CHAPTER 3

PROTEIN EXPRESSION PATTERN AND ANALYSIS OF DIFFERENTIALLY EXPRESSED PEPTIDES DURING VARIOUS STAGES OF TAIL REGENERATION IN *HEMIDACTYLUS FLAVIVIRIDIS* AND *POECILIA LATIPINNA*.

INTRODUCTION

Regeneration is the field of study wherein the loss of the tissue or appendage of an adult species is redirected to the development process which ultimately leads to the tissue restoration both structurally and functionally. The goal of regeneration studies is to understand the mechanisms driving the process in complicated biological structures so that a regenerative response can be stimulated in the humans who are deprived of such enormous capacity. Amongst the different types of regeneration, one of the types is called as epimorphic regeneration. Epimorphic regeneration involves the de-differentiation of existing tissue and recruitment of proliferative cells to form the new structure to restore the lost part. Such type of appendage regeneration is observed in a number of vertebrates like fish, salamander, and lizard (Bellairs and Bryant, 1985; Poss *et al*., 2000a; Brockes *et al*., 2001; McLean and Vickaryous, 2011). Conventionally epimorphic regeneration can be divided into three distinct phases namely (1) wound epithelium: wherein epithelial cells from the amputation site migrate and cover wound surface, 2) Blastema: after wound healing, a cone-shaped blastema is formed beneath the multilayered wound epidermis which is a pool of undifferentiated proliferating cells and 3) Differentiation: the final stage wherein redevelopment of the lost structure is achieved through differentiation and patterning (Bryant and Gardiner *et al.,*, 2002).

Over the years several studies have been carried out in various models of regeneration to unravel the finer mechanisms of epimorphic regeneration (Poss *et al*., 2000a; Brockes *et al*., 2001). Initial studies were focused on the morphological, histological and biochemical changes associated with regeneration (Zika, 1969; Alibardi, 2009; McLean and Vickaryous, 2011). With the advent of modern analytical tools, the focus of the study of lately shifted to understand the cellular and molecular

mechanisms of regeneration. This led to the understanding of roles played by some of the important signalling molecules during regeneration like FGF, Wnt, BMP, Shh and many more (Riddle *et al*., 1993; Poss *et al.,* 2000b; Quint *et al.,* 2002; Jaźwińska *et al*., 2007; Tal *et al*., 2010). However, all the cellular and molecular processes involve the controlled expression of these signalling molecules at varied combination and concentrations. These signals facilitate various cellular events like proliferation, apoptosis, and morphogenesis during development. Epimorphosis too involves the coordination of these above mentioned cellular events which takes place in a coordinated manner. Nonetheless, in order to manage each step, it inevitably requires a large scale of protein turnover. Despite their significance in the regulation of a myriad of cellular events which are critical for the initiation and progression of epimorphosis, protein turnover during regeneration has attracted only minimum research interest. Moreover, their interaction with each other and how they modulate the stage specific physiological events during regeneration remains to be unfolded. Thus there is a great need to identify the proteins which govern the process of epimorphic regeneration.

Nowadays, studies have been initiated to elucidate the globally expressing genes, peptides, and transcription factors at one particular point of one specific stage/phase or event. Amongst all the cellular and molecular studies, a proteomic study is a valuable tool which elucidates the final products - the peptides that actually regulate the biological process of regeneration. Recently, few attempts have been made to understand the proteomic profile in regeneration models which are aimed at understanding the conserved and diverse mechanisms amongst them (Knapp *et al.,* 2013; Looso *et al.,* 2013; Wu *et al*., 2013; Hui *et al.,* 2014).

However, proteomic studies in the field of regeneration were initiated by Slack in 1982. He studied the protein expression pattern during few stages of axolotl limb regeneration both by 2D gel electrophoresis and by histochemistry. Moreover, a proteomic study of axolotl regenerating limb was carried out with quantitative label-free liquid chromatography and tandem mass spectrometry by Rao and associates (Rao *et al*., 2009). They have identified almost 309 proteins with significant fold change in comparison to resting stage, which is involved in different biological

processes. Subsequently, few other proteomics studies have also been carried out. While Jhamb and coworkers attempted network based transcription factor analysis of regenerating axolotl limbs (Jhamb *et al*., 2011), a comparative analysis of fibroblastema formation in the regenerating limb of *Xenopus laevis* froglet and axolotl was attempted by Rao *et al*., (2014).

In 2009, King *et al*. compared the profiles of abundant proteins in larval limbs of the *Xenopus laevis* at the time of amputation (0day post-amputation – 0dpa) and later at 3dpa, when the limb reached the blastema stage. They have observed a total of 1517 peptides, of which 1067 were identified and amongst them, 489 showed a significant change in their level of expression between the two groups. Additionally, they focused on genes which were found elevated at 3dpa and found heightened expression of genes belonging to the members of annexin family (e.g. ANXA1, ANXA2, ANXA5) which are known for their immunoregulatory roles. Additionally, they found the abundance of keratins that might facilitate cytoprotection and growth regulation possibly at the time of wound closure.

Furthermore, proteome profile of regenerating caudal fin of zebrafish was studied using 1D followed by LC-MS/MS and two-dimensional gel electrophoresis coupled with MALDI MS/MS (Singh *et al*., 2011). Based on their work they have identified 101 proteins, out of which 90 proteins were identified as differentially expressed in the stages of regeneration when compared to the resting stage. However, studies of proteome profile of lizards which are evolutionarily closer to humans were still lagging behind. Recently, attempts have been initiated in the reptilian group as well, and the differential protein expression pattern was studied during the tail regeneration in green anoles, *Anolis carolinensis*. The tail of anolis at 72 hours post amputation (72hpa) revealed 326 differentially expressed genes, many of them are found to be actively involved in multiple developmental and repair mechanisms. Among them, the major genes were known for their participation in the stress response, hormonal regulation, musculoskeletal development. Further, it was noted that components of Wnt and FGF signaling pathways too were expressed differentially. Additionally, multiple tissue-type specific clusters of proliferating cells were observed along the regenerating tail of *Anolis* (Hutchins *et al.,* 2014).

It is evident from the above review of literature that stage-specific proteome profile study is still to be undertaken in a reptilian model to understand the involvement of peptides during different stages of regeneration. The attempt has not yet been initiated in the lizard model of regeneration, northern house gecko, *Hemidactylus flaviviridis. H. flaviviridis* is an excellent model from the reptilian lineage to study the regeneration process and has been used in our laboratory for many years (Pilo and Suresh, 1994; Sharma *et al*., 2011; Yadav *et al.*, 2012; Buch *et al*., 2017). Hence, herein studies were designed to evaluate the protein expression profile during various stages of tail regeneration in *H. flaviviridis* using two-dimensional gel electrophoresis, followed by differentially expressed peptide identification and their transcriptional level confirmation using real-time PCR. The major goal of the study was to examine changes in the proteome of the re-growing tail that is triggered by amputation.

Moreover, it was also thought pertinent to check the extent of evolutionary conservation, if any, in the regulation of epimorphic regeneration amongst the vertebrates that possess the regenerative ability. In order to achieve this notion, parallel studies of the parameters mentioned earlier were conducted in teleost fish *Poecilia latipinna* (anamniote model of regeneration evolved early on in the vertebrate lineage) and in lizard *Hemidactylus flaviviridis* (representative of amniote model of regeneration originated late in the evolutionary time scale). Teleost fish *P. latipinna* was selected as a representative of anamniote model for tail fin regeneration, since it was found easy to maintain them in our laboratory condition and we have recorded sufficient historical data regarding the basics of caudal fin regeneration from our previous studies using this model organism over the years (Pillai *et al*., 2011; Saradamba *et al*., 2013, Rajaram *et al*., 2016).

MATERIAL AND METHODS

(A) Evaluation of the protein expression pattern using two-dimensional gel electrophoresis during the tail regeneration of *H. flaviviridis* and identification of representative proteins which are expressed differentially.

Northern House Geckos, *Hemidactylus flaviviridis*, of both sexes with normal intact tail were collected and acclimatized for a week before the commencement of the

experiments. The animals were maintained in the animal house as per conditions described earlier in the chapter Material and Methods.

In this study, total 16 animals were used. Autotomy in all the animals was induced three segments away from the vent by employing mild thumb pressure on the intact tail. Intact tail from three animals was collected and considered as a resting stage (RES). All the animals were examined daily for their progression of regeneration, and the tissue samples for a particular stage were collected from animals that achieved the given stage on a given day. Different stages of regeneration from where tissues collected were (1) wound epithelium stage (WE): which can be recognised as a smooth, shining surface upon the wound surface usually achieved on 4dpa; (2) blastema stage (BL): a small cone shaped stump of 2-3mm achieved on 6dpa and (3) fully differentiated stage or regenerated stage (REG): a completely regenerated unsegmented tail accomplished after 40dpa.

Experiment I

SDS-PAGE and Two-dimensional gel electrophoresis

For this experiment, all the stages *viz*. RES (resting tail), WE (wound epithelium stage), BL (a cone shape blastema) and REG (fully differentiated regenerated tail), from the animals, were collected and 10% homogenate was prepared with 2D lysis buffer containing (3-cholamidopropyl) dimethylamino-1-propane sulfonate (CHAPS), dithiothreitol (DTT) and protease inhibitor. Samples were centrifuged at 8000 rpm at 4°C for 15 minutes, and the supernatant was collected and stored at -20°C. All the samples were estimated for protein concentration using Bradford method (Bradford, 1976). Optical density was measured at 595 nm with an ELISA plate reader (Metertech Σ960), and concentration was obtained with the help of the standard curve.

