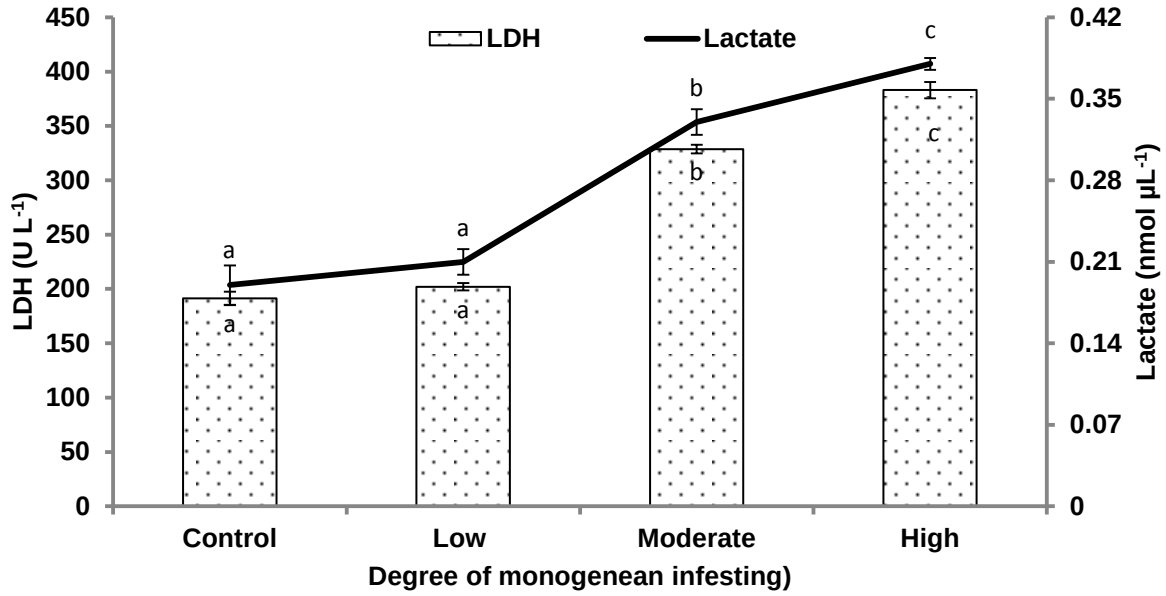


Figure 24: Effect of experimental infection of *Thaparocleidus* sp. on lactate dehydrogenase and lactate level in *P. hypophthalmus*. (Values with different superscript differed significantly ($p < 0.05$) and expressed as mean ± SE) (n = 9).

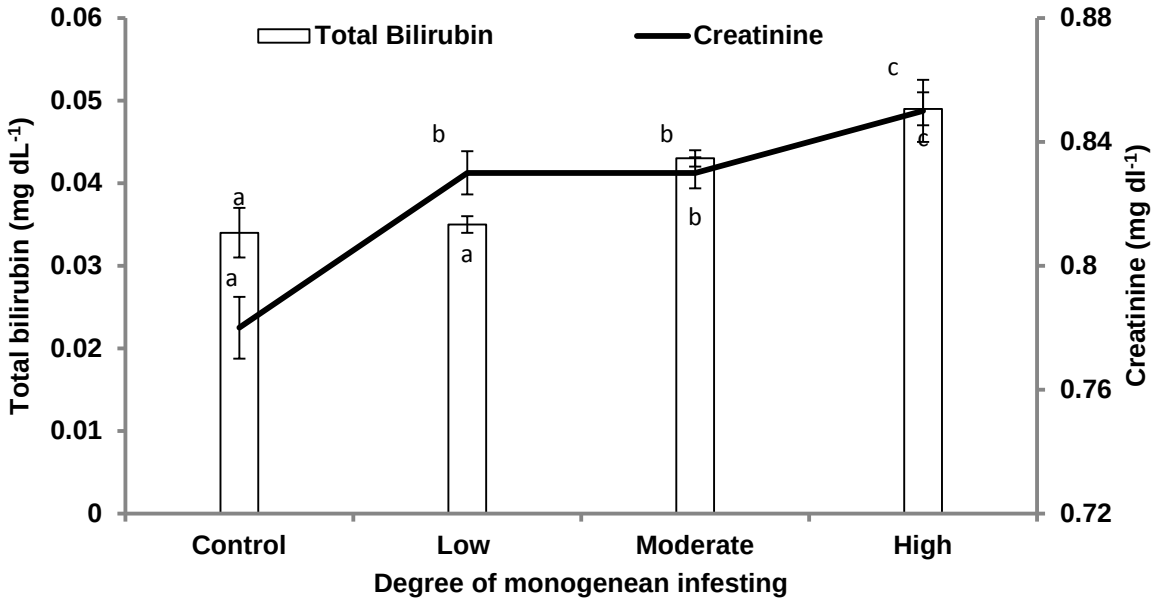


Figure 25: Effect of experimental infection of *Thaparocleidus* spp. on serum total bilirubin and creatinine level in *P. hypophthalmus*. (Values with different superscript differed significantly ($p < 0.05$) and expressed as mean ± SE) (n = 9).

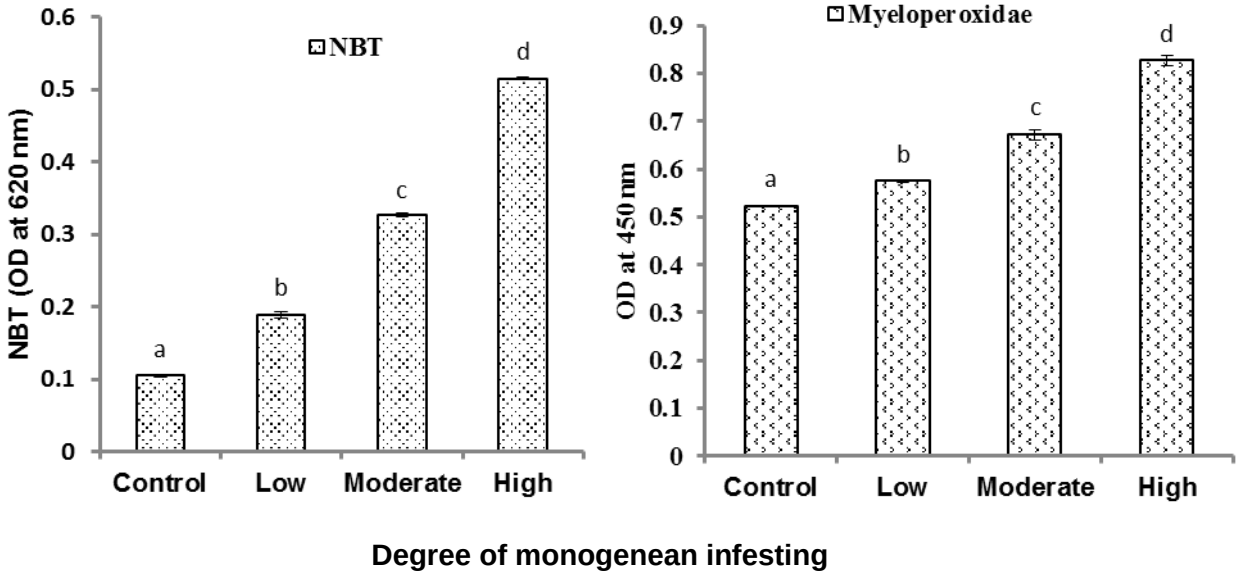


Figure 26: Effect of experimental infection of *Thaparocleidus* spp. (gill monogenean) on respiratory burst activity (NBT test) and myeloperoxidase level in *P. hypophthalmus*. (Values with different superscript differ significantly ($p<0.05$) and expressed as mean ± SE) (n=9).

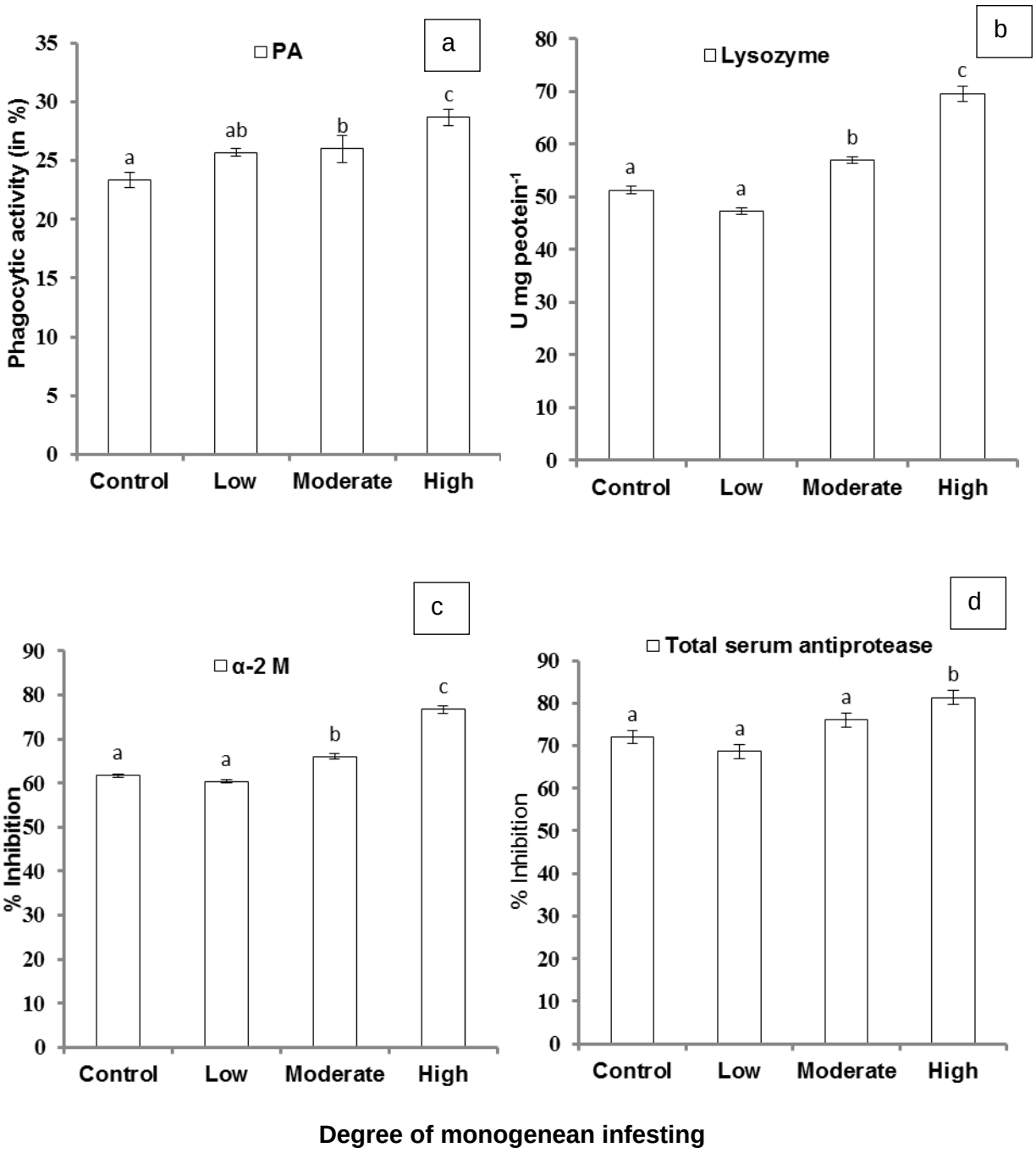


Figure 27: Effect of experimental infection of dactylogyrid (*Thaparocleidus* spp.) on (a) phagocytic activity, (b) serum lysozyme, (c) α-2 macroglobulin and (d) total serum antiprotease activity in *P. hypophthalmus*. (Values with different superscript differ significantly ($p<0.05$) and expressed as mean ± SE) (n=9).

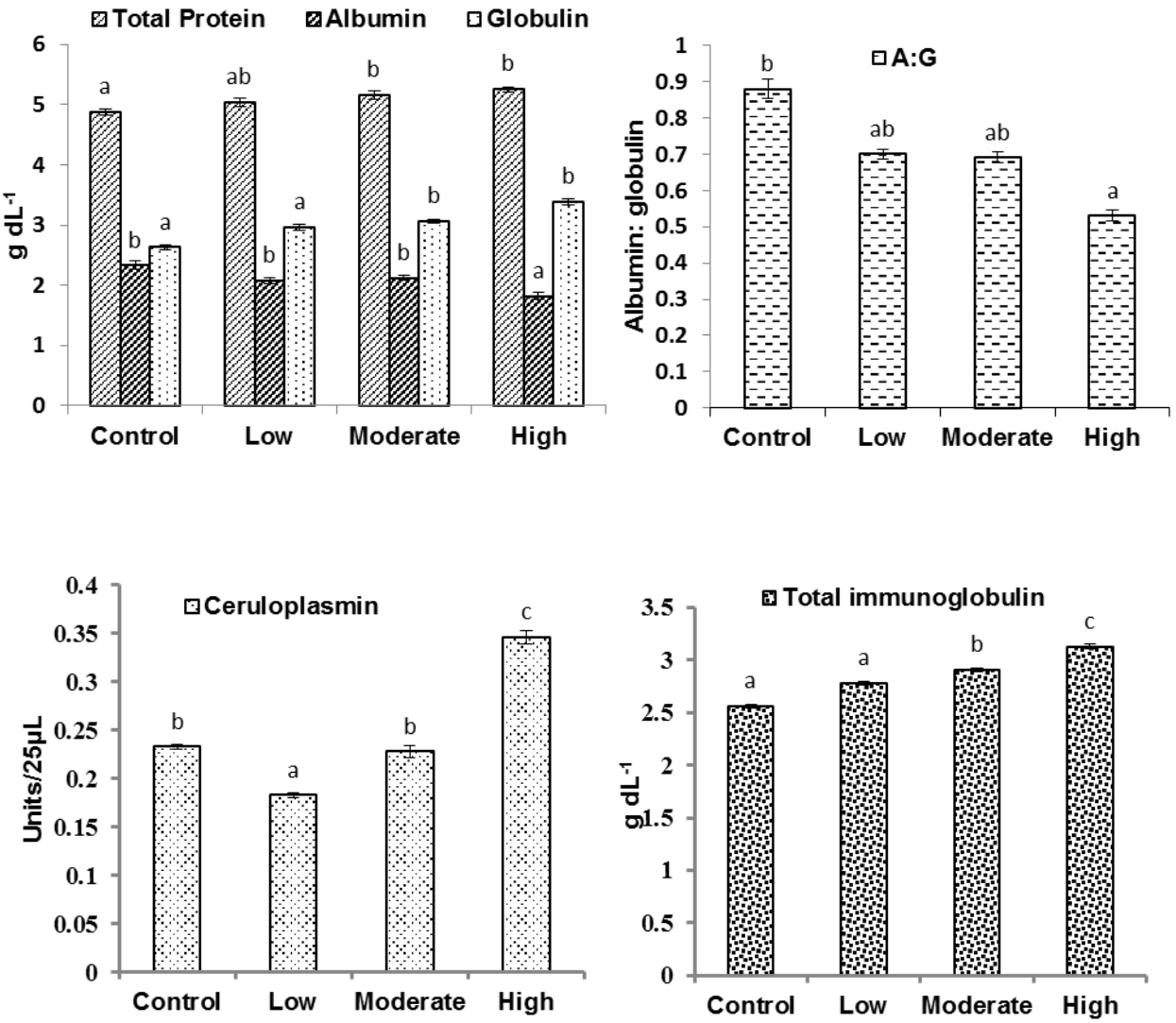


Figure 28: Effect of experimental infection of dactylogyrid (*Thaparocleidus* spp.) on (a) serum protein profiles (total serum protein, albumin, globulin and (b) A:G ratio), (c) ceruloplasmin level and (d) total immunoglobulin in *P. hypophthalmus*. (Values with different superscript differ significantly ($p<0.05$) and expressed as mean ± SE) (n=9).

Table 14: Effect of experimental infection of *Thaparocleidus* spp. on gills, liver and serum superoxide dismutase and catalase activity in *P. hypophthalmus*. Values with different superscript differ significantly ($p<0.05$) and expressed as mean ± SE) (n=9).

Treatment groups	Superoxide dismutase (U mg protein^{-1})			Catalase (mM H_2O_2 decomposed min^{-1} mg^{-1} protein)		
	Gills	Liver	Serum	Gills	Liver	Serum
Control	42.08^{a}±0.57	54.12^{a} ±0.33	34.66^{a}±0.67	6.76^{b}±0.12	8.73^{a}±0.46	4.27^{a}±0.33
Low	40.67^{a}±0.66	53.34^{a} ±0.66	35.33^{a}±0.67	6.30^{a}±0.15	9.40^{b}±0.35	4.43^{a}±0.12
Moderate	47.33^{b}±0.67	58.66^{b} ±0.67	46.67^{b}±0.87	10.53^{c}±0.48	12.56^{c}±0.82	5.67^{b}±0.36
High	66.15^{c}±0.57	71.33^{c} ±0.67	52.10^{c}±1.15	12.73^{d}±0.46	15.43^{d}±0.74	7.46^{c}±0.17

Table 15: Effect of experimental infection of *Thaparocleidus* spp. on gills, and liver glutathione-S-transferase and Na^{+}-K^{+}-ATPase activities in *P. hypophthalmus*. Values with different superscript differ significantly ($p<0.05$) and expressed as mean±SE) (n=9).

Treatment groups	Glutathione-S-transferase (U mg protein^{-1})		Na+-K+-ATPase activity (µM ADP mg^{-1} protein h^{-1})	
	Gills	Liver	Gills	Liver
Control	53.66^{a}±0.88	86.34^{a}±0.89	0.86^{c}±0.01	1.25^{c}±0.01
Low	60.63^{b}±0.67	95.67^{b}±0.33	0.99^{c}±0.01	1.20^{bc}±0.01
Moderate	67.45^{c}±0.34	109.02^{c}±2.08	0.62^{b}±0.04	0.97^{b}±0.01
High	82.08^{d}±1.15	126.67^{d}±3.52	0.55^{a}±0.07	0.84^{a}±0.03

5. Discussion

Aquaculture, during the last few decades has witnessed tremendous growth and played a significant role in the economic growth and rural livelihood. Nevertheless, incidence of disease has remained as one of the major constraints, and the success of aquaculture depends principally on effective health management, and disease control. Outbreaks of diseases in aquatic animals are usually associated with aetiological agents coupled with predisposing factors (Bondad-Reantaso *et al.,* 2005). In Indian aquaculture, farming of *Pangasianodon hypophthalmus* (popularly known as pangasius) has been adopted to bridge the gap between domestic fish demand and supply due to its remarkable growth rate in monoculture, polyculture and cage culture (Rahman *et al.,* 2006; Lakra and Singh, 2010). However, intensive farming practices are always prone to disease risk, leading to epizootic outbreaks. Bacterial diseases associated with aetiology of *Enterobacter cloacae*, *Aeromonas jandaei*, and *Edwardsiella tarda* have been reported in grow out systems of *P. hypophthalmus* in India (Kumar *et al.,* 2013, 2015; Shetty *et al.,* 2014). In India, infestation of protozoan and metazoan parasites in early larval stages of pangasius has also been reported by Lakra and Singh (2010). Further, high prevalence of monogenean infestation (60-90%) was recorded in *P. hypophthalmus* farms (Singh and Lakra, 2012). Although taxonomic identification and molecular characterization of these gill monogeneans (dactylogyrids) were carried out (Tripathi *et al.,* 2014; Chaudhary *et al.,* 2014a; Rajvanshi and Agrawal, 2014), the pathological implications of these monogeneans and host responses are not studied. In the present study, an attempt was made to identify the species of the monogenean parasites and pathology associated with infection in *P. hypophthalmus*. Further, an artificial challenge experiment was performed to assess the haemato-immunological responses of *P. hypophthalmus* to dactylogyrids.

Isolation and identification of monogenean parasite(s) infesting on *P. hypophthalmus*

Epidemiological parameters

In the present study, high prevalence (72%) of monogenean in *P. hypophthalmus*, with abundance of 20 parasites per individual and mean intensity of 39 parasites per infected fish were recorded. The present finding is in agreement within the reported prevalence (60 to 90%) of monogenean in *P. hypophthalmus* (Singh and Lakra, 2012) in India. Further, Tripathi *et al.* (2014) reported 40-80% and 100% prevalence of *Thaparocleidus* sp. in *P. hypophthalmus* collected from aquarium and pond, respectively. However, there is no report available from India on systemic epidemiological investigation related to monogenean parasite in *P. hypophthalmus*. Singh and Lakra (2012), without referring the taxonomic status of monogeneans from *P. hypophthalmus*, also reported comparatively higher mortality during the first week post stocking in *P. hypopthalmus* due to gill fluke infection. Subsequently, Tripathi *et al.* (2014) reported significantly high intensity of *T. siamensis* (>200 parasites per aquarium-kept *P. hypophthalmus* and 250 parasites per pond-reared fish), and stressed upon adverse effect on the host survival due to high parasitic burden. Besides, the sporadic mortality of *P. hypophthalmus* due to *T. siamensis* and *T. caecus* infestation has been documented in Vietnam (Thuy and Buchmann, 2008a). In Vietnam, Dong (2008) reported an association of *Thaparocleidus siamensis* in pale gill and pale liver of *P. hypophthalmus*.

