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EFFECT OF PHOTOPERIOD ON TAIL FIN REGENERATION IN *GAMBUSIA AFFINIS*

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ABSTRACT
Circadian rhythm is the endogenous biological clock of various daily physiological processes that show rhythmic oscillations, governed by internal and external cues. Light is one of the major external cues that regulate the circadian clock and thus may hamper a lot of biological events like sleep cycle, hormonal regulation etc. An attempt was made to find out the correlation, if any, between photoperiod, different sexes and regeneration ability of vertebrates using *Gambusia affinis,* a fresh water fish as a model system. Regeneration of amputated tail fins of male and female fish were studied for seven days post-amputation under either natural light-dark cycle or continuous dark conditions. The results indicate no significant difference in the regeneration ability between male and female fish that were exposed to normal conditions of light and dark. When the regeneration ability of female fish kept in natural light-dark cycle was compared with the female fish kept in continuous dark during regeneration period, the regeneration was slowed down in fish with dark conditions, though it was not significant. On the other hand, male fish showed significant reduction in the regeneration of tail fins under dark conditions as compared to male kept in normal light-dark cycle. Thus, the present results suggest gender specific differences in regeneration ability of *Gambusia affinis* if the photoperiod is experimentally altered.

KEYWORDS: Circadian rhythm, *Gambusia affinis*, Photoperiod, Regeneration, Tail fin.

INTRODUCTION

The natural ability of some living organisms to re-form the lost or damaged part of its body is known as regeneration. It is a developmental process that involves growth, morphogenesis and differentiation (Gilbert, 2013). Reparative and restorative are mainly the two different mechanisms by which an animal can regenerate the lost part. Reparative regeneration is also known as physiological regeneration as it is limited to day to day damages to the body due to its own physiological processes like replacement of skin cells, blood cells, repair of a cut etc. It involves cell proliferation and migration which mostly occurs in almost all animals in the form of healing of wound or replacement of old/damaged cells. On the other hand, restorative regeneration can replace the lost body part or produce a complete organism from a tissue fragment which is mostly observed in lower animals like Hydra (Gilbert, 2013).

Fish is one of the vertebrates that has the capacity to regenerate damaged organs such as heart, spinal cord, retina and fins (Shao *et. al*., 2009). If surgically amputated, fish can completely regenerate the tail fin with organized musculature, endoskeleton and scales (Shao *et. al.*, 2009). *Gambusia affinis,* a fresh water fish belongs to order Cyprinidontiformes and family Poeciliidae. These are small, robust-bellied, surface-swimming fish that prefer shallow water with grass and other aquatic plants. *Gambusia* are easily available, exhibit distinct sexual dimorphism and are easy for experimental manipulations. Females are bigger in size of about 7 cm as compared to males that grow up to 4 cm length. Male *Gambusia* possess highly extended anal fin in comparison with that of the females (Baird and Girard, 1853). Females usually have translucent body and the gravid females can be identified by the presence of a large black spot on their belly just over the anus. Males are very active with much colorful body than females (Baird and Girard, 1853). Due to its admirable capacity of regeneration, *Gambusia affinis* has been extensively used as a model organism for the study of tissue regeneration in vertebrates (Borgave *et. al*., 2016).

Circadian rhythm is any biological/physiological process that displays an endogenous oscillation of about 24 hour that can be modulated by external cues like temperature, sunlight etc. Circadian rhythmicity is observed in the sleeping, feeding patterns, body temperature, brain wave activity, hormone production, cell regeneration and other biological activities. The 24 hour rhythms are maintained by a circadian clock, widely observed in animals. Circadian rhythm regulates various body functions through a series of clock gene clusters. The dys-regulation of any of these clock genes often lead to pathology of various kinds of diseases like obesity, lipid disorders, diabetes, skin regeneration, hematopoiesis and cancer (Hadadi *et. al.,* 2018; Kimura *et. al.,* 2018; Nakashima *et. al.,* 2018; Steffens *et. al.,* 2017). Circadian rhythms regulate cell proliferation and differentiation (Lowe *et. al.,* 2018) and are shown to significantly influence corneal epithelium renewal, repair in mice (Xue *et. al.*, 2017) and intestinal regeneration (Stokes *et. al.*, 2017). Fibroblasts, which are primary cells of wound healing process, also exhibit circadian timekeeping (Hoyle *et. al.*, 2017).

