STUDY OF CONCOMITANT EXPOSURE OF *CARASSIUS AURATUS* TO *ARGULUS* AND *AEROMONAS HYDROPHILA* WITH REFERENCE TO TEMPERATURE

Thesis submitted in partial fulfillment
of the requirements
for the degree of

Ph.D. (Aquatic Animal Health Management)

by

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Dedicated to my Ummi, Vappi and Abi

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ICAR-CENTRAL INSTITUTE OF FISHERIES EDUCATION
(A University Established Under Sec. 3 of UGC Act 1956)
Ministry of Agriculture & Farmers Welfare,
Govt. of India.

Date: 15 February, 2022

CERTIFICATE

Certified that the dissertation entitled "**STUDY OF CONCOMITANT EXPOSURE OF *CARASSIUS AURATUS* TO *ARGULUS* AND *AEROMONAS HYDROPHILA* WITH REFERENCE TO TEMPERATURE**" is a record of independent research work carried out by **Ms. SHAMEENA S.S.** during the period of study from September, 2017 to August, 2021 under our supervision and guidance for the degree of **Doctor of Philosophy (Aquatic Animal Health Management)** and that the dissertation has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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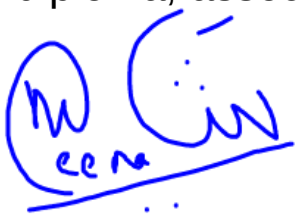
DECLARATION

I hereby declare that the dissertation entitled "**STUDY OF CONCOMITANT EXPOSURE OF *CARASSIUS AURATUS* TO *ARGULUS* AND *AEROMONAS HYDROPHILA* WITH REFERENCE TO TEMPERATURE**" is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.



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15th February, 2022

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सारांश

वर्तमान अध्ययन अलग - अलग तापमान व्यवस्थानों पर एरोमोनास हाइड्रोफिला तथा आर्गुलस स्पी. की अलग-अलग खुराक के साथ गोल्डफिश (कैरेसिथस औरेटस) में सह-संक्रमण के प्रभाव को निर्धारित करने के उद्देश्य से आयोजित किया गया । प्रायोगिक मछली कैरेसिथस औरेटस के आठ उपचार समूहों (T1-T8) डुप्लिकेट में बेवरतीब (रैंडमली) ढंग से वितरित किया गया । इसके पश्चात ए. हाइड्रोफिला को T2, T4, T5 और T6 समूहों को इंजेक्ट किया गया । बैक्टीरियल संक्रमण बाद 24,72 तथा 168 घंटे में सैम्पलिंग की गयी और उपचार समूहों की तुलना में संकमण समूह T8 में ए. हाइड्रोफिला की उच्च दर की रोगजनकता के लिए बढ़ी हुई मृत्यु दर पाया गया । अध्ययन में यह पाया गया कि सह- संक्रमण रहित समूहों की तुलना में परजीवी उपद्रव ने आरबीसी, पीसीबी और एचबी के मान में गिरावट का पैटर्न दर्शाता है । इसके अलावा प्रयोगात्मक अवधि के दौरान अन्य सह- संक्रमित समुहों के सापेक्ष बैक्टेरिया की उप घातक खुराक से सह- संक्रमित न्युट्रोफिल का मान घटा हुआ पाया गया । इसके अतिरिक्त, परिक्षण अवधि के दौरान T1और T2 समुहों की तुलना में T8 समूह में जन्मजात प्रतिरक्षा के मानकों एवं एंटीआक्सिडेंट इकाइयों में कमी देखी गई । इसी प्रकार सभी प्रायोगिक समुहों ने विभिन्न घंटों के सैंपलिंग में प्रतिरक्षा जीन के अलग-अलग एक्सप्रेशन स्तर को दिखाया । इन निष्कर्षों से यह संकेत मिलता है कि आर्गुलस संक्रमण की खुराक में वृध्दि ए. हाइड्रोफिला के गोल्डफिश में उपनिवेशीकरण (कोलोनाइजेशन) में सुधार बढ़ोत्तरी लाता है, साथ ही जन्मजात प्रतिरक्षा प्रणाली में दमन तथा उच्च मृत्यु दर में योगदान देता है । पुन: सह- संक्रमण पर अलग-अलग तापमान के प्रभाव को निर्धारित करने के लिए प्रायोगिक मछली को छह उपचार समूहों (T1-T6) में बेतरतीव (रैन्डमली) ढंग से वितरित किया गया । फिर सैंपलिंग 24, 72 तथा 168 घंटों के चैलेंज के बाद किया गया । प्रत्येक प्रायोगिक समूहों में नियंत्रण मछली की तुलना में सह- संक्रमित समूह में आरबीसी, एचबी तथा पीसीबी के मानों में महत्वपूर्ण कमी देखी गई । इसके अलावा, T4- समूह ने अन्य सह- संक्रमण की तुलना में काफी अधिक नाइट्रोब्लू टेट्राजोलियम परीक्षण, माइलोपेरॉक्सिडेंज और लाइसोजाइम सक्रियता दिखाई । नियंत्रण समूह के मछलियों में जो कि 330 (T5) के संपर्क में थी उनमें एंटीऑक्सीडेंट इंजाइमों की सक्रियता काफी बढ़ी हुई दर्ज की गई ।

इन परिणामों से यह सिध्द होता है कि तापमान में वृध्दि न केवल सह- संक्रमण की तीव्रता को बढ़ाता है, अपितू इनके प्रतिरक्षात्मक और शारीरिक मानकों में अवरोध डालकर इन्हें हानिकारक पक्ष को और अग्रसित करता है, जिससे मछलियों की स्वास्थ्य स्थिती असंतुलित होती है ।

ABSTRACT

The present study was conducted with an aim to determine the impact of co-infection in goldfish (*Carassius auratus*) with varying doses of *Aeromonas hydrophila* and *Argulus* sp at varying temperature regimes. The experimental fish *C. auratus infested with different grade of Argulus* were randomly distributed into eight treatment groups (T1 –T8) in duplicates. After this, *A. hydrophila* was injected into T2, T4, T6, and T8 groups. Sampling was done at 24, 72, and 168 h after the bacterial challenge. In the co-infection group T8, a high degree of enhanced pathogenicity of *A. hydrophila* was noticed with increased mortalities (84.2%) in comparison to other groups. The study showed a declining pattern in RBC, PCV, and Hb values with the degree of parasite infestation without co-infection groups. Furthermore, a decreased value for WBC, monocyte, and neutrophil was found in the higher parasite group co-infected with a sub-lethal dose of bacteria relative to other co-infected groups during the experimental period. Also, a decrease in innate immune parameters and antioxidative stress enzymes were observed in the T8 group compared to T7 and T2 groups throughout the trial period. Similarly, all the experimental groups showed varying expression levels of immune genes at different sampling hours. These findings indicate that a rise in the dose of *Argulus* infection improves *A. hydrophila* colonization in goldfish and contributes to suppression of the innate immune system and increased mortality. Further, to determine the effect of varying temperature on co-infection, the experimental fish were assigned randomly to six treatment groups (T1-T6). Sampling was done at 24, 72, and 168 h post-challenge. A significant decrease of RBC, Hb, and PCV values was observed in the co-infected group when compared to control fish in each of the experimental groups. However, a significant reduction in WBC, neutrophil, and monocyte was observed in co-infected fish exposed to 33°C during the progression of infection. Furthermore, the T4 group showed a significantly higher myeloperoxidase, and lysozyme activity compared to other co-infection groups. A significantly increased activity of antioxidative enzymes was recorded in control fish exposed to 33°C (T5). This result implied that an increase in temperature not only accelerated the intensity of co-infection but also imbalance the health status of the fish by hampering the immunological and physiological parameters towards a more detrimental side.

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1. INTRODUCTION

The aquatic environment is home to a diverse range of microorganisms, both parasitic and non-parasitic, and co-infection is a common occurrence (Kotob *et al.,* 2017). Several pieces of evidence clearly suggest that co-occurring infections on the animal can have synergistic or antagonistic interactions (Cox, 2001; Bradley and Jackson, 2008).Before implementing any health management treatments, host susceptibility to co-infection, as well as changes in host-pathogen dynamics, infection biology, illness severity, infection duration, and host pathology following co-infection incidence, must be thoroughly examined (Graham *et al*., 2007; Telfer *et al*., 2008). Most investigations on host-pathogen interactions, on the other hand, focus on a single infection. In this scenario, research into multi-pathogen infections in fish is becoming extremely relevant in culture practises, as secondary or opportunistic infections can cause severe disease episodes with negative consequences, as well as delays in diagnosis and treatment, obstructing host recovery and potentially resulting in financial loss (Griffiths *et al.,* 2011; Eswarappa *et al.,* 2012).

There has been growing evidence in recent years that multi-species co-infections in fish add to the severity of various infectious diseases, particularly bacterial diseases (Busch *et al*., 2003). Co-infection between parasitic and bacterial infections is important among the many bacterial diseases researched in fish, because parasitic infections boost the risk of secondary bacterial diseases and can act as a vehicle for transmitting bacterial pathogens (Holzer *et al.*, 2006). Furthermore, Xu *et al.* (2012 a) hypothesised that some parasites could promote bacterial infection indirectly by lowering the host's immunological defences. Regardless of the fact that published studies have proven that parasite infection can increase bacterial invasion, the exact causes of this phenomena are still unknown. *Argulus*, a crustacean ectoparasite, and *Aeromonas hydrophila*, a bacterium, are common diseases of goldfish *Carassius auratus*, causing severe mortality and significant economic losses. *Argulus* spp. are also harmful ectoparasites of fish that have caused various epizootics around the world (e.g. Menezes *et al*., 1990; Hakalahti *et al*., 2004). Argulids use suckers and spines to adhere to fish skin and migrate across it, causing damage to the mucus layer. The parasite continually enters the fish's skin to feed on blood, causing sores and

ulcers, as well as providing a potential entry point for secondary microbial invasions (Singhal *et al.,* 1990). Localized inflammation is often seen at the site. Opportunistic bacteria such as *Aeromonas* or *Pseudomonas* can sometimes infect these damaged areas leading to skin ulceration (Yıldız, *et al.,* 2002). Argulids also often attach and detach from their hosts, making them potential vectors for the spread of diseases between fish (Cusack and Cone, 1986). Parasites have been studied as vectors for other diseases including Aeromoniasis and Pseudomoniasis in a number of researches (Cusack and Cone, 1986; Liu, and Lu, 2004; Harrus and Baneth, 2005; Novak *et al*., 2016). Argulids alone rarely cause mortality in adult rainbow trout (Lester and Roubal, 1995), but they can considerably affect the survival of small-sized fish (Lester and Roubal, 1995). Ectoparasitic infections have been shown to produce a variety of physiologic abnormalities, including mortality, in fish. Ectoparasitic infections have been demonstrated to cause numerous alterations in fish physiology, resulting in lower disease resistance (Tully and Nolan, 2002). When additional confinement stress was given, rather than when the parasite was utilised as the sole stress component, extended *Argulus foliaceus* infections (6 parasites/fish) resulted in reduced immune response among infected rainbow trout (Ruane *et al.,* 1999).

Motile Aeromonas spp. are the most hazardous bacteria, and they play a substantial role in causing serious problems in freshwater ornamental fish of all ages. *Aeromonas hydrophila* is the aetiological agent of motile aeromonad septicemia (MAS), which causes disease in a wide range of freshwater fish species and is often associated with stressed or immunocompromised hosts (Sreedharan, 2008). Infection with *A. hydrophila* is a worldwide scourge of fresh and warm water fish farming and is seen as a big economic problem, notably in China and India in recent years (Viji *et al*., 2011). MAS infect several species producing stress-related diseases with the common symptoms of ulcerations, exophthalmia, and abdominal distension. *Aeromonas* spp. are known to be opportunistic pathogens for fish, and under conditions of stress, such as an increase in water temperature, presence of other pathogens, poor water quality, excessive handling, *etc*. they cause epidemic outbreaks. Mostly during summer, when the parasite load is high, goldfish and koi carp are vulnerable to *A. hydrophila* infection (Dixon and Issvoran, 1993).

There is a wide range of opportunistic pathogens like bacteria or parasites in aquatic environments, which expand disease susceptibility and cause important losses in intensive aquaculture (Marcogliese & Pietrock, 2011). Further, environmental stressors such as water temperature and chemical pollutants, as well as biological factors like age or co-infection of other pathogens, were reported to have drastic effects on the pathogenicity of certain bacteria, parasites, and viruses (Dorson and Torchy, 1981). The current rise in water temperature as a result of global warming is concerning for aquaculture, as it is a major stress element in many disease outbreaks in the aquatic environment (Green and Haukenes, 2015; Islam *et al.,* 2019). Furthermore, studies have shown that environmental temperatures above or below the optimum level can affect the growth and physiological processes of any fish species (Fu et al., 2018), and can have negative effects on fish physiology, immunology, behaviour, fish abundance, and parasite-avoidance strategies, as well as severe alterations in healthy ecosystem patterns and interactions (Lohmus *et al*., 2015).On other hand, it was proved beyond doubt that, temperature influences the pathogens in establishing or maintaining infection, latency as well as aggressiveness in the host-pathogen system (Fels and Kaltz, 2006). Elevation in ambient temperature may have a two-way effect on pathogenic transmission. Firstly, the pathogen metabolism could be improved directly, resulting in a higher number of transmission phases, leading to rapid spread of the disease in a single outbreak. Secondly, a rise in temperature at either end of the disease's natural phase of occurrence could lengthen the transmission season, resulting in a greater overall spread of the disease among the host population (Karvonen *et al*., 2010).

Currently, it is uncertain how temperature-induced changes in immune parameters will disturb and/or modify the combined immune response toward infections in most of the infections and co-infections in fish, but latent bacterial, fungal, and parasitic infections have been shown to increase their susceptibility at such connected episodes of higher/lower temperatures (Magnadotir *et al*., 1999). The epizootiology and pathogenicity of such co-infection at temperature stress is poorly understood in most of the economically important diseases in fishes, while, climate-mediated, physiological stresses had shown to compromise host resistance and increase the frequency of opportunistic infections (Harvell *et al.,* 1999). In this scenario, the study was planned to explore the effect of *Argulus*

spp parasitism on infection of the bacterium *A. hydrophila* with a concomitant effect of temperature stress in goldfish, *Carassius auratus.* The study was designed with the following aims, recognizing the centrality of natural co-infections and rising climate change-related stresses and disease episodes in fish;

1.2 Objectives

- To study the host immune response of *Carassius auratus* during co-infection to *Argulus* and *Aeromonas hydrophila.*
- To determine the effect of temperature on co-infection.

2. REVIEW OF LITERATURE

2.1 Aquaculture status in India

Aquaculture continues to dominate aquatic food production in Asia and around the world, with Asia accounting for more than 91 percent of worldwide aquaculture output (102.9 million tonnes in 2017) (FAO 2020). Aquaculture currently accounts for over 50% of the global food fish consumption (Subasinghe et al., 2009). The Indian fisheries and aquaculture sector is a major contributor to the country's economy and is emerging as a thriving sector with a variety of resources and potentials, providing nutritional security, livelihood support, and gainful employment to more than 14 million people, furthermore, contributing to agricultural exports (NFDB 2007). Presently, India is the second-largest fish producing and second-largest aquaculture nation in the world after China (DADF, 2018). Indian aquaculture has demonstrated a six and half fold growth over the last two decades, with freshwater aquaculture contributing over 95% of the total aquaculture production. India is bestowed with 3.15 million ha of reservoirs, 2.36 million ha of ponds and tanks as well as 0.19 million ha of rivers and canals. Freshwater aquaculture with a share of 34% in inland fisheries in the mid-1980s has increased to about 80% in recent years (DADF, 2017). During the financial year 2017-18, the total fish production in India is estimated as12.61 Million Metric tonnes (DADF, 2018). Freshwater carps and cyprinids currently dominate aquaculture fish production, accounting for nearly 53.1 percent of total fish production, followed by other freshwater fish species (19.5%), tilapia, and other cichlids (11.0 Percent) (Tacon., 2020).

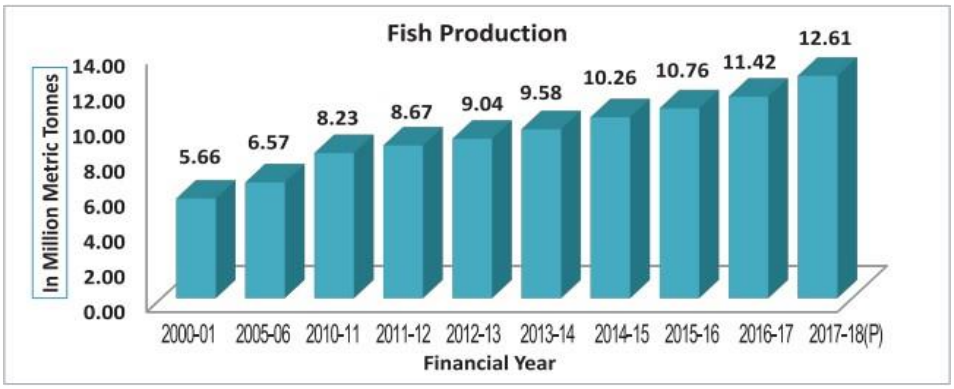


Fig 1: Total fish production of India (Million metric tonnes) from 2000-2018. (Source: DADF, 2018)

Fisheries and aquaculture at higher production result in a deteriorating environment owing to worsening water and soil quality. Maintaining constant welfare in this deteriorating environmental quality forms the raising concern in the aquaculture industry. In this scenario, the disease is a primary constraint to the growth of many aquaculture species and is responsible for impeding both economic and socio-economic development in many countries of the world leading to millions of dollars lost annually (Bondad-Reantaso *et al.,* 2005). In Asia, the disease has emerged as a major constraint to the sustainable development of the aquaculture sector. Much of the epizootic episodes in aquaculture were directly correlated to deteriorated environmental quality/altered hydrographic parameters of the aquatic ecosystem. For instance, the rise of water temperature due to global warming is considered as one of the major constraints in total aquaculture production (Idris *et al.,* 2014; Hamdan *et al.,* 2015).

2.2 Co-infections in natural aquatic environment

Fish in the aquatic ecosystem are supposed to have a close contact with their surroundings, exposing them to a variety of pathogens such as parasites and bacteria at any particular time. Co-infections are prevalent in this scenario and are more likely to develop when hosts are infected by two or more distinct pathogens, either concurrently or as secondary infections. Co-infections have a variety of interactions that might be agonistic or synergistic. In the case of multiple infections, the interaction can result in a variety of outcomes, such as an

increase or decrease in the load of one or both pathogens, or one pathogen being elevated while the other is suppressed (Cox 2001). Despite the fact that such infections occur frequently in nature, there has recently been a lot of interest in understanding the interactions that occur between different pathogenic species during mixed infections, as well as the negative effects of multi-infections on fish disease pathogenesis, prognosis, and treatment (Johnson and Hoverman, 2012; Kotob *et al.,* 2017; Ma *et al.,* 2019).

During the episodes of co-infections, the pathogens may compete for the resources or target site within the host. This is achieved by either suppressing or triggering the immune system and/or one pathogen can alter the host's immune response to subsequent infections by other pathogens (Lello *et al.,* 2004). This may alter host-pathogen dynamics, infection physiology, illness severity, and infection period (Telfer *et al*., 2008). As a result, the combination of infections may have either synergistic or antagonistic effects on the fish's health (Bradely *et al.,* 2008). When the first pathogen causes immunosuppression in the host, the immune response to future infections is hampered, resulting in an increase in infection frequency and mortality (Telfer *et al.,* 2008). However, antagonistic effects may be created as a result of direct pathogens competing for nutrients and space, which may diminish the infectious agent's population number and, in some situations, change the infection site (Andrews *et al*., 1982). In other cases, antagonistic outcomes occur when the pathogen's initial infection stimulates and alters the host immune system, preventing the second pathogen from invading (Chen *et al*., 2013). Co-infections of fish by homologous and heterologous pathogens have been studied by a number of scientists (Kotob *et al*., 2017). The development of new effective preventive and disease control measures in aquatic health management is expected to be aided by research into various co-infection models.

2.3 Co-infections involving homologous pathogens

2.3.1 Parasitic co-infections

A dynamic equilibrium exists in the host-parasite interaction, and any imbalance in the relationship alters the host-parasite equilibrium leading to disease outbreak (Iwanowicz, 2011). In addition, parasite co-infections can be antagonistic, neutral, or facilitative, and they play an important role in

epidemiology, disease dynamics, and virulence evolution in the host (Karvonen *et al*., 2019). Fish parasites increase fish's susceptibility to secondary invaders by acting as a vehicle or carrier for their transfer to the fish, as well as suppressing the host's immune response or reducing disease resistance (Bowers *et al*., 2000; Holzer *et al*., 2006).

Researchers hypothesize that co-infection by multiple parasites can be sequential, rather than concurrent; in both natural and man-made environments (Karvonen *et al.,* 2019). Nonetheless, several parasite co-infections have a significant impact on the host-parasite ecosystem (Klemme *et al*., 2016). Community ecology provides a more mechanistic framework for evaluating parasite interactions, which can have direct effects, like competition for attachment sites, competition for host resources like nutrients, and predation upon one another (Lello *et al*., 2004, Mideo 2009). However, such multi-parasitic interactions can also have an indirect or "host-mediated" effect, often involving changes in immunity such as cross-immunity and immune suppression in hosts (Cattadori *et al.,* 2008, Jolles *et al.,* 2008). However, association between parasites can be modulated by host behavior, residing ecology, exposure history to infections, and pathology (Poulin 2011, Behnke 2008, Telfer *et al.,* 2008), interfering both direct and indirect interaction in parasitic co-infections like host immunity and between-host transmission processes (Hawley and Altizer 2011).

Several studies have been conducted to understand the host immune responses in aquatic parasitic co-infections. Kotob *et al*., 2018 evaluated the immune modulation of rainbow trout in response to myxozoan parasites *Tetracapsuloides bryosalmonae* and *Myxobolus cerebralis* co-infections. Alarcon *et al*., 2016 studied the co-infection of a Microsporidian parasite *Nucleospora cyclopteri* and a Myxozoan parasite *Kudoa islandica* in farmed lumpfish, *Cyclopterus lumpus*. *Kudoa* spores found in the skeletal muscle, kidney, spleen, and liver revealed significant necrotic alterations with intracellular *N. cyclopteri* in the affected organs. Schmidt *et al.,* 2013 reported the co-infection of myxozoan *Tetracapsuloides bryosalmonae*, the causative agent of proliferative kidney disease (PKD), and the nematode *Raphidascarius acus* in brown trout. Bustos *et al.,* 2011 reported high mortalities in *Atlantic salmon* farms due to the co-infection by *Caligus rogercresseyi* and *Neoparamoeba perurans*, the

causative agent of amoebic gill disease. Similarly, *Lepeophtheirus salmonis*, a salmon louse associated to *C. rogercresseyi*, was discovered to be a vector in the transmission of *N. perurans* in *Atlantic salmon*, influencing the disease's epizootiology and increasing mortality in *Atlantic salmon* farms in the United States (Nowak *et al.,* 2010). The presence of five myxozoans in farmed *Salmo trutta* was investigated by Holzer *et al.*, in 2006.

2.3.2 Bacterial co-infections

The subject of bacterial co-infections in fish has yet to be examined more and includes dual, triple, or multiple bacterial infections (Kotob *et al.,* 2017). An experimental approach was done by Roy *et al.,* 2019 to delineate the possible effects of interaction between two pathogenic Aeromonads when co-infected within a fish model. The host fish *Labeo rohita* was challenged by a native (*Aeromonas hydrophila*) and an exotic (*Aeromonas salmonicida*) pathogenic *Aeromonad* individually and simultaneously. *Flavobacterium columnare* and *Edwardsiella ictaluri* infections in striped catfish, *Pangasianodon hypophthalmus*, were examined by Dong *et al.,* in 2015. The first report of a bacterial super-infection in fish was published in 2002, by Nusbaum and Morrison. Artificial infection of channel catfish, *Ictalurus punctatus*, with the *enterobacterium Edwardsiella ictaluri* causes bacteremia with the motile aeromonad species *Aeromonas hydrophila*, as per their study. Crumlish *et al.,* 2010 reported that artificial co-infection of Vietnamese catfish with both bacteria (*Edwardsiella ictaluri* and *Aeromonas hydrophila)* using an immersion route resulted in higher cumulative mortalities (95%) in the co-infected group and (80%) in *E. ictaluri* only infected fish, compared to the very low mortalities (10%) in the fish exposed to both bacteria. In parallel to artificial challenges, similar findings were seen in Chinook salmon, *Oncorhynchus tshawytscha*, where *A. hydrophila* was detected in a higher quantity than would have been predicted by chance alone in fish infected with *Renibacterium salmoninarum* (Loch *et al.,* 2012). Because of the immunosuppressive properties of *R. salmoninarum* (Grayson *et al.,* 2002), the researchers assume that facultative pathogenic motile Aeromonas spp. operate as an opportunistic pathogen and interact synergistically with *R. salmoninarum* in this situation as well (Loch *et al.,* 2012).

2.3.3 Viral co-infections

Mixed infections involving two or more viral pathogens are reported in fish. Jitrakorn *et al.,* 2020 reported the unexpected discovery of dual infections of Megalocytivirus ISKNV and nervous necrosis virus (NNV) in a single Asian sea bass (*Lates calcarifer*) farm experiencing 50% cumulative fish mortality. Xu *et al.,* 2019 reported the co-infection of infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV) in farmed Rainbow trout which leads to high mortality. Snakehead retrovirus (SnRV) was reported to increase the infection titter and the cytopathic effects (CPE) of grouper nervous necrosis virus (GNNV), a member of the Nodaviridae in grouper fin cell line, GF-1 (Lee *et al*., 2002). Ma *et al.,* (2012) reported the co-infection of megalocytivirus and viral nervous necrosis virus in mass mortality of juvenile orange-spotted groupers (*Epinephelus coioides*). Skotheim, (2009) co-infected Chinook Salmon Embryo Cells (CHSE-214) with Salmonid Alphavirus (SAV) and Infectious Pancreatic Necrosis Virus (IPNV) and examined infections using IFAT, real-time RT-PCR, and viral end-point titration. SAV reduced IPNV replication to some amount, according to IFAT and end-point titration, whereas IPNV had little effect on SAV infections. Furthermore, in SAV-infected cells, the IFN and Mx mRNAs were up- regulated, but not in IPNV-infected cells. Surprisingly, viral interference has been observed in fish in several researches. Lin *et al.,* 2017 reported the experimental co-infection of Chinese perch, *Siniperca chuatsi* with infectious spleen and kidney necrosis virus (ISKNV) and *Siniperca chuatsi* rhabdo virus (SCRV). When Chinese perch brain cells (CPB cells) were infected with the same Multiplicity of Infection (MOI) of SCRV and ISKNV, the replication of SCRV overwhelmed the replication of ISKNV. When the MOI of ISKNV was 10,000 times of MOI of SCRV (0.001 MOI), the dynamic virus loads of the two viruses in CPB cells indicated that co-infections could synergistically stimulate both viruses replication at the later time points but not at early time points. The incidence of viral interference during co-infection with channel catfish reovirus (CRV) and Ictalurid herpesvirus 1 (CCV 1) was studied by Chinchar *et al.,* 1998. Prior infection with CRV significantly reduced subsequent CCV protein synthesis and virus yield. CRV- mediated interference was greatest when CRV infection preceded CCV infection by 16 hr and was least when cell cultures were simultaneously infected with both

viruses. In another study, the simultaneous infection with two piscine rhabdoviruses, infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) in a susceptible host rainbow trout, *Oncorhynchus mykiss* results in some degree of interaction at the cell level, leading to a reduced systemic distribution of IHN (Brudeseth *et al.,* 2002). This interference and antagonistic effect, as per the scientists, may be due to competition for the same receptors on the cell surface. The interactions between infectious pancreatic necrosis virus (IPNV) and infectious haematopoietic necrosis virus (IHNV) are perhaps the best-studied example of viral interference in fish. Alonso *et al.,* 1999 reported that the dual infection of IHNV and IPNV reduces the yield of IHNV, while it does not affect IPNV. Furthermore, the same scientists later discovered that dual infection decreases both viral concentrations in fish leukocytes (Alnoso *et al.,* 2003). Recently, Xu *et al.,* 2020 reported that IHNV is inhibited at the early stage of infection by IPNV and in a time-dependent manner during co-infection. Moreover, the authors reported that IPNV did not affect the amount of IHNV entering the cells.

2.4 Co-infections involving heterologous pathogens

2.4.1 Bacterial and viral co-infections

Several infection episodes in cultured fish species were recorded, and causal agents were isolated and characterised, revealing the presence of bacteria and virus in the affected fish. Several infection episodes in cultured fish species were recorded, and causal agents were isolated and characterised, revealing the presence of bacteria and virus in the affected fish. Amal *et al.,* 2018 reported a case of natural co-infection in Malaysian red hybrid tilapia (*Oreochromis niloticus* * *O. mossambicus*) by tilapia lake virus and *Aeromonas veronii* experiencing high mortality. Similarly Nicholson *et al.,* 2020 demonstrated the experimental co-infection of tilapia lake virus and *A. hydrophila* in tilapia (Oreochromis spp.).Co-infection between TiLV and *A. hydrophila* resulted in 93% cumulative mortality compared to 0%, 34%, and 6.7% in the control, single TiLV, and single *A. hydrophila* infection, respectively. Ma *et al.,* 2019 elucidated the co-infection of rainbow trout with infections hematopoietic necrosis virus (IHNV) and *Flavobacterium psychrophilum.* This combined infection caused high

mortality (76.2%–100%), while mortality from a single pathogen infection with the same respective dose was low (5%–20%). The onset of mortality was earlier in the co-infected group (3–4 days) when compared with fish infected with *F. psychrophilum* alone (6 days) or IHNV (5 days), confirming the synergistic interaction between both pathogens. García-Rosado *et al.,* 2007 revealed the co-occurrence of viral and bacterial pathogens in disease outbreaks affecting newly cultured sparid fish. The bacteria were identified as *Vibrio* spp. and *Photobacterium damselae subsp. damselae*, with the presence of viral nervous necrosis virus (VNNV) and viral nerve hemorrhagic syndrome virus (VHSV) confirmed in the same infected fish samples. Secondary infection of acutely infected *Atlantic salmon* with ISAV resulted in reduced mortality than infection with ISAV alone, indicating that IPNV has an antagonistic effect on ISAV (Johansen and Sommer, 2001). The experimental combination infection of aquabirnavirus with VHSV, *Edwardsiella tarda*, or *Streptococcus ininae* in *Japanese flounder*, *Paralichthys olivaceus*, was explained by Pakingking *et al.*, 2003.The results indicated that the primary ABV infection in flounder suppresses the secondary viral infection but facilitates the secondary bacterial infection.

2.4.2 Parasitic and viral co-infections

There is scarcity in the co-infection studies of viral and parasitic interaction in fish. Gorgoglione *et al.,* 2019 developed a sequential co-infection model with myxozoan parasite *Tetracapsuloides bryosalmonae*, and *Novirhabdovirus* in brown trout (*Salmo trutta*) to assess if the responses elicited in co-infected fish are modulated when compared to fish with single infections. The role of *salmon lice* (*C. elongates* and *L. salmonis*) as a vector for the transmission of ISAV, including the occurrence of skin injury and immunosuppression, culminating in epizootic outbreaks and mortalities, was investigated by Nylund *et al.*, 1993. The vector potential of the salmon louse *Lepeophtheirus salmonis* in the transmission of infectious hematopoietic necrosis virus (IHNV) was clarified by Jakob *et al.*, 2011. IHNV-positive salmon lice spread IHNV successfully after being exposed to water or parasitizing infected *Atlantic salmon*, with 76.5 and 86.6 percent of exposed *Atlantic salmon* testing positive for IHNV, respectively.

2.4.3 Parasitic and bacterial co-infections

Parasitic infections increase the risk of secondary bacterial infections and may act as a vehicle for the spread of bacterial pathogens (Holzer *et al.,* 2006). This synergistic interaction has been demonstrated in numerous studies, with greater mortality rates in parasitized / bacterium co-infected fish (Kotob *et al*., 2017). This synergistic impact was explained by parasite-induced stress, which reduced the fish's resistance to secondary bacterial infections, as well as the parasite's destructive effects, which offered an entry path for bacteria to invade (Bowers *et al.,* 2000). To reduce fish mortality owing to secondary bacterial infection, more emphasis on parasite infection prevention in fisheries is required.

Scanning electron microscopy revealed the existence of bacterial colonies on the surface of *Gyrodactylus* in 1985, albeit the precise role of these bacteria was unclear, and it was vague whether the bacteria were pathogenic to fish. Bandilla *et al.,* 2006 reported experimentally for the first time an ectoparasite can enhance the severity of bacterial infection in fish. Rainbow trout were more susceptible to *F. columnare* after being infected with *Argulus coregoni*, and cumulative mortality was greater in the co-infected group than in the single infected group. In addition, disease and death occurred earlier. Xu *et al*., 2007 investigated whether parasitism in Tilapia with *Gyrodactylus* affected susceptibility and death after immersion infection with *S. iniae* in another investigation. During the first two weeks following exposure, a co-infected group had a greater mortality rate (42.2%) than a *S. iniae*-only infected group (6.7%), and no mortalities were detected in a *G. niloticus*-only infected group. Also, it has been shown that the infestation of channel catfish (*Ictalurus punctatus*) fry with *Tricodina* sp. resulted in increased susceptibility to streptococcosis (Evans *et al*., 2007). Xu *et al.,* 2009 investigated the effect of *I. multifiliis* load and trophont size on *Nile tilapia* mortality following *Streptococcus iniae* exposure. They demonstrated that the mortalities increased in fish with a high parasite load (88%) when compared to fish having a low parasite load (38%) after Tilapia were exposed to an equal number of *Streptococcus iniae* and subjected to the same exposure conditions. This study also found that when fish were exposed to well-developed trophonts, the epithelium was damaged more and bacterial invasion was increased, resulting in higher mortalities than when fish were exposed to

young little trophonts. Fish mass mortalities have been recorded during the concurrent infection of earthen pond reared Nile tilapia with *F. columnare* and *Myxobolus tilapiae* during the early summer especially with deteriorated water quality parameters (Eissa *et al.,* 2010). Shoemaker *et al*., 2012 investigated the effects of parasitism by the protozoan parasite *I.multifiliis* on survival, haematological, and bacterial load in channel catfish exposed 1 day previously to *E. ictaluri*, the causative agent of catfish enteric septicemia. When compared to single infected individuals, the co-infected group (71.1 percent) had a larger bacterial load in several organs, a change in haematological parameters, and a higher mortality rate. They assumed that the increased mortality in the co-infected group may be due to stress caused by the parasite infection influencing the immune response of catfish. Another prior investigation by Xu *et al*., 2012a verified that *I. multifiliis* parasitized catfish had much higher mortality (80%) and a higher load of *A. hydrophila* in the internal organs after being exposed to *A. hydrophila*. Lhorente *et al.,* 2014 used intracellular bacteria, *Piscirickettsia salmonis*, as a primary pathogen and the sea louse, *C. rogercresseyi*, as a secondary pathogen 4 days after bacterial exposure at high and low doses to study co-infections in *Atlantic salmon*. After 53 days, death in the two treatments of co-infected groups reached 100 percent, compared to 46 percent in the single infection group. In another study, the susceptibility of hybrid tilapia to *F. columnare* was considerably improved following *I. multifiliis* infection (Xu *et al.,* 2014). Furthermore, it was found that the treatment of *Trichodina* sp. using formalin solution has considerably improved the survival of hybrid tilapia and reduced the load of *F.columnare* after the experimental exposure (Xu *et al.,* 2015). When young Chinook salmon, *Oncorhynchus tshawytscha* are exposed to freshwater diseases *Flavobacterium columnare* or *Aeromonas salmonicida*, Roon *et al*., 2015 investigated if infection by the trematode *N. salmincola* increased mortality in the same way. They discovered that cumulative percent mortality from *F. columnare* infection was higher in *N. salmincola*-parasitized (80%) fish than in non-parasitized (40%) fish, but that cumulative percent mortality from *A. salmonicida* infection did not vary. They assumed the change in mortality between the two bacterial challenges might be due to dissimilarities in bacterial pathogenesis and the relative virulence of isolates. However, the lack of a synergistic effect following *A. salmonicida* challenge is in

contrast with the increased mortality observed in a co-infection challenge with *N. salmincola* and *V. anguillarum* (Jacobson *et al.*, 2003). Zhang *et al*., 2015 investigated a concurrent experimental infection model of *Carassius auratus* in which the fish were infected with *D. intermedius*, a monogenean, and subsequently challenged with *Flavobacterium columnare*, pathogenic bacteria. When compared to non-parasited goldfish, the mortality rate in *D. intermedius* parasitized goldfish after exposure to *F. columnare* was higher (63.9%). Furthermore, the parasitized fish's gill tissue had the greatest bacterial burden of the four tissues studied at all observation points. According to Zhang *et al.,* 2015, this ectoparasite mostly parasitizes fish gills, triggering gill injury and serving as a conduit for invasive bacteria through mechanical damage to the fish gill.

Moreover, *F. columanrae* also occurs predominantly on the gills. Carvalho *et al.,* 2019 evaluated the effect of co-infection in Atlantic salmon with different doses of sea lice *Lepeophtheirus salmonis and a* single dose *of Moritella viscosa.* The result of this study showed higher mortalities, more severe skin lesions, and significant up-regulation of immune genes in the high lice co-infected group compared to the medium lice co-infected group of *M. viscose* only infection. These findings demonstrate that while *M. viscosa* infection may cause skin lesions in salmon, co-infection with a large number of lice might magnify this effect and decrease the ability of these lesions to heal, resulting in higher mortality. Recently, Abdel-Latif and Khafaga, 2020 reported that the synergistic co-infection of *A. hydrophila* and *G. cichlidarum* in cultured tilapia with deteriorated water quality parameters could induce exaggerated fish mortalities during hot weather.