An additional disease risk is involved, especially in Indian conditions; where the culture of *P. hypophthalmus* in cages are one of the most preferable practices and very close to open water (Singh and Lakra, 2012). Further, there is a chance for spreading and colonisation of *P. hypophthalmus* and associated monogeneans to sympatric wild fish as previously known to have established in the lake Kinneret, Israel as an aquarium escapee (Snovsky and Golani, 2012). Moreover, a thorough examination of the literature reveals that *T. caecus* can actually exploit a range of pangasiid hosts. Evidently, *T. caecus* is a common parasite with wide host specificity, and thus most likely to represent a natural threat to native fish biodiversity of importing countries.

In the present study, two species of dactylogyrids (*Thaparocleidus siamensis* Lim, 1990 and *T. caecus* (Mizelle and Kritsky, 1969) Lim, 1996 was observed in *P. hypopthalmus*, based on morphometric traits. Similarly, several authors reported these two *Thaparocleidus* spp. exclusively infesting *P. hypophthalmus* (Lim, 1996; Komarudin, 1999; Pariselle *et al.,* 2005; Lerssutthichawal and Lim, 2005; Thuy and Buchmann, 2008a; Witkowski and Grabowska, 2012; Šimková, 2013; Tripathi *et al.,* 2014; Tripathi, 2014; Chaudhary *et al.,* 2014a, b; Rajvanshi and Agrawal, 2014). Interestingly, Chaudhary *et al.* (2013) extensively reviewed the monogenean diversity in India and listed 17 species of *Thaparocleidus* isolated from siluridae except *T. siamensis* and *T. caecus. Thaparocleidus caecus* was originally described as *Ancylodiscoides caecus* from an unidentified aquarium fish collected in United States (Mizelle and Kritsky, 1969). Subsequently, this species described and transferred to *Silurodiscoides* as *Silurodiscoides caecus* (Mizelle and Kritsky, 1969) n. comb (Gusev, 1978). Further, *S. caecus* along with a new species, *S. siamensis* was redescribed based on new morphometric traits which were collected from *P. hypophthalmus* in Malaysia (Lim, 1990). Subsequently, based on the law of priority, Lim (1996) designated *Silurodiscoides* Gusev, 1978, as synonym of *Thaparocleidus* Jain, 1952. Accordingly, the scientific names of *S. caecus* and *S. siamensis* were designated as *T. caecus* (Mizelle and Kritsky, 1969) Lim, 1996 and *T. siamensis* Lim, 1990, respectively.

Identification and characterization of monogenean parasite(s)

In monogeneans, species identification is generally based on the morphology of two sclerotized organs, the attachment organ (the haptor) and the reproductive organ, including the copulatory organ and the vagina (Gusev, 1985). The morphology of the haptor is considered useful for parasite determination at the genus level, while the reproductive organ is more suitable for identification at the species level, probably because of its higher rate of change (Pouyaud *et al.,* 2006; Wu *et al.,* 2008). The morphometric traits of isolated *T. siamensis* and *T. caecus* were measured and compared with specimen reported from India (Tripathi *et al.,* 2014) and Malaysia (Lim, 1990). The general morphology and measurements of male and female reproductive organs and haptoral parts of specimen of present monogenean are consistent with the details provided by Lim (1990) and Tripathi *et*

al. (2014), and the key provided by Lim *et al.* (2001) for identification of *Thaparocleidus*. The mean body length of the present specimen appears to be different from that of *T. siamensis* and *T. caecus*. The difference in body length might be due to the difference in the specimens selected for the study, number of sample and even the fixation procedure followed. Additionally, orientation of sclerite part of the present specimen showed similarity with the previously reported one. Remarkably, longer marginal hook (mean length 22 and 20 μm for *T. siamensis* and *T. caecus,* respectively) was observed in the present study in comparison to documented specimen (12-14 μm for both spp.) by Lim (1996) and Tripathi *et al.* (2014). This might be attributed to the adaptive constraints of parasite to host. Moreover, the evolution of sclerotized organs is not neutral and seems to be under adaptive constraints in case of the attachment organ (Morand *et al.,* 2002). Elongated and spirally coiled posterior region of copulatory organ and details of female organ are described in the present study which is in agreement of previously described report. However, in the present study inverted cone shaped vagina could not be observed as supported by Tripathi *et al.* (2014) in the case of *T. siamensis*.

The measurements of hard parts are serving as a more effective method of validation of species as they are important part in discrimination between different species. In the present study, the results of principal component analysis (PCA) of morphometric traits of *T. siamensis* and *T. caecus* reveal the large amount of variation mainly relies on width parameters like dorsal bar, testis, ovary, whereas length related to vagina, testis and dorsal recurved point. Further, less variation was noticed in the length of haptor, body and hook length. Similarly, Chaudhary *et al.* (2016) analysed ten morphometric traits of 17 Indian *Thaparocleidus* spp. (excluding *T. siamensis* and *T. caecus*) by linear discriminant analysis (LDA), PCA and multivariate analysis of variance (MANOVA) and reported two morphometric parameters *i.e.,* dorsal outer length and ventral inner length, are responsible for the discrimination between these species. However, these two parameters were not estimated in the present investigation.

In some cases, identification is problematic at the generic as well as species level. Therefore, molecular identification is a helpful tool in resolving taxonomic problems in cases where the morphological “boundaries” among monogenean genera or species groups are ambiguous (Pouyaud *et al.,* 2006; Wu

et al., 2008). Molecular analysis and phylogenetic analysis demonstrated that the specimens of *T. siamensis* from reported in the present study and previously reported one from Malaysia are similar. The specimens of *T. siamensis* from India and Malaysia have 100 % similarity and are thus theoretically identical species. Though, the segment of sequence of aligned 28S rDNA is not large enough. Similarly, Rajvanshi and Agrawal (2014) found a high degree of similarity in molecular link of Indian and Malaysian *T. siamensis* sequences of 28S rDNA. Further, no consequential variation except difference in five bases of 28S rDNA sequence of *T. siamensis* was noticed in Indian and Malaysian isolates (Tripathi *et al.,* 2014).

Aggregation of *Thaparocleidus* spp. on gills of *P. hypophthalmus*

In the present study, single infection with monogenean and co-occurrence of monogenean and metacercariae (unidentified) in few samples of gills of *P. hypophthalmus* were noticed in cage-reared fish. This might be due to cages in reservoir are proximate to wild condition where first intermediate host of trematode, molluscs (snails) are available.

The non-random distribution and aggregation of monogenean parasites on gill filaments irrespective of infection of trematode metacercariae was noticed in the present study. Interestingly, there are several studies explaining the interactions between parasites in closely related species, such as congeneric monogeneans (El Hafidi *et al.,* 1998; Simkova *et al.,* 2000; Buchmann and Lindenstrom, 2002; Koskivaara *et al.,* 2009) but very few literatures are available on co-occurrence of various parasite groups in fish (Rohde, 2013; Zolovs *et al.,* 2017). Simková *et al.* (2000, 2001) studied the distribution of congeneric *Dactylogyrus* species and found that interspecific competition played only a minor role in their niche specialization, despite having similar microhabitat and/or feeding preferences. However, parasite abundance might affect the extent of interspecific interactions, niche breadth and overlap. High densities of *Dactylogyrus* individuals promoted interspecific interactions, expanded niche breadth and overlap, whereas dactylogyrid infracommunities at low densities behaved in a non-interactive fashion (Koskivaara and Valtonen, 1992; Koskivaara *et al.,* 2009). Moreover, distantly related parasites (e.g. species belonging to different major taxa) can also interact if they exploit similar resources. However, such interactions have rarely been studied,

due to the aggregation of parasites among hosts where most hosts harbour a few parasite species and only some host species harbour large infracommunities (Shaw and Dobson, 1995).

Host and tissue preferences of *Thaparocleidus* spp.

In the present study, a noticeable positive relation was estimated between host standard length and parasite intensity. Interestingly, a low level of *Thaparocleidus spp.* infection was recorded in fish below and above standard length of 10 and 15 cm. Several authors reported a positive significant correlation between host total body length and parasite load (Frankland, 1955; Paperna *et al.,* 1984; Buchmann, 1989; Cedrick *et al.,* 1998; Lizama *et al.,* 2007). Buchmann (1989) reported a high *Pseudodactylogyrus* infestation in larger fish host (*Anguilla anguilla*). Cedrick *et al.* (1998) assessed a positive relationship between abundance of ectoparasites and host body length, but also mentioned the relationship can be changed under culture condition. Further, Saha *et al.* (2010) reported a positive relationship between *L. rohita* fingerlings size and monogenean infection. Lizama *et al.* (2007) pointed both possibility of positive or negative correlation between the host length and monogenean parasitism in *Piaractus mesopotamicus*. Hockley *et al.* (2011) reported significantly high infection of *Onchocleidus dispar* (monogenean) in adult *Lepomis gibbosus* in comparison to immature one. They provide possible reason for this as longer exposure of the adults to infective stage resulted in higher infection. However, they found no correlation between host size and infection intensity. In contrast, Poulin and Mouillot (2005) reported a negative correlation between host size and parasite abundance. In the present study, no correlation and interaction were found between host body mass and parasite intensity. The possible reason might be due to body weight depends on various factor, like body size, food intake, health status, life stages and physiological conditions (Bolger and Connolly, 1989). Hockley *et al.* (2011) reported lack of direct relationship between infection of *Onchocleidus dispar* (monogenean) and *Lepomis gibbosus* body weight. Similarly, Ruehle *et al.* (2017) reported a positive relationship between helminth parasite assemblages and host standard length in two cyprinid fish (*Cyprinella venusta* and *Notropis volucellus*) but not between host weight and biomass indices *i.e.,* gonadosomatic indices and parasite abundance.

Host specificity, intraspecific aggregation and microhabitat specificity appear to be a common trait among monogeneans. In present study, *Thaparocleidus* spp. exhibited some degree of habitat selection for the first two (outer) gill arches of its host and subsequent decreased preference towards third and fourth gill arch (GA1≅GA2 > GA3 > GA4). The increased number of *Thaparocleidus* spp. may also be reflective of the larger size of the outer gill arches, therefore providing a greater gill surface for attachment (Gutiérrez and Martorelli, 1999; Hockley *et al.,* 2011). Similar pattern of habitat specificity of *Onchocleidus dispar* (monogenean) was found in *Lepomis gibbosus*, where *O. dispar* demonstrated certain degree of habitat selection for the first (outer) gill arch of its fish host, with decreasing preference towards the second, third and fourth (Hockley *et al.,* 2011). Similarly, other authors have recorded a higher number of monogeneans on the first (Hanek and Fernando, 1978; Ramsay and Dotson, 1995) and second gill arches (Gutiérrez and Martorelli, 1999; Chapman *et al.,* 2000; Raymond *et al.,* 2006; Saha *et al.,* 2010). An apparent preference for this location may merely reflect the first point of contact for the larvae when they crawl into the gill chamber. Alternatively, there may be a habitat tradeoff between high oxygen availability and avoiding becoming dislodged in the centre of the gill cavity (gill arches 2 and 3) where there is a higher water current (Paling, 1965; Hanek and Fernando, 1978; Gutiérrez and Martorelli, 1999). Raymond *et al.* (2006) concluded that microhabitat specificity in *Afrodiplozoon polycotyleus* for 2nd gill arch of 4 species of barbus was highest when oxygen availability was low, and site specificity was more lowered when oxygen was more available.

More precisely, in the majority of fish sampled, intensity of *Thaparocleidus* spp. and its aggregation was found to be high in posterior-central and dorsal-medial regions of the gills arch. Although chances of parasites are being dislodged from the posterior regions, which are more unstable due to greater movement of the filaments in this region, however, thick epithelial layer and more surface area might be favourable for parasite aggregation. Similar microhabitat specificity was studied for *O. dispar* in *Lepomis gibbosus* and found the number of parasites on 9 regions (combination of dorsal-ventral and anterior-posterior) of the gills increased with increased intensity of parasite. However, they reported a high parasite load in the anterior and central regions which are closest to the gill rakers

and attributed the selection of these regions might be a stable site for attachment as there is less movement of the filaments in those regions. However, this will be particularly apparent when intensity increases and competition for stable sites on the gills is increased. Clustering of parasites in specific areas in the gills will also increase intraspecific contact. This will increase the likelihood of locating and selecting mates of higher fitness to facilitate cross fertilisation (Zolvos *et al.,* 2017).

Pathology associated with monogenean parasite infesting on *P. hypophthalmus*

Histo-morphological changes in gills of *P. hypophthalmus* associated with dactylogyrids (*Thaparocleidus* spp.)

Histopathology is concerned with the study of alteration of tissue architecture and function that occur in disease condition and the correlation of these with the clinical signs. The main objective of histopathological techniques is to reveal the changes in the tissue structures from the normal at the cellular level. In the present study, cellular changes and pathology (focal to multifocal) in gill tissues caused by dactylogyrids, the monogenean parasite alone and concurrent with trematode larva (metacercaria) were observed with the diseased fish *P. hypophthalmus*.

The degree of severity of the gill lesions caused by *Thaparocleidus* spp. (dactylogyrid), a gill monogenean ranged from mild to severe and focal to multifocal as intensity of parasites increased in the observed samples. Proliferative epithelial response, high number of mucous cells, infiltrating inflammatory cells, distortion of the lamellar structure, fusions of secondary lamellae, epithelial rupture and depression in gill filaments were observed in histological sections of the gills of *P. hypophthalmus*. Similar pathology in gills was also reported in amberjack (*Seriola dumerili*) infected by a polyopisthocotylean monogenean *Zeuxapta seriolae* (Montero *et al.,* 2004), in gilthead seabream (*Sparus aurata*) artificially infected with gill monogenean, *Sparicotyle chrysophrii* (Sitja-Bobadilla and Alvarezo-Pellitero, 2009), in spotted rose snapper (*Lutjanus guttatus*) artificially infected with dactylogyrid monogenean (Del Rio-Zaragoza *et al.,* 2010). Jerônimo *et al.* (2014) reported severe hyperplasia, sub-epithelial edema, fusion of the secondary lamellae, focal and multifocal necrosis in highly parasitized *Piaractus*

mesopotamicus by monogenean. Further, Molnár *et al.* (2016) reported depression in the epithelium of the filaments at the site of monopisthocotylean gill parasite *Ancyrocephalus paradoxus* attached to the gill of pikeperch (*Sander lucioperca*). They also observed fusion in gill filaments, clubbing and epitheloid-type regeneration of tissue in heavily infected fish.

In the present study, we observed relatively high degree of depression in lamellae and increased hyperplasia, and infiltration of inflammatory cells in infected gill in comparison to low level infection. In contrast, only the occurrence of severe epithelial hyperplasia of the gills was detected during attachment of dactylogyrids to the gills of the *Cyprinus carpio* (Paperna, 1996) and to *Glaucosoma hebraicum* (Pironet and Jones, 2000; Kritsky and Stephens, 2001). The high level of infection by dactylogyrids increased the number of mucous cells in the gills in the present study. This might be due to self-curing response of host by means of excessive production of mucus leads to disengage the monogenean. Buchmann (1999) reported that infection by monogenean can stimulate mucus production in the host as a protective mechanism in response to parasitism. However, the secretion of excess mucus can cause hypoxia and induce gill dysfunction in host (Montero *et al.,* 2004; Chavez *et al.,* 2006).

In the present study, severe damage of gill filaments caused by the insertion of hamuli and hooklets into the epithelium was observed. However, a low degree of pathology (hyperplasia and fusion of secondary gill lamellae) associated with the less intensity of dactylogyrids was also noticed in the present study. In agreement to this, Paperna (1963) and Molnár (1972) reported that in less acute cases a host response to dactylogyrids with hyperplasia of the epithelial cells accompanied by massive proliferation of goblet cells. The gills were covered by an abundant amount of thick mucus only partially originating from the increased number of goblet cells but mainly from the cytoplasm of disrupted cells and the destroyed vascular network. Due to intensive proliferation of the interlamellar epithelium, the lamellae were overgrown by the proliferating epithelial cells, and the remnants of damaged capillaries were seen deep inside this regenerated tissue.

The neighbouring filaments fused and formed projections denuded of lamellae, a process generally known as clubbing. Although this host reaction eliminated most of the parasites, many fish died of suffocation. A similar pattern of

infection was found in cultured sheatfish fingerlings with *Thaparocleidus* (*Ancylodiscoides*) infection. In constrast, in *Dactylogyrus* infection, most of the worms survived and could be found on and within the clubbed filaments. Buchmann (2012) reported hyperplasia of mucous cells and gill epithelial cells with excess production of mucus and fusion of the gill lamellae and primary filaments, which led to clubbing and fusion of the neighbouring filaments in *Pseudodactylogyrus anguillae* and *P. bini* infected European eel.

The infection of freshwater fishes with monopisthocotylean monogeneans showed severe degenerative and proliferative changes which are mostly restricted to the gill epithelium (Paperna, 1963; Molnár, 1972, 1980; Buchmann, 2012). Interestingly, more complex host reactions were reported in marine fishes against blood-feeding polyopisthocotylean monogeneans. Ogawa (2012) found a development of severe anaemia, inflammation, hyperplasia, necrosis in the buccal cavity wall and a dense granular cell infiltrate with monocytes and granulocytes in olive flounder during *Heterobothrium hirame* infection. Only local changes at the attachment sites of the worms with haemorrhages, mucoid exudates, erosion of the lamellar epithelium, and local hyperplasia on the filaments were reported in marine fishes infected with monogenean parasites (Gonzalez-Lanza *et al.,* 1991; Roubal, 1995; Dezfuli *et al.,* 2007; Sitja-Bobadilla and Alvarez-Pellitero, 2009; Merella *et al.,* 2009; Yardimci and Pekmezci, 2012). Clubbing of filaments was not observed but all these authors reported fusion of the secondary lamellae besides degenerative changes at the attachment points of monogeneans. Although severe damage of the gills was reported, these infections can be assigned to the category of local damage rather than disease.

In the present study, a concurrent infection of dactylogyrids and metacercariae in gill tissue was also observed. The encysted metacercariae at various regions were found to be associated with cartilaginous part of the gill filament and cause local deformation of cartilaginous filament ray but did not cause any alteration in the linear orientation of gill filament. Moreover, these metacercariae were surrounded by young chondroblats and chondrocytes resulting a swelling of lamellae. Similarly, Molnar *et al.* (2016) reported encysted *Echinochasmus* sp. (metacercaria) in gill cartilaginous filament, caused bifurcation of the gill filaments and severe pathological condition in the gill. Further, in the

present study, contentional hyaline parasitic capsule (CPC) of fibrous connective tissue formed fusiform cells as fibroblasts around the metacercariae. In agreement of the present finding, Silva *et al.* (2016) also observed CPC around the cysts of metacercariae of *Bucephalus margaritae* in gill of *Hypleurochilus fissicornis*. Several authors recorded that the tegumental specializations of the metacercaria (as scale-like tegumental spines) might promote irritation or erosion on host's tissues. Faliex (1991) reported different organizational features of CPC structure in response of *Labratema minimus* (Bucephalidae) metacercaria in the liver of *Liza ramada* and *Atherina boyeri*.

Clinical pathology of *P. hypophthalmus* naturally infected by dactylogyrids (*Thaparocleidus* spp.)

Haematological parameters

Haematological and serum biochemical profiles can be used as a direct or inferential indicator for monitoring the fish health condition. This provides information related to the tolerance to a stressor agents or/and illness of the fish (Schreck and Moyle, 1990; Del Rio-Zaragoza *et al.,* 2011; Kumar *et al.,* 2013). Further, the evaluation of blood cells can be useful for the measurement of physiological disturbances in parasitized fish and therefore, provides information about the level of damage in the host and the prognosis for the diseases (Tavares-Dias and Moraes, 2003; Tavares-Dias *et al.,* 2007). In the present study, effect on haemto-serum biochemical parameters of *P. hypophthalmus* naturally infested by dactylogyrids (*Thaparocleidus* spp.) was evaluated.

The elevated TEC found in high level in *Thaparocleidus* spp. infested fish suggests a positive relationship between parasitic infection and red blood cells of *P. hypophthalmus*. Gill monogenean causes severe damage in epithelial layer of gill lamellae and lead to hypoxic condition due to clubbing of secondary lamellae (Modu *et al.,* 2012). This might be associated with fish adaptive response and release of more erythrocyte in blood to carry and maintain oxygen. Further, Bayley *et al.* (2015) reported a considerable plasticity in *P. hypophthalmus* gills, and can able to perform efficient oxygen transmission during hypoxic condition with the secondary lamella become almost partially embedded by an interlamellar. Del Rio-Zaragoza *et al.* (2011) reported elevation in haematocrit value in dactylogyrid

infected *Lutjanus guttatus*. In contrast, several authors found a reduction in erythrocyte, Hb and PCV during monogenean infection in fish (Genc *et al.,* 2005; Shah *et al.,* 2009; Ali and Ansari, 2012). Similarly, the decrease of RBC concentration was reported in *Rita rita*, rainbow trout, magur and Japanese flounder infected with trematode (Agarwal, 1989); *Proteocephalus neglectus* (Engelherdt *et al.,* 1989); helminth (Sinha, 2000) and *Neoheterobothrium hirame* (Mushiake *et al.,* 2001), respectively.

