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1,3-1-6 ß-glucans enhance tissue regeneration in zebrafish (Danio rerio): Further advantages for aquaculture applications

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1,3-1-6 ß-glucans enhance tissue regeneration in zebrafish (*Danio rerio*): Further advantages for aquaculture applications

Running title: ß-glucans enhance fish tissue regeneration

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ABSTRACT

High aquacultural rearing density and fish handling may result in frequent fish tissue damage and skin wounds, thereby facilitating the onset of secondary infections. The capacity of the zebrafish to regenerate tissues, as well as fins and other organs, makes it an ideal animal model for studying the mechanisms of tissue regeneration. Since macrophages are involved in tissue regeneration, a diet including ß-glucans might positively affect the process through activation of macrophages and other immune pathways. Consequently, the aim of the present study was to investigate the effects of inclusion in feed of two differently extracted 1,3-1,6 β-glucans on the caudal fin regeneration process in zebrafish.

One hundred twenty zebrafish were randomly distributed into 4 groups with 3 replicates each: an untreated non-amputated group (CNA), an untreated amputated group (CA), and two treated groups (MI and MII); each treated group received a different ingredient containing 1,3-1,6 ß-glucans, both administered at a dose of 12.5 mg kg^{-1} of body weight.

Results showed that 1,3-1,6 ß-glucans decreased fish mortality rate and enhanced both daily and cumulative regenerated fin area, independent of the specific extraction method used. Based on the mechanisms similarities of the innate immune system and tissue regeneration, these results may likely be extended to species of interest for the aquaculture sector.

Keywords: aquaculture, fish nutrition, immune-stimulant, 1,3-1,6 β-glucans, tissue regeneration, zebrafish

1 INTRODUCTION

Due to high aquaculture rearing density and/or handling of fish for several purposes (e.g., fish grading, culling, tank transfers, transportation), fish tissue damage and skin wounds frequently occur. Injuries may result in the onset of secondary infections that may impair fish growth and survival (Castanheira et al., 2017). To cope with tissue ruptures and wounds, the innate immune system is the primary defence mechanism and also plays key roles in the acquired immune response and homoeostasis. Several factors are involved in the activity of the innate immune system; temperature, handling and crowding stress can have suppressive effects, whereas several feed additives or ingredients (such as immuno-modulators) can enhance the immune defences (Magnadóttir, 2006). Macrophages are the main responding cells, due to the surface expression of pathogen-recognition receptors (PRRs) and their interactions with pathogen-associated patterns (PAMPs) on pathogens.

The PRRs-PAMPs binding mechanism activates macrophages and consequently host disease resistance (Soltanian, Stuyven, Cox, Sorgeloos & Bossier, 2009; Thompson, Oyston & Williamson, 2010). Moreover, macrophages have a defined and functionally important role in the tissue repair process. When injury or tissue damage occurs, a complex cascade of signals activates inflammatory responses (Whitehead, Makino, Lien & Keating, 2005). Specifically, macrophages secrete growth factors and cytokines that attract keratinocytes and fibroblasts to trigger both tissue repair and scar formation (Sandvik et al., 2007; Gurtner, Werner, Barrandon & Longaker, 2008; Yoshinari & Kawakami, 2011). Several authors (Li, Yan, Shi, Zhang & Wen, 2012; Petrie, Tsung-Yang, Rabinowitz & Moon, 2014; Przybylska-Diaz, Schmidt, Vera-Jiménez, Steinhagen & Nielsen, 2013) have reported that macrophages directly stimulate the wound healing process in common carp (*Cyprinus carpio* L.) and zebrafish (*Danio rerio*).

All phases of the wound-repair process of adult mammals have also been documented in

adult zebrafish. In this species, wound re-epithelialization is notably fast and starts with no apparent lag-phase; the starting phase is quickly followed by the migration of inflammatory cells and the formation of granulation tissue consisting of macrophages, fibroblasts, blood vessels, and collagen (Richardson et al., 2013). Furthermore, zebrafish have an outstanding ability to regenerate amputated fins and lesioned internal organs, such as the heart, brain, retinas, spinal cord and other tissues. The zebrafish is thus considered one of the most important animal models for tissue regeneration studies (Thatcher, Kimberly, Anderson & Patton, 2008; Singh, Holdway & Poss, 2012; Sousa, Valerio & Jacinto, 2012) and aquaculture research (Ribas & Pifferer, 2013; Ulloa, Medrano & Feijoo, 2014).