2.5 Implications of experimental studies on co- infections in fish

2.5.1 Goldfish as a model experimental organism

Goldfish (*Carassius auratus* Linnaeus, 1758) is a freshwater fish that belongs to the order Cypriniformes and the Cyprinidae family. It is the world's most common ornamental fish and is traded in over 100 nations (Maceda-Veiga *et al.,* 2013). More than 100 goldfish varieties are discovered throughout the world. The red list of goldfish is in the category of least concern (Brown, 2018). Goldfish is an omnivore fish. It feeds on wide varieties of food. Goldfish are

oviparous and produce adhesive eggs which attach to the vegetation. The spawning generally occurs at a temperature range of 15 – 20°C. The hatched larvae are pelagic (Kottelat and Freyhof, 2007). Goldfish is popular due to its visual features, robustness, and very low keeping requirements (Adamek *et al.,* 2018). Goldfish exhibits a range of social behaviors and is often discovered in other goldfish companies (Pitcher and Magurran, 1983). Indian ornamental fish trade mostly deals with freshwater fish (90%) of which 98% are cultured and 2% are captured from the wild. The remaining 10% are marine fishes of which 98% are captured and 2% culture (NFDB, 2007). Goldfish has the highest preference among hobbyists and hence its breeding dominates the Indian Ornamental Fish Sector (Raja *et al.,* 2019) For more than 500 years, human translocation has facilitated the spread of goldfish globally, which has enabled numerous and repeated introductions of pathogen taxa that infect them (Trujillo-Gonzalez *et al*.,2018). The pathogen fauna assemblage of goldfish is generally well documented, but few studies provide evidence of co-infection in goldfish.

2.6 Argulus

2.6.1 Scientific classification of *Argulus*

Kingdom: Animalia

Subphylum: Crustacea

Subclass: Branchiura

Order: Arguloida

Family: Argulidae

Genus: *Argulus*

Species: *A. japonicus*

2.6.2 Morphology

Argulus, generally known as fish lice, is the largest and most varied genus of the Argulidae family (Fryer, 1968; Kabata, 1970; Byrnes, 1985; Moller, 2009). Argulosis, a parasitic disease affecting both wild and cultivated fish populations, is caused by members of this genus (Rahman, 1995; Sahoo *et al.,* 2013). *Argulus foliaceus, Argulus japonicus,* and *Argulus coregoni* are the most studied species

from freshwater systems (Steckler and Yanong, 2012). *Argulus* is a macro-ectoparasite measuring about 5-10 mm in size and consists of a head, thorax, and abdomen (Yildiz and Kumantas, 2002). The head is covered by a flattened horseshoe-shaped carapace, maxillipeds, preoral sting, and basal glands. The thorax has four segments, each bearing a pair of swimming legs and a simple bilobed abdomen bearing spermatheca in female and testes in males. Suckers help the parasite in aggressive attachment and feeding by inserting a long spine-like structure into the skin, which breaks down tissues through the secretion of enzymes (Baker 2007; Kumari *et al.*, 2019).

2.6.3 Impact of Argulosis on fish health

In the freshwater aquaculture industry argulosis is one of the major parasitic infections that cause serious threats to fishes (Sahoo *et al*., 2013). Though numerous other *Argulus* species have been documented from India, *A. siamensis* is the most common species found in carp culture farms (Sahoo *et al.,* 2013). When factors like reduced growth, mortality, and pharmacological expense for the treatment of argulosis are taken into account, a loss of 29524.40 INR (US$ 615) is incurred annually in Indian aquaculture systems due to argulosis per hectare of water area each year (Sahoo *et al.,*2013). The male and female sexes of *Argulus* are parasitic. The fish lice have a direct life cycle and worldwide distribution. This parasite repeatedly attaches and detaches from fish fins and skin leading to tissue damage, stress, and secondary infections by fungi, bacteria, or viruses in infected fish (Mousavi *et al*., 2011).

Mucus, tissue fluids, externally digested substances, and blood are all known sources of food for these parasites (Kar *et al.,* 2015). Because of the mechanical movements of the pre-oral spine and the enzymatic action/toxicity of secretory chemicals produced by the spinal gland, the parasite's feeding activity causes far more injury to the host's skin (Bandilla *et al*., 2005; Walker, 2008). The mandibles of the mouth tube also cause mechanical damage during the feeding process, acting as vectors for various infections like as bacteria and viruses, limiting growth and altering the host's immune physiology, but in many cases, resulting in death (Kumar *et al*., 2012). The most notable immune response by the host against *Argulus* infestation is localized inflammation which appears as a small red spot on the skin of fish (Walker *et al.,* 2004). With the aid of two

suckers, *Argulus* attach to fish. They use their slender pre-oral sting to pierce the fish skin and inject the cytolytic toxin. They use their mouth and mandibles to consume the blood, mucous, tissue at the puncture site (Steckler and Yanong, 2012). It will also release a chemical messenger that attracts other fish lice which attach primarily to the caudal peduncle of the host

2.6.3.1 Pathology

Argulus infected fishes are observed with clinical signs like excessive mucus secretion, discoloration, redness and pale coloration of fins, skin and scales, frayed fins, cloudy eyes, black and white spots on the external skin of fish, scraping and scratching against hard objects (Kaur and Pandey, 2014). Major changes include the intense irritation and tissue damage due to the feeding behaviour leading to pinpoint hemorrhages which in turn make an entry route for opportunistic bacteria such as *Aeromonas* or *Pseudomonas* (Richards 1977). Other than physical damage, infected fish subjected to severe stress, which often leads to secondary parasitic infestations like white spot and costia sp., and may also act as a mechanical vector for some viruses like spring viremia of carp (Gurney, 1948).

2.6.3.2 Host response against *Argulus*

There is a paucity of information on the immunological responses of host fish to *Argulus* sp. *Argulus* is one of the most important fish ectoparasites that cause heavy economic loss to the aquaculture industry (Sahoo *et al.,* 2013). *Argulus* species cause skin lesions on the host by their suckers and proboscis while feeding. These lesions often lead to secondary infections by bacteria and fungi (Walker *et al.,* 2004). The infection also causes reduced appetite, weight loss, and anemia in fish. Skin is the primary target organ during any ectoparasitic infection, the local or systemic inflammation governs the susceptibility or resistance to any infection (Kar *et al.,* 2016). Localized inflammation, which appears as little red spots on the fish's skin, is usually the most noticeable immunological response to argulid infections (Walker *et al.,* 2004). After being inoculated with an antigen extract from *Argulus foliaceus*, Ruane *et al.* (1995) observed humoral antibody response in rainbow trout (*Oncorhynchus mykiss*). The number of monocytes and particular granulocytic cells in the periphery blood

of pacu, *Piaraclus mesopolamicus*, parasitized with *Argulus* sp., increased according to Tavares-Dias *et al*., 1999. The skin expression of the chemokine CXCa, the chemokine receptor CXCR1, and the interleukin TNFa in *Cyprinus carpio* exposed to *A. japonicus* was investigated (Forlenza *et al.,* 2008). The non-specific immune responses of the Indian main carp, *L. rohita*, naturally infested with varied loads of the freshwater fish louse, *A. siamensis*, were studied by Saurabh and Sahoo (2010). Saurabh *et al*., 2011 showed that exposure to *A. siamensis* at a single time point after a co-habitational challenge with adult parasites altered the innate immune response and the expression of immune-related genes in *L. rohita*. Furthermore, the changes in various innate immune parameters and the sequential changes in the expression pattern of some immune-related genes in *Labeo rohita* infected with *Argulus siamensis* through the course of infection were analyzed for a better understanding of the pathogenesis of this parasite (Kar *et al.,* 2015).In another study, Kar *et al.,* 2015 evaluated the changes in the adaptive immune response by quantification of the expression of Ig heavy chains in the skin, head kidney, and mucus of *Argulus* infected rohu. Saurabh *et al*., 2012 discovered immunodominant polypeptides varying from 75.78 to 79.6 kDa in freshwater fish lice *Argulus siamensis*, and these protein fractions could be used to create immunoprophylaxis against this terrible ectoparasitic infection. Panda *et al.,* 2014 elucidated the mRNA expression profile in immune-relevant tissues of carps and suggested the participatory role of TLR22 during lice infection. Histological studies showed increased melanization in the dermis and mild inflammatory cellular reactions in pre-adult rohu fish whereas, massive subcutaneous myositis with engorged blood vessels were observed in fingerlings of rohu infested with *Argulus* (Parida *et al.,* 2018). Furthermore, expression levels of various inflammation and innate immune-related genes viz., interleukin (IL)-8, IL-10, IL-11, IL-15, natural killer enhancing factor, toll-like receptor 4, apolipoprotein A-I, and immunoglobulin Z were significantly high in skin samples of infected fingerlings.

2.7 Genus *Aeromonas*

Member of genus *Aeromonas* is a gram-negative cell, straight, short rods with rounded ends. Aeromonas cells have a diameter of 0.3–1 m and a length of

1-3.5 m. It can appear as a single strand, a couple of strands, or even a small chain (Altwegg, 1999). The majority of the individuals are motile thanks to a single 1.7m wavelength polar flagellum. They exhibit many fine structures like the S layer, flagella, piled, and capsule outside the cell membrane and cell wall (Austin *et al.,* 1996). They are facultatively anaerobic and chemoorganotrophic organisms. They can perform oxidative and fermentative metabolism of D-glucose, acid and often produces acid with gas from many carbohydrates. In addition to glucose, aeromonads also utilize several other carbohydrates (Popoof, 1984; Renaud *et al*., 1988; Carnahan *et al*., 1989; Kujper *et al*., 1989; Altwegg *et al*., 1990; Abbott *et al.,* 1992). Most isolates utilize ammonium salt as the sole source of nitrogen. They are positive for both oxidase and catalase. They are enzymatically very active and reduce nitrates. They are reported to produce amylase, DNAase, chitinase, elastase, esterases, peptidases, arylamidases, and other hydrolytic enzymes (Joseph *et al*., 1988). Two biochemical studies were done by Abbott *et al*. (1992, 2003) for all known hybridization groups reported that apart from the above-said characters *Aeromonas* shared some common features like production of D-trehalose, inability to utilize malonate or mucate as a sole source of carbole, inability to ferment adonitol, dulcitol, erythritol, inositol and D-xylose and growth in nutrient broth containing 0 and 3% NaCl. The ideal temperature for growth is between 22 and 37°C. Temperatures for growth can range from 0 to 45°C. They are generally resistant to the vibriostatic drug 2, 4 diamino 6, 7 disisopropyl pteridine (O/129) in concentrations of up to 150 g.

2.7.1 Classification of genus *Aeromonas*

The genus *Aeromonas* belongs to the family aeromonadacea within class gamma proteobacteria (Carnahan M and Joseph, 2005). Over the past 2 decades, bacterial taxonomy was in a path of expansion, and the same reflected in the genus *Aeromonas* from 1943 to 2015 (Janda and Abbott, 2010). Initially based on phenotypic expression, genus *Aeromonas* was allocated to the family vibrionacea. Molecular genetic studies using 16S rRNA cataloging, 5S rRNA gene sequence comparisons and rRNA DNA hybridization reveal that *Aeromonas* is sufficiently different from the vibrionacea family (Colwell *et al*., 1986; Kita-Tsukamoto *et al.,* 1993; Ruimy *et al*., 1994). Following phylogenetic

analysis, it was discovered that it is not closely linked to vibrios, but rather forms a monophyletic unit in the class Proteobacteria's -3 subgroup (Martinez- Murcia *et al.,* 1992). Finally, *Aeromonas* has been transferred from the Vibrionaceae family to the Aeromonadaceae family (Colwell *et al*., 1986).

Super kingdom	: Bacteria
Phylum	: proteobacteria
Class	: Gammoproteobacteria
Order	: Aeromonadales
Family	: Aeromonadacea

2.7.2 *Aeromonas* infection in fish

A. hydrophila is an important causative agent of disease conditions affecting ornamental fishes in India (Walczak *et al*., 2017). The disease conditions such as dropsy, ulcer, fin rot, tail rot, gill rot, septicemia, and popeye are associated with *A. hydrophila* infection and are mainly affected by ornamental fishes belonging to family *Cyprinidae* and *Poecilidae* (Mohapatra and Swain, 2012). During summer, Goldfish and koi carp are susceptible to *A. hydrophila* infection, when secondary infection results from a high parasite load (Dixon and Issvoran, 1993). CRI 14 is one of the *A. hydrophila* strains that causes goldfish haemorrhage (Tang *et al*., 1998). *A. hydrophila* has been implicated as a causative agent of motile aeromonad septicemia (MAS), which is associated with disease conditions mainly in fish and humans and causes gastrointestinal and extra intestinal infections in humans, such as septicemia, wound infections, gastroenteritis, hepato-biliary tract infections, and hemolytic uremic syndrome (Kang *et al*., 2005).

The etiological factors of MAS include *A. hydrophila* and *A. sobria,* which cause serious bacterial illnesses in a variety of warm-water (Leung and Stevenson, 1988) and freshwater fishes (Cipriano *et al.,* 2001) and ornamental fishes (Swann and White, 1989). In farmed aquatic animals, outbreaks of motile aeromonad septicemia can reach epidemic proportions, with substantial fatality rates (Liles *et al.,* 2011*).* A bacteriological investigation of healthy goldfish *C. auratus* revealed the presence of *A. hydrophila* on a regular basis (Van Impe,

1977). A number of tropical freshwater ornamental fishes are affected by *A. hydrophila* (Swann and White, 1989). *Astronotus ocellatus,* a MAS-infected ornamental fish, had a substantial amount of red-ascitic fluid accumulating in the abdominal cavity, as well as liver and kidney haemorrhages (Soltani *et al.,* 1998). *A. hydrophila* is a gram-negative bacteria (Nordmann and Poirel, 2002), that causes infections in food and ornamental fishes, thereby posing a threat to the development of the aquaculture enterprise (Viji *et al*., 2011). Aerolysin and hemolysin genes are reported to be the putative virulence genes of *A. hydrophila* (Shome *et al*., 2005). Aerolysin, produced by some strains of *A. hydrophila*, is an extracellular, soluble, hydrophilic protein exhibiting both hemolytic and cytolytic properties. Fish diseases produced by *A. hydrophila* are a huge economic issue, but it's difficult to differentiate between direct losses and those caused by secondary infections (Amin *et al*., 1985; Aguilera-Arreola *et al*., 2005). Several putative virulence factors have been identified in several species of the genus Aeromonas, including hemolysins, cytotoxins, enterotoxins, proteases, lipases, DNases, and adhesins, all of which may play a role in disease development (Sen and Rodgers, 2004). Aeromonas sp. pathogenic genes and the cytolytic enterotoxin aerolysin were discovered (Kingombe *et al*., 1999; Albert *et al*., 2000; Watanabe *et al*., 2004).The two important spp. of A*eromonas* i.e. *A. hydrophila* and *A. sobria* causing diseases in warm water aquarium fishes have been reported by Mc Garey *et al*., (19991). Several disease conditions such as septicemia, tail rot, fin rot, ulcers, dropsy, and abnormal distension, etc. of a wide variety of freshwater ornamental fishes worldwide have been reported to be due to *A.hydrophila* (Samal *et al*., 2014).

2.7.2.1 Host response against *A. hydrophila* infection

The innate defense mechanism of fish against bacterial pathogens mainly includes the production of antimicrobial substances and acute-phase proteins complement activation, inflammation, phagocytosis, and release of cytokines (Ellis *et al.,* 1999). The pathogenic strains of *A. salmonicida* are less susceptible to anti-bacterial peptide cercopin p1 than non-pathogenic strains which lack the A-layer (Henry and Secombes, 2000). Similarly, Jia *et al.,* 2000 reported that synthetic plerocidin gives protection to *Coho*

salmon from *V.anguillarium* infection. In the skin mucus of *Japanese flounder*, Aranishi and Mano (2000) found trypsin-like proteases as well as cathepsin L and B proteases. The expression levels of TNFα, IL-1β, and IFNγ were up-regulated in the kidneys of *A. hydrophila* infected zebra fish with viable bacteria, heat-killed bacteria, and ECPs (Rodríguez *et al.,* 2008). Mu *et al.,* 2010 elucidated the transcriptome and comparative expression profiles of the large yellow croaker infected with *A. hydrophila*. Their results revealed that the inflammatory response may play an important role in the early stages of infection. Furthermore, the signalling cascades such as the Toll-like receptor, JAK-STAT, and MAPK pathways are regulated by *A. hydrophila* infection. Lu *et al.,* 2015 studied the transcriptional profiling of the skin immune response to *Aeromonas hydrophila* infection of the zebra fish, *Danio rerio.* Reyes-Becerril *et al.,* 2011 evaluated the changes in cellular innate immune parameters of gilthead sea bream (*Sparus aurata*) after 24 and 48 hr post-infection with *A. hydrophila*. Simultaneously, the expression levels of nine immune-relevant genes were also measured in the head kidney, spleen, intestine, and liver of sea bream. Gong *et al.,* 2017 reported the differential expression of TLRs in common carp after *Aeromonas hydrophila* infection. Relative percent survival (RPS) and immunomodulating effect of lipopolysaccharide (LPS) were studied in *Cyprinus carpio* against the bacterial pathogen, *Aeromonas hydrophila* (Selvaraj *et al*., 2009). Following LPS injection, they noticed a considerable increase in total leucocyte count as well as an increase in monocyte and neutrophil population. Moreover, the evaluation of interleukin-1 mRNA showed elevated expression in kidney macrophages on day 16 in fish injected with 50 and 100 µg of LPS/fish, which presumably aided efficient killing of the bacterial pathogen. Parameters of non-specific immune response and level of specific and natural antibodies were investigated after an experimental challenge with *Aeromonas hydrophila* in genetically different common carp (*Cyprinus carpio*) families (Ardo *et al.,* 2010).

Goldfish infected with *A. hydrophila* decreased innate immunity level in peripheral blood and expressed the cathepsin D in tissues (Harikrishnan *et al.,* 2010). Sahoo *et al.,* 2008 reported that ceruloplasmin level could potentially be a marker for resistance to aeromoniasis in rohu. Li *et al.,* 2013 evaluated the early mucosal response in blue catfish (*Ictalurus furcatus*) skin to *A. hydrophila* infection. In another study, Li *et al*., 2013 reported the evasion of

mucosal defense in channel catfish skin during *A. hydrophila* infection. The early alterations in blood parameters and characteristics of innate immune parameters in *Puntius sarana* infected with *A. hydrophila* were examined by Das *et al.,* 2011. Yang *et al.,* 2016 revealed the transcriptome patterns of grass carp spleens from both healthy and *A. hydrophila* infected individuals. During the infection of *Schizothorax prenanti* with *A. hydrophila,* important functional genes related to complement and coagulation cascades, chemokine signalling pathway, toll-like receptor signalling pathway, NOD-like receptor signalling pathway, and leukocyte transendothelial migration were regulated, according to Ye *et al*., 2018. Fu *et al.,* 2019 provides the findings regarding the differently expressed genes and proteins in the spleen of pufferfish, *Takifugu obscurus* infected with *A.hydrophila*.

2.8 Importance of environmental predisposing factors on infection/co-infection

Environmental stress is a significant psychosomatic factor in the outbreak of fish diseases. Many significant contributions have been made to the analysis of environmental stress and its impact on fish health (Raman *et al.,* 2013). Stress modifies numerous defense mechanisms, inhibits some, and exacerbates others. Depending on the intricate interactions between stress causes and the physical status of the animal, which ultimately affects how well the animal responds to the condition, this may be useful or damaging to the organism in question.There are many stress factors which are either excessive or deficient quantity can adversely affect the fish and predispose them to disease outbreak. Broadly, they are of four types: (A) Physical, (B) Chemical, (C) Biological, and (D) Procedural (Raman *et al.,* 2013). Both nitrogen and oxygen are dissolved in water and both are involved in fish diseases if present in too low or too high concentrations (Demeke and Tassew, 2016). The lack of optimum amount of free carbon dioxide demand on the buffering capacity of blood results in a destabilization of the acid-base balance and during their maintenance there is a respiratory alkalosis, or after the first uptake of protein-rich food, there is excessive metabolic acidosis (Schaperclaus, 1991). The toxicity of ammonia is the highest of all nitrogen compounds. The higher the pH of the water, the higher will be the ammonium

toxicity. The damage due to ammonia is confined to the respiratory organs, blood, and nervous tissues of fish (Levit, 2010). In eutrophic waters, there are frequent and wide fluctuations in dissolved oxygen. Certain species of freshwater algae, if present in large quantities produces powerful toxins which can seriously affect aquatic life (Lee and Jones, 1991). The procedural stress factors like stocking, handling, hauling, treatment procedures, and feeding methods are mostly ignored during culture operations but they can cause a serious outbreak of diseases by activating latent infections (Raman *et al.*, 2013). Changes in these physicochemical factors can be stressful for many organisms including fishes and can potentially induce adaptive stress responses (Tine, 2017). Among the various stress factors the physical stress or changes in temperature important in outbreaks or reduction of fish diseases (FA0, 2018; Green and Haukenes, 2015).

2.8.1 Temperature

One of the world's most important environmental dangers is climate change. Water temperature rises as a result of global warming are concerning for aquaculture and are a major stress element in many disease outbreaks (Green and Haukenes, 2015; Islam *et al*., 2019). Climate change has been shown to have an impact on the fishing industry in several studies (Williams and Rota, 2011; Winfield *et al.,* 2016; Golam *et al*., 2017; Adhikari *et al*., 2018). Any fish species' growth and physiological processes can be affected directly or indirectly by temperatures that are above or below the optimum threshold (Fu *et al*., 2018). Fish physiology, immunology, behaviour, and parasite-avoidance techniques will all be affected by rising temperatures, which could lead to ecosystem changes (Lohmus *et al.*, 2015). Temperature change is a complex amalgam of stressors in and of itself, but synergy with other biotic stressors may exacerbate temperature's impact in aquatic organisms (Woodward *et al.,* 2010).

2.8.2 Temperature effect on pathogenesis

Disease development in fish is a complex process involving the interaction of a susceptible host, a virulent microorganism, and environmental factors (Hedrick, 1998). The third requirement is particularly important in the aquaculture industry since the high densities and stress to which fish are commonly exposed

favor the appearance of diseases that are rare or even non-existent in natural environments (Guijarro *et al*., 2015). Temperatures above or below a fish's physiological optimum may change the immune response and increase infection susceptibility (Abram *et al*., 2017). Elevation in ambient temperature could have a two-way effect on pathogenic transmission. Firstly the pathogen metabolism could be improved directly, resulting in a higher number of transmission phases, leading to improved fitness and rapid spread of the disease in a single outbreak. Secondly, the increase in temperature at either end of the natural period of the occurrence of the disease could extend the transmission season, resulting in a wider overall spread of the disease within the host population. Both of these mechanisms alone or in combination would result in an increased prevalence of the disease (Pounds *et al*., 2006; Karvonen *et al*., 2010). Several authors have suggested that the increase in temperature will enhance the multiplication of bacteria and parasite which have a direct impact on the degree and nature of pathology experienced by hosts (Rahman *et al*., 2001; Karvonen *et al*., 2010; Callaway *et al*., 2012; Zhang *et al*., 2015). Similar to other organisms, aquatic pathogens do need specific temperature optima for the completion of their life cycles (Marcogliese, 2001). At higher temperatures, *the Argulus* parasite will reproduce frequently and the intensity of their attachment with fishes will also increase (Kumari *et al*., 2019; Saha and Bandyopadhyay, 2015; Sahoo *et al*., 2013). Warmer temperatures may increase the energetic demands and growth rates of parasites, or increase the number of hatched parasites, and thus leads to an over-exploitation of the host (Barber *et al*., 2000). Moreover, temperature acts as a significant environmental element that influences protein secretion or gene expression in the bacteria and enables the bacteria to adapt to the changing environment (Shapiro *et al*., 2012; Guijarro *et al*., 2015). A key environmental stress factor in outbreaks of most fish bacterial diseases in fish farms is water temperature (Snieszko, 1974).

It has been shown that the abiotic ecosystem has an impact on the developments in pathogenic lives (Blanford; Fels and Kaltz, 2006). Temperature variation is one of the omnipresent sources of environmental variation and is known to have a significant impact on the biochemical, physiological, and behavioral processes of organisms (Vaumourin and Laine, 2018). Pathogens with a free transmission phase are considered particularly susceptible to temperature

fluctuations (Truscott and Gilligan, 2003). In every host-pathogen system, temperature influences the pathogens in establishing or maintaining an infection, latency as well as aggressiveness (Burdon, 1987; Blanford *et al.,* 2003; Fels and Kaltz, 2006). In addition to the surrounding abiotic environment, the impact of the biotic environment on the maintenance of variations in pathogen populations has also been highlighted (Vaumourin and Laine, 2018). When numerous pathogens infect the same host, the outcome of infection might be unpredictable (Susi *et al*., 2015). Pathogens compete for the same limited resources offered by the host in a mixed infection, leading to a higher pace of host exploration and increased pathogen pathogenicity than in a single infection (Alizon, 2013). Although both abiotic and biotic settings may have a significant impact on infection outcomes, little is known about their relative relevance or whether temperature influences co-infection outcomes (Marcais *et al*., 2017).

2.9 Evaluation of immune responses in fish; relevant immunological indicators

2.9.1 Innate Immune system v/s infection in fish

Despite certain distinctions, the immune systems of fish and higher vertebrates are biologically comparable (Rombout *et al*., 2005). Innate and adaptive immune defence systems exist in fish. Innate parameters are at the forefront of immune defence and play an important role in disease resistance (Magnadottir, 2006). Fish's adaptive response is often delayed, but it is critical for long-term immunity and is a crucial determinant in vaccination success. Because of the tremendous development in aquaculture in recent decades, researchers have focused more on the immune system and disease defences of fish (Secombes and Wang, 2012).

Nonspecific immunity is a fundamental defense mechanism in fish. It also plays a crucial role in the acquired immune response and homeostasis via a receptor protein system. These receptor proteins identify molecular patterns typical of pathogenic microorganisms such as polysaccharides, lipopolysaccharides (LPS), peptidoglycan bacterial DNA, viral RNA, and other molecules not normally found on the surface of multicellular organisms. The response is divided into physical barriers and cellular and humoral immune

responses (Tort *et al*., 2003). And the immunological parameters include growth inhibitors, lytic enzymes, the classic complement pathways, the alternative and lectin pathway, agglutinins and precipitins (opsonins and primary lectins), antibodies, cytokines, chemokines, and antibacterial peptides (Uribe *et al*.,2011). Various internal and external factors may influence the parameters of the innate immune response. Temperature changes, stress management, and density may have suppressive effects on this type of response, while several food additives and immunostimulants may improve their efficacy (Magnadottir, 2006).

2.9.2 Innate immune parameters

Innate immunity is made up of both constitutive and receptive defensive systems that provide immediate and long-term protection against a wide spectrum of infections (Ellis *et al.*, 1999; Magnadottir *et al.,* 2006; Saurabh and Sahoo, 2008). Temperature is also thought to have a significant impact on a fish's immunological response (Langston *et al*., 2002). Phagocyte respiratory burst activity, myeloperoxidase enzyme antibacterial activity, and bactericidal activity of lysozyme have all been utilised as indicators of nonspecific immunity in fish (Anderson and Siwicki, 1995; Ellis *et al*., 1999). In fish, phagocytosis was recognized as one of the important elements in the host defense against invading micro-organisms (Rehulka., 1998), and thus the quantification of oxidative radical (primarily Reactive Oxygen Species or ROS) production from neutrophils and monocytes as a defense mechanism was done using nitrobluetetrazolium (NBT) assay. The major role of fish phagocytic cells is to limit the dissemination and growth of pathogens. Once contained the other immune cells are also recruited for destroying the infectious agents. Both neutrophils and macrophages contain a repertoire of antimicrobial molecules stored within the granule and lysosomes of these cells. During pathogen phagocytosis, leukocytes increase their oxygen consumption via NADPH oxidase and produce a variety of reactive oxygen species (ROSs) such as superoxide anion radical (O2), hydrogen peroxide (H2O2), singlet oxygen (1O2), and hydroxyl radical (OH-) in a process known as the respiratory burst (Biller-Takahashi *et al*., 2013). Superoxide and hydrogen peroxide are highly hazardous reactive oxygen species (ROSs) that are at the heart of a powerful antibacterial system (Klebanoff, 1999). Myeloperoxidase

enzyme (MPO) is located in the primary granules of neutrophils, which deliver the MPO and other bactericidal compounds to invading pathogens by fusing with phagocytic vesicles accelerating pathogen destruction (Klebanoff *et al*., 2013). MPO enhances the microbicidal potential of neutrophils by converting hydrogen peroxide (H2O2) into the highly toxic antimicrobial compound hypochlorous acid (HOCl) and by forming radicals by oxidating substrates including phenols, nitrate, and tyrosine residues (Kettle and Winterbourn., 1997). Estimation of NBT activity and myeloperoxidase levels significantly reflect the immunological status and leucocyte performance (Kumar *et al*., 2013). Likewise, Lysozyme is an essential innate immune system defence molecule that plays a role in mediating protection against microbial invasion (Saurabh & Sahoo., 2008). The lytic activity of fish lysozyme against Gram-positive and Gram-negative bacteria has been thoroughly characterised. It is also opsonic, activating the complement system as well as phagocytes. The non-specific cellular humoral response of fish subjected to temperature stressors has been studied in a number of ways. For example, after lowering the temperature from 18 to 11°C at a rate of 1°C per day, both serum complement and lysozyme activity of sea bream S. aurata considerably decreased (Tort *et al*., 2004). After being transported from 27 to 19 °C for 12-96 hours, both lysozyme activity and ACH50 were drastically reduced in tilapia (Ndong *et al*., 2007). Increased lysozyme activity was connected to a larger number of leucocytes in Atlantic halibut exposed to higher temperatures (Langston *et al*., 2002). In *Labeo rohita* infected with *E. tarda*, a rise in serum lysozyme activity and a decrease in myeloperoxidase activity were found (Mohanty and Sahoo, 2010). During bacterial (Biller-Takahashi *et al*., 2013; Xia *et al.,* 2017; Lazado *et al*., 2018; Khan *et al*., 2018) and parasite infections, several authors have documented an increase in NBT, MPO, and lysozyme activity (Souza *et al*., 2019; Kumar *et al.,* 2013; Munoz *et al.,* 2007; Alishahi *et al.,* 2006).

2.9.3 Adaptive immune parameters

The specific immune response happens via mechanisms involving a complex network of cells, proteins, genes, and biochemical messages which provide the means necessary for the body to react specifically to antigens, antibodies, and effector cells with high specificity and affinity (Uribe *et al.,* 2011).

The adaptive immune system will be engaged if a pathogen persists despite innate immune defences (Smith *et al.,* 2019). The adaptive immune system, like the innate immune system, consists of both humoral and cellular components. B cells are essential components of the humoral adaptive immune response. The primary purpose of B cells is to create high-affinity immunoglobulins (Ig) against foreign antigens and to serve as a professional antigen-presenting cell (pAPC) by presenting processed antigen to T cells for activation (Albert *et al*., 2002). Antibodies exist in two types: a soluble form that is secreted from the cell and a membrane-bound form that forms the B cell receptor (BCR) when combined with the signalling molecules Ig/Ig (Flajnik and Kasahara, 2010). T cells play an important role in cellular adaptive immunity. The T cell receptor (TCR) is always membrane-bound, and once stimulated by antigen presented by the pAPC, the T cell can be activated to serve as a helper (CD4+) T cell, a regulatory (CD4+) T cell, or a cytotoxic (CD8+) T cell in the presence of co-stimulation (Mutoloki *et al*., 2014).

2.9.4 Anti-oxidative stress enzymes

Oxidative stress is a condition that refers to the imbalance between levels of Reactive Oxygen Species (ROS) and their antioxidants (Schieber and Chandel 2014). Fish make many ROSs as part of their regular metabolism, but they have an antioxidant defence system that keeps these oxidative chemicals at a safe level (Biller and Takahashi, 2018). In some cases, the generation of oxidants is exceedingly high, and the cells might be severely damaged and die as a result (Dong *et al*., 2016, Finkel 2011, Kiley and Storz 2004, Li *et al*., 2016). Catalase (CAT), superoxide dismutase (SOD), and glutathione-dependent enzymes (glutathione peroxidase, GPX, and glutathione reductase, GR) have been found in the majority of fish species studied to date (Albuquerque *et al.,* 2017). Together with these enzymes, lower molecular- weight antioxidants, such as carotenoids, vitamins E, K, and C, amino acids, and peptides (glutathione), have been detected in antioxidant defenses in fish (Martínez-Álvarez *et al.,* 2005). During phagocytosis, leukocytes consume more intracellular oxygen, resulting in the creation of numerous reactive oxygen species, which improve the antioxidative stress enzyme pathway's functioning, resulting in improved antioxidant enzyme activity (Biller and Takahashi, 2018). SOD is a type of antioxidative enzyme

present in the cytosol (Cu2+-SOD and Zn^2+-SOD) and mitochondria (Mn^2+-SOD) that works on superoxide anion to convert it into H_2O_2, preventing the accumulation of O^2•-, which is extremely reactive and harmful to the cell. Catalases (CAT), GPx, and peroxidases attached to thioredoxin can remove the H_2O_2 generated, or it can be discharged into the cytoplasm of cells (Lambeth, 2004, Brand, 2010, Schieber and Chandel, 2014, Li *et al.,* 2016). CAT (generated from mitochondria and peroxisomes) catalyses the decomposition of H_2O_2 into H_2O and O_2, which is abundant in phagocytes, and maintains the perfect balance of ROS generation and removal, which is critical for the innate defence system's proper functioning (Wang *et al.,* 2013). Catalase and SOD, on the other hand, are key oxidative stress biomarkers (Kumar *et al.,* 2013). A sudden increase in temperature caused oxidative stress in goldfish tissues, according to Lushchak and Bagnyukova, 2006. Fathead minnows *Pimephales promelas* were shown to have considerably increased superoxide dismutase (SOD) activity when kept at 25°C for 6 hours compared to fish kept at 7°C or 32°C (Clotfelter *et al*., 2013). The presence of a considerable increase in the level of stress enzyme in azadirachtin-treated experimental fish at higher concentrations indicates a negative impact on the operation of important tissues and the physiology of the host (Kumar *et al.,* 2013). In the serum of *Labeo rohita* infected with *E. tarda*, Mohanty and Sahoo (2007) found a substantial reduction in superoxide. The release of reactive oxygen species (ROS) from *L. rohita* phagocytic cells infected with native *Aeromonas hydrophila* and an exotic *Aeromonas salmonicida* caused a simultaneous increase in the antioxidative stress enzyme pathway, resulting in increased activity of superoxide dismutase (SOD), catalase (CAT), and significantly higher concentrations of CAT (Roy *et al*., 2019).

2.9.5 Hematological parameters

The complete blood cell count (CBC) is an important and effective diagnostic tool and a minimum database component. It can be used to monitor the health status of fish in response to changes related to nutrition, water quality, and disease and in response to therapy (Fazio F., 2019). As a disease Diagnostic method hematological analysis is particularly important because it can provide

reliable assessment by non-lethal means (Satheesh kumar *et al*., 2012). Both human and veterinary medicine is equipped with well-established laboratory protocols and reference ranges for the Hematology profile (Hrubec *et al*., 2000). A wide range of pathogens (viruses, bacteria, parasites, etc.), environmental factors (temperature), and even husbandry factors have caused heavy losses in aquaculture facilities (Noga, 2000). The presence of various pathogenic infections single or co-infected, habitat change, and change in surrounding water temperature and climate, and stress factors can influence the normal range of hematological profile (Tavares-Dias *et al*., 2008; Fazio F., 2019). Moreover, the differences among hematological values are affected by a multitude of intrinsic and extrinsic factors (Clauss *et al*., 2008). Changes in these parameters depend on several factors such as species (Anthony *et al*., 2010), temperature (Magill and Sayer, 2004), stress (Cnaani *et al*., 2004), health condition (Vazquez and Guerrero, 2007), water quality (Fazio *et al*., 2012), and microbial infection and parasitism (Martins *et al*., 2004: Azevedo *et al*., 2006; Jamalzadeh *et al*., 2009). Combined with other routine diagnostic methods the haematological profile can be used to identify and assess conditions that cause stress and/or diseases that affect production performance (Pavlidis *et al*., 2007). The most important diagnostic results are the qualitative and quantitative variability of the haematological parameters (Martins *et al*., 2004). Different investigators in fish physiology and pathology identified normal ranges for different blood parameters in fish (Xiaoyun *et al*., 2009). In particular, an examination of the RBC, PCV, Hb, and WBC values is recommended on a routine basis for monitoring the health of stocks in fish farms (Fazio F., 2019). In addition to the identification of the causative agents, understanding the haematological changes in infected fish will be helpful in the identification and subsequent control of the disease (Rajapakshe *et al*., 2012). Thus database of the haematological parameters of fish is an important tool that can be used as an effective and sensitive index to monitor the health status of fishes. Kumar *et al.* (2013) investigated the impact of azadirachtin solution treatment on the haematological and biochemical profiles of goldfish infested with *Argulus* spp. Another study reported a reduced RBC and hematocrit value in channel catfish, *Ictalurus punctatus* co-infected with *Ichthyophthirius multifiliis* (Ich) and *Edwardsiella ictaluri* (Shoemaker *et al.*, 2012). Several researches has found that as the temperature rises, the oxygen

affinity of the blood decreases, resulting in an increase in RBC count and dependant parameters such Hb and PCV (Ashaf-Ud-Doulah *et al*., 2019; Langer *et al.*, 2013; Radoslav *et al*., 2013).

2.10 Innate immune relevant genes in fish

Fish immune-relevant genes have received considerable attention due to their role in improving the understanding of both fish immunology and the evolution of immune systems (Zhu *et al*., 2013). The innate immune response involves a coordinated system of induced gene products, preformed immune effectors, biochemical signalling cascades, and specialized cells. However, the multidimensional stimulation of these protective actions may disrupt or overshoot and if left uncontrolled, inundate the host (Rebl and Goldammer, 2018). Due to the constraints on adaptive immunity in suboptimal conditions, innate immunity plays a larger role in teleosts' initial defence against pathogen invasion (Ullal *et al.,* 2008). Many commercial fish species, such as rainbow trout, (*Oncorhynchus mykiss*) have been studied for innate immunity genes such as those encoding pattern recognition receptors, antimicrobial peptide, complements, lectins, and cytokines (Casadei *et al*.,2009; Sangrador-Vegas *et al*., 2000; Falco *et al*.,2008), pufferfish (*Tetraodon nigroviridis*) (Wu *et al*., 2008), large yellow croaker (Pseudosciaena crocea) (Wang *et al.,*2009; Xiao, *et al.,*2011) channel catfish (Ullal *et al*.,2008; Wang *et al.,* 2006; Pridgeon *et al.,* 2010), etc. They are an evolutionary older defence system that provides instant protection against infection (Gao *et al*., 2012). The identification of genes linked to immunity in fish, as well as the analysis of their expression patterns, receives a lot of interest. Increased immune gene expression is usually seen as an indication of immunological stimulation or an improved immune response (Abo Al Ela and Haitham, 2018). Numerous investigations have discovered individual differences in genes, resulting in variable gene production activities (Whyte, 2007; Zhou *et al.,* 2012; Rajendran *et al*., 2012). These disparities in resistance or susceptibility to disease, which is responsible for the host's health when exposed to pathogenic organisms, may account for variances in resistance or susceptibility to disease in different individuals.

