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CONTENT

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ABSTRACT

Argulosis is one of the constrain in ornamental fishery as ectoparasite infestation. *Allium sativum* is recognized as a potential anti-parasitic agent against *Argulus japonicus*. The present study was aimed to investigate anti-parasitic effect of *Allium sativum* against *Argulus japonicus* in *Carassius auratus* recorded after 5 days. The parasite was cultured under laboratory condition to infest the host. Host fish was concurrently subjected to *Allium sativum* at the rate of 0.125g/L, 0.25g/L and 0.5g/L. Morphometric, growth, survival, biochemical, histopathological, haematological and micropathological indices were recorded after the challenge test. *A. sativum* reduced intensity of infestation(0.62$\pm$0.38), behavioral morbidity and increased the parasite mortality rate in addition to the haemotological and biochemical indices of the host. However, increasing the concentration to *A. sativum* to 0.5g/L was found to be fatal resulting in declining survival rate (84.00$\pm$21.90). Low concentration of *A. sativum* (78.00$\pm$3.74) stimulated feed intake. Overall, 0.25g/L of *A. sativum* offered to *C. auratus* was found to be optimum, promoting the growth rate during the *A. japonicus* challenge test.

Keywords: *Allium sativum*, *Argulus japonicas*, *Carassius auratus*; ecto-parasite, ornamental fishery.

INTRODUCTION

India is rich in freshwater fish resources (Sandilyan, 2016), thus aquariculture in India is contributing about 1% of the global ornamental fish trade with an export of 69.26 tons pricing 566.66 crores in 2014-15. Major exports are from Kolkata, Mumbai and Chennai (Ahilan *et al*., 2008). North-eastern regions contribute a lot for native species. Moreover,

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300 exotic ornamental species are in trade out of which, 200 species are exclusively bred within the country. The Goldfish, *Carassius auratus* is used and valued as ornamental fish for aquarium and ponds is edible but rarely eaten as food. It is also used as an experimental species. As a member of the minnow family, *Cyprinidae*, native to East Asia, the fish is an omnivorous forager (Maitland, 2004) and is susceptible to many ectoparasites and endoparasites including the gill monogean parasite (Ekanem *et al*., 2004), *Trypanosoma, Dactylogyrus sp., A. foliceus, Trachelobella torquata, Contracaecum sp* (Tekin-Özan and Kir, 2005) and *sphaerospora sp* (Eszterbauer and Székely, 2004). *A. japonicus* is an obligate crustacean ectoparasites of the genus *Argulus* causing Argulosis and are also known to be vectors for several viral, bacterial, fungal and nematode diseases (Walker, 2008). *Allium sativum*, a perennial plant is reported to have antimicrobial activity against many genera of bacteria (Tsao, and Yin, 2001), fungi and viruses. *Allium sativum* also contains a higher concentration of sulfur compounds that are responsible for its medicinal effects. In aquacultural operations, garlic promotes growth, enhances immunity, stimulates appetite, and strengthens the control of bacterial and fungal pathogens (Shakya, and Labh. 2014). Therefore in the present study the efficacy of *Allium sativum* in 3 different concentrations was tested against the infestation of *Argulus japonicus* in *Carassius auratus*.

MATERIALS AND METHODS

Sample collection

A total of 100 *C. auratus* were collected from the local aquarium shops in and around Coimbatore district. Healthy fishes of 5.58$\pm$ 0.26 cm in length and 2.60$\pm$0.32 gm in weight were selected for the study. Selection was ensured for devoid of *Argulus japonicus* infestation in *C. auratus*.

Transportation and maintenance of collected specimens

The collected specimens transported to the research area in oxygenated plastic bags. They were acclimatized under laboratory condition with atmospheric temperature of 28°C, water temperature of 25°C and water pH of 7.5 for 7 days. Maintenance of the collected specimens were carried out in plastic containers of 19 x 35 (L X W) diameter. Continuous aeration provided throughout the acclimatization and experiment. To avoid starvation and the following misconception, the fishes fed *ad libitum* with commercial feed pellets of 0.3mm in size.

Morphometric indices

The total length was measured in centimeters (cm) and the weight was measured in gram (gm). Healthy fishes of 5.58$\pm$ 0.26 cm in length and 2.60$\pm$ 0.32 gm in weight were selected for the study. The study was carried out irrespective to sex selection.