Recently, to cope with injuries occurring during fish rearing and handling, the "aquafeed" industry has been able to employ a wide range of immuno-active feed ingredients and additives to induce innate immune system and macrophage activation (Kiron, 2012). One of the most studied group of immuno-active feed ingredients are the β-glucans (Novak & Vetvicka, 2008; Robertsen, Engstad & Jorgensen, 1994), homopolymers of glucose having a linear structure (1,3-β-D-glycosidic linkages) or a branched one with bound side chains (1,6-β-D-glycosidic linkages). ß-glucans are the main constituents of cell walls of some plants, fungi, bacteria, mushrooms, yeast, and seaweeds. A common source of ß-glucans is the cell wall of baker's yeast *Saccharomyces cerevisiae*, and these carbohydrates are distinguished by an array of stimulatory effects on the immune system (Novak & Vetvicka, 2008; Sandvik et al., 2007); several studies illustrate their effects on stress and disease resistance (Bridle, Carter, Morrison & Nowak, 2005; Fronte et al., 2013; Gatesoupe, 2007; Kumari & Sahoo, 2006; Meshram, Murthy, Ali, Swain & Ballyaya, 2015), and 1,3-1,6 ß-glucans are considered to constitute the most effective series (Meena et al., 2013; Soltanian et al., 2009). The ability of yeast β-glucans to promote wound repair was first described by Leibovich & Danon (1980). β-glucan complexes have been shown to be practical and effective dressings to

improve healing of sunburn wounds (Delatte et al., 2001). Yeast 1,3-1,6 ß-glucans have been used for *in vitro* and *in vivo* experiments to study the degranulation of primary granules in fish neutrophils (Palic, Andreasen, Herolt, Menzel & Roth, 2006). However, few studies have addressed the effects of ß-glucans on tissue regeneration and the wound healing process (Przybylska-Diaz et al., 2013), even though these compounds are valuable indicators of the efficiency of immune cells involved in tissue repair and regeneration. Therefore, the aim of the present study was to investigate the effects of 1,3-1,6 β-glucans, obtained using two different extraction processes, on zebrafish fin regeneration.

2 MATERIALS AND METHODS

The experiment was performed at the Laboratory of Aquatic Animal Health, College of Veterinary Medicine, Chungnam National University (Daejeon, South Korea) and performed in accordance with the Institutional Animal Care and Use Committees of Chungnam National University (CNU-00927).

2.1 Zebrafish and fish husbandry

One hundred twenty adult zebrafish (wild type, AB line) were purchased from a local aquarium (Seoul Aquarium, Daejeon, Korea). Only male fish were used for the experiment to reduce variability among individuals (sex effect). On their arrival, all fish were treated with a solution of sodium hypochlorite (Sigma, Aldrich), 0.0075% chlorine final concentration, for removing possible external parasites, such as gill flukes. The fish were then randomly assigned to 12 tanks (3.5 L capacity), 10 individuals per tank, and kept for one month (acclimation period). Rearing water temperature was 26 °C (± 0.5 °C) throughout the whole duration of the experiment, and each tank was provided with a porous stone for air distribution. From each tank, faeces and debris were manually removed daily by syphoning out 80% of the water and restoring the initial water volume. A 12:12-h light cycle was

employed, and the fish were fed Aqua Tech© commercial feed (ß-glucan free), distributed 4 times per day (9:00AM, 12:00AM, 3:00PM, and 6:00PM) *ad libitum* according to the "five minute rule" described by Lawrence (2007).