As a result, these features could be employed in breeding programmes to select disease resistance, and they're vital for health management (Gao *et al.,* 2012). The type of receptors employed to sense pathogens is the major difference between innate and adaptive immunity (Medzhitov, 2007). Several receptors are involved in innate immune recognition, including pattern recognition receptors (PRRs) with broad specificity that has evolved to detect pathogen-associated chemical patterns (PAMPs) (Janeway and Medzhitov, 2002). PAMPs include lipopolysaccharide (LPS) or peptidoglycan (PGN) in bacterial cell walls, 1, 3-glucan on fungal cell walls, and double-stranded RNA from viruses. Pattern recognition receptors (PRRs) are responsible for recognising microbial pathogens based on sensing structures conserved across microbial species, known as pathogen-associated molecular patterns (PAMPs) (Mogensen., 2009). Toll-like receptors are the well-studied of the several functionally different groups of PRRs (TLRs). Complement components, which are made up of many soluble membrane-bound proteins, play a similar role in the innate immune system. Microbial death, phagocytosis, inflammatory responses, immune complex clearance, and antibody synthesis are just a few of its many roles (Merle *et al.,* 2015). The classical pathway, the mannan-binding lectin pathway, and the alternative pathway have all been discovered to activate the complement system. Antibodies are involved in the traditional route. Host lectins activate the mannan-binding lectin pathway when they recognise and bind to microbial carbohydrate patterns. The alternative pathway, on the other hand, is constantly active and is activated by a variety of foreign molecule (Ricklin *et al.,* 2010; Dunkelberger *et al.,* 2010).

2.10.1 Toll-like receptors (tlr)

Toll-like receptors (TLRs), the first and best understood innate immune receptors, play a notable role in the innate immune system by sensing pathogenic agents and initiating appropriate immune responses. TLRs got their name and were first discovered because of their resemblance to the Toll protein in Drosophila melanogaster (Medzhitov *et al.*, 1997), which is involved in dorsoventral patterning during embryogenesis as well as the antifungal response (Lemaitre *et al.,* 1996). Structurally, TLRs are integral glycoproteins characterized

by extracellular or luminal ligand-binding domain-containing leucine-rich repeat (LRR) motifs and a cytoplasmic signaling Toll/interleukin-1 (IL-1) receptor homology (TIR) domain (O'Neill and Bowie 2007). Ligand binding to TLRs through PAMP-TLR interaction induces receptor oligomerization, which subsequently triggers intracellular signal transduction. The type of receptors employed to sense pathogens is the major difference between innate and adaptive immunity (Medzhitov, 2007). Many receptors play a role in innate immune recognition, including pattern recognition receptors (PRRs) that have evolved to identify pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002). Toll-like receptors (TLRs) are the well-studied of the several functionally different groups of PRRs (Alvarez-Pellitero, 2008). PRRs are divided into two categories: cytosolic and membrane-bound receptors. Inside the endosomal compartment, where many viruses lose their coat and expose their genome for replication and transcription, membrane-bound receptors, notably TLRs, detect viral nucleic PAMPs. TLR signalling enhances the production of pro-inflammatory cytokines such IL1 (interleukin 1), TNF (tumour necrosis factor), IL8 (Rauta *et al*., 2014), and IFN molecules, which alert adaptive immune cells to an existing pathogen and trigger direct defence responses (Iwasaki and Medzhitov, 2004; Kawai and Akira, 2010).

Twenty TLR types have been reported, so far in teleosts (Rauta *et al.,* 2014) (TLR1, 2, 3, 4, 5M, 5S, 7, 8, 9, 13, 14, 18, 19, 20, 21, 22, 23, 24, 25, 26). The first piscine full-length TLR sequence was reported from goldfish (*Carassius auratus*) having no recognizable sequence homology with any mammalian TLR genes (Stafford *et al*., 2003). TLR22 is the only TLR found in aquatic creatures, and it is activated by ligands such as peptidoglycans and ployI: C. TLR22 is a receptor that detects dsRNA infection and provides antiviral defence in fish (Su *et al*., 2012). TLR 22 can also be greatly influenced by bacterial and parasite ligands, showing that it has diverse roles (Kar *et al.,* 2015).TLR22 is believed to be associated with innate immunity against ectoparasite infection (Panda *et al.,*2014). Two isolates of TLR22 were detected in goldfish (Stafford *et al.,* 2003) and rainbow trout (Rebl *et al.,* 2007). The presence of the TLR22 gene was also identified from large yellow croaker (Xiao *et al*., 2011), grass carp (Lv *et al.,* 2012), orange-spotted grouper (Ding *et al.,* 2012), gilthead seabream (*Sparus aurata*) (Munoz *et al.,* 2014) and turbot

(Hu *et al.,* 2015). Several authors have also reported the TLR22 expression in different tissues of finfish after a challenge with pathogens. Saurabh *et al.* (2011) studied the TLR22 expression in different tissues of rohu after *Argulus siamensis* infection. Ding *et al.* (2012) reported significant up-regulation of TLR22 in the spleen of orange-spotted grouper after injection with *V. alginolyticus*. Expression patterns of TLR22 in different tissues of gilthead sea bream after *V. anguillarum* infection were elucidated by Munoz *et al.* (2014). Tu *et al*., 2016 investigated the expression pattern of TLR22 in different tissues of goldfish in response to the infection of the ectoparasitic monogenean *Dactylogyrus intermedius*. Another study reported the significant up-regulation of TLR22 in the skin and liver tissue of catla infested with *Argulus spp*. (Panda *et al.,* 2014). Zhang *et al.,* 2015 elucidated the significant up-regulation of TLR22 in goldfish injected with *A. hydrophila* thus suggesting the importance of TLR22 in the immune response against bacterial infection. Comparative mRNA expression study profile in immune relevant tissues of Indian major carp viz, catla, rohu, mrighal artificially infested with *Argulus* also suggested the participatory role of TLR22 during lice infection (Panda *et al.,* 2014). Samanta *et al*., 2014 studied the expression profile of TLR22 in various tissues of *Labeo rohita* infected with *A. hydrophila*. Kar *et al.,* 2015 elucidated the TLR22 expression in different tissues of *Labeo rohita* infected with *Argulus siamensis*. Li *et al.,* 2017 discovered a considerable up-regulation of TLR22 in multiple tissues of common carp when challenged with poly I: C or *A. hydrophila*, showing that TLR22 plays a vital role in systemic as well as mucosal defence after viral or bacterial stimulation or infection. TLR22 expression during the co-infection of any fish species has yet to be studied.

2.10. 2 Complement C3 gene

Complement component 3 (C3) is a central component of the complement system. All three pathways converge at the formation of C3 convertases and share the terminal pathways of membrane attack complex (MAC) formation (Holland and, Lambris, 2002). The complement system is an important link between innate and adaptive immunity in vertebrates, playing a pivotal role in the defense against pathogens infection (Boshra and Sunyer, 2006). It is generally accepted that the complement system consists of more than 30 plasma proteins

and cell surface receptors that participate in opsonization, cell lysis, phagocytosis, inflammation, and B cell response (Carroll.,2004). The complement systems can be activated through three pathways: classical, lectin, and alternative pathways. Among the three activation pathways, complement component 3 (C3) is the central molecular in all the known proteins of the complement system, and its activation is essential for the formation of membrane attack complex (Boshra and Sunyer, 2006). The C3 emerged over 700 million years ago, belongs to the Alpha-2-macroglobulin (A2M) family (Sunyer *et al.,* 1998). Pushpa *et al.,* 2014 studied the tissue-level expression of complement C3 in healthy and *A. hydrophila* infected *Labeo rohita*. Xu *et al.*, 2018 studied the expression pattern of the C3 component in dojo loach (*Misgurnus anguillicaudatus*) infected with *A. hydrophila*. Mohanty and Sahoo, 2007 elucidated the expression of C3 in the head kidney of *Labeo rohita* infected with *E. tarda*. The differential expression of the C3 gene was studied in the kidney, skin, and liver tissue of *Labeo rohita* infected with A*rgulus siamensis* (Saurabh *et al*., 2011). Induction of C3 transcription level was evident in the skin and spleen of Rainbow trout infected with parasitic ciliate *Ichthyophthirius multifiliis* (Sigh *et al*., 2004). Similarly, a significant up-regulation of complement factor C3 in the liver of common carp infected with *Ichthyophthirius multifiliis* was reported by Gonzalez *et al.,* 2007. Zhou *et al*., 2018 elucidated the expression profile of C3 in the skin of goldfish during the primary infection and re-infection with monogenean parasite *Gyrodactylus kobayashii.* Das *et al*., 2011 studied the expression of C3 in immune-relevant organs like the liver and kidney of *Puntius sarana* experimentally infected with *A. hydrophila*. Expression of the C3 gene in gilthead sea bream (*Sparus aurata*) was reported by Reyes-Becerril *et al.,* 2017. Significant up-regulation of the c3 gene in gills and kidney of *Catla catla* infected with *Flavobacterium columnare* was reported by Pradhan *et al.,* 2019.

The expression profile of C3 in co-infection studies was reported by Zhang *et al.,* 2015. They evaluated the expression pattern of different immune genes of goldfish co-infected with monogenean *Dactylogyrus intermedius* and the bacterium *Flavobacterium Columnare.* Their result elucidated the down-regulation of complement component C3 in the gill and kidney of parasite-infected fish compared to the non-parasitized controls.

3. MATERIAL AND METHODS

In the present study, live ornamental fish *Carassius auratus*, were maintained at the central wet laboratory, ICAR- Central Institute of Fisheries Education (ICAR-CIFE), and microbiological and molecular studies were conducted in laboratories of Aquatic Environment and Health Management Division ICAR- CIFE, Versova, Mumbai during the year 2016-19.

3.1. Sterilization of glasswares

The glassware was kept immersed in a solution of non-corrosive detergent for about 8-10 h and then washed with clean water. These were then kept immersed in a disinfectant K_2Cr_2O7 for few hours and were rinsed 3-4 times with distilled water followed by drying in a hot air oven at 180 °C for one hour. Finally, these were wrapped in brown paper followed by aluminum foil and thereafter were sterilized in an autoclave at temperature 121˚C and 15 psi pressure for 15minutes.

3.1.2 Media and reagents

The common bacteriological media were procured from Hi-MEDIA Pvt. Ltd, Mumbai, India.

The media used in the present study were as follows:

1. Nutrient agar
2. Nutrient broth
3. Brain heart infusion agar
4. Brain heart infusion broth
5. Luria bertani broth
6. Luria bertani agar
7. Aeromonas selective media
8. Aeromonas isolation media
9. Triple sugar iron agar
10. Motility agar

11. EDTA (ethylene diamine tetra acetic acid)
12. TAE (Tri acetate EDTA)
13. Tris base
14. Glacial acetic acid

According to manufacturer's instruction, preparation and sterilization was done.

The reagents used in this study were as follows:

1. Hydrogen peroxide
2. PBS (Phosphate Buffered Saline)
3. Safranin 1% (*w/v*)
4. Xylene
5. Crystal Violet
6. Glycerol
7. Ampicillin supplement
8. Aeromonas selective supplement
9. *DNAZOL* reagent

The biochemical discs used in the present study are as follows:

1. O-129 disc
2. Oxidase disc

3.2 Sampling

Goldfish (*Carassius auratus*) were procured from different aquarium shops of Mumbai *viz*, Kurla, Seven bungalows, and Borivali. *Argulus* infected and diseased goldfish were selected for isolation of *Argulus spp.* and *Aeromonas* spp., respectively. The sampling was done twice a week from October 2017 to August 2018. The goldfish were brought in plastic bags with proper aeration and maintained in the central wet laboratory, ICAR-CIFE, Mumbai. Fish were fed with the pelleted feed and the water quality parameters were tested every 48 hours.

3.3 Experimental setup

The experiment was carried out in the Central Wet Laboratory, ICAR-CIFE. The infected fish were stocked in FRP tanks of 500-L capacity. All the tanks used in the experiments were washed with normal detergent and then disinfected using potassium permanganate (5ppm) overnight before using the experiment. The tanks were thoroughly rinsed with fresh water, dried and then filled with filtered freshwater up to the desired leveland and aerated overnight.

3.4 Experimental fish

Healthy goldfish (*C. auratus*) of size 9.0 ± 0.02 cm and 7.2 ± 1.2 g were procured from aquarium shops located at Kurla, Maharashtra. Fish were transported in transparent polythene bags with sufficient oxygen (15 fish/ bag). Upon reaching the laboratory, fish were disinfected with 5 ppm potassium permanganate ($KMnO_4$) solution and were carefully transferred to FRP tanks of 700-L capacity and acclimatized for 15days with continuous aeration in the Central Wet Laboratory of ICAR-CIFE.

3.5 Experimental procedure

3.5.1 Isolation of bacteria

Clinically infected live or moribund fishes were collected from aquarium shops at different locations for the isolation of *Aeromonas hydrophila*. Fishwere anesthetized using 50 ppm clove oil for 2 – 3 minutes and wiped lightly with tissue paper. The external surface was disinfected using a cotton ball soaked with 70% alcohol to avoid external contamination. Under the septic condition, each fish was dissected from the dorsal side after removing the dorsal fin with a sterile scalpel to reach up to the kidney. Using a sterilized loop, the kidney was touched and streaked onto fresh Aeromonas isolation media (AIM) plates containing ampicillin supplement. The plates were incubated at 28° C for 24-48 hours in an inverted position. *Aeromonas* colonies appeared dark green, opaque with or without a black center. From the primary culture plate, colonies of typical of *Aeromonas* spp. were streaked onto Luria Bertani (LB) plates to obtain pure colonies.

3.5.2 Biochemical identification of *Aeromonas hydrophila*

. The colonies showing dark green opaque morphology with or without a dark center on Aeromonas selective agar (AIM) were subjected to biochemical tests for presumptive identification of the Genus *Aeromonas*. The biochemical tests included, following Gram staining, motility test, oxidase test, catalase test, fermentation of glucose withgas production, triple sugar iron (TSI)agar test,resistance to vibriostatic agent O/129 (2,4–diamino-6,7 disopropylpteridine,150 µg).

3.5.2.1. Gram staining

The Gram staining was done using the Gram-staining kit (HiMedia, Mumbai). The stained slides were observed under 100 X oil immersion objective (Zeiss, Germany)

3. 5.2.2 Motility test

Motility was observed using hangining drop technique in a compound light microscope under40X objective (Microscope name). A semi-solid motility test medium was also used for the motility test. The motility medium was stabbed with a small number of inoculums, incubated overnight at room temperature. If the bacterial cultureswere motile, the medium became turbid with growth that radiated away from the line of inoculation. If bacteria were not motile, only the stab line was visible.

3. 5.2.3 Cytochrome oxidase test

The oxidase test is used to identify bacteria that produce cytochrome C oxidase, an enzyme of the bacterial electron transport chain. Readymade oxidase disc containing 1% Kovacs oxidase reagent was used for the oxidase test (Hi-Media, Mumbai). Oxidase disc was placed over the glass plate and 0.1-0.2 µl of 24 h broth culture of bacteria was placedover the disk. Development of deep purple coloration within 20 seconds was considered as thepositive result.

3. 5.2.4 Catalase test

The catalase test determines the production of the catalase enzyme by the bacteria. A drop of fresh hydrogen peroxide (3% reagent grade) was placed on a microscopic slide. With a sterile loop, the bacterial sample of 18-24 hours was picked

up and placed on the slide. Production of effervescence due to the liberation of O_2 gas was considered catalase-positive.

3.5.2.5. O/129 discs

This test determines the sensitivity of a bacterial organism to the vibriostatic agent 0/129 (2, 4-diamino-6, 7 diisopropylpteridine).The bacterial culture weregrown in nutrient broth overnight. Nutrient agar plates were inoculated with the bacterial culture by soaking sterile cotton swabs in the broth culture and streaking on the plates.The O/129 discs were placed onthefreshly inoculated platesusing sterile forceps and the plates were incubated at 37 ˚C for 24-48 hours. Zone of inhibition around the disc indicated susceptibility of bacteria to vibriostatic agent 0/129.

3.5.2.6 Triple sugar iron (glucose fermentation)

TSI test determines the ability of an organism to utilize specific carbohydrates incorporated in a basal growth medium, with or without the production of gases along with the determination of hydrogen sulfide (H**2**S) production. With a sterile needle, triple sugar iron agar slants were inoculated by stabbing to the bottom of the tube and then streaking the surface of the slant as the needle was drawn out of the stab. The cap was loosely screwed and incubated at 30 ºC for 24-48 hours. TSI agar contains three sugars in varying concentrations: glucose (1X), lactose (10X), and sucrose (10X). It also contains phenol red as a pH indicator. If sugar fermentation occurred, glucose was initially used and the butt will be acidic (yellow). After glucose utilization, the organisms may continue to ferment the remaining sugars and if this occurs, the entire tube becomes acidic. Certain bacteria, which are unable to utilize any sugar, will break down the peptone present. Peptone utilization caused an alkaline (red) shift in the medium that causes a color change from orange to red. Blackening of the medium was caused by hydrogen sulfide production, which changes ferrous sulfate to ferric sulfide. In addition, the splitting of the medium or presence of bubbles in the butt of the tube indicated the gas production

3.6 Biochemical identification of *Aeromonas* up to complex level

Aeromonas isolates were further classified into complex level as *Aeromonas hydrophila, Aeromonas caviae* and *Aeromonas sobria* complex based four different biochemical tests as described by Abbott *et al*. (2003).

3.6.1. Esculin hydrolysis

This test was used to determine the bacterial ability to hydrolyze esculin by β-glucosidase or esculinase enzyme. Esculin agar without bile is used for the identification of oxidase-positive facultative anaerobic *Aeromonas* species. Bacteria hydrolyze esculin into glucose and esculentin. The development of black coloration indicated a positive reaction. Esculin agar media was prepared by dissolving 41.5 g of esculin agar in 1000 mL of distilled water and boiling to dissolve the media completely, followed by by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Esculin agar was preparedas slants. With a sterile loop, slants were inoculated with the test bacterium by stabbing to the bottom of the tube and then streaking on the surface of the slant. The inoculatedslants were incubated at 37˚C for 24-48 h. Formation of black coloration in the esculin agar indicated positive hydrolysis test.

3.6.2. Indole test

This test was used to determine the ability of bacteria to hydrolyse theamino acid tryptophan to form indole using the enzyme tryptophanase. Tryptophan broth was prepared by dissolving 15 g of tryptophan broth in 1000ml of distilled water and boiling to dissolve the media completely, followed byautoclaving at 15 lbs pressure (121°C) for 15 minutes. Sterile tryptone broth was inoculated with test bacterium and incubated at 37 °C for 24- 48 hrs. Indole production was detected by Kovac's reagent which contains 4 (p) - Dimethyl-amino benzaldehyde. A volume of 5 drops of Kovac's reagent was added into the test tube.Formation of a cherry red ring at the surface indicated a positive result.

3.6.3. Voges-Proskauer (VP) test

VP test was used to determine the ability of bacteria to produce 2, 3-butane-diol as a fermentation product from glucose. 2,3-butan-diol is an end product of the fermentation pathway but it is not easy to detect. So intermediate named acetoin is determined in this reaction and thus indirectly the end product is detected. Methyl red Voges - Proskauer (MR-VP) broth was inoculated with light inoculums from 18-24 hr culture and was incubated for 2-3 days at 37°C. After incubation, 3 ml of Barritt reagent A (5% α – naphthol in absolute alcohol) and 1 ml of Barritt reagent B (40% KOH) were added. In the presence of KOH, the intermediate acetoin is oxidized to diacetyl, a reaction which was catalyzed by α–naphthol. Diacetyl reacts with the

guanidine group, which isassociated with molecules contributed by peptone in the medium to form a port winecolored product. Formation of Eosin pink color within 30 minutes after addition of reagents indicated a positive result.

3.6.4. Arabinose utilization

This test was used to determine ifthe bacterium was able to ferment the carbohydrate arabinose resulting in acid formation.Phenol red agar broth base was used as a medium to which 1 % arabinose was added. The arabinose-phenol red broth was inoculated with the test bacterium and incubated at 28°C for 24 hours. A positive test showed color change from red to yellow, indicating a pH change due to arabinose fermentation.

3.7 Molecular characterization of *A. hydrophila*

3.7.1 Bacterial DNA isolation

Genomic DNA was isolated using *a DNAzol* reagent. This method is based on the use of novel guanidine–detergent lysing solution which permits selective precipitation of DNA from a cell lysate. After centrifugation of culture at 8000 rpm for 5 minutes, the supernatant was discarded. The pellet was re-suspended in 100 µl nuclease-free water in Eppendorf tubes and was heated for 10 minutes in a boiling water bath, cooled on ice and 1.0 ml of *DNAzol* reagent was added to the tubes. Tubes were centrifuged for 10 min at 10,000 xg to pellet cell debris. Supernatant fluids were transferred to a new Eppendorf tube and 0.5 ml 100% ethanol was added to each tube. Tubes were mixed thoroughly by inverting several times and then allowed to stand for 15 min at 4 °C to precipitate the DNA. Precipitated DNA was pelleted by centrifuging the tubes for 10 min at 14,000 rpm and supernatant was removed. The DNA was washed in 0.8 ml 95% ethanol and centrifuged at 14,000 rpm for 2 min to pellet the DNA. The supernatant was discarded and the washing step was repeated. The final DNA pellet was dissolved in 20 µl TE buffer.

3.7. 2 Amplification of 16SrRNA by PCR

PCR amplification of *16SrRNA gene of Aeromonas isolates* was done using the universal primers 27F (forward) and 1492R (reverse) primers. The thermocycling conditions consisted of aninitial denaturationof 94 °C for 5 min, () followed by35 cycles

of 94 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 1.30 minutes, and a final cycle of 72 °C for 10 minutes.

Table 1: Primers used for PCR amplification of 16SrRNA.

16S rRNA	Primer sequence	Length	Product (bp)	Melting temperature(Tm)	Reference
27 F	5'-GTTGATCATGGCTCAG-3'	16	1500	52.3	Sarkar *et al*.,2012
1492 R	5'-GGTTCACTTACGACTT-3'	19		54	

3.7.3 Agarose gel electrophoresis of PCR products

The PCR products were analyzed on 1% agarose gel. Agarose gel was prepared by boiling to dissolve1 g agarose in 100 ml of 0.5X TAE(diluted from a 50X stock solution: 242 g tris base, 57.1 ml glacial acetic acid, 100 ml of .5M EDTA). The solution was cooled to approximately 50 °C and ethidium bromide was added to a final concentration of 0.5 µg/mL and mixed. The gel was poured into the electrophoresis tray and allowed to solidifyfor 20 min. The gel was then placed into the electrophoresis tank (BioRad, USA) and the wells were loaded with 8 µL of PCR product was mixed with 2 µL of 6X DNA loading dye. A 1 Kbp DNA ladder (Thermo Scientific GeneRuler) was used as a molecular size reference. The electrophoresis was carried out at 80V for 30 to 45 min and the gel was photographedusing a Gel Documentation System (BioRad, USA).

3.7.4. 16SrRNA by PCR product purification

Purification of PCR products was done using a GeneJET gel extraction kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. For purification, the PCR product was mixed with the binding buffer at a 1:1 ratio in a 1.5 ml microcentrifuge tube and mixed by pipetting. Then the mixture was transferred to a silica column provided with the kit and centrifuged at 10,000 rpm for 1 min. The

exudate, collected in the collection tube, was discarded and the collection tube was placed back. After this, 700 µl of wash buffer was added and centrifuged at 10,000 rpm for 1 minute. The collected exudate was removed, the collection tube was placed back and the silica column was dried by centrifuging at 10,000 rpm for 1 min. The column was placed in a fresh centrifuge tube and 30 µl of pre-warmed (65^{o}C) elution buffer was added. The tube was kept for 1 min at room temperature and centrifuged at 10,000 rpm for 1 min to elute the DNA. All DNA samples were labeled and stored at -20^{o}C until further use.

3.7.5. Agarose gel electrophoresis of purified 16SrRNA PCR product

The purified *16SrRNA* PCR product was analyzed on a 1% agarose gel (1 gm agarose in 100 ml .5X TAE) before sending the purified product for sequencing.

3.7.6. Confirmatory test for *Aeromonas hydrophila*

The purified PCR products of representative isolates were subjected to 16S rRNA sequencing using a forward primer from a commercial sequencing facility. The sequences were blast analyzed with the NCBI (National Centre for Biotechnology Information) database and Ribosomal database project. For further confirmation species-specific PCR has been used in which the DNA of *Aeromonas* isolate, along with a positive and negative control was subjected for PCR and agarose gel electrophoresis is performed. The species-specific primer used for the PCR is given in 2 Table 2.

Table 2: Species specific primers used for PCR amplification of *A. hydrophila*

Target	Primer sequence (5'-3')	Product(bp)	Annealing temperature	Reference
F	GGCCTTGCGCGATTGTATAT	103	55.5	Trakhan *et al.*,2009
R	GTGGCGGATCATCTTCTCAA		55	

3.8 Determination of lethal dose (LD_{50}) of *A. hydrophila*

To determine the LD_{50} (the lowest bacterial dose which caused 50% mortality), fish were divided into five groups with 20 fish in each group. The experiment was conducted in 25L glass aquarium tanks. The bacterial isolates (n=how many) were sub-cultured in 5 ml BHI broth and the suspension was static-incubated at 28 °C for 16 hours. After the incubation, the individual culture was centrifuged for 5 minutes at 5000 rpm. The supernatant was discarded and pelletwaswashed twice with PBS and resuspended in 5 ml PBS. The bacterial count was determined by standard dilution and surface plating method. The suspension was 10-fold serially diluted to obtain the bacterial concentration of 10^4 to 10^7CFU/ml. The fish in the group (1-4) were injected with 0.1 mL of bacterial suspension intraperitoneally with a concentration of 10^4 to 10^7 CFU/fish, respectively. The control (5^{th}) group was injected with 0.1 ml sterilePBS. The experiment was carried out in duplicate. Fish mortality was recorded every 24 hours for 7 days. The injected bacterial isolates were re-isolated from the kidney of moribund fish to fulfilKoch's postulates. Dead fish were subjected to bacteriological examination immediately after death. The LD_{50} values were calculated based on the cumulative mortalities following the method of Reed and Meunch (1938),

3.9 Isolation of parasite

Fish were examined for the presence of *Argulus sp*. The identification of *Argulus* infestation in goldfish was done by keeping the fish in Petri plate containing water and watching for the presence of a parasite. The caudal fin, skin, belly region, and head or opercula part of the body were generally, observed for the presence of parasites, as the parasites mostly attach to these parts of fish body.

3.10 Identification of *Argulus* species

Identification of *Argulus* species was done by observing the parasite under microscope at 10x for their morphometric characteristics based on the key provided by Fryer (1982).

3.11 Artificial infection in healthy goldfish with *Argulus sp.*

3.11.1 Cohabitation method

Cohabitation was done following the method by (Saurabh *et al.,* 2010). In this method, the heavily or moderately infected goldfish were kept together with healthy goldfish in the ratio of 1:5 and 1:3 in separateglass aquaria. The fish were observed daily for one week to determine the increase in the number of *Argulus* inhealthy fish and spread of the infection. During this artificial infection, the water quality parameter was maintained optimal with dissolved oxygen maintained at 5.5 -7.5 mg/L using proper aeration and the fish were fed with commercial pelleted feed.

3.11.2 Exposing goldfish to the egg clutches of *Argulus*

The method used in the present study was a modification of Kumar *et al.* (2012b) and Sharma *et al.* (2016) in which artificial infection was carried out by exposing the healthy goldfishes with eyespot stage of *Argulus sp.* eggs. Clutches of *Argulus* eggs of five-days old were scraped with the help of a scalpel and observed under a microscope at 10x before experimenting. The eggs were distributed in 3 aquaria containing approximately 200 eggs for 5 fish in each. Moderate aeration was provided. Siphoning was not performed for 20 days until the eggs have hatched and were attached to the fish. Once the infection is developed; all the infected goldfish were transferred to the 500 L FRP tank and maintained as the stock tank.

Objective 1: To study the host immune response of *Carassius auratus* during co-infection to *Argulus* and *Aeromonas hydrophila*

3.12 Experimental fish

Five hundred healthy goldfish *C. auratus* with an average weight of 7.2 ± 1.2 g were procured from the local Aquarium market, Mumbai, India. The fish were acclimatized in laboratory condition in three Fibre-reinforce plastic (FRP) tanks of 700-L capacity for 15 days. The fish were fed with a commercial pelleted diet at 3% of their body weight. About one-tenth of water was exchanged daily to remove waste feed and fecal materials. The physicochemical parameters of water were maintained at optimum condition *viz*., dissolved oxygen (5.80±0.85 mg L-1); pH (7.5-7.8);

ammonia (0.095±0.055 mg L-1); nitrite (0.020±0.005 mg L-1) throughout the experiment. The ethical guidelines for the animal care of the Central Institute of Fisheries Education (CIFE), Mumbai were strictly followed during the experiment. Ten goldfish were randomly selected from the acclimatized tanks for screening the pathogen-free status of selected parasites and bacteria before the experiment. The examined fish were ensured to be negative for the parasites and fish pathogenic bacteria

3.13 Experimental infection with *Argulus* to determine the different grades of infection

Experimental infection of *C. auratus* with *Argulus* was performed according to the cohabitation method of Saurabh *et al*., 2010 by maintaining *Argulus* infested and healthy goldfish in the same experimental tanks for two weeks. Before the experiment, all the healthy fish were screened for the absenceof pathogens. One-hundred fifty *Argulus* infested goldfish with an average body weight 10 ± 1.5 g were procured from Kurla ornamental fish market, Maharashtra, India and maintained in FRP tanks (500 L). The mean intensity of *Argulus* infection on *C. auratus* was determined as 15± 6 parasites per fish. For the cohabitation challenge study, these fish were equally distributed (n=175 each) in two experimental tanks of 700-L capacity (440 cm dia* 100cm height). After acclimatization, 350 healthy goldfish were randomly selected and stocked into two experimental FRP tanks used for cohabitation study. For two weeks, fish were observed daily to determine the increase in the number of *Argulus* and spread of infection. Based on the observed intensity of infection, the three levels of parasitic infection were defined as low (1–10 lice fish-1), moderate (10–20 lice fish-1), and high (>20 lice fish-1) according to Saurabh and Sahoo*,* (2010).

3.14 Experimental design

To elucidate the effect of different levels of intensity of *Argulus* sp. infestation during co-infection with a single sub-lethal dose (1/10 of LD50) of *A. hydrophila* in goldfish, the experimental fish were distributed into eight treatment groups (fig 1): T1 (Control group without *Argulus* and *A. hydrophila* infection), T2 (Fish exposed to 1/10 of LD50 dose of *A. hydrophila*), T3 (Fish exposed to the low grade of *Argulus* infestation), T4 (T3 + *A. hydrophila*), T5 (Fish exposed to moderate grade

of *Argulus* infestation), T6 (T5 + *A. hydrophila*), T7 (Fish exposed to the high grade of *Argulus* infestation) and T8 (T7+ *A. hydrophila*) (fig: 1). Eighty healthy goldfish from the acclimatization tanks were equally distributed into T1 and T2 treatment tanks in duplicate. Twenty fish of each infection grade were randomly selected from stock tanks and introduced into the respective tanks. Each grade of infection was assigned to with four experimental tanks, duplicated for each co-infection and single grade of the parasite infestation study. Fish assigned for the T2 and co-infection treatments in T4, T6, and T8 groups with different grades of *Argulus* infection received an intraperitoneal injection with100 µl of 4.7 x 10^5 CFU *A. hydrophila.* Fish belonging to T1 treatment (control) were injected with 100µl of PBS. One set of all the treatment groups' was maintained separately for observing the mortality throughout the trial period (168 h). Ten goldfish were examined and cultured to verify the pathogen-free status of parasites and bacteria before the trial. All the fish were negative for non-targeted parasites and pathogenic fish bacteria other than *Argulu*s and *Aeromonas hydrophila.*

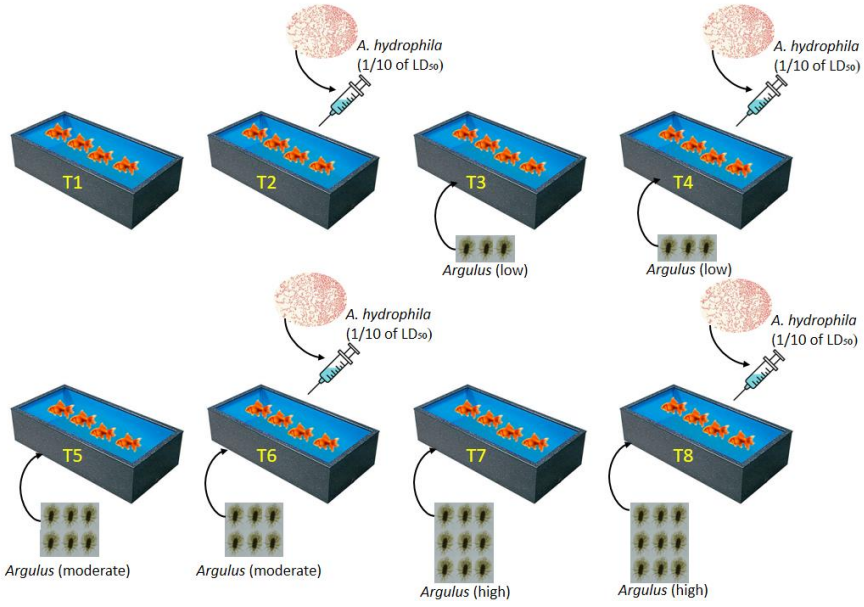


Fig 2: Experimental design

3.15 Sampling framework

The hematological and enzyme parameters of fish were analyzed after the bacterial challenge. For this; four fish from each treatment group (T1-T8) were randomly sampled at 24, 72, and 168 hrs. Before the sampling, fish were anesthetized with clove oil (50 µlperliter). Blood was collected aseptically by severing the caudal peduncle using a 1 mL tuberculin syringe rinsed with 2.7% EDTA solution. The collected blood was transferred immediately to EDTA-containing test tubes. Serum was obtained by collecting the blood without the anticoagulant and the supernatant was collected after centrifuging for 10min at 3000×g at 4ºC (REMI, Mumbai, India). The serum samples were stored immediately at -70°C until further analysis for various enzyme parameters.

For the gene expression study, skin from the base of the pectoral fin (the area found with major parasitic load in infected fish), liver and kidney tissue samples were collected at 0, 6, 12, 24, 48,72hours and on day-7 post-infection (p.i.) after anesthetizing the fish with clove oil. The samples were immediately stored in *RNA later* (Ambion, Austin, TX) at -20°C for extraction of total RNA.

3.16 Total RNA extraction

Total RNA was extracted using *Trizol* reagent (Thermo Fisher Scientific, USA) following the manufacturer's instructions. Tissue samples weighed around 100mg were sliced out from the tissue kept in *RNALater*, blotted using lint-free tissue paper, and transferred to a homogenizing tube containing 800µL of *Trizol* reagent and homogenization beads. The sample was then subjected to homogenization using a microsmasher for 20 sec. 200µL of *Trizol* was added to the sample to make up the total volume 10 times of the sample volume. The homogenate was incubated at room temperature for 5 minutes, and 200µL of chloroform was added. Following vigorous shaking of the homogenate, it was kept undisturbed in room temperature for 5-15 minutes and centrifuged at 12000×g for 15 minutes at 4°C. To a fresh microfuge tube, the clear aqueous phase was transferred and 500µL of isopropanol was added, gently mixed the contents before incubating for 10-15 minutes at room temperature. The tubes were centrifuged at 12000g for 10 minutes at 4°C and the pellets obtained

were washed with 1 mL of 70% ethanol by vortexing, followed by centrifugation at 7500×g for 5 minutes. Pellets were air-dried for 15-20 minutes to remove the alcohol residue and RNA pellet is dissolved in 20-30 µL of nuclease-free water.

3.16.1 Quantification of RNA

The concentration of the isolated RNA was measured using a NanoDrop™ spectrophotometer; (Thermo Fisher Scientific, USA).The Nucleic acid concentration was obtained directly regarding ng/µl along with the 260:280 ratios. The ratio of the absorbance at 260 nm and 280 nm provides an estimate of the purity of the isolated DNA/RNA.

3.16.2 DNase treatment

To remove the genomic DNA contamination, total RNA isolated using *Trizol* reagent was treated with *RNase-free DNase* I (Thermo Fisher Scientific, USA) according to manufacturers' instructions before cDNA synthesis. Briefly, the reaction mixture was prepared in a PCR tube by adding 2000 ng/µL of total RNA, 2µL of 10X reaction buffer, 2 µL of *DNase* I, and the final volume was made to 18µL with nuclease-free water. This reaction mixture was incubated for 30 minutes at 37°C in a thermal cycler (Applied biosynthesis, USA). The reaction was terminated by adding 2µL of 50 mM EDTA followed by a 5 minutes incubation at 65°C.

3.16. 3 Quantification of DNase treated RNA

The concentration of the *DNase* treated RNA was measured using a Nanodrop spectrophotometer (Thermo Scientific, USA). RNA concentration was obtained directly in terms of ng/µL along with the 260:280 ratios for checking the purity.

3.16.4 cDNA synthesis

The mRNA pool was converted into its complementary DNA using the first-strand *cDNA* synthesis Kit (Thermo Scientific, USA) as per the manufacturer's instructions. Briefly, a 2000 ng/µL *DNase* treated RNA sample was mixed with 1µL of *oligo dT* in a PCR tube and make up the final volume up to 12 µL using nuclease-free water. This mixture was incubated at 65°C for 5 min in a thermal cycler and immediately chilled on ice for 2 min. The reaction volume was made up to 20 µL by adding 4 µL of 5X reaction buffer, 2 µL of *dNTP* mix (10 mM each *dNTP*), 2µL reverse transcriptase, 2µL *RNase* inhibitor, mixed gently by tapping, spun and incubated at

42°C for 60 min and 70°C for 5 min. The synthesized first-strand *cDNA* was stored at -20°C.

