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Original Article

The regeneration capacity of caudal fin in the common tooth-carp, *Aphanius dispar* (Rüppell, 1829) (Teleostei: Cyprinodontidae)

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Abstract: Regeneration ability is known for several Actinopterygii or ray-finned fishes. In order to assess the universality of regenerative potencies in this group of fishes, we have examined for the first time, caudal fin regeneration in tooth-carp, *Aphanius dispar* (Rüppell, 1829). The caudal fin is used because of its accessibility, simple structure and fast regeneration. The results revealed the regeneration ability in caudal fin of *A. dispar*. The initiation of the regenerative outgrowth was differing in three examined water temperatures. It is started approximately 5 days post amputation (5 dpa) at room temperature, and 2 dpa at 25°C and 28°C. Our finding indicates that water temperature more than 25°C promotes procedure in caudal fin regeneration of *A. dispar*. By considering the high regeneration ability in *A. dispar*, and also the relatively short life span of the members of the genus *Aphanius*, we concluded that these fishes could probably be used in regeneration researches. However, details of this mechanism in *Aphanius* need further examinations.

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Introduction

The teleost fish together with some groups of amphibians among the vertebrates represent remarkable capability to regenerate their various organs (Pfefferli and Jazwinska, 2015). Due to the simplicity in manipulation and macroscopic examination, most studies on regeneration potential focused on external appendages such as fins and limbs in abovementioned animal groups. The first report on fish regeneration was documented for the gold fish (Broussonet, 1786). He discovered that in regeneration experiment of the fishes, caudal fin has advantage over other fin types, because it regenerates faster than pectoral, ventral and dorsal fin (Pfefferli and Jazwinska, 2015).

Among the teleost fishes, zebrafish *Danio rerio* (Hamilton, 1822) has been used as powerful model for regeneration studies (e.g., Nechiporuk and Keating, 2002; Pfefferli and Jazwinska, 2015). Besides its simple architecture, its accessibility and its fast and robust regeneration, it is able to regenerate fins, scales, retina, spinal cord and heart among other internal organs (Iovine, 2007; Azevedo et al., 2011).

In contrast to amphibians and fishes, other vertebrate such as reptiles, birds and mammals have a more limited capacity to regenerate complex body structures (Brockes and Kumar, 2008; Galis et al., 2003; Unguez, 2013). The limited capacity of humans to regenerate tissues and evolutionary loss of regenerative ability in animal groups attracts many biologists from medical perspective (Bely and Nyberg, 2010; Unguez, 2013), and therefore researchers always attempt to elucidate the mechanisms responsible for the extensive regenerative capacities in specific groups of vertebrates.

Caudal fin is composed of segmented bony rays, mesenchymal tissue, blood vessels and nerve axons. Each bony ray consists of two concave hemi-rays that define an inner space filled with intra-ray mesenchymal cells. The bifurcations are responsible for generating the characteristic shape of the caudal fin (Poss et al., 2003). Bony rays are produced and maintained by osteoblasts (also called scleroblasts), skeletogenic cells that secrete bone matrix (Hall, 2005).

Based on the study of Poss et al. (2003), an amputation of caudal fin triggers a regenerative

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program that occurs in three phases: (i) wound healing: the first phase after amputation started immediately after injury and continues within 12 hour-post-amputation (hpa). The injury is healed through migration of epidermal cells that cover and close the wound. (ii) blastema formation: the wound epithelium thickens forming an apical epidermal cap (AEC). Formation of blastema, is accomplished by the dedifferentiation and proliferation of cells in the disorganized mesenchymal tissue under AEC. (iii) Regenerative outgrowth: the onset of regenerative outgrowth starts after establishment of the blastema, at 48 hpa. The third phase is characterized by the extensive proliferation of cells.

Up to now, no information is available on the regeneration phenomenon in tooth-carp, genus *Aphanius* Nardo, 1827. To determine whether *Aphanius* can regenerate its body parts, we used *Aphanius dispar* from southern Iran as model, and removed its caudal fin from the basal part.

Materials and Methods

Study model: The common tooth-carp, *A. dispar* inhabits brackish and freshwater environments almost in the desert regions (Wildekamp, 1993) (Fig. 1). More especially, its principal habitats are coastal lagoons, but owing to its high tolerance to ecological changes, it can live in inland waters and hot sulphuric springs (Wildekamp, 1993; Teimori et al., 2012a, b).

In this study, *A. dispar* specimens were captured from Bandar Abbas city in southern Iran and transferred to the laboratory aquariums equipped with heater, thermometer and constantly aerated water. The animals were fed every day during experiment period with freeze-dried live food and also dry food that contain macronutrients, trace elements and vitamins necessary to keep captive fish in good health.