2.2. Feed preparation and experimental design

During the last week of the acclimation period, the fish voluntary feed intake (FI) in 3 different tanks (10 fish each) was measured and estimated to be approximately 14.7 ± 0.64 mg per day (mean ± sd). Furthermore, the day before the beginning of the experiment (last day of the acclimation period), fish body weight (BW) was measured at 391 ± 68 mg (mean ± sd). Considering a feed intake ratio equal to 3.76% of BW and a 12.5 mg kg^{-1} BW ß-glucan supplementation, 352 mg of 1,3-1,6 ß-glucans per kg of feed were included (0.35 g kg^{-1} of feed). Two different commercial sources of 1,3-1,6 ß-glucans (MacroGard® and Experimental MacroGard®, Biorigin©, Sao Paulo, Brazil) extracted from *Saccharomyces cerevisae* cell walls were used; the difference between MacroGard® and experimental MacroGard® was not disclosed by the company, which referred only to a different 1,3-1,6 ß-glucans extraction process. For this study, ß-glucans were suspended in distilled water at a concentration of 20 mg mL^{-1} and sonicated (2 times x 30", pulse 2); afterwards, the suspension was dispersed into a pre-determined quantity of ground control feed (AquaTech©), and the mixture was amalgamated; the dough was then re-pelleted by means of a syringe, dried at 40 °C for 24 hours and finally re-ground to restore the original particle size. The same procedure was used for all three tested feeds (control, MI and MII).

The 12 tanks were randomly assigned to the 4 experimental treatments (3 replicates each): i) CNA (control - not amputated); ii) CA (control - amputated); iii) MI (MacroGard®, amputated); and iv) MII (Experimental MacroGard®, amputated). The trial was carried out

according to the "blind" methodology; neither the feeds nor the tanks were disclosed to the operators.

2.3 Caudal fin amputation

To perform the caudal fin amputation, fish were anesthetised in a 0.2% tricaine (MS 222 – Sigma Aldrich©, USA) solution, and then the following procedure was used: a) fish were placed under the stereoscope; b) the whole caudal fin of each fish was photographed before its amputation; c) fins were amputated 1 mm below the fork using a sterilized blade; d) the amputated fins were again photographed; e) fish were transferred into clean water tanks to recover from anaesthesia; and f) fish were then transferred into their initial tanks. Photos of the regenerating caudal fins were then taken at days 1, 4, 5, 6, 7, 12 and 14 after the amputation to evaluate their regeneration (Figure 1). No antibiotic treatment was required or administered to the fish after fin amputation.

2.4 Detection of the fin regeneration process

The caudal fin digital images taken before and immediately after fin amputation were used to individually identify each fish by observing unique fin patterns. After each fish was imaged, the full fin area (before amputation) and the regenerated fin area (after amputation) were digitally measured on the 1st, 4th, 5th, 6th, 7th, 12th and 14th day post-amputation (DPA) using ImageJ® software (Institute of Health, Bethesda, MD). The fin regeneration performance was then calculated and described using the following parameters: i) cumulative fin regenerated area (RA = (fin area at day n/pre-amputation fin area) x 100) and ii) daily regenerated area (DRA = (n DPA fin area – n-1 DPA fin area) x 100 / pre-amputation fin area). The regenerated fin area was measured only on non-blurred images; thus, the number of observations (n) does not match the number of live fish in the tank.

2.5 Statistical analysis

To estimate the number of fish required for observing differences among treatments, Statistical Power Analysis (expected difference between means: 3.5 mm^2, observed standard deviation: 3.8 mm^2, alpha value: 0.05) was performed. In addition to the fish of the CNA group (n = 30), there were a total of 90 fish used for detecting the regeneration process.

A chi-square test was used to investigate the treatment effect on mortality rates. Differences between treatments were then tested by Yates' Chi-square test (differences were considered significant when P values were lower than 0.05). ANCOVA nested by initial (pre-amputation) fin size was used to investigate the treatment effect on regenerated fin area, at different categorized times. Differences between treatments were tested by mean Tukey-Kramer; differences were considered significant when P values were < 0.05, even if values < 0.01 and < 0.001 were also observed (JMP, 2008).