3.17 Validation of housekeeping genes

For the validation of internal controls, three commonly used housekeeping genes namely, β-actin, elongation factor 1α (EF1a), and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) were selected for standardization in this study. The expression stability of these three selected housekeeping genes was evaluated in the kidney, liver, and skin tissues of healthy (T1) and treatment group T6 (moderate parasite and sub-lethal dose of bacteria) and T8 (high parasite and sub-lethal dose of bacteria) at three-time points viz, 24, 48 and 72 h p.i. Total RNA from the tissues was extracted using *TRIzol* reagent following the manufacturer's instructions and the RNA was subjected to *DNase* treatment followed by *cDNA* synthesis. Specific primers for each gene were taken from published papers (Table 3). The optimal annealing temperature of each primer was about 60 °C and the amplicons were within the range of 100–150 bp. PCR specificity was confirmed with a single melt peak in dissociation curve analysis. PCR efficiency was calculated based on the slope of a standard curve generated using tenfold serial dilutions (10, 10−1, 10−2, 10−3, and 10−4) of kidney cDNA. The validation of these housekeeping genes was done by using statistical algorithms like *the Delta Ct* method (Silver *et al*., 2006), *Best keeper* (Pfaffl *et al.*, 2004), *Normfinder* (Anderson *et al.,* 2004), and *geNorm* (Vandesompele *et al.,* 2002). The comprehensive ranking of the expression stability was evaluated using the *RefFinder* program (Xie *et al.,* 2012). According to *the Delta Ct* method, the gene which shows the lowest value should be considered as the most stable and the gene which displays the highest value is to be ranked as the least stable. As per the *Best keeper* software, the gene which shows the least value of the standard deviation, the better is the stability. According to *NormFinder* statistical algorithm, the gene with the lower value indicates higher stability. Each program may give a different result. Therefore, comprehensive gene stability was carried out which calculates the overall stable reference gene for gene data normalization.

Table 3: primer sequence and efficiency of primers used for validation of control genes

3.18 Quantitative Real-Time PCR

Comparative quantification of the mRNA of genes of interest was performed

GENE	Primer sequence (5′-3′)	Reference	Efficency E (%)
β- actin	F: GATGATGAAATTGCCGCACTG R: ACCGACCATGACGCCCTGATGT	Zhang *et al.,* 2015.	101
GAPDH	F: GCCAGTCAGAACATTATCCCAGCCT R:GGTCCTCAGTGTATCCCAGAATGCC	Hao *et al*., 2008	103
Ef-alpha	F: 5 CCGTTGAGATGCACCATGAGT R: TTGACAGACACGTTCTTCACGT	Grayfer*et al.,* 2008	100

using LC 96 (Roche, Germany) with SYBR green as DNA intercalating dye. The primers used for the study were taken from published papers and the sequence and optimum annealing temperature for primers are given in Table 3. The constitutively expressed house-keeping gene, EF- alpha was used both as a positive control and for sample normalization. Reference gene was selected after validating CT values of three housekeeping genes concerning treatments. Each well of a 96 well plate contained a final volume of 10 µL containing 5 µL of SYBR Green I master mix (Thermo Fisher Scientific, USA), 1 µ L of (10 pM) each forward and reverse primers, 1µl cDNA and 2 µl nuclease-free water. The reaction was performed in triplicates along with non-template controls to rule out the cross-contamination. The thermal profile for the reaction consisted of hot start at 95 °C for 3 minutes followed by 40 cycles of denaturation (95°C for 20 s), annealing (20 s at its respective annealing temperature), and extension (60°C for 30 s). A melting curve analysis was carried out after every amplification reaction. The mRNA level was expressed in terms of 2-ΔΔCT, where CT is threshold cycle and ΔΔCT is calculated by the following formula;

$$\Delta CT = CT_{\text{target gene}} - CT_{\text{reference gene}}$$

$$\Delta\Delta CT = \Delta CT_{\text{test sample}} - \Delta CT_{\text{control}}$$

GENE	Primer sequence (5′-3′)	Annealing temperature (°C)	Reference
C3	F: CTGTGCTGGCGGTTGT R:ATCCTCCATAATGAGACTGTTG	56	Zhang *et al.,* 2015
TLR22	F: TGGTCTTTCTCACCCTGCTC R: AAATGAAGGCGTCGTACTGG	60	Tu *et al*., 2016

Table 4: List of primers used in real time RT-PCR

3.19 Innate immune parameters

3.19.1 Respiratory burst activity (RBA)

Nitroblue tetrazolium (NBT) assay was performed to estimate RBA using the method described by Anderson and Siwiki (1995). Briefly, One-hundred microliters of blood were mounted in the wells of a flat microtitre plate and incubated at 37 °C for 1 h to promote cell adhesion. 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 another 1 h and washed once with 100% methanol and thrice with 70 % methanol, followed by air drying. Potassium hydroxide (KOH) (2N; 120 µL) and dimethyl sulphoxide (DMSO) (140 µL) were added into each well to form a blue formazone precipitate. The absorbance of the wells was measured in an ELISA reader (Quant, Universal microplate spectrophotometer, USA) at 620 nm.

3.19.2 Myeloperoxidase (MPO) activity

Serum myeloperoxidase activity was measured according to Quade and Roth (1997) with some modifications. Breifly, 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'- tetramethylbenzidine hydrochloride (TMB) (Hi-media, India) and 25 µL of 5 mM H_2O_2 (Qualigens, India) (Both substrates of MPO). The reaction was stopped after 2 minutes by adding 50 µl of 4 M sulphuric acid (H_2SO_4). The plate was centrifuged (400×g) for 10 minutes, 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 microplate reader (Quant, Universal microplate spectrophotometer, USA).

3.19.3 Serum lysozyme activity

The serum lysozyme activity was measured using the 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 the 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 intervals. 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.

3.20 Hematological study

Hematological parameters *viz*., total erythrocytes counts (TEC), total leukocyte counts (TLC), hemoglobin (Hb), packed cell volume (PCV), monocytes, and neutrophiles were analyzed using automated blood analyzer (Pentra XL 80, Pentra 60C+, BIORAD D-10HPLC, Automated coagulometer, Japan).

3.21 Enzymes of oxidative stress

3.21.1 Superoxide dismutase (SOD)

The SOD activity was measured using the colorimetric measurement assay kit (Cat no. 706002, Cayman Chemicals Ltd, USA), following the manufacturers protocol. The SOD activity was reported as units per ml.

3.21.2 Catalase (CAT)

The colorimetric measurement of CAT activity was done using catalase assay kit (Cat no. 707002, Cayman Chemicals Ltd, USA), following the manufacturers protocol. The activity was reported in nmol/min/ml.

3.21.3 Glutathione peroxidize (GPx)

GPX activity was measured using a Glutathione peroxidize assay kit (Cat no. 703102, Cayman Chemicals Ltd, USA)following the manufacturer's protocol. The activity was reported in nmol/min/ml.

Objective 2: To determine the effect of temperature on co-infection

3. 22 Experimental Fish

Healthy goldfish *Carassius auratus* with an average weight of 7.2 ± 1.2 g (n=180) were procured from the local fish market, Mumbai, India. The fishes were acclimatized in 700-L Ferro cement tanks with aerated freshwater in a wet laboratory for two weeks and fed with a commercial pelleted diet at 3% of their body weight. About one-tenth of water was exchanged daily to remove waste feed and fecal materials. The Physico-chemical parameters of water were assessed regularly to maintain its optimum 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.

3.23 Artificial infection of goldfish with *Argulus*

Argulus infested goldfish *Carassius auratus* weighing 7.5 ± 2.0 g (n=150) procured from the local fish market were acclimatized in six plastic crates of 60 L capacity containing25 fish in each crate. In subsequent days, an egg laid by the adult parasites on the sides of the tank was kept undisturbed with less exchange of water.

The water condition was monitored periodically and the eggs were observed for hatching every day until 100% hatching was obtained in12- 14 days. The hatched metanaupli remained in the same condition unless it attached to the exposed goldfish. On the 25th day, each fish was observed for the parasitic intensity and prevalence. Based on the observed intensity of infection, three levels of infection were defined; low (1–10 lice fish−1), moderate (10–20 lice fish−1), andhigh (>21 lice fish−1). *Argulus* from each goldfish was collected using forceps and used for the experiment.

3.24 Experimental design

To elucidate the effect of three different water temperatures *viz*., 23, 28, 33°C on the co-infection of a moderate dose of *Argulus* and sub-lethal dose of *A. hydrophila* in goldfish, the experimental fish were distributed into six treatment groups (Fig:2): (T1): Healthy fish exposed to 23°C, (T2): Co-infected group exposed to 23°C, (T3): Healthy fish exposed to 28°C, (T4): Co-infected group exposed to 28°C, (T5): Healthy fish exposed to 33°C, (T6): Co-infected group exposed to 33°C. The chosen temperatures were nonlethal but close to the thermal limits of goldfish. The water at required temperatures viz, 23, 28, and 33 °C was conditioned in separate well labelled experimental crates using 300 W thermostats, and the temperature was monitored throughout the experimental period by using a standard mercury thermometer (G H ZEAL, England). After acclimatization to the laboratory settings for two weeks, one hundred eighty goldfish from the stock tanks were randomly distributed in all the treatment tanks in duplicate following a randomized block design. Further, acclimatization was done by altering the water temperature with a maximum of 1°C/ day. Following the acclimatization period, all experimental groups except the respective temperature control groups (T1, T3, and T5) were exposed with the expected number of *Argulus* to get a moderate grade of infection. Within an hour of post-exposure, all the released *Argulus* were found attached to the fish. Further, fish assigned for the co-infection treatments in T2, T4, and T6 groups received an intraperitoneal injection containing one hundred microliters of 4.7 x 105 CFU fish^{-1} *A. hydrophila*. One set of all treatment groups were maintained separately for observing the mortality and the intensity (T2, T4, and T6 group) throughout the trial period.

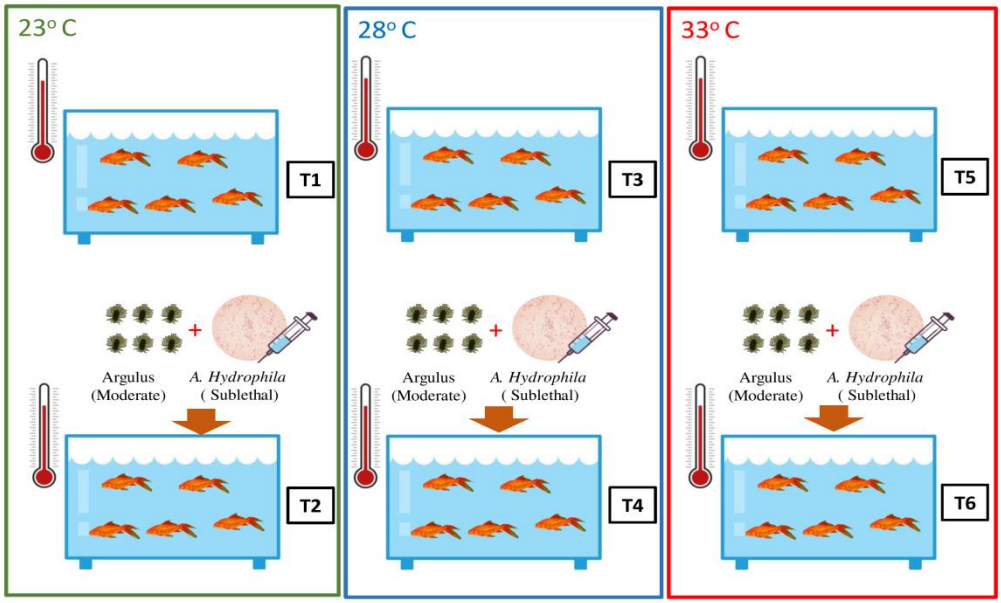


Fig 3: Experimental design

3.25 Sampling procedure

For the hematological and enzyme parameter analysis, sampling was done at 24 hr, 72 hr, and 168hr post-challenge in all experimental groups. Each fish was anesthetized with clove oil at a rate of 50 µL L−1 of water before taking blood. The blood sample was collected under aseptically by severing the caudal peduncle using a 1 mL tuberculin syringe, which was previously rinsed with 2.7% EDTA solution, and collected blood was then transferred immediately to EDTA test tubes. To obtain the serum, blood was collected without anticoagulant and centrifuged for 10min at 3000 ×g in a refrigerating centrifuge (REMI, Mumbai, India). The following parameters are measured: TEC, TLC, Hb, PCV using automated blood analyzer (Pentra XL 80, Pentra 60C+, BIORAD D-10HPLC, automated coagulometer, Japan).

3.26 Innate immune parameters

Same procedure as followed for objective 1

3.27 Hematological study

Same procedure as followed for objective 1

3.28 Enzymes of oxidative stress

Same procedure as followed for objective 1

3.29 Statistical analysis

All data expressed in the text, figures, and tables are mean ± standard error, and statistical significance for all statistical tests was set at 5%. Data obtained for co-infection study were analyzed by one-way ANOVA using statistical tool Statistical Package for the Social Sciences SPSS 16.0 22.0 version computer program (IBM statistic 16.0). Whereas, differences in the mean value of the parameters estimated for the treatment groups and controls at three different temperatures were subjected to multivariate analysis of variance in ANOVA. Duncan's multiple range test (DMRT) was used for *post-hoc* analysis. Survivor curve among the entire experimental group was done using Kaplan Meier curve in graph pad prism (p=0.02343*).

4. RESULTS

4.1. Targeted sample

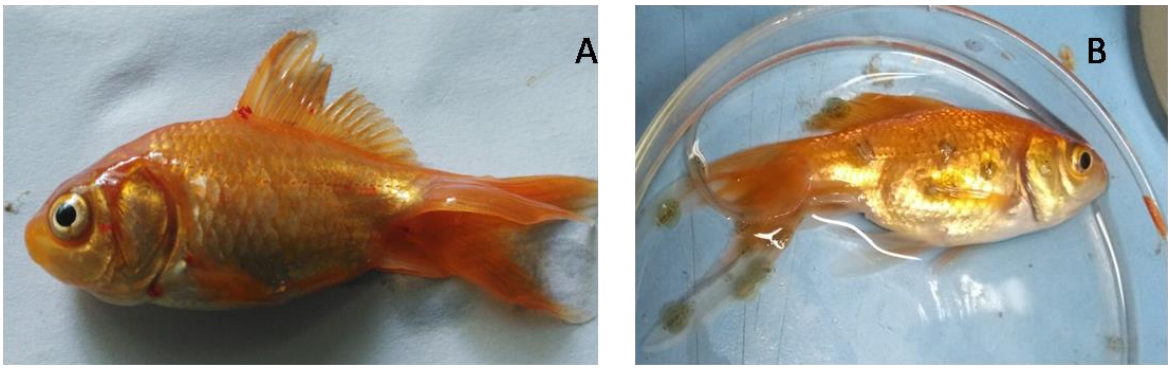


Plate 1: Diseased goldfish (A) *Argulus* infested goldfish (B)

4.2 Isolation of bacteria from infected gold fish

The presumptive *Aeromonas* colonies which appeared smooth, round or green with a black tinge in Aeromonas isolation media (AIM) plate after overnight incubation at 28°C were selected for the study.

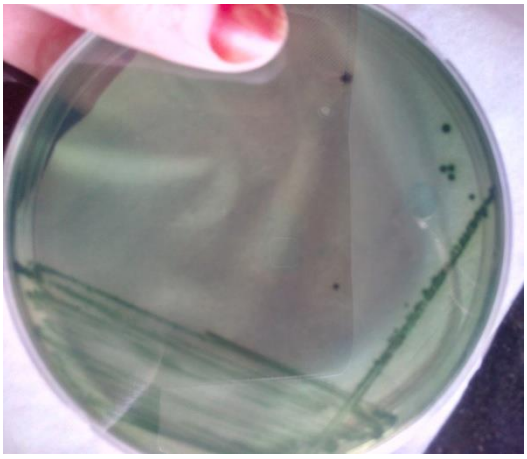


Plate2: Typical green colonies

4.3 Presumptive biochemical identification of *Aeromonas* isolates

A total of 32 isolates of bacteria were recovered from 50 diseased goldfish on Aeromonas isolation medium (AIM). Typical colonies of *Aeromonas* spp. were subjected to a series of biochemical tests. All the isolates were Gram-negative, motile, and fermented glucose in TSI agar (Table: 5). A few isolates showing susceptibility to O/129 were eliminated as possible *Vibrio spp*, while a few other

oxidase-negative isolates were also excluded from further study. Of 32 isolates selected from the preliminary screening, 15 isolates were presumptively identified as belonging to the Genus *Aeromonas*. These isolates were further subjected to identification up to the species and *Aermonas* complex levels (Table: 6).

Table 5: Biochemical identiifcation of *Aeromonas* isolates

Isolate No	Gram staining	Motility	Oxidase	Catalase	Fermentation of glucose	Resistance to 0/129.
1	N	M	P	P	P	R
2	N	M	P	P	P	R
3	N	M	N	P	P	R
4	N	M	P	P	P	R
5	N	M	N	P	P	R
6	N	M	P	P	P	R
7	N	M	P	P	P	R
8	N	M	P	P	P	S
9	N	M	N	P	P	R
10	N	M	P	P	P	R
11	N	M	N	P	P	R
12	N	M	P	P	P	S
13	N	M	P	P	P	R
14	N	M	P	P	P	R
15	N	M	P	P	P	S
16	N	M	P	P	P	R
17	N	M	N	P	P	R
18	N	M	P	P	P	R
19	N	M	N	P	P	R
20	N	M	N	P	P	R

21	N	M	P	P	P	S
22	N	M	N	P	P	R
23	N	M	P	P	P	R
24	N	M	P	P	P	R
25	N	M	N	P	P	R
26	N	M	P	P	P	S
27	N	M	P	P	P	R
28	N	M	P	P	P	R
29	N	M	N	P	P	R
30	N	M	P	P	P	R
31	N	M	P	N	P	R
32	N	M	N	P	P	R

Abbrevations: N: negative, P :positive, M: motile, R:resistant, S: susceptable**.**

4.4 Biochemical identification of *Aeromonas* upto complex level

Fifteen *Aeromonas* isolates identified based on the biochemical tests were further grouped into *A.hydrophila, A.caviae* and *A. sobria* complex based on four biochemical tests as recommended by Abbott *et al.* (2003).

Table 6 : Grouping of isolates into complex level

Test	*A.hydrophila complex* N = 4	*A.caviae complex* N = 3	*A.sobria complex* N = 8
Esculin hydrolysis	100%	67%	0%
Voges- Proskauer	75%	0%	70%
Glucose (gas)	100%	0%	89%
L-Arabinsoe	50%	50%	60%

Plate 3: Esculin hydrolysis

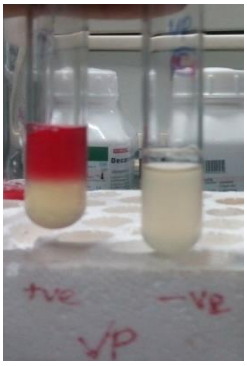


Plate 4: Voges – Prousker test

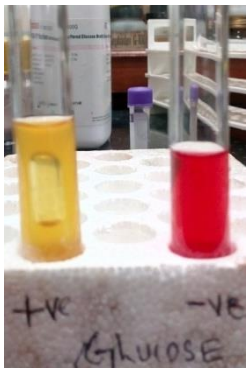


Plate 5: Gas from glucose

Plate 6: Arabinose utilization

Based on esculin hydrolysis, Voges-Proskauer test, gas from glucose fermentation, and arabinose utilization, four isolates were grouped under *A. hydrophila complex,* three under *A. caviae complex,* and eight under *A. sobria complex.* Four *A. hydrophila* isolates were subjected to molecular characterization.

4.5 Molecular characterization of *Aeromonas hydrophila*

4.5.1 Genomic DNA extraction

The Genomic DNA of four isolates was extracted using a *DNAZOL* reagent. The purity of the extracted DNA was in the range of 1.7 – 1.8.

4.5.2 PCR amplification of 16SrRNA

The 16SrRNA gene of *Aeromonas hydrophila* was PCR amplified using of 27F and 1492R universal primers. The concentration of PCR product was in the range of 300-900 ng/µl. The amplicon of ~1400bp from *16SrRNA* PCR was purified using a PCR purification kit and sequenced.

4.5.3 Sequencing of partial 16S rRNA gene

Partial sequencing of *16SrRNA* PCR amplified product was done using forward primer and reverse universal primers 27F (5' AGAGTTGATCATGGCTCAG 3') and 1498R (5' GGTTCACTTGTTACGACTT 3'). The sequence was analyzed using BLAST (Basic Local Alignment Search Tool) software of the National Biotechnology Information Center and Ribosomal Database project.

4.5.4 Identification of *Aeromonas* spp. based on *16SrRNA* gene sequence analysis and confirmatory PCR

The *16S rRNA* sequence data were BLAST searched against Gene bank database and the Ribosomal Database Project. The sequence identities were in the range of 98 – 100 % (Table 7). The sequences were submitted in the GenBank database (NCBI) under the accession numbers shown in the Table 7. *A. hydrophila* (A1) isolate with accession number AB473004 was chosen for further confirmation using species-specific primer.

PCR amplification using *Aeromonas hydrophila*-specific primers (16SrRNA-F and 16SrRNA-R, Trakhan *et al.*, 2009) yielded 103 bp products with three isolates confirming the identity of these isolates as *A. hydrophila*.

Table 7: BLAST results of partial 16SrRNA sequences of Aeromonas isolates

Isolates	Sequence Similarity with GenBank sequence (%)	Identity of the isolate	GenBank Accession no.
A1	100	*A. hydrophila*	AB 473004
A2	98	*A. hydrophila*	AB 532759
A3	99	*A. veronii bv. sobria*	GQ 4927906
A4	100	*A. veronii bv. sobria*	KC 583582.

4.6 Determination of LD_{50}.

The virulence of *A. hydrophila* was assessed *in vivo* in goldfish.. Among the isolates of *A. hydrophila strain* A1 (AB 473004) was selected for determining the LD_{50} in goldfish. The pathogenicity of the selected strain was further confirmed by the challenge test. The isolate A1 was found to be pathogenic based on observed clinical signs and mortality (Plate 7).The LD_{50} value of the isolate was 4.7 x 10*6 CFU $fish^{-1}$.Post infection, the clinical signs of infected fish were observed. Reddening at the site of injection was observed in both experimental and control groups after one hour of injection with the pathogen. After 24 hours, haemorrhage at the surface and swelling of the abdomen was observed in experimental fish. The control fish injected with PBS did not exhibit any clinical signs or mortality throughout the experimental period. Re-isolation of bacteria was done from the haemorrhagic sites and the kidney of infected fish on AIM agar and its characterization was carried out.

Plate 7: Artificially injected (intraperitoneal) gold fishes(*C. auratus*) with *A. hydrophila* (A1) exibiting hemorrages and dropsy.

4.7 Identification of *Argulus* spp.

Based on the morphological traits, the ectoparasites from gold fish were identified as branchiuran, belonging to the family Argulidae, genus *Argulus*, and species *Argulus japonicus* and *Argulus foliaceous.* Since *Argulus japonicus* was the dominant ectoparasite observed in this study, it was selected for further experimentation. Fish with a high prevalence of *Argulus foliaceous* were excluded from the experiment. The morphological characterization of *Argulus japonicus* was carried out.

4.7.1 Morphological characteristics

The morphological description of *Argulus japonicus* can be expressed as follows: body with oval shape, dorso-ventrally flattened (plate 9 A), with 2 noticeable compound eyes (dorsally) and 2 sucking discs (ventrally). There are four pairs of thoracic legs. The carapace covers the base of the fourth pair of legs and the small spines marginally. The abdomen consists of two acutely rounded lobes, in which the abdominal incision reaches more than half its length. There is a pair of respiratory areas on the ventral surface of the lateral lobes, with the anterior region being smaller and almost circular, having less than half the size of the larger kidney-shaped posterior one. Further, the posterior incisures of the abdomen reaching the middle of the body of the parasite were observed with no leg pigmentation. The anterior spine of the first antenna was equipped with a hook and the coxal spines of the second maxilla were observed as long and finger-like sharp projections. A series of long

closely arranged setae on the basal plate of second maxillae was also observed for the parasite. In addition to these, spermatheca was observed in females and a pair of testes was observed in the male parasite. In the case of males, the cephalic appendages and carapace structure are similar to those of females. Differences accounted for the copulatory structures located on the second, third, and fourth legs. All the collected specimens of adult *Argulus japonicus* were measured as 4-5 mm in length and width of 3.5 to 4 mm (Plate 8).

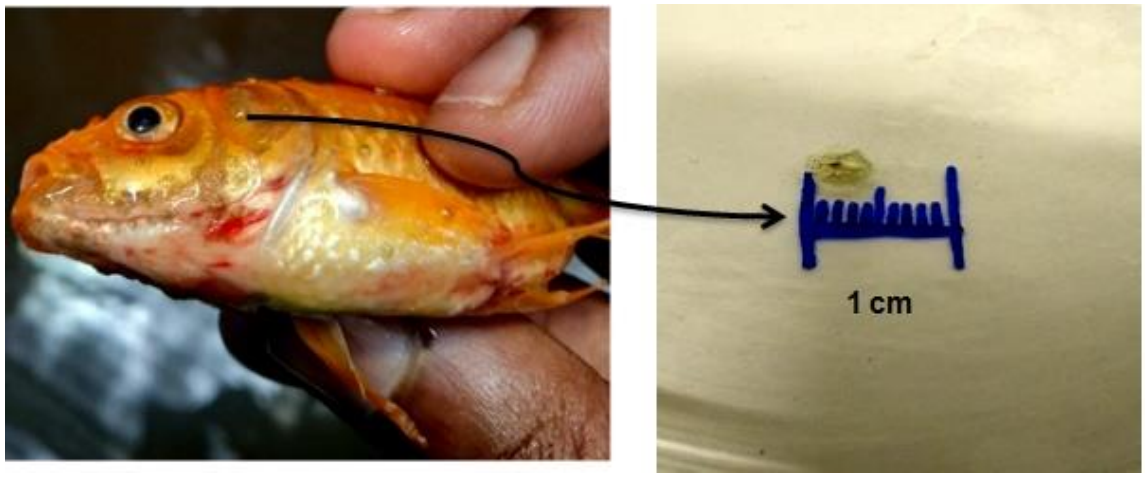
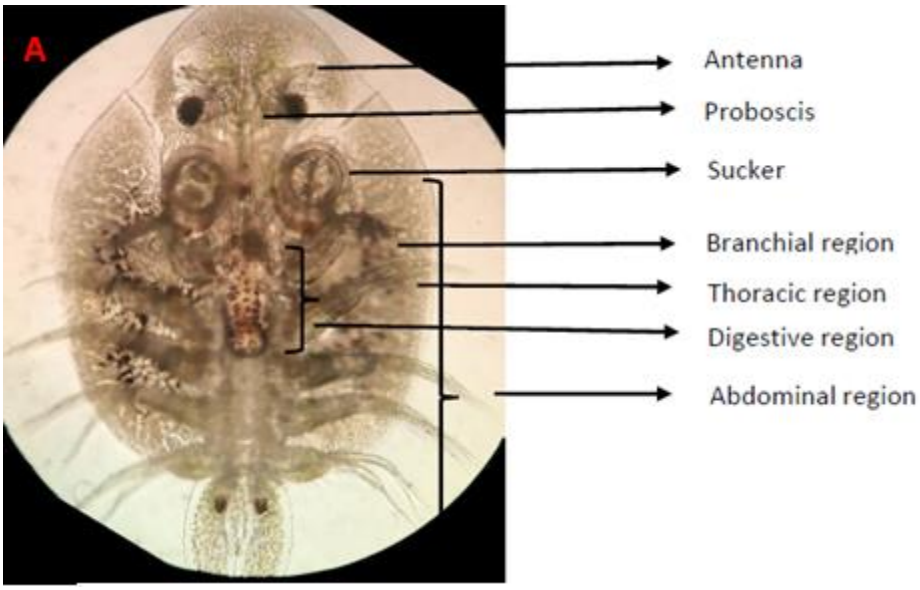


Plate 8: Parasitic examination in goldfish and observation of *Argulus,* length-width measurement on millimeter scale (length range: 4-5 mm and width range: 3-4 mm



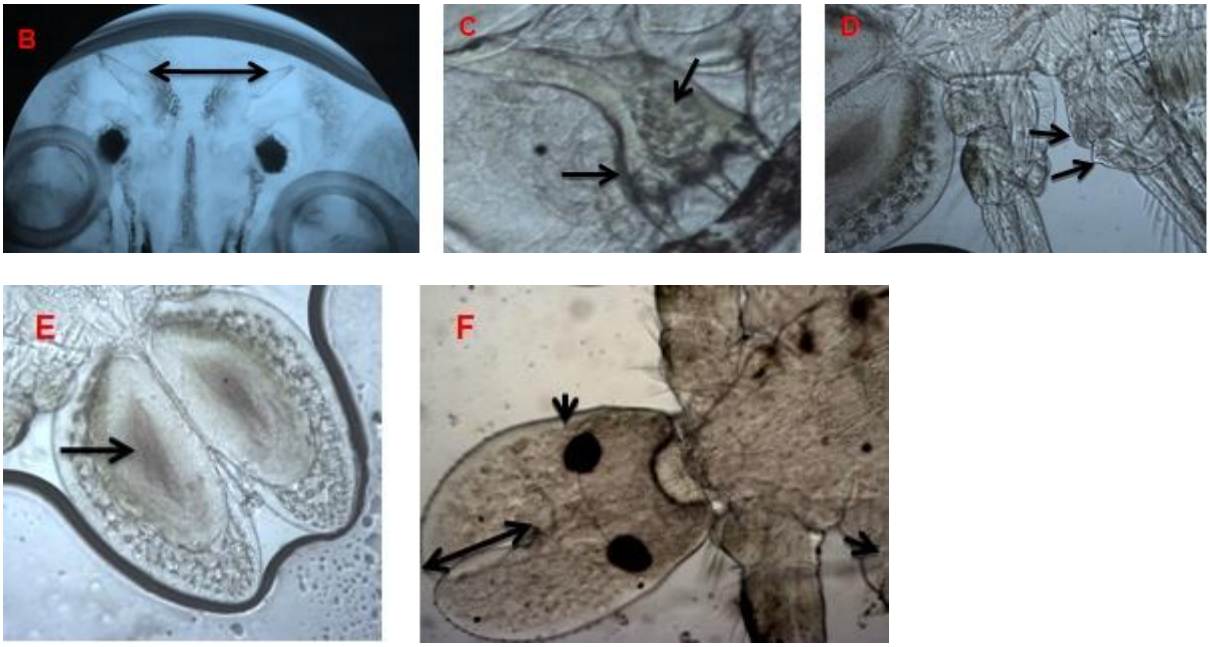


Plate 9: Microscopic examination of adult *A. japonicus*

A: Schematic representation of *A. japonicus* (ventral view); B: arrow indicates the presence of a hook on the anterior spine of the first antenna; C: arrow indicating coxal spines with series of setae on basal plate of second maxillae D: arrows indicating characteristic spines on swimming legs of male *Argulus* (accessory copulatory structure); E: Male with string like testes; F: Female with spermatheca at 10X.

4.8 Artificial infection of goldfish with *Argulus japonicus*

4.8.1 Cohabitation method

In the first experiment, the challenge study was performed by co-habiting heavily *Argulus* infested goldfish with healthy goldfish. The horizontal distribution of *Argulus* from infected to naive goldfish was observed. The infested fish showed a non-uniform distribution of *Argulus japonicus* on the body surface. Various sites of attachment for parasite was observed on the fish body, which included the caudal fin, snout, throat, head, dorsal body surface, pelvic region, belly region, and other fins (Plate 10). The caudal fin was observed as the most preferred site of attachment by the *Argulus sp*. in the present experiment *(*Plate 11).

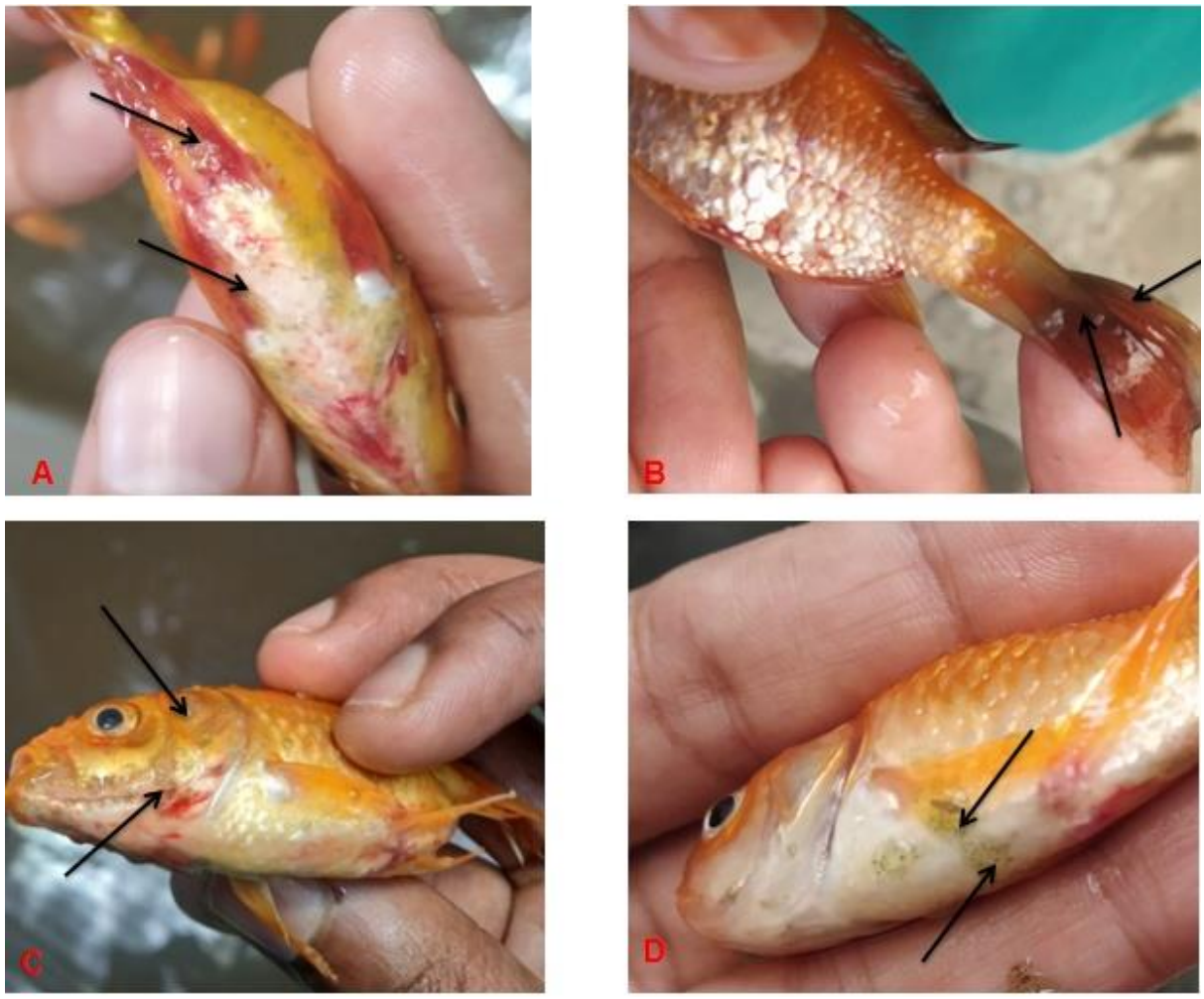


Plate 10: Adult *Argulus japonicus* infestation of goldfish showing different sites of attachment marked with the arrows: snout, head region, ventral region, and belly region, tail part and throat region.

Plate 11: Juvenile *Argulus* attached to the caudal fin of the goldfish.

4.8.2 Effect of exposing goldfish to the eggs clutches of *Argulus*

Egg collecting substrates were used to collect the egg clutches of *A. japonicus*. The eggs were collected from the aquarium wall, as wall of the glass aquarium was observed to be the most preferred site for laying eggs by *A. japonicus* (Plate 12). `Other major sites of egg clutches were observed to be on the filter pipe, air stone, thermometer, and the aeration tube (Plate 13). The preference criteria for egg collection were based on the length of the egg clutches, the number of clutches as well as rows and eggs per clutch. The clutches of eggs was collected gently by using a scalpel without damaging it. Collected eggs were used as a source for carrying out artificial infestation experiments (Plate 14).

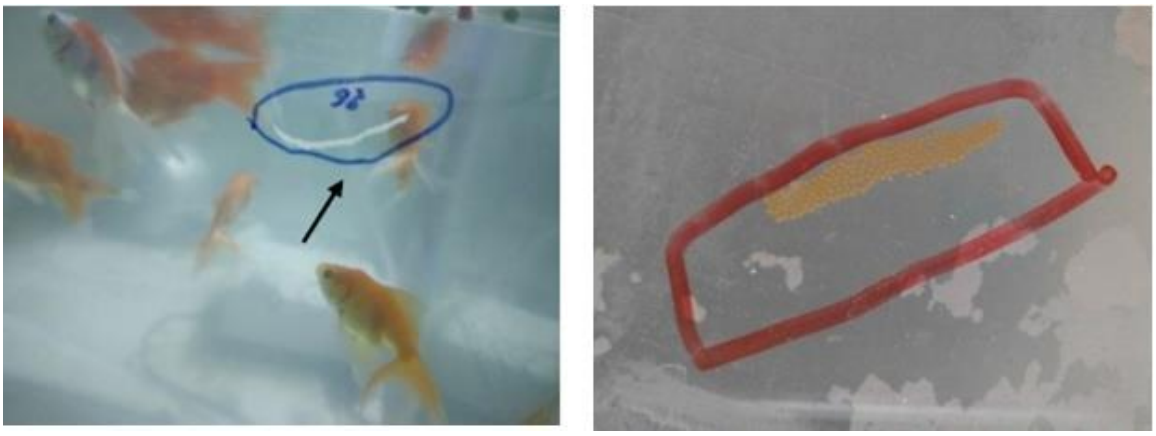


Plate 12: Egg clutches of *A. japonicus* on the side walls of glass aquarium

Plate 13: Egg clutches on the aeration tube, filter pipe, air stone and heater.

