Journal of Aquatic Biology & Fisheries | 2021 | **9** | pp. 22-28

ISSN 2321–340X

Regulation of Zebrafish (*Danio rerio*) Tail Fin Regeneration by Temperature Variations

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Abstract

The caudal fin of Zebrafish is one of the most commodious tissues to study regeneration. To investigate the daily changes of caudal fin regeneration in Zebrafish, the selected fishes of size range 4.337±0.71 cm were reared at different temperature treatments *viz.* 22±1°C (T22), 29±1°C (T29), 33±1°C (T33), with three replications of each treatment for 12 days under laboratory condition. Three fishes (n=5, Mean caudal fin length 8.359±0.56 mm) were stocked into each glass aquarium. The different physico-chemical features like turbidity, temperature, pH, dissolved oxygen, total alkalinity, TDS, total hardness, ammonia, EC, and microbial load of water samples were analysed. All surgical procedures were performed under anesthesia, and the amputation of the caudal fin was done using a sterile scalpel blade. The study recorded that length increment (mm) is slower in T22 (0.73±0.12) treatment group, while T29 (1.83±0.35) and T33 (2.02±0.39) shown faster growth. Similar results were also received in length gain percentage where T22 (11.9±2.07) showed lower length gain while two-fold length gain percentage was achieved with those of the T29 (29.67±6.08) and T33 (33.64±6.69) groups. No significant response (P>0.05) was recorded in survival (%) among the treatment groups. The length increment (mm) and percent weight gain were found significantly (P < 0.05) higher in T33 and T29 treatment group compared with the T22 treatment group. The results indicated that high temperature has an overall stimulating impact on the structural recovery of the caudal fin of *Danio rerio* and may help consider the fish as model fish for biomedical studies. Thus, this study brings out the first evidence that higher temperature like T33 is a critical temperature factor that regulates tissue regeneration of the caudal fin of *Danio rerio*.

Keywords: Tissue Regeneration, Amputation, Tail fin, Alternative Animal Model

1. Introduction

Zebrafish (*Danio rerio*), have emerged extraordinary capacity to regenerate fins, scales, retina, spinal cord, and heart (Iovine, 2007). Zebrafish, being given considerable importance as an experimental model due to its accessibility, fast and robust regeneration, its caudal fin is one of the most potent tools for regenerative studies. Zebra's caudal fin comprises several segmented bony rays and inter-ray mesenchymal tissue, all enclosed by an epidermis. The fins' simple architecture consists of numerous segmented bony fin rays surrounded by connective tissues, nerves, and blood vessels (Ferretti and Géraudie, 1995).

Fish, being cold-blooded animals, is affected by the temperature of the surrounding water, which influences the body temperature, growth rate, development, food consumption, feed conversion, and other body functions (Britz *et al*., 1997; Azevedo *et al*., 1998). The temperature has a powerful influence on the transformation of food energy to net energy. Thus, the scope for growth and development increases. It is clear from this that water temperature is a dynamic force in regulating fish growth, development, and maintenance. It has been found that every fish species exhibits a suitable temperature range within which it grows rapidly (Brett and Groves, 1979). Zebrafish caudal fins undergo isometric growth throughout life. Hence it is necessary to understand the regulatory mechanisms for controlling such growth. The fin, which is composed of multiple bony rays, growing autonomously, is made up of bony segments. Each ray consists of is two hemirays that create a protective shell around nerves, blood vessels, and mesenchymal cells. Fin growth occurs through the addition of bone to the distal tip of the fin, and it has been noted that regeneration proceeds through wound healing, mesenchymal disorganization or reorganization, blastema formation, outgrowth, and termination. The biomechanism of regeneration has been widely studied but poorly understood because of its different extents in various animals. Understanding the primary mechanism of regeneration in the wound environment is of high significance because it can lead to an applied possibility of making non regenerating to a regenerating system. Consider the above parameters, and the study was to determine how varied temperature ranges upon the caudal fin regeneration influences.