The quality of the samples was checked using SDS-PAGE as described in the chapter material and methods. Thereafter, the equal amount of each sample was subjected to first-dimensional gel electrophoresis with a 2D marker (BIO-RAD, USA). Proteins were allowed to separate on the basis of their iso-electric point in the first-dimensional gel electrophoresis called isoelectric focusing with broad range immobilized pH gradient strip (IPG) 3-10 (BIO-RAD, USA) followed by equilibration

with DTT and Iodoacetamide (IAA). The strips were then embedded on the precast SDS gels, and the peptides were allowed to separate further based on their molecular weight. Gels were fixed with methanol:glacial acetic acid solution, stained with silver staining kit (Thermo Fisher, USA). All the protocols were followed according to the instruction manual. All the technical replicates were run in the similar manner. Gel pictures were taken and analyzed using BIO-RAD PDQuest analysis software. All the samples were run with the narrow range IPG strip (pH4 to pH7) also for the proper selection of the resolved spots. Gel pictures were captured and analsed in the PDQuest software version 8.0, and the spots showed significant stage specific change in expression pattern were selected after careful computational analysis.

LC-MS/MS

Selected spots were excised out and sent to proteomics facility of C-CAMP (Centre for Cellular and Molecular Platforms), Bangalore for the peptide identification and further characterization. Samples were subjected to in-gel digestion followed by1D nano-LC setup connected to the mass spectrometer. For the detection of the ions, LTQ Orbitrap Discovery was used. Data were recorded and searched on the MASCOT search engine with the available database SWISS-PROT and TrEMBL of *Anolis carolinensis*. The database was searched against the *Anolis* database because of want of peptide database for *Hemidactylus*.

Label free Protein analysis

Tissues of regenerates were homogenized with 2D lysis buffer in pre-chilled mortar pestle. 10% homogenate was prepared and supernatant from each samples by centrifugation was collected in the new tube to send it for in solution digestion followed by MS analysis. To find out the protein quantification present in each stage of lizard regenerating stage, each sample were subjected to the in solution digestion as per the protocol mentioned in on their site (http://www.ccamp.res.in / mass_spectrometry_services/sites/default/files/Digest%20Protocols.pdf). Trypsin buffer was re-dissolved in 1.5ml ice cold 1mM HCl (13ng/µl Trypsin prepared) and 100µl aliquots stored at -20˚C. Also, 100mM ammonium bicarbonate in water was freshly made and 5% Formic acid (vol/vol) was made in water.

In brief, the prcedure steps of in sloution digestion are as follows; pH of the protein sample was adjusted by adding 50mM ammonium bicarbonate to ~8.5. after vortex mixing with short spin 13ng/µl ice cold trypsin was added in 1:30 ratio. Mixed well. Tubes were places into thermostat and incubated at at 55^{o}C for 2hours/37^{o}C for overnight. Tubed were chilled to room temperature and 5% formic acid were added till the pH reaches nearly 3. Digested peptides were subjected to MS analysis. (http://www.ccamp.res.in/mass_spectrometry_services/sites/default/files/Digest%20P r otocols.pdf) (with additional alkylation and reduction). Digested peptides were vaccum dried and reconstituted in 15 µL of 2% ACN with 0.1% formic acid and 1 µL of the same was injected on to the column. Digested peptides were subjected to 180 minute RPLC gradient, followed by acquisition of the data on LTQ-Orbitrap-MS. Generated data was searched following standard approach for the identity using MASCOT 2.4 as search engine on Proteome discoverer 1.4. The data was searched against UniprotSwiss-Protdatabase (non redundant database with reviewed proteins), *Hemidactylus flaviviridis* database downloaded from NCBI. Minimum of two High confident peptides was used as a prerequisite to identify the proteins. 20 fmoles of Standard BSA digest was analyzed in parallel to the sequence to check the performance of the instrument. 20 fmoles of standard BSA was successfully found back with area of 2.001E8 and 2.618E8 and sequence coverage of 38.71% & 48.11% respectively. Due to insuficient database of *H. flaviviridis* corresponding search against different databases was made.

Experiment II

Real-time PCR

For this study, a total of 16 animals were used and divided into groups as described earlier in experiment I. Only those animals were selected who attained the respective stages *viz*., WE, BL and REG on a given day along with the amputated resting stage of the original tail of *H. flaviviridis*. Tissue was collected in the TRIzol reagent.

RNA isolation from the tissue collected in the TRIzol reagent was achieved using the TRIzol method, and their purity was checked by the ratio of absorbance at 260 nm, and 280 nm and quality of RNA was ascertained with 1% agarose gel

electrophoresis. To avoid contamination of genomic DNA, RNA samples were treated with RNase-free DNase (Thermo Fisher Scientific, USA). 1µg RNA was subjected to reverse transcription with cDNA synthesis kit (Applied Biosystems, USA). Sequences used for qRT-PCR are as follows:

krt15 Forward; 5'-GAGGCTGACATCAACGGTCT-3',
krt15 Reverse; 5'-TCCTGGGGCAGCATCCATTT-3',

myl-1 Forward; 5'-GCAACCCCAGCAATGAGGAA-3',
myl-1 Reverse; 5'-GAGTTCAGCACCCATGACTGT-3',

18S Forward;5'-GGCCGTTCTTAGTTGGTGGA-3' and
18S Reverse; 5'-TCAATCTCGGGTGGCTGAAC-3'.

cDNAs were subjected to qRT-PCR analysis using select SYBR green mix (Applied Biosystems, USA) and specific primers for real-time PCR as described in the chapter material and methods. Samples were measured in triplicate with 18SrRNA endogenous control. $\Delta\Delta$Cq values were calculated with reference to resting stage. The fold change in the target gene relative to the 18SrRNA endogenous control gene was also calculated as described earlier in material and methods.

(B) Study of the expression profile of peptide in the teleost fish, *Poecilia latipinna* during tail fin regeneration and the analysis of the expression pattern of *krt15* and *myl-1* during selected stages of fin regeneration.

Sailfin molly, *Poecilia latipinna,* (Lesueur, 1821), of both the sexes of the same age with an average size of 4-5 cm and weigh about .5 g were purchased from a commercial supplier. They were acclimatized and maintained in optimal conditions as described in the chapter material and methods.

One-third part of the fins from all the animals was amputated in a sterile condition and allowed to regenerate. Normal fin from six animals was collected and considered as a resting stage (RES). All the animals were examined periodically for their regeneration stages. As they reach a particular stage of regeneration in a given day six of them were collected, and the tissue samples were excised under hypothermic anaesthesia. The stages of regenerating fish fin collected were (1) wound epithelium

stage (WE): which can be visually recognised as a smooth during epithelial lining on the outer surface of the wound. Under ideal lab condition this stage is achieved in 24hpa; (2) blastema stage (BL): by 60hpa the blastemal cells accumulate as a bulge at the distal end of each lepidotrichium and (3) differentiation stage (DIF): characterised by the presence of *de novo* actinotrichia, the hallmark of early differentiation, beyond the cut end of lepidotrichia. In the case of *Poecilia latipinna,* this stage is achieved on 5dpa under optimal laboratory condition.

Experiment III

Two-dimensional gel electrophoresis

In this study, a total of 40 fishes were used. For this experiment, tissue samples were collected from all the selected stages *viz*. R (resting tail), WE (24hpa), BL (60hpa) and DF (differentiation stage) (5dpa) and 10% homogenate was prepared as described previously. All the run conditions were same as that of lizard tissue samples. During this experiment, samples from ten fishes were taken and pooled as one group, and multiple runs were made for accuracy.

Experiment IV

Real-time PCR

For this study, total 24 animals were used and divided into four groups as described in the earlier experiment. Animals were collected as and when they attained the respective stages of regeneration namely at 24hpa, 60hpa, and 5dpa along with the amputated resting stage (0dpa) of the original fin of *P. latipinna*. The tissue was excised and transferred to the TRIzol reagent.

All the protocols were followed as per the conditions which are given earlier. RNA purity was checked optically and ascertained with 1% agarose gel electrophoresis. 1µg RNA was subjected to reverse transcription with cDNA synthesis kit (Applied Biosystems, USA). Same sequences of lizards were used even for the *P. latipinna* after careful verification with semi quantitative PCR analysis. Their quantitative expressions were determined as described earlier with 18S as endogenous control. The $\Delta\Delta$Cq values were calculated with reference to resting stage.

Statistical Analysis

All the data were subjected to same way as described earlier using One-Way Analysis of Variance (ANOVA) followed by Bonferroni's multiple range test using GraphPad Prism 5.0, statistical analysis package. The values are expressed as mean ± SEM. A 'p' value of 0.05 or less was accepted as being statistically significant.

RESULTS

SDS-PAGE analysis of all the regeneration stages (RES, WE, BL and REG Stage) along with standard medium range molecular weight marker with the 98kDa, 68kDa, 44kDa, 29kDa, and 16kDa revealed differentially expressed peptides among them (Figure 1). Since the focus of this study was to identify few proteins that were either positively or negatively correlated with the regeneration process during the *H. flaviviridis* tail regeneration, protein extract was analyzed on a 7cm IPG strip followed by analysis on SDS-PAGE gels. Two-dimensional gels of regeneration stages (WE, BL and REG), when compared to the resting stage (RES) of lizard *H. flaviviridis* tail showed differentially expressed peptides (Figure 2, 3 & 4A). It is apparent that this is a very small fraction of the protein content of tissues of regenerate. Since the focus of the present study was to detect differences between the proteome of distinct stages, loading of a greater amount of protein on the strips would make resolution of individual spots more difficult. Figure 2 and 3 shows the expression pattern of the peptide at various stages of tail regeneration in lizard.