Quantitative estimation of haemoglobin provides index of red blood cell (RBC) function and can be determined by the severity of anemia. In the present study, a significant increase, but within optimum level of haemoglobin contents were observed in parasitized fish and this could be attributed to adaptation towards hypoxia and increase in number of erythrocyte in peripheral blood circulation. In agreement to the present finding, increased Hb level in blood was reported in black scorpion fish (*Scorpaena porcus*) naturally infected by *Trachelobdella lubrica* (Hirudinea) (Celik and Aydin, 2006). In contrast, a reduction in haemoglobin content has been reported in *C. carpio* (Ivasik and Virepo, 1969), cut throat trout (Evans, 1974), *Clarias batrachus* (Sinha, 2000) and Japanese flounder (Yoshinaga *et al.,* 2001) infected with Sanguinicolosis, *Sanguinicola kiamathensis*, helminthes and *Neoheteriobothrium hirame*, respectively. However, Soberon *et al.* (2014) did not find any significant difference in Hb in *Colossoma macropomum* naturally parasitized by monogenean (*Anacanthorus spathulatus*).

Although in the present study, a significant increase in PCV was recorded in high level *Thaparocleidus*-infested *P. hypophthalmus* but within the optimum range of PCV in fish. Further, there was no significant difference in PCV between low level infested and uninfested group. In agreement to our finding, Del Rio-Zaragoza *et al.* (2011) found high but within the reference level for healthy fish haematocrit value in dactylogyrid infected *L. guttatus* in comparison to non-parasitized group. Further, Soberon *et al.* (2014) reported no significant difference in values of PCV in low level dactylogyrids naturally infected *C. macropomum*. In contrast, a decrease in PCV level was reported in *C. carpio* infected with monogenean parasite (Ali and Ansari, 2012). In contrast, decreased values of haematocrit were found in black scorpion fish (*Scorpaena porcus*) and bogue fish (*B. boops*) naturally infected with *Trachelobdella lubrica* (Hirudinea) (Celik and

Aydin, 2006) and isopods (*Ceratothoa oestroides*) (Özdemir *et al.,* 2016), respectively in comparison to healthy group.

In the present study, significant lower values of MCV, MCH and MCHC were observed in parasitized fish in comparison to uninfested group. A decrease in MCV and MCHC could be attributed to microcytic anaemia, a condition of shrinking of erythrocytes that might be due to more number of immature red blood cell (RBC) formations with inadequate level of haemoglobin synthesis. Further, no significant difference in blood indices was noticed between low level *Thaparocleidus* spp. infestation and uninfested group. There are few reports available on blood indices of monogenean infected fish related to natural infection. Soberon *et al.* (2014) found no significant difference in MCV, MCH and MCHC in *C. macropomum* naturally parasitized with *Anacanthorus spathulatus* (monogenean). Similarly, Azevedo *et al.* (2006) did not observe change in blood indices in *O. niloticus* parasitized with *Trichodina* sp., *Lamproglena* sp., and *Cichlidogyrus sclerosus*. In agreement with the present findings, a significant lower blood index was reported in curimbas, (*P. lineatus*) naturally infected with acanthocephala (*Neoechinorhynchus curemai*) (Belo *et al.,* 2013). The variation in response of blood indices could be due to form of parasitism, intensity, type of host, and nature of infection etc.

In the present study, total leucocyte count was found to be high in parasitized fish in comparison to uninfested group. Blood leucocytes seem to represent an important defense line in the hosts. The elevated leucocyte in parasitized fish may be attributed to inflammatory and non-specific immune response to invaders (Passantino *et al.,* 2005). Similar results have been reported in European eel (*Anguilla Anguilla*) (Sahan *et al.,* 2007); in *Schizothorax* spp. and *Cyprinus carpio* (Shah *et al.,* 2009) infected with helminth parasites; in *C. carpio* infected with monogenean parasite (Ali and Ansari, 2012), in *Ceteopharyngodon idella* infected with *Gyrodactylus* and *Dactylogyrus* spp. (Rastiannasab *et al.,* 2016).

The differential leucocyte counts (DLC) are one of the important reliable haematological indices used in clinical pathology. In the present study, increase in proportion of neutrophil, eosinophil and monocytes in DLC of parasitized fish exhibits inflammatory and protective response of host against *Thaparocleidus* spp. However, decrease in lymphocytes percentage in DLC was noticed in parasitized fish group in comparison to uninfested group. One hypothesis that could

explain the observed effect refers to pathological alterations resulting from the *Thaparocleidus* infection, because the inflammatory process in the brachial system releases chemotactic stimuli to activate the granulocyte-macrophage stimulating factor, which favours the increase of granulocyte and monocyte responsiveness and granulocytosis in case of acute and monocytosis often occurs during chronic inflammation (Belo *et al.,* 2005). Neutrophils represent the body's primary line of defence against external agents and in the circulation of healthy animals, neutrophils exist in a resting state which can become activated by foreign invaders that include microbes, parasites, toxins and cytokines or chemokines (Hallett and Loyds, 1995), and activated neutrophils are then mobilized to the site of infection or inflammation, where they encounter activating signals to trigger destruction of external agents (Wright *et al.,* 2010). The elevation in levels of monocyte and eosinophil in peripheral blood indicates to an improvement of cell defense system (Sopinska, 1984). Monocytes participate in tissue healing, clearance of foreign invaders and dead cells, and initiation of adaptive immunity (Ingersoll *et al.,* 2011). In agreement to our findings, Belo *et al.* (2013) reported increase in monocyte, and eosinophils in *Prochilodus lineatus* infected with *N. curemai*. Similarly, Ali and Ansari (2012) found significant high number of neutrophils, monocytes and eosinophils in monogenean infected *C. carpio*. The decrease in lymphocytes of DLC in parasitized fish group exhibits stimulation of granulocytes synthesis and release in peripheral blood. The primary function of lymphocytes seems to be to act as the cells of specific immune system via antibody production (Evelyn, 2002). The result of DLC in the present study attributes to inflammatory response of the fish against *Thaparocleidus,* and the recruitment of more granulocytes and monocytes at the gill site in response to inflammation and healing the infection.

Serum and plasma proteins are termed as circulating mobile proteins and are divided into two groups, albumin and globulins. Albumin is synthesized by liver and creates an osmotic force that maintained fluid volume within the vascular space. The albumin is an easily available protein reserve and a protein transporter (Anderson *et al.,* 1979). The gamma globulin fraction is the source of almost all the serum biochemical active protein of the blood. Globulins like gamma are essential for maintaining a healthy immune system. Increase in albumin and globulin levels is considered a strong innate response in fishes (Wiegertjes *et al.,* 1996). In the

present study, a significant elevation in the level of total protein and globulin was observed. Since, gamma fraction makes the largest portion of globulin, it can be inferred that subsequent enhanced globulin level in parasitized groups may be a protective immune response of *P. hypophthalmus* against *Thaparocleidus* spp. and phase towards preparatory stage for stress mitigation. In contrast, several authors found a lower value of serum total protein in naturally parasitized fish (mainly ectoparasites which feed on blood and mucus materials) and attributed to the consumption of the nutrient materials (Eissa *et al.,* 2012; Ozdemir *et al.,* 2016). Del Rio-Zaragoza *et al.* (2011) reported low level of total protein in dactylogyrids infected *L. guttatus*. Similarly, Özdemir *et al.* (2016) reported a decreased level in total protein and globulin values in bogue fish (*Boops boops*) naturally parasitized by isopoda (*Ceratothoa oestroides*). The form of parasitism (feeding on tissue or blood) and pathological response could be the main probable reason for varying findings in the present study. The elevated albumin and A to G ratio showed the adaptive response to maintain the blood osmolality and increased inflammatory response (Çelik and Aydın, 2006).

Oxidative stress biomarkers

Parasitism represents one form of biological stress on the host body and therefore affects the natural balance of oxidative stress parameters, thereby causing sever oxidative pressure on the host. Reactive oxygen species are capable of damaging biological macromolecules such as DNA, carbohydrates, or proteins, thus compromising an organism (Sies, 1991). All cells possess enzymatic and non-enzymatic antioxidant defenses to inactivate these damaging molecules. In the present study, the increase in activities of SOD and CAT of high level *Thaparocleidus*-infested *P. hypophthalmus* results the cellular oxidative response towards parasitism. Similar to the present result, Al-Olayan and Metwally (2015) found an increased level of SOD and CAT in *Tilapia zilli* naturally infected with trematode metacercaria of *Diplostomum tilapiae* and *Heterophyes* species. However, they did not find significant difference in SOD and CAT activities in gills, liver and muscle of *Sarotherodon galilaeus* naturally infected by *Diplostomum tilapiae*, *Centrocestus formacenus* and *Heterophyes* species. Interestingly, Neves *et al.* (2000) reported no significant difference in CAT but reduced activity of SOD in gills of shrimp, (*Palaemonetes argentinus*) infected with isopoda, Boypyridae

(*Probopyrus ringueleti*). Further, Dautremepuits *et al.* (2003) reported increase in activity of catalase in naturally parasitized *C. carpio* by *Ptychobothrium* sp. The difference might be attributed to mode of parasitism; intensity of parasite on host and also host responses differs with parasite types.

Serum biochemical enzymes related to metabolism

The protein metabolism is regulated by two key enzymes AST and ALT aminotransferases and they catalyze the intermolecular transfer of the amino groups between α-ketoacidosis and amino acids (Jee *et al.,* 2006). In the present study, increased activity of sGOT and sGPT of parasitized group but within the optimum range of healthy fish is evident in total serum protein. The activity of these enzymes is upregulated for producing responses against parasitic infection. In agreement to our findings, Celik and Aydin (2006) reported an increased activity of sGOT and sGPT in black scorpion fish (*Scorpaena porcus*) naturally infected by *Trachelobdella lubrica* (Hirudinea). Similarly, an increased sGOT and sGPT activity was reported in *Clarias gariepinus* and *C. anguilaris* fish naturally parasitized by ectoparasites (Nnabuchi *et al.,* 2015) in comparison to healthy fish. However, a significant decrease in the GOT and GPT values was found in the serum of *C. oestroides* infected with *B. boops* in comparison to the non-parasitized ones. The decrease observed in the AST activities was attributed as a result of fasting in infected fish caused by the parasites. This can be attributed to the decreased protein regeneration in the malnourished fish under the influence of the decline in gluconeogenesis in the amino acids and the amino acid deficiency in the digestive system (Tlak *et al.,* 2008).

ALP is an enzyme produced in body tissues, liver and gall bladder cells (Agrahari and Gopal*,* 2008). ALP is a good stress indicator in biological systems and has an important role in cell transport and phosphate hydrolysis as well. ALP measurement is valuable in assessing the cellular membrane health and liver dysfunctions (Banaee *et al.,* 2011; Banaee, 2013). The increase in ALP in the high level parasitized *P. hypophthalmus* found in this study might be related to the tissue damage in the gills or increased stress on liver caused by the parasite. Although, the effect of gill monogenean parasites on fish ALP is not perceived.

Similar reports of elevated ALP activity were found in *Clarias gariepinus* and *C. anguilaris* fish naturally parasitized by ectoparasites (Nnabuchi *et al.,* 2015) and in bogue fish (*Boops boops*), naturally infested with isopods (*Ceratothoa oestroides*) (Özdemir *et al.,* 2016).

A significant increase in the LDH value of the *Thaparocleidus*-parasitized *P. hypophthalmus* was observed in comparison to uninfested group. An increase in LDH level indicates metabolic changes like glycogen catabolism and synthesis of glucose from lactate in distressed fish (Simon *et al.,* 1983). Various stress conditions (temperature, starvation and infection) tend to induce LDH activity (Grigo, 1975; Vijayaraghaban and Rao, 1986; Kumar *et al.,* 2013). Increase in LDH activity can be attributed to the damage in muscle tissue and liver (Rui and Zuzuki, 1997). Similarly, Özdemir *et al.* (2016) reported a significant increase in LDH level in bogue fish (*Boops boops*), naturally infested with isopods (*Ceratothoa oestroides*). Moreover, a similar increase in LDH level was recorded in the *Tilapia* infested with the *Pygidiopsis geneta* and *P. summa* parasites (Elnemaki, 2003).

Serum biomarkers of by-product of metabolism

Glucose is the chief sources of energy for all the animals including fish. It has been reported as a biomarker of stress, caused by various means primarily physical factors (Manush *et al.,* 2005). However, glucose is a sensitive marker, and its measurements show many inconsistencies and should be a complement of stress tests rather than the primary indicator. In the present study, significant elevated blood glucose in high level parasitized fish group was observed when compared with uninfested group. Rise in the blood glucose might have been indicated a high caused rate of metabolism responsible for high glucose turnover in fish due to parasitism (Wright *et al.,* 1989). In agreement of our findings, Soberon *et al.* (2014) reported an increment in blood glucose level in *C. macropomum* naturally infected by dactylogyrids. Further, significant hyperglycemia was observed in *Dolops carvalhoi* infected hybrid tambacu fish (Tavares-Dias *et al.,* 2007) and in *O. mykiss* infected by *Lepeophtheirus salmonis* (Ruane *et al.,* 2000). In contrast, a decrease in blood glucose level was found in black scorpion fish (*Scorpaena porcus*) and bogue fish (*B. boops*) naturally infected by *Trachelobdella lubrica* (Celik and Aydin, 2006) and *C. oestroides* (Özdemir *et al.,* 2016), respectively.

Lactate produced by anaerobic glycolysis in the muscles moves to the liver and is converted to glucose, which then returns to the muscles and is metabolized back (Cori cycle) to lactate (Nelson and Cox, 2005). In the present study, significantly elevated serum lactate concentration but no difference in bilirubin level was noticed in high level *Thaparocleidus*-infested *P. hypophthalmus* in comparison to control group. The reduced capacity of anaemic fish to deliver oxygen to the body may cause anaerobic metabolism and consequent lactate production (Wells and Pankhurst, 1999). Lactate level and LDH activity in the high degree of infested fish suggests anaemic condition and reduction of oxygen supply due to the high level of monogenean infestation alter the normal gill physiology. The effect of gill monogenean on fish total bilirubin levels is not known as it can be attributed to liver and specially gall bladder dysfunction (Arthur and John, 1986). However, in isopoda *C. oestroides* infested bouge fish, Özdemir *et al.* (2016) reported a significant increase in total bilirubin.

Haemato-Immunological responses of *P. hypophthalmus* artificially infected by dactylogyrids (*Thaparocleidus* spp.)

Haematological responses

Non-specific defense mechanism plays a significant role in protection of the fish during parasitic infestations, especially inflammatory responses, and cellular metabolism (Bayne and Gerwick, 2001; Secombes, 1996, 1997; Sahoo and Saurabh, 2008). In the present study, significant increase in total erythrocyte, haemoglobin and total leucocyte counts was recorded in the high and moderately infested groups when compared with low and uninfested groups. However, no significant difference was observed in packed cell volume of the infested group in comparison to control. The increased number of erythrocyte and hemoglobin level might be indicative of adaptive response towards oxygen demand which altered due to infestation of gill monogenean on host lamellae. In constrast, Rana and Das (2016) found during the progression of infection, a significant decrease in RBC and Hb level in a high degree of *Paradactylogyrus*-infested *Catla catla*. Similarly, Ali and Ansari (2012) recorded significant low RBC and Hb level in monogenean infected *C. carpio* in comparison to uninfested control group. But, Del Rio-zaragoza *et al.* (2010) did not find the significant change in blood parameters viz. RBC, Hb, PCV in low, moderate and high level of dactylogyrids on experimentally infected spotted

rose snapper (*Lutjanus guttatus*). The significant increase in TLC in all *Thaparocleidus*-infested *P. hypophthalmus* reveals the protective (innate immune) response of fish and recruitment towards inflammation. In conformity to this, an increase in TLC in monogenean infested fish were recorded in *Trachinotus marginatus* (*Chavez et al.,* 2006), *L. guttatus* (Del Rio-zaragoza *et al., 2010*), and *C. catla* (*Rana and Das, 2016*).

The blood indices (MCH, MCV, and MCHC) were recorded significantly low in the high and moderately monogenean-infested group when compared with control. A decrease in MCV and MCHC were also found in naturally infected *P. hypopthalmus* in the present study and this could be attributed to more number of immature red blood cell (RBC) formations without the adequate level of hemoglobin synthesis. Similarly, low level of blood indices was observed in experimentally infested *Sparus aurata* with *Sparicotyle chrysophrii* (Sitja-Bobadilla and Alvarez-pellitero, 2009), in *C. auratus* infested with *Argulus* sp. (Kumar *et al.,* 2013). However, no significant change in MCH, MCV and MCHC was recorded in low, moderate and high level of dactylogyrids infested *L. guttatus* (Del Rio-zaragoza *et al.,* 2010).

The significant increase in erythrocyte osmolality brittleness activity of high degree of infected fish showed a decrease in hemolytic resistance of erythrocyte, giving an indication of alteration in membrane composition and integrity as well as the cells sizes or the surface area to volume ratio of erythrocytes (Bain *et al.,* 2011). The effect of gill parasite on EOB in fish is not documented. However, a significant effect on EOB was detected in case of bacterial (*Aeromonas hydrophila*) and viral (Cyprinid herpesvirus 2) infected *C. carpio* and *C. gibelio* respectively, (Wenxiang and Xiaomin, 2006; Lu *et al.,* 2016).

Serum biochemical responses

In the present study, a significant increase in the blood glucose level was recorded in high and moderately experimentally monogenean-infested fish. The results are in agreement with the elevation of blood glucose level in *L. guttatus* infected with a different degree of dactylogyrids (Del Rio-zaragoza *et al.,* 2010); in *C. auratus* artificially infested with *Argulus* sp. (Kumar *et al.,* 2013). Further, in the present study, sGPT, sGOT and ALP activities were significantly high in

Thaparocleidus-infested group in comparison to control. These enzymes specifically regulate the metabolism and are predominantly expressed in the liver. However, sGOT is present in lesser extent in kidney and skeletal muscle while sGPT and ALP in all body tissue especially liver, heart and skeletal muscle. Elevated activities of these enzymes in the serum of parasite infested group, in the absence of acute necrosis or ischemia of other organs except gill, indicates an increase in synthesis of these enzymes and leaching into the blood. Similarly, elevated activity of GOT and GPT in serum was reported in roach (*Rutilus rutilus*) infected by the digenean *Rhipidocotyle fennica* (Jeney, 2002) and in *Argulus*-infested *C. auratus* (Kumar *et al.,* 2013). In the present study, the change in activity of sGPT was found to be more sensitive to the different degree of *Thaparocleidus* infestation in *P. hypophthalmus* which might be more responsive to parasitic infection.

In the present study, significantly elevated LDH and serum lactate concentration was noticed in high and moderately *Thaparocleidus*-infested *P. hypophthalmus* in comparison to control group. Similar result was found in naturally parasitized fish in the present study. The results might be attributed to increased demand of energy in anaemic fish which leads to elevation in LDH responses. Lactate dehydrogenase is the terminal enzyme of glycolysis pathway and interconverts lactate to pyruvate in the presence of coenzyme NADH oxidize to NAD^+. However, during anaerobic condition or lack of oxygen supply in tissue, LDH perform reverse reaction (Cori cycle) in the liver and converts pyruvate (a final product of glycolysis) into lactate. In agreement to present finding, Mansell *et al.* (2005) reported an increase in the blood lactate in monogenean infested kingfish in comparison to uninfested group and observed increasing trend with the progression of infestation and decrease in hemoglobin level. Further, the reduced capacity of anaemic fish to deliver oxygen to the body may cause anaerobic metabolism and consequent lactate production (Wells and Pankhurst, 1999). Lactate level and LDH activity in the high infested fish suggests anaemic condition and reduction of oxygen supply due to the infestation of high monogenean parasite.

Significant increase in serum bilirubin and creatinine concentration was noticed in high and moderately infested fish in comparison to uninfested and low infested group. Bilirubin, also known as hematoidin, is a degraded product formed as a result of heme catabolism in the liver. High levels of bilirubin can result

in liver disease, hemolysis or blood disorder (Bain *et al.,* 2011). Whereas, creatinine release through the kidney and their detection in blood is useful as the test for kidney function. Similarly, Özdemir *et al.* (2016) reported a significant increase in bilirubin and creatinine kinase in *B. boops*, infested with *C. oestroides*. However, Floyd-Rump *et al.* (2017) did not observe a significant difference in creatinine in *Ichthyophonus* infested Chinook salmon. In the present study, the significantly high level of creatinine was observed in only the high degree of parasitized fish, indicating increased activity and dysfunction of kidney.

Immunological responses of *P. hypophthalmus* to *Thaparocleidus* spp.

Innate immunity is paramount in defence of teleost fish and several workers highlighted the importance of changes in the innate immune parameters in relation to different parasitic infections (Dickerson and Clark, 1996; Secombes and Chappell, 1996; Buchmann *et al.,* 2001; Woo, 2001; Xu *et al.,* 2004; Cuesta *et al.,* 2006; Sitja-Bobadilla *et al.,* 2006; Saurabh *et al.,* 2010; Kumar *et al.,* 2013). The results of the present study reveal an immunomodulory effect on innate parameters of *P. hypophthalmus* during different degrees of artificial infection, which may be useful for understanding host-parasite interaction.

The oxygen dependent defence mechanism in vertebrate phagocytic cells is termed as respiratory burst activity, wherein there is generation of reactive oxygen intermediates molecules such as superoxide anion radical ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radical (OH^-) with powerful antimicrobial activity (Babior, 2000; Secombes and Fletcher, 1992). In the present study, significantly high respiratory burst activity (RBA) and phagocytic activity were recorded in fish with different degrees of dactylogyrid infection when compared with un-infected control fish. Muñoz *et al.* (1998) reported higher levels of NBT activity of cells from *Dicentrarchus labrax* parasitized by *Sphaerospora dicentrarchi*, compared with non-parasitized fish. Higher NBT activity was reported in *Argulus*-infected *C. auratus* in comparison to un-infected control fish (Kumar *et al.,* 2013). Similarly, induction in respiratory burst response in pink salmon exposed to secretory products from *L. salmonis* (Lewis *et al.,* 2014). However, Fast *et al.* (2002) reported no significant differences in RBA and phagocytic capacity of macrophages isolated from *L. salmonis*-infected coho salmon and rainbow trout when compared to control fish.