To mathematically model the fin regeneration process, DRA values were transformed (to normalize residues, ln) and submitted to non-linear analysis (square regression). Differences between intercepts, slopes and quadratic slopes were tested using alpha level 0.05 (JMP, 2008).

3 RESULTS

Mortality was limited throughout the whole experimental period; nevertheless, statistically significant differences (P = 0.0354) between the CA group and the remaining groups were observed (Table 1). Notably, mortality was observed for the CA group on the 5th DPA (1 fish) and on the 14th DPA (last experimental day; 2 fish), another fish died in the MII group on the 14th DPA, while no fish died in the CNA or MI groups.

On the 14th DPA, none of the tested groups attained the initial fin area (pre-amputation fin size). Nevertheless, differences between groups in fin regeneration performance were observed (Table 2). On the 6th DPA, group MI exhibited an RA value (70.43%) significantly

(P = 0.0475) higher than that of the CA group (67.29%); no differences were observed between groups MI and MII (69.54%) or between MII and CA groups. On the 7th DPA, significant differences (P = 0.0271) were observed between groups MI (74.81%) and CA (71.20%), and again, no differences were observed between groups MI and MII (73.36%) or between MII and CA. On the 14th DPA, group MI reached 90.87% of the pre-amputation fin area, group MII 89.82% and group CA 85.75%; the differences between the treated groups and the CA group were statistically significant (P = 0.0092), while no difference was observed between groups MI and MII.

A similar trend was observed for DRA values (Table 3); on the 5th DPA the highest daily fin growth was observed in group MII (4.16% of the pre-amputation fin size) followed by group MI (3.81%); both of these groups significantly (P = 0.0019) differed from CA (2.58%), which exhibited the lowest DRA value. Similar results were observed on the 6th DPA; in this case, only group MI (4.51%) significantly differed (P = 0.0484) from group CA (3.34%), and no difference was observed between groups MI and MII (4.30%).

From a biological perspective, the fin regeneration process was closely represented by a parabolic curve (quadratic regression; Figure 2). The equation's parameters i) intercept (origin of the curve), ii) slope (inclination of the curve in its growing portion = growth-speed of fin), iii) quadratic (curve inclination in its descending portion), iv) maximum x value, and v) maximum y value are shown in Table 4. As expected, the intercept was not exactly equal to zero due to the amputation and differences (P<0.001) between the supplemented groups, (MI, 0.66; MII, 0.66) and the CA group (0.6). The slope values, which estimate the fin growth rate, were higher (P<0.001) in the MI (1.032) and the MII (1.030) groups than in the CA group (0.828). The quadratic coefficients confirmed the faster and earlier (P<0.001) fin regeneration process observed for groups MI (-0.0731) and MII (-0.0729) compared to group CA (-0.0592). The maximum velocity (X = DPA) of regeneration observed in the CA group

(6.2457), suggested that this group reached its highest daily regenerated area (y value) earlier than ($P<0.05$) did the ß-glucans treated groups (MI 6.5167; MII 6.3857). Similarly, the CA group (1.1713) exhibited the lowest ($P<0.05$) y value (maximum area regenerated in a day) in comparison to groups MI (1.3502) and MII (1.3626).

4 DISCUSSION

Recently, several studies have investigated the roles and mechanisms of macrophages and other immune cells in the fin regeneration of zebrafish (Li et al., 2012; Singh et al., 2012; Sousa et al., 2012). To the best of our knowledge, no studies evaluating the effect of 1,3-1,6 ß-glucans on zebrafish fin regeneration have been published. Nevertheless, the zebrafish is considered a valid animal model (Petrie et al., 2014; Richardson et al., 2013), and several authors suggest its use in aquaculture research (Dahm & Geisler, 2006; Ulloa, Iturra, Neira & Araneda, 2011).