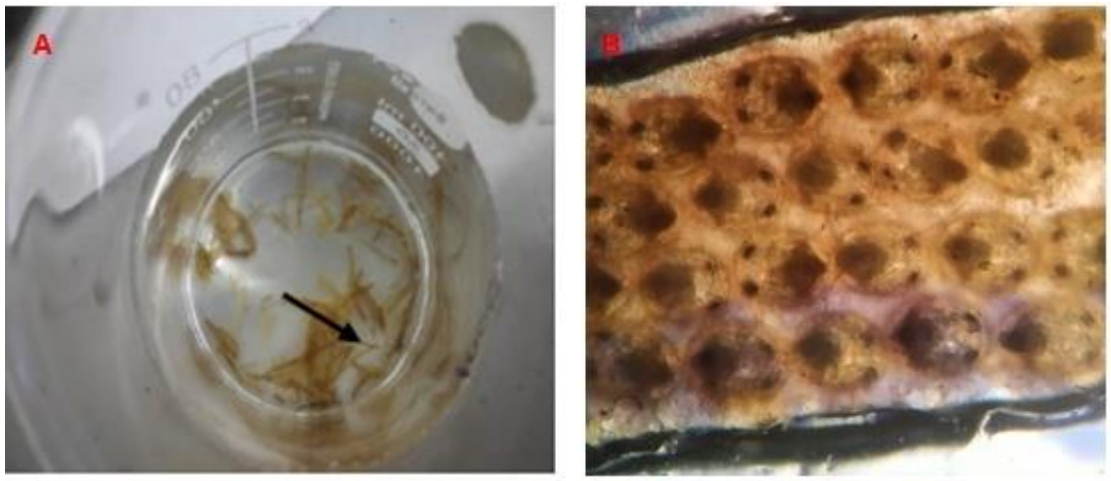


Plate 14: A: Scraped egg clutches of *A. japonicus* B: Microscopic examination of *A. japonicus* eggs

The collected eggs were experimentally exposed to goldfish. Heavy infestation of *A. japonicus* was observed from the third week of artificial infestation in all goldfish after setting up the artificial infestation challenge with the eyespot stage of collected eggs. The mean intensity of an average of 24 juvenile parasites per fish was recorded from randomly selected 10 experimental fish. Based on the observed level of intensity of parasite infestation, three ranges, *viz*. low (1–10 lice fish−1), moderate (10–20 lice fish−1), and heavy (>20 lice fish−1) were defined. Further, fish with a heavy infestation of *A. japonicas* showed clinical signs such as lethargy, settling at the aquarium bottom, reduced feeding, isolation from other fish and haemorrhages on fins and skin. The infection eventually resulted in the mortality of experimental fish.

Objective 1

4.9 Clinical signs and mortality

The parasite was found to infest new hosts within one to two hours of co-habitational challenge. A careful periodical examination was carried out to ensure the infestation of parasites on all fishes under study. The level of infestation was categorized based on the previously described criteria. The intensity of parasite infestation varied between each fish and was defined as low (<10 *Argulus* on the host), moderate (10-20 *Argulus* on the host), and high (> 20 *Argulus* on the host). Further, fish categorized to each grade of infestation with the parasite were individually exposed to a sub-lethal dose of *Aeromonas hydrophila*. The first clinical

sign in the entire co-infected fish group was observed within 24h post-infection (p.i.) as minute red spots on the ventral side. Haemorrhages appeared in moderate and high parasite co-infection groups at 72 h p.i., which subsequently developed into lesions, particularly at bases of pectoral fins at 96 h p.i. On visual examinations throughout the challenge period, all co-infected fish appeared to show a greater severity of acute clinical signs compared to those infested only with either bacteria or different grades of the parasite alone (exposure to a single type of pathogen). Among the co-infection groups, the moderate and heavy *Argulus* infested groups exhibited behavioural abnormalities accompanied with irritation, off food, excessive production of mucus, focal to distinct haemorrhages at the sites of parasite attachment, fin erosion, loss of skin, presence of skin ulcers often surrounded by a bright red rim of tissue and reddish appearance throughout the body, especially around the fins and the head portion (Plate 15).

In the experimental group T8, a high degree of enhanced pathogenicity of *A. hydrophila* was noticed during co-infection in goldfish with increased mortalities (84.2%), while single parasite and bacterial infection with the same dose resulted in 75% and 33%, mortality respectively after 168 h (Fig 3). In all the co-infection groups and single pathogen groups of moderate and heavy infection, the mortalities were severe (66.6%, 50%; 84% and 75%, respectively).In the low parasite-infested group, the mortality was less (16.6%) and the onset of mortality was reported after168 h p.i.

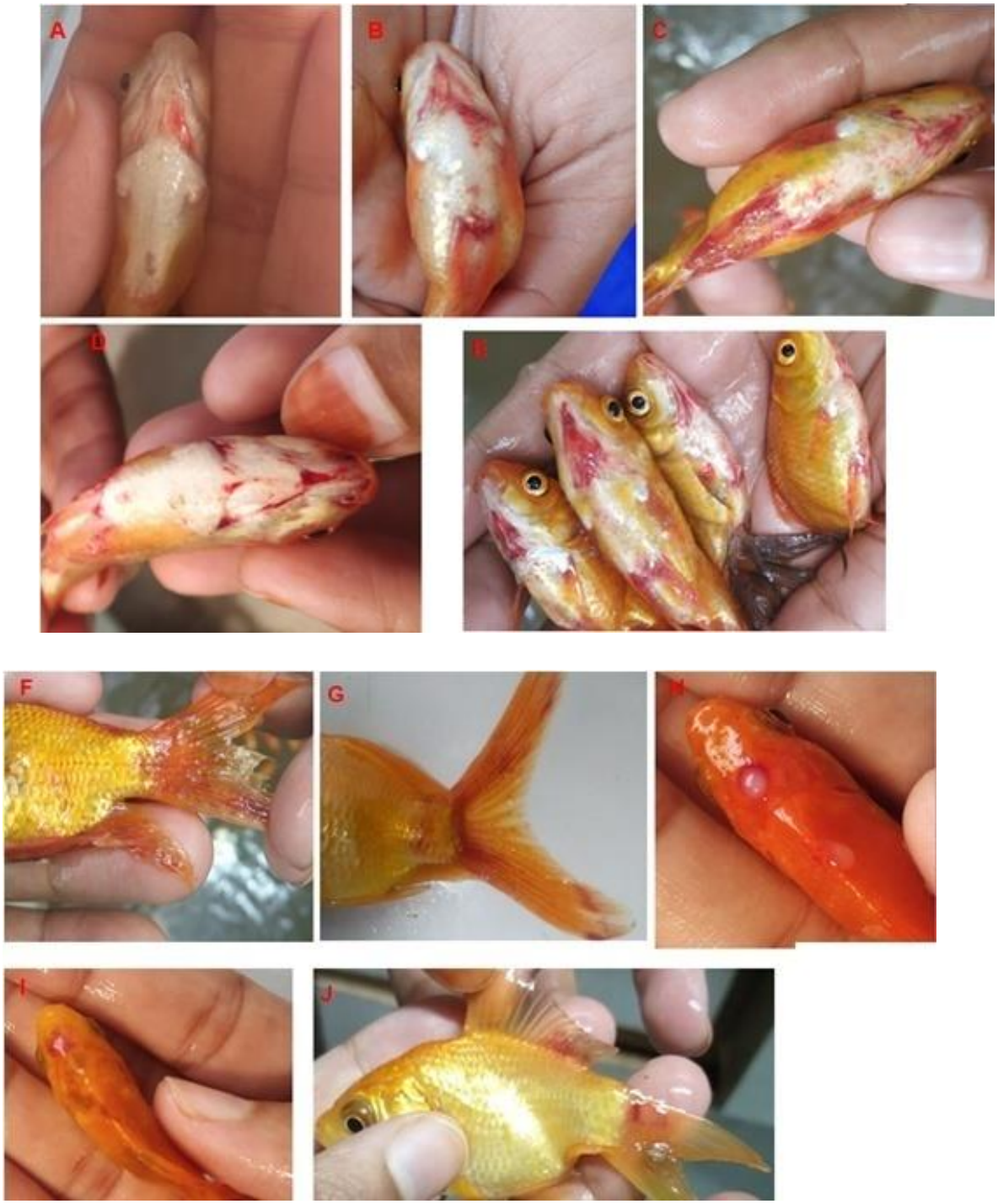


Plate 15: Clinical signs of goldfish co-infected with three doses of *Argulus* and sub-lethal dose of *A. hydrophila*. A and B represent minute red spots on the ventral side of goldfish; C, D and E show haemorrhages and lesions on the pectoral side of moderate and heavy *Argulus* co-infected goldfish. F and G show haemorrhage on the tail region. H and I show skin ulcers surrounded by a bright red rim of tissue at the head region. J shows minute clinical signs in low parasite co-infected goldfish.

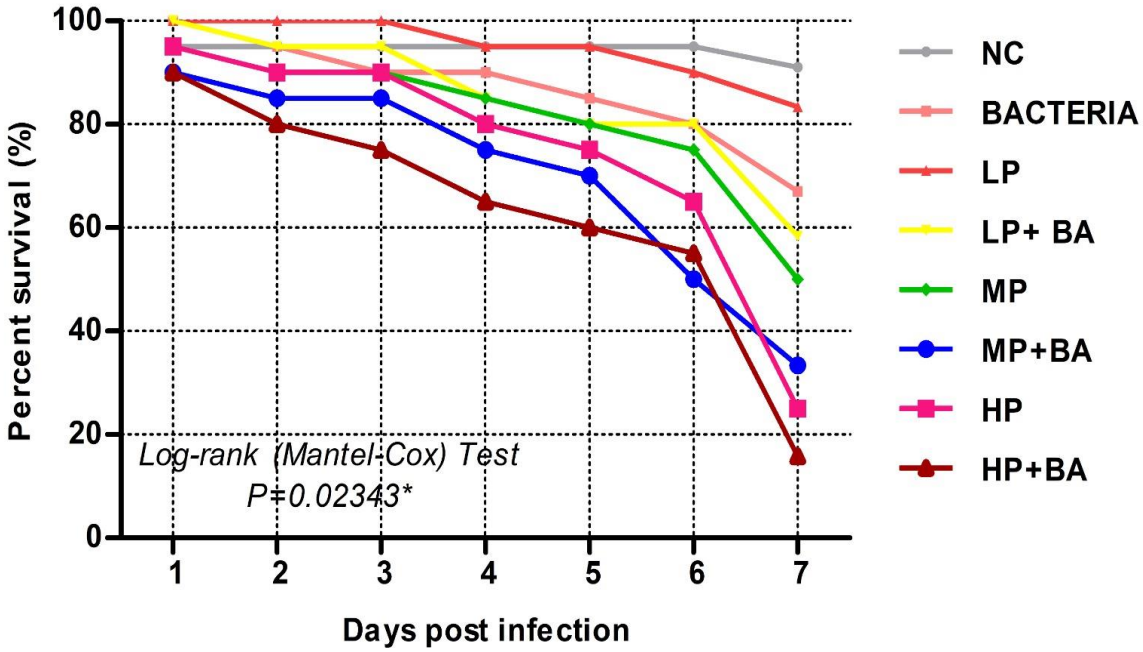


Fig 4: Survival curve of goldfish experimental groups co-infected with different degrees of *Argulus* and a sub-lethal dose of *A. hydrophila.*

NC: control group without *Argulus* and *A. hydrophila* infection; LP: Fish exposed to low grade of *Argulus* infestation; LP+ BA: LP+ sub-lethal dose of *A. hydrophila*; MP: Fish exposed to moderate grade of *Argulus* infestation; MP+ BA: MP+ sub-lethal dose of *A. hydrophila*; HP: Fish exposed to high grade of *Argulus* infestation; HP+ BA: HP+ sub-lethal dose of *A. hydrophila***.**

Low grade *Argulus* infection= fish carrying 1-10 number of *Argulus*

Moderate grade *Argulus* infection= fish carrying 11- 20 number of *Argulus*

High grade *Argulus* infection= Fish carrying >20 number of *Argulus*

4.10 Innate immune parameters

4.10.1 Respiratory burst activity (RBA)

In the present study, there was a significant (p<0.05) difference in the NBT values among the different parasite-infested and co-infected groups compared to un-infested control (Fig 4). Among parasite infested groups, T5 (moderate parasite infested) group showed a significantly (p<0.05) higher NBT value throughout the sampling periods, whereas the T7 group (high parasite infested) exhibited a reduced NBT activity. Among the co-infected groups, a significantly (p<0.05) higher NBT activity was shown by the T6 (moderate parasite and sub-lethal dose of bacteria) group than T4 (low parasite and sub-lethal dose of bacteria) and T8 (high parasite and sub-lethal dose of bacteria) groups.

Similarly, the MPO value was also significantly (p<0.05) different among the parasite-infested and co-infected groups compared to the control and single dose bacterial group (Fig 5). Even though the absolute values were comparable, there was a statistically significant (p<0.05) difference between the T2 (sub-lethal dose of bacteria) and T3 (sub-lethal dose of bacteria) groups. T6 (moderate parasite and sub-lethal dose of bacteria) group showed a significantly higher MPO value among the different parasite-infested group. Among the co-infected groups, the T8 group had a reduced MPO value compared to other groups throughout the sampling period.

In the present study, a significant (p<0.05) difference was observed in the serum lysozyme activity among different parasite and bacterial co-infected groups compared to the uninfected control (Fig 6). The T4 (low parasite and the sub-lethal dose of bacteria) and T6 (moderate parasite and the sub-lethal dose of bacteria) groups showed significantly (p<0.05) higher lysozyme activities compared tothe respective bacteria and parasite groups (T2, T3, and T5).The T8 (high parasite and the sub-lethal dose of bacteria) group showed a significantly (p<0.05) reduced lysozyme activity compared to T2 and T7 groups throughout the sampling period.

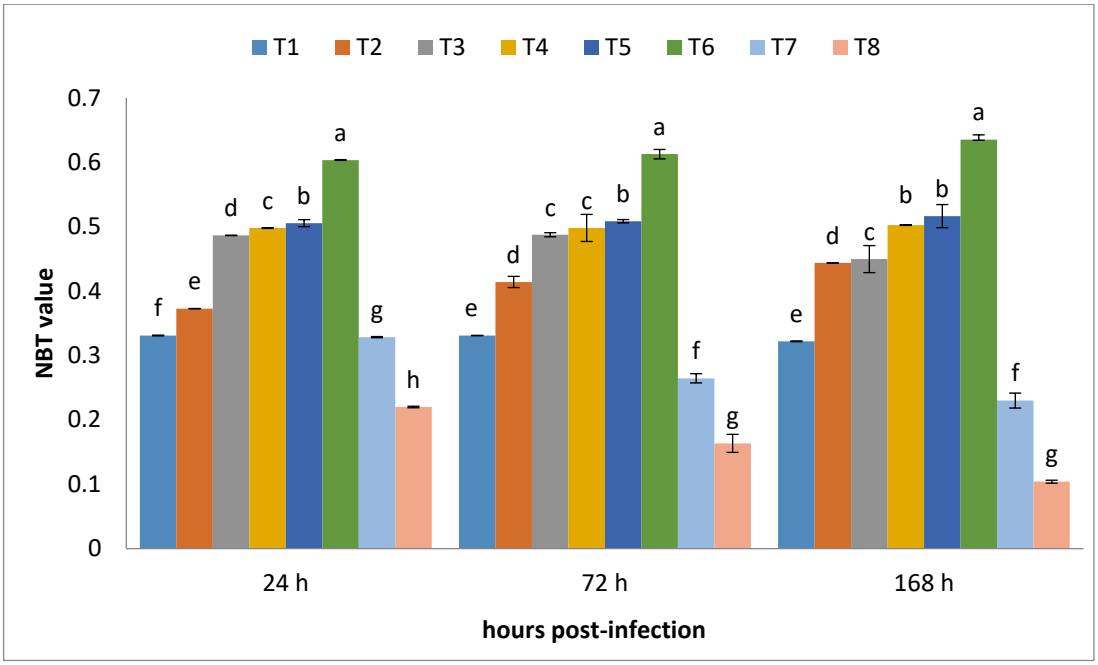


Fig 5: NBT value of different treatment groups. Mean values in the treatment group with different superscript differ significantly ($P < 0.05$). Data were expressed as Mean ± SE.

Abbreviations T1: Control group without *Argulus* and *A. hydrophila* infection, T2 (Fish exposed to 1/10 of LD50 dose of *A. hydrophila*), T3 (Fish exposed to low grade of *Argulus* infestation), T4 (T3 + *A. hydrophila*), T5 (Fish exposed to moderate grade of *Argulus* infestation), T6 (T5 + *A. hydrophila*), T7 (Fish exposed to high grade of *Argulus* infestation) and T8 (T7+ *A. hydrophila*).

Low grade *Argulus* infection= fish carrying 1-10 number of *Argulus*

Moderate grade *Argulus* infection= fish carrying 11- 20 number of *Argulus*

High grade *Argulus* infection= Fish carrying >20 number of *Argulus*

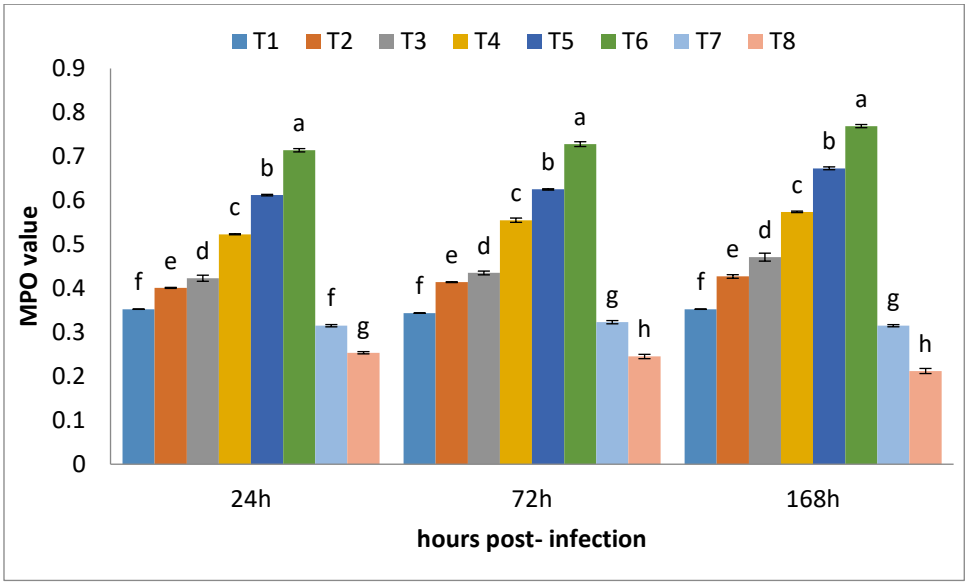


Fig 6: MPO value of different treatment groups. Mean values in the treatment group with different superscript differ significantly ($P < 0.05$). Data were expressed as Mean ± SE.

Abbreviations T1: Control group without *Argulus* and *A. hydrophila* infection, T2 (Fish exposed to 1/10 of LD50 dose of *A. hydrophila*), T3 (Fish exposed to low grade of *Argulus* infestation), T4 (T3 + *A. hydrophila*), T5 (Fish exposed to moderate grade of *Argulus* infestation), T6 (T5 + *A. hydrophila*), T7 (Fish exposed to high grade of *Argulus* infestation) and T8 (T7+ *A. hydrophila*).

Low grade *Argulus* infection= fish carrying 1-10 number of *Argulus*

Moderate grade *Argulus* infection= fish carrying 11- 20 number of *Argulus*

High grade *Argulus* infection= Fish carrying >20 number of *Argulus*

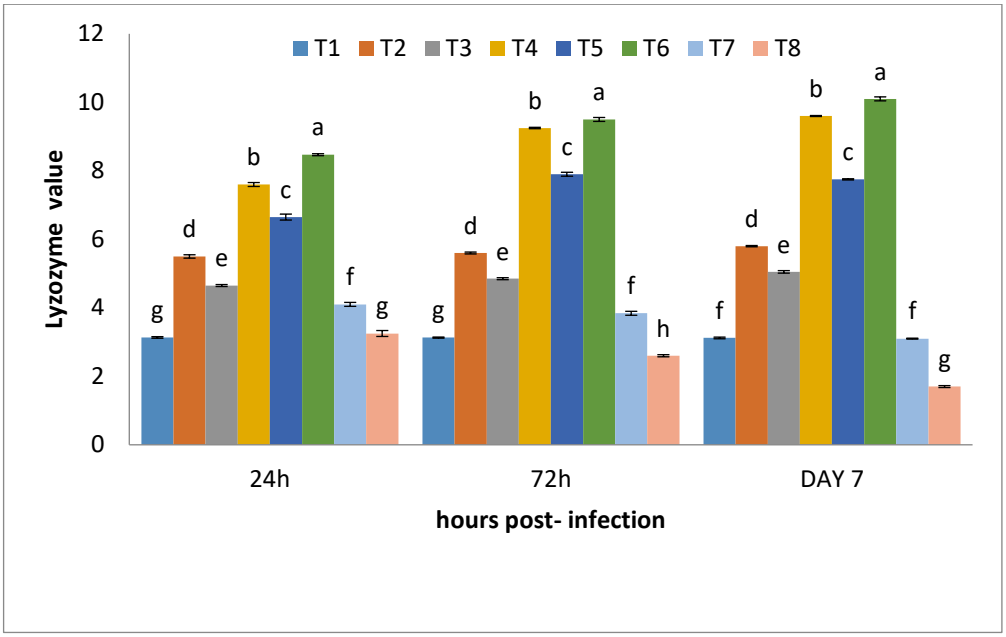


Fig 7: Lyzozyme values of different treatment groups. Mean values in the treatment group with different superscript differ significantly ($P < 0.05$). Data were expressed as Mean ± SE.

Abbreviations T1: Control group without *Argulus* and *A. hydrophila* infection, T2 (Fish exposed to 1/10 of LD50 dose of *A. hydrophila*), T3 (Fish exposed to low grade of *Argulus* infestation), T4 (T3 + *A. hydrophila*), T5 (Fish exposed to moderate grade of *Argulus* infestation), T6 (T5 + *A. hydrophila*), T7 (Fish exposed to high grade of *Argulus* infestation) and T8 (T7+ *A. hydrophila*).

Low grade *Argulus* infection= fish carrying 1-10 number of *Argulus*

Moderate grade *Argulus* infection= fish carrying 11- 20 number of *Argulus*

High grade *Argulus* infection= Fish carrying >20 number of *Argulus*

4.10.2 Haematological parameters

In the present study, a significant ($p<0.05$) reduction in RBC, PCV, and Hb was observed in all the treatment groups (T2-T8) when compared with the uninfested control (T1) group. A decreasing trend was observed in RBC, PCV, and Hb values as observed among the treatment groups with different degrees of parasite infestation. However, it was observed that exposure to a sub-lethal dose of bacteria in the T8 (high parasite-infested host) group has shown a drastic reduction in recorded parameters in comparison to the T7 (high parasite-infested host) group (Fig. 7, 8 and 9).

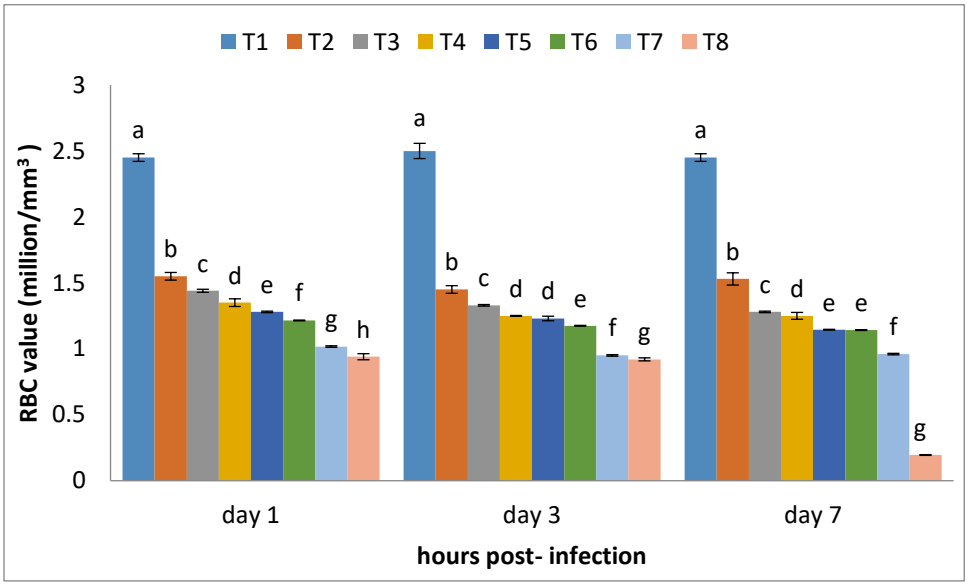


Fig 8: Red Blood Cell (RBC) values of different treatment groups. Mean values in the treatment group with different superscript differ significantly ($P < 0.05$). Data were expressed as Mean ± SE.

Abbreviations T1: Control group without *Argulus* and *A. hydrophila* infection, T2 (Fish exposed to 1/10 of LD50 dose of *A. hydrophila*), T3 (Fish exposed to low grade of *Argulus* infestation), T4 (T3 + *A. hydrophila*), T5 (Fish exposed to moderate grade of *Argulus* infestation), T6 (T5 + *A. hydrophila*), T7 (Fish exposed to high grade of *Argulus* infestation) and T8 (T7+ *A. hydrophila*).

Low grade *Argulus* infection= fish carrying 1-10 number of *Argulus*

Moderate grade *Argulus* infection= fish carrying 11- 20 number of *Argulu*

High grade *Argulus* infection= Fish carrying >20 number of *Argulus*

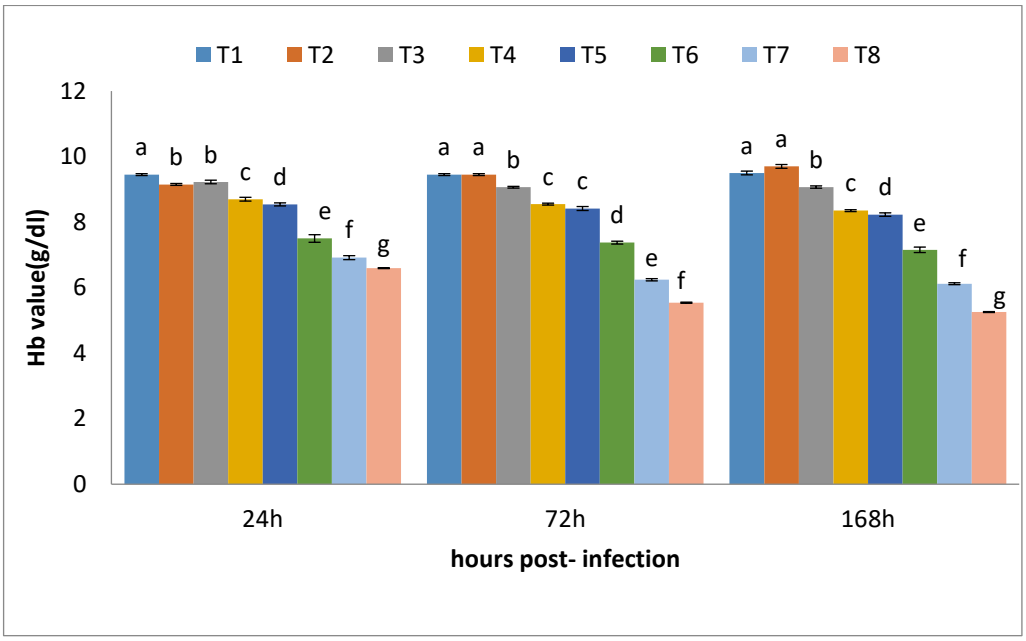


Fig 9: Haemoglobin (Hb) values of different treatment groups. Mean values in the treatment group with different superscript differ significantly ($P < 0.05$). Data were expressed as Mean ± SE.

Abbreviations T1: Control group without *Argulus* and *A. hydrophila* infection, T2 (Fish exposed to 1/10 of LD50 dose of *A. hydrophila*), T3 (Fish exposed to low grade of *Argulus* infestation), T4 (T3 + *A. hydrophila*), T5 (Fish exposed to moderate grade of *Argulus* infestation), T6 (T5 + *A. hydrophila*), T7 (Fish exposed to high grade of *Argulus* infestation) and T8 (T7+ *A. hydrophila*).

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High grade *Argulus* infection= Fish carrying >20 number of *Argulus*

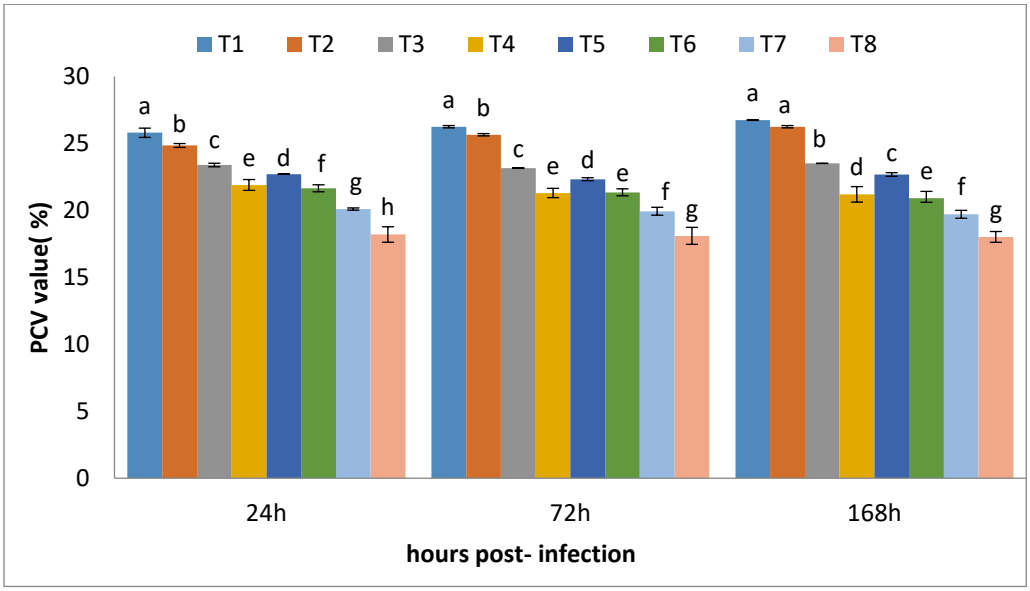

Fig 10: Packed Cell Volume value of different treatment groups. Mean values in the treatment group with different superscript differ significantly ($P < 0.05$). Data were expressed as Mean ± SE.

Abbreviations T1: Control group without *Argulus* and *A. hydrophila* infection, T2 (Fish exposed to 1/10 of LD50 dose of *A. hydrophila*), T3 (Fish exposed to low grade of *Argulus* infestation), T4 (T3 + *A. hydrophila*), T5 (Fish exposed to moderate grade of *Argulus* infestation), T6 (T5 + *A. hydrophila*), T7 (Fish exposed to high grade of *Argulus* infestation) and T8 (T7+ *A. hydrophila*).

Low grade *Argulus* infection= fish carrying 1-10 number of *Argulus*

Moderate grade *Argulus* infection= fish carrying 11- 20 number of *Argulus*

High grade *Argulus* infection= Fish carrying >20 number of *Argulus*

There was a significant ($p<0.05$) difference in WBC, monocyte, and neutrophil counts among different parasite-infested groups compared to non-infested control throughout the sampling period (Fig: 10, Fig: 11, and Fig: 12). Among the parasite-infested group, T5 (moderate parasite-infested) group showed a significantly higher value of WBC and related parameters compared to T3 (low parasite infested) and T7 (High parasite infested) groups. Among the co-infected groups, T4 and T6 groups showed significantly higher values of monocytes compared to T3 and T5 groups. The T8 (High parasite and a sub-lethal dose of bacteria) group showed lower WBC, monocyte, and neutrophil values compared to T4 and T6 groups throughout the sampling days.

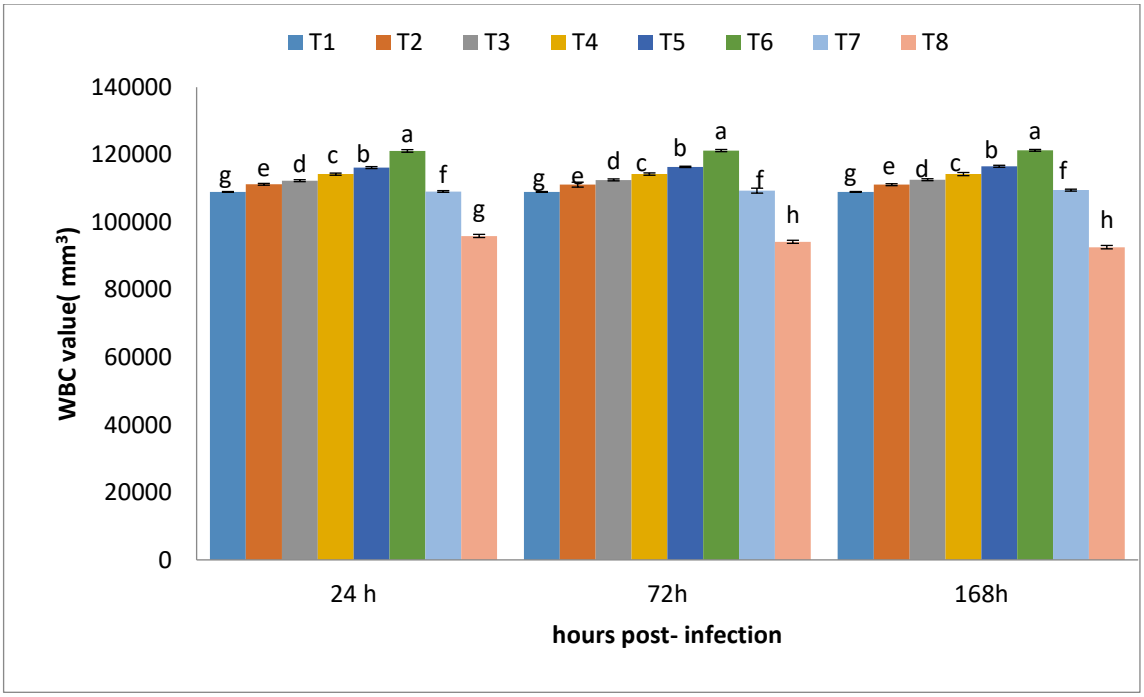

Fig 11: WBC value of different treatment groups. Mean values in the treatment group with different superscript differ significantly ($P < 0.05$). Data were expressed as Mean ± SE.

Abbreviations T1: Control group without *Argulus* and *A. hydrophila* infection, T2 (Fish exposed to 1/10 of LD50 dose of *A. hydrophila*), T3 (Fish exposed to low grade of *Argulus* infestation), T4 (T3 + *A. hydrophila*), T5 (Fish exposed to moderate grade of *Argulus* infestation), T6 (T5 + *A. hydrophila*), T7 (Fish exposed to high grade of *Argulus* infestation) and T8 (T7+ *A. hydrophila*).

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High grade *Argulus* infection= Fish carrying >20 number of *Argulus*

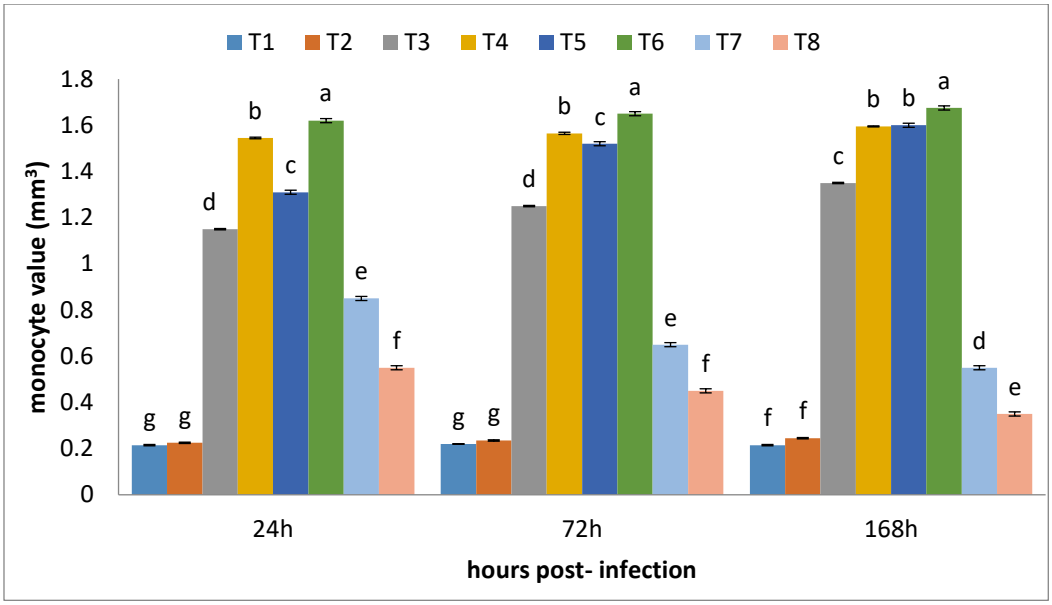

Fig 12: Monocyte value of different treatment groups. Mean values in the treatment group with different superscript differ significantly ($P < 0.05$). Data were expressed as Mean ± SE. Abbreviations T1: Control group without *Argulus* and *A. hydrophila* infection, T2 (Fish exposed to 1/10 of LD50 dose of *A. hydrophila*), T3 (Fish exposed to low grade of *Argulus* infestation), T4 (T3 + *A. hydrophila*), T5 (Fish exposed to moderate grade of *Argulus* infestation), T6 (T5 + *A. hydrophila*), T7 (Fish exposed to high grade of *Argulus* infestation) and T8 (T7+ *A. hydrophila*).