Experimental setup

100 *C. auratus* were divided to 4 groups for triplicate analysis. *A. sativum* was offered to *C. auratus* on a regular basis of one serving / day. 0.125 g *A. sativum* was offered per litre of water in group - 1. 0.25g of *A. sativum* was offered per litre in group - 2. 0.5g of *A. sativum* was offered per litre of water in group-3. The group which was not offered *A. sativum*, served as control. Water was renewed daily. The *C. auratus* were fed *ad libitum* with commercial feed pellets. The amount of unfed feed was checked in the third hour of the day to calculate the amount of feed intake. To each group the parasite *A. japonicus* was introduced at the rate of 1:1. The experimental setup was kept undisturbed throughout the experiment of 5 days. The analyses were done after 5 days of the trial. The parasite mortality and host survival rate was also checked during the trial.

Feed intake rate

Feed intake rate was calculated using the formula:

$$\text{Feed intake rate (\%)} = \frac{\text{Amount of unfed feed–Amount of feed offered}}{\text{Amount of feed offered}} \times 100$$

Feed was offered as floating food pellets. Pellets of 3 mm in size was used. Pellets were offered in numbers, considering the dis-intergity of the food pellets in water over time. Unfed feed pellets were enumerated after 3 hours of feeding. Feeding was *ad libitum* to eliminate any misconception.

Host survival rate

Host survival rate was calculated using the formula:

$$\text{Survival rate (\%)} = \frac{\text{Total number of fishes –Number of dead fish}}{\text{Total number of fish}} \times 100$$

Parasite mortality rate

The parasite mortality rate was calculated by using the formula:

$$\text{Mortality rate (\%)} = \frac{\text{Total number of parasites –Number of alive parasites}}{\text{Total number of parasites}} \times 100$$

Intensity of infestation

The intensity of infestation was calculated using the formula:

$$\text{Intensity of infestation} = \frac{\text{Total number of parasites examined in 1 host}}{\text{No. of infected host}}$$

Behavioral Morbidity

Behavioral Symptoms of morbidity was studied in *C. auratus* by observing behavioral changes after *A. japonicus* infestation. Symptoms such as repeated rubbing of fins, lethargic, restlessness are to be observed.

Biochemical Indices

Proteins, carbohydrates and lipids in the muscle of *C. auratus* were estimated after 5 days of challenge test. Proteins were estimated using Lowry *et al*., (1951); Carbohydrate content of the sample was estimated by Roe *et al*., (1955); Lipids were estimated by Folch *et al.,*1957.

Haematological indices

0.5ml of blood from each fish was collected using 2mm gauze needle pre rinsed with anticoagulant. The collected blood was transferred to a sterile vial of 1ml, pre-rinsed with the anticoagulant heparin. The blood was collected using heart puncture method. The withdrawn blood was subjected to various haematological parameters such as RBC count, haemoglobin estimation, WBC count (lymphocytes & monocytes), platelet count and plateletcrit volume.

Histopathology of *A. japonicus* in *C. auratus*

Histological sections of epidermis, fins and skeletal muscles showing observable pathological symptoms, were done using microtome (Leica RM 2125) and stained with Haematoxylin and Eosin. Photomicrographs were taken from a compound microscope (Labomed CXR 2) under 40x illumination and interpretations were made.

Micropathology of *A. japonicus* in *C. auratus*

Mucus was scrapped from the infected epidermal region of the fish and subjected to investigation. Fungal infection owing to *A. japonicus* infection was studied by culturing the fungus with Potato Dextrose Agar (PDA) medium. Fungal hyphae stained with Grocotts-Gomori methenamine silver stain method (Grocott, 1955), observed and identified under microscope.

Culture of *A. japonicus*

20 Adult male and female *A. japonicus* parasites were collected from the local aquarium shops in and around Coimbatore district. The species and sex identification was done by the method as described by Kar, (2016) and Radkhah, (2017) (fig-2). Fertilization was allowed in presence of host. After few days, the eggs were laid by the adult female *A. japonicus* on the hard surface of the tank, which were collected carefully and incubated at 37°C in the incubator. The eggs were frequently analyzed under compound microscope for the embryogenesis of developing *A. japonicus*. After 10 days, the eggs hatched out. The hatched out metanauplius was metamorphosed into adult by undergoing 6 repeated moults. An USB digital microscope was

used to study parasite morphology, parasite - host interaction, parasite reproduction (egg laying activity of females, egg morphology).

Statistical analysis

The growth and survival indices, haematological indices and biochemical analysis data were analyzed statistically using SPSS statistics version 20 package software (Zar, 2009). Data were obtained as Mean± Standard deviation.