Myeloperoxidase is a hemoprotein, utilizes hydrogen peroxide during respiratory burst to produce hypochlorous acid, which are potent oxidants known to have several cytotoxic effects on mammalian and bacterial cells (Dalmo *et al.,* 1997). In the present study, a significant difference in MPO activity was observed in fish infested with different degrees of monogenean. Similar induction of serum peroxidases level was reported in *E. leei*-exposed sharpsnout seabream (Munoz *et al.,* 2007) and in gilthead seabream during cohabitation, but a decrease occurred in subsequent samplings and no differences were detected between parasitized and non-parasitized fish (Cuesta *et al.,* 2006). Similarly, during experimental exposure of *Argulus* in *L. rohita*, no significant difference in MPO activity was observed among different degrees of parasite-infected and uninfected fish (Saurabh *et al.,* 2010). In contrast, Sitja-Bobadilla *et al.* (2008) reported depletion of serum peroxidase levels in *Enteromyxum leei*-infected and non-parasitized gilthead sea bream fish.

Serum lysozyme is used as an indicator of innate immune response in fish (Tort *et al.,* 2003). Lysozyme is an important defence molecule of the innate immune system that disrupts bacterial cell walls by splitting glycosidic linkages in the peptidoglycan layers (Yano, 1997; Saurabh and Sahoo, 2008). Serum lysozyme levels can change in fish in response to parasitic infections (Alvarez-Pellitero, 2008). In our study, significantly elevated serum lysozyme levels were found in moderately and highly infested fish. In sharpsnout seabream, no significant difference in lysozyme level was detected in either infected or healthy animals during *E. leei* infection (Golomazou *et al.,* 2006). An increase of lysozyme activity was detected in guppies following immunization with *T. lysate* along with Freund's complete adjuvant (Chettri *et al.,* 2009), in rainbow trout immunized with live theronts of *Ichthyophthirius multifiliis* (Alishali and Buchmann, 2006), and in Chinook salmon infected by *Ceratomyxa shasta* (Foott *et al.,* 2004). On the other hand, lysozyme activity was diminished in gilthead seabream parasitized by *Polysporoplasma sparis* (Karagouni *et al.,* 2005) and *E. leei* (Sitja-Bobadilla *et al.* 2008).

In the present study, the total antiprotease activity showed a significant difference between the high degree of *Thaparocleidus*-infested fish and uninfected control fish. Basically antiproteases are glycoproteins, synthesized in the liver and secreted into blood which is observed in the form of α-1-protease inhibitor,

α-2 antiplasmin and α-2M in teleost (Ellis, 1987; Zuo and Woo, 1997). Host protease inhibitors modulate the protease activity of the parasite and control a variety of protease-mediated processes, including resistance to invasion by infectious agents (Munoz *et al.,* 2007). Chettri *et al.* (2009) reported that antiprotease was not affected in guppies following immunization with Tet-NI, a culture-attenuated *Tetrahymena* lysate. Further, during artificial *Argulus* infection study in *L. rohita*, antiprotease activity did not show variation in different degrees of lice-infected fish and uninfected fish (Saurabh *et al.,* 2010).

The prime function of α-2M antiproteases family is the clearance of active proteases from the tissue fluids (Armstrong and Quigley, 2001). Alternatively, they can also contribute in intermingle with innate and adaptive mechanisms (Dalmo *et al.,* 1997). In the present study, a significantly elevated level of α-2M activity was observed in high and moderate degree *Thaparocleidus*-infested fish which indicates that the infected fish might be producing a higher amount of α-2M (chief protease inhibitors) under parasitic induction. Similarly, a higher level of α2-macroglobulin was reported in *E. scophthalmi* exposed turbot and *Argulus* challenged *L. rohita* compared to control fish (Sitja-Bobadilla *et al.,* 2006; Saurabh *et al.,* 2010).

Ceruloplasmin is one of the vital positive acute-phase proteins, having important anti-inflammatory roles (Cousins, 1985; Arnaud *et al.,* 1988). In the present study, ceruloplasmin level was correlated with degrees of monogenean infestation on fish, but it was significantly lower in low degree and elevated in high degree of monogenean-infested fish in comparison with control fish. The increase in concentration of ceruloplasmin might be due to inflammatory response of fish to parasite infection and triggering the liver to synthesize the positive acute phase protein to maintain the homeostasis. The effect of gill monogenean on ceruloplasmin of fish is not documented however, it is found with other group of ectoparasite. Saurabh *et al.* (2010) reported decreased ceruloplasmin levels during differential degrees of *Argulus* infection in *L. rohita*.

Increase in albumin and globulin levels are considered a strong innate response in fishes (Wiegertjes *et al.,* 1996). In the present study, elevated total protein and globulin in high and moderate degree of infested group confers the upregulated synthesis of acute phase protein or lysis of cellular component by

parasitic infection that release in serum. Similar finding of elevated level in total protein was reported in olive flounder infected with *Philasterides dicentrarchi* (Harikrishnan *et al.,* 2012). The main reason for increased TP level is stress. However, no difference in total protein concentration was reported between *Argulus* infested and uninfested rohu fish (Saurabh *et al.,* 2010), *Neoparamoeba*-infected Atlantic salmon (Gross *et al.,* 2005). The lower albumin-globulin ratio in the parasitized groups may be due to reduced synthesis of albumin protein and more conversion of globulin to cope the immunological function.

The function of natural antibodies (immunoglobulin) in a variety of defence-related activities such as limiting the dispersal of infectious agents, killing of microbes, and other potential pathogens, repair of tissue damage, and restoration of the healthy state are recorded. We found a significant high level of total immunoglobulin in serum of *Thaparocleidus*-infested fish in comparison with non-infested fish. A higher amount of immunoglobulin was estimated in the plasma of *Ptychobothrium* infected *C. carpio* in comparison to non-infected fish (Studnicka and Siwicki, 1990). In contrast, reduction in Ig level was reported in silver catfish immunized with live theronts of *I. multifiliis* by intra peritoneal injection and bath immersion (Tancredo *et al.,* 2015).

Oxidative stress responses

Parasitic infections are often associated with an oxidative stress in hosts. Such response was also noticed in fish by Bello´ *et al.* (2000) who suggested that the fish response to parasitic infection could involve reactive oxygen intermediates and, therefore, an induced oxidative stress, but without inducing significant differences in the superoxide dismutase and catalase activities (antioxidant enzymes). In the present study, we observed induction of oxidative stress biomarkers (SOD, CAT, and GST) in gills, liver and serum in fish infested with different degrees of *Thaparocleidus* in comparison to uninfested fish. Induction in the activities of antioxidant enzymes might be due to the response of gill tissues to parasite infestation which cause a cellular stress and release of severe reactive oxygen molecules (ROS) to counteract the infection. Subsequently, the cellular protective defence system of host against ROS, and immune evasion mechanism of parasite (Dzik, 2006) both trigger the antioxidant enzymes activity in gills. Dautremepuits *et al.* (2003) reported significantly higher GST, CAT and glutathione

reductase (GR) activities in liver and head kidney in *Ptychobothrium* (cestode) infected carp than un-infected fish. No significant difference in catalse activity was reported in the head kidney between normal and *Ptychobothrium*-infected fish. Increase or improvement in antioxidant status of parasitized fish is related to host response against oxidative stress. This may be explained as modulation in antioxidant enzymatic activities related to metabolic variations (Wilhelm Filho *et al.,* 1993). As parasitism can disturb the metabolic pathways of its host, an increase in the different antioxidant defence found in infected fish can probably be linked to the modulated metabolic activity in relation to the parasitic load (Buchmann and Lindenstrøm, 2002). Radovanovi´c *et al.* (2015) reported higher glutathione-dependent enzyme activities in barbel (*Barbus barbus*) infected with intestinal parasite *Pomphorhynchus laevis* (Acanthocephala). Similarly, low SOD and catalase activities were reported in *Argulus*-infested fish when compared to healthy un-infected goldfish (Kumar *et al.,* 2013).

In the present study, we found significantly decreased activity of Na^+-K^+-ATPase in gills and liver of *Thaparocleidus*-infested fish when compared with control fish. Reduction in the activity of Na^+-K^+-ATPase in the tissues of the parasite infested fish could be a consequence of cellular stress and degradation by toxin released by parasite which impaired the synthesis of Na^+-K^+-ATPase in the system. Ferguson et *al.* (2012) reported the multispecies parasitism (*Nanophyetus salmincola*, *Apophallus* sp.) and one myxozoan (*Myxobolus insidiosus*) inhibited the Na^+-K^+-ATPase in *Oncorhynchus kisutch*. On the contrary, a significantly elevated gill Na^+-K^+-ATPase activity was reported in low level sea lice (*L. salmonis*) infected *Salmo salar* (Nolan *et al.,* 2000).

In the present study, a description of the morphological and molecular characteristic of *Thaparocleidus* spp. infesting on *P. hypophthalmus* is presented. Subsequently, we demonstrated the host and tissue specificity of *Thaparocleidus* and recordes in first the co-occurrence of trematode larvae (metacercariae) and dactylogyrids infection in *P. hypophthalmus* from India. Further, clinical and histopathological investigation of blood and gills respectively, reveals a severe alteration in haematological and serum biochemistry of *P. hypophthalmus* that could cause reduction in overall health status of infected fish. The artificial challenge of *Thaparocleidus* to fish suggested that varying degree of *Thaparocleidus* infestation

not only modulated the innate immune response of *P. hypophthalmus* by lowering albumin, total serum antiprotease, ceruloplasmin and Na^{+}-K^{+}-ATPase and inducing NBT, MPO, lysozyme, PA, α-2M, total protein and total immunoglobulin but also induce oxidative stress biomarkers (SOD, CAT and GST).

6. SUMMARY

Aquaculture provides high-quality animal protein to growing population and sustains income and employment for rural communities. India produces about 10.79 million tonnes of fish annually and there is a tremendous potential to increase fish production through aquaculture using advanced technologies and cultural practices. Diversification of species in aquaculture is an important step towards enhancing fish production in order to meet the increasing demand. The introduction and popularization of *Pangasianodon hypophthalmus* in various parts of India is an example of successful diversification of aquaculture species. Owing to the strong domestic and foreign market demand, remarkable growth and relatively higher resistance to infections; pangasius has become the 3rd most cultured freshwater fish in India. However, few studies have been conducted to assess the risk and benefits of the pangasius culture in India highlighting the disease risk including introduction and establishment of alien pathogens in our indigenous system.

Disease outbreaks are a major bottleneck in the development of sustainable aquacultural practices including pangasius farming. Majority of reports of bacterial diseases in wild and grow out ponds of *P. hypophthalmus* from various countries have been documented. In India, *P. hypophthalmus* is also noticed for infection with bacterial pathogens viz. *Enterobacter clocae, Aeromonas hydrophila, A. jandaei, Pseudomonas sp.* and *Edwardsiella tarda*. In addition, severe parasitic infestation is also found to have severe infection in early larval stages of pangasius fish which hampers the production. Gill fluke infection is commonly seen in *P. hypophthalmus* farms with prevalence ranging from 60% to 90% with intensity as high as 200 parasites per individual. Further, high mortalities due to gill fluke are generally noticed during the first week after stocking. Despite the fact that gill flukes contribute to significant losses due to the secondary bacterial infections that follow the initial infections with the parasite, this receives little attention. The investigation on the monogenean parasite infesting *P. hypophthalmus*; their pathogenicity and host-parasite interaction are important in terms of developing the guidelines for prevention, control and treatment of the disease epidemics.

In the present study, an attempt was made to investigate and identify species of the monogenean parasite infesting *P. hypophthalmus* and study the

pathology associated with infection. Further an artificial challenge experiment with monogenean parasite was performed to assess the haemato-immunological responses of *P. hypophthalmus* to parasite.

The epidemiological parameters of monogenean parasite infection in *P. hypophthalmus* showed 72% of only gill monogenean prevalence in *P. hypophthalmus*, with abundance approximately 20 parasites per individual and mean intensity (39 parasites per infected fish). In the present study, we found two distinct gill monogenean species belonging to genera *Thaparocleidus* of dactylogyrids infesting on *P. hypopthalmus*, on the basis of morphometric traits. Further, the morphometric traits of isolated *Thaparocleidus* spp. were measured and compared with specimen reported from India and Malaysia. The identification features of *Thaparocleidus* spp. along with orientation of general morphology and measurements of male and female reproductive organ, and haptoral parts of our specimens were consistent with the details of previous reported specimen. In this regard, we identified these two specimens as *T. siamensis* and *T. caecus*. However, the mean values of measurements of morphometric traits of our specimen showed a marginal higher side in case of *T. siamensis* and lower side in case of *T. caecus* in comparison to previously reported one. Remarkably, longer marginal hook (mean length 22 and 20 μm for *T. siamensis* and *T. caecus,* respectively) was observed in the present study in comparison to documented specimen (12-14 μm for both spp.). Further sequencing analysis of the PCR amplified 28S rDNA (partial segment) gene of monogenean parasite isolated from infected *P. hypophthalmus* revealed 100% similarity with Malaysian *T. siamensis*.

The gross pathology of infected fish and gill biopsy displayed varying degrees of changes in body colouration, severe mucus secretion, gasping behaviour and lethargy. The behaviour irregularities, shown by the infected fish increased with increase in intensity of monogenean parasite. We observed single parasite group, monogenea infecting gills of *P. hypophthalmus* in most of the cases whereas, co-occurrence of monogenean and trematode larvae (metacercaria) in some cases was also recorded from the fish collected from cages. This might be due to the fact that cages in reservoirs are in proximity of wild conditions where there are chances of miracidium infected molluscs being consumed by *P. hypothalamus* or may be due to transfer of metacercariae larvae by a carrier to

second intermediate host resulting in metacercariae encysted in the gill filaments of fish. Further, we observed non-random distribution and aggregation of monogenean parasites on gill filaments irrespective of infection of trematode metacercariae. Water flow, the interplay between the morphological properties of gill filaments and the attachment apparatus of the parasites on the host might be influenced the aggregation and distribution of monogenean parasite in specific host.

In the present study, the results of the host and tissue preference of *Thaparocleidus* spp. revealed a noticeable positive relation between host standard length and parasite intensity. Interestingly, a low level of *Thaparocleidus spp.* infection was recorded in fish below and above standard length of 10 and 15 cm. This might be attributed to exposure of parasite to host and acquired immunity in host to reduce the burden, as well as resistance to parasite for their preferable attachment. There was lack of relationship and interaction found between host body mass and parasite intensity.

Host specificity, intraspecific aggregation and microhabitat specificity appears to be a common trait among monogeneans. In present study, *Thaparocleidus* spp. exhibited some degree of microhabitat selection for the first two (outer) gills arch of its host and subsequently decreased preference towards third and fourth gill arch (GA1≅GA2 > GA3 > GA4). Briefly, significantly high (69.31%) number of total *Thaparocleidus* individuals was recorded on gill arch1 and 2 and subsequently a decreased (30.69%) of total parasite recorded on gill arch 3 and 4. The increased number of *Thaparocleidus* spp. may also be reflective of the larger size of the outer gill arches, therefore providing a greater gill surface for attachment. More precisely, a significantly high percentage of parasite number 48.84 and 48.47 was estimated in medial and central region of gill arch, respectively, in comparison to dorsal (31.84), posterior (35.04), ventral (20.05) and anterior (17.48) regions.

The histopathological technique revealed the changes in the tissues at the cellular level. The degree of severity of the gill lesions caused by *Thaparocleidus* spp. (gill monogenean) ranged from mild to severe and focal to multifocal as intensity of parasites increased in the samples. A proliferative epithelial response, a high number of mucous cells, infiltrating inflammatory cells, distortion of the lamellar structure, fusions of secondary lamellae, rupture epithelia and

depression in gill filaments were observed in histological sections of the gills of parasitized *P. hypophthalmus*. In the present study, we also observed a concurrent infection of dactylogyrids and trematode larvae (metacercaria) in gill tissue. The encysted metacercaria at various regions was found to be associated with cartilaginous part of the gill filament and caused local deformation of cartilaginous filament ray.

In the present study, based on monogenean parasite intensity, the sampled fish were categorized into three distinct group viz. uninfested, low (<35 parasite per gill), and high (>35 parasite per gill). There was a significant ($p<0.05$) elevation in TEC, TLC, Hb, PCV, heterophils, eosinophils and monocytes whereas reduction in MCV, MCH, MCHC and lymphocyte of high *Thaparocleidus* spp. infested groups in comparison to low level infested and uninfested fish. Further the first component (PC1) from principal component analysis depicts approximately 76.71% of the total variance which indicate the separation among the groups through the distribution of the significantly high but negatively related MCV, MCH and positively related TEC, Hb and monocytes of the high level parasitized fish. The considerable changes might be a physiological adaptation that result due to infection in gill which reduces the respiratory physiology and create a hypoxic condition. In the present study, there was a significant elevation in level of total protein and globulinin parasitized groups. It can be inferred that subsequent enhanced globulin level in parasitized groups may be protective immune response of *P. hypophthalmus* against *Thaparocleidus* spp. and this may be an initial phase towards preparatory stage for stress mitigation.

Parasitism represents one form of biological stress on the host body and therefore affects the natural balance of oxidative stress parameters, thereby causing sever oxidative pressure on the host. We observed the increase in activities of SOD and CAT of high level of parasitized *P. hypophthalmus* which exhibited the cellular oxidative response towards parasitism. Further, an increased but within optimum level sGOT and sGPT activity of parasitized group exhibits significant increase in protein synthesis evident from elevated total serum protein. The increase in ALP in the high level parasitized *P. hypophthalmus* found in this study can be related to the tissue damage in the vital gills or increased workload of liver caused by the parasite. However, an increase in LDH level indicates metabolic

changes like glycogen catabolism and glucose changes direction towards lactate form in distressed fish. Noticeable elevated blood glucose, and lactate content in high level parasitized fish group indicates a higher rate of metabolism and high glucose turnover under anaerobic condition.

Different degree of infestation of monogenean was accomplished by an artificial challenge of parasite to healthy pangasius fish. Further responses of haemato-immunological and oxidative stress biomarkers were assessed to evaluate host responses to different degree of parasitism. In the present study, significant increase in haematological parameters except blood indices were recorded in the high and moderately infested group when compared with low and uninfested group. This might be indicative of cellular adaptive response towards oxygen demand, active phagocytosis and changes caused by infestation of gill monogenean on host lamellae. The significant increase in erythrocyte osmolality brittleness of high degree of infected fish showed a decrease in hemolytic resistance of erythrocyte, giving an indication of alteration in membrane composition and integrity as well as the cells sizes or the surface area to volume ratio of erythrocytes.

Total protein, globulin and blood glucose were elevated in the high and moderate parasitized groups suggesting that increase in metabolism to liberate active energy substrate to various tissues is a primary step in order to cope with the increased energy demand and maintain the osmolality of blood plasma. The high degree of parasitism also led to the elevation of sGOT, sGPT, LDH, bilirubin and creatinine in the infected fish serum that suggests that higher protein synthesis and active liver function.

The increase in activity of innate immune parameters i.e. RBA, myeloperoxidase, serum lysozyme and phagocytosis in moderate and high level of parasitized groups suggest an induction of effector function of innate immune system. Further elevation in activity of humoral innate system i.e. antiprotease activity, α-2 macroglobulin, ceruloplasmin and total immunoglobulin in the moderate and high level parasitized fish contribute in effector function, elicit the cellular defence mechanism and intermingle with innate and adaptive immune mechanisms. Changes in gill and serum oxidative markers i.e SOD, CAT, GST and Na^+-K^+ ATPase, either by increasing or decreasing their activities are mostly interpreted as the adaptive responses to oxidative stress.

In conclusion, we recorded that the morphological and molecular characteristic of *Thaparocleidus* spp. infesting *P. hypophthalmus* and found *Thaparocleidus* preference towards 10 to 15 cm of host standard length and gill 1-2 (posterior-central, and dorsal-medial). The study provides first report on co-occurrence of trematode larvae (metacercariae) and dactylogyrids infection in *P. hypophthalmus* from India. Further, clinical and histo-pathological investigation of blood and gills respectively, reveals a severe alteration in haemato-serum biochemistry and gill filaments of *P. hypophthalmus* that may be a cause for reduction in overall health status of infected fish. The artificial challenge of *Thaparocleidus* to fish suggested that varying degree of *Thaparocleidus* infestation, not only modulated the innate immune response of *P. hypophthalmus* by lowering albumin, total serum antiprotease, ceruloplasmin and Na^+-K^+-ATPase and inducing NBT, MPO, lysozyme, PA, α-2M, total protein and total immunoglobulin but also induced oxidative stress biomarkers (SOD, CAT and GST) in the infected fish. The baseline data obtained in the present study will be valuable in understanding the host-parasite relationship and thereby, in formulating effective management strategies for the parasite which has significant impact on the health of farmed *P. hypophthalmus*.