Computational analysis of these 2D gel images with PDQuest software revealed a number of spots. In the resting stage, a total number of 301 protein spots were present, while in wound epithelium, blastema and regenerated tail 230, 220 and 411 spots were recognized respectively (Figure 4A). Comparison of each stage with resting stage of lizard tail showed that between them they shared 138 spots with wound epithelium stage, 132 spots with blastema stage and 204 spots with the regenerated stage. In order to see whether the analysis was significant, correlation of each stage with resting was done which revealed a significance of 0.89, 0.79 and 0.96 for WE, BL and REG respectively. This has been represented by correlation graphs made in PDQuest software version 8.0 in figure 4B. The Venn diagram in figure 5 shows the comparison made between resting tail with that of different stages

of regeneration namely wound epithelium, blastema and regenerated stage. While there were 81, 79 and 119 number of spots up-regulated in WE, BL and REG stage respectively, it was also observed that 57, 53 and 87 peptides were down-regulated in the WE, BL and REG stage with respect to resting stage. After standardization of 2-Dimensional gel electrophoresis, a complete proteome profiling was necessary as the results from 2D suggested many peptides to be differentially expressed in the regeneration stages. In order to get the proteome profile of regenerating lizard tail for RES, WE, BL and REG stages, the analytical strategy was chosen for this was label-free quantification by high-resolution MS/MS acquisition. In fact, this type of approach is robust and provides the ideal alternative to traditional gel-based approaches, which remain the methods of choice in this type of study. Protein analysis of complex samples poses some issues. In our case, the main one was that *Hemidactylus flaviviridis* is a non-sequenced organism. Therefore protein identification must rely on homology, namely on sequences similarity of experimental proteins to the sequences of homologous proteins from phylogenetically related species. For this reason, experimental spectra were searched against certain specific protein sequence database, derived from SWISS-PROT and TrEMBL, which provides a huge data record of evolutionary closely related organisms, such as those belonging to the Squamata order.

The present experiment was devised to comprise a quantitative analysis as well, which would allow better comparing and help in characterizing the differences between the samples, in this case between the stages of regeneration in lizard tail. In the entire experiment, a total of 261 proteins were identified. The qualitative description of the whole proteome of all the samples has been listed down in Annexure I. The quantitative data provided differentially expressed proteins amongst the samples where 4, and 7 proteins were found to be up-regulated for WE and BL stage respectively. Few proteins were found to be down-regulated in all the stages, but most of the proteins remained unaffected (Table 4). All the differentially expressed proteins were categorized based on their involvement in the particular cellular process. A heat map was generated from the protein expression across all the stages of regeneration which is depicted in figure 6.

Moreover, it was observed that the major peptides are found in the range of pH 4 to 8 in all the stages. Therefore, the study was extended to the narrow range IPG strip (4-

7) and spots were selected from the more resolved peptides from the gel sets of narrow range (Figure 3). After careful analysis of the gels, we chose two spots for identification of peptides. The first spot, selected was differentially expressed (annotated by yellow circle in the gel in Figure 4A) and intensity was observed higher in the proliferative stages (*viz*. wound epithelium stage and blastema stage; where remodeling of the tissue takes place, and undifferentiated cells accumulate) in comparison to resting and regenerated stages. Second spot (annotated by green triangle in the gel image presented in Figure 4A) which was present in the resting stage and regenerated stage but not present in the WE and BL stage. Table 1 shows the peptides of interest selected during the study with their molecular weights and pI. All the identification steps were carried out at C-CAMP proteomics facility, Bangalore, involving in-gel digestion of spots. Data was searched on the MASCOT search engine with the available database SWISS-PROT and TrEMBL of *Anolis carolinensis*. The *Anolis* database was searched since it is the closest available database in terms of evolutionary hierarchy to that of the currently used model system - *Hemidactylus flaviviridis*. Two selected spots were identified as Keratin type 1 cytoskeletal 12/15 like and Myosin-light chain-1/3 skeletal isoform respectively (Table 2 and 3). According to the database search, Keratin type 1 cytoskeletal 12/15 like has almost the same score of 52.31 with a molecular weight of 50.94 and 53, and pI of 4.97 and 5.08 respectively. While the myosin light chain-1 showed 210 score with low molecular weight (20.6) and pI (4.89). However, the myosin light chain-3 isoform presented the same coverage and molecular weight of 16.6kDa and pI 4.6.

Nonetheless, to reaffirm the presence of these two proteins during the respective stages mentioned, further transcript level analysis was carried out using quantitative PCR. It is apparent from the analyzed results that *krt15* showed a significant increase in its expression during wound epithelium stage with a mean fold change of 5.472 compared to the resting stage. However, the same was found significantly decreased during the BL and REG stage of the tail (mean fold change 0.0032 and 0.0029 respectively**)** compared to the resting stage (Table 5; Figure 7). Conversely, *myl-1* showed a subtle but significant increase during WE stage. However, its level dropped substantially in the blastema stage followed by a sharp hike of 503.943-fold in the differentiated tail (Table 5; Figure 8).

As mentioned previously, in order to have a comparative understanding of the protein expression pattern between two distantly related vertebrate groups of animals with large scale regenerative ability a similar set of the study was also conducted in teleost fish *Poecilia latipinna.* Not surprisingly, a visual comparison of two-dimensional gel images of regeneration stages (WE, BL and DF) and resting stage (R) of *P. latipinna* caudal fin showed striking variation in the temporal expression pattern of peptides (Figure 9A). Further, computational analysis of these 2D gel images with PDQuest software revealed the presence of 182, 196, 201 and 193 spots in the resting, wound epithelium, blastema, and differentiated stages of tail fin respectively (Table 6; Figure 9A). Correlation graphs were made for the stages of regeneration for *Poecilia latipinna* wherein 0.83, 0.87 and 0.92 correlation was found between R stage and WE, BL and DF respectively. These graphs were made in the PDQuest software version 8.0 represented in Figure 9B.

Like the analysis performed in lizards, here too the comparison of the expression pattern of each regeneration stage with resting stage was performed, and the result revealed that 83 spots in WE, 87 in BL and 92 in DF stage were found commonly expressed between the stages mentioned and resting tail tissue. Figure 10 represents the Venn diagram of the comparison made amongst them, and it was observed that there were 40, 57 and 37 numbers of spots remained up-regulated in WE, BL and DF stage respectively. However, it was also noted that 40, 30 and 62 spots were down-regulated in the WE, BL, and DF stage with respect to their resting stage.

In order to further ponder the existence of an evolutionarily conserved pattern of peptide expression during epimorphosis and therefore the chance of existence of a common regulatory mechanism of epimorphosis amongst the vertebrates of diverse lineage, the peptides which were found differentially expressed as well as characterized during lizard tail regeneration were searched at transcript level in the regenerating fish fin as well. The analysis of the real-time PCR results revealed beyond doubt that *krt15* showed a definite hike in its expression during wound epithelium stage (24hpa) compared to the resting stage. However, the same was found to be significantly decreased during the late BL (60hpa) and differentiation stage (5dpa) of the tail as exemplified by the fractional mean fold change of 0.069

and 0.0146 respectively when compared to the resting stage (Table 7; Figure 11). On the other hand, *myl-1* showed only basal levels of expression during 1dpa and 4dpa but its level increased manifold during differentiation stage (7dpa) as evident from the mean fold change value of 15.9959 (Table 7; Figure 12).

DISCUSSION

A critical introspection into the finely tuned mechanisms of cellular events that culminate in the restoration of the lost structure, it is but logical to surmise that regeneration involves the controlled expression of peptides which are critical for achieving stage specific milestones of regeneration. Therefore, the current study was envisaged, wherein the stage-specific expression of peptides were studied using 2-dimensional gel electrophoresis as the tool. As a prelude, SDS-PAGE was performed for the selected stages of regeneration, and the analysis of the result showed that the expression levels of many proteins as indicated by the grey scale intensity of bands in the gel image were found different at a particular stage of regeneration. Hence, the study was extended to more detailed analysis. To achieve this, two-dimensional gel electrophoresis was performed which separates peptide of same molecular weight by focusing them on the basis of their isoelectric point. When the digital images of the stained two-dimensional gels were analysed and compared the resolution pattern of peptides of the selected stages of regeneration with that of the resting stage using an appropriate image analysis software, we found that some of the spots were significantly up-regulated at a particular stage of regeneration. In addition, it was also noticed that few spots were down-regulated, and their presence was not observed at a given time point or an event of regeneration. In other words, a stage specific signature expression pattern of peptides was quite apparent. Nonetheless, among all the spots, few remained constantly present in all the stages which largely belong to the class of structural proteins. From our historical data as well as from the vast amount of published data (some are discussed in the chapter Introduction), it is well perceived that several cellular and physiological events like cell proliferation, apoptosis, morphogenesis, repatterning and immune response occur during the course of regeneration (Endo *et al.,* 2004; Alibardi, 2017). And to regulate these events (many of them are stage specific), characteristic regulatory peptides, especially the components of signal transduction and transcriptional regulators, need to be expressed in a stage specific manner. The list of peptides identified from a label