RESULTS

Behavioral morbidity

The control group, which was not offered *A. sativum*, was found exhibit behavioral morbidity of Argulosis. The fish were found to exhibit symptoms such as flashing, rubbing on tank surfaces, rubbing over aerators in 5 days of infestation.

Histopathology of *A. japonicus* in *C. auratus*

The Control group showed acute infestation with *A. japonicus*. Microscopic comparison was done between healthy dorsal fin of *C. auratus* and infested dorsal fin of *C. auratus* (fig. 3 & -4). As a result haemorrhagic necrosis of dorsal fin (fig. 5) and skeletal muscles (fig. 6) showed dystrophic calcification.

Micropathology of *A. japonicus* in *C. auratus*

The cultured fungi from infected host was identified to be *Mucor mycosis* (fig. 7).

Table 1: Growth and Survival indices of C. *auratus* in a challenge test against *A .japonicus* when treated with *A. sativum* at various concentrations.

Parameters	Control (0 g/L)	Group 1 (0.125g/L)	Group 2 (0.25g/L)	Group 3 (0.5g/L)
Feed intake rate (%)	63.60±15.05	78.00±3.74	66.60±11.73	58.60±34.81
Growth rate (%)	0.00±0.00	6.51±3.12*	7.67±3.45*	4.23±1.67
Host survival rate (%)	84.00±21.90	84.00±21.90	80.00±20.00	64.00±40.98
Parasite mortality rate (%)	0.00±0.00	32.00±36.33*	44.00±26.07	56.00±35.77
Intensity of infestation	1.26±0.36	0.75±0.27	0.66±0.19	0.62±0.38*

Data obtained as a result of triplicate analyses expressed as Mean ± S.D. All values are significant at P≤0.05. *-values not significant at P≤0.05.

Culture of *A. japonicus*

The culture of parasites under laboratory condition provided 250 eggs grown into adult parasites used for the experiment. The developmental stages of the parasites *A. japonicus* includes: Egg, Metanauplius and Adult. An adult female laid eggs in tank surfaces (fig. 8), the eggs were laid in linear strands (fig. 9). Photomicrographs were taken using USB digital microscope. Development of eggs after incubation (fig.10), finally hatched out to Metanauplius resembling like adult (fig. 11).

Table 2: Hematological indices of C. *auratus* in a challenge test against *A.japonicus* when treated with *A. sativum* at various concentrations

	Parameters	Control (0 g/L)	Group 2 (0.25g/L)
RBC	RBC Count (million/ul)	1.20±0.10	1.36±0.06
	Haemoglobin (gm/dl)	2.16±0.15	2.20±0.10
WBC	Lymphocytes (%)	61.00±1.00	64.00±1.00
	Monocytes (%)	6.00±1.00	11.00±1.00
Platelets	Platelet count (lakhs/ul)	97,665.66 ±576.48	1,11,000±1.00
	Plateletcrit volume (%)	0.074±0.001	0.096±0.001

Data obtained as a result of triplicate analyses expressed as Mean±S.D.
All values are significant at $P \leq 0.05$.

Table 3: Biochemical Indices of C. *auratus* in a challenge test against *A.japonicus* when treated with *A. sativum* at various concentrations.

Parameters	Control (0 g/L)	Group 1 (0.125g/L)	Group 2 (0.25g/L)	Group 3 (0.5g/L)
Protein (mg/g)	113.38±0.901	145.81±0.586	164.08±0.01	163.22±0.882
Carbohydrate (mg/g)	65.11±0.055	133.59±0.036	155.15±0.011	145.04±0.010
Lipid (mg/g)	22.80±0.010	38.03±0.015	47.92±0.026	37.92±0.025

Data obtained as a result of triplicate analyses expressed as Mean±S.D. All values are significant at $P \leq 0.05$.