The results of the present study clearly showed that including 1,3-1,6 ß-glucans in zebrafish diet enhances tissue regeneration and consequently the wound healing process. The fin re-growth value (RA) of the amputated control group (CA) was significantly lower than that observed for both groups treated with ß-glucans. These findings are well-supported and described by the modelled fin generation equation, which clearly shows that the CA group reached its maximum daily fin regeneration before both treated groups. It is reasonable to expect that this is due to the limited fin growth observed in the control group in comparison to those observed in the treated groups. However, in the present study, none of the tested groups fully completed the regeneration process. Other authors (Azevedo, Grotek, Jacinto, Weidinger & Saúde, 2011; Singh et al., 2012) have observed total fin regeneration at approximately 14 days after amputation, but the different water temperature and different extension of the amputation may have influenced the regeneration process. The full fin

regeneration process may have lasted longer in the present study than expected due to the relatively low water temperature (26 °C) used and the larger fin amputation (1 mm below the fork). However, the almost full fin regeneration observed just after 14 days post-amputation confirms that caudal fin amputation in zebrafish does not represent a permanent and severe injury (Azevedo et al., 2011). Furthermore, it is remarkable to notice that no antibiotic treatment was administered to the fish after the amputation of the fin; they were immediately swimming and eating, and no signs of distress were observed. Rather, the observed mortality events might have been related to the stress caused by the handling and repeated anaesthesia during the experiment, rather than to the amputation injury itself. From this perspective, it is also possible to conclude that the lower mortality rates observed for the groups fed 1,3-1,6 ß-glucans may be due to an enhanced stress resistance of these fish relative to the CA group; in fact, similar findings related to stress resistance enhancement after 1,3-1,6 ß-glucans administration have been previously reported by other authors (Soltanian et al., 2009; Fronte et al., 2013).

Consistent with our findings, a positive effect of the use of 1,3-1,6 ß-glucans on tissue regeneration has been reported by Przybylska-Diaz et al. (2013) in common carp (*Cyprinus carpio* L.). In this study, experimentally injured fish that received 1,3-1,6 ß-glucans exhibited a faster wound-healing response than did non-treated fish. Moreover, the effect of β-1,3-d-glucans has been studied in db/db mice to evaluate if it stimulates wound healing in diabetes; in this case, as well, the results showed that when β-1,3-d-glucans were administered, macrophage function was stimulated and the wound healing process enhanced (Berdal et al., 2007). Udayangani et al. (2017) tested an oat-derived nano-scale ß-glucans (NBG) preparation and investigated its immunomodulatory properties on zebrafish larvae; here too, the results showed that the survival rate of zebrafish larvae in the presence of the pathogenic bacterium *Edwardsiella tarda* increased when NBG were added to the water (500 mg/mL).

Moreover, quantitative real time PCR (qRT-PCR) analysis showed an up-regulation of immune functional genes, including TNF-a, IL-1b, ß-defensin, lysozyme, IL 10, IL 12 and C-Rel (Udayangani et al., 2017). However, Schmidt et al. (2016) did not observe a significant difference in the wound healing process in rainbow trout (*Oncorhynchus mykiss*). In this latter case, the authors explain that during the experimental period, water temperatures were extremely low and variable (lack of thermal insulation for tanks and an unusually cold winter); therefore, the lack of standardized rearing conditions and an excess of their variability may have negatively affected the accuracy of the experiment and might account for the lack of observed effects.

Future investigations may assess whether a dosage different than 12.5 mg kg^{-1} BW of 1,3-1,6 ß-glucans may result in performances differing from those observed in the present study. Similarly, it would be relevant to investigate further the effects of 1,3-1,6 ß-glucans on wound-healing performances of other teleost species of interest for aquaculture. One may speculate that due to similarity in tissue regeneration, as well as in innate immune system mechanisms, positive results on wound healing might be observed in other teleosts. However, tissue regeneration performance might vary according to the specific capability for tissue regeneration of the species in question.