Figure 1. A fresh male common tooth-carp, *Aphanius dispar* from southern Iran, studied as model in this study (Scale bar is about 1 cm; fish photo provided by H.R. Esmaeili).

The individuals were kept in three separate aquariums in pH 6.8-7, and each settled with specific temperature (Room Temperature (RT)=23°C, 25°C and 28°C). Throughout the examination, temperature was recorded twice a day.

Fin amputation: In total, 12 fish individuals were examined for this experiment. They placed in 180 ppm of aqueous clove (*Eugenia caryophyllata*) solution, and the anesthesia was induced after approximately 3 minutes. Once fish asleep and immobile, it was moved to the dry lid of the Petri dish, oriented so the head was positioned to the left, the caudal fin was fanned out with a clean razor blade. The fish was then transferred back to a tank of fresh water, observed for gill movement, and allowed to awaken. The caudal fins of four fish individuals (two males and two females) in each temperature were imaged 2, 5, and 7 days post amputation (dpa) (Fig. 2).

Each captured image was used for measurement in

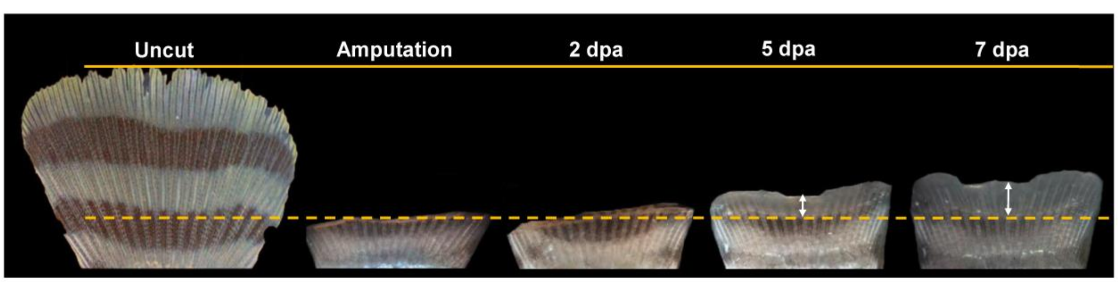

Figure 2. Stages of the caudal fin including amputation: Uncut (before cutting), Amputation (immediately after cutting) and 2, 5, and 7 days after amputation (dpa). The dashed line indicates position of the cutting and the regeneration processes. The white arrows show the measurements from point of amputation.