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ABBREVIATION

%: Percent

∆A: Change in absorbance

°C: Degree centigrade

µL: Microliter

$^1O_2$: Singlet oxygen

A: Absorbance

A: Albumin

A: Anterior

Ab: Antibody

ADP : Adenosine diphosphate

AFA: Alcohol-formalin-acetic acid

AGD : Amoebic gill disease

ALP: Alkaline phosphatase

ANOVA: Analysis of Variance

APL : Accessory piece length

ATP: Adenosine triphosphate

B: Blank

BAPNA: Sodium-benzoyl-DL-arginine-p-nitroanilide

BCG: Bromocresol Green

BL : Body length

BLAST: Basic Local Alignment Search Tool

BNP: Bacillary necrosis of pangasius

bp: base pair

BW : Body width

C: Central

CAT: Catalase

Cb: Chondrioblast

cc: cartilaginous cells

CC: Chondriocyte

CCV: Channel catfish virus

CD : Cluster of designation

CDNB: 1-chloro-2,4-dinitrobenzene

CIFE: Central Institute of Fisheries Education

cm: Centimetre

CPC: Contentional hyaline parasitic capsule

cr: cartilaginous ray

CRE: Creatinine

CTL : Copulatory tube length

cy: encysted

D: depression

DAHDF: Department of Animal Husbandry, Dairying and Fisheries

DBL : Dorsal bar length

DBW : Dorsal bar width

DC: distal clubbing

DIR : Inner root length of dorsal anchor

dL: Desiliter

DLC: Differential leucocyte count

DMRT: Duncan's multiple range test

DMSO: Dimethyl sulphoxide

DNA: Deoxyribonucleic acid

dNTPs: Deoxynucleotides triphosphates

DOR : Outer root length of dorsal length

DRP : Recurved point of dorsal anchor

DTL : Total length of dorsal anchor

EDTA: Ethylenediaminetetraacetic acid

ELC : Eosinophils-like cells

ELISA: Enzyme linked immunoassay

EOB: Erythrocyte osmolarity Brittleness

EUS: Epizootic ulcerative syndrome

FAO: Food and Agriculture Organization

fL: Femtoliters

FRP: Fibre Reinforced Plastic

g L^{-1} : gram per liter

G: Globulin

g: Gram

GPx : Glutathione peroxidise

GSH: Glutathione

GST: Glutathione-S-transferase

H : Hyperplasia

H&E: Haematoxylin and Eosin

h: Hour

H_2O_2 : Hydrogen peroxide

H_2SO_4 : Sulphuric acid

Ha: hamulus

Ha: hectare

Hb: Haemoglobin

HBSS: Hanks balanced salt solution

HL : Haptor length

HL: Hook Length

HW : Haptor width

i.e.: that is

IC: inflammatory cells

ICAR: Indian Council of Agricultural Research

Ig : Immunoglobulin

IGS: Intergenic spacers

IL 1β : interleckin 1 beta

iNOS : inducible nitrogen oxide synthetase

KCl: Potassium chloride

Kg: Kilogram

KOH: Potassium hydroxide

L: Litre

LDA: Linear discriminant analysis

LDH: Lactate dehydrogenase

LF: Lamellar fusion

LYM : Lymphocyte

M: Mole/Molarity

M: Monocyte

MAF: Ministry of Agriculture and Forestry

MANOVA: Multivariate analysis of variance

MAS: Motile aeromonand septicaemia

MC: mucous cell

MCH: Mean cell haemoglobin

MCHC: Mean cell haemoglobin concentration

MCV: Mean cell volume

mg: Milligram

$MgCl_2$: Magnesium chloride

MHC: Major histocompatibity complex

Min.: Minute

mm: Millimeter

mM: Millimole

MMCs: Melano-macro phage centres

MPD : Main part length of dorsal anchor

MPO: Myeloperoxidase

MPV : Main part length of ventral anchor

N : Neutrophils

N : Nitrogen

n: Number

N: Total number

NaCl: Sodium chloride

NADH: Nicotinamide adenine dinucleotide

NaN_3 : Sodium azide

NBF: Neutral buffer formalin

NBT: Nitroblue tetrazolium

NCBI: National Center for Biotechnology Information

NJ: Neighbour-Joining

NK: Natural Killer

nM: nanomole

$O^{\cdot}_2$: Superoxide

o/oo : Parts per thousand

OD: Optical density

$OH^{\cdot}$: Hydroxyl radical

OIE: World Organization of Animal Health

OL : Ovary Length

OW : Ovary width

P: Posterior

PA: Phagoctic activity

PBS: Phosphate buffer saline

PC: Principal component

PCA: Principal component Analysis

PCR: Plymerase chain reaction

PCV: Packed Cell Volume

PEC: Proliferative epithelial cells

PEG: polyethylene glycol

pg: Picograms

Pi: inorganic phosphate

PL : Pharynx length

pM: Picomole

PPD: p-phenylene diamine

ppm: Parts per million

PW : Pharynx width

R: Correlation coefficient

RAPD: Random amplified polymorphic DNA

RBA: Respiratory burst activity

RBC: Red Blood Cell

rDNA: Ribosomal Deoxyribonucleic acid

RFLP: Restriction fragment length polymorphism

RNA: Ribonucleic acid

ROS: Reactive oxygen species

rpm: Revolutions per minute

S: Standard

SDS: Sodium dodecyl sulfate

Sec.: Second

sGOT: Serum glutamate oxaloacetate transaminase

sGPT: Serum glutamate pyruvate transaminase

SOD: Superoxide dismutase

sp. : Species

SPSS: Statistical Package for Social Sciences

T: Test

TAE: Tris-acetate-EDTA

TCA: Trichloroacetic acid

TCR : T-cell receptor

TEC: Total Erythrocyte Count

TGF : Transforming growth factor

TL : Testis length

TLC: Total Leucocyte Count

TMB: 3,3'-5,5'-tetramethyl benzidine hydrochloride

TNF-α : Tumor necrosis factor-alpha

TP: Total protein

Tris-HCl: Tris-hydrochloride

TW : Testis width

U: Unit

UDN : Ulcerative dermal necrosis

US: United state

UV: Ultraviolet

V/V: Volume by volume

VBL : Ventral bar length

VBW : Ventral bar width

Vis: Visible

Viz.,: as follows

VL : Vaginal length

VRP : Recurved point of ventral anchor

VTL : Total length of ventral anchor

W/V: Weight by Volume

WBC: White Blood Cell

α -2 M: α-2 macroglobulin

ε : Extinction coefficient

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Full length article

Modulation of innate immune responses and induction of oxidative stress biomarkers in *Pangasianodon hypophthalmus* following an experimental infection with dactylogyrid monogeneans

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Pangasianodon hypophthalmus

ABSTRACT

Modulation of innate immune activity and oxidative stress response of *Pangasianodon hypophthalmus* through experimental infection with (*Thaparocleidus* sp.) dactylogyrid monogenean was studied. A standard cohabitation method was used to infect healthy experimental fish. After 14 days, dactylogyrid (gill monogenean) infested fish were sampled and categorised into three different infected groups namely (T1) low (<10 mean dactylogyrid per gill arch per fish), (T2) moderate (10–49 mean dactylogyrid per gill arch per fish) and (T3) high (>50 mean dactylogyrid per gill arch per fish) along with a control group T0 (un-infested fish). Serum and tissues (gills and liver) were collected from experimental fish and analyzed for markers of innate immune and oxidative stress, respectively. The results showed that respiratory burst activity, myeloperoxidase level, serum lysozyme, α-2 macroglobulin and total serum immunoglobulin level were significantly ($p < 0.05$) elevated in fish with different degrees of parasite infestation compared to the control (un-infested group). Similarly, cellular oxidative biomarkers superoxide dismutase, catalase, glutathione-S-transferase and Na^{+}-K^{+}-ATPase activities of gills and liver were significantly ($p < 0.05$) elevated in dactylogyrid infested fish in comparison to the control. However, significantly decreased level of albumin, albumin to globulin ratio, total serum antiprotease and ceruloplasmin were observed in fish infested with low degree of dactylogyrids, while no significant differences in these parameters were observed between moderately infested and the control groups. The results suggested that varying degree of gill monogenean dactylogyrid infestation not only modulated the innate immune response of *P. hypophthalmus* by lowering albumin, total serum antiprotease and ceruloplasmin and inducing respiratory burst activity, phagocytic activity, myeloperoxidase, lysozyme, α-2 macroglobulin and total immunoglobulins, but also the oxidative stress biomarkers. The baseline data obtained in the present study will be valuable in understanding the host-parasite relationship and the dynamics of innate, oxidative stress responses and susceptibility of *P. hypophthalmus* to different degrees of parasitosis.

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1. Introduction

Aquaculture is recognized as one of the potential food producing sectors which provides high-quality animal protein to growing population and generates income and the employment. India produces about 8.66 million tonnes of fish annually [1] and with vast aquatic resources available in the country, there is a

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tremendous potential to increase fish production. Diversification of species in aquaculture is an important step towards enhancing fish production in order to meet the increasing demand. The introduction and popularisation of catfish pangasius (*Pangasianodon hypophthalmus*) in various parts of India is an example of successful diversification of aquaculture species [2]. Owing to the strong market demand, fast growth and standardized culture practices, pangasius has quickly become the 3rd most cultured freshwater fish in India. Currently, pangasius is also being cultured in cages in reservoirs [3]. However, few studies have been conducted to assess the risk and benefits of the pangasius culture in India [2]. In recent years, farming practices have intensified to increase production and maximize the profit. It is widely recognized that this intensification is generally accompanied by an increase in diseases and problems caused by various infectious agents.

Disease is the primary constraint to the all types of culture systems including pangasius farming. Few reports of bacterial diseases caused by *Enterobacter cloacae, Aeromonas jandaei* and *Edwardsiella tarda* have been reported in grow out systems of *P. hypophthalmus* in India [4–6]. In addition, severe parasitic infestation can occur in early larval stages and hamper the production [2]. Gill fluke infection is commonly seen in *P. hypophthalmus* farms with prevalence ranging from 60% to 90% [7]. High mortalities due to gill fluke are generally noticed during the first week after stocking. It has been observed that the prevalence and intensity of parasites on the fish correlates positively with high stocking density, water temperature and organic load in culture ponds. Although monogenea is not considered as a great hurdle in aquaculture, the growth reduction due to biological stress, severely affects the economies of aquaculture.

Fish has a strong immune system to protect it from various infectious agents. However, information on the innate and adaptive immune responses to monogenean parasites by the fish hosts is meagre. In general, fish largely rely on the innate immune system as the first defence barrier to monogenean infestation in the form of localized inflammation which appears as hyperplasia and severe mucus secretion on the fish gills and skin [8,9]. Reports of gill monogenean infestation on pangasius species and associated health risk are available. However, no systematic studies have been conducted on the host immune responses against this parasite. Therefore, it is important to recognize the changes in the immune system of host occurring during monogenean infestation. Moreover, host responds to biological stressors by an antistress or oxidative response mechanism such as catalase, superoxide dismutase (SOD), and glutathione-S-transferase (GST) and levels of total protein, globulin, and albumin [10,11]. Catalase and SOD are considered as important biomarkers of oxidative stress. Fish are endowed with defense mechanisms to counteract the impact of reactive oxygen species (ROS) resulting from the catabolism of various antioxidant defense enzymes such as SOD, Catalase and GST family possessing detoxifying activities [12]. Thus, the present work aimed to study the effect of different degrees of dactylogyrid (*Thaparocleidus* sp.) infestation on the innate immune responses and oxidative responses of *P. hypophthalmus*.

2. Materials and methods

2.1. Experimental fish and maintenance

Healthy *P. hypophthalmus* (n = 120) having mean weight 20.2 ± 2.56 g and a mean standard length of 9.4 ± 1.02 cm were procured from Kurla Aquarium shop, Mumbai, India. The stock was acclimatized in 500 L water under aerated conditions in 1.52 × 0.65 × 0.62 m Fibre Resin Plastic (FRP) tank for a period of 14 days and fed with commercial pelleted diet at 3% of body weight. In order to ameliorate the handling stress, the fish were given a mild salt treatment and ascorbic acid (vitamin C). About one tenth of water was replaced daily in order to remove uneaten feed and faecal matter. The physico-chemical parameters of water were measured weekly with a view to ascertain its optimal condition (temperature 26.8 ± 1.2 °C; dissolved oxygen: 5.80 ± 0.85 mg L^{-1}; pH: 7.5 ± 0.64; ammonia: 0.095 ± 0.055 mg L^{-1}; nitrite: 0.020 ± 0.005 mg L^{-1}) throughout the experiment.

Infected *P. hypophthalmus* (n = 40) were obtained from cage culture of Murbagh, Mumbai, India, with a mean weight of 18.0 ± 5.28 g and a mean standard length of 9.2 ± 1.13 cm. Fish were transferred to the Central Wet Laboratory, ICAR-CIFE, Mumbai for acclimatization, and randomly selected 5 fish were biopsied and their gills, skin and fins were examined to ascertain the presence of the monogenean parasite. These fish had a mean intensity (MI) of 30.5 ± 4.0 gill monogenean parasite. Infected fish (n = 30) were used as a source of parasitic infection in the cohabitation trial. These fish were naturally infected under prior culture conditions. The presence or absence of adult dactylogyrid (gill monogenea) on the infected fish and uninfected fish during the trials was confirmed by observation of the parasite on gills of fish, using a stereomicroscope (Leica Microsystems, Germany) [13]. During 15-days acclimatization period and throughout the experiment, fish were reared in 500 L tanks supplied with saturated and aerated ground water. Fish were exposed to a natural (12 h light: 12 h dark) photoperiod, a temperature of 26.6 ± 1.5 °C, pH of 7.6 ± 0.4 and dissolved oxygen of 5.3 ± 0.8 mg L^{-1}, and fed to apparent satiation with a commercial pellets diet (35% protein) twice per day.

2.2. Infection by cohabitation

The artificial experimental challenge was performed by cohabitation method with a slight modification [14]. Healthy fish (120 in number) were given a bath treatment with 2 ppt saltwater for 15 min, followed by a bath treatment with mebendazole (4.5 mg L^{-1} Sigma, India) for 24 h against helminthic parasites [13]. Each bath treatment was repeated for 3 consecutive days. The treated healthy fish were used for the experiment after 7 days of acclimatization. Infected fish were marked by making a cut on the tip of the caudal fin to differentiate them and these fish were not sampled. In order to demonstrate the immunomodulation in the host (*P. hypophthalmus*) caused by differential degree of monogenan parasite infestation, experimental infection trials were conducted in 500 L FRP tanks containing 300 L of fully aerated ground water. Healthy fish were challenged by cohabitation mode for artificial infestation for a period of 14 days. The whole experiment was conducted with complete randomized design with triplicates of each treatment. In the control group (T0), 30 healthy fish were kept without introducing any monogenean infested fish. In the artificial infection challenge group, 90 fish were kept in nine tanks with each tank containing 10 healthy fish, cohabited with 2, 4 and 6 infected fish. After 14 days, three grades of infections were defined as (T1) low (<10 mean dactylogyrid per gill arch per fish), (T2) moderate (10–49 mean dactylogyrid per gill arch per fish) and (T3) high (>50 mean dactylogyrid per gill arch per fish). The serum and gill samples were collected from 9 fish with each of the three infection grades in the experimental tanks and from 9 uninfected fish in the control tanks.

2.3. Collection of blood, serum and tissue sample

Fish were anesthetized with clove oil (Merck, Germany) at the rate of 50 μL per litre of water before taking blood from fish. Blood was drawn from the caudal vein without using the anticoagulant and the serum was separated from blood by keeping the tubes in

slanting position for around 2 h. The blood was centrifuged at 1500 *g* for 10 min at 4 °C and the serum was collected and stored at −20 °C for further use. Subsequently, fish were dissected under aseptic conditions and the tissues such as gills and liver were collected. Further, the tissues were homogenized (5% w/v) separately in chilled sucrose solution (0.25 M) in a glass tube using Teflon coated mechanical tissue homogenizer (Remi, India). The homogenates were centrifuged at 3500 × *g* for 20 min at 4 °C. The supernatants were collected and the protein contents were quantified following the method of Lowry et al. [15] and stored at −20 °C until further analysis.

2.4. Innate immune parameters

2.4.1. Respiratory burst activity (RBA)

Nitroblue tetrazolium (NBT) assay was carried out to estimate RBA using the method of Anderson and Siwiki [16]. One-hundred microliters of blood was placed into the wells of a flat bottomed microtitre plate and incubated at 37 °C for 1 h to facilitate adhesion of cells. The supernatant was removed and the wells were washed three times with phosphate buffered saline (PBS, pH 7.4), followed by the addition of 100 μL of 0.2% NBT. The plate was incubated for further 1 h and washed once with 100% methanol and thrice with 70% methanol. The plates were allowed to air dry. Potassium hydroxide (2 N; 120 μL) and dimethyl sulphoxide (DMSO) (140 μL) were added into the wells to form blue formazone precipitate. The absorbance of the wells on was measured in an ELISA reader (Quant, Universal microplate spectrophotometer, USA) at 620 nm.

2.4.2. Phagocytic activity

Phagocytic activity was detected using *Staphylococcus aureus* as described by Anderson and Siwicki [16]. A sample (0.1 mL) of blood was placed in a microtiter plate well into which 0.1 mL of *Staphylococcus aureus* 1×10^7 ($A_{450} = 0.5–0.6$) cells suspended in phosphate buffered saline pH 7.2 was added and mixed well. The bacteria–blood solution was incubated for 20 min at room temperature. Five microliters of this mixture was taken on a clean glass slide and a smear was prepared. The smear was air dried, fixed with ethanol (95%) for 5 min and air dried, followed by staining with Giemsa (7%) stain for 10 min. Two smears were made from each fish. A total of 100 phagocytic cells (peripheral monocytes and neutrophils) from each smear were observed under the light microscope (Carl Zeiss, Axioskope 40, Germany) and the number of phagocytising cells and the number of bacteria engulfed by the phagocytes were counted. Phagocytic activity was determined by dividing the number of phagocytising cells by the total number of phagocytes counted.

2.4.3. Myeloperoxidase content

Total myeloperoxidase content present in serum was measured according to Quade and Roth [17] with some modifications [18]. About 15 μL of the serum was diluted with 135 μL of hank's balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+} in 96 well plates. The wells were added with 25 μL of 20 mM 3,3′-5,5'- tetramethyl benzidine hydrochloride (TMB) (Hi-media) and 25 μL of 5 mM H_2O_2 (Qualigens) (Both substrates of MPO). The reaction was stopped after 2 min by adding 50 μL of 4 M sulphuric acid (H_2SO_4). The plate was centrifuged (400×*g*) for 10 min, and 150 μL of the supernatant from each well was transferred into a new 96-well plates. The absorbance was measured at 450 nm in a micro plate reader (Quant, Universal microplate spectrophotometer, USA).

2.4.4. Serum lysozyme activity

The serum lysozyme activity was measured using colorimetric method [19]. In a cuvette, 3 mL of *Micrococcus luteus* suspension in phosphate buffer (A 450 = 0.5–0.7) was taken, to which 50 μL of serum sample was added. The content of cuvette was mixed well for 15 s and reading was taken in a spectrophotometer at 450 nm. The lysis of bacteria was recorded immediately at 15, 30 and 270 s interval. A unit of lysozyme activity was defined as the amount of sample causing a reduction in absorbance of 0.001 per min expressed as U min^{-1}.

2.4.5. α-2 macroglobulin (α-2M) activity

The activity of α-2M in fish serum was estimated as per the procedure of Zuo and Woo [20] with slight modification. Ten microliter serum was mixed with 100 μL of trypsin (bovine pancreas type I (Sigma, India); 100 μg mL^{-1} of 0.1 M Tris-HCl (pH 8.2). Trypsin was replaced with 100 μL of Tris-HCl in blank and in positive control; no serum was added to trypsin. All tubes were made up to 200 μL with Tris-HCl and incubated at 25 °C for 40 min. After incubation, 2 mL of Sodium-benzoyl-DL-arginine-*p*-nitroanilide HCl (BAPNA, Sigma) dissolved in Tris-HCl containing 20 mM $CaCl_2$ was added to all the tubes and incubated for 20 min at 25 °C. The reaction was stopped by the addition of 500 μL of 30% (v/v) acetic acid. The optical density (OD) was measured at 410 nm in a UV-Vis spectrophotometer (Thermo Spectronic, USA). The OD of the positive control was taken as 100%. The OD value of the serum blank was deducted from the trypsinized sera and the percentage of trypsin inhibition by the serum was calculated.

2.4.6. Ceruloplasmin level

The level of ceruloplasmin in the fish serum sample was measured as *p*-phenylene diamine (PPD) oxidase activity (Sigma, USA) according to method of Pelgron et al. [21] with slight modifications. Twenty five microliter serum or standard of ceruloplasmin (Sigma, USA) was mixed with 500 μL (1.2 M, pH 5.0) acetate buffer containing 0.1% PPD as the substrate. Further, each sample was incubated in the presence of 0.5 mL of 0.5% NaN_3 (Sodium azide). The reaction was terminated by the addition of 0.5 mL of 0.5% NaN_3. One unit of ceruloplasmin was defined as the amount of oxidase that catalysed a decrease in absorbance of 0.001 min^{-1} at 550 nm in a UV-Vis spectrophotometer (Thermo Spectronic, USA).