Light acts as a major source of energy for almost all types of biological processes. Light also affects circadian rhythm in animals which reflects in hormonal changes (Johnsson, 2008). An environmental rhythm of light and darkness is as termed photoperiodism. The daily cycle of light and dark is called photoperiod. The terms 'photophase' and 'scotophase' are used to explain the period of light and darkness, respectively. Different animals have evolved different morphological, physiological, behavioral and ecological adaptations during the course of evolution to varying photoperiods. Photoperiodism, the physiological reaction of organisms to the length of day or night, is vital to both plants and animals.

The circadian system plays an important role in the measurement and interpretation of day length. Regeneration process involves hormonal regulation. Most of hormones secreted by endocrine glands are regulated by circadian rhythms apart from other physiological factors (Gamble *et. al.*, 2014). A recent study has shown that the time of injury significantly affects the healing process in burnt cases in humans. The daytime wounds heal 60% faster than the nighttime wounds suggesting circadian regulation of the wound-healing efficiency (Hoyle *et. al.*, 2017). If an animal is exposed to light, it leads to rapid activation of stem cells from hair follicle, resulting in significant hair regeneration. Light is thought to regulate tissue stem cells via an ipRGC-SCN autonomic nervous system circuit, thus it may facilitate rapid adaptive responses to external light in many other homeostatic tissues also (Fan *et. al.*, 2018). Zebra fish, a fresh water fish with highest regeneration ability, show onset of circadian rhythms due to exposure to light. Light induces expression *per2* (*period of internal clock* gene) mRNA, that is involved in the development and maturation of the pineal clock. Pineal gland is a photoreceptor organ related to the retina in terms of expression of similar sets of genes, whose expression levels alter in zebra fish according to daily photo rhythm, with higher mRNA levels during the night (Ben-Moshe *et. al.*, 2014). In zebra fish, most tissues and organs including the heart and central nervous system possess the remarkable ability to regenerate after severe injury and the important role of the circadian clock in proliferation of the epidermal cells in response to injury has been demonstrated (Idda *et. al.*, 2012). Light stimulus is shown to regulate the cell cycle in zebra fish (Poss *et al*., 2003).

As described earlier, there are hints about possible co-relation between photoperiod and regeneration in animals. The present work evaluates this aspect of regeneration and highlights the role of light in the process of regeneration using *Gambusia affinis* as a model system.

MATERIALS AND METHODS

Procurement of animal:

Gambusia affinis were collected from the local pond of S.N. Arts, D.J.M Commerce and B.N.S Science College, Sangamner, Ahmednagar, India. Fish were collected randomly in container and transported to laboratory. Then fish of same size and color were separated in another container for experiment.

Amputation of tail fin:

Fish were anesthetized with 0.2% MS222; an aquatic anesthetizer (Fisher Scientific). The anesthetized fish were taken on a glass slide one by one. Tail fin amputation was carried out using a sharp blade taking a single sharp cut leaving behind intact tail fin of 2mm. Fish with amputated tails were randomly distributed into control and treated groups. These fish were maintained in glass bowls with daily change of pond water throughout the experimental period. All selected fish were of length of 5cm. Both male and female fish were used separately for experiments.

Experimental set up:

Each experimental set had of 2 glass bowls containing 5 fish each. Pond water was renewed after every 24 hours. Control animals were kept in open and exposed to natural light-dark cycles whereas the experimental fish were kept in dark for all 7 days of experiment. Males (pigmented fish) and females (un-pigmented) were used in separate experiments just to check if there is any gender specific response to photoperiod in context to regeneration ability. The tail fin of each fish from control and treated groups were measured daily for 7 days post-amputation. All the experiments were repeated for five times with 25 fish as the sample size for control and treated groups.