free analysis of protein samples extracted from various stages of tail regeneration in a lizard (table 4) gives credence to the above notion. The label-free approach provided more comprehensive protein identification, with good analytical reproducibility and sample throughput. Moreover, label free quantification is inexpensive and can be applied to any kind of biological material, with high proteome coverage of quantified proteins (Piovesana *et al*., 2016). In total, 261 proteins were identified through label-free detection analysis. Both qualitative and quantitative data were obtained for RES, WE, BL and REG stages. Out of which some proteins were found to be expressed differentially in all the stages. Those differentially expressed proteins were categorized based on their involvement in cellular processes. During WE and BL stages, Bcl-2, an anti-apoptotic protein was found to be up regulated whereas BAD; a pro-apoptotic protein was down regulated signifying a tight regulation of cell proliferation and apoptosis. Such controlled activity of pro- and anti-apoptotic proteins during fin regeneration has been reported in zebrafish (Hasegawa *et al*., 2015). Other than apoptosis related proteins, noticeable changes in the levels of inflammatory mediators were reported. Anti-inflammatory proteins, IL10, and platelet-derived growth factor receptor alpha precursor were found to be elevated during early stages of regeneration. Expression of tumor necrosis factor was found to be decreased as it acts as pro-inflammatory mediator. There are reports on balanced inflammation for successful epimorphic regeneration in Xenopus limb and lizard *Podarcis muralis* (Harty et al., 2003; King et al., 2012, Vitulo *et al*., 2017). Many of the structural proteins were found to be unaltered during lizard tail regeneration from their proteins levels obtained by label-free detection. No significant changes in their levels throughout regeneration suggest their involvement in maintaining the structural integrity of such complex tissue which is regenerating lizard tail in this case. Moreover, protein levels of certain metabolic intermediates also remained unaffected showing a great cop up with the situation for an amputated animal for its appendage. Lastly, regulatory proteins were also recorded to be more or less constant for all the stages of regeneration. Similar results were observed during regeneration of zebrafish caudal fin in a screening study for differentially expressed genes (Padhi *et al*., 2004). However, we could not rely more on this result since the bioinformatic search was far from complete due to lack of suitable species-specific databank. Hence, further study in this direction had to be suspended as of now and is not discussed in detail. Notwithstanding the above technical hitch, the result so

generated is good enough to explain the reasons behind the differential expression pattern of peptides observed during the defined stages of regeneration in lizard and hence, presumably in teleost fish as well.

Further, our aim of the present study was to identify those peptides which are specifically found up-regulated in the early (proliferative) stages of regeneration *viz*. WE and BL, and hence they are assumed to play a significant role in the initiation of regeneration via triggering important signalling cascade involved during the epimorphic regeneration. Hence, after careful analysis, the spot which showed the significantly elevated level of expression was excised and characterised using LC MS/MS. It was identified as Keratin type I cytoskeletal 12/15 like peptide.

Keratin type I cytoskeletal 12/15 like is one of the epithelial keratins (soft alpha-keratins) which are an important constituent of intermediate filaments of the intracytoplasmic cytoskeleton of epithelial cells (Alibardi and Toni, 2006). It is documented that keratin is a structural protein which is important for the maintenance of the structure and hence provides robustness to tissues (Roop, 1995; Morasso and Tomic-Canic, 2005). Of lately, the valued role of keratin proteins was reported from the studies on the healing responses of several model systems of wound healing (Ishida-Yamamoto *et al.*, 1998; Wojcik *et al*., 2000; Patel *et al*., 2006). It has also been observed that various keratins are expressed at high levels in regenerating tissues (Smoller *et al*., 1989; Ferretti and Brockes, 1991; Tsonis *et al*., 1992; Ferretti *et al*., 1993; Martorana *et al*., 2001). Imboden *et al*., (1997), identified Cytokeratin 8 as a suitable epidermal marker during zebrafish caudal fin regeneration. In a study of regenerating axolotl limb, Keratin 5 (KRT5) and Keratin 17 (KRT17) were analyzed by Moriyasu and coworkers in the year 2012, and they reported that the KRTs were found expressed in the regenerating limb but not in differentiating limb. Regulated expression of KRT5 was observed during blastema stage, wherein the expression was found suppressed in the basal layer of the AEC. Based on the observations they have concluded that KRT5 and KRT17 can be used as markers for AEC formation during limb regeneration (Moriyasu *et al*., 2012). Knockout study of Keratin 17 in mice showed delayed wound healing (McGown *et al*., 2002) and its reintroduction leads to improved healing. From the results of an *in-vitro* study, Kim *et al*., (2006) suggested

that keratin plays an important role in improving the speed of wound healing. Moreover, there is ample evidence to prove that keratin formulation can improve epidermal migration during wound healing by upregulating keratin gene expression (Perez *et al.*, 2009; Pechter *et al.*, 2012).

Moreover, it has also been reported that the expression pattern of different cytokeratin polypeptides varies with the biological state of vertebrates like development, adulthood, regeneration, and hyperproliferation, suggesting that each one plays a distinct role in cell fate (Fuchs *et al*., 1987; Kallioinen *et al*., 1995). Additionally, Keratins can also regulate the growth and protein synthesis of epithelial cells. Kim *et al.,* (2006) have reported that Keratin 1 lacking cells showed depressed protein synthesis with decreased *Akt* signaling. Moreover, since they are prevalently found expressed during the wound healing process, recently keratins have been proposed as a potential treatment for wounds (Than *et al*., 2012). Further, understanding about the role of keratin during wound healing, as in the case of its role in WE stage of regeneration, might give the much-needed fillip to the clinical trials leading to the potential use of keratin based biomaterial for wound healing.

As mentioned previously, the first phase of epimorphic regeneration is always achieved by the formation of a wound epithelium with the migration of keratinocytes by digesting the basal membrane with the help of matrix metalloproteinases (Ferretti and Géraudie, 1998; Kawasumi *et al.,* 2013). Few of the mammalian type keratin markers such as Keratins 6, 16, and 17 have also been reported from lizard (Alibardi, 2000; Alibardi and Toni 2005, 2006). Therefore, herein the observed expression of keratins during the early stage of regeneration suggests their possible role in the formation of a multilayered wound epithelium – the apical epithelial cap. The AEC acts as one of the major organizing center during early epimorphosis and maintains the mesenchymal cells beneath in a state of continued proliferation (Ferretti, 2013).

Considering the significance of AEC formation during regeneration, the expression level of its possible inducer - the cytoskeletal keratin 12/15 like was studied further at transcript level during various stages of tail regeneration in *H. flaviviridis*. For the transcript level study, several primers, from different animal models on the basis of their conserved region, were designed for the quantitative expression study. Due to

the paralog nature of *krt12* and *krt15,* we designed primers for both based on their conservation sequence. Analysis of the quantitative PCR result revealed that there is a definite change in the transcript level expression of *krt12* and *krt15* during the regeneration. Elevated levels of mRNAs were found at WE stage, which again confirms the role of Cytoskeletal keratin 12/15 like in the formation of a functional wound epithelium during epimorphosis.

Notwithstanding the above observation, from the literature search, it was found that *krt12* is generally observed in the corneal epithelium of mammalian test systems during Meesmann Corneal Dystrophy (Kao *et al.,* 1996; Irvine *et al*., 1997; Allen *et al*., 2016). However, in other vertebrates, the role of keratin 12 is still inconclusive. On the other hand, Keratin 15 is reported to be present in the epithelial cells and is proposed as an epidermal stem cells marker (Bose *et al*., 2013)**.** Based on the above discussion, we presume that the identified peptide Cytoskeletal keratin 12/15 like could be Cytoskeletal keratin 15. However, the further species-specific analysis needs to be conducted in future to ratify the present notion.

The second spot we selected for the current study was the one found overtly expressed the differentiated stage but was inconspicuous in terms of spot intensity during the early proliferative stages of regeneration. In other terms, it can be said that the selected peptide spot was negatively regulated during the important events of regeneration i.e. WE and BL in the *H. flavivridis* tail. Hence, the selection of the second spot was made by considering the fact that it might negatively regulate the initiation of regeneration. Subsequent analysis using LC MS/MS it was identified as Myosin light chain 1/3 skeletal muscle isoform (MLC1/3).

MLC-1/3, a small polypeptide alkali light chain and is a functional unit of myosin. This light chain (MLC-1/3) along with two heavy chains and two regulatory chains form a complex unit of myosin hexamer – a structural component of fast skeletal muscles (Periasamy *et al*., 1984). The same authors have also reported that MLC-1/3 is encoded by the gene myl-*1* and that exists in two transcript variants. Their isoform diversity is greatly controlled at transcription level with cis-acting regulatory modules which control tissue-specificity and the spatiotemporal regulation of gene expression (Kelly and Buckingham, 2000). However, they are not considered as ‘myosins’ but

are recognised as components of the macromolecular complexes that make up the functional myosin enzymes (Kelly and Buckingham, 2000). It has also been reported that along with myosin heavy chain, myosin light chain forms a functional unit for smooth muscle contractility, migration, and proliferation (Gallagher *et al*., 1997). Moreover, it has been documented that in all types of smooth muscles, the contractile response involves activation of the myosin to a force generating stage upon phosphorylation of myosin regulatory light chains by kinases (Gallagher *et al*., 1997). Rao and his coworkers (2009), have reported the presence of myosin light chains in the limb tissues of the axolotl. Based on their proteomic study of regenerating limb of axolotl, they have noted that about one-third of the cytoskeletal proteins were found significantly down-regulated during the initial proliferative stages of regeneration, and that include sarcomeric proteins of skeletal muscle such as TNNT3A, TM7, myosin light chain 3 (MYL3) and myosin light chain 5 (MYL5) (Rao *et al.,* 2009).

The above observation gives credence to our finding that MLC-1/3 is conspicuously expressed only in the differentiated tissues of lizard tail and during WE as well as BL stages the expression of the same is significantly down-regulated. However, in order to further confirm our result, we also conducted a transcript level analysis. Nonetheless, it has been documented that, the isoforms of Myosin light chain 1/3 skeletal muscle are formed from a single gene (*myl-1*) in humans as well as in mouse (Robert *et al*., 1984; Barton and Buckingham, 1985). Therefore, the primers were designed for *myl-1* gene, and the transcript study was carried out based on their evolutionarily conserved sequence among the different species. It is also reported that their orthologs are found in all vertebrates (Barton and Buckingham, 1985). The qRT-PCR results also followed the same trend as that of LC-MS/MS result. The expression of *myl-1* transcripts at the WE and BL stage was found to be at the basal level, while the same at the differentiation stage was observed to be significantly up-regulated. The results suggest that the Myosin light chain 1/3 might be playing a significant role in the contractility of differentiated tail muscles in lizard. However, it has been reported that after autotomy the integrity of the muscle, at the site of amputation, is altered enzymatically to make the muscles undergo cellularization (Brockes and Kumar, 2002; Morrison *et al.,* 2006). This change in state of muscle explains the reason for the observed down-regulation of myosin light chain protein during WE and BL stages of lizard tail regeneration.