2.4.7. Serum antiprotease activity

Total serum antiprotease in fish serum was estimated as per Zuo and Woo [20] with slight modification. Serum (10 μL) was mixed with 100 μL of trypsin (bovine pancreas type I, 200 μ g mL^{-1} of PBS; Sigma, India). In the positive control, serum was replaced by 10 μL of PBS buffer solution. All tubes were incubated at 25 °C for 30 min. After incubation, 1 mL of casein dissolved in PBS (2.5 mg mL^{-1}) was added to all tubes and incubated further for 15 min at 25 °C. the reaction was terminated with the addition of 500 μL of 10% trichloroacetic acid. The sample was centrifuged at 10000 *g* for 5 min to remove protein precipitates. The OD of the supernatant was measured in a UV-Vis spectrophotometer (Thermo Spectronic, USA) at 280 nm and the percentage of trypsin inhibition was calculated.

2.4.8. Serum protein profiles

Plasma protein was estimated by Biuret reagents using a commercial kit (Merck, Germany) [22]. Albumin was estimated by bromocresol green binding method [23]. Albumin in a buffered medium binds with bromocresol green (BCG) and produces a green colour whose absorbance is proportional to the albumin concentration. Globulin content was calculated by subtracting albumin values from total serum protein. A:G ratio was calculated by dividing values of albumin by globulin.

2.4.9. Total immunoglobulin level

The modified method of Anderson and Siwicki [16] was followed for the estimation of total immunoglobulin in plasma. The serum (0.1 mL) was placed in a plastic serum vial and 0.1 mL of 12% PEG (Polyethylene glycol) was added and suspended in deionised water. Incubation was done at room temperature for 2 h under constant mixing, followed by centrifugation at 5000×*g* for 10 min. The protein concentration in the supernatant was determined by Biuret method using a commercial kit (Merck, Germany). Protein reading from supernatant was the amount of protein taken out by absorption into the polyethylene glycol. The total immunoglobulin expressed as unit g dL^{-1} was calculated by the formula given below.

$$\text{Total immunoglobulin} = \text{Total protein in individual samples plasma} - \text{Total protein taken out by absorption to polyethylene glycol.}$$

2.5. Enzymes of oxidative stress

2.5.1. SOD (superoxide dismutase)

Superoxide dismutase was assayed as described by Misra and Fridoyich [24], based on the oxidation of epinepherine–adreno chrome transition by the enzyme. Fifty-microliters of the sample (gills, liver and serum) was taken in the cuvette and 1.5 mL of 0.1 M carbonate–bicarbonate buffer containing 57 mg dL^{-1} EDTA (pH-10.2) and 0.5 mL (3 mM) epinephrine was added and mixed well. Change in the optical density at 480 nm was read immediately for 3 min in a UV-Vis spectrophotometer (Thermo Spectronic, USA). One unit of SOD activity was the amount of protein required to give 50% inhibition of epinephrine auto oxidation. SOD was expressed as unit activity (amount of protein required to give 50% inhibition of epinephrine auto oxidation).

2.5.2. Catalase (CAT)

Catalase activity in gills, liver and serum was assayed according to the method described by Takahara et al. [25]. The reaction mixture of 3 mL consisted of 2.50 mL phosphate buffer (50 mM, pH 7.0), 50 μL tissue homogenate (enzyme source) and the reaction was started by the addition of 1.0 mL of H_2O_2 solution. The decrease in absorbance was measured at 240 nm at 15 s intervals for 3 min. An enzyme blank was run simultaneously with 1.0 mL distilled water instead of H_2O_2 solution. Enzyme activity was expressed as nano-moles H_2O_2 decomposed min^{-1} mg^{-1} protein.

$$\text{Catalase activity} = \frac{\Delta OD/min \times (total\ reaction\ volume \times 1000)}{equivalent\ weight\ of\ H_2O_2 \times sample\ volume \times protein\ concentration}$$

2.5.3. Glutathione-S-transferase

The activity of glutathione-S-transferase (GST) of gills and liver was determined spectrophotometrically [26] following the formation of glutathione (GSH) conjugate with 1-chloro-2, 4-dinitrobenzene (CDNB) at 340 nm using extinction coefficient of 9.6 mM^{-1} cm^{-1}. The reaction mixture contained 3 mL volume of 0.3 M sodium phosphate buffer (pH 6.5), 30 mM GSH, and 30 mM CDNB in ethanol, 0.1 mL tissue homogenate and 1.3 mL of distilled water. The mixture was incubated at 37 °C for 5 min. The change in absorbance was measured at 340 nm for 3 min at 60 s intervals. GST activity was expressed as nM CDNB conjugates formed min^{-1} mg of $protein^{-1}$ where ε = 9.6 mM^{-1} is extinction coefficient for CDNB conjugate at 340 nm for test in 1 mL cuvette.

$$\text{GST activity} = \frac{\Delta OD_{340}/min \times (total\ reaction\ volume \times 1000)}{\varepsilon \times sample\ volume \times protein\ concentration}$$

2.5.4. Na^+-K^+-ATPase activity

Na^+-K^+-ATPase activity of gills and liver was measured by liberating PO_4 (Pi) from a hydrolysis reaction with ATPase, according to Svobaca and Mossinger [27] with slight modification [28]. The activity in the gill and liver was assayed in reaction mixture of 1 mL of 0.1 M Tris-HCl buffer (7.8), 0.1 mL 100 mM NaCl, 0.1 mL 20 mM KCl, 0.1 mL 3 mM $MgCl_2$, 0.5 mL 5 mM ATP and 0.1 mL tissue homogenate (test). For control experiments, Trichloroacetic acid (TCA) (Sigma, USA) was used instead of tissue homogenate. The reaction mixture was incubated for 15 min and the reaction was terminated by adding 10% TCA. The liberated phosphate was estimated at 660 nm according to Fiske and Subbarow [29] and the values were calculated and expressed as μM Pi liberated mg^{-1} protein h^{-1} in the tissues using the following formula.

$$\text{ATPase activity} = \frac{OD\ at\ 660\ nm(Test - Control) \times concentration\ of\ standard \times 0.5}{OD\ of\ standard \times volume\ of\ sample \times protein\ concentration \times time}$$

2.6. Statistical analysis

The data of immune parameters and oxidative stress biomarkers were tested for their normality using Shapiro-Wilk test and were statistically analyzed using statistical package SPSS version 16 in

which data were subjected to one-way ANOVA. Duncan's multiple range tests (DMRT) was used to determine the significant differences between the means of treatment and control. Comparisons were made at 5% probability level.

3. Results

3.1. Innate immune responses

The respiratory burst activity measured by NBT assay (OD at 620 nm) of the infected groups differed significantly ($p < 0.05$) in comparison to the control (uninfected fish). Significantly elevated NBT level and phagocytic activities were recorded in high and moderately infected groups compared to control (Figs. 1 and 2). However, no significant ($P > 0.05$) difference in phagocytic activity was observed between low degree infected group and the control group. The myeloperoxidase content of the dactylogyrid infected groups measured at 450 nm differed significantly ($p < 0.05$) from the control group and elevated MPO values was noticed in the high degree of parasite infected group (Fig. 3).

The serum lysozyme activity and α-2 macroglobulin (α-2M) activity was significantly ($p < 0.05$) elevated in moderate and high degree monogenean infested fish in comparison to low and un-infested control fish. However, no significant ($P > 0.05$) difference in lysozyme and α-2M activity was noticed in low degree monogenean infested fish when compared with the control (Figs. 4 and 5). The level of ceruloplasmin in serum was found to be dependent on the degree of infestation of fish with the monogenean. However, the level of ceruloplasmin in serum was significantly ($p < 0.05$) decreased in low infested fish and increased in high infested fish in comparison to the un-infested control fish (Fig. 6).

No significant difference ($p > 0.05$) in total serum antiprotease activity was found between control and different degrees of monogenean infested fish except high degree of parasite infested group (Fig. 7). Total protein and globulin contents (g dL^{-1}) were significantly ($p < 0.05$) elevated in high and moderate degree of parasite infested group in comparison to low and un-infested control group (Fig. 8). However, the albumin content (g dL^{-1}) and albumin to globulin ratio were significantly ($p < 0.05$) decreased in all the treatment groups when compared with the control (Figs. 8 and 9).

The increase levels of total serum immunoglobulin (g dL^{-1}) were observed in all the treatment groups when compared with the control (Fig. 10). However, a significantly ($p < 0.05$) elevated concentration of serum IgM was noticed in high and moderately infested fish in comparison to low and un-infested fish.

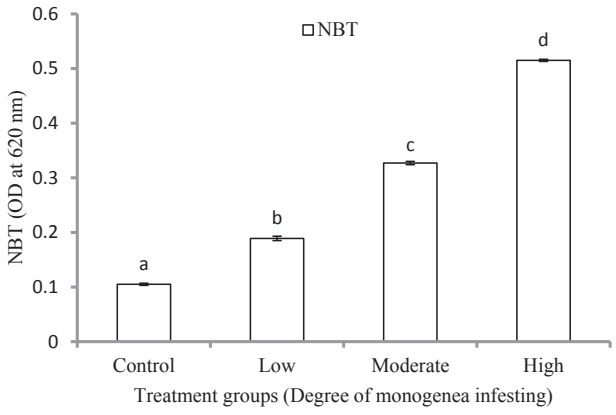


Fig. 1. Effect of experimental infection of dactylogyrid (gill monogenean) on respiratory burst activity (RBA) in *P. hypophthalmus*. (Values with different superscript differ significantly ($p < 0.05$) and express as mean ± SE) ($n = 9$).

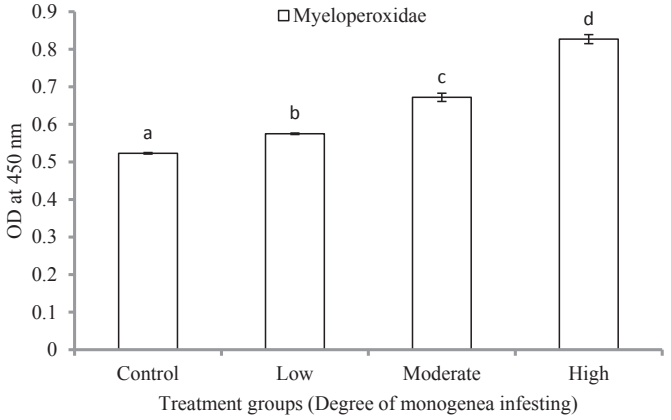


Fig. 3. Effect of experimental infection of dactylogyrid (gill monogenean) on myeloperoxidase level in *P. hypophthalmus*. (Values with different superscript differ significantly ($p < 0.05$) and express as mean ± SE) ($n = 9$).

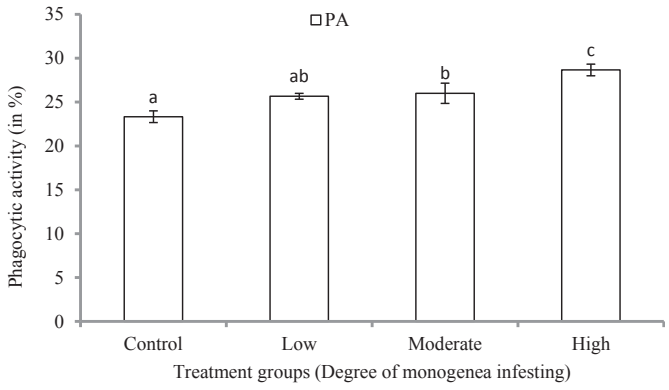


Fig. 2. Effect of experimental infection of dactylogyrid (gill monogenean) on phagocytic activity in *P. hypophthalmus*. (Values with different superscript differ significantly ($p < 0.05$) and express as mean ± SE) ($n = 9$).

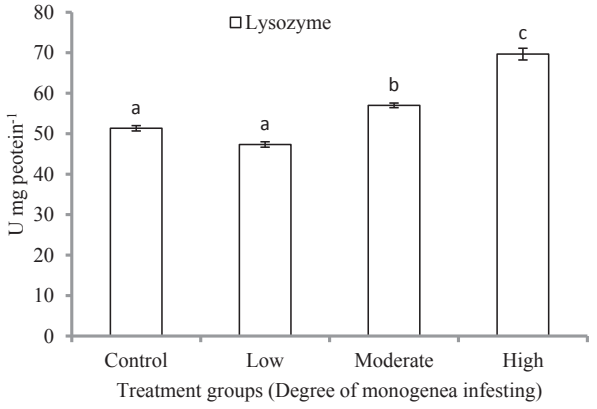


Fig. 4. Effect of experimental infection of dactylogyrid (gill monogenean) on serum lysozyme in *P. hypophthalmus*. (Values with different superscript differ significantly ($p < 0.05$) and express as mean ± SE) ($n = 9$).

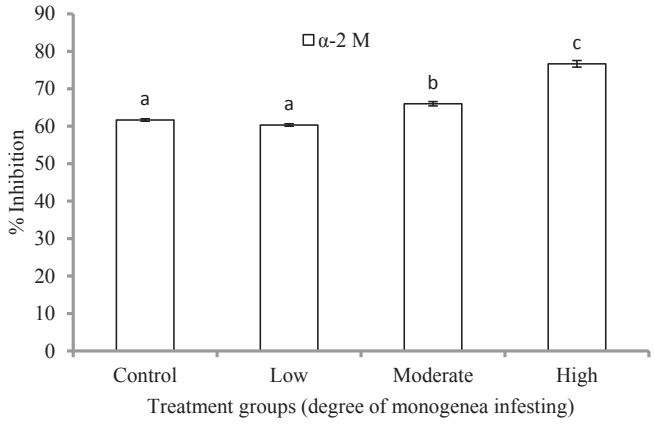


Fig. 5. Effect of experimental infection of dactylogyrid (gill monogenean) on α-2 macroglobulin in *P. hypophthalmus*. (Values with different superscript differ significantly ($p < 0.05$) and express as mean ± SE) ($n = 9$).

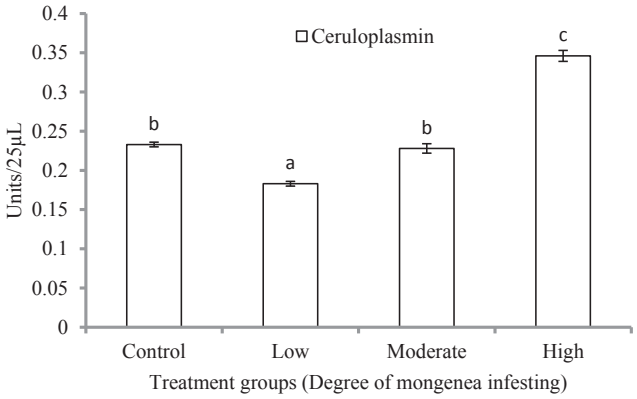


Fig. 6. Effect of experimental infection of dactylogyrid (gill monogenean) on ceruloplasmin activity in *P. hypophthalmus*. (Values with different superscript differ significantly ($p < 0.05$) and express as mean ± SE) ($n = 9$).

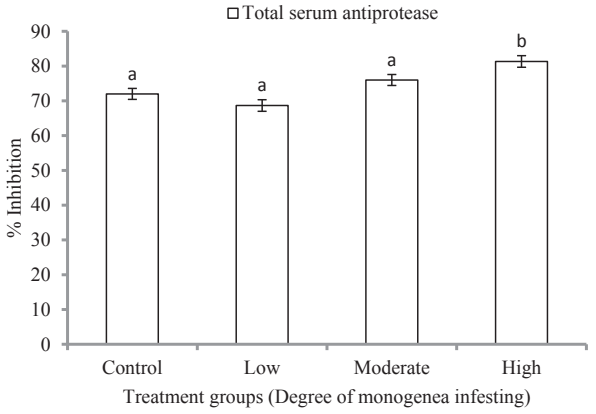


Fig. 7. Effect of experimental infection of dactylogyrid (gill monogenean) on total serum antiprotease activity in *P. hypophthalmus*. (Values with different superscript differ significantly ($p < 0.05$) and express as mean ± SE) ($n = 9$).

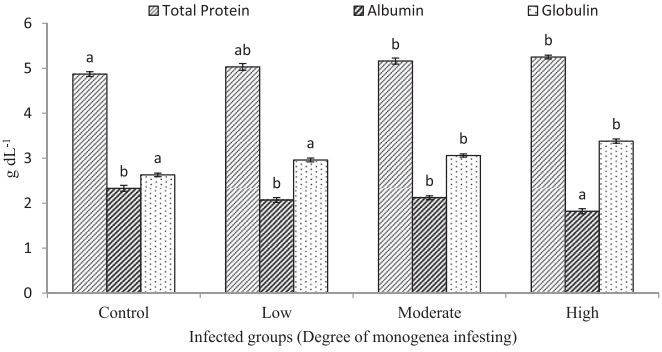


Fig. 8. Effect of experimental infection of dactylogyrid (gill monogenean) on serum protein profiles (total serum protein, albumin, and globulin) in *P. hypophthalmus*. (Values with different superscript differ significantly ($p < 0.05$) and express as mean ± SE) ($n = 9$).

3.2. Enzymes of oxidative stress (SOD, CAT, GST and Na^+-K^+-ATPase activity)

Significantly ($p < 0.05$) elevated activities of SOD, catalase and GST were observed in dactylogyrid infested fish when compared with un-infested control fish. Increased activities of SOD and CAT were observed in gills, liver and serum samples of moderately and highly infested fish when compared with low and un-infested fish (Table 1). However, decrease in the activity of Na^+-K^+-ATPase was estimated in gills and liver of fish with different degrees of parasite infestation when compared with un-infected control fish (Table 2).

4. Discussion

Innate immunity plays a very important role in the defense mechanism of teleost fish. Several past studies have highlighted changes in the innate immune parameters in relation to different parasitic infections in fish [30–37]. Yet, the immune status of *P. hypophthalmus* against gill monogenean parasites (dactylogyrid) remains unstudied. Concomitantly, monogenean infection in pangasius farming has been reported to be a serious threat during risk analysis [2]. Till date, no adequate prophylactic measures are available for complete eradication of this metazoan parasite. Thus, the present study was conducted to evaluate the immunomodulation of innate parameters of *P. hypophthalmus* during different degrees of artificial infestation, which might be useful for understanding host-parasite interaction and for developing potential control measures by means of prophylaxis or chemotherapy.

Oxygen dependent defence mechanism in vertebrate phagocytic cells is termed as respiratory burst activity, wherein there is generation of reactive oxygen intermediates molecules such as superoxide anion radical (radical $O_2\cdot$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radical (OH^-) with powerful antimicrobial activity [38,39]. In the present study, significantly high respiratory burst activity (RBA) and phagocytic activity were recorded in fish with different degrees of dactylogyrid infection when compared with un-infested fish. Muñoz et al. [40] reported higher levels of O_2^- (NBT activity) of cells from *Dicentrarchus labrax* fish parasitized by *Sphaerospora dicentrarchi* when compared with non-parasitized fish. Higher NBT activity was reported in *Argulus*-infected *Carassius auratus* in comparison to non-infected control fish [11]. Similarly, induction in respiratory burst response in pink salmon exposed to secretory products from *Lepeophtheirus salmonis* [41]. However, Fast et al. [42] reported no significant differences in respiratory burst activity and phagocytic capacity of macrophages isolated from *L. salmonis*-infected coho salmon and rainbow trout when compared with control fish, respectively.

Myeloperoxidase is a haemoprotein, utilizes hydrogen peroxide during respiratory burst to produce hypochlorous acid [43], which are potent oxidants known to have several cytotoxic effects on mammalian and bacterial cells [44]. In the present study, a

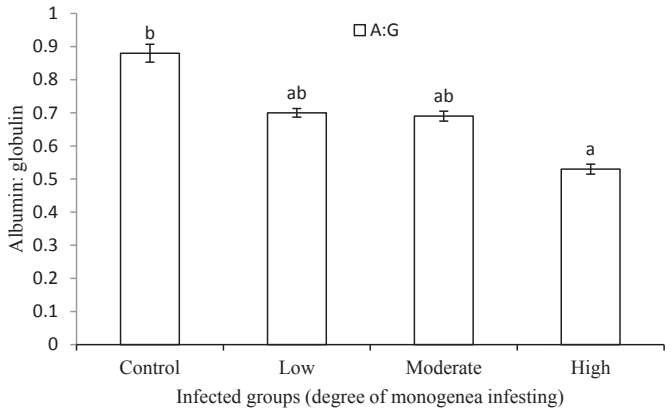


Fig. 9. Effect of experimental infection of dactylogyrid (gill monogenean) on ratio of albumin to globulin in *P. hypophthalmus*. (Values with different superscript differ significantly ($p < 0.05$) and express as mean ± SE) ($n = 9$).

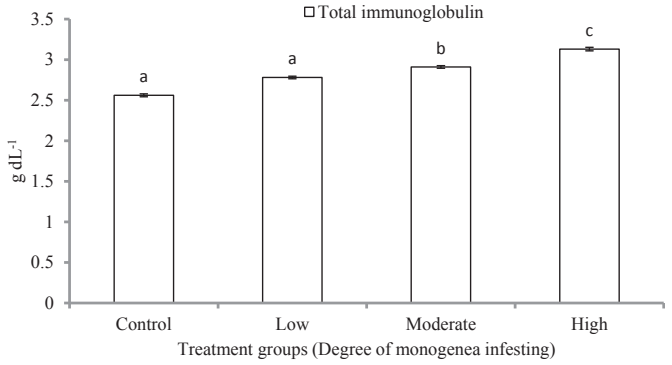


Fig. 10. Effect of experimental infection of dactylogyrid (gill monogenean) on total immunoglobulin in *P. hypophthalmus*. (Values with different superscript differ significantly ($p < 0.05$) and express as mean ± SE) ($n = 9$).

significant difference in MPO activity was observed in fish infested with different degrees of monogenean (*Thaparocleidus* sp.). Similar induction of serum peroxidases level was reported in *E. leei*-exposed sharpsnout seabream [45] and in gilthead seabream during cohabitation, but a decrease occurred in subsequent samplings and no differences were detected between parasitized and non-parasitized fish [35]. Similarly, during experimental exposure of *Argulus* in *L. rohita*, no significant difference in MPO activity was observed among the different degrees of parasite-infected and uninfected fish [37]. However, Sitja-Bobadilla [46] reported depletion of serum peroxidase levels in *E. leei*-infected and non-parasitized gilthead sea bream fish.