2. Materials and Methods

2.1 Zebrafish maintenance and regeneration experiments

Wild *Danio rerio* obtained from local farmers was housed and reared under standard conditions in sterile distilled water (RODM 20) (Brand *et al*., 2002). Male and female adult Zebrafish between the ages of 6 to 9 months (size range 4.337±0.71 cm) were selected and designed a consecutive repeated amputation experiment to evaluate whether amputated caudal fin regeneration influences temperature or not. The caudal fin (Mean caudal fin length 8.359±0.56 mm) of all the experimental adult zebrafish

Received on: 02-11-2020; Revised on:01-05-2021; Accepted on: 03-05-2021

was subjected to four amputations every three days interval for a spanning period of approximately 12 days. Caudal fin tissues of the same size were amputated from the distal part of the caudal fin using sterile blades (a little away from the caudal fork) after anesthetizing the animals one by one for 3 to 5 min in 0.1% Tricaine (Sigma). After recovering from anesthesia, each experimental aquarium tank was stocked with five nos. of fish (n=5) with three replicates for each temperature treatment and allowed to regenerate the amputated caudal fin under different conditions of temperature such as 22±1°C (T22), 29±1°C (T29), 33±1°C (T33). Water temperature conditions were regularly monitored throughout the experimental days with an automatic temperature sensor probe. Every two days, water was changed at the rate of 30-50% of the total unit volume with water that had similar temperatures and siphoned to remove the fecal matters. Regenerating fin tissues were further collected by amputating the fins in the same way for different time points (days) such as 1, 3, 6, 9, and 12 to record the total caudal length and released back in the tanks concerned. They were fed twice daily with Tetra bits complete fish food. All fish were individually measured in each experimental setup before and after amputation. The water quality parameters were analyzed every three days (APHA, 2005) in the morning hours. The amputated zebrafish caudal fin on the 0th hour is considered the control (0hpa) and imaged under the stereomicroscope (10 x). The amputated fins are allowed to regenerate for the time mentioned above points. Again, the image of the regenerated tissue was taken individually using a stereomicroscope (please write model) to observe the structural regeneration patterns and evaluated the effect of consecutive repeated amputations on regenerative outgrowth. After that, allowed the caudal fin to regenerate for 12 days to ensure complete regeneration. The remarkable difference among temperature exposure is that the growth parameters such as length increment (mm) and length gain percent have been undertaken. On Day 12, all the survived fishes were counted and recorded. The experimental data were statistically analyzed, and the remarkable difference among each treatment means (P<0.05) was determined by multiple-range tests using SPSS (Version 16.0) statistical software. Outcomes presented as mean ± SE (standard error).