Furthermore, the role of *myl*-1 in fast-twitch fibres in zebrafish embryo and in the mouse has also been reported. Kelly and Buckingham in 1997 have reported the expression of *myl-1* in mouse fast-twitch fibres. In 2011, Burguie`re, and his colleagues had reported *myl-1* as the earliest expressed marker in the fast-twitch precursor cells in the zebrafish embryo. They also observed that *myl-1* knockdown disrupts myogenesis. Hence, the *myl-1* expression during the differentiation phase of lizard tail regeneration points its possible involvement in the muscle differentiation, which is a predominant cellular activity at that stage of tail regeneration.

Additionally, we conducted a parallel study of the expression pattern of *keratin 15* and *myl-1* in the regenerating caudal fin of teleost fish *P. latipinna* to find out whether these peptides play a similar role in epimorphosis, as observed in lizard, the other model of vertebrates, as well. The conduct of the study was kept same as it was designed for the northern house gecko. The results of the study revealed that as observed in the regenerating tail of a lizard, *keratin 15* showed a definite hike during the WE as well as BL stages of the fish fin regenerate. Moreover, it was further observed that during the caudal fin regeneration of fish the *myl-1* expression remained high during the differentiation stage compared to the early stages (WE and BL) of regeneration, again showing a similar pattern of expression for the studied transcript in the tail regenerates of teleost fish and that of the regenerating tail of lizard. When a careful review of the available literature was made, it was found that several keratins have been reported to be conserved evolutionarily. It has been documented that the chromosomal arrangement of *keratins 8*, *keratin 18*, and a second *type II keratin*, as a cluster of three genes, has remained conserved in the vertebrate lineage (Krushna *et al*., 2006). Based on the comparative expression pattern of the selected peptides (KRT 12/15 and MLC-1/3) in the regenerating tissues of fish and lizard, there appears to have a parallelism in the regulation of epimorphosis amongst vertebrates. Furthermore, we could observe similarity in the expression pattern of a lot more peptides in the annotated images of the 2-D gels and the trend of two of them (MMP2 and 9) are discussed in the next chapter.

In brief, heightened expression of Keratin type I cytoskeletal 15 like was found during wound healing stage of appendage regeneration in our selected animal models *H.*

flaviviridis and *P. latipinna,* indirectly suggesting the evolutionarily conserved mechanisms of regeneration. In addition to the above results, MLC 1/3 isoform was observed to be at its basal level during the proliferative stages (WE and BL) of regeneration. However, its expression increased significantly with the advent of differentiation in both the regeneration models, suggesting its role in the initiation of muscle differentiation and subsequent build up to the myosin hexamer – the functional unit of fast striated muscle that helps in the mobility of the tail or fin. The results so far consolidate our notion of evolutionary conservation of the mechanisms that regulate epimorphosis in vertebrates, however, still more such comparative analysis is to be conducted before confirming this hypothesis beyond doubt. Keeping this in mind, we studied two isotypes of one protein in the regenerating appendage of both fish as well as a lizard, and the results are presented in the following chapter.

Protein	Molecular Weight	pI
MMP2	74.9kDa	5.2
MMP9	76.5kDa	5.6
Keratin, type 1 cytoskeletal 12 like	50.9kDa	4.9
Myosin light chain 1	20.6kDa	4.9

Table 1: Spots of interest of two-dimensional gels during the study during *H. flaviviridis* tail regeneration.

Accession	Description	Score	Coverage	MW [kDa]	calc. pI
327275722	PREDICTED: Keratin, type I cytoskeletal 12-like isoform X1/X2/X3/X4[*Anolis carolinensis*]	52.31592879	4.03	50.9435029	4.97021484
637314966	PREDICTED: Keratin, type I cytoskeletal 15-like [*Anolis carolinensis*]	52.31592879	3.76	53.2517173	5.08447266

Table 2: Identification details of selected spot 1 with nano-LC-MS/MS

Accession	Description	Score	Coverage	MW [kDa]	calc. pI
327260713	PREDICTED: myosin light chain 1/3, skeletal muscle isoform isoform X1 [*Anolis carolinensis*]	210.8668829	31.22	20.6353527	4.89404297
327260715	PREDICTED: myosin light chain 1/3, skeletal muscle isoform isoform X2 [*Anolis carolinensis*]	210.8668829	39.33	16.6861483	4.64013672

Table 3: Identification details of selected spot 2 with nano-LC-MS/MS

Table 4: Fold change in protein level across the regeneration stages in *Hemidactylus flaviviridis* tail. Data is obtained from the label-free analysis.

PROTEIN	FOLD CHANGE		
	WE	BL	REG
Apoptosis			
BAD	0.391 (-)	0.394 (-)	0.916
Bcl2	3.287 (+)	3.842 (+)	1.044
Caspase 3	0.961	0.906	0.980
Inflammation			
tumor necrosis factor (TNF superfamily, member 2)	0.332 (-)	0.296 (-)	0.772
IL17	0.819	0.789	0.982
IL-1-beta	0.627	0.599	1.118
cyclo-oxygenase 2	0.714	0.698	0.965
platelet-derived growth factor receptor alpha precursor	2.243 (+)	0.862	1.059
IL10	2.883 (+)	2.858 (+)	1.004
Structural proteins			
Actin, Gamma	1.091	2.302 (+)	1.176
Calmodulin	1.141	1.088	1.070
Keratin type I cytoskeletal 15	5.031 (+)	0.137 (-)	0.210
Keratin, type I cytoskeletal 9 isoform X5	1.385	0.416 (-)	0.956
Myosin light chain 1/3, skeletal muscle isoform	0.857	0.344(-)	13.590(+)
Myosin-4	0.641	0.563	1.272
Myosin-7	0.707	0.508	1.006
Tropomyosin alpha-1 chain	1.305	2.188 (+)	0.464 (-)
Tropomyosin alpha-3 chain	1.164	0.537	1.197
Tropomyosin alpha-4 chain	0.916	0.947	0.909
Troponin C, skeletal muscle	1.054	1.040	1.030
Tubulin alpha-1 chain	1.158	1.427	1.018
Tubulin alpha-5 chain-like isoform X1	0.878	1.550	1.065
Tubulin beta-7 chain	1.143	1.254	1.883

PROTEIN	FOLD CHANGE		
	WE	BL	REG
CALM3	1.141	1.088	0.892
Metabolism			
Creatine kinase	0.815	8.623 (+)	1.026
Enolase 1, (Alpha)	0.569	1.722	1.396
Enolase 3 (Beta, muscle)	0.334 (-)	1.002	1.062
Fructose-bisphosphate aldolase	1.016	1.054	1.190
Glyceraldehyde-3-phosphate dehydrogenase	0.723	0.551	1.020
Fatty Acid-Binding Protein, Adipocyte	0.872	0.855	0.978
Pyruvate Kinase PKM	1.228	1.191	0.993
Regulatory proteins			
Elongation factor 1-alpha 1	1.042	1.188	0.977
Serine/threonine-protein phosphatase 4 catalytic subunit B	1.355	1.525 (+)	0.986
Eukaryotic Translation Initiation Factor 1	1.198	3.752 (+)	1.055
Far Upstream Element-Binding Protein 2	1.034	0.982	1.012

Stages of Regeneration	***keratin type 1 cytoskeletal 15 like (krt15)*** **(Mean ± SEM)**	***myl-1*** **(Mean ± SEM)**
Wound Epithelium Stage	$5.472 \pm 0.856^{***}$	$3.15 \pm 0.61^{***}$
Blastema Stage	$0.0032 \pm 0.00045^{***}$	$0.292 \pm 0.08^{***}$
Regenerated Stage	$0.0029 \pm 0.00038^{***}$	$503.943 \pm 60^{***}$

Table 5: *keratin type 1 cytoskeletal 15 like* and *myl-1* light chain transcript fold change expression during *H. flaviviridis* tail regeneration. SEM=standard errors of the mean.**p<0.01,***p<0.001.n=3.

Stages of Regeneration	Spots
Resting stage	182
Wound Epithelium stage	196
Blastema stage	201
Regenerated Tailfin	193

Table 6: Number of peptides expressed during *P. latipinna* fin regeneration at resting, Wound epithelium, Blastema and Regenerated stage.

Stages of Regeneration	***keratin type 1 cytoskeletal 15 like (krt15)*** **(Mean ± SEM)**	***myl-1*** **(Mean ± SEM)**
Wound Epithelium Stage (24hpa)	$2.34 \pm 0.45^{***}$	$0.0138 \pm 0.00326^{***}$
Blastema Stage (60hpa)	$0.069 \pm 0.0089^{***}$	$0.186 \pm 0.01241^{***}$
Differentiated Stage(5dpa)	$0.0146 \pm 0.004^{***}$	$15.9959 \pm 5.0580^{***}$

Table 7: *keratin type 1 cytoskeletal 15 like* and *myl-1* light chain transcript fold change expression during *P. latipinna* fin regeneration. SEM=standard errors of the mean. **p<0.01, ***p<0.001. n=6.