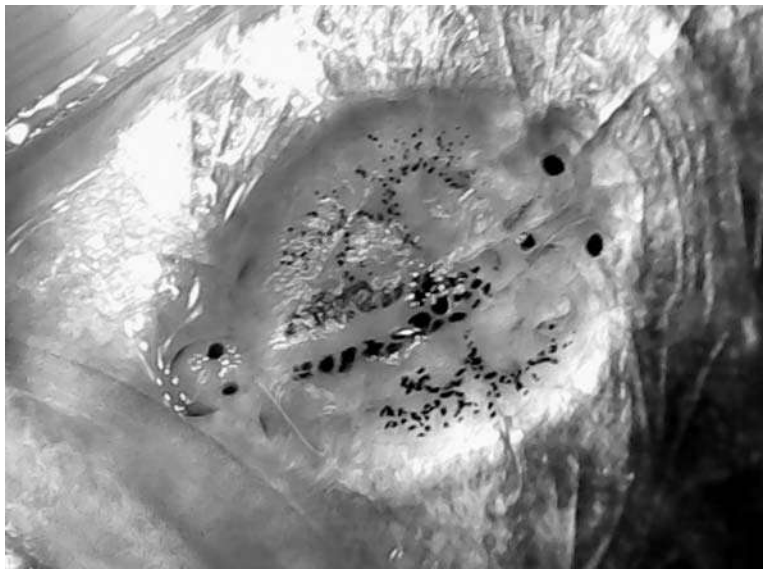

Fig. 1. *A. japonicus* infestation in Epidermis of *C. auratus*

Fig. 2. ventral view of *A. japonicus*
1. Compound eye; 2. Suckers; 3. Proboscis; 4. Abdominal appendages