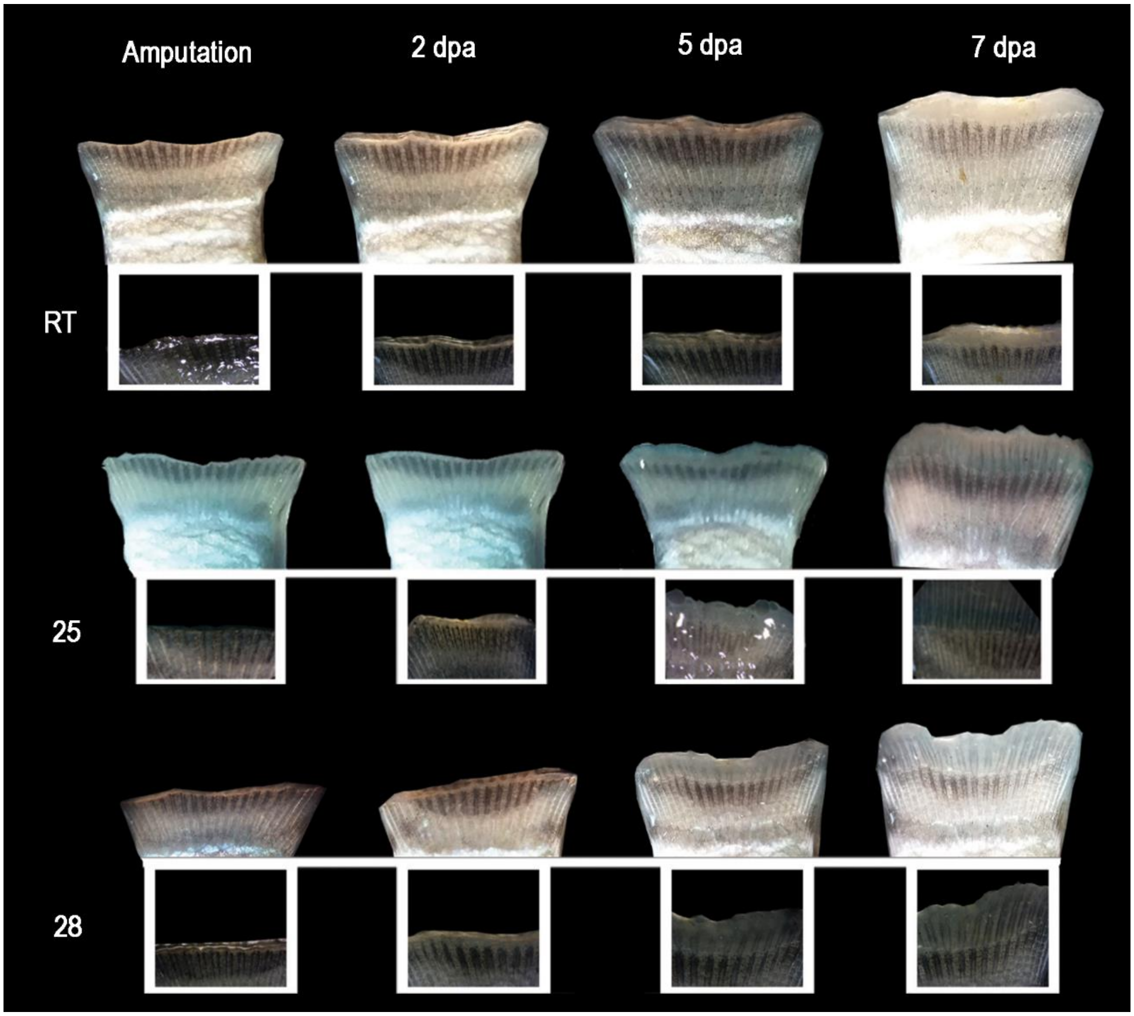

Figure 3. The regeneration processes in caudal fin of *Aphanius dispar* in different days at three water temperatures.

Image-J software (Schneider et al., 2012). The length of caudal fin regeneration from point of amputation was measured in three times (in millimeter, mm) to obtain an average values of the growth rate. Eventually the obtained data was analyzed using IBM SPSS Statistics 24. The T-test (P<0.05) was used to show if significant differences exist for the regeneration rates among different days at different water temperatures.

Results

The results clearly indicate for the first time the regeneration ability in tooth-carp, *A. dispar*. The results reveal that *A. dispar* can obviously repair its caudal fin a short time (approximately 2 days) after traumatic injury as described below.

There was little bleeding after caudal fin amputation. Few hours after amputation, the wound is covered by epidermal layer which is called wound healing phase. Next step in *A. dispar* fin regeneration is blastema formation which is occurred in 12-48 hours after amputation (2 dpa in Fig. 3).

During blastema formation, epidermis accumulates additional layers by cell migration. The hall mark of blastema is a proliferative mass of mesenchymal cells which sits atop each fin ray and gives rise to the new structures of the caudal fin. The last step in fin regeneration is switch from blastema formation to regenerative outgrowth. This transition occurs at approximately 5 dpa at RT, and 2 dpa at 25°C and 28°C (Fig. 3).

Therefore, for quantitative analysis, we only compared regeneration rates for 2 and 7 days post amputation at 25°C and 28°C water temperature in males and females separately. Descriptive analysis of regeneration rate in different days and water

Table 1. Descriptive analysis of the regeneration rates between males and females of the *Aphanius dispar*.

Sex	T°C	Day 2		Day 5		Day 7	
		Mean	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**
Male	RT	0.20	0.085	0.61	0.064	0.66	0.106
	25	0.23	0.059	0.83	0.114	1.86	0.328
	28	0.22	0.006	0.78	0.178	2.05	0.375
Female	RT	0.32	0.162	0.44	0.080	0.45	0.160
	25	0.31	0.014	0.59	0.050	1.98	0.070
	28	0.32	0.028	0.73	0.028	2.89	1.163

The rate of regeneration is shown for different days at three water temperatures. RT=Room Temperature and S.D. = standard deviation.

Table 2. T-test analysis to show significant differences (P<0.05) of the regeneration rates for different days at three water temperatures in the examined *Aphanius dispar*. The analysis was carried out for the males and females separately. RT=Room Temperature and S.D.= standard deviation.