3. Results

3.1 Rate of regeneration at different temperatures

Physico-chemical factors of water such as water temperature (°C), pH, dissolved oxygen (mg/l), total alkalinity (mg/l), total hardness (mg/l), ammonia (NH3+-N, mg/l), electric conductivity, total dissolved solids, turbidity, and microbial load observed are presented in Table 1. In the present study, all the water quality parameters tested were within the acceptable limits except pH and ammonia showed trace level variations. The pH and ammonia level observed was T22 (6.65±0.31), T29 (6.68±0.33), T33 (6.74±0.33) and T22 (0.23±0.05), T29 (0.22±0.03), T33 (0.23±0.053) respectively. The obtained results of various physicochemical parameters also indicated that the tested water samples were within the WHO and ICMR standards limits. Zebrafish are known to have an average pH of 6.3. The pH of the system water checked daily and maintained between 6.5 and 7.5. In the present study, it was noted some minor fluctuations in the pH value of experimental water. The highest value of pH was recorded in T33 (6.74±0.33), and the lowest was recorded in T22 (6.65±0.31). The optimum water temperature for Zebrafish survival has been reported to be between 26 - 28^{o}C. The outcome of this work showed that temperature values are ranging from 22.3±0.22^{0}C to 33.2±0.32^{0}C. The reported results were also within the standard ranges of WHO and ICMR. It was also noted that the increment of temperature strongly influenced the capacity of regeneration. The range of electrical conductivity (EC) in the present study was between 88.73±38.58 to 89.38±26.37 ìs. The EC was recorded maximum at 29^{0}C and a minimum at 22^{0}C. The values obtained fell within WHO, and ICMR limits and the water would be regarded as safe. The turbidity was found to be recorded at a maximum of 22^{0}C (5.5±3.35 NTU**)** and a minimum at 33^{0}C (2.13±1.32 NTU). These results also within the WHO limit, so the values obtained within this range makes these water suitable for regeneration. The DO received from this study ranged from 1.9±0.81 to 2.1±0.79 mg/L. The DO was recorded maximum at 22^{0}C and a minimum at 29^{0}C. These values come under the WHO limit so that the water would be considered free of pathogens. In the present study, negligible hardness values were observed from experimental tanks. The obtained alkalinity ranged from 14.5±4.43 to 16.5±6.61mg/lL. The alkalinity values were maximum in T22 (16.5±6.61) and minimum in T33 (15.5±6.19). The experimental Zebrafish do not seem to be overtly impaired by ammoniac nitrogen 0.23±0.053 mg/L and total coliform (11MPN/100) level. The total regenerated caudal fin length of *Danio rerio* reared in three different temperatures was analyzed and depicted in Table 2 and Fig. 1. To evaluate the regenerative efficiency after consecutive repeated amputations, measured full regenerated caudal fin area of each fish after amputations. As a control, each fish measured the uncut caudal fin area just before initiating the consecutive amputation experiment. The regeneration experiment indicates that the regeneration rate at T33 and T29 approximates twice the rate of regeneration at T22. The results indicate that increment of temperature will enhance the caudal fin regeneration of Zebrafish (Fig. 2). These results also showed that the regenerative outgrowth of the caudal fin does not decline with repeated amputations. At T22, the total length of the caudal fin immediately after amputation did not exhibit any significant difference (p>0.05) in the treatment groups in 3 days and achieved only a length increment rate of only 0.73±0.12 compared to that of initial caudal fin length (8.57±0.53mm). From day six onwards, the same effect was monitored, but after that, it was found that the total caudal fin length was significantly growing at a moderate level (p<0.05) on days 9 and 12 (p<0.05). As contrary to that of T22, the T29 groups showed a higher significant growth rate (29.67±6.08%) compared to the initial length (8.31±0.42mm) from day 6 (p<0.05), and a complete caudal fin growth (1.83±0.35 mm) was regained within 9th day itself. In T33 groups, compared to initial length

Table 1. Physico-chemical parameters of *Danio rerio* in different temperature ranges

Parameters	22±1 ⁰C	29±1 ⁰C	33±1 ⁰C
Temperature (°C)	22.3±0.22	29.1±0.37	33.2±0.32
pH	6.65±0.31	6.68±0.33	6.74±0.33
Electrical conductivity (µS/cm)	88.73±38.58	95.35±37.51	89.38±26.37
Total Dissolved Solids (mg/L)	44.17±19.41	46.45±17.43	45.33±15.46
Turbidity (NTU)	5.5±3.35	2.75±1.91	2.13±1.32
Alkalinity(mg/L)	16.5±6.61	14.5±4.43	15.5±6.19
Total Hardness (mg/L)	0±0	0±0	0±0
Dissolved Oxygen (mg/L)	2.1±0.79	1.88±0.91	1.9±0.81
Ammoniacal Nitrogen (mg/L)	0.23±0.05	0.22±0.03	0.23±0.053
Total Coliforms (MPN/100 ML)	9.1±0.06	9.9±0.09	11.02±0.05

'±' indicate standard error (S.E.)

(8.2±0.7mm), significantly ($p<0.05$) higher length gain percentage (33.64±6.69) and faster mode of growth increment (2.02±0.39) was noted compared to the rest of the groups. Nested 'T' test values further confirm a significant difference ($p<0.05$) in day 0 and day 6 of T29 and T33, respectively. During day 9, the highest caudal fin length gain (8.2±0.46mm) was noted in T33, and the lowest was in T22 (6.78±0.35mm) group. A similar trend was also found during the 12th day of observation. On the 9th day, the regeneration was completed in the T33 group, whereas the progress was slower in T29 followed by T22. In this study, length increment (mm) was found slower in T22 (0.73±0.12) treatment group while T29 (1.83±0.35) and T33 (2.02±0.39) shown a faster growth rate (Fig. 3). Almost the same outcomes were also obtained in length gain percentage where T22 (11.9±2.07) exhibited lower length gain while two-fold length gain percentage was achieved with those of the T29 (29.67±6.08) and T33 (33.64±6.69) groups. No significant response ($P>0.05$) was recorded in survival (%) among the treatment groups.