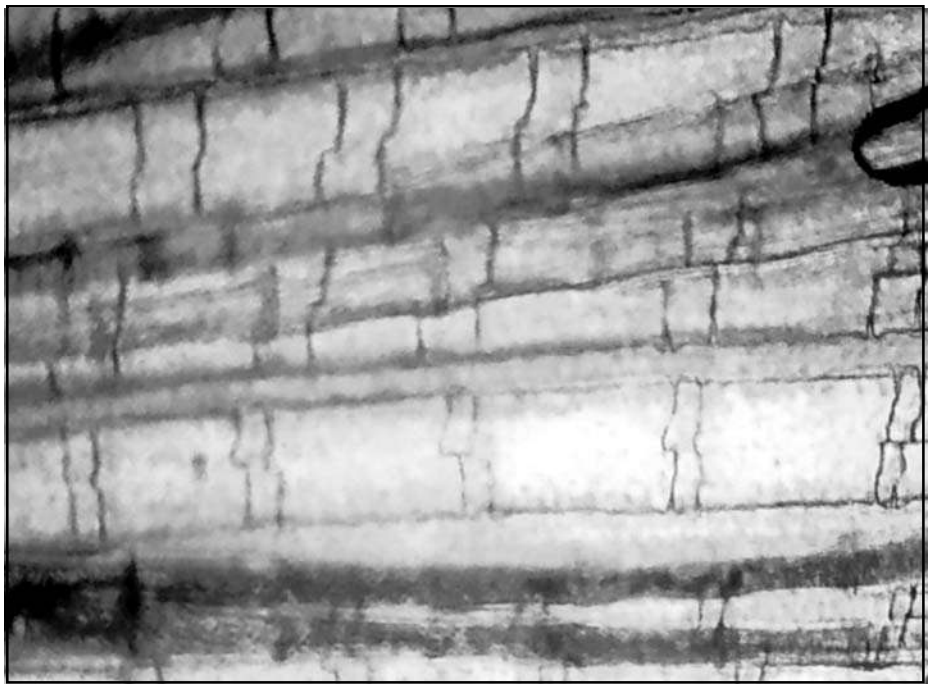

Fig. 3. Microscopic view of dorsal fin in healthy *C. auratus*

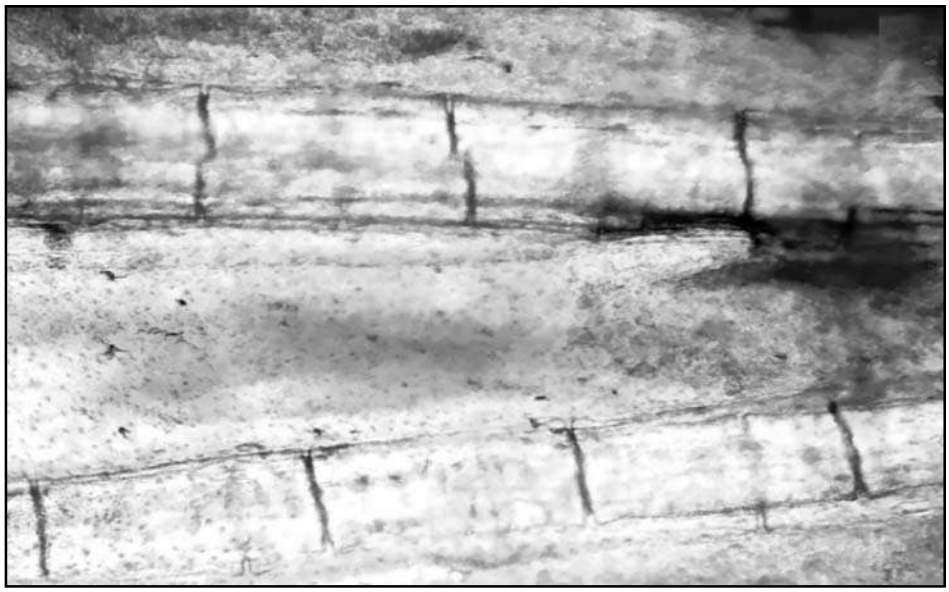

Fig. 4. Microscopic view of haemorrhagic spot in dorsal fin in infested fish

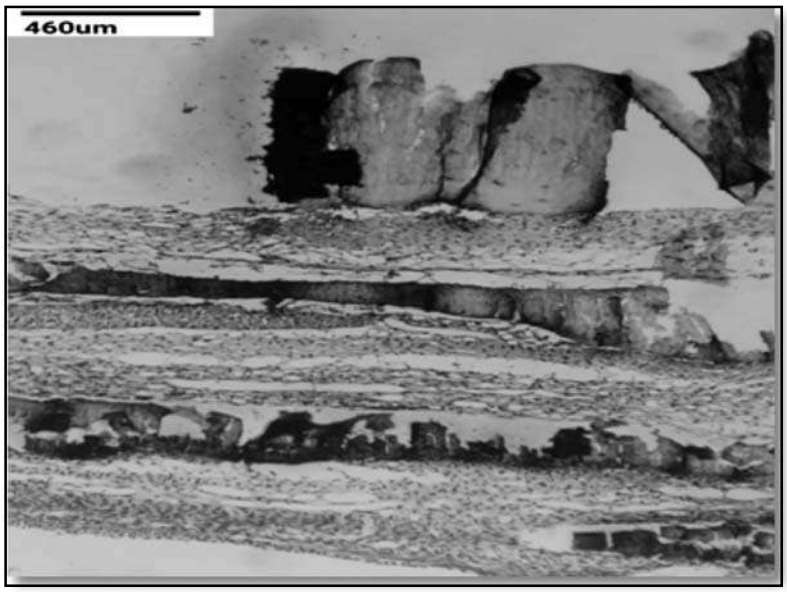

Fig. 5. Histopathology of *A. japonicas* infestation in *C. auratus* showing Dystrophic calcification in dorsal fin

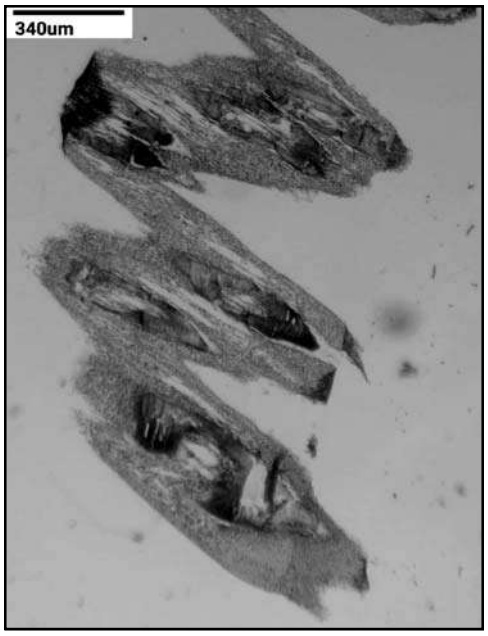

Fig. 6. Histopathology of *A. japonicas* infestation in *C. auratus* Dystrophic calcification in skeletal muscle