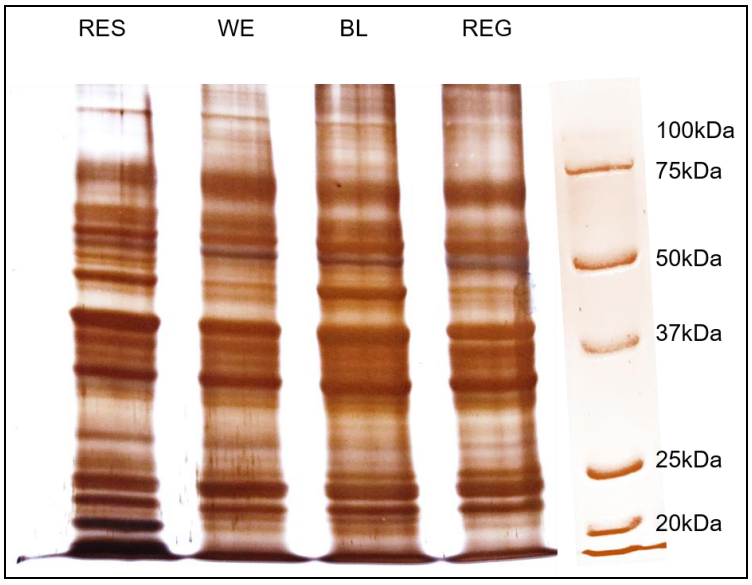


Figure 1. SDS-PAGE gel images of different stages in tail tissues of *H. flaviviridis*. Lane RES: protein extract from resting stage; Lane WE: protein extract from wound epithelium stage; Lane BL: protein extract from blastema stage; Lane REG: protein extract from regenerated stage. Lane 5 has the standard molecular weight marker.

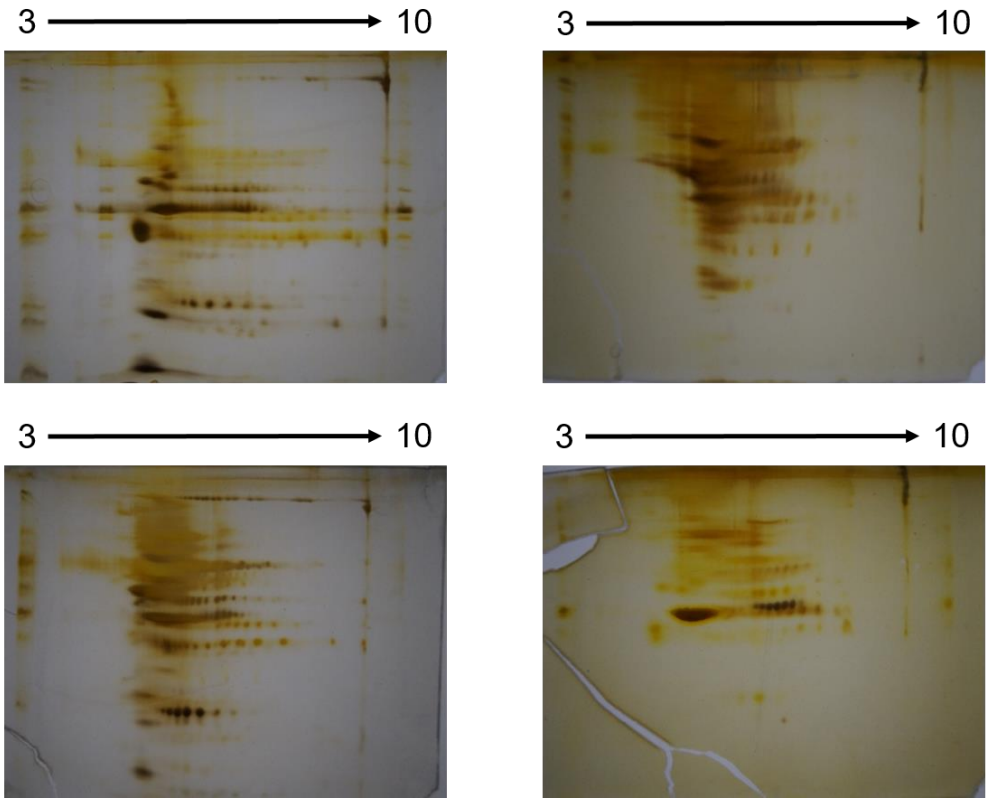


Figure 2: Two-dimensional gel images of different stages in tail tissues of *H. flaviviridis.* 1) protein spots from resting stage; 2) protein spots from wound epithelium stage; 3) protein spots from blastema stage; 4) protein spots from regenerated stage. IPG strip: pH 3-10 and the gels were silver stained.

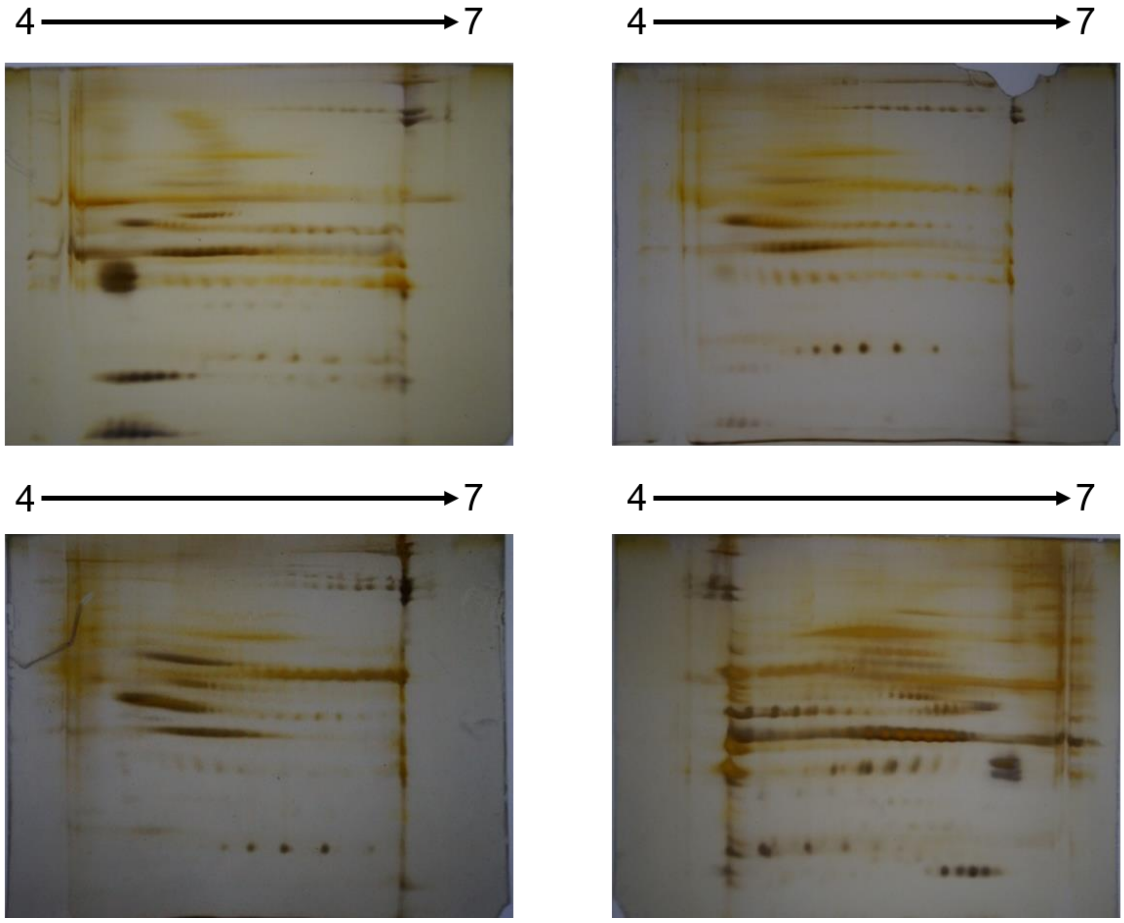
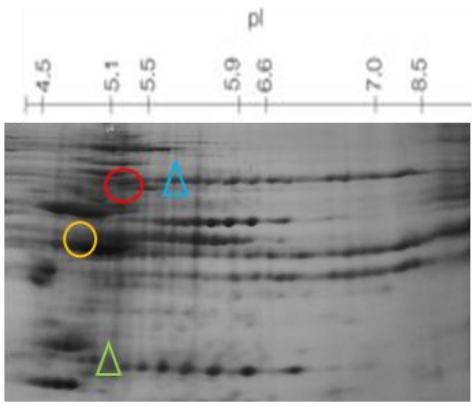
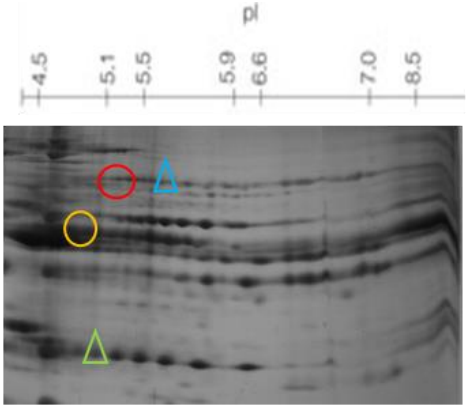


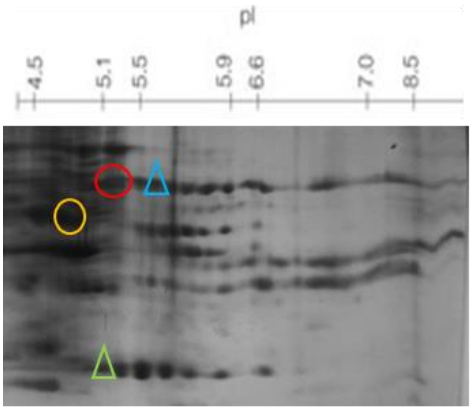
Figure 3: Two-dimensional gel images of different stages in tail tissues of *H. flaviviridis.* 1) protein spots from resting stage; 2) protein spots from wound epithelium stage; 3) protein spots from blastema stage; 4) protein spots from regenerated stage. IPG strip: pH 4-7 and the gels were silver stained.