4. Discussion

Experimental values of different physico-chemical parameters of the water sample's results were mostly within the WHO and ICMR standard limits (Jain *et al*., 2016). Physio-chemical parameters such as temperature (°C), pH, Electrical conductivity (µS/cm), total dissolved solids (mg/L), turbidity (NTU), dissolved oxygen (mg/l), total alkalinity (mg/l), total hardness (mg/l), ammonia nitrogen(NH_3^+-N, mg/l) and total coliforms (MPN method) were within the acceptable limits except pH and ammonia level showed variations, and this may be due to water exchange, aeration and siphoning from the aquariums during the experiment. In the present study, it was noted some minor fluctuations in the pH value of experimental water. The pH of water in aquatic systems exerts profound effects on biological processes in fish and the function of the microbial community that supports them. In the present study, the highest pH value was recorded in T33 (6.74±0.33), and the lowest was recorded in T22 (6.65±0.31). These values are tending towards neutrally, that also within the values for optimum fish survival (Stevens, 2007). The optimal pH for Zebrafish in captivity has never been determined so far. However, the maintenance pH that most Zebrafish facilities strive between 6.5-8.0 (Alabaster and Lloyd, 1980; Brand *et al*., 2002). It is also within the observed range. The results were also found within the permissible limits of WHO and ICMR standards.

Previous studies of Cortemeglia and Beitinger (2005) indicated that Zebrafish have a maximal thermal tolerance range of 6.7–41.7 °C. The optimum water temperature for Zebrafish survival has been reported earlier to be between 26 - 28^{o}C (Jonassen *et al*., 1999). The results from this showed temperature values that range from 22.3±0.22^{0}C to 33.2±0.32^{0}C. These reports were also within standards (WHO and ICMR) (Hemlata Mahobe, 2013). Here, it also noted that an increment of temperature much influences the range of regeneration. Verheust (1997) has stated that conductivity can be used as a sign of primary productivity, affecting fish production. Sikoku and Veen (2004) observed that fishes vary in their ability to maintain osmotic pressure. Thus, the optimum conductivity for fish production differing from species to species. The range of electrical conductivity (EC) in the present study was between 88.73±38.58 to 89.38±26.37µs.

Table 2. Results of caudal fin regeneration of *Danio rerio* before amputation

Initial caudal fin length (mm)	8.57±0.53	8.31±0.42	8.2±0.7
After amputation			
Initial caudal fin length (mm)	6.18±0.36	6.2±0.22	6.22±0.36
3rd day caudal fin length (mm)	6.22±0.35	6.4±0.17	6.5±0.39
6th day caudal fin length (mm)	6.46±0.31	7.57±0.34	7.94±0.5
9th day caudal fin length (mm)	6.78±0.35	7.87±0.37	8.2±0.46
12th day caudal fin length (mm)	6.91±0.39	8.03±0.34	8.24±0.49
Length increment (mm)*	0.73±0.12	1.83±0.35	2.02±0.39
Length gain (%)**	11.9±2.07	29.67±6.08	33.64±6.69
Survival (%)***	100%	100%	100%

* Length increment (mm) = Final length – initial length;
**Length gain (%) = 100 [(final length – initial length)/ initial length];
***Survival (%) = 100 [(Number of surviving fish/ Total number of fish stocked).