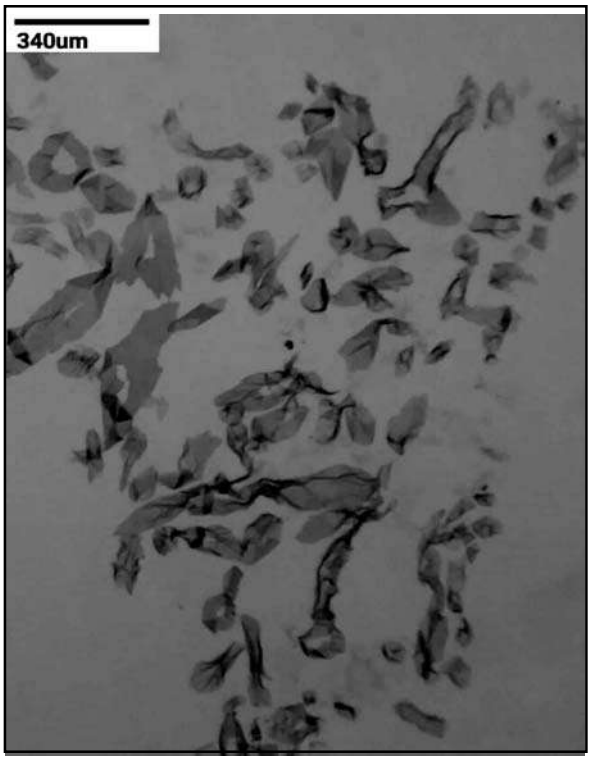

Fig. 7. Micropathology of *A. japonicus* in *C. auratus* Fungus- *Mucormycosis*

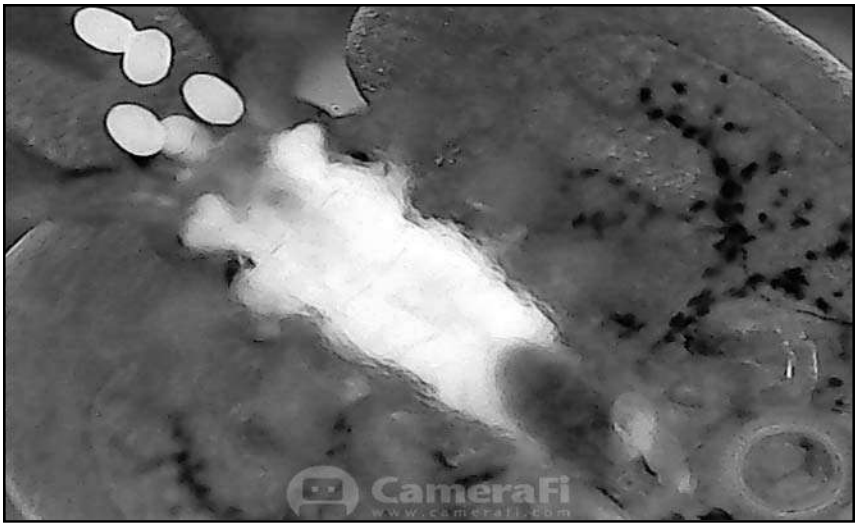

Fig. 8. A female *A. japonicus* laying eggs in tank surface

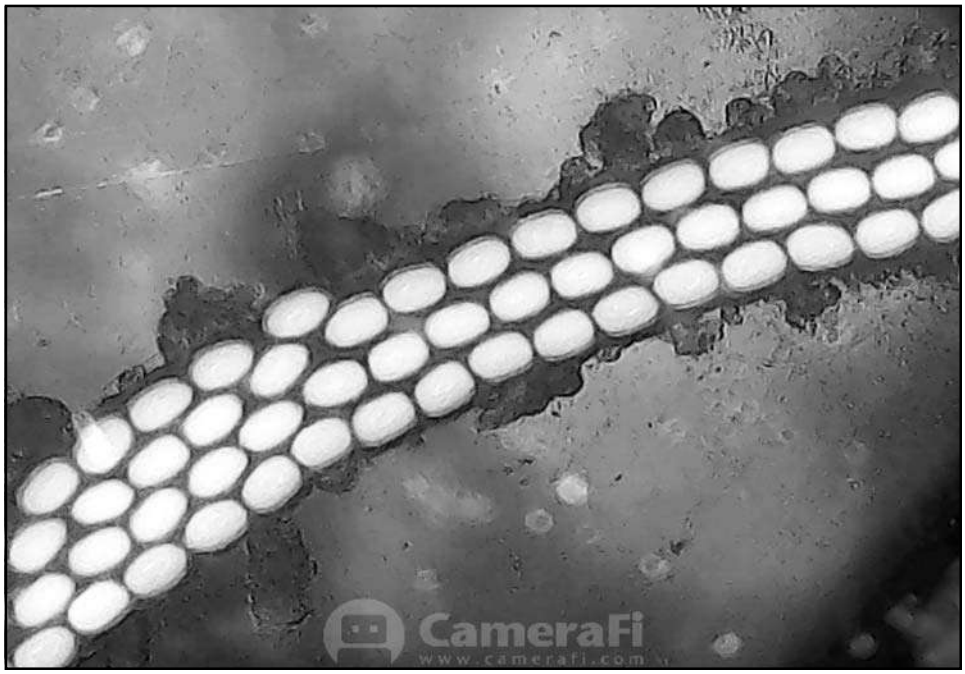

Fig. 9: A strand of *A. japonicas* eggs

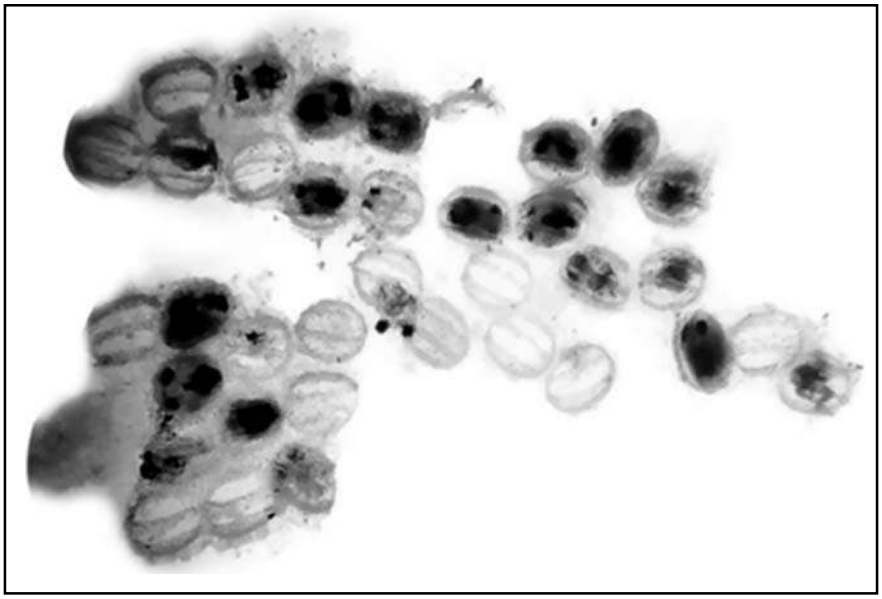

Fig. 10: *A. japonicus* eggs showing embryogenesis

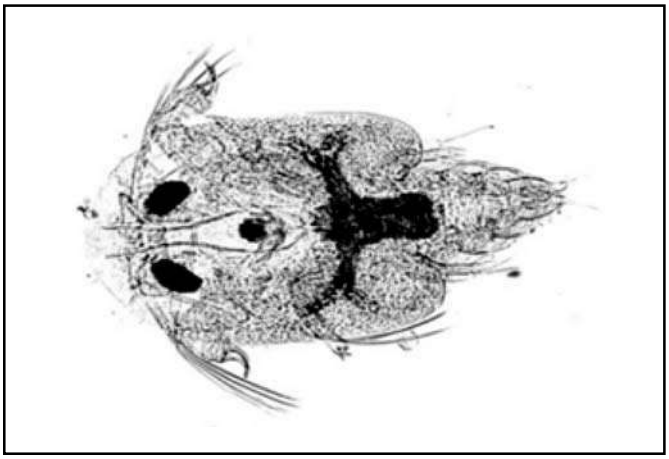

Fig. 11. Post embryonic stage in *A. japonicus* – Metanauplius

DISCUSSION

A. sativum obviously controlled Argulosis infestation by lowering the infestation rate, morbidity rate and increasing the parasite mortality rate. However, *A. sativum* at higher concentration was found to be fatal for *C. auratus.*

A positive correlation is found to exist between increasing *A. sativum* concentrates and host survival index. The increased concentration of *A. sativum* increased the mortality rate of *C. auratus*. However, this is not always true as of Sasmal *et al.,* (2005), reporting no mortality in increasing the concentrations of *A. sativum* paste when mixed with basal feed ingredients and fed to C. *auratus* orally. However, an optimum concentration (0.25g/L) was found to increase the feed intake (66.60±11.73) and the subsequent tissue biochemical (protein-164.08±0.01; carbohydrates-155.15±0.011 and lipid-47.92±0.026) compared to control (protein-113.38±0.901; carbohydrate-65.11±0.055, lipids-22.80±0.010) having only 63.60±15.05% of feed intake. Farahi *et al*., (2010) affirmed that, protein content in *Oncorhynchus mykiss* were significantly higher with optimal level of garlic concentration. Daghar *et al.,* (2017) observed that, 0.5% *A. sativum* is capable to elevate the survival rate of *C. auratus*.