Low grade *Argulus* infection= fish carrying 1-10 number of *Argulus*

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High grade *Argulus* infection= Fish carrying >20 number of *Argulus*

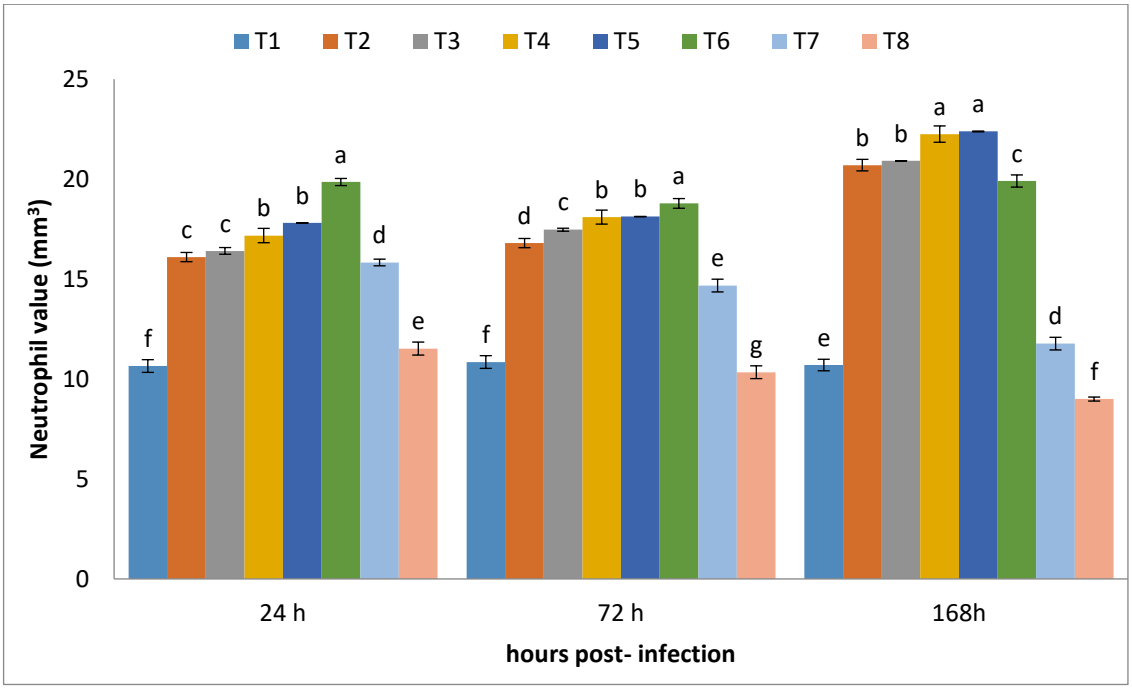

Fig 13: Neutrophil values of different treatment groups. Mean values in the treatment group with different superscript differ significantly ($P < 0.05$). Data were expressed as Mean ± SE. Abbreviations T1: Control group without *Argulus* and *A. hydrophila* infection, T2 (Fish exposed to 1/10 of LD50 dose of *A. hydrophila*), T3 (Fish exposed to low grade of *Argulus* infestation), T4 (T3 + *A. hydrophila*), T5 (Fish exposed to moderate grade of *Argulus* infestation), T6 (T5 + *A. hydrophila*), T7 (Fish exposed to high grade of *Argulus* infestation) and T8 (T7+ *A. hydrophila*).
Low grade *Argulus* infection= fish carrying 1-10 number of *Argulus*

Moderate grade *Argulus* infection= fish carrying 11- 20 number of *Argulus*

High grade *Argulus* infection= Fish carrying >20 number of *Argulus*

4.10.3 Enzymes of oxidative stress

The parasite-infested groups displayed significant ($p<0.05$) differences in SOD, GPx, and catalase activities compared to the control (Fig. 13, 14, and 15). Reduced SOD, GPx, and catalase activities were observed in T7 (High parasite infested) group compared to T3 and T5 (Low and moderate parasite infested) groups. A significantly higher ($p<0.05$) SOD, catalase, and GPx activities were observed inT4 (low parasite and sub-lethal dose of bacteria) and T6 (moderate parasite and sub-lethal dose of bacteria) groups compared to the respective bacteria and parasite-infested groups (T2, T3 and T5), while the T8 (high parasite and sub-lethal dose of bacteria) group showed reduced antioxidant enzyme activities compared to the T7 (high parasite) group

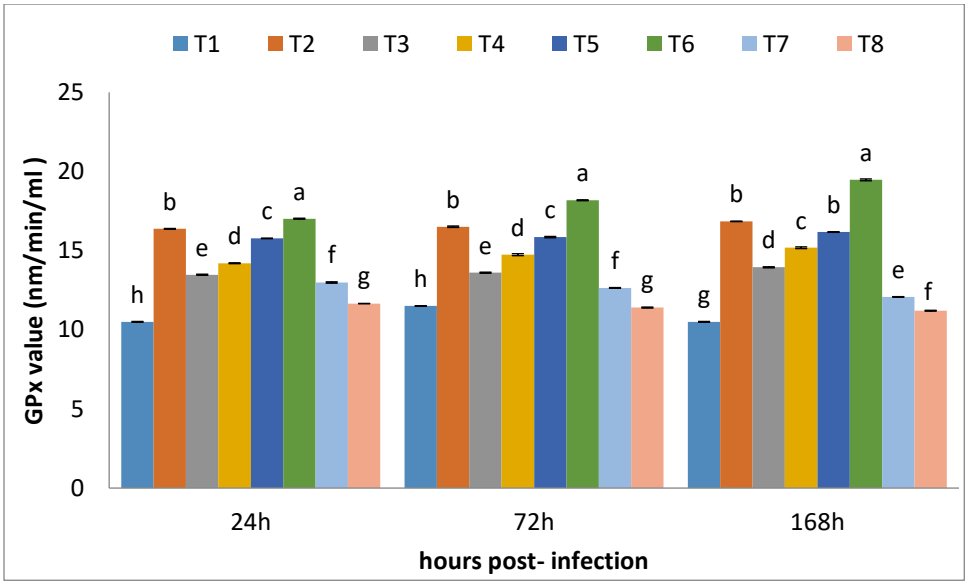

Fig 14: GPx value (nm/min/ml) of different treatment groups. Mean values in the treatment group with different superscript differ significantly ($P < 0.05$). Data were expressed as Mean ± SE.

Abbreviations T1: Control group without *Argulus* and *A. hydrophila* infection, T2 (Fish exposed to 1/10 of LD50 dose of *A. hydrophila*), T3 (Fish exposed to low grade of *Argulus* infestation), T4 (T3 + *A. hydrophila*), T5 (Fish exposed to moderate grade of *Argulus* infestation), T6 (T5 + *A. hydrophila*), T7 (Fish exposed to high grade of *Argulus* infestation) and T8 (T7+ *A. hydrophila*).

Low grade *Argulus* infection= fish carrying 1-10 number of *Argulus*

Moderate grade *Argulus* infection= fish carrying 11- 20 number of *Argulus*

High grade *Argulus* infection= Fish carrying >20 number of *Argulus*

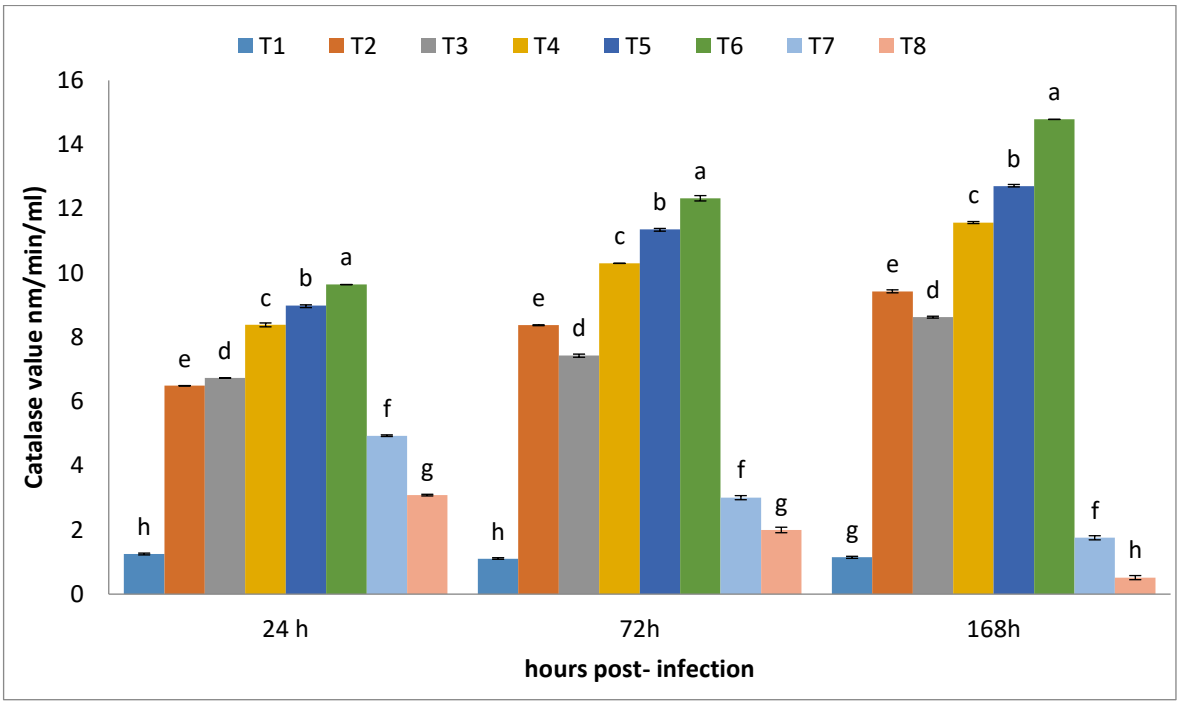

Fig 15: Catalase activity (nm/min/ml) of different treatment groups. Mean values in the treatment group with different superscript differ significantly ($P < 0.05$). Data were expressed as Mean ± SE.

Abbreviations T1: Control group without *Argulus* and *A. hydrophila* infection, T2 (Fish exposed to 1/10 of LD50 dose of *A. hydrophila*), T3 (Fish exposed to low grade of *Argulus* infestation), T4 (T3 + *A. hydrophila*), T5 (Fish exposed to moderate grade of *Argulus* infestation), T6 (T5 + *A. hydrophila*), T7 (Fish exposed to high grade of *Argulus* infestation) and T8 (T7+ *A. hydrophila*).

Low grade *Argulus* infection= fish carrying 1-10 number of *Argulus*

Moderate grade *Argulus* infection= fish carrying 11- 20 number of *Argulus*

High grade *Argulus* infection= Fish carrying >20 number of *Argulus*

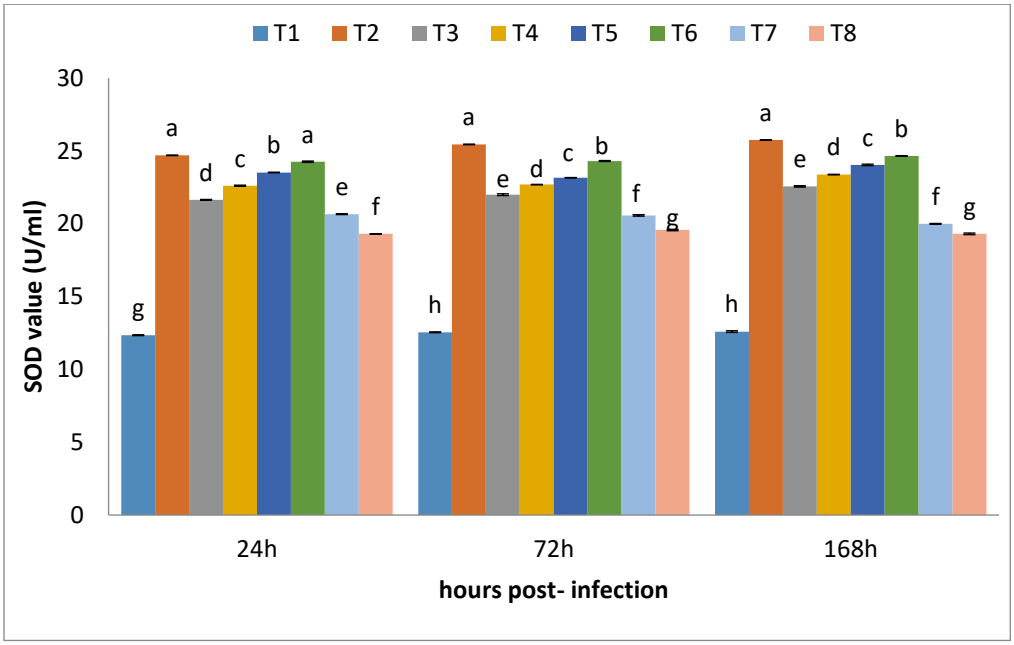

Fig 16: SOD value (U/ml) of different treatment groups. Mean values in the treatment group with different superscript differ significantly ($P < 0.05$). Data were expressed as Mean ± SE.

Abbreviations T1: Control group without *Argulus* and *A. hydrophila* infection, T2 (Fish exposed to 1/10 of LD50 dose of *A. hydrophila*), T3 (Fish exposed to low grade of *Argulus* infestation), T4 (T3 + *A. hydrophila*), T5 (Fish exposed to moderate grade of *Argulus* infestation), T6 (T5 + *A. hydrophila*), T7 (Fish exposed to high grade of *Argulus* infestation) and T8 (T7+ *A. hydrophila*).

Low grade *Argulus* infection= fish carrying 1-10 number of *Argulus*

Moderate grade *Argulus* infection= fish carrying 11- 20 number of *Argulus*

High grade *Argulus* infection= Fish carrying >20 number of *Argulus*

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4.11 Validation of housekeeping gene

The three housekeeping genes, *viz*., β-actin, EF1α, and GAPDH were amplified by qPCR from three different tissues of the control (T1) and treatment groups viz, T6 (moderate parasite and sub-lethal dose of bacteria) and T8 (high parasite and sub-lethal dose of bacteria). The amplified products ranged from 100 to150 bp. The PCR efficiency was calculated using the equation:

PCR efficiency (E %) = (10–1/slope − 1) ×100.

All reactions displayed efficiency between 100%and 105% (Table 5). For expression stability analysis, the stability ranking for the four genes obtained from the delta Ct method, *Bestkeeper, geNorm*, and *NormFinder* programs was compared. EF-alpha was found to be the most stable expressed gene by the delta Ct, *Normfinder*, and *Bestkeeper*, whereas, actin-β by the *geNorm* (fig: 15). Comprehensive stability ranking according to *refFinder* suggested that EF-alpha was the most suitable gene with a geomean value of 1.0, and the overall stability order was EF1α > actin-β > GAPDH (Table 8, Fig: 16)

Table 8: Geomean values of three housekeeping genes

Genes	**Geomean value**
Elongation factor (EF 1a)	1.00
B-actin	1.68
Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)	3.00

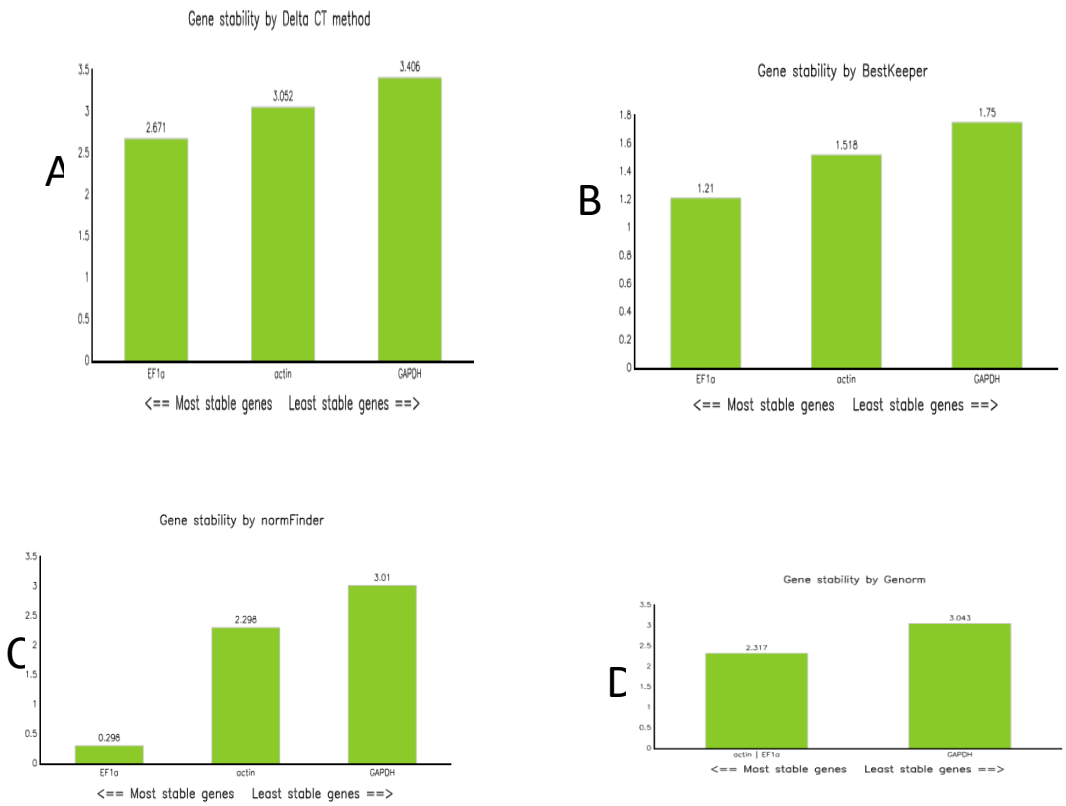

Fig 17: Determination of the expression stability in co-infected goldfish tissues evaluated using different programs (*viz*., Delta Ct, *Bestkeeper, NorrnFinder*, and *geNorm*)

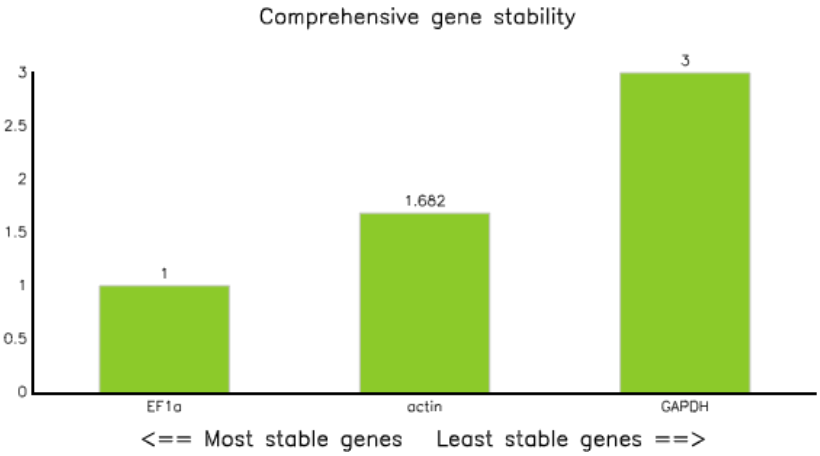

Fig 18: Comprehensive stability ranking of housekeeping genes

4.12 Gene expression analysis in kidney

The expression kinetics of two immune-relevant genes was investigated in the kidney of goldfish co-infected with different doses of *Argulus* and a sub-lethal dose of *A. hydrophila* at different time periods post-infection. Transcription levels of these genes relative to EF-alpha are shown in Fig : 18. In this experiment, a significant up-regulation of TLR 22 was observed in the T2 group (sub-lethal dose of bacteria) during the early time point with a peak value at 24 h p.i. (10.5 times),which declined thereafter. However, at 168 h p.i., the expression level of this immune gene in the T2 group was similar to the control fish. Also, there was a significant difference in TLR 22 expression among the different parasite-infested groups compared to un-infested control. At 24 hp.i, the maximum up-regulation of TLR 22 was observed in the T3 (low parasite infested) and T5 groups (moderate parasite infested) (9.04 and 11.72 times respectively), whereas a significant down-regulation was observed in the T7 (High parasite infested) group from 72 hr p.i. Among different co-infected groups, T4 (low parasite and sub-lethal dose of bacteria) and T6 (moderate parasite and sub-lethal dose of bacteria) groups displayed a significant up-regulation of TLR 22 with a peak value at 24h p.i.,whereas a significant down-regulation was observed in the T8 (high parasite and sub-lethal dose of bacteria) group from 12 h p.i.

Intraperitoneal injection of *A. hydrophila* induced significant ($p<0.05$) up-regulation of complement component C3 in the kidney of the T2 (sub-lethal dose of bacteria) group with a peak value at 24 hr (10.4 times) p.i. compared to the control group. An increased C3 expression was obvious in the kidney of the T3 (low parasite-infested) and T5 (moderate parasite-infested) group at 24h (8.7 times) at48 h p.i., whereas a significant down-regulation of C3 was observed in T7 (high parasite-infested) from 72 hp.i. compared to un-infested control. Among the co-infected groups, C3 expression showed significant up-regulation in T4 (low parasite and sub-lethal dose of bacteria) and T6 (moderate parasite and sub-lethal dose of bacteria) groups from 6 hp.i, which peaked at 24 h p.i. In contrast, the T8 (high parasite and sub-lethal dose of bacteria) group showed a down-regulation trend for C3 from early time points (6 h p.i.).

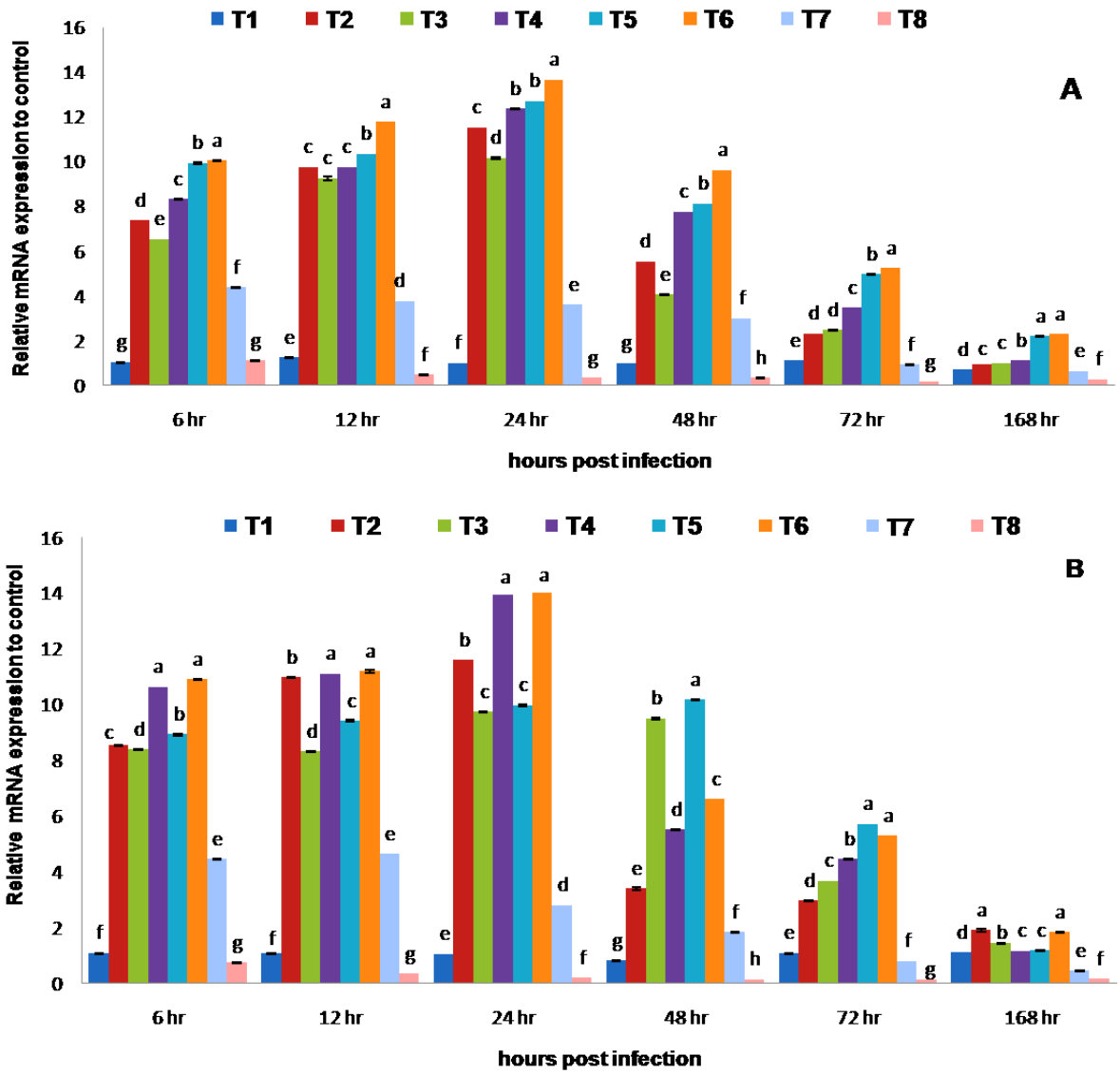

Fig 19: Relative mRNA transcription of TLR22 (A) and c3 genes (B) at different time-points in kidney tissue of *Carassius auratus* co-infected with different degree of *Argulus* and a sub-lethal dose of *A. hydrophila* determined by quantitative real-time PCR. Bars represent the mean ± SEM. Significant differences between the treatment and the control at each sampling point were indicated with different alphabets ($P \leq 0.05$). Abbreviations T1: Control group without *Argulus* and *A. hydrophila* infection, T2 (Fish exposed to 1/10 of LD50 dose of *A. hydrophila*), T3 (Fish exposed to low grade of *Argulus* infestation), T4 (T3 + *A. hydrophila*), T5 (Fish exposed to moderate grade of *Argulus* infestation), T6 (T5 + *A. hydrophila*), T7 (Fish exposed to high grade of *Argulus* infestation) and T8 (T7+ *A. hydrophila*).

4.13 Gene expression analysis in skin

Changes in expression levels of two immune-relevant genes were also monitored in skin tissue of goldfish at different time points after the co-infection with different doses of *Argulus* and sub-lethal doses of *A. hydrophila*. (Fig.19). The transcription profiles of these genes relative to respective EF-alpha transcript showed that expressions of TLR22 and C3 were significantly different in treatment groups as compared with the control (Fig: 18). In the present study, a significant up-regulation of TLR22 gene was observed in the T2 (sub-lethal dose of bacteria) group compared to control at all the time points with a peak value at 48 h (3.7 times) p.i. Among the different parasite-infested groups, T3 (low parasite-infested) and T5 (moderate parasite-infested) groups have shown comparable up-regulation of TLR22 at all the time points, albeit no significance (P > 0. 05) observed between the groups. In contrast, the T7 (high parasite infested) group showed an interesting transcription profile in which a significant down-regulation of TLR22 gene was observed as early as 6 hr (0.13 times), followed by 12 hr (0.2 times), and 24 hr (0.73 times) p.i., but increased at 48 hr (1.2 times) p.i. The same trend of early down-regulation and a later up-regulation (1.1 times at 48 hr p.i.) was observed in the T8 (high parasite and sub-lethal dose of bacteria) group compared to other co-infection groups. A significant up-regulation of the TLR22 gene was observed at all-time points in T4 and T6 groups with a peak value at 24 hr (6.6 and 9.8 times) p.i.

In the skin, a significant ($p<0.05$) up-regulation of complement component C3 was observed in the T2 (sub-lethal dose of bacteria) group with a peak value at 24h (9.2 times) p.i. compared to the control fish. Among different parasite-infested groups, T3 (low parasite-infested) and T5 (moderate parasite-infested) groups showed a significant ($p<0.05$) up-regulation of the C3 gene having a peak value at 48 h (11.6 and 11.9 times respectively) p.i. Similarly, T7 (high parasite-infested) group showed a significant ($p<0.05$) up-regulation at early point (3.3 times), and a down-regulation was observed from 168 h p.i. Among the co-infected groups, T4 (low parasite and sub-lethal dose of bacteria) and T6 (moderate parasite and sub-lethal dose of bacteria) groups showed a significant up-regulation trend having a peak at 48 h p.i (12.3 and 13 times, respectively). Whereas; in T8 (high parasite and sub-lethal dose

of bacteria) group a significant down-regulation of C3 gene was observed from 24 h p.i.

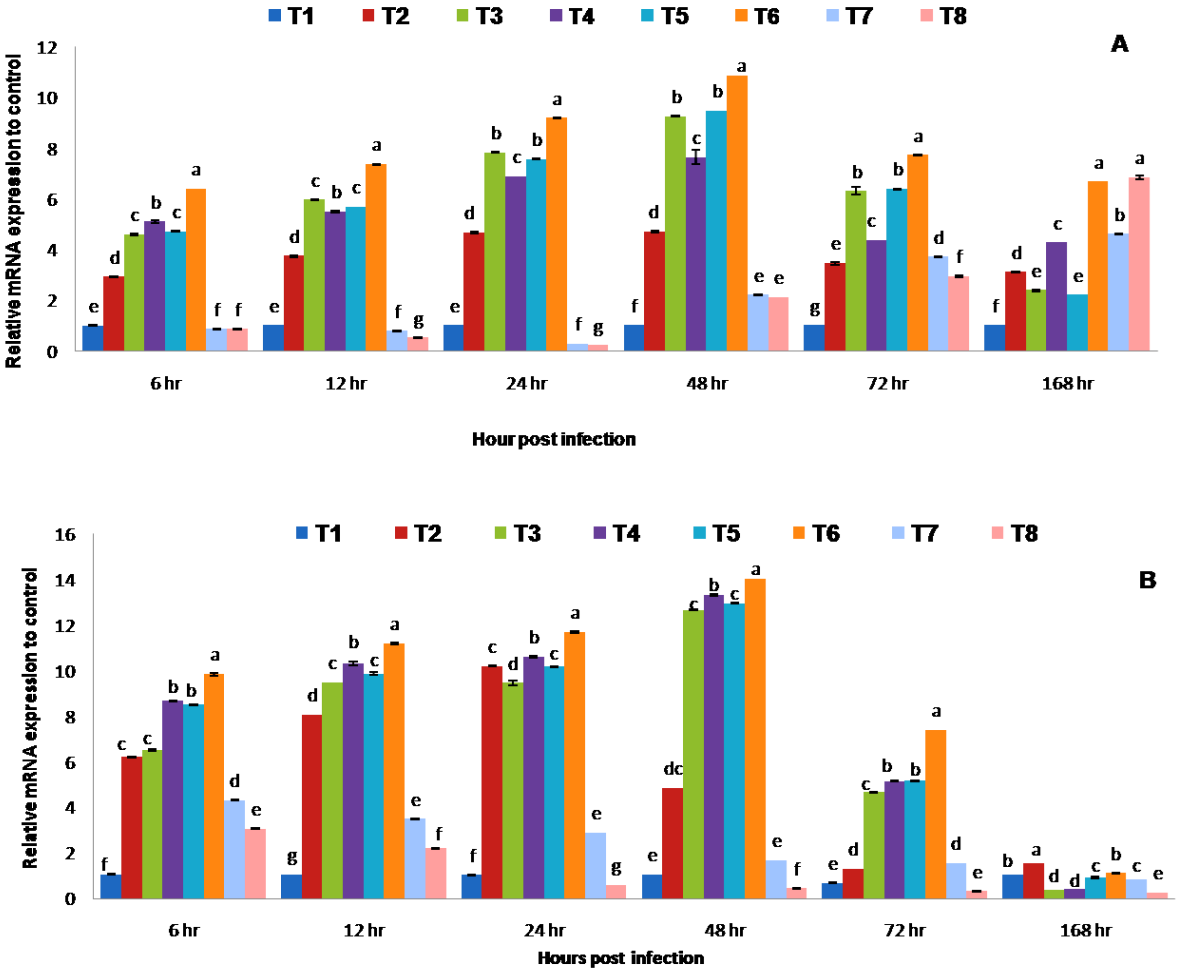

Fig 20: Relative mRNA transcription of TLR22 (A) and c3 genes (B) at different time-points in skin tissue of *Carassius auratus* co-infected with different degree of *Argulus* and a sub-lethal dose of *A. hydrophila* determined by quantitative real-time PCR. Bars represent the mean ± SEM. Significant differences between the treatment and the control at each sampling point were indicated with different alphabets ($P \leq 0.05$). Abbreviations T1: Control group without *Argulus* and *A. hydrophila* infection, T2 (Fish exposed to 1/10 of LD50 dose of *A. hydrophila*), T3 (Fish exposed to low grade of *Argulus* infestation), T4 (T3 + *A. hydrophila*), T5 (Fish exposed to moderate grade of *Argulus* infestation), T6 (T5 + *A. hydrophila*), T7 (Fish exposed to high grade of *Argulus* infestation) and T8 (T7+ *A. hydrophila*).

4.14 Gene expression analysis in liver

The expression kinetics of two immune-relevant genes was investigated in the liver of goldfish co-infected with different doses of *Argulus* and a sub-lethal dose of *Aeromonas hydrophila*, at different time periods post-infection. Transcription levels of these genes relative to EF-alpha are shown in Fig : 20. In the present study, a significant up-regulation of the TLR22 gene was observed in the T2 (sub-lethal dose of bacteria) group having an elevated value at 24 h (9 times) compared to control. Among the different parasite-infested groups, the T3 group (low parasite infested) and T5 (moderate parasite infested) group showed a significant up-regulation in all-time points compared to the control. Among the co-infected groups, T4 (low parasite and sub-lethal dose of bacteria) and T6 (moderate parasite and sub-lethal dose of bacteria) group exhibited a significant up-regulation of TLR22 gene, with apeak value at 24 h p.i. (9.7 and 11.9 times respectively). Whereas; a significant down-regulation was observed in the T8 (high parasite and sub-lethal dose of bacteria) group at all time points.

In the liver, a significant ($p<0.05$) up-regulation of complement component C3 was seen in the T2 (bacteria alone) group with a peak value at 24h (13.3 times) p.i. compared to control fish. Even though a significant ($p<0.05$) up-regulation of C3 was shown at early time points in all the parasite infested groups, a down-regulation trend was observed in T3 (low parasite infested), T5 (moderate parasite infested), and T7 (high parasite-infected) group from 72 h, 168 h, and 48hp.i, respectively. Among the co-infected group, a significant up-regulation was observed in the T4 (low parasite and sub-lethal dose of bacteria) and T6 (moderate parasite and sub-lethal dose of bacteria) group at all time points. Incontrast, a down-regulated C3 expression was observed in T8 (high parasite and sub-lethal dose of bacteria) group from 48 h p.i.

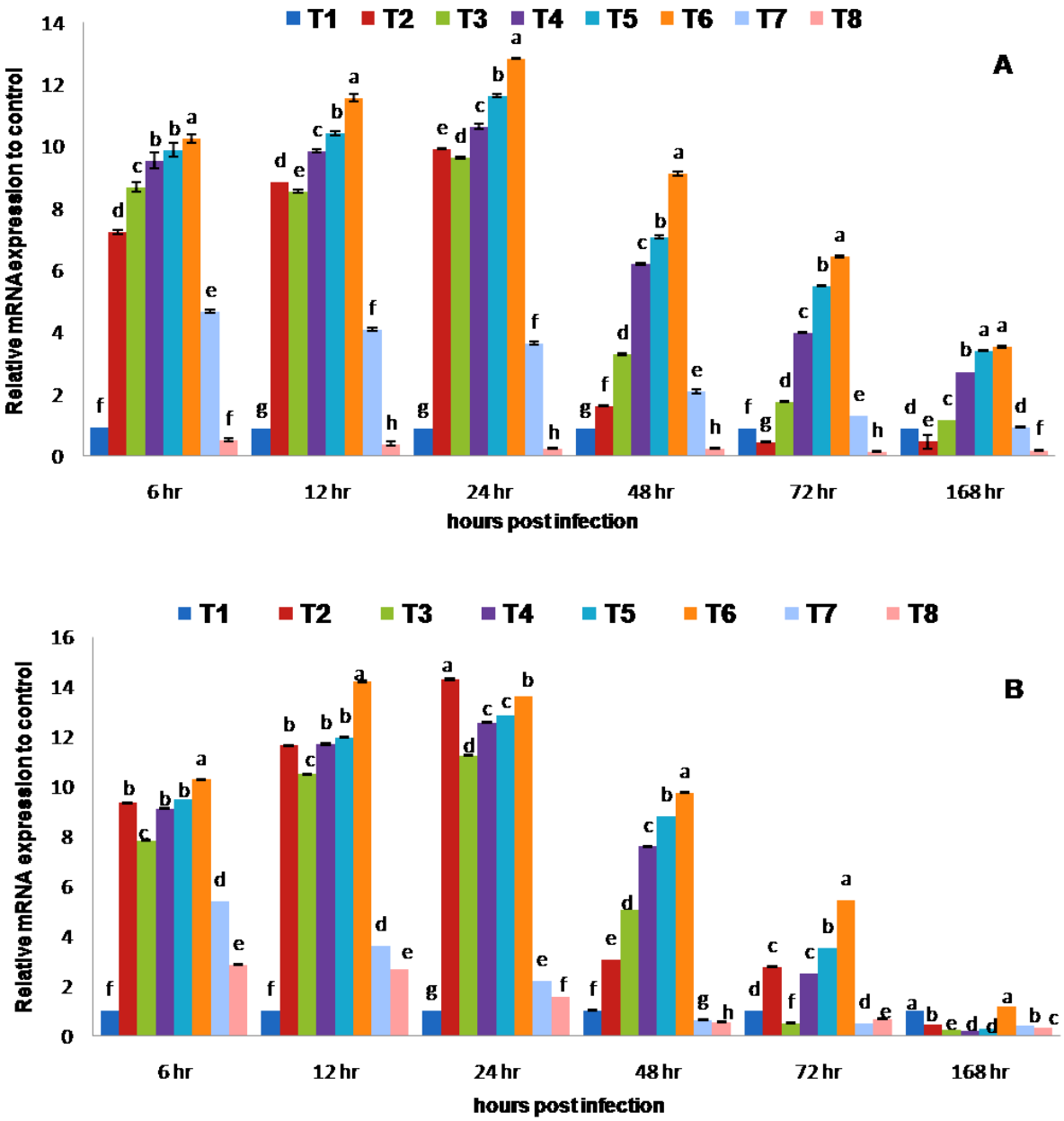

Fig 21: Relative mRNA transcription of TLR22 (A) and c3 genes (B) at different time-points in liver tissue of *Carassius auratus* co-infected with different degree of *Argulus* and a sub-lethal dose of *A. hydrophila* determined by quantitative real-time PCR. Bars represent the mean ± SEM. Significant differences between the treatment and the control at each sampling point were indicated with different alphabets ($P \leq 0.05$). Abbreviations T1: Control group without *Argulus* and *A. hydrophila* infection, T2 (Fish exposed to 1/10 of LD50 dose of *A. hydrophila*), T3 (Fish exposed to low grade of *Argulus* infestation), T4 (T3 + *A. hydrophila*), T5 (Fish exposed to moderate grade of *Argulus* infestation), T6 (T5 + *A. hydrophila*), T7 (Fish exposed to high grade of *Argulus* infestation) and T8 (T7+ *A. hydrophila*).

Objective 2

4.15 Mean intensity of *Argulus*

Intensity profile of *Argulus* in treatment groups T2, T4, and T6 was observed throughout the sampling period. A temperature-dependent increase in the intensity of *Argulus* was observed in goldfish. The intensity of *Argulus* was lower in the T3 group (23°C) compared to the T4 (28°C) and T6 (33 °C) group (fig: 21).