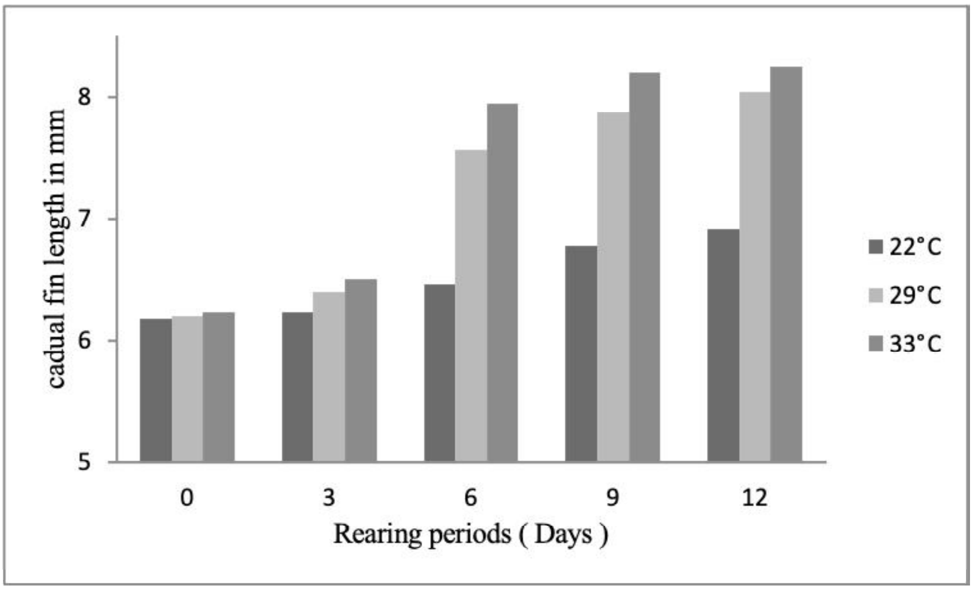

Fig. 1. Total caudal fin length (mm) of *Danio rerio* reared in different temperature
Data are based on means (±SE) ($p<0.05$) of triplicate tanks in each group (T22, T26 and T30)

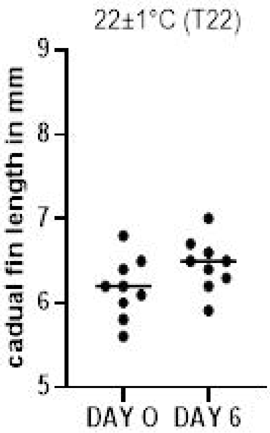

Fig. 2a.

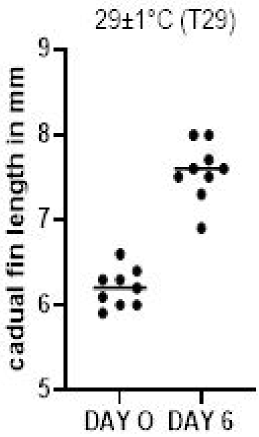

Fig. 2b.

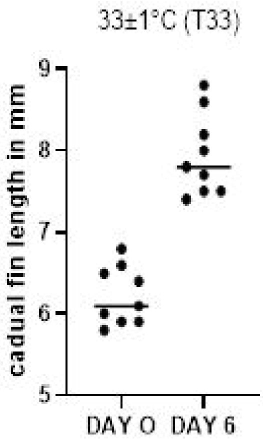

Fig. 2c.

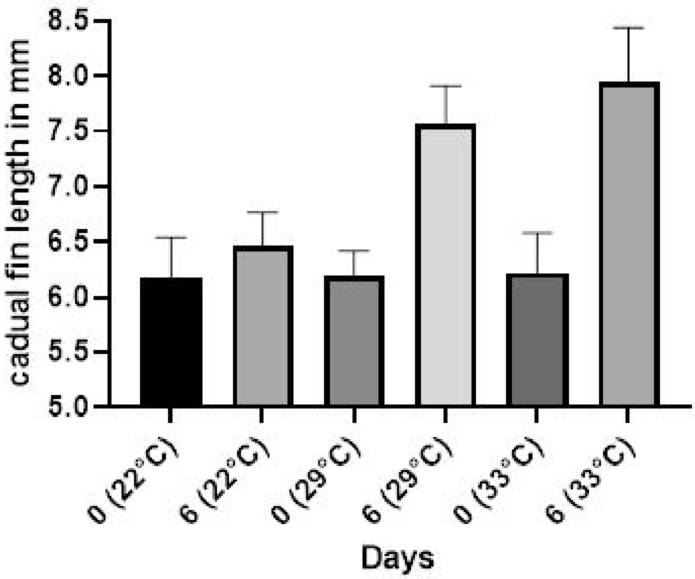

Fig. 2d.