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RESULTS

In both male and female fish from the control group that were exposed to natural light-dark cycles, the fin regeneration began on 4th day. Complete regeneration of the amputated fin was achieved by 7th day post-amputation. Daily observation and measurement of tail fin of each fish from control and treated groups was recorded for 7 days.

Tail fin regeneration in male and female fish kept under natural light-dark cycle: When the rate of regeneration in male and female control groups was compared to each other over the regeneration period of 7 days, there was highly any difference in the regeneration rates. These results indicate no significant gender-specific difference in the regeneration capacity of animals that were exposed to natural light-dark cycle (please refer Table 1 and Figure 1).

Effect of photoperiod on tail fin regeneration in *Gambusia* female fish: The female fish with amputated tail fins when kept in dark continuously for 7 days, exhibited slightly lower rate of regeneration as compared to female fish kept under natural photoperiod. However, the reduction in regeneration rate due to lack of light was not statistically significant as compared to respective control female fish (Table 2 and Figure 2).

Effect of photoperiod on tail fin regeneration in *Gambusia* male fish: On the other hand, in another set of experiments wherein the male fish with amputated tail fins were exposed to either natural light-dark conditions or dark throughout the regeneration period, exhibited significant difference in the length of the regenerated tail fin in both the groups (Table 3). The fish kept in dark showed remarkable reduction in the length of the regenerated tail fin (Figure 3); suggesting that males are more susceptible to photoperiod during regeneration process as compared to females.

Table. 1. Comparison between regeneration of tail fins in male and female fish kept in natural light-dark cycle throughout the regeneration period.

Fish	Length of the regenerated tail fin under natural light-dark cycle (mm)			
	Day 4	Day 5	Day 6	Day 7
Female	0.86±0.35	1.18±0.35	2.86±0.35	3.93±0.41
Male	0.866±0.35	1.86±0.35	2.93±0.25	3.86±0.39

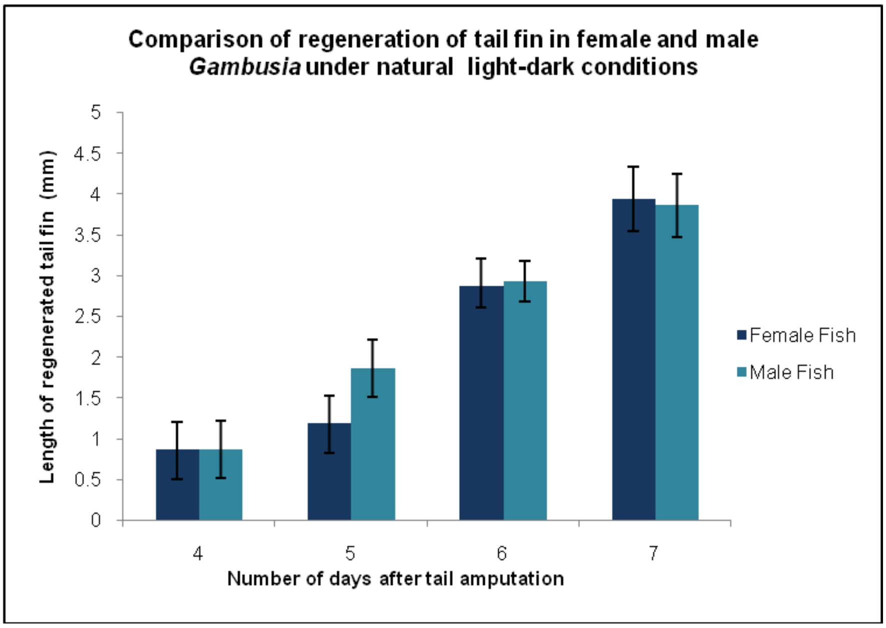

Figure. 1. *Graphical representation of length of regenerated tail fin in male and female fish from 4th to 7th day post-amputation under natural light-dark cycle. Note no significant difference in the male and female fish in the day-wise length of the regenerated tail.*