Sex	Day	T-test	Mean differences	Sig.
Female	**Day 2**	RT vs 25	0.0066	0.98
		RT vs 28	0.0030	0.99
		28 vs 25	0.0100	0.11
	Day 5	RT vs 25	0.1575	0.91
		RT vs 28	0.2925	0.03
		28 vs 25	0.1350	0.61
	Day 7	RT vs 25	1.5250	0.56
		RT vs 28	2.4375	0.03
		28 vs 25	0.9125	0.27
Male	**Day 2**	RT vs 25	0.0216	0.89
		RT vs 28	0.0183	0.92
		28 vs 25	0.0033	0.99
	Day 5	RT vs 25	0.2200	0.97
		RT vs 28	0.4625	0.24
		28 vs 25	0.0575	0.81
	Day 7	**RT vs 25**	1.2009	0.00
		RT vs 28	1.3895	0.00
		28 vs 25	0.1885	0.48

temperatures is shown in Table 1. In the 5 dpa, caudal fin regeneration was 0.77 mm at 25°C and 0.73 mm at 28°C in the males vs. 0.59 mm at 25°C and 0.83 mm at 28°C in the females. In the 7 dpa, at both 25°C and 28°C, the regeneration rate in the males was lower than the females (1.86 mm at 25°C and 2.05 mm at 28°C in the males vs 2.0 mm at 25°C and 2.90 mm at 28°C in the females) (Table 1).

In the males, there were not significant differences for the regeneration rates at different water temperatures in 2- and 5 dpa, while the regeneration rates significantly differ between RT vs 25°C and RT vs. 28°C in 7 dpa (T-test, P<0.05) (Table 2 and Fig. 4). In the females, the regeneration rates differ significantly only between RT vs 28°C in both 5- and 7 dpa (T-test, P<0.05) (Table 2 and Fig. 4).

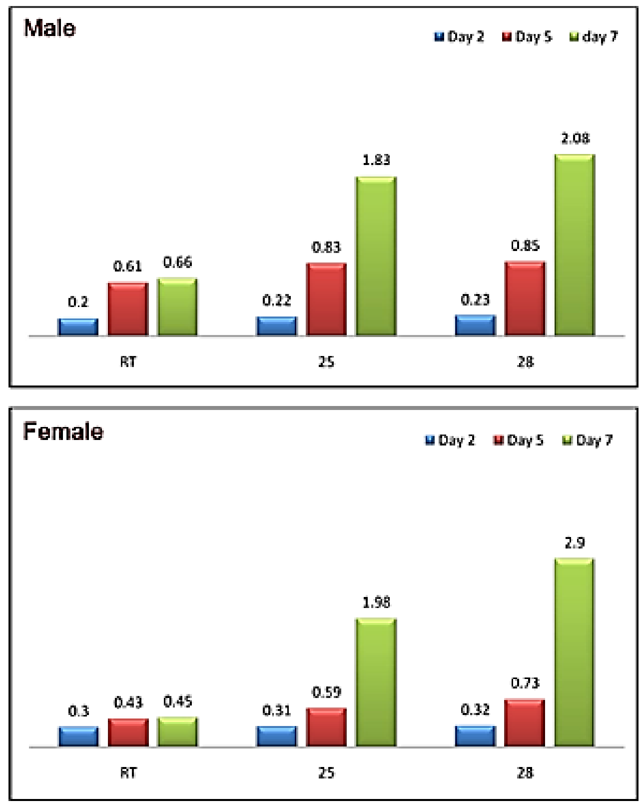

Figure 4. Comparison of the rate of caudal fin regeneration in the male (above), and the female (below) specimens housed at three water temperatures over 7 days.

Fin regeneration measurement revealed an overall increase in growth with increasing of water temperature. Regeneration outgrowth was seen after 5 dpa in 25°C and 28°C. However, in Rome Temperature (RT) it is detected after 7 dpa (Figs. 3-4). The increasing rate of regeneration was significant in

5 and 7 days post amputation in comparison to the 2 dpa (T-test, $P<0.05$). Also, our results indicate that no clear growths happened in the 2 dpa comparing the time of amputation (Figs. 2-4).

Discussion

Regenerative capacity in different organs and organisms is interesting for biologists, and clinicians. There are many studies which attempt to discover various aspect of regeneration (e.g. Poss et al., 2003; Azevedo et al., 2011; Rink, 2013; Hoppe et al., 2015; Kim et al., 2016). Therefore, it is in the center of attention to reveal regenerative capacity in organisms from evolutionary point of view, the role of stem cells, signals which initiate regeneration and its controlling mechanisms.

Among teleost fishes, various degrees of regeneration abilities have been reported. Good examples are cyprinid fish, *D. rerio* which can regenerate lost parts repeatedly (Azevedo et al., 2011), and short-lived African turquoise killifish, *Nothobranchius furzeri* (e.g. Hoppe et al., 2015; Kim et al., 2016). However, some teleost fishes exist with total absence of regeneration such as some members in the family Blennidae (Wagner and Misof, 1992). In the present study, the possible occurrence of the regeneration ability in caudal fin of another relatively short-lived killifish, *A. dispar* is examined. The outcome of all these studied could help researchers to assess the universality of regenerative potencies in Actinopterygii fishes.