Serum lysozyme is used as an indicator of innate immune response in fish [47]. Lysozyme is an important component of the innate immune system which disrupts bacterial cell walls by splitting glycosidic linkages in the peptidoglycan layers [48,49]. Serum lysozyme levels can change in fish in response to parasitic infections [50]. In our study, significantly elevated serum lysozyme levels were found in moderately and highly infested fish. In sharpsnout sea bream, no significant difference in lysozyme level was detected in either infected or healthy animals during *E. leei* infection [51]. An increase of lysozyme activity was detected in guppies following immunization with *T. lysate* along with Freund's complete adjuvant [52], in rainbow trout immunized with live theronts of *Ichthyophthirius multifiliis* [53], in European seabass immunized with *S. dicentrarchi* [54] and in Chinook salmon infected by *Ceratomyxa shasta* [55]. On the other hand, lysozyme activity diminished in gilthead seabream parasitized by *E. leei* [46], *Polysporoplasma sparis* [56] and *L. rohita* infected with *Dactylogyrus catlaius* [57]. In a separate study, lysozyme activity did not differ in *Lernanthropus kroyeri*-infected seabass [58] and *Argulus*-infected *L. rohita* compared with non-parasitized fish [37].

In the present study, the total antiprotease activity showed a significant difference between high degree of *Thaparocleidus*-infested fish and un-infested control fish. However, the total antiprotease activity did not show a significant difference between control and low and moderate *Thaparocleidus*-infested fish. Basically antiproteases are glycoproteins synthesized in liver and secreted into the blood which are observed in form of α-1-protease inhibitor, α-2 antiplasmin and α-2M in teleost [20,59]. Host protease inhibitors modulate the protease activity of the parasite and control a variety of protease-mediated processes, including resistance to invasion by infectious agents [45]. Chettri et al. [52] reported that antiprotease was not affected in guppies following immunization with Tet-NI, a culture-attenuated *Tetrahymena* lysate. Further, during artificial *Argulus* infection study in *L. rohita*, antiprotease activity did not vary in different degrees of lice-infected fish and uninfected fish [37]. In the present study, a significantly elevated level of α-2M activity was observed in high and moderate degree *Thaparocleidus*-infested fish as compared with low degrees and un-infested control fish. High α-2M level in highly monogenean-infested fish indicated that the infected fish might be producing a higher amount of α-2M, which is chief protease inhibitors under parasitic induction, as total antiprotease varied significantly among monogenean-infested fish as compared with the control fish. Similarly, a higher level of α2-macroglobulin was reported in *E. scophthalmi* exposed turbot and *Argulus* challenged *L. rohita* with respect to control fish [36,37].

In the present study, ceruloplasmin level was correlated with degrees of monogenean infestation on fish, but it was significantly decreased in low degree and elevated in high degree of monogenean-infested fish in comparison to control fish. The increase in concentration of ceruloplasmin might be due to inflammatory response of fish to parasite infection and triggering the liver to synthesize the positive acute phase protein to maintain the homeostasis. Similarly, Studnicka and Siwicki [60] reported an elevated level of ceruloplasmin during *Eimeria subepithelialis* infection in *Cyprinus carpio*. On the contrary, Saurabh et al. [37]

Table 1
Effect of experimental infection of dactylogyrid (gill monogenean) on gills, liver and serum superoxide dismutase and catalase activity in *P. hypophthalmus*. Values with different superscript differ significantly ($p < 0.05$) and express as mean ± SE) ($n = 9$).

Treatment groups	Superoxide dismutase (U mg protein^{-1})			Catalase (mM H_2O_2 decomposed min^{-1} mg^{-1} protein)		
	Gills	Liver	Serum	Gills	Liver	Serum
Control	42.08^{a}±0.57	54.12^{a} ±0.33	34.66^{a} ± 0.67	6.76^{b} ± 0.12	8.73^{a}±0.46	4.27^{a}±0.33
Low	40.67^{a}±0.66	53.34^{a} ±0.66	35.33^{a} ± 0.67	6.30^{a}±0.15	9.40^{b} ± 0.35	4.43^{a}±0.12
Moderate	47.33^{b} ± 0.67	58.66^{b} ± 0.67	46.67^{b} ± 0.87	10.53^{c}±0.48	12.56^{c}±0.82	5.67^{b} ± 0.36
High	66.15^{c}±0.57	71.33^{c} ±0.67	52.10^{c}±1.15	12.73^{d} ± 0.46	15.43^{d} ± 0.74	7.46^{c}±0.17

Table 2
Effect of experimental infection of dactylogyrid (gill monogenean) on gills, and liver glutathione-S-transferase and Na + -K + -ATPase activities in *P. hypophthalmus*. Values with different superscript differ significantly ($p < 0.05$) and express as mean ± SE) (n = 9).

Treatment groups	Glutathione-S-transferase (U mg protein^{-1})		Na + -K + -ATPase activity (μM ADP mg^{-1} protein h^{-1})	
	Gills	Liver	Gills	Liver
Control	53.66^{a}±0.88	86.34^{a}±0.89	0.86^{c}±0.01	1.25^{c}±0.01
Low	60.63^{b} ± 0.67	95.67^{b} ± 0.33	0.99^{c}±0.01	1.20^{bc}±0.01
Moderate	67.45^{c}±0.34	109.02^{c}±2.08	0.62^{b} ± 0.04	0.97^{b} ± 0.01
High	82.08^{d} ± 1.15	126.67^{d} ± 3.52	0.55^{a}±0.07	0.84^{a}±0.03

reported decreased ceruloplasmin levels during differential degrees of *Argulus* infection in *L. rohita*.

Albumin is an easily available protein reserve and a protein transporter [61] and globulins like gamma are essential for maintaining a healthy immune system. Increase in albumin and globulin levels are considered as an indicator of strong innate response in fish [62]. In present study, elevated total protein and globulin in high and moderate degree of infested group confers the unregulated synthesis of acute phase protein or lysis of cellular component by parasitic infection that release in serum. Similar finding regarding total protein (TP) was reported in olive flounder infected with *Philasterides dicentrarchi* [62]. The main reason for increased total protein level is stress. For instance, chum salmon infected with erythrocytic necrosis virus had increased TP level due to the destruction of red blood cells and resultant release of cell contents into the blood stream [63]. However, no difference in total protein concentration was reported between *Argulus* infested and uninfested rohu fish [37], *Neoparamoeba*-infected Atlantic salmon [64]. The lower albumin-globulin ratio in the treatment groups may be due to less synthesis of albumin protein and more conversion of globulin to cope with the immunological function. High levels of TP and globulin suggested the secondary infection owing to *Thaparocleidus* infestation and might be due to the resultant immunomodulatory activity.

The function of natural antibodies (immunoglobulin) in a variety of defence-related activities such as limiting the dispersal of infectious agents, killing of microbes, and other potential pathogens, repair of tissue damage, and restoration of the healthy state. We found a significantly high level of total immunoglobulin in serum of *Thaparocleidus* -infested fish in comparison with the non-infested fish. A higher amount of immunoglobulin was estimated in the circulating fluid (plasma) in *Ptychobothrium* infected *C. carpio* in comparison with non-infected fish. In contrast, a decrease in the Ig levels in liver and head kidney was measured in infected fish [60]. Muñoz et al. [54] reported higher non-specific immune response while no significant elevation in total serum Ig in *Dicentrarchus labrax* immunized with spores of *Sphaerospora dicentrarchi*. Similarly, reduction in Ig level was reported in silver catfish immunized with live theronts of *I. multifiliis* by intra peritoneal injection and bath immersion [65].

Parasitic infections were often associated with an oxidative stress in hosts. Such response was also noticed in fish by Bello' et al. [66] who suggested that the fish response to parasitic infection could involve reactive oxygen intermediates and, therefore, an induced oxidative stress, but without significant induction of superoxide dismutase and catalase activities (antioxidant enzymes). In the present study, we observed induction of oxidative stress biomarkers (SOD, CAT, and GST) in gills, liver and serum in fish infected with different degrees of *Thaparocleidus* sp. when compared with un-infected fish. Induction in the activities of antioxidant enzymes might be due to the response of gill tissues to parasitic infestation which causes a cellular stress and release of severe reactive oxygen molecules (ROS) to counteract the infection. Subsequently, the cellular protective defence system of host against ROS and immune evasion mechanism of parasite [67] both trigger the antioxidant enzymes activity in gills. Dautremepuits et al. [68] reported significantly higher GST, CAT and glutathione reductase (GR) activities in liver and head kidney in *Ptychobothrium* (cestode) infected carp than un-infected fish. No significant difference in catalase activity was reported in the head kidney between normal and *Ptychobothrium*-infected fish. Increase or improvement in antioxidant status of parasitized fish is related to host response against oxidative stress. This may be explained as modulation in antioxidant enzyme activities related to metabolic variations [69]. As parasitism can disturb the metabolic pathways of its host, an increase in the different antioxidant defence found in infected fish can probably be linked to the modulated metabolic activity in relation to the parasite load [70]. Radovanovi'c et al. [71] reported higher glutathione-dependent enzyme activities in barbel (*Barbus barbus*) infected with intestinal parasite *Pomphorhynchus laevis* (Acanthocephala). Similarly, low SOD and catalase activities were reported in *Argulus*-infested fish when compared with healthy uninfected goldfish (*C. auratus*) [11].

In the present study, we found significantly decreased activity of Na^{+}-K^{+}-ATPase in gills and liver of dactylogyrid-infected fish in comparison to the control fish. Reduction in the activity of Na^{+}-K^{+}-ATPase in the tissues of the parasite infested fish could be a consequence of cellular stress and degradation by toxin released by the parasite which impair the synthesis of Na + -K + -ATPase in the system. Ferguson et al. [72] reported that multispecies parasitism (*Nanophyetus salmincola*, *Apophallus* sp.,) and one myxozoan (*Myxobolus insidiosus*) inhibited the Na^{+}-K^{+}-ATPase in *Oncorhynchus kisutch*. On the contrary, a significantly elevated gill Na^{+}/K^{+}-ATPase activity was reported in low level sea lice (*L. salmonis*) infected *Salmo salar* [73]. Similarly, increased gill Na^{+}/K^{+}-ATPase activity with elevated plasma Na^{+} was reported in *S. salar* exposed to *L. salmonis* and handling stress [73].

The present study suggests that varying degree of gill monogenean *Thaparocleidus* sp. infection not only modulates the innate immune response of *P. hypophthalmus* by lowering albumin, total serum antiprotease, ceruloplasmin and Na^{+}-K^{+}-ATPase and inducing RBA, MPO, lysozyme, PA, α-2M, total protein and total immunoglobulin, but also induces oxidative stress biomarkers (SOD, CAT and GST). Further studies are required to elucidate the mechanisms involving innate immune genes and their regulations in the host fish in response to the monogenean ectoparasitic infection. The primary data obtained here could be useful in understanding the susceptibility of pangasius to *Thaparocleidus* sp., a gill monogenean, and might be help to control this ectoparasite responsible for persistent economic losses in the aquaculture sector.

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Effects on haematological and serum biochemical parameters of *Pangasianodon hypophthalmus* to an experimental infection of *Thaparocleidus* sp. (Monogenea: dactylogyridae)

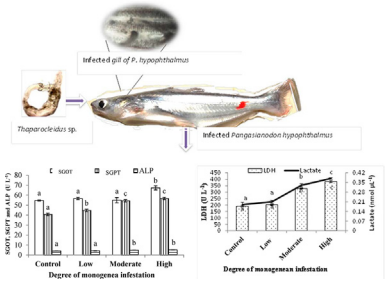
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HIGHLIGHTS

- Haematological and serum biochemical parameters of *P. hypophthalmus* were studied under various degrees of *Thaparocleidus* sp. infection.
- Significant increase in TEC, TLC, Hb and EOC were noticed in moderate and high infested fish.
- A decrease in MCH and MCV were estimated in high *Thaparocleidus*-infested fish suggests the anaemic condition.
- Moderate to high *Thaparocleidus*-infestations induce the blood glucose, SGOT, SGPT and ALP level.
- LDH activity, lactate, creatinine and bilirubin level in serum of *P. hypophthalmus* were elevated under various degrees of *Thaparocleidus*-infections.

GRAPHICAL ABSTRACT



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ABSTRACT

Monogenea (gill parasite) is a major problem in aquaculture that reduces the growth of cultured fish and adversely affects the economy. The present study was performed to evaluate the impact of various degrees of *Thaparocleidus* sp. (dactylogyrids, monogenean) infestation on haematological and serum biochemical parameters of *Pangasianodon hypophthalmus*. A standard cohabitation study, following complete randomized design in triplicate, was conducted to obtain low, moderate and high degrees of infestation in *P. hypophthalmus* along with the control (uninfested) group. Blood and serum were studied for haematological (total erythrocyte count (TEC), haemoglobin (Hb), packed cell volume (PCV), total leucocyte count (TLC) and indices viz. mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC) and erythrocyte osmolarity brittleness (EOB)) and serum biochemical parameters (serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate

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transaminase (SGPT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), lactate, total bilirubin and creatinine. Significant ($p < 0.05$) increase in TEC, Hb, TLC, EOB, SGOT, SGPT, LDH, lactate, bilirubin, and creatinine were noticed in moderate to high monogenean-infested group in comparison to the control group. However, significant ($p < 0.05$) reduction in MCH, and MCV and no difference ($P > 0.05$) in PCV were noticed in high degree parasitized group in comparison to the control group. The results of altered haematological and serum biochemical parameters in various degrees of monogenean-infested groups signify the density dependent physiological responses and changes in cells of the blood. The data of serum enzymes in the present study would be valuable for assessing the health status of the host and facilitate as a potential biomarker in relation to various degrees of monogenean infestation.

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1. Introduction

Disease is a primary constraint for aquaculture systems, particularly in intensive practices, where entry of input ingredients into the system cannot be avoided that create stress on fish and increase the chances of disease outbreaks. Pangasius farming in India has been adopted to bridge the gap between domestic fish demand and supply due to its remarkable growth rate in monoculture, polyculture and cage culture (Rahman et al., 2006; Lakra and Singh, 2010). However, intensive farming practices are always prone to disease risk, leading to epizootic outbreaks. Bacterial diseases associated with the etiology of *Enterobacter cloacae*, *Aeromonas jandaei*, and *Edwardsiella tarda* have been reported in grow out systems of *P. hypophthalmus* in India (Kumar et al., 2013, 2015; Shetty et al., 2014). In addition, infestation of protozoan and metazoan parasites in early larval stages of pangasius has also been reported (Singh and Lakra, 2012). Among a wide array of parasites, prevalence of monogenean infestation was found from 60% to 90% in *P. hypophthalmus* farms (Lakra and Singh, 2010). Although risk analysis and impact of monogeneans infestation have not been evaluated in aquaculture, the growth reduction due to physiological stress severely affects the economics of aquaculture (Singh and Lakra, 2012).

Cellular and humoral components of blood play an important role during stress and maintain the homeostasis in the host, and fight against pathogens during infection. Additionally, haematological and serum biochemical profiles provide an array of valuable information related to the health status and act as a monitoring tools (Del Rio-Zaragoza et al., 2010). However, information on blood cell and serum biochemical profiles of monogenean infested fish in relation to different degrees of parasite infestation is scarce. In general, fishes greatly rely on non-specific immune responses to fight monogenean infestation. Monogenean infestation shows localized inflammation which appears as hyperplasia and severe mucus secretion on the fish gills and skin (Mansell et al., 2005; Del Rio-Zaragoza et al., 2010). Recently, we described the innate immune responses and oxidative stress biomarkers of monogenean infested *P. hypophthalmus* (Kumar et al., 2017). Although, alteration in haematological and biochemical parameters of fish infested with parasite are reported (Ghiraldelli et al., 2006; Del Rio-Zaragoza et al., 2010; Ferreira and Avenant-Oldewage, 2013; Rastiannasab et al., 2016; Yin et al., 2015; Rana and Das, 2016), there is no systematic information available on changes in haemato-serum biochemical parameters of pangasius infested with *Thaparocleidus* sp. (dactylogyrids) a gill monogenean. Alterations in gill physiology (gaseous exchange and osmotic regulation) due to gill parasites lead to anemic condition and reduction of oxygen supply to vital organs (Montero et al., 2004). Further, this condition influences the normal serum biochemistry in infected fish (Mansell et al., 2005). In several cases, mortality in host is observed due to the impairment of the normal physiological functions of the gill organs (Buchmann and Bresciani, 2006). Moreover, the host responds to biological stressors by mobilizing energy, active phagocytosis and agglutinins, and synthesizes active proteins to maintain the homeostasis. Red blood cell (RBC) counts, haemoglobin (Hb), packed cell volume (PCV), leucocytes counts (TLC) and haematological indices (MCH, MCV and MCHC), including erythrocyte osmotic brittleness (EOB) are suitable measures of the physiological responses and damage to organs in human clinical medicine as well as in fish (Dacie and Lewis, 2011; Kumar et al., 2012). Serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) are considered important biomarkers of cellular physiological stress (Winkaler et al., 2007; Kumar et al., 2012). At the same time, serum biomarker like blood glucose, lactate and creatinine can be viewed as part of a stress response triggered by the presence of biological stressors in water (Grutter and Pankhurst, 2000; Mansell et al., 2005; Wells et al., 2006). Therefore, it is important to recognize the changes in the haematological and serum biochemical parameters occurring during monogenean infestation. Thus, the present work aimed to study the effect of different degrees of infestation by *Thaparocleidus* sp. (gill monogenean) on haematological and serum biochemical parameters of *P. hypophthalmus*.

2. Materials and methods

2.1. Experimental fish and maintenance

Forty infected *P. hypophthalmus* having a mean weight of 18.0 ± 5.28 g and a mean standard length of 9.2 ± 1.13 cm were obtained from cage culture of Murbad, Mumbai, India. Fish were stocked in the tanks of Central Wet Laboratory, ICAR-CIFE, Mumbai, acclimatized for one week and randomly selected five fish were biopsied and their gills, skin and fins were examined to ascertain the presence or absence of the monogenean parasite. These fish had a mean intensity (MI) of 30.5 ± 4.0 gill monogenean parasite. Infested fish ($n = 36$) were used as a source of parasitic infestation in the cohabitation trial. Further, the presence or absence of adult dactylogyrid (gill monogenean) on the infested fish and uninfested fish during the experiment was confirmed by observation of the parasite on gills of fish, using a stereomicroscope (Leica Microsystems, Germany).

One hundred twenty healthy *P. hypophthalmus* fish with a mean weight of 20.2 ± 2.56 g (mean weight ± SD) were used for experimental purposes and were procured from an aquarium shop in Mumbai, India. The fish was acclimatized in 500 L water under aerated conditions in $1.52 \times 0.65 \times 0.62$ m fibreglass reinforced plastic (FRP) tank for two weeks following standard protocol of best management practice (BMP) and were fed at the rate of 3% of body weight with a commercial pelleted diet containing 35% crude protein. The physico-chemical parameters of water were measured weekly intending to ascertaining its optimal condition

(temperature 26.8 ± 1.2 °C; dissolved oxygen: 5.80 ± 0.85 mg L^{-1}; pH: 7.5 ± 0.4; ammonia: 0.095 ± 0.055 mg L^{-1}; nitrite: 0.020 ± 0.005 mg L^{-1} and exposed to a natural (12 h light: 12 h dark) photoperiod) throughout the experiment.

$$TEC\left(cells\ mm^{-3}\right) = \frac{total\ number\ of\ cells\ counted\ \times dilution\ factor\ (200)}{\frac{1}{5}\times\ volume\ factor\ (0.1)}$$

$$TLC\left(cells\ mm^{-3}\right) = \frac{total\ number\ of\ cells\ counted\ \times dilution\ factor\ (50)}{4\ \times\ volume\ factor\ (0.1)}$$

2.2. Infection by cohabitation

The artificial experimental challenge was performed by the cohabitation method with slight modifications (Kumar et al., 2012). To ascertain the removal of helminthic parasites, healthy fish were treated with 2‰ saltwater for 15 min followed by a bath treatment with mebendazole (4.5 mg L^{-1} Sigma, India) for 24 h and each bath treatment was repeated for three consecutive days (Fajer-Avila et al., 2007). The treated healthy fish were used for the experiment one week post acclimatization. Infested fish were marked by making a cut on the tip of the caudal fin to differentiate them and these fish were not sampled. To demonstrate an effect of differential degree of monogenean parasite infestation on haemato-serum biomarkers in *P. hypophthalmus*, experimental infection trials were conducted in 500 L FRP tanks containing 300 L of sufficiently aerated groundwater. Healthy fish were challenged by cohabitation model for artificial infestation for two weeks. Complete randomized design (CRD) with triplicates of each treatment including control was adopted for conducting the experiment. In the control group, 30 healthy fish were equally distributed in three tanks without introducing any monogenean infested fish. In the artificial infection challenge group, 90 fish were kept in nine tanks with each tank containing 10 healthy fish, cohabited with 2, 4 and 6 infected fish with adjusting water volume. After two weeks, three grades of infestations were defined as low (<10 mean dactylogyrid per gill arch per fish), moderate (10–49 mean dactylogyrid per gill arch per fish) and high (≥50 mean dactylogyrid per gill arch per fish). The blood and serum samples were collected from randomly selected 9 fish specimens with each of the three infestation grades in the experimental tanks and from 9 uninfested fish in the control tanks.