Differing ß-glucans extraction techniques, as well as particle size, sonication, solubility, and stability, could have direct effects on the degree of immune stimulation. Novak & Vetvika (2008), Jaafar et al. (2011) and Sirimanapong et al. (2015), suggested variation in the extraction process could vary the potency of a ß-glucans based product from a negligible to an extremely high level. These findings suggest further research is needed to reduce the particle size and prevent glucans re-aggregation (clumping) into larger particle sizes when exposed to water in the digestive process (Hunter, Gault & Berner, 2002). In relation to the two different ß-glucan preparations tested in the present study, MacroGard® enhanced fin

regeneration slightly more efficiently than the Experimental MacroGard®, although the difference was never statistically significant. However, a comprehensive discussion of this aspect is not possible, since the producer did not disclose the extraction processes.

In conclusion, the use of zebrafish as an animal model to investigate the relationships among nutrition, immune stimulation and tissue regeneration (e.g., in wound healing) could be considered an innovative and effective approach in aquaculture research.

The results presented in this study suggest that 1,3-1,6 ß-glucans have a positive effect on the zebrafish fin regeneration process. Notably, the administration of 12.5 mg kg^{-1} fish body weight of ß-glucans enhanced tissue regeneration in zebrafish and promoted wound healing. Conversely, the differing extraction processes of the two 1,3-1,6 ß-glucan preparations used did not affect their efficacy. Based on the mechanism of the innate immune system and the tissue regeneration process of teleosts (Magnadóttir, 2006; Richardson et al., 2013), these results could potentially be extended to species of interest for the aquaculture sector.

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1 **TABLES**

2 Table 1: Cumulative fish mortality in relation to treatments

	CNA		CA		MI		MII		Chi^2	P
Number of dead	0	b	3	a	0	b	1	b	3.257	0.0354
%	0		10		0		3.3			

3 Note: Different letters within row denote significant differences among treatments (P<0.05)

Table 2: Regenerated area (RA†) values (%)

Group		CA		MI		MII		SEM	P
DPA§	n	mean		n	mean	n	mean		
0	29	35.41		30	33.40	30	33.60	0.2105	0.1747
1	29	50.88		29	52.08	30	51.07	0.2348	0.5838
4	27	61.31		29	62.35	30	61.19	0.2340	0.7186
5	27	64.35		30	66.15	30	65.29	0.2413	0.4927
6	28	67.29	b	28	70.43 a	30	69.54 ab	0.2346	0.0475
7	28	71.20	b	28	74.81 a	30	73.36 ab	0.2433	0.0271
12	25	85.11		28	88.55	30	87.47	0.2816	0.2845
13	23	86.23		23	88.83	22	88.61	0.3156	0.3747
14	23	85.26	b	28	90.87 a	30	89.82 a	0.2892	0.0092

Note: Different letters within a row denote significant differences among treatments (P<0.05)

† RA = (fin area at day n/pre-amputation fin area) x 100

§ DPA = days post-amputation

Table 3: Daily regenerated area (DRA‡) values (%)

Group		CA		MI		MII		SEM	P
DPA§	n	mean		n	mean	n	mean		
1	29	2.26		29	2.46	29	2.42	0.0838	0.4191
5	24	2.58	b	30	3.81 a	30	4.16 a	0.1444	0.0019
6	27	3.34	b	29	4.51 a	30	4.30 ab	0.1551	0.0484
7	28	3.90		28	4.04	29	3.95	0.1630	0.8656
13	18	1.21		21	1.22	22	1.34	0.1390	0.8628
14	19	0.74		19	1.29	22	1.21	0.1370	0.1039

Note: Different letters within row denote significant differences among treatments (P<0.05)

‡ DRA = (n DPA fin area – n-1 DPA fin area) x 100/ pre-amputation fin area

§ DPA = days post-amputation

Table 4: Descriptive parameters of the fin regeneration equation, in relation to treatment

Group	Intercept	Slope	Quadratic	Max X value	Max Y value
CA	0.60 a	0.828 a	-0.0592 a	6.2457 a	1.1713 a
MI	0.66 b	1.032 b	-0.0731 b	6.5167 b	1.3502 b
MII	0.66 b	1.030 b	-0.0729 b	6.3857 b	1.3626 b

Note: Different letters within a column denote significant differences among treatments ($P<0.001$)