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Table. 2. Effect of photoperiod on regenerating tail fins in female fish kept in either natural light-dark cycle or kept in dark throughout the regeneration period

Female Fish	Length of the regenerated tail fin (mm)			
	Day 4	**Day 5**	**Day 6**	**Day 7**
Regeneration under natural light-dark cycle	0.86±0.35	1.18±0.35	2.86±0.35	3.93±0.41
Regeneration under dark conditions	0.625±0.22	1.16±0.30	2.25±0.45	3.00±0.60

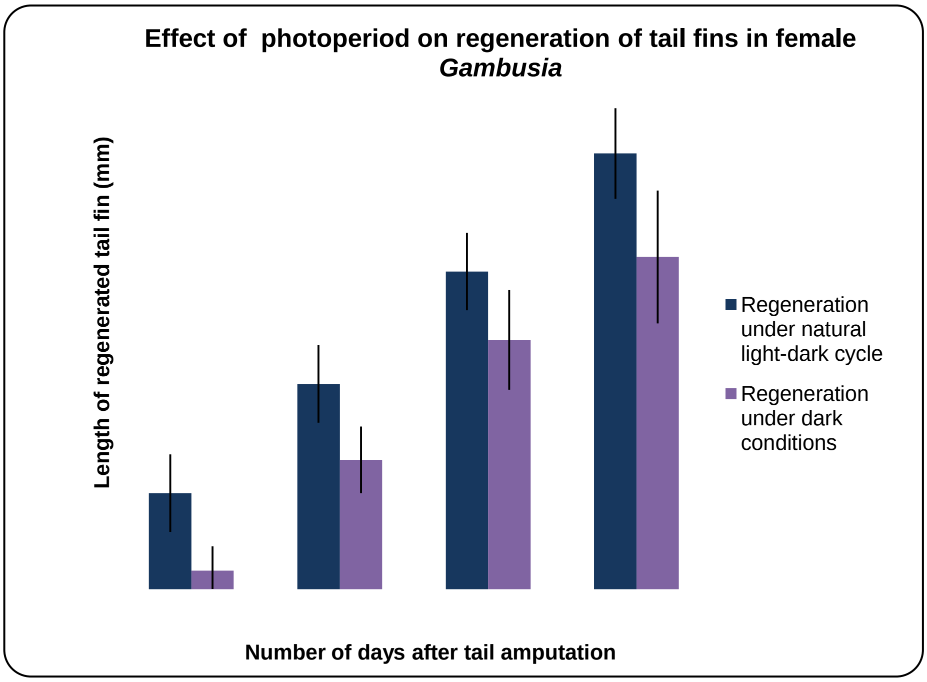

Figure. 2. *Graphical representation of length of regenerated tail fin in female fish from 4th to 7th day post-amputation. Note accelerated rate of regeneration in fish exposed to natural light-dark cycle as compared to fish kept in dark; however the acceleration does not seem to be statistically significant.*

Table.3. Effect of photoperiod on regenerating tail fins in male fish kept in either natural light-dark cycle or exposed to dark throughout the regeneration period.