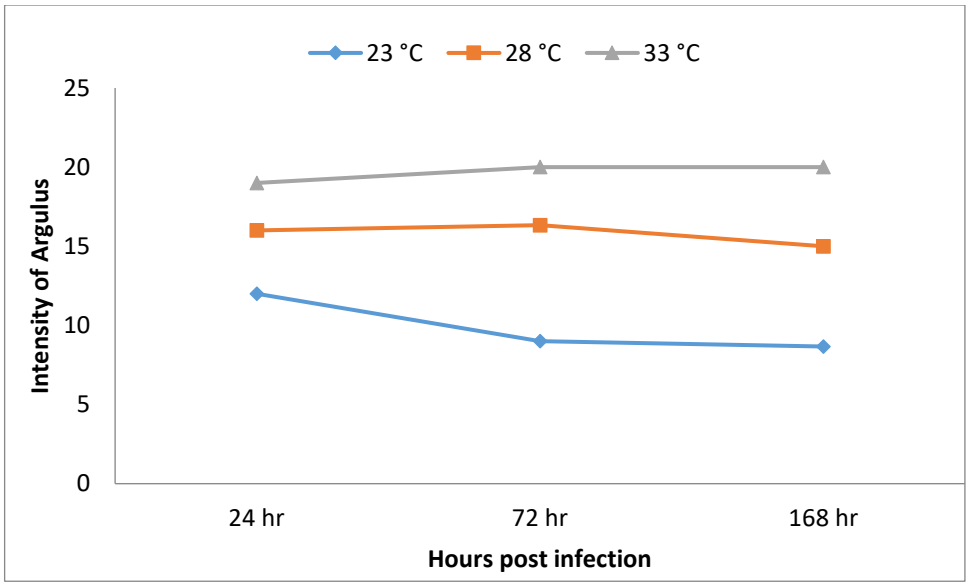

Fig 22: Temperature wise mean intensity of *Argulus* spp. in goldfish.

4.16 Cumulative mortality of goldfish

In the present study, experimental healthy *Carrasius auratus* maintained at 33°C temperature (T5) showed 20 % cumulative mortality, whereas no mortality was observed in the other two groups. Further, in the co-infected group, the highest mortality was observed for fish exposed to 33°C, T6 (60%) followed by the fishes held at T4 (co-infected group exposed to 28°C) and T2 (co-infected group exposed to 23°C) groups (fig: 22).

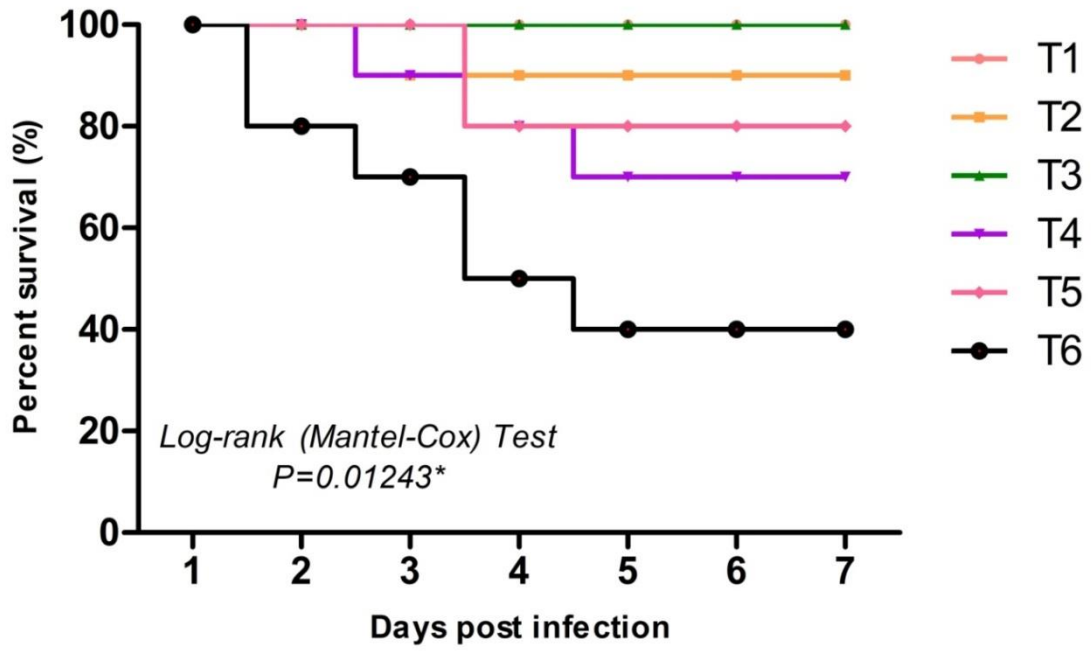

Fig 23: Survivor curve of *Carassius auratus* co-infected with *Argulus* and *Aeromonas hydrophila* exposed to different temperatures.

Abbreviations; (T1): healthy fish exposed to 23°C, (T2): co-infected group exposed to 23°C, (T3): healthy fish exposed to 28°C, (T4): co-infected group exposed to 28°C, (T5): healthy fish exposed to 33°C, (T6): co-infected group exposed to 33°C.

4.17 Haematological parameter

Temperature-dependent increase in RBC, Hb, and PCV values of *C. auratus* was observed in the control group; however, there was a significant ($p<0.05$) decrease in RBC, Hb, PCV values in the co-infected group when compared with to control fish in each of the experimental group.At 23°C, a significant increase in RBC value was observed in theco-infected group (T2) during the progression of infection

Table 9: RBC value of *C. auratus* experimentally co-infected with *Argulus* and *Aeromonas hydrophila* and exposed at three different temperatures.

Temperature (°)	Co-infection	day1	day3	day 7
23	0	1.2^{cB}	1.25^{cB}	1.4^{cA}
23	1	1.15^{cC}	1.25^{cB}	1.35^{cA}
28	0	1.45^{bC}	1.65^{bA}	1.55^{bB}
28	1	1.215^{cB}	1.15^{dB}	1.25^{dA}
33	0	2.055^{aB}	2.025^{aB}	2.15^{aA}
33	1	0.985^{dA}	0.995^{eA}	0.985^{eA}
SEM		0.031	0.024	0.024
Effect of temperature (°)				
23		1.175^{c}	1.25^{c}	1.375^{b}
28		1.333^{b}	1.4^{b}	1.4^{b}
33		1.52^{a}	1.51^{a}	1.568^{a}
SEM		0.022	0.017	0.017
Effect of co-infection				
control		1.568	1.642	1.7
Co-infection		1.117	1.132	1.195
SE		0.018	0.14	0.014
Source of variation (p value)				

Temperature	0.000	0.000	0.000
Co-infection	0.000	0.000	0.000
Temp* co-infection	0.000	0.000	0.000

Abbreviations: 23, 28 and 33 refer to three exposed temperatures. O refers no infection and 1 refers to co-infection. Data is represented as mean ± SE, p value < 0.05 and various superscripts represent significant differences (a, b, c for columns and A, B, C for rows).

Table 10: Hb value of *C. auratus* experimentally co-infected with *Argulus* and *Aeromonas hydrophila* and exposed at three different temperatures.

Temperature(°)	Co-infection	day1	day3	day 7
23	0	8.15^{cC}	8.25^{cB}	8.35^{cA}
23	1	7.25^{eB}	7.275^{eA}	7.15^{eB}
28	0	8.45^{bA}	8.45^{bA}	8.5^{bA}
28	1	7.7^{dA}	7.44^{dB}	7.37^{dB}
33	0	10.15^{aC}	10.595^{aB}	10.99^{aA}
33	1	6.85^{fA}	6.55^{fB}	6.45^{fC}
SEM		0.035	0.041	0.032
Effect of temperature (°)				
23		7.7^{c}	7.762^{c}	7.75^{c}
28		8.075^{b}	7.945^{b}	7.935^{b}
33		8.5^{a}	8.573^{a}	8.72^{a}

SEM	0.025	0.029	0.023
Effect of co-infection			
control	8.917	9.098	9.28
Co-infection	7.267	7.088	6.99
SEM	0.02	0.024	0.019
Source of variation (p value)			
Temperature	0.000	0.000	0.000
Co-infection	0.000	0.000	0.000
Temp* co-infection	0.000	0.000	0.000

Abbreviations: 23, 28 and 33 refer to three exposed temperatures. O refers no infection and 1 refers to co-infection. Data is represented as mean ± SE, p value < 0.05 and various superscripts represent significant differences (a, b, c for columns and A, B, C for rows).

Table 11: PCV value of *C. auratus* experimentally co-infected with *Argulus* and *Aeromonas hydrophila* and exposed at three different temperatures.

Temperature(°)	Co-infection	day1	day3	day 7
23	0	24.25^{cA}	24.15^{c}	23.75^{c}
23	1	23.75^{dA}	23.45^{dB}	23.15^{cD}
28	0	25.8^{bB}	26.25^{bB}	26.75^{bA}
28	1	21.25^{fA}	21.2^{fA}	20.885^{fB}
33	0	28.4^{aC}	28.8^{aB}	29.325^{aA}
33	1	22.7^{eA}	22.45^{eB}	22.25^{eC}
SEM		0.15	0.053	0.085
Effect of temperature (°)				
23		24^{b}	23.8^{b}	23.45^{c}
28		23.525^{c}	23.725^{b}	23.818^{b}
33		25.55^{a}	25.625^{a}	25.787^{a}
SEM		0.106	0.037	0.06
Effect of co-infection				

control	26.15	26.4	26.608
Co-infection	22.567	22.367	22.095
SEM	0.087	0.03	0.049
Source of variation (p value)			
Temperature	0.000	0.000	0.000
Co-infection	0.000	0.000	0.000
Temp* co-infection	0.000	0.000	0.000

Abbreviations: 23, 28 and 33 refer to three exposed temperatures. O refers no infection and 1 refers to co-infection. Data is represented as mean ± SE, p value < 0.05 and various superscripts represent significant differences (a, b, c for columns and A, B, C for rows).

Also, a temperature-dependent increase in WBC, neutrophil, and monocyte value was observed in control fish. Further, a significantly ($p<0.05$) higher value of WBC, neutrophil, and monocyte was observed in the co-infected group exposed to 23 and 28° C (T2 and T4) compared with the respective control (T1 and T3). In contrast, a significant ($p<0.05$) reduction in WBC, neutrophil, and monocyte was observed in co-infected fish exposed to 33°C during the progression of infection (T6).

Table 12: WBC values of *C. auratus* experimentally co-infected with *Argulus* and *Aeromonas hydrophila*and exposed at three different temperatures.

Temperature(°C)	Co-infection	day1	day3	day 7
23	0	107629^{eB}	107629.5^{eB}	107632.5^{eA}
23	1	117411.5^{cA}	117500.5^{cA}	117088.5^{cA}
28	0	108999.5^{dA}	108999.5^{dA}	108999.5^{dA}
28	1	121999^{bA}	122001.5^{bA}	121495^{bA}
33	0	132541^{aC}	132552.5^{aB}	132604^{aA}
33	1	97828.5^{fA}	94621.5^{fB}	91698^{fC}
SEM		288.267	288.267	282.342
Effect of temperature (°C)				
23		112520.25^{b}	112565^{c}	112360.5^{b}
28		115499.25^{a}	115500.5^{a}	115247.25^{a}
33		115184.75^{a}	113587^{b}	112151^{b}
SEM		203.835	199.646	187.729
Effect of co-infection				
control		116389.833	116393.833	116412
Co-infection		112413	111374.5	110093.833
SEM		166.431	163.01	153.28
Source of variation (p value)				
Temperature		0.000	0.000	0.000
Co-infection		0.000	0.000	0.000
Temp* co-infection		0.000	0.000	0.000

Abbreviations: 23, 28 and 33 refer to three exposed temperatures. O refers no infection and 1 refers to co-infection. Data is represented as mean ± SE, p value

< 0.05 and various superscripts represent significant differences (a, b, c for columns and A, B, C for rows).

Table 13: Neutrophil values of *C. auratus* experimentally co-infected with *Argulus* and *Aeromonas hydrophila* and exposed tothree different temperatures.

Temperature (°C)	Co-infection	day1	day3	day 7
23	0	9.98^{eA}	9.765^{eA}	9.35^{eB}
23	1	15.35^{bC}	16.6^{bB}	16.93^{bA}
28	0	10.65^{dA}	10.85^{dA}	10.7^{dA}
28	1	18.085^{aB}	19.065^{aA}	19.315^{aA}

33	0	12.365^{cB}	12.42^{cA}	12.34^{cB}
33	1	6.315^{fA}	5.98^{fB}	5.835^{fB}
SEM		.133	.137	0.138
Effect of temperature (°C)				
23		10.65	10.683	10.64
28		14.368	14.958	15.007
33		9.34	9.2	9.088
SEM		.094	.097	.097
Effect of co-infection				
control		10.998	11.012	10.797
Co-infection		11.917	12.215	12.36
SEM		.077	.079	.079
Source of variation (p value)				
Temperature		0.000	0.000	0.000
Co-infection		0.000	0.000	0.000
Temp* co-infection		0.000	0.000	0.000

Abbreviations: 23, 28 and 33 refer to three exposed temperatures. O refers no infection and 1 refers to co-infection. Data is represented as mean ± SE, p value < 0.05 and various superscripts represent significant differences (a, b, c for columns and A, B, C for rows).

Table 14: Monocyte values of *C. auratus* experimentally co-infected with *Argulus* and *Aeromonas hydrophila* and exposed to three different temperatures.

Temperature (°)	Co-infection	24h	72h	168h
23	0	$.205^{cA}$	$.185^{dB}$	$.2^{cA}$
23	1	1.04^{bC}	1.25^{bB}	1.525^{bA}
28	0	$.215^{cA}$	$.22^{dA}$	$.215^{cA}$
28	1	1.35^{aC}	1.55^{aB}	1.85^{aA}
33	0	$.235^{cB}$	$.255^{cA}$	$.26^{cA}$
33	1	$.19^{cA}$	$.17^{dA}$	$.13^{dB}$
SEM		.019	.017	.022
Effect of temperature (°)				
23		.622	.718	.863
28		.783	.885	1.033
33		.212	.212	.195
SEM		.013	.012	.016
Effect of co-infection				
control		.218	.22	.225
Co-infection		.86	.99	1.168
SEM		.011	.01	.013
Source of variation (p value)				
Temperature		0.000	0.000	0.000
Co-infection		0.000	0.000	0.000
Temp* co-infection		0.000	0.000	0.000

Abbreviations: 23, 28 and 33 refer to three exposed temperatures. O refers no infection and 1 refers to co-infection. Data is represented as mean ± SE, p value < 0.05 and various superscripts represent significant differences (a, b, c for columns and A, B, C for rows).

4.18 Innate immune response

A temperature-dependent increase in NBT, lysozyme, and myeloperoxidase was observed in the control group. Among the co-infected group, the T4 group (co-infected group exposed to 28°C) showed a significantly ($p<0.05$) higher NBT, MPO, and lysozyme activity compared to other co-infection groups. Further, a significantly decreased NBT, MPO, and lysozyme activity was observed in co-infected fish exposed to 33°C (T6) from 72 hrs of stress exposure

Table 15: NBT activity of *Carassius auratus* experimentally co-infected with *Argulus* and *Aeromonas hydrophila* and exposed to three different temperatures.

Temperature (°)	Co-infection	24h	72h	168h
23	0	$.255^{dA}$	$.24^{eB}$	$.215^{eC}$
23	1	$.308^{cA}$	$.316^{dB}$	$.325^{dC}$
28	0	$.335^{cA}$	$.345^{cA}$	$.335^{cA}$
28	1	$.435^{bB}$	$.426^{bB}$	$.453^{bA}$
33	0	$.615^{aA}$	$.626^{aA}$	$.62^{aA}$
33	1	$.249^{eA}$	$.245^{eA}$	$.208^{fB}$
SEM		.003	.004	.006
Effect of temperature (°)				
23		.297	.278	.145
28		.385	.386	.394
33		.427	.435	.439
SEM		.002	.003	.005
Effect of co-infection				
control		.402	.404	.357
Co-infection		.337	.329	.295
SEM		.002	.002	.004

Source of variation (p value)			
Temperature	0.000	0.000	0.000
Co-infection	0.000	0.000	0.000
Temp* co-infection	0.000	0.000	0.000

Abbreviations: 23, 28 and 33 refer to three exposed temperatures. O refers no infection and 1 refers to co-infection. Data is represented as mean ± SE, p value < 0.05 and various superscripts represent significant differences (a, b, c for columns and A, B, C for rows).

Table 16: Lyzosyme activity of *Carassius auratus* experimentally co-infected with *Argulus* and *Aeromonas hydrophila* and exposed at three different temperatures.

Temperature (°)	Co-infection	day1	day3	day 7
23	0	5.53^{e}	5.545^{e}	5.395^{eB}
23	1	6.8^{cA}	6.58^{c}	6.575^{c}
28	0	6.43^{d}	6.25^{dB}	6.3^{d}
28	1	7.45^{bC}	8.45^{bB}	8.7^{bA}
33	0	10.45^{aC}	12.7^{aB}	13.3^{aA}
33	1	4.55^{fA}	4.3^{fB}	4.05^{fC}
SEM		.041	.04	.054
Effect of temperature (°)				
23		6.165	6.063	5.985
28		6.94	7.35	7.5
33		7.5	8.5	8.67
SEM		.029	.028	.038
Effect of co-infection				
control		7.47	8.165	8.332
Co-infection		6.267	6.443	6.442
SEM		.023	.023	.031
Source of variation (p value)				
Temperature		0.000	0.000	0.000
Co-infection		0.000	0.000	0.000
Temp* co-infection		0.000	0.000	0.000

Abbreviations: 23, 28 and 33 refer to three exposed temperatures. O refers no infection and 1 refers to co-infection. Data is represented as mean ± SE, p value < 0.05 and various superscripts represent significant differences (a, b, c for columns and A, B, C for rows).

Table 17: MPO activity of *Carassius auratus* experimentally co-infected with *Argulus* and *Aeromonas hydrophila* and exposed at three different temperatures.

Temperature (°)	Co-infection	day1	day3	day 7
23	0	$.23^{fA}$	$.24^{eA}$	$.245^{eA}$
23	1	$.316^{dC}$	$.321^{dB}$	$.332^{dA}$
28	0	$.425^{cA}$	$.452^{cA}$	$.439^{cA}$
28	1	$.55^{bB}$	$.56^{bB}$	$.58^{bA}$
33	0	$.733^{aB}$	$.734^{aB}$	$.739^{aA}$
33	1	$.295^{eA}$	$.255^{eA}$	$.243^{eA}$
SEM		.004	.011	.01
Effect of temperature (°)				
23		.293	.275	.233
28		.487	.506	.484
33		.514	.495	.491
SEM		.003	.008	.007
Effect of co-infection				
control		.463	.475	.471
Co-infection		.4	.375	.335
SEM		.002	.007	.006
Source of variation (p value)				
Temperature		0.000	0.000	0.000
Co-infection		0.000	0.000	0.000
Temp* co-infection		0.000	0.000	0.000

Abbreviations: 23, 28 and 33 refer to three exposed temperatures. O refers no infection and 1 refers to co-infection. Data is represented as mean ± SE, p value < 0.05 and various superscripts represent significant differences (a, b, c for columns and A, B, C for rows).

4.19 Enzymes of oxidative stress (SOD, CAT, and GPx activity)

A significantly increased activity of SOD, GPx, and catalase was recorded in control fish exposed to 33°C (T5), whereas no significant difference was observed in the activity of catalase and GPx in control fish (T1 and T3 group). Among the co-infected groups, a significant increase in SOD, catalase, and GPx activity was recorded in fishexposed to 23 and 28°C (T2 and T4) compared to their respective control groups. In addition, there was no significant difference in SOD, catalase, and GPx activities in each treatment group with the progression of exposure to temperature and co-infection.

Table 17: SOD activity of *Carassius auratus* experimentally co-infected with *Argulus* and *Aeromonas hydrophila* and exposed at three different temperatures.

Temperature (°)	Co-infection	day1	day3	day 7
23	0	11.55^{eA}	11.65^{eA}	11.55^{eA}
23	1	13.35^{dA}	13.55^{dA}	13.95^{dA}
28	0	10.35^{fB}	10.55^{fA}	10.6^{fA}
28	1	22.15^{bA}	22.5^{bA}	23.3^{bA}
33	0	28.41^{aC}	29.05^{aB}	29.55^{aA}
33	1	18.3^{cA}	18.4^{cA}	18.05^{cB}
SEM		.052	.062	.041
Effect of temperature (°)				
23		12.45	12.6	12.75
28		16.25	16.52	16.95
33		23.35	23.72	23.8
SEM		.037	.044	.029
Effect of co-infection				
control		16.77	17.08	17.23
Co-infection		17.93	18.15	18.43
SEM		.03	.036	.024

Source of variation (p value)			
Temperature	0.000	0.000	0.000
Co-infection	0.000	0.000	0.000
Temp* co-infection	0.000	0.000	0.000

Abbreviations: 23, 28 and 33 refer to three exposed temperatures. O refers no infection and 1 refers to co-infection. Data is represented as mean ± SE, p value < 0.05 and various superscripts represent significant differences (a, b, c for columns and A, B, C for rows).

Table 19: Catalase activity of *Carassius auratus* experimentally co-infected with *Argulus* and *Aeromonas hydrophila* and exposed at three different temperatures.

Temperature (°)	Co-infection	day1	day3	day 7
23	0	3.35^{eA}	3.45^{eA}	3.35^{eA}
23	1	4.475^{cB}	4.48^{cB}	4.91^{cA}
28	0	3.25^{eA}	3.1^{eB}	3.15^{eB}
28	1	5.4^{bA}	5.5^{bA}	5.45^{bA}
33	0	7.3^{aB}	7.25^{aB}	7.7^{aA}
33	1	4.2^{dA}	4.1^{dA}	4.18^{dA}
SEM		.047	.052	.034
Effect of temperature (°)				
23		3.912	3.965	4.13
28		3.825	3.8	3.8
33		5.75	5.67	5.94
SEM		.033	.037	.024

Effect of co-infection			
control	.218	.22	.225
Co-infection	.86	.99	1.168
SEM	.011	.01	.013
Source of variation (p value)			
Temperature	0.000	0.000	0.000
Co-infection	0.000	0.000	0.000
Temp* co-infection	0.000	0.000	0.000

Abbreviations: 23, 28 and 33 refer to three exposed temperatures. O refers no infection and 1 refers to co-infection. Data is represented as mean ± SE, p value < 0.05 and various superscripts represent significant differences (a, b, c for columns and A, B, C for rows).

Table 20: GPx activity of *Carassius auratus* experimentally co-infected with *Argulus* and *Aeromonas hydrophila* and exposed at three different temperatures

Temperature (°)	Co-infection	day1	day3	day 7
23	0	11.5^{eA}	11.3^{eA}	11.5^{eA}
23	1	15.35^{cB}	15.4^{cB}	15.6^{cA}
28	0	11.3^{eA}	11.5^{eA}	11.6^{eA}
28	1	16.5^{bB}	16.65^{bB}	16.7^{bA}
33	0	20.15^{aC}	20.35^{aB}	20.55^{aA}
33	1	13.25^{dA}	13.2^{dB}	13.03^{dC}
SEM		.169	.169	.17
Effect of temperature (°)				

23	13.92	13.95	14.55
28	13.5	14.07	13.6
33	16.7	16.77	16.79
SEM	.12	.12	.12
Effect of co-infection			
control	14.38	14.78	14.85
Co-infection	15.03	15.08	15.11
SEM	.098	.098	.098
Source of variation (p value)			
Temperature	0.000	0.000	0.000
Co-infection	0.000	0.000	0.000
Temp* co-infection	0.000	0.000	0.000

Abbreviations: 23, 28 and 33 refer to three exposed temperatures. O refers no infection and 1 refers to co-infection. Data is represented as mean ± SE, p value < 0.05 and various superscripts represent significant differences (a, b, c for columns and A, B, C for rows).

5. DISCUSSION

Co-infections are demarcated by the concurrent presence of more than one pathogen in the host (Cox, 2011), and the interactions between the pathogens can be either synergistic or agonistic (Telfer *et al*., 2008; Chen *et al.,* 2013). Further, such interactions among the invading pathogens can often drastically alter the host susceptibility to infection, disease course, severity, and duration of the infection (Graham *et al*., 2007; Telfer *et al*., 2008). Many studies have suggested that parasites may have a role in encouraging secondary pathogen infections in fish, such as bacteria and viruses (Cusack and Cone, 1986, Busch *et al.,* 2003, Pylkko *et al.,* 2006), wherein the parasites facilitate an entry route for secondary pathogens (Buchmann and Bresciani, 1997) or may function as vectors of these pathogens (Cusack and Cone, 1986). Reduced host immunocompetence as a result of parasite infestation is one of the major methods by which a parasite may indirectly promote bacterial infections (Bowers *et al.,* 2000). Dual-infection studies under laboratory conditions have attempted to establish a link between the two co-infecting pathogens in fish (Xu *et al.,* 2009; Xu *et al*., 2014; Xu *et al*., 2015). However, a very few studies have investigated the interactions between parasites and pathogenic bacteria and their implications on fish health and immunity (Busch *et al*., 2003, Suomalainen *et al.,* 2005, Pylkko *et al.,* 2006).

It is anticipated that the susceptibility of fish to different pathogens may get altered due to fluctuations in water temperature, causing sudden disease outbreaks. The alterations in ambient temperatures are also reported to have drastic effects on the pathogenicity of certain bacteria, parasites, and viruses (Dorson and Touchy, 1981). Temperature plays a critical role in establishing or maintaining an infection, latency as well as its aggressiveness (Burdon, 1987; Blanford *et al*., 2003; Fels and Kaltz, 2006). However, little is known about their relative importance and the effects of altered temperature conditions on the outcomes of co-infection. Thus, understanding interactions between co-infecting pathogens and the abiotic environments, and establishing their effect on host physiological and immunological responses is crucial for developing health management strategies in aquaculture as well as in ornamental fish culture systems.

Argulus spp. are harmful ectoparasites of fish, particularly the ornamental fish. These ectoparasites impact severe disease outbreaks throughout the world and their co-infection with *A. hydrophila* can cause major epizootics (Menezes *et al*., 1990, Hakalahti *et al*., 2004). However, how *Argulus* infections modulate or facilitate bacterial infections remains unknown. Considering this gap in the understanding of complex mechanisms of parasite and consecutive bacterial infections, the present study was framed to investigate the immune response of goldfish in events of co-infection with different doses of *Argulus*, followed by a single sub-lethal dose of *A. hydrophila.* Further, the effect of environmental temperature in such co-infections was studied by performing co-infection of *Argulus* followed by a sub-lethal dose of *A. hydrophila* infection at varying temperature conditions.

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5.1 Isolation and identification of *A. hydrophila* from infected goldfish.

Aeromonas spp. are the etiological agents of diseases in freshwater ornamental fishes with clinical signs such as fin rot, tail rot ulceration, exophthalmia, dropsy, *etc* (Sreedharan, 2008; Cizek *et al.,* 2010). Among different *Aeromonas* spp, infecting fish, *A. hydrophila, A.caviae, A.veronii* are the most pathogenic Aeromonas species, and they constitute the major part of isolates from diseased fish (Janda and Abbott, 1998). Several biochemical tests have been proposed for the identification and differentiation of *Aeromonas* species. Identification of *Aeromonas* to species level is intricated by the lack of a clear phenotypic identification for distinguishing these groups (Abbott *et al.*, 2003). Presumptive identification was done using six major biochemical tests *i.e.* Gram staining, motility, oxidase, catalase, fermentation of glucose, resistance to vibriostatic agent 0/129.

Of 32 strains isolated in the present study using AIM, only 15 isolates were found to be positive for all presumptive biochemical tests performed. Further identification of *Aeromonas* up to the complex level was done according to Abbott *et al. (*2003). Four tests were performed to group the isolates into *A.hydrophila, A.caviea, and A.sobria* complex. *A. caviae* complex was negative to Voges-Proskauer test and was positive to esculin hydrolysis,

produced gas from glucose, and utilized arabinose. *A. hydrophila* complex was positive for all four tests, whereas *A. sobria* complex was found to be negative for esculin hydrolysis. In this study, 4 isolates that came under *A. hydrophila* complex were subjected for further molecular identification as many authors suggested ambiguity in phenotypic identification (Beaz -Hidalgo *et al*., 2010; Figueras *et al*., 2011; Beaz – Hidalgo and Figueras, 2012). The *16S rRNA* gene sequencing, the most commonly used molecular technique for genus and species identification, was carried out with specific primers as suggestedby many authors (El-ghareeb *et al.,* 2019; Duman *et al*., 2018; Hafez *et al.,* 2018; Mukherjee *et al*., 2017; Soltani *et al.*, 2016). The isolates with 98 to 100% homology to *A. hydrophila* were selected and sequences were submitted to NCBI Genbank and acquired with accession numbers (AB 473004.1 and AB 532759.1). The isolates with 16SrRNA sequences showing100% similarity were further subjected to species-specific PCR along with positive and negative controls. In 1% agarose gel electrophoresis, 103 bp products were found confirming the isolates as *A. hydrophila*.

5.2 Identification of *Argulus* species

Studies on goldfish confirm that the *Argulus* is the most prevalent ectoparasite infecting goldfish leading to severe economic losses (Noga 2010).In the present study, *Argulus japonicas was identified from C. auratus* based on morphological traits. The posterior lobes of the cephalo-thoracic region of the specimen extend beyond the beginning of the abdomen. The carapace was found covering the fourth pair of legs and covers marginally with small spines. The posterior incisures of the abdomen reaching the middle of the body are observed with no leg pigmentation. The presence of a hook on the anterior spine of the first antenna was also noticed. Coxal spines of the second maxilla were observed as long and finger-like sharp projections. A series of long closely arranged setae on the basal plate of second maxillae was also observed. The morphological characters like the shape of the abdominal lobe and extent of abdominal incision were used to differentiate *A. japonicas* from related species like *A. foliaceus* and *A. coregoni* (Yildiz and Kumantas, 2002; Noaman *et al.,* 2010). The morphological characteristics of the *Argulus sp.* observed were the acutely rounded abdominal lobe with abdominal incision more than half of the

abdominal length characteristic of *A. Japonicus*. All morphological characters observed in the present study were in agreement with features of *A. japonicus* proposed by different authors (Fryer 1982; Rushton-Mellor, 1994; Noaman *et al.,* 2010; Soes *et al*., 2010; Wafer *et al.,* 2015). The identified parasite was confirmed as *A. japonicus.*

5.3 Artificial infestation of goldfish with *A. japonicus*

Artificial infestation of goldfish with *A. japonicas* was carried out as a preliminary step, to facilitate subsequent secondary bacterial infection. Artificial infection of goldfish was carried out in two ways; i) using *Argulus* parasitic stage as a source of infection by the co-habitation method (Kumar *et al.,* 2012), and ii) by exposing naïve fish to *Argulus* eggs (Kumar *et al.* (2012b) and Sharma *et al.* (2016).Both the experiments were performed separately for an optimized period of 3 weeks under normal laboratory conditions. The results showed an average intensity of 15± 6 parasites per fish in the co-habitation method. However, studies have reported varying mean intensity of parasites. Kumar *et al.* (2012) and Sharma *et al.* (2016) used the co-habitation approach to demonstrate the artificial infection of *Argulus* to *C. auratus*, reporting moderate to high intensity of 15-20 and 40-50 *Argulus* per fish, respectively, on experimentally challenged fish. Furthermore, Kone *et al*. (2014) discovered an *Argulus* intensity of 9.9 to 31.8 per fish following a 6-month challenge infection of *Argulus* in healthy tilapia using the co-habitation approach. At different phases of rohu confronted with the parasite, Parida *et al*. (2018) discovered varying degrees of *Argulus* intensity. After 20 days of post-infection with *Argulus* at 1 number/ 8 g of fish, the pre-adult stage had the largest load of *Argulus*, followed by juvenile and fingerlings (Parida *et al.,* 2018). Similarly, the infection of healthy *C. auratus* with *A. japonicas* through the co-habitation method showed an average intensity of 20 to 26 juvenile *Argulus* per fish (Kumari *et al.*, 2019). Different researchers have taken up different strategies for co-habiting fish with parasites, in which the incubation period ranged from few hours to several days, with differences in pathogens as well as in the environmental parameters (Hemaprashant *et al.*, 2012; Kumar *et al*., 2017; Stewart *et al*., 2018; Lira *et al*., 2020). Thus, the possible variations in mean intensity of parasite infections can be attributed to the difference in species, environmental parameters, and the incubation period.

The second challenge method using eggs of *Argulus* showed a hatching percentage of 60 to 70% with an average of 24 juvenile parasites per fish. Also, in the present study, glass walls of aquaria, air stone, aeration tube, and filter pipe were found to be the preferred site for laying eggs by *A. japonicus.* Similar observations were made by many authors, in which hard substratum like stones, plastic boards, wooden sticks, rocks, plants like hydrilla, and concrete etc were preferred by female *Argulus* to lay eggs (Hakalathi *et al.*, 2004; Sahoo *et al.,* 2013b, and Sharma *et al.,* 2016).

In the present study, the attempts to artificially infect goldfish using eggs of *A. japonicus* to were successful. In the present study, fish heavily infested with *A. japonicas* exhibited lethargy, irritation, settling at the aquarium bottom, off feed, frayed fins with opaque skin, presence of haemorrhages on the body surface and fins along with the incidence of mortality. These findings were in concurrence with the observations of Sharma *et al.* (2016) in goldfish.

5.4 Mortality and clinical signs

Under natural conditions, hosts rarely encounter a single pathogen and are often infected with more than one pathogen (Xu *et al.,* 2012). The variations in the dose of infection and genetic diversity of pathogens present in a host can enhance the severity of infection (Kinnula *et al.,* 2016). Currently, co-infection studies with parasites and bacteria are gaining attention in fish health; as such studies contribute to understanding the epidemiology and the effect of multi-pathogens, and eventually contribute towards the control and prevention of disease (Xu *et al.,* 2012). The interactions between *Argulus* and the secondary opportunistic pathogen *A. hydrophila* have been documented in earlier studies (Cusack and Cone, 1986). But, the information on the contribution of *Argulus* infectious dose in pathological events followed by co-infection with *A. hydrophila* is scarce. The present study aimed at understanding how different doses of *Argulus* affect the immune response of goldfish during subsequent co-infection with an opportunistic pathogen, *A. hydrophila.*

To understand the pathological sequelae in parasite-bacteria co-infection, challenge experiments were carried out in goldfish. In this study, the co-infection was established by exposing healthy goldfish infected with different levels (15± 6

parasites per fish) of *Argulus* to a single sub-lethal dose of *Aeromonas hydrophila.* Following the co-infection challenge, the clinical signs were observed in each challenged fish to understand pathological developments. The clinical signs observed in goldfish in the present study were similar to those reported by earlier workers. *Argulus* spp. have earlier been reported to cause direct damage to the fish skin like craters and ulcers by their attachment and feeding mechanisms (Lester & Roubal 1995; Saurabh *et al.,* 2011). Adult *Argulus* parasites can glide over the integument of their host using their maxillary suckers and cause injuries at multiple sites (Forlenza *et al.,* 2008). Several authors have reported the appearance of red inflamed spots, lesions, and skin ulcers observed after the infestation with *Argulus* as the natural clinical sign (Walker *et al* . 2004; Saurabh *et al*., 2011; Kumar *et al.,* 2012; Kar *et al.,* 2015; Kumar *et al.,* 2017). Furthermore, these parasites inject cytolytic toxins (LaMarre & Cochran 1992) and digestive enzymes (Shimura & Inoue 1984) through the oral sting and labial spines which may result in damage to the skin as well as other internal organs of the host. Previous studies have also reported typical clinical symptoms of *A. hydrophila* infections, which include fin loss and hemorrhagic ulceration at the fin base (Rahman *et al.,* 2001; Harikrishnan *et al.,* 2010). Apart from various clinical signs, it was also observed that high parasite load in experimental co-infection had led to increased mortalities compared to low parasite load, where all the experimental fish were exposed to identical sub-lethal dose of *A. hydrophila*. The foraging nature of the parasites created more lesions and ulcers in the skin, which in turn could have helped secondary pathogens like *A. hydrophila* to enter fish tissues easily. This was evident in a previous study with the different parasites, where enhanced mortality was observed in tilapia co-infected with the higher load of *ich* and identical dose of *Streptococcus* (Xu *et al*., 2009).

5.4.1 Haematological parameters

Erythrocyte count, WBC count, packed cell volume (PCV), and haemoglobin (Hb) levels have all been utilised as indications of disease and stress, as well as a marker for evaluating fish health (Fallah *et al*.,2015). Various haematological indices and innate immunological markers in serum were investigated in the study to understand the alterations in the immune response to

co-infection with different doses of *Argulus* followed by a sub-lethal dose of *A. hydrophila*. During the development of the disease, fish exposed to high *Argulus* infection showed a drastic and significant decline in RBC, Hb, and PCV values, both in single and co-infected groups. The parasite-infested fish's reduced RBC count, Hb value, and packed cell volume can be linked to parasitic infestation, which frequently results in anaemia (Martins *et al.,* 2004). During primary stages of stress from parasite infestation, the PCV changes due to the release of catecholamine, which can mobilize RBC from the spleen (Wells & Weber, 1990) or otherwise induce red blood cell swelling as a result of fluid shift into the intracellular compartment (Chiocchia & Motais, 1989) affecting the hematocrit value (Panjvini *et al.,* 2016). In contrast, Wendelaar (1997) and Lebelo *et al.* (2001) reported a significant rise in Hb value and packed cell volume and non-significant increase in RBCs count Henneguyosis. These differences could be attributed to the mode of parasite infection followed by different investigators. Kumar *et al*., (2013) hypothesized that continual feeding of fish using the oral string of *Argulus* and haemorrhage from injuries in the skin that occurred during the attachment may adversely affect the haematological parameters like RBC, PCV, and Hb. In the present study, the lytic activity of *A. hydrophila* might have further contributed to the pathological changes as *A. hydrophila* are reported to have red blood cell lytic activities for nutrient acquisition, causing anemia in affected populations (Uma *et al*., 2010). Further, recent studies have reported that a significant decrease in haemoglobin concentration in co-infected fishes due to depression/exhaustion of hemopoietic potential of the fish (Ray *et al.,* 2016; Roy *et al*., 2019).

Un-infected fish have reduced WBCs, which can be used as a sign of infectious disorders (Fallah *et al.,* 2015). During each experimental sample day, the moderate and low *Argulus* co-infected groups had a substantial rise in WBC, lymphocyte, neutrophil, and monocyte levels compared to their respective single *Argulus* infection. The rise in WBC can be thought of as the host's cellular immune system's active response to infection. Increased WBC may play an important role during parasite and bacterial infection by triggering inflammatory responses in hemopoietic tissues and strengthening the immune system by creating antibodies that can fight the infection (Mohaved *et al*., 2016). The significant fall in WBC and neutrophil found in fish exposed to a strong dosage of

Argulus and bacteria, on the other hand, could be attributed to acute stress lymphopenia (Plusford *et al.*, 1994; Larsson *et al.*, 1980; Davis *et al.,* 2008).