Fig. 2a-d. Significant difference ($p<0.05$) showing Day 0/Day 6 in different temperature

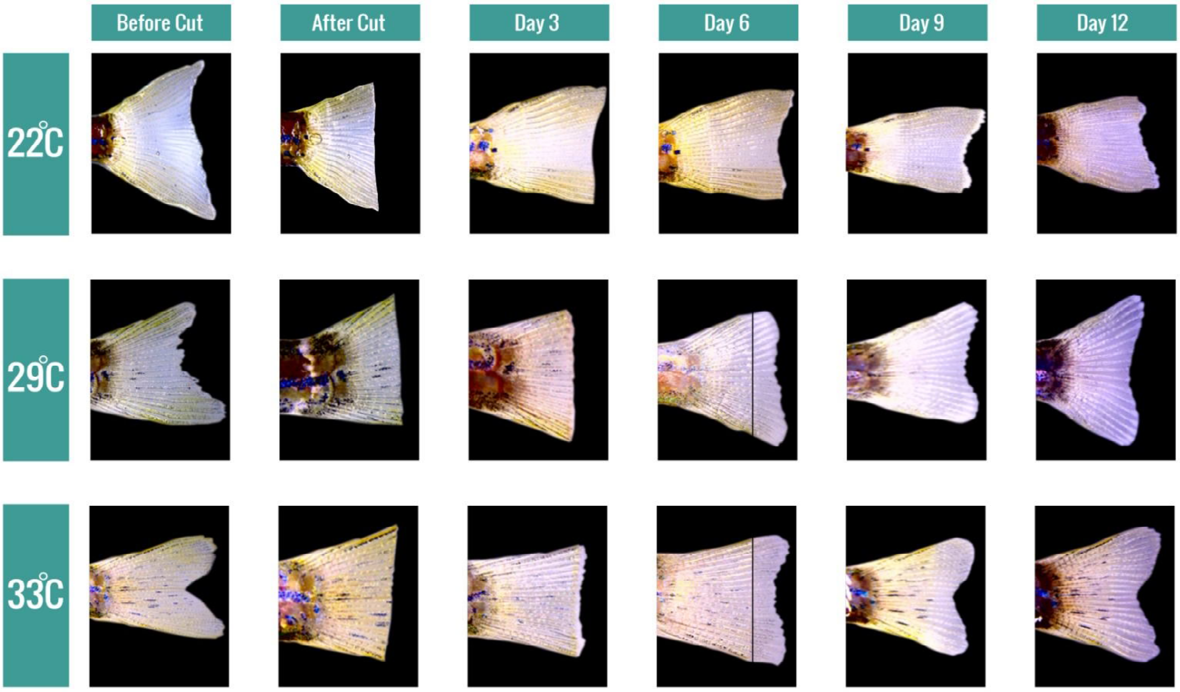

Fig. 3. Repeated amputations and regenerative growth of caudal fin of *Danio rerio*

The EC was recorded maximum at 29^{0}C and a minimum at 22^{0}C. It is also found that water would become safe, as the above values fall within WHO's limit. The turbidity was found to be recorded maximum at 22^{0}C (5.5±3.35 NTU**)** and a minimum at 33^{0}C (2.13±1.32 NTU). These results also within the WHO limit and indicate this range makes water suitable for regeneration. Similar results were also observed by Bhavimani and Puttaiah (2014). According to Zweigh (1989), 20-30 NTU is ideal for fish culture. Findings from this study even within the WHO limit, which makes these water suitable for regeneration studies.

According to Timmons *et al*. (2002), low dissolved oxygen levels are responsible for more fish mortalities in culture than any other parameter. The DO obtained from the present study had ranged between 1.9±0.81 to 2.1±0.79 mg/L. The DO was recorded maximum at 22^{0}C and a minimum at 29^{0}C. The water would be free of pathogens, as the above values are within the limit of WHO. Likely, warm water species such as Tilapia (Popma and Masser, 1999) are tolerant of lower levels of dissolved oxygen, and it may be possible that Zebrafish fall into this category. In the present study, negligible hardness values were observed from experimental tanks. Likely, water hardness values are not consistent across zebrafish facilities, principally because the methods used to buffer pH in recirculating systems are not compatible. Regulation of alkalinity via the direct addition of sodium bicarbonate, without concomitant addition of calcium and/or magnesium salts, can result in low hardness values (Buttner *et al*., 1993).