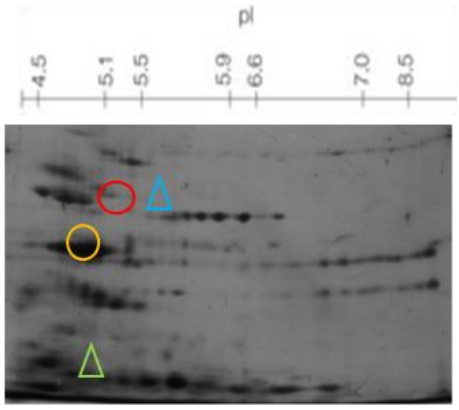
Resting tail



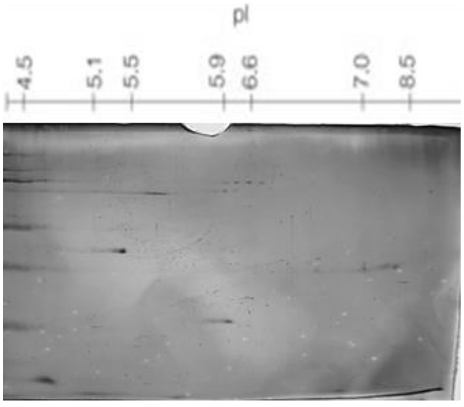
Wound Epithelium



Blastema



Regenerated Tail



2D Marker

1	pI 5.5 to 6.9	76kDa
2	pI 5.5	66kDa
3	pI 5.1	43kDa
4	pI8.5	31kDa
5	pI 5.9	31kDa
6	pI4.5	22kDa

Figure 4A: Representative two-dimensional gel images for the Selected spots and their comparison in all the stages of *H. flaviviridis* regenerating tail for further analysis. The table shows values of 2D molecular weight marker in kDa.

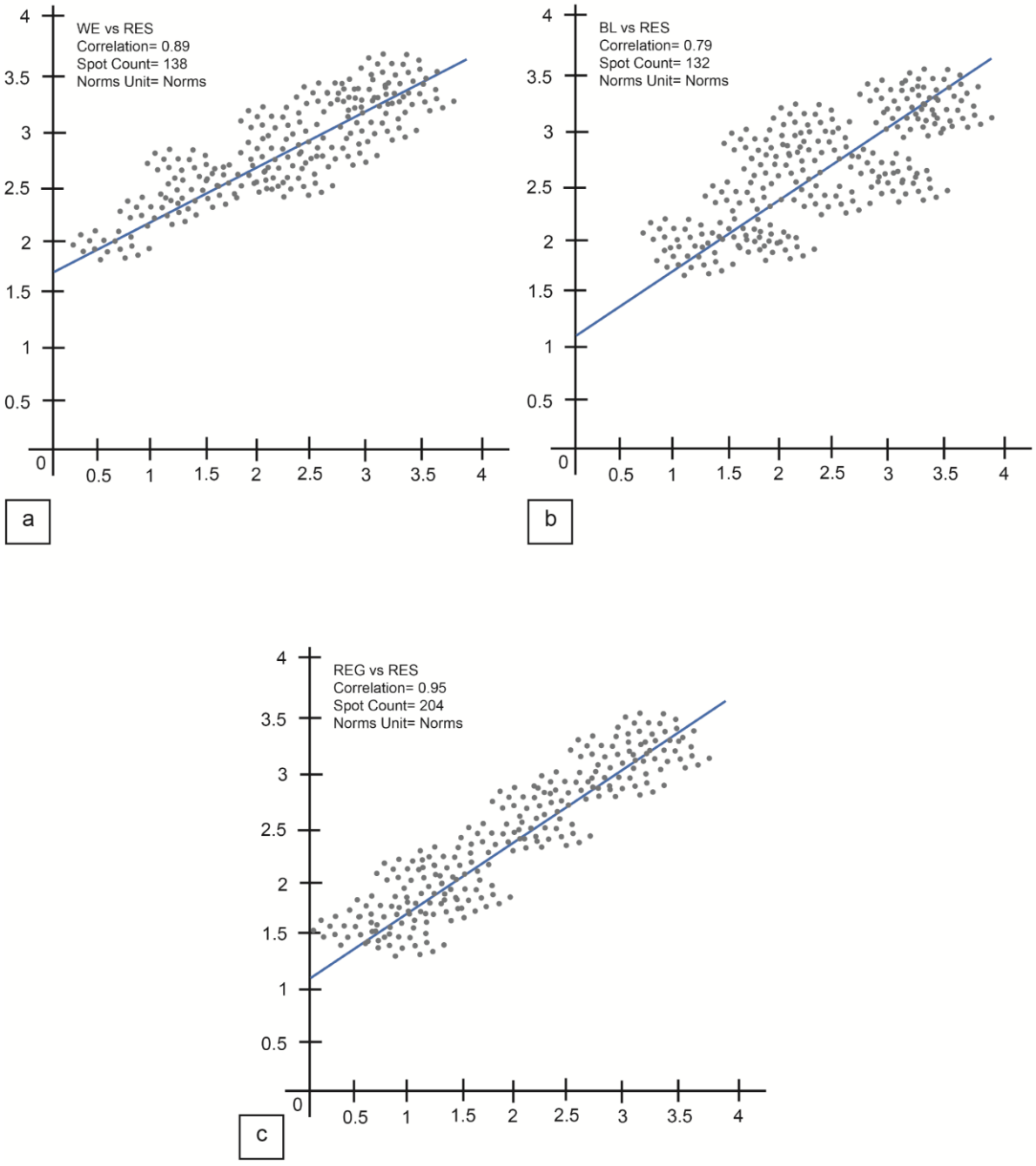
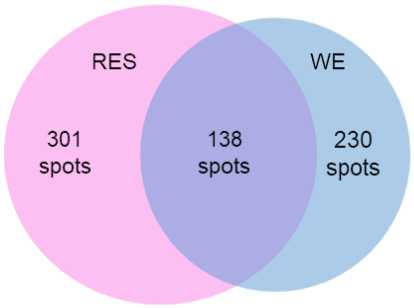
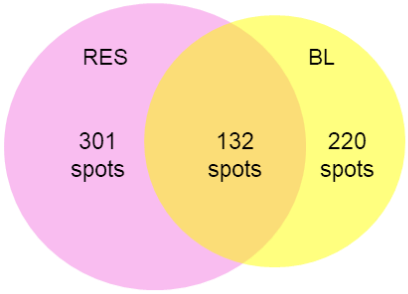


Figure 4B: Correlation graphs of 2D-gel electrophoresis analysis: a) correlation graph of wound epithelium stage vs. resting, b) correlation graph of blastema stage vs. resting and c) correlation graph of regenerated stage vs. resting in *Hemidactylus flaviviridis*.

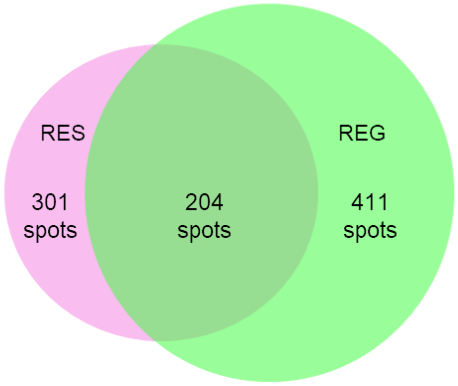
Stages	Spots
RES	301
WE	230
BL	220
REG	411



WE vs RES	Spots
New	92
Shared	138
a. Up	81
b. Down	57



BL vs RES	Spots
New	88
Shared	132
a. Up	79
b. Down	53



REG vs RES	Spots
New	207
Shared	204
a. Up	119
b. Down	85

Figure 5: Venn diagram representing the overlapping spots in the gel when resting tail is compared with other stages. The table shows the newly expressed proteins and from the shared proteins the ones that are up-regulated and the ones which are down-regulated. All the stages are compared with the resting tail of *H. flaviviridis*. (RES: resting stage, WE: wound epithelium stage: BL: blastema stage; REG: Regenerated stage).