5.4.2 Innate immune parameters

Non-specific immunity in fish has been measured using respiratory burst activity of phagocytes, antimicrobial activity of myeloperoxidase enzyme, and bactericidal activity of lysozyme (Anderson and Siwicki, 1995; Ellis *et al.*, 1999). The potential of active phagocytic cells to produce reactive oxygen species (ROS) and haloperoxidase molecules, which are harmful to a variety of pathogens and host cells, is represented by respiratory burst (RB) activity and MPO (Schrader and Fahimi, 2006, Valko *et al.*, 2007). Similarly, lysozyme is a blood enzyme that actively lyses Gram-positive bacteria's cell wall, and a higher quantity has been thought to represent a natural defensive mechanism in fish (Grinde, 1989). A variety of immune changes have been documented after applying a multitude of stressors in fish (Pruett, 2003). Both immune system enhancing and suppressive processes have been reported following the stress episodes, even though the majority of changes often results in deleterious effects. Immediate response during the activation phase enhances the humoral innate immunity such as increased level of phagocytic cells, lysozyme, and C3 proteins after the acute stress (Demers and Bayne, 1997; Sunyer *et al.,* 1995). Similarly, an increase in NBT, MPO, and lysozyme activity was observed in fish co-infected with low to moderate *Argulus* and *A. hydrophila* compared to their respective single *Argulus* and *A. hydrophila* groups, indicating an immediate host innate immune response to mixed/co-infection to destruct or eliminate the invaded pathogens. Several authors have documented an increase in NBT, MPO, and lysozyme activities during bacterial (Biller-Takahashi *et al.*, 2013; Xia *et al.,* 2017; Lazado *et al.,* 2018; Khan *et al.*, 2018;) and parasite infection, supporting the findings of the current investigation (Souza *et al.*, 2019; Kumar *et al.*, 2013; Munoz *et al.,* 2007; Alishahi *et al.*, 2006). Further, the increase in ROS in the phagocytic cells of infested fish corresponds to the recruitment of active neutrophils and monocytes and a gradual increase in the response of these cells associated with invader-induced injury and localized inflammation (Kumar *et al.,* 2013). However; when a stress response develops it may be assumed that the outcome will depend on the intensity and duration of the stressor.

Researchers have reported that stress response can enhance or suppress certain pathways of the immune response (Dhabhar, 2002, 2009; Small and Bilodeau, 2005; Dios *et al*., 2007). The low intensity of RBA, MPO, and serum lysozyme activity seen in the high *Argulus* infested group and corresponding co-infected group could be attributed to the host's innate immune response being exhausted as a result of significant acute damage and inflammation. When the intensity and duration of stress increases, it may significantly affect the fish immune system and can have an impact on the energy machinery (Fast *et al*., 2008). Thus, significant amounts of energy may be continuously used leading to growth arrest and making the fish more prone to infections by opportunistic pathogens. In addition, some responses of the immune system can be inactivated, particularly the synthesis of immunocytes which might increase the pathogen susceptibility and lead to immunosuppression (Tort, 2011).

5.4.3 Antioxidative stress enzymes

Antioxidant systems form one of the crucial protective systems in the organism against oxidative stress caused by biotic and abiotic factors. Pathogen infection imbalances the metabolism of infected fish and stimulate oxidative stress manifested by an increase in free radical and peroxide processes modulating the antioxidant status of the host (Skuratovskaya and Zavyalov, 2006, 2008; Bello *et al.,* 2000; Dautremepuits et al., 2003; Martinez-Alvarez *et al*., 2005). Further, increased ROS production in the host is ascribed to a natural elimination mechanism against pathogens, especially parasites which can lead to the inhibition of the host antioxidant enzyme activities (Mikrjakov *et al.*, 2014; Skuratovskaya and Zavyalov, 2006, 2008; Bello *et al*., 2000; Dautremepuits *et al*., 2003; Martinez-Alvarez *et al*., 2005). However, the character and direction of these modulations in antioxidant systems depend on the species of host and pathogens. In the current investigation, fish co-infected with low to moderate *Argulus* + *A. hydrophila* showed a considerable increase in SOD, GPx, and catalase activity. This could be a host adaptive response, as antioxidants are a critical biological defence against oxidative stress in cell detoxification and are crucial for comprehending the harmful effects of free radicals created within the cell (Ahmad *et al.,* 2000). Excess ROS, on the other hand, causes metabolic

events in the cells that result in impaired cellular function owing to oxidative damage to proteins, carbohydrates, and lipids, which can lead to apoptosis and the build-up of oxidised molecular aggregates. Aged cells have lower levels of antioxidative enzymes including SOD, CAT, and GPx, and have a higher level of free radical build-up (Scandalios 2005, Canizzo *et al.* 2011). This can be related to the reduced antioxidant enzymes activity observed in the co-infection of a heavy dose of the parasite with a sub-lethal dose of bacteria (co-infection). Furthermore, the presence of free radicals causes the constant activation of granulocytes, macrophages, and dendritic cells, with permanent production of ROS due to the leukocytes' respiratory burst activity, as well as the subsequent activation of innate defence compounds such as complement system proteins activation and leukocyte adhesion protein expression. This continual activity can, in some situations, lead to immune system depletion (Vider *et al.*, 2001, Lotze *et al.,* 2007, and Gill and Tuteja., 2010). Another possible reason for the reduced antioxidant activity can be the high content of parasites metabolites causing oxidative stress, presenting a great danger for fish health (Skuratovskaya *et al.*, 2013). Such a decrease in antioxidant enzymes was observed in several studies with high intensity of parasite infestation (Skuratovskaya *et al.,* 2015). Further, co-infections found to worsen the antioxidant system of fish and several studies have reported the oxidative stress response related to *Argulus* and *A. hydrophila* co-infections, and those observations were in agreement with the current findings (Xia *et al.*, 2017; Abdel-Magid *et al.,* 2019*;* Kumar *et al.*, 2013; Saurabh *et al.,* 2010).

Altogether a significant reduction in innate as well as cellular parameters like NBT, MPO, and lysozyme activities was observed in co-infection with a heavy dose of *Argulus* and *A. hydrophila.* This might be due to the exhausted lymphocytes and hematopoietic potential of fish leading to immune suppression in the host, as evident by, the immunosuppressive effects of *L. salmonis* in *Atlantic salmon,* where the authors suggested that immunosuppressive secretions from *L. salmonis* might cause a decrease in cellular and innate immune responses (Mustafa *et al.,* 2000). In the present study, the heavy *Argulus*-infected goldfish which exhibited lower innate immune parameters compared to control fish may be more sensitive to the burden of multiple stressors under co-infection. These cumulative effects of stressors have

previously been reported to affect the innate immune parameters as observed in fish administered cortisol and infected with *Argulus spp*. (Ruane *et al.*, 1999 and Van der Salm *et al*., 2000) and in fish infected with *L. Salmonis* followed by crowding stress (Ruane *et al.,* 2000).

5.4.4 Expression of immune genes

Transcriptional analyses have contributed a great deal to the understanding of changes in the levels of immune-related proteins present in the blood or tissues (Saurabh *et al*., 2011). In this study, two major immunocompetent organs namely kidney and liver tissues along with dermal tissue at different time points were collected during experimental co-infection of *A. japonicus* and *A.hydrophila* for the transcriptional analysis of selected immune-related genes. Two important immune genes namely TLR22 and C3 genes in were targeted throughout the study,

Toll-like receptors (TLRs) are type I transmembrane proteins involved in the recognition of the pathogens based on pathogen-associated molecular patterns (PAMPs) and alert the host about the pathogens to induce immunity in a host (Uma *et al.*,2012). Its involvement in the anti-bacterial and antiparasitic responses in fishes has also been documented by several authors (Saurabh *et al*., 2011; Zhao *et al*., 2013; Panda *et al*., 2014; Kole *et al*., 2017). However, among different TLRs characterized so far, TLR22 forms one of the important receptors with the crucial role, where their expression can be modulated by various PAMPS (Sundaram *et al.,* 2012; Salzar *et al*., 2016). In the present study, up-regulated expression of TLR 22 observed in skin, liver, and kidney post-infection with a low and moderate dose of *Argulus* and sub-lethal dose of *A. hydrophila* suggest the immune responses induced by these two pathogens. The up-regulation at sub-lethal co-infections indicated their important role in recognizing bacterial and parasitic ligands during infection. This result was inconsistent with Zhang *et al.* (2015) and Tu *et al*. (2016) who reported up-regulated expression of TLR22 in the spleen and skin following challenge with *Aeromonas hydrophila* and *Dactylogyrous intermedius* in goldfish. Also, in goldfish infected with *Dactylogyrous intermedius, the expression of TLR22 was examined at different time intervals.* A significant up-regulation of TLR22 was observed in the liver, spleen, and kidney on days 16, 18, and 28p.i. A varying

tendency of TLR22 expression was even more evident after the second challenge with *D. intermedius,* showing a maximum expression at 7 days p.i. in spleen and kidney. Recently, modulation of TLR22 gene was reported in Soiny mullet (*Liza haematocheila*) following *Streptococcus dysgalactiae* infection. At 12h and 24h p.i., TLR22 remained up-regulated in the skin, head kidney, spleen, and intestine (Qi *et al.*, 2020). Also, sturgeon TLR22 was significantly up-regulated at 12 h after LPS stimulation (Qi *et al*., 2018). Similar results were also observed in channel catfish and rohu following infection with *Edwardsiella ictalurii* and *Aeromonas hydrophila* (Zhang *et al*., 2013; Samanta *et al.,* 2014). Recently, modulation of the TLR22 gene was analyzed in different tissues of *Lates calcarifer* following stimulation with dsRNA viral analog, poly (I: C) and Gram-negative bacterium, *Vibrio alginolyticus (*Paria *et al.,* 2018*).* Similarly, the modulatory role of TLR22 in different tissues of Indian major carps Catla, rohu, and mrighal after the infection with *Argulus siamensis* was investigated by Panda *et al.* (2014), and the results showed that TLR22 was constitutively expressed in all studied tissue/organs, but the level of expressions varied among them. Expression of TLR22 was found to be significantly down-regulated at 15 dpi in gill tissue of rohu parasitized with *D.catalaius* (Dash *et al*., 2014). Whereas, after natural infection with the ectoparasite *A. ocellatum*, the expression levels of TLR22 in yellowtail (*Seriola lalandi)* was potently induced at 72 h in the spleen (1461 fold), muscle (173 fold), skin (553 fold), and gill (132 fold) compared with the healthy group (Reyes-Becerril *et al*., 2015). Taken together, these observations indicate that fish TLR22 was involved in innate immunity against a broad range of infectious agents including bacteria, ectoparasites, and even viruses.

On the contrary, down-regulation of TLR 22 was observed after infection with high dose *Argulus* in liver and kidney throughout infection. This might be due to the toxic effect of the parasite. It is documented that the parasitic toxins suppress leucocytic activities (Saurabh *et al.,* 2011). Thus, a possible explanation for TLR 22 down-regulation could be the decrease of leucocytes levels in the kidney that are involved in TLR expression. Also, another species of the genus *Argulus, A. coregoni,* is known to lower leukocyte counts in *Onchorhynchus masou* (Shimura *et al.,* 1983). However, according to Dash *et al*. (2014), the changes in the expression pattern for a single TLR may not

indicate the status of the whole TLR pathway. Further, the same authors described that the down-regulation of TLR22 could be the result of an effective escaping mechanism shown by the pathogen out of host machinery, to establish themselves in the host.

Although the TLR22 gene expression pattern in the skin is down-regulated until 48 h.p.i., it exhibited a sudden up-regulation later. The damaged skin tissue which is self-molecule may act as a danger signal possessing PAMP (Dalmo & Bogwald, 2008). This might act as PRR for the TLRs during the heavy *Argulus* infection. This may explain the up-regulation of TLR 22 in the skin at later time points (72 h.p.i). This is also inconsistent with the studies of Saurabh *et al.* (2011) and Kar *et al.* (2015), who reported this dynamic pattern of TLR 22 expression in the skin only after heavy *Argulus* infection.

The complement system is a key component of innate immunity in teleost and its activation leads to opsonization and neutralization of pathogens (Pushpa *et al.,* 2014). Further, complement factor C3 is the central component of all three activation pathways and plays a key role in the formation of membrane attack complex (Janeway, 2001). The activation of the complement cascade is an important function in the immune reactions against pathogenic, especially parasitic infections in teleost fish (Buchmann & Bresciani 1999; Saeij *et al.,* 2003, Boshra *et al.,* 2006). In alignment with several reports, the expression pattern of C3 examined in this study revealed an up-regulation of its transcription in skin, liver, and kidney after the infection with a low and moderate dose of *Argulus* and a sub-lethal dose of *A. hydrophila*. Such an acute hike in complement factors was observed by Wang *et al.* (2015) in liver tissues of large yellow croaker (*Larimichthys crocea*) under *V. alginolyticus* infection. Similarly, enhancement of C3 was also observed in the liver and kidney of *Labeo rohita* and skin tissue of loach infected with *Aeromonas hydrophila* (Xu *et al.*, 2018). Such an up-regulation in C3 activity is observed in many bacterial and parasitic infections associated with teleost fish. In parallel to our observations, an up-regulation of C3 was also discovered in the skin of Zebra fish (Lu *et al.*, 2015 and *Cyprinus carpio* (Gonzalez *et al.*, 2007,) after the infection with parasite *Citrobacter freundii* and *Ichthyophthirius multifiliis* respectively.

In contrast, the skin, kidney, and liver of goldfish showed a downregulation in C3 expressions after the co-infection with a heavy dose of *Argulus* and a sub-

lethal dose of *A. hydrophila* infection. This implies that a heavy dose of *Argulus* may develop strategies to selectively inhibit host antimicrobial defense to establish the infection. Additionally, the toxic substance released by *Argulus* may be damaging to the liver tissue, which is the primary site of C3 synthesis, and this can be correlated with the reduced complement activity during heavy infection. This result is in consistent with the study of Saurabh *et al*., 2011 and Kar *et al*., 2015 who reported reduced C3 activity during the different grades of *Argulus* infection. This finding is particularly interesting because C3 transcription is reported to be elevated in most bacterial and parasitic infections (Alvarez-Pellitero, 2008).

Interestingly, the transcription of the TLR22 and C3 genes was differently regulated in the study, concerning the different doses of infection, and more intensive expression changes were observed after the higher dose of parasitic infection. This is in consistent with previously described expressions of immune relevant genes in Rohu (Saurabh *et al*., 2011), goldfish (Lu *et al*., 2013), and Nile tilapia (Zhi *et al.,* 2018), in which differently regulated expression of the genes was observed after a low, moderate and high dose of *Dactylogyrous intermedius* and *Gyrodactylus cichlidarum* infections, respectively. However, a low dose of *G. salaris* infection in rainbow trout did not cause any significant change in the expression of immune-related genes (Jorgenson *et al.,* 2009). But in this study, an up-regulation of the concerned genes was observed. Moreover, a synergistic interaction was revealed in the present study between a higher dose of *Argulus* and a sub-lethal dose of *A. hydrophila*, which is found to suppress the immune expression of challenged goldfish drastically. This mode of interaction between these two pathogens at the particular dose might be ascribed to the immunosuppression caused by infection of either species, since significantly down-regulated TLR 22 and C3 expressions were observed. An event of immunosuppression following a high dose of *Argulus* might have facilitated an apparent increase in susceptibility to *A. hydrophila*. In the same manner, goldfish infected with *D. intermedius* had down-regulated the cytokine TGF-β expression and became more susceptible to *F. columnare* infection (Zhang *et al*., 2015). Also, down-regulation of immune genes was observed during the co-infection of two monogenean parasites in Nile tilapia (Zhi *et al*., 2018).

When co-infected with a lower dose of *Argulus* and sub-lethal dose of *A. hydrophila,* a rise in the expression pattern of TLR22 and C3 genes were observed to occur, especially during the early phase. We hypothesis that the presence of low or moderate parasite intensity could enhance the immune response in the studied fish and a higher dose of infection may down-regulate the immune response due to the immunosuppressive effect. Difference in infectious dose of pathogens in the study may have serious consequences on the virulence of pathogens infecting a common host. Increasing the dose of *Argulus* not only accelerated the frequency of co-infection, but also altered the goldfish immune response allowing the easier colonization by *A. hydrophila.*

Abiotic environmental conditions, particularly water temperatures above or below the physiological optimum of fish, might change the immune response and increase infection susceptibility (Abram *et al.*, 2017), while the temperature mediated physiological stresses may compromise host resistance and alleviate the frequency of opportunistic infections (Harvell *et al.*, 1999). As a result, infections become more harmful to hosts, resulting in high mortality, especially in multi pathogen infections (Macnab and Barber 2012). Numerous studies have conclusively shown that pathogenicity increases with increase in temperature during infection by single pathogens (Gilad *et al.,* 2003; Kim *et al.*, 2010; Cutuli *et al.,* 2015). Though temperature has been advocated as an important attribute in many epizootic events, there is a scarcity of studies on the impact of temperature on co-infection in fish. The host immune strategies to counteract *Argulus-Aeromonas* confection has been already addressed in the primary objective of the present study. To fill the gap of the interaction of temperature and its contribution towards host immune resistance and disease susceptibility in co-infections, the second objective of the present study was envisaged within a co-infection framework, where moderate parasite infestation was followed by a sub-lethal dose of bacteria at varying temperatures.

5.5 Mortality

Several authors have suggested that an increase in temperature will enhance the multiplication of bacteria and parasites which has a direct impact on the degree and nature of pathology experienced by hosts (Rahman *et al.,* 2001;

Karvonen *et al*., 2010; Callaway *et al.,* 2012). There was an increasing trend in mortality was linear with the temperature in this study. In the present investigation, the increased mortality observed in the co-infected group of fish at high temperature could be attributed to the increased virulence of the bacterium and parasite at changed ambient temperatures for fish survival and metabolic activities (Cutuli *et al.,* 2015). Aquatic pathogens do need specific temperature optima for the completion of their life cycles (Marcogliese, 2001). Elevated temperatures may enhance parasite energetic needs, enzymatic activity, and growth rates, as well as parasite hatching rates, thus leading to over-exploitation of the host (Barber *et al*., 2016). Moreover, temperature acts as a significant environmental element that influences protein secretion or gene expression in bacteria, which facilitates their rapid proliferation (Shapiro and Cowen, 2012). As evidenced in some bacterial epizootics, outbreaks occur when the water temperature drops to a certain value like cold water vibriosis, (Enger *et al*., 1991), cold water disease (Cipriano and Holt, 2005), Red-mouth disease (Fernandez *et al*., 2007) and on another hand, increased frequencies of disease episodes at elevated temperatures such as Lactococcus (Vendrell *et al*., 2006), hemorrhagic septicemia (Austin and Austin, 2007), and edwardsiellosis (Mohanty and Sahoo, 2007) have been reported. The Argulus parasite will multiply more frequently at higher temperatures, and the intensity of their attachment to fish will also increase (Kumari *et al.,* 2019; Saha and Bandyopadhyay, 2015; Sahoo *et al*., 2013). However, a decreased intensity of *Argulus* infestation was observed in the study at lower experimental temperatures. Several studies have found that increasing water temperatures affect the incubation time of Argulus eggs as well as juvenile and adult development rates (Hakalahti *et al*., 2006). This may be the probable reason for the decreased intensity of *Argulus* in the group exposed to low temperature (23°C). In line with these observations, several authors have reported a low prevalence and intensity of *Argulus* infection during post-monsoon season (low temperature), and a higher prevalence in summer (high temperature) (Kumari *et al*., 2019; Saha and Bandyopadhyay 2015; Trujillo *et al*., 2018).

5.5.1 Haematological parameters

Haematological parameters are often used as important indices to the health status of fish (Islam *et al*., 2019).Measuring the parameters of blood cells

can determine the physiological changes induced by stressors (Wu *et al.,* 2015). It Higher temperature fluctuations can lead to reduction in blood cell counts in teleost (Qi *et al*., 2013; Cheng *et al.,* 2017). Temperature was observed to influence certain haematological parameters in the current co-infection experiment, including hematocrit value, RBC, and WBC counts. Several studies have found that as the temperature rises, the oxygen affinity of the blood decreases, the affinity of Hb to bind oxygen decreases, and the count of RBC and dependent components such as Hb and PCV rises (Ashaf-Ud-Doulah *et al.,* 2019; Langer *et al*., 2013; Radoslav *et al*., 2013). In the current study, control fish exposed to higher temperatures had significantly greater RBC, Hb, and PCV levels than control fish housed at lower temperatures.

However, when compared to their respective temperature controls, the entire co-infected group had lower Hb, RBC, and PCV values in the current study. Similar to this observation, a significant decrease in RBC and hemoglobin concentration and the occurrence of anemia was reported in channel catfish co-infected with *Ichthyophthirius multifiliis* and *Edwardsiella ictaluri* (Shoemaker *et al.,*2012), and *Labeo rohita* co-infected with *A. hydrophila* and *A. salmonicida* (Roy *et al.,* 2019). The loss of peripheral blood from capillaries and arteries, as well as depression/exhaustion of the fish's hemopoietic potential, can be ascribed to the reduction in RBC and related parameters in co-infected fish (Ray *et al*., 2016). In addition, reduction in RBC, Hb, and Hct values and the occurrence of anemia are associated with *Argulus infection,* since they feed on blood, mucus and epithelial cells using the suckers and proboscis (Shimura, 1983; Saurabh *et al.,* 2010; Kumar *et al*., 2013; Patra *et al.,* 2016). Similarly, decreased levels of RBCs, and PCV were noticed in rainbow trout, carp, Nile tilpia, and *Channa striatus*, injected with *A. sobria* and *A. cavie* (Rehulka *et al*., 2002), *A. hydrophila* (Harikrishnan *et al*., 2003), *Streptococcus iniae* (Chen *et al*., 2004) and *A. hydrophila* (Podeti *et al.*,2017). As previously stated, low temperatures affect the *Argulus* parasite's multiplication and intensity, which could explain why *Argulus* had a smaller influence on RBC values in co-infected fish exposed to low temperatures compared to other co-infected groups in the current study. Furthermore, during the course of infection, a considerable rise in RBC was detected in co-infected fish at a lower temperature (23°C), indicating a decreased prevalence or null effect of *Argulus* attachment. In comparison to other

groups exposed to lower temperatures, co-infected fish exposed to a higher temperature (32°C) showed a significant reduction in RBC, PCV, and Hb levels. Higher temperatures encourage *Argulus* infestation and active feeding behaviour, resulting in blood loss in the host, which, in combination with bacterial infection, may have led to the co-infected fish's lower RBC and hematocrit levels. As a result, the current study shows that temperature has a different influence on goldfish haematological alterations following co-infection with *Argulus* and *Aeromonas hydrophila*.

In the *Carassius auratus* control group (without co-infection), there was a temperature-dependent rise in WBC, neutrophils, and monocytes. The increase in WBC and innate and adaptive immunity of fish may help them survive at temperatures higher than their optimum (Rahman *et al.,* 2019), and an increase in neutrophil number in the higher temperature group also indicates immune system disturbance, which could lead to infectious disease in thermally stressed fish (Shahjahan *et al*., 2018). Ellis (1991) found that white blood cell count could be used to evaluate some diseases and injuries in the fish body, such as the rearing environment affecting the number of circulating leukocytes. It was found that there was an increase in the white blood cell count in rainbow trout (*Oncorhynchus mykiss*) (Houston et al., 1996) and carp (Engelsma *et al.,* 2003) at elevated temperature and after cold shock, respectively. Ndong *et al*. (2007) found that when Mozambique tilapias (*O. mossambicus*) was transferred from 27°C to 19°C or 35°C, white blood cell counts decreased significantly. WBC counts of sea bass *Dicentrarchus labrax* and tench were significantly elevated at suitable water temperature in summer than in winter (Pascoli *et al*., 2011). During parasite and bacterial infection, there is a rise in WBC, neutrophils, and monocytes (Lebelo *et al*., 2001; Sebastiao *et al.,* 2011*;* Panjvini *et al*., 2016). In the present study, the result of differential WBC count revealed that percentage of all types of cells *viz*. neutrophil, lymphocyte, and monocyte increased significantly among the co-infected groups exposed to 23 and 28°C temperature compared to their respective control group, that the innate immunity of the fish indicated that the body elicited immune response to fight against the pathogens as the primary line of defense (Sovlo *et al*., 1981). Roy *et al*. (2019) reported an increase in leucocytes among the co-infected fish, which produced a huge

amount of leucocytes to protect the fish by phagocytosis and the antibacterial chemicals to stop the pathogens spread in the system.

In contrast, a reduced WBC, neutrophil, and monocyte count was observed in co-infected fish exposed to high temperatures during the progression of infection. Ambient temperature fluctuations could affect the animal's physiological state causing animal stress and resulting in the “general adaptation syndrome” (Selye, 1950; Iwama *et al.,* 2004). In the teleost fish, any change in the culture water temperature could affect the fish survival, physiological conditions, and immune responses (Bowden, 2008; Li *et al*., 2014).In channel catfish, the *in vitro* cell proliferation of leukocytes is inhibited when fish is maintained at 11 °C (Bly and Clem, 1991). Suboptimal temperatures adversely impact B lymphocytes in rainbow trout (Kollner and Kotterba, 2002). Temperature decrease from 25° to 16 °C over 3 h reduces significantly the B-lymphocytes counting in common carp (Engelsma *et al*., 2003). In spite of particular differences in the responsiveness, T-lymphocytes are also reactive to temperature decrease in channel catfish. The counting of T-cells in fish maintained at 11 °C is lower compared to 24 °C (Bly and Clem, 1991). It appears plausible to infer that the relationship of increasing temperature and greater pathogenicity is related to lymphocytic cell fatigue, rendering the fish more immunocompromised to infection and so reducing disease resistance (Barbosa *et al.,* 2020). Therefore, decrease in temperature can impact lymphocytes and the teleost immune system as a whole.

5.5.2 Innate immune parameters and antioxidative stress enzymes

The temperature affects several physiological and immune parameters of fish (Tort *et al*., 2004). Further, the respiratory burst activity of phagocytes, antimicrobial activity of myeloperoxidase enzyme, and the bactericidal activity of lysozyme have been used frequently as indicatosr of nonspecific immunity in fish (Anderson and Siwicki, 1995; Ellis *et al*., 1999). Non-specific humoral defenses in teleost are considerably similar to those in mammals (Yano, 1996). Lysozyme and complement are relevant biomarkers of the innate immune response in fish. A more detailed study on the immune functioning at the cellular level was focused on different attributes such as MPO, lysozyme, NBT, antioxidative stress

enzymes like catalase, oxidase, and Gpx to gain insight into the interaction of temperature and co-infection on the immune system functioning of goldfish. All these targeted attributes had a different role in host immune responses against pathogen invasions. The neutrophil enzyme myeloperoxidase generates antimicrobial and immunomodulatory compounds, notably hypochlorous acid (HOCl), amplifying their capacity to destroy pathogens and regulate inflammation (Kettle and Winterbourn., 1997; Buchan *et al*., 2019). By breaking glycosidic connections inside the peptidoglycan layers, lysozyme breaks bacterial cell walls and stimulates phagocytosis (Harrison., 1991; Saurabh and Sahoo., 2008). It is well established that the immune system of fish can be severely affected by various stress conditions. Some stressful environmental factors influence the activity of lysozyme. For example, the water temperature seems to affect negatively the lysozyme concentration in plasma (Langston *et al.*, 2002). It reduces drastically the level of serum lysozyme in plaice (Fletcher and White, 1973) and carp (Studnicka *et al*., 1986) among other species. However, the effect of low water temperature varies even into different strains of the same species (Langston *et al*., 2002). Usually, several stress sources are immunosuppressive (Saurabh and Sahoo, 2008). In the present study, increase in a respiratory burst, MPO, and lysozyme activity observed in the experimental co-infected fish exposed to 23 and 28°C can be related to a higher number of phagocytic cells and potential induction of the phagocyte respiratory burst and lysozyme activities against the pathogen attack (Marnila and Lilius, 2015). The non-specific cellular humoral response of fish subjected to temperature stress has been studied in a number of ways. For example, after lowering the temperature from 18 to 11°C at a rate of 1°C per day, both serum complement and lysozyme activity of sea bream *S. aurata* considerably decreased (Tort *et al*., 2004). After being transported from 27 to 19 °C for 12-96 hours, both lysozyme activity and ACH50 were drastically reduced in tilapia (Ndong *et al.,* 2007). Increased lysozyme activity was linked to a increased number of leucocytes in Atlantic halibut exposed to higher temperatures (Langston *et al*., 2002). Several authors have also reported an elevated NBT, MPO, and lysozyme activity during bacterial (Biller-Takahashi *et al*., 2013; Xia *et al*., 2017; Lazado *et al*., 2018; Khan *et al*., 2018) and parasitic infection (Souza *et al*., 2019; Kumar *et al*., 2017; Chettri *et al*., 2009) signifying their role in destruction and elimination of invading pathogens

(Sharma *et al*., 2017). Rapid temperature fluctuations cause severe physiological stress in fish (Crawshaw, 1979) and eventually lead to variations in immune response (Lillehaug *et al*., 1993).

Similarly, oxidative stress in fish is also affected by water temperature (Bagnyukova *et al*., 2007). During phagocytosis, leukocytes consume more intracellular oxygen, resulting in the creation of numerous reactive oxygen species, which improve the antioxidative stress enzyme pathway's functioning, resulting in improved antioxidant enzyme activity (Biller and Takahashi (2018). SOD catalyses the dismutation of superoxide radical (O^{2-}) into H_2O_2 and O_2 to protect the cell from oxidative damage, which explains the considerable increase in SOD activity found in the co-infected group exposed to 23 and 28°C (Barreiros *et al*., 2006). Furthermore, the considerable increase in catalase and GPx activity could be linked to their function of catalysing the decomposition of H_2O_2 into H_2O and O_2, thereby maintaining the perfect balance of ROS generation and removal required for the innate immune system's proper functioning (Wang *et al*., 2013). Several studies have reported oxidative stress response related to *Argulus* and *A. hydrophila*, which are in agreement with the findings of this study (Xia *et al*., 2017; Abdel-Magid *et al*., 2019*;* Kumar *et al*., 2013; Saurabh *et al*., 2010). High temperature within the physiological range of the fish species could affect the immune function (Bly and Clem, 1992). For example, exposure of Catfish (*Heteropneustes fossilis*) to high temperature leads to mitochondrial superoxide (O2) production in the gills (Prakash *et al*., 1998) and induced antibody activity in Atlantic cod, *Gadus morhua* L. (Magnadottir *et al*., 1999). Exposure of Atlantic halibut to high water temperature did not affect phagocytosis, but inhibited the immune response against bacteria (Avtalion, 1981). Elevated environmental temperature causes metabolic activation and increased oxygen consumption, which may stimulate ROS production, cellular constituent oxidation, and the antioxidant-associated enzyme system's response (Lushchak and Bagnyukova 2006). This could be the rationale for the enhanced antioxidant enzyme activity seen in high-temperature fish groups compared to low-temperature fish groups. Increased catalase activity has also been documented in *Liza ramada* (Madeira *et al.,* 2013) and European seabass (*Dicentrarchus labrax)* (Vinagre *et al.,* 2012), increased levels of lipid peroxides and superoxide dismutase (SOD) activity in *Carassius auratus* were reported at

higher temperatures (Lushchak &Bagnyukova, 2006). As discussed above, in normal conditions, ROS production and elimination are well managed in regular cell metabolism. Although this balance between ROS production and antioxidant defense system is disturbed due to environmental stress like higher temperature leading to ROS overproduction which has toxic effects on cells (Achard-Joris *et al*., 2006; Qiu *et al*., 2011). This can be a possible reason for the reduced antioxidative stress enzyme activities observed in the group exposed to co-infection and higher temperature as the overproduction of ROS leads to damage the cellular metabolites such as protein, lipids, and DNA which eventually cause impaired cellular functions and thus making the host more immunocompromised (Biller and Takahashi., 2018).

5.6 Conclusion

The effects of different doses/combinations of infection observed in the present study strongly suggest synergistic effect of pathogens on a common fish host. The present study further affirmed that parasite infection facilitates increased colonization and virulence in subsequent bacterial infections. Higher numbers of *Argulus* not only accelerates the frequency of co-infection, but also alter the goldfish immune response allowing the easier colonization by *A. hydrophila* resulting in a synergistic negative impact on the health of goldfish. As observed in many previous studies, higher mortality patterns and increased appearance of clinical signs were observed in heavily infected experimental groups. There were enhanced immune responses in all co-infected groups except that in heavy parasitic infestations. The study indicated a decreased immune response as well as a lag in immune gene expressions at higher parasitic doses. The possible immune suppressions and parasitic evasion mechanisms can be attributed to such a decreased immune response. In contrast to most of the prior studies that focused on the impact of temperature on the immune system of fish using a single pathogen, the present study investigated the importance of "temperature" as a critical abiotic factor on the modulation of the innate immune system and infection pattern, determining the severity of co-infection by *Argulus* and *Aeromonas hydrophila* in goldfish. An increase in temperature not only accelerates the intensity of co-infection as observed in the present study, but also causes a major imbalance in the health status of the fish by debilitating the immunological and physiological parameters towards disease susceptibility. Further, the combined amount of energy spent on resistance and tolerance against the elevated temperature and co-infection might contribute to increased mortality. Thus, the study becomes more relevant in the prevailing in climate change scenario, wherein infection with multiple pathogens is more likely to occur in climate-vulnerable natural ecosystems.

6. SUMMARY

Aquaculture is a fast growing food production sector, which is expected to pay a central role ensuring food safety in the developing world (FAO, 2020). Fish farming involving ornamental fish is also a major industry and is an attractive sector for potential entrepreneurs. The success of aquaculture depends on the balance among the health and physiology cultured fish, the environment, and the pathogens in the farm environment that include parasites, bacteria and the viruses. Freshwater ornamental fish farming is often prone to several infections caused by bacteria, fungus, parasites, and even viruses. Fish diseases often occur following an initial stressor and of the ensuing infections generally involve more than one pathogen in the aquatic environment (Kotob *et al*., 2017). The susceptibility of fish to different pathogens may also alter due to the change in water temperature, resulting in sudden disease outbreaks. Temperature plays a major role in establishing or maintaining an infection, and also has a direct effect on pathogen metabolism. Similarly, the multi-pathogenic infections might alter the host's immune response to subsequent infections by other pathogens either by suppressing or by priming the immune system (Kotob *et al.,* 2017). This can lead to a subsequent increase in the severity of infection with a higher mortality rate (Eissa *et al*., 2013). Since the detrimental effects of bacteria and parasites are exacerbated when their hosts are stressed, it is relevant to determine the combined effects of abiotic stressors such as water temperature and biotic stressors such as parasites and bacteria.

The present study aimed to investigate the effect of prior parasitism by *Argulus spp* on infection by the bacterial pathogen *A. hydrophila, and the effect of* h varying temperatures on the susceptibility of goldfish (*Carassius auratus) to combined infection by the bacterium and the parasite.*

In the present study, *Aeromonas hydrophila* isolated from diseased goldfish and characterized using biochemical and molecular techniques was used as the bacterial pathogen. The *Argulus* parasite isolated from goldfish and identified as *Argulus japonicus* by analyzing the morphological traits was used in the infection studies. The experimental infection of *Argulus japonicus* was done by two methods, viz, co-habitation method and exposing the egg clutches to healthy goldfish. The results of the co-habitation method showed an average intensity of 15± 6 parasites per fish, whereas in the second challenge method using eggs of *Argulus* showed a

hatching percentage of 60 to 70% with an average of 24 juvenile parasites per fish. Based on the observed intensity of infection through the co-habitation method, three levels of parasitic infection defined as low (1–10 lice fish-1), moderate (10–20 lice fish-1), and high (>20 lice fish-1) were identified. The experimental fish were divided randomly into eight treatment groups and the fish of each grade of parasitic infection received intraperitoneal injection of a sub-lethal dose of *A. hyrophila*. Along with the co-infection groups, a single dose of bacteria and an un-infested control group were also maintained. After the bacterial challenge, four fish from each treatment group are subjected to hematological, innate immune parameters, antioxidative stress enzymes, and immune gene transcription analysis. The mortality rate was also observed for 7 days. The co-infection groups expressed a higher mortality rate than the single pathogen-infested group. Among the co-infected groups, the group which received a heavy dose of parasite and a sub-lethal dose of bacteria showed a reduction in hematological parameters, innate immune parameters, and antioxidative stress enzymes. Also, a down-regulation of immune genes TLR22 and C3 was observed in the group infested with a heavy dose of parasite and sub-lethal dose of bacteria. Furthermore, to determine the effect of temperature on co-infection, the experimental fish were exposed to moderate grade of co-infection with a sub-lethal dose of bacteria, along with the varying temperature. To elucidate the effect of three different water temperatures viz. 23, 28, and 33°C, the experimental fish were divided into six treatment groups consisting of the fish exposed to experimental temperature alone as the control and the groups of fish exposed to both co-infection and temperature. One set of all treatment groups were maintained separately for observing the mortality and the intensity throughout the experimental period. A temperature-dependent increase in the intensity of *Argulus* was observed in goldfish. Similarly, the experimental group exposed to higher temperatures experienced higher mortality than those exposed to lower temperatures. For the haematological and enzyme parameter analysis, sampling was done at 24, 72, and 168h post-challenge in all experimental groups. Temperature-dependent increase in RBC, Hb, and PCV values of *C. auratus* was observed in each of the control groups; however, there was a significant ($p<0.05$) decrease of RBC, Hb, PCV, WBC, neutrophil, and monocyte values in co-infected fish exposed to 33°C during the progression of infection. Similarly, a temperature-dependent increase was observed in innate immune

parameters and anti-oxidative stress enzymes in each of the control groups, whereas, a reduction was observed in the co-infected group exposed to a higher temperature.

In the present study, effect of a dose-dependent co-infection of *Argulus* and *Aeromonas hydrophila* along with varying temperature in the innate immune response of goldfish was studied. Increasing the dose of *Argulus* and change in temperature not only accelerate the frequency of co-infection but also alter the goldfish immune response, allowing the easier colonization by *A. hydrophila,* thus resulting in a synergistic effect on the health of goldfish. To conclude, this study reveals that water temperature is an important abiotic factor that has a significant bearing on the modulation of the innate immune system of fish, susceptibility of fish to infection and the severity of co-infection involving *Argulus* and *Aeromonas hydrophila* in goldfish.

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