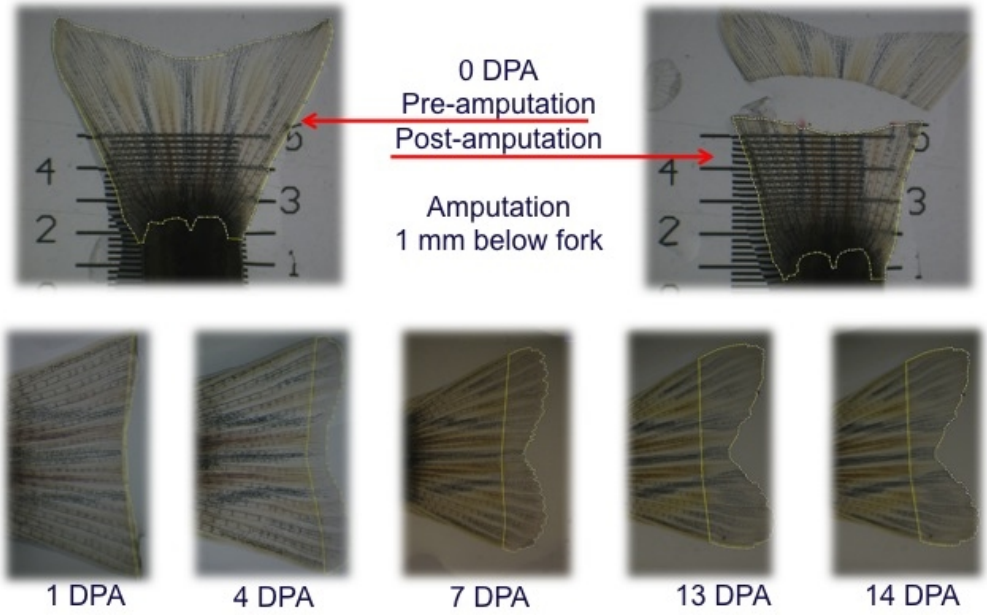

Figure 1: Caudal fin amputation and detection of the regeneration process

222x138mm (72 x 72 DPI)

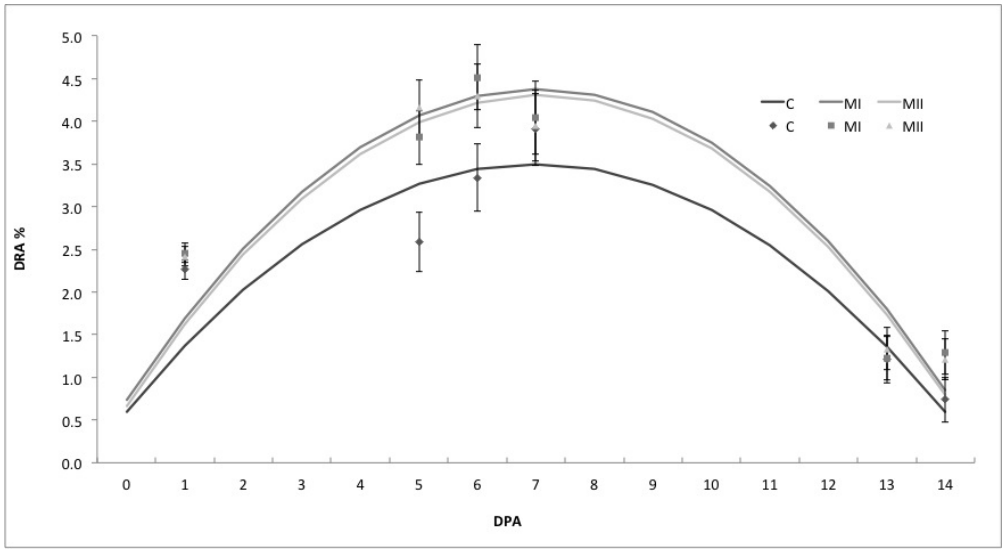

Figure 2: Quadratic regression of the Daily Regenerated Area (DRA)

309x168mm (72 x 72 DPI)