The obtained alkalinity ranged from 14.5±4.43 to 16.5±6.61mg/lL. The alkalinity values were maximum in T22 (16.5±6.61) and minimum in T33 (15.5±6.19). A higher level of alkalinity registered in T33 might be due to the presence of an excess of the free CO_2 product due to the decomposition process coupled with the mixing of fecal matter. The low alkalinity in T22 may be due to dilution (Jain and Seethapati, 1996). The experimental Zebrafish do not seem to be overtly impaired by ammoniacal nitrogen 0.23±0.053 mg/L and total coliform (11MPN/100) level. According to Wilkie (2002), nitrite is toxic to fish and can be problematic in freshwater systems at concentrations above 1 ppm. Buttner *et al*. (1993) observed that small-bodied fish seem to be less sensitive to nitrite toxicity than larger fish. In our study, Zebrafish does not seem to be overtly impaired by chronic nitrate levels of up to 100 ppm (personal observation).

The present study investigated the structural recovery of caudal fin in a model organism *Danio rerio* to find the most favorable temperature conditions to follow better growth and developments. *Danio rerio* were reared under three temperatures conditions such as 22±1°C (T22), 29±1°C (T26), and 33±1°C (T30) for about 12 days under laboratory condition in triplicates. The outcome of the study clearly showed that there was a significantly higher length increment (mm) in T29 and T33 treatment groups compared with the T22 ($p<0.05$) group.

Similar observations were reported in electric fish, *Apteronotus leptorhynchus*, where temperature fluctuations profoundly influenced spinal cord injury recovery and structural regeneration of caudal fin after amputation (Sîrbulescu and Zupanc, 2010). They could find remarkable improved structural and functional recovery of caudal fin in *Apteronotus leptorhynchus* after amputation. The present study results also found a significantly higher structural and functional recovery of caudal fin after amputation with the higher temperatures. It was also observed that on the 9th day, the regeneration was completed in T33 treatment group whereas the progress was a little slower in T29 followed by T22 treatment group. According to Brett and Groves (1979), increasing temperature leads to a faster growth rate and developments, parallel with higher food intake and organ regeneration, also strongly dependent on water temperature where the fish lives.

According to Gabillard (2005), environmental temperature promotes growth through direct influence on growth hormone (GH) secretion that leads to the rise of plasma IGF (Insulin-like growth factor) levels in connection with the development. This study indicates that fish kept at 29±1°C and 33±1°C showed length gain (%) compared to fish kept at 22±1°C. The present result also said that high temperature has a stimulating effect on cellular metabolism in poikilotherm organisms. This study is in harmonious with goldfish, wherewith increasing temperature (up to 35°C), the proliferation rate of primary cells also increased simultaneously (Kondo and Watabe, 2004). The low recovery of caudal fin in *Danio rerio* at a lower temperature (T22) might be due to lower metabolism, which subsequently decreased cell proliferation. It also reduces metabolic rate, which affects GH secretion that leads to late regeneration of the caudal fin. Our studies provide the first evidence that T33 is a critical temperature factor for regulating vascular regeneration of the caudal fin of *Danio rerio.*

5. Conclusion

In conclusion, this study suggested that the caudal fin of *Danio rerio* regenerates better in high temperatures such as 33±1°C and 29±1°C compare to low temperature (22±1°C). This work provides baseline data for the conservation and monitoring of water quality of the freshwater system. This study firmly concludes that the tested water samples fall within the stipulated range of acceptability. However, the present study explores the beneficial influence of ambient temperature on the rapid recovery of the caudal fin of *Danio rerio*. Hence, we provided a piece of the necessary information about the critical temperature factor that enhances the caudal fin regeneration of Zebrafish and highlights the importance of considering the species as a model fish for regenerative studies.

Acknowledgements

We thank Kerala State Plan fund 2016-2017, Kerala Biotechnology Commission, YIPB program, KSCSTE; HRD scheme, Dept. of Health Research-Start up Grant, Govt. of India, and the University of Kerala.

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