Male Fish	Length of the regenerated tail fin (mm)			
	Day 4	**Day 5**	**Day 6**	**Day 7**
Regeneration under natural light-dark cycle	0.866±0.35	1.86±0.35	2.93±0.25	3.86±0.39
Regeneration under dark conditions	0.56±0.25	1.03±0.39	1.6±0.63	2.6±0.63

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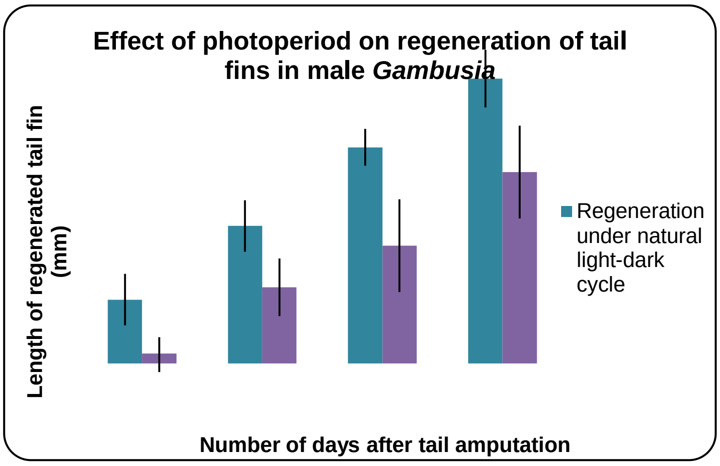

Figure. 3. *Graphical representation of length of regenerated tail fin in male fish from 4th to 7th day post-amputation. Note significantly reduced rate of regeneration in fish exposed to dark as compared to fish kept in natural light-dark cycle.*

DISCUSSION

As reported earlier, regeneration of the amputated tail fin in *Gambusia* began after 3 days of amputation with blastema formation and complete regeneration was achieved by the end of 7 days post-amputation (Borgave *et. al.*, 2016). The effect of photoperiod, means period of exposure to light and dark, on the regeneration phenomenon was evaluated in the present study to check the role of photoperiod in regeneration. Also, gender specific differences in the regeneration capacity, if any, in male and female fish, were also studied. The present data does not exhibit any significant difference in the regeneration capacity of male and female suggesting lack of gender specific differences on the efficiency of regeneration in both the control groups; i.e. males and females exposed to natural light-dark cycle.

The present study highlights an important role of light in the regeneration process. Fish exposed to light regenerated the cut tail fins at a higher rate as compared to fish kept in dark, however it was significantly observed in male fish as compared to corresponding female fish. The circadian clock, present in brain, is controlled by specialized cells in the superachaismatic nucleus of the hypothalamus in the brain. Circadian clock controls the regulation of hormones such as cortisol and melatonin, which act as signals for maintaining the biological clock within animal body. Circadian rhythm, maintained by the circadian clock, not only regulates the sensitivity of different hormones but also regulates the natural ups and downs that these hormones throughout day and night (Cajochen *et. al.*, 2010). Circadian clock is set by variety of external factors including light. The light and dark cycle, thus is one of the most important factors regulating circadian clock (Duffy and Czeisler, 2009). Sunlight exposure during day supports the normal production of melatonin in the evening (Nagashima *et. al.*, 2018). A recent finding on the molecular involvement of melatonin in the regeneration of various tissues like nervous system, liver, bone, kidney, bladder, skin and muscle has highlighted importance of appropriate photoperiod for maintenance of circadian clock (Majidinia *et. al.*, 2018).

The present results, thus, underline the co-relation between photoperiod and circadian rhythmicity which may have resulted into delayed regeneration in male *Gambusia affinis* that were kept in dark throughout the regeneration period. In human studies, the endogenous frequency of the circadian clock and the amplitude of the melatonin production have been shown to differ between men and women (Santhi *et. al.*, 2016). These reports have formed a strong platform to study the role of steroids and sex differences in the circadian timing pattern. The overall circadian changes affect all behavioral and biological responses in an animal. Furthermore, steroid hormones can modulate various aspects of circadian responses (Yana and Silver, 2016). Different sexes in mammals have been thought to possess different circadian timing systems which have potential implications in disease (Bailey and Silver, 2014). Our study supports the earlier observations emphasizing an important role of light in the regeneration process and forms a frame work for further research on the role of photoperiod in crucial biological process like regeneration. Elucidation of the mechanism by which light regulates fin regeneration is in progress.

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