In the present study, the optimum concentration of *A. sativum* resulted in increased RBC count (1.36±0.06 million/ul), haemoglobin (2.20±0.10%), lymphocytes (64.00±1.00%), monocytes (11.00±1.00%), platelet count (1,11,000±1.00/ul) and plateletcrit volume (0.096±0.001%) in group-2 (0.25g/L) when compared to the control (1.20±0.10million/ul, 2.16±0.15%, 61.00±1.00%, 6.00±1.00%, 97,665.66±576.48/ul and 0.074±0.001%). The similar results of Martins *et al.,* (2002), verified the addition of garlic to fish diets increased the erythrocyte number, hemoglobin content, leukocyte number, and thrombocyte number. Moreover, Aly *et al*., (2010) stated that

A. sativum improved the immune response of *O. niloticusvia* a rapid increase in monocytes.

Alternatively, higher concentration of *A. sativum* (0.5g/L) resulted in lesser feed intake (58.60±34.81), growth rate (4.23±1.67) and host survival rate (64.00±40.98) when compared to other groups. The results are parallel with Ndong *et al*., (2007) declaring high concentration of *A. sativum* extract is harmful to fish health resulting in reducing or lowering the survival rate.

Lee *et al.,* (2014) reported higher weight gain, feed efficiency, specific growth rate, protein efficiency ratio and decreased hepatosomatic index with garlic powder supplementation in Starlet Sturgeon (*Acipenser ruthenus*). Moreover**,** increasing concentrations of NaCl is reported to increase survival rate, when compared to the control.

A negative correlation is recorded to exist between increasing *A. sativum* concentration and parasite mortality rate. The parasite mortality rate is 0% for control, 32.00±36.33% for 0.125g/L, 44.00±26.07% for 0.25g/L and 56.00±35.77% for 0.5g/L. Absence of *A. sativum* in control groups, resulted in 0% parasite mortality rate and obviously interfered with the growth rate of the fish showing no observable growth rate in control (0.00±0.00%) in comparison with group-2 (7.67±3.45%). Walker *et al*., (2011) reported a higher off-host survival rate of *A .japonicus* compared *A. foliaceus*. *A. japonicus* is also reported to persist more resistant to starvation at higher temperatures. Presumably, *A. sativum* can also be effective against *A. foliaceus* infestation.

Anti-parasitic effect of *A. sativum* is well recognized in the present study by the reducing intensity of infestation (0.62±0.38) on increasing *A. sativum* concentration (0.5g/L). *A. sativum* is reported to have a negative effect on harmful bacteria and coliformes in the water medium (Shalaby, 2006). *A. japonicus* is reported to cause histopathological changes such as inflammation, epidermis erosion, congestion, ballooning and haemorrage in comet fish (*Carassius auratus*) skin tissues and infestation level of *A. japonicus* was directly proportion with the level of comet fish tissue damages (Aly and Mohamed, 2010). Singh *et al*., (2018) reported seasonal co-relation with *A. japonicus* infestation in cultivable carp fish. In the present study, the infestation rate was directly proportional to the behavioral morbidity and these were all together inversely proportional to the concentration of *A. sativum*. *Argulus* infested *C. auratus* exhibited symptoms of Argulosis including: flashing, rubbing on tank surfaces and rubbing against aerators, which is similar to the report given by Ahamed *et al*., (2016) on pathomorphology of argulosis in freshwater carps. Therefore, the study establish the anti-parasitic effect of *A. sativum* on the parasite *A. japonicus* against the host *C. auratus* by increasing the parasite mortality rate and lowering the intensity of infestation and behavioral morbidity.

CONCLUSION

Argulus japonicus is a major threat to ornamental fish especially to *Carassius auratus*, the most popular ornamental fish across the Globe. “Argulosis,” the state of mortality and morbidity with Argulus can possibly be reduced with *A. sativum*, a well-documented and widely distributed

herb in India. The present study clearly states the efficacy of *A. sativum* in controlling *A. japonicus* infestation, parasite mortality and morbidity, which is attributed to the presence of sulphur compounds in *A. sativum*. Moreover, *A. sativum* at optimum level provides median survival rate and enhances the morphological, biochemical, haematological and infestation resistance. Therefore, it is suggested that an optimum level of 0.25g/L of *A. sativum* reduces morbidity of argulosis in *C. auratus.*

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