The studied taxon is a small tooth-carp with a maximum length up to 8 cm (usually 45-55 mm in total length). It is widely distributed throughout the coastal environments of the Mediterranean Sea, Red Sea and Persian Gulf regions (Wildekamp et al., 1999; Hrbek and Meyer, 2003; Reichenbacher et al., 2009; Ferrito et al., 2013; Teimori et al., 2016; Esmaeili et al., 2017). Most of the *Aphanius* species tolerate euryhaline and eurythermal habitats as well as relatively low levels of oxygen and wide range of pollution. In addition, they are good candidates as biological control of malaria because they feed on mosquito larvae of Anopheles (Homski et al., 1994).

Based on the finding of the present study, the regeneration phenomenon is happening in *A. dispar*, and probably could be occurred also in the other members of the genus *Aphanius*. The killifishes with special biological properties such as short life span and having regeneration ability are very interesting models for biomedical researchers. Therefore, besides the already known killifish, *Nothobranchius furzeri* (Hoppe et al., 2015; Kim et al., 2016), here we present genus *Aphanius* as a new candidate model organism to be used to study the mechanisms of regeneration.

According to the previous study, the regeneration procedure contains the wound healing, blastema formation and growth (Pfefferli and Jazwinska, 2015). Blastema is a heterogeneous group of dedifferentiated/proliferative cells, able to regrow and pattern a new complex organ (Pfefferli and Jazwinska, 2015).

In this study, after the caudal fin amputation, the wound is healed in few hours. Thereafter, blastema formation is occurred in 12-48 hours. It is documented that *B*-catenin is one of the earliest molecular markers for fin regeneration in wound epidermal cells, induced in the first few hours after amputation and maintained through regeneration (Poss et al., 2000). Initiation of regenerative outgrowth is differing in three examined water temperatures of this study. It is started approximately 5 dpa at RT, and 2 dpa at 25°C and 28°C. Our finding indicates that temperature more than 25°C promotes procedure in caudal fin regeneration of *A. dispar*.

As results, members of the genus *Aphanius* can probably be a suitable candidate to study regeneration phenomenon. A noticeable biological characteristic of the *Aphanius* is its mid-life span (12-24 months) in compare to the *D. rerio* (3-5 years), and this would probably make *Aphanius* to be suitable for the aging researches, even though this assumption need further evaluation in future.

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چکیده فارسی

توانایی ترمیم باله دمی در ماهی گورخری معمولی *Aphanius dispar* (Rüppell, 1829) (ماهیان استخوانی عالی: کپورماهیان دندان‌دار)

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چکیده:
توانایی ترمیم در تعدادی از ماهیان شعاع باله شناخته شده است. برای ارزیابی قابلیت‌پذیری توانایی‌های ترمیم در این گروه از ماهیان، برای نخستین بار، ترمیم باله دمی در ماهی گورخری معمولی *Aphanius dispar* مورد بررسی قرار گرفت. در این مطالعه، باله دمی به‌دلیل دسترسی راحت، ساختار ساده و ترمیم سریع مورد استفاده قرار گرفت. نتایج نشان داد که ماهی گورخری معمولی از توانایی ترمیم باله دمی برخوردار است. آغاز رشد ترمیمی در سه درجه حرارت آب مورد مطالعه، متفاوت می‌باشد. در دمای اتاق، ترمیم حدوداً ۵ روز پس از اولین برش (۵dpa) و در دمای ۲۵ و ۲۸ درجه سانتی‌گراد ۲ روز پس از برش ترمیم شروع می‌شود. یافته‌های این مطالعه نشان می‌دهد که دمای آب بیش از ۲۵ درجه سانتی‌گراد موجب افزایش سرعت ترمیم در ماهی گورخری معمولی می‌گردد. با در نظر گرفتن توانایی بالای ترمیم در ماهی گورخری معمولی و همچنین عمر نسبتاً کوتاه اعضای این ماهیان، می‌توان نتیجه‌گیری نمود که این ماهیان احتمالا می‌توانند در مطالعات مربوط به ترمیم مورد استفاده قرار گیرند. با این حال، برای شناخت جزئیات مکانیسم ترمیم در این ماهیان نیاز به مطالعه بیشتر می‌باشد.
کلمات کلیدی: ترمیم، بلاستما، باله دمی، کپورماهیان دندان‌دار، آفانیوس.