2.3. Collection of blood and serum

Before collection of blood, fish were anesthetized with clove oil (Merck, Germany) at the rate of 50 μL per liter of water. Blood was drawn from Vena caudal by using a tuberculin medical syringe with needle number 24, which was previously rinsed with 2.7% EDTA solution (Hi-Media, Mumbai, India). The collected blood was immediately transferred to a test tube coated with a thin layer of anticoagulant sodium citrate and mixed well. Further, serum was collected without using anticoagulant and separated from blood by keeping the tubes in slanting position for about 4 h and centrifuged at 1500 *g* for 10 min at 4 °C followed by a collection of straw colored serum with the micropipette and stored at −20 °C for further use.

2.3.1. Haematological profiles and indices

The total erythrocyte counts (10^6 mm^{-3}) and total leucocyte counts (10^3 mm^{-3}) were estimated following the standard protocol (Schaperclaus et al., 1991). TEC and TLC were calculated using the following formula as.

Packed cell volume was determined by drawing non-dotted blood by capillary action into microhaematocrit tubes. One end of the tube was sealed with a synthetic sealant. The sealed tube was centrifuged in a microhaematocrit centrifuge for 5 min at 8000 *g*. The PCV was measured using microhematocrit reader and expressed as a percentage (%). The haemoglobin level of blood was analyzed by the cyano-methemoglobin method using Drabkin's fluid (Himedia, India). The derived haematological profile of the mean cell volume (MCV; fl), mean cell haemoglobin (MCH; pg) and mean cell haemoglobin concentration (MCHC; %) were calculated according to the equation suggested by Haney et al. (1992).

$$MCH\ (pg) = \frac{PCV\ (\%) \times 10}{RBC\ (million\ mm^{-3})}$$

$$MCV\ (fl) = \frac{Hb\ (g\ dL^{-1}) \times 10}{RBC\ (million\ mm^{-3})}$$

$$MCHC\ (\%) = \frac{Hb\ (g\ dL^{-1}) \times 100}{PCV\ (\%)}$$

The erythrocyte osmotic brittleness was estimated following the standard method (Bain et al., 2011). Briefly, 50 μL of anticoagulant blood was added to a series of 5 mL buffered sodium chloride (NaCl) solutions that ranged in concentration from 1.0 to 9.0 g L^{-1} in 1.0 g L^{-1} increments including a tube contained only distilled water. After incubation for 2 h at room temperature, these solutions were mixed again and then centrifuged for 5 min at 1200 *g*. The supernatants were tested for the amount of lysis in each using a spectrophotometer at a wavelength of 540 nm and supernatant of 9.0 g L^{-1} was taken as blank. The minimum concentration of NaCl solution containing no obvious intact erythrocytes was taken as the value of erythrocyte osmotic brittleness.

2.3.2. Physiological stress biomarkers

Blood glucose level was estimated by the method of Nelson and Somogyi (1945), as described by Oser (1965) in a blood glucose diagnostic kit (Merck, Germany). SGOT and SGPT activity (U/L) in serum were determined by using SGOT (AST) and SGPT (ALT) diagnostic kit (Merck, Germany), respectively. The diagnostic kits were based on Reitman and Frankel (1957) method. Alkaline phosphatase (ALP) activity was determined following the method of Ross et al. (2000). LDH activity (U L^{-1}) in serum was determined by using LDH diagnostic kit (Merck, Germany). The kit was based on Wroblewski and La Due (1955) method. One unit of LDH activity is defined as the amount of enzyme that catalyzes the conversion of lactate into pyruvate to generate 1.0 μM of NADH per minute at 37 °C. Lactate (Santulli et al., 1999), bilirubin (Pearlman and Lee,

1974), and creatinine (Bowers, 1980) in serum were estimated using lactate, bilirubin total and direct and creatinine kit (Erba, India) respectively.

2.4. Statistical analysis

The data of haematological and physiological biomarkers were analyzed using statistical package for social science (SPSS) version 16 and their normality tested using Shapiro-Wilk test. The data were subjected to one-way ANOVA, and Duncan's multiple range tests (DMRT) was used to determine the significant differences between the means of the various degree of monogenean infested and control groups. Comparisons were made at 5% probability level.

3. Results

Haematological profiles (TEC, Hb, and PCV) and indices (MCV, MCH, MCHC, RBD and EOB) of various degrees of infested groups differed in comparison to the control (uninfested) group. Significant increase in TEC and Hb levels were observed in moderate to highly infested groups compared to control and low infested group. However, no significant but increase in PCV values were recorded in high infested group in comparison to control and significant elevated level of PCV was recorded in low and moderate infested groups in comparison to control (Table 1). Further, MCV and MCH indices recorded significant decrease in moderate to highly infested groups in comparison to the control. In case of MCHC, significant low value was observed in low and moderate infested groups when compared with control and high infested group. The parasite intensity dependent increase in TLC was recorded in the present study. The erythrocytes cell integrity index, EOB of high degree of monogenean infested group was found significantly elevated when compared with all other groups including control (Table 1).

Blood glucose level was found to be significantly high in moderate and highly infested groups compared to the control and low infested group. However, no significant difference was observed in blood glucose level between low degree infested group and the control (Fig. 1). Significantly elevated SGPT and ALP was recorded in high and moderately infested groups in comparison to the control and low infested group (Fig. 2). Significant elevated SGPT activity was found in low infested group in comparison to control but not in case of ALP. However, SGOT activity showed significant increase in high infested group in comparison to others. No significant difference was recorded in SGOT activity between moderate and low infested groups as well as the control (Fig. 2). The lactate dehydrogenase activity and lactate level were found to be significantly elevated in the high and moderately infested group in comparison to the control and low infested group (Fig. 3). Significantly elevated serum bilirubin and creatinine level were recorded in the high and moderately monogenean infested group when compared with the control. However, no significant difference was observed in bilirubin level in between low and the control group, similarly, in between low and moderately infested group for creatinine level (Fig. 4).

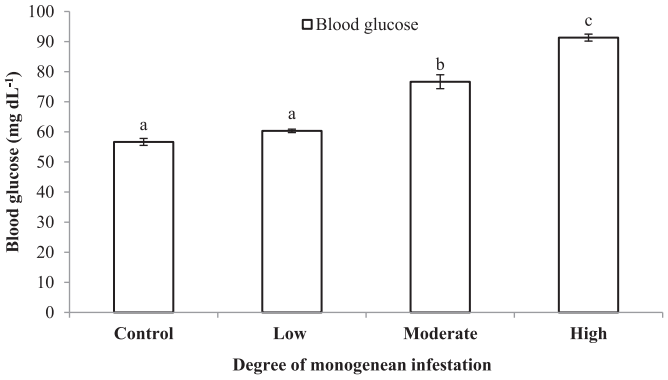


Fig. 1. Effect of experimental infection of *Thaparocleidus* sp. on blood glucose level in *P. hypophthalmus*. (Values with different superscript differ significantly ($p < 0.05$) and express as mean ± SE) ($n = 9$).

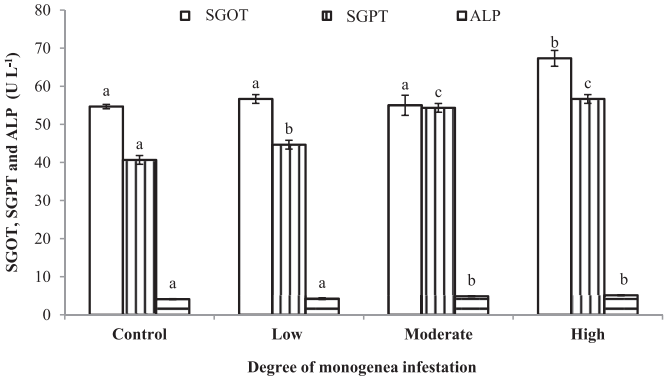


Fig. 2. Effect of experimental infection of *Thaparocleidus* sp. on serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) and alkaline phospatase (ALP) in *P. hypophthalmus*. (Values with different superscript differ significantly ($p < 0.05$) and express as mean ± SE) ($n = 9$).

4. Discussion

Haematological and serum biochemical profiles can be highly valuable for monitoring the fish health and to diagnose cellular homeostasis and metabolic disturbances in response to parasitic

Table 1
Haematological profiles (total erythrocyte count (TEC), haemoglobin content (Hb), packed cell volume (PVC), mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), total leukocyte count (TLC) and Erythrocyte osmolality brittleness (EOB)) of various degree of monogenean-infested *P. hypophthalmus*. Values are expressed as mean ± SE ($n = 9$) in the columns with different superscripts differ significantly ($p < 0.05$).

Parameters	Degree of monogenean infestation			
	Control	Low	Moderate	High
TEC(10^6 mm^{-3})	1.03^{a}±0.015	1.04^{a}±0.005	1.13^{b} ± 0.005	1.14^{b} ± 0.015
Hb (g dL^{-1})	8.43^{a}±0.057	8.53^{a}±0.057	8.93^{b} ± 0.057	8.96^{b} ± 0.152
PCV (%)	26.33^{a}±0.57	27.37^{bc}±1.15	28.67^{c}±0.83	26.67^{ab} ± 1.15
MCV (in fl)	254.92^{bc}±8.81	261.11^{c}±9.622	250.01^{ab} ± 5.83	232.67^{a}±3.12
MCH (pg)	81.62^{b} ± 1.41	81.52^{b} ± 0.507	78.82^{a}±0.891	78.21^{a}±2.052
MCHC (%)	32.03^{ab} ± 0.82	31.25^{a}±1.25	31.53^{a}±0.56	33.66^{b} ± 1.43
TLC (10^3 mm^{-3})	83.66^{a}±1.15	87.37^{b} ± 1.57	95.00^{c}±1.73	101.67^{d} ± 1.52
EOB	0.434^{a}±0.004	0.467^{a}±0.004	0.467^{a}±0.002	0.633^{b} ± 0.022

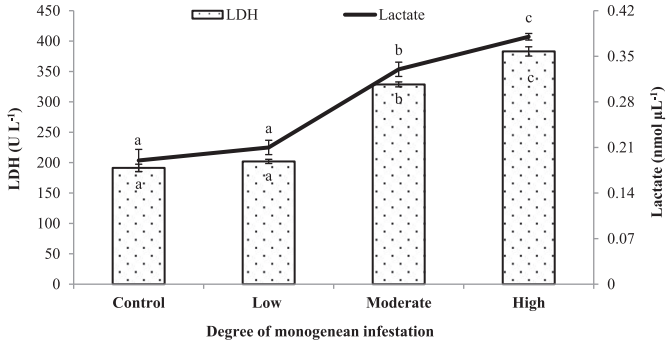


Fig. 3. Effect of experimental infection of *Thaparocleidus* sp. on lactate dehydrogenase (LDH) and lactate level in *P. hypophthalmus*. (Values with different superscript differ significantly ($p < 0.05$) and express as mean ± SE) ($n = 9$).

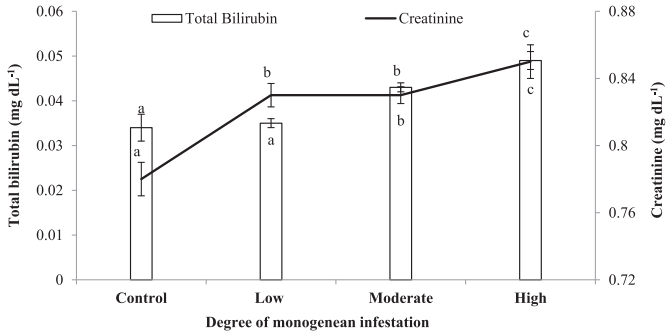


Fig. 4. Effect of experimental infection of *Thaparocleidus* sp. on serum total bilirubin and creatinine level in *P. hypophthalmus*. (Values with different superscript differ significantly ($p < 0.05$) and express as mean ± SE) ($n = 9$).

infection (Ghiraldelli et al., 2006; Del Rio-Zaragoza et al., 2010; Ferreira and Avenant-Oldewage, 2013; Rastiannasab et al., 2016; Yin et al., 2015; Rana and Das, 2016). The haemato-serum biochemical parameters of *P. hypophthalmus* against *Thaparocleidus* sp. (dactylogyrid) remain unstudied. Thus, the present study was conducted to evaluate markers of a haematological and physiological stress of *P. hypophthalmus* during different degrees of artificial infestation which could be useful for monitoring the health status of fish and understanding the host-parasite interactions.

Non-specific defense mechanism involving inflammatory responses and cellular effector response plays a significant role in protection of the fish during parasitic infestations (Bayne and Gerwick, 2001; Secombes, 1996, 1997; Sahoo and Saurabh, 2008). In the present study, the increased number of erythrocyte and haemoglobin levels might be indicative of adaptive response towards altered oxygen demand due to the infestation of host lamellae by the gill monogenean. In contrast, Rana and Das (2016) found during the progression of infection, a significant decrease in RBC and Hb level in a high degree of *Paradactylogyrus*-infested *Catla catla*. Similarly, Haidar and Ansari (2012) recorded significant low RBC and Hb levels in monogenean infected *Cyprinus carpio* in comparison to uninfested control group. However, Del Rio-Zaragoza et al. (2010) did not find any significant change in blood parameters such as RBC, Hb, PCV in low, moderate and high level of dactylogyrids on experimentally infected spotted rose snapper (*Lutjanus guttatus*). The significant increase in TLC in all *Thaparocleidus*-infested *P. hypophthalmus* reveals the protective (innate immune) response of fish and recruitment towards inflammation. In a similar study, the elevated acute phase proteins and the humoral components of the innate immune system were noticed in the high and moderately monogenean-infested groups (Kumar et al., 2017). In conformity with this, increase in TLC in monogenean infested fish has been recorded in *Trachinotus marginatus* (Chaves et al., 2006), *C. carpio* (Haidar and Ansari, 2012), *L. guttatus* (Del Rio-Zaragoza et al., 2010) and *C. catla* (Rana and Das, 2016). Further, blood indices (MCH, MCV, and MCHC) were significantly low in the high and moderately monogenean-infested group when compared with the control. A decrease in MCV and MCHC could be attributed to shrinking of erythrocyte and at the same time indicate microcytic anemia that might be due to the formation of more number of red blood cells (RBC) without an adequate level of haemoglobin synthesis. Similarly, low levels of blood indices were observed in experimentally infested *Sparus aurata* with *Sparicotyle chrysophrii* (Sitjà-Bobadilla and Alvarez-Pellitero, 2009), in *Carassius auratus* infested with *Argulus* sp. (Kumar et al., 2012). However, no significant change in MCH, MCV and MCHC was recorded in low, moderate and high level of dactylogyrids infested *L. guttatus* (Del Rio-Zaragoza et al., 2010). A significant increase in erythrocyte osmolality brittleness in high degree infected fish suggests a decreased hemolytic resistance of erythrocyte. This also indicates alteration in membrane composition and integrity as well as the cell sizes or the surface area to volume ratio of erythrocytes (Bain et al., 2011). Similarly, a significant decrease in hemolytic resistance was reported in common carp (*C. carpio*) infested with *Aeromonas hydrophila* (Wenxiang and Xiaomin, 2006) and in prussian carp (*C. gibelio*) infested with cyprinid herpesvirus 2 (Lu et al., 2016).

Glucose has been reported as a biomarker of stress caused by various means, primarily the physical factors (Manush et al., 2005). However, glucose is a sensitive marker and its measurement shows many inconsistencies and should be a complement of stress tests rather than a primary indicator. In the present study, a significant increase in the blood glucose level was recorded in high and moderately monogenean-infested fish. The results are in agreement with the elevation of blood glucose levels in *L. guttatus* infected with different degrees of dactylogyrids (Del Rio-Zaragoza et al., 2010); in *C. carpio* infested with gill monogenean parasites (Haidar and Ansari, 2012); in *C. auratus* infested with *Argulus* sp. (Kumar et al., 2012). Rise in the blood glucose might have been caused by high rate of metabolism due to parasitism stress (Wright et al., 1989). Contradictory to this, the blood glucose level continued to decrease in *C. carpio* infested with *A. hydrophila* (Harikrishnan et al., 2003) and *C. gibelio* infested with cyprinid herpesvirus 2 (Lu et al., 2016).

Assessment and profiling of the serum biochemical parameters is considered a valuable tool of clinical pathology indicative of tissue or organ damage and disease progression (Del Rio-Zaragoza et al., 2010). An elevated enzyme activity and concentration above the baseline level often reflects tissue or organ disorder leading to an increase in the release of enzymes into the peripheral blood (Meyer and Harvey, 1998). In the present study, SGPT, SGOT and ALP activities were significantly high in monogenean infested group in comparison to the control. These enzymes specifically regulate the metabolism and are predominantly expressed in the liver. However, SGOT is present in a lesser extent in kidney and skeletal muscle, while SGPT and ALP are present in all body tissues especially the liver, heart and skeletal muscles (Srivastava and Chosdol, 2007). Elevated activities of these enzymes in serum of parasite infested group, in the absence of acute necrosis or ischemia of other organs except gill, indicates an increase in the synthesis of these enzymes. Haidar and Ansari (2012) reported an increase in serum enzyme (SGOT, SGPT and ALP) in monogenean infested *C. carpio*. Similarly, elevated activities of GOT and GPT in serum were reported in roach (*Rutilus rutilus*) infected by the digenean

Rhipidocotyle fennica (Jeney et al., 2002) and in *Argulus*-infested *C. auratus* (Kumar et al., 2012). Further, the low tissue and organ specificity and high variations in levels of these enzymes in different tissues and organs have also been reported in various animals (Ramsay and Dotson, 1995; Fauquier et al., 2008; Anderson et al., 2013). In the present study, the change in activity of SGPT was found to be more sensitive to the different degrees of *Thaparocleidus* infestation in *P. hypophthalmus* which might be useful in the diagnosis of infected fish.

Lactate dehydrogenase is the terminal enzyme in the glycolytic pathway that interconverts lactate to pyruvate in the presence of coenzyme NADH. However, during anaerobic condition or lack of oxygen supply in the tissue, LDH performs reverse reaction (Cori cycle) in the liver and converts pyruvate (a final product of glycolysis) into lactate (Nelson, 2005). In the present study, significantly elevated LDH and serum lactate concentrations were noticed in high and moderately *Thaparocleidus*-infested *P. hypophthalmus* in comparison to the control group. Various stress conditions such as temperature, starvation and infection tend to induce LDH activity (Grigo, 1975; Vijayaraghavan and Rao, 1986; Kumar et al., 2012). Mansell et al. (2005) reported an increase in the blood lactate in monogenean infested kingfish in comparison to the uninfested group and observed increasing trend with the progression of infestation and decrease in haemoglobin level. Similarly, Özdemir et al. (2016) reported a significant increase in LDH in bogue fish (*Boops boops*) infested with isopods (*Ceratothoa oestroides*). The reduced capacity of anemic fish to deliver oxygen to the body may cause anaerobic metabolism and consequent lactate production (Wells and Pankhurst, 1999). Lactate level and LDH activity in high degree infested fish suggests anemic condition and reduction of oxygen supply due to the altered the gill physiology (Mansell et al., 2005).

Significant increase in serum bilirubin and creatinine concentration was noticed in high and moderately infested fish in comparison to uninfested and low infested group. Bilirubin, also known as hematoidin, is a degradation product formed as a result of heme catabolism in the liver circulating in the blood either in unconjugated or conjugated forms (total/direct). High levels of bilirubin can result in liver dysfunction, haemolysis or blood disorder (Bain et al., 2011). Creatinine release through the kidney and its detection in blood is useful as a test for the kidney function (Srivastava and Chosdol, 2007). Lu et al. (2016) reported an increase in the level of total bilirubin and creatinine in the blood of *C. gibelio* infested with cyprinid herpesvirus 2. Similarly, Özdemir et al. (2016) reported a significant increase in bilirubin and creatinine kinase in *B. boops,* infested with *C. oestroides*. However, Floyd-Rump et al. (2017) did not observe a significant difference in creatinine in *Ichthyophonus* infested Chinook salmon. In the present study, the significantly high level of creatinine was observed only in the group infested with high degree of parasites indicating dysfunction of kidney and loss of osmoregulation (Rastiannasab et al., 2016).

The present study reports the effect of various degrees of *Thaparocleidus* sp. (gill monogenean) infestation on haematological and serum biochemical parameters of *P. hypophthalmus*. The results of haematology and serum biochemical parameters reveal the density-dependent alteration in serum chemistry and cells of the blood of the host. The change observed in blood profiles could offer a direct or indirect analytical tool to assess the physiological response of the host. Evaluation of haematological parameters could help in detection of infestation levels that negatively impact aquaculture production. In aquaculture, pathophysiological profiles of fish are not routinely studied due to difficulties in collecting samples from large water bodies. The haematology and serum enzyme tests can be applied for assessing the health status of cultured fish. The present study shows that infestation by dactylogyrids represents a potential health risk to *P. hypophthalmus* and need to be critically evaluated and appropriate preventive measures should be applied during the culture.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.exppara.2018.02.007.

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