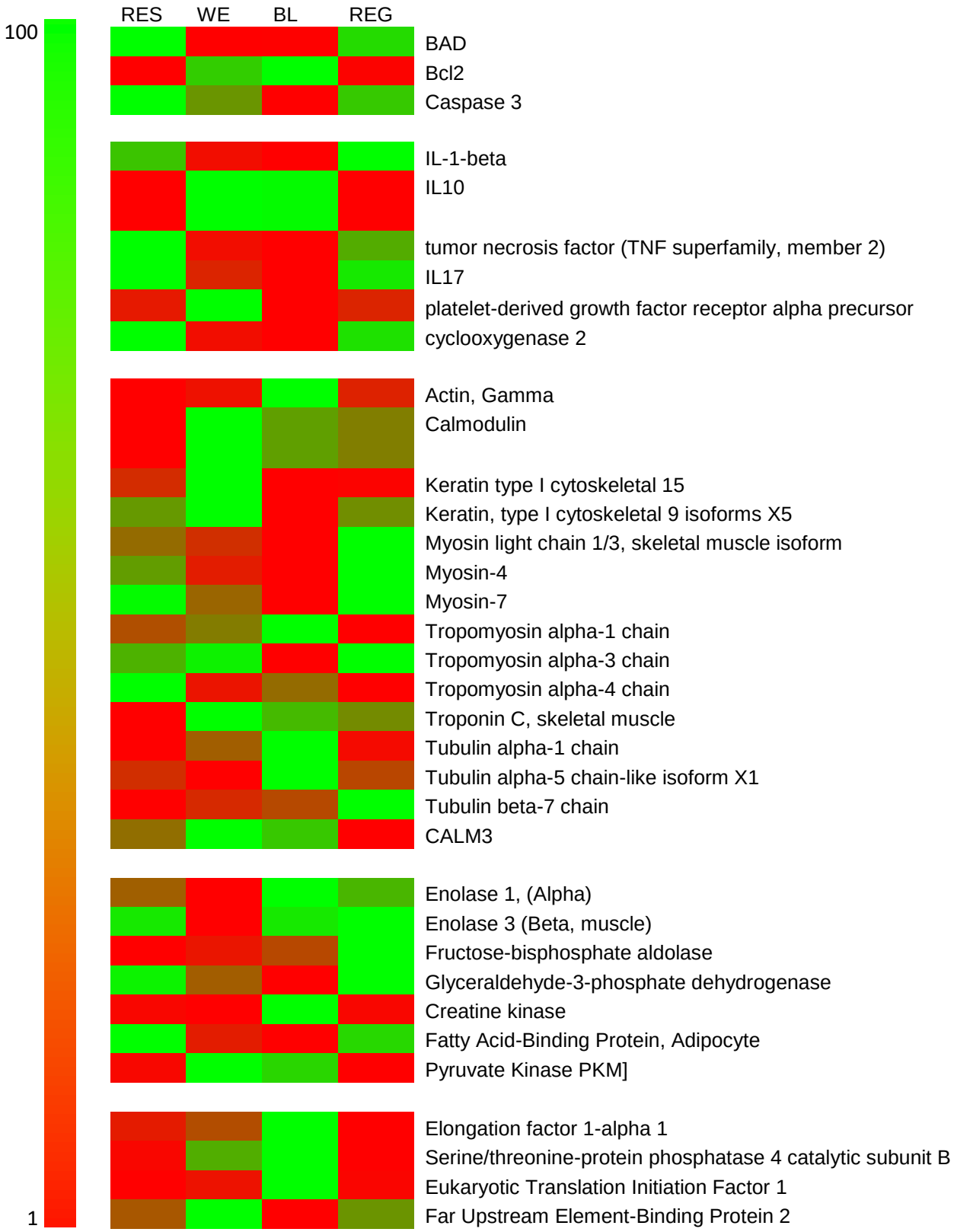


Figure 6: Heat map generated for protein expression levels obtained from label-free analysis in all the stages of regeneration in *Hemidactylus flaviviridis.*

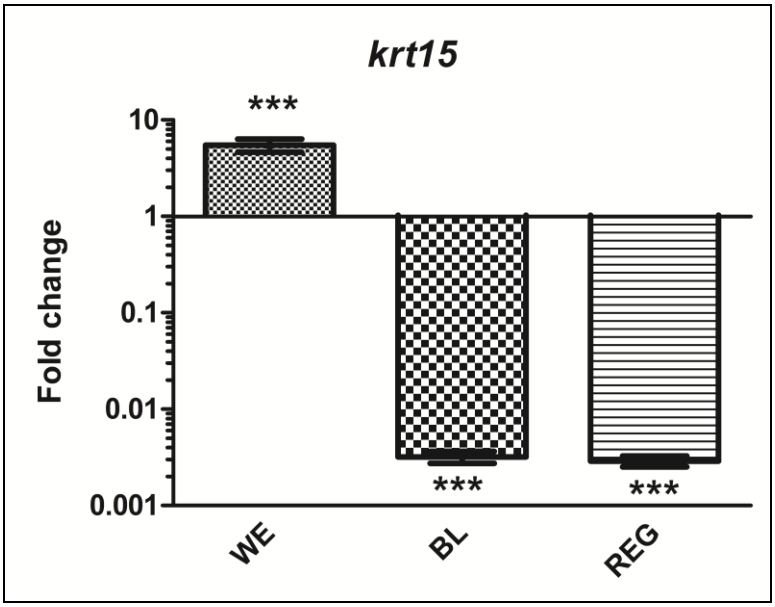


Figure 7: *Keratin type 1 cytoskeletal 15 like* transcript fold change expression during *H. flaviviridis* tail regeneration. Error bars represent standard errors of the mean. **p<0.01, ***p<0.001. n=3.

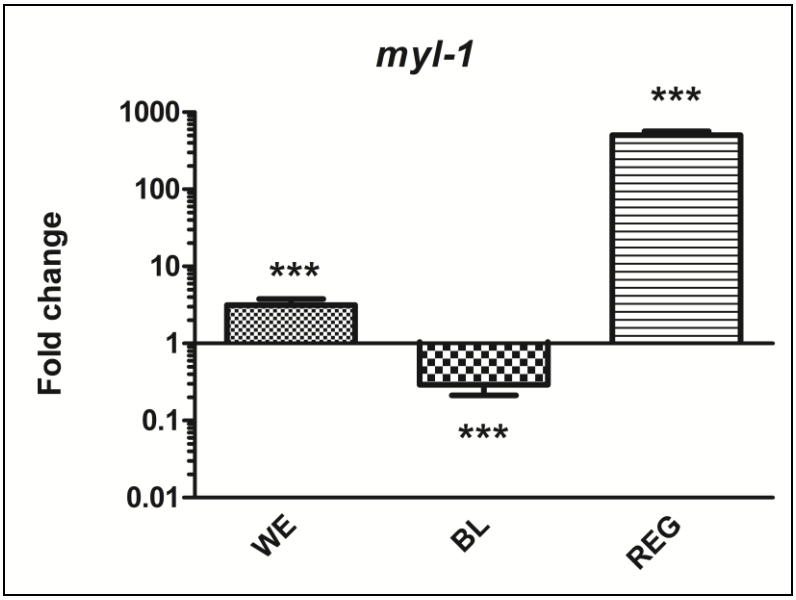


Figure 8: *myosin light chain-1* transcript fold change expression during *H. flaviviridis* tail regeneration. Error bars represent standard errors of the mean. **p<0.01, ***p<0.001. n=3**.**

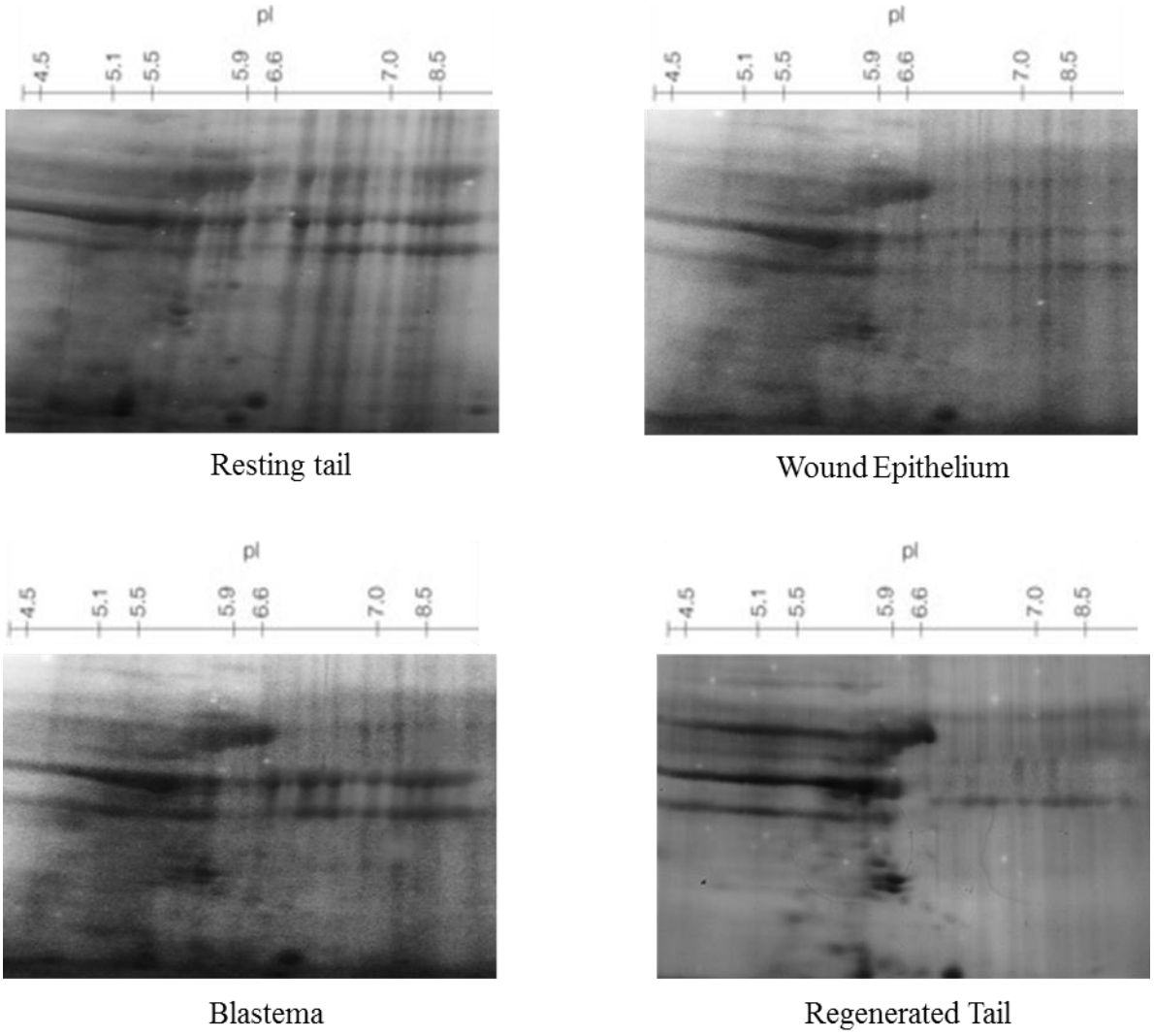


Figure 9A:Representative two-dimensional gel images for the selected spots and their comparison in all the stages of *P. latipinna* regenerating tail fin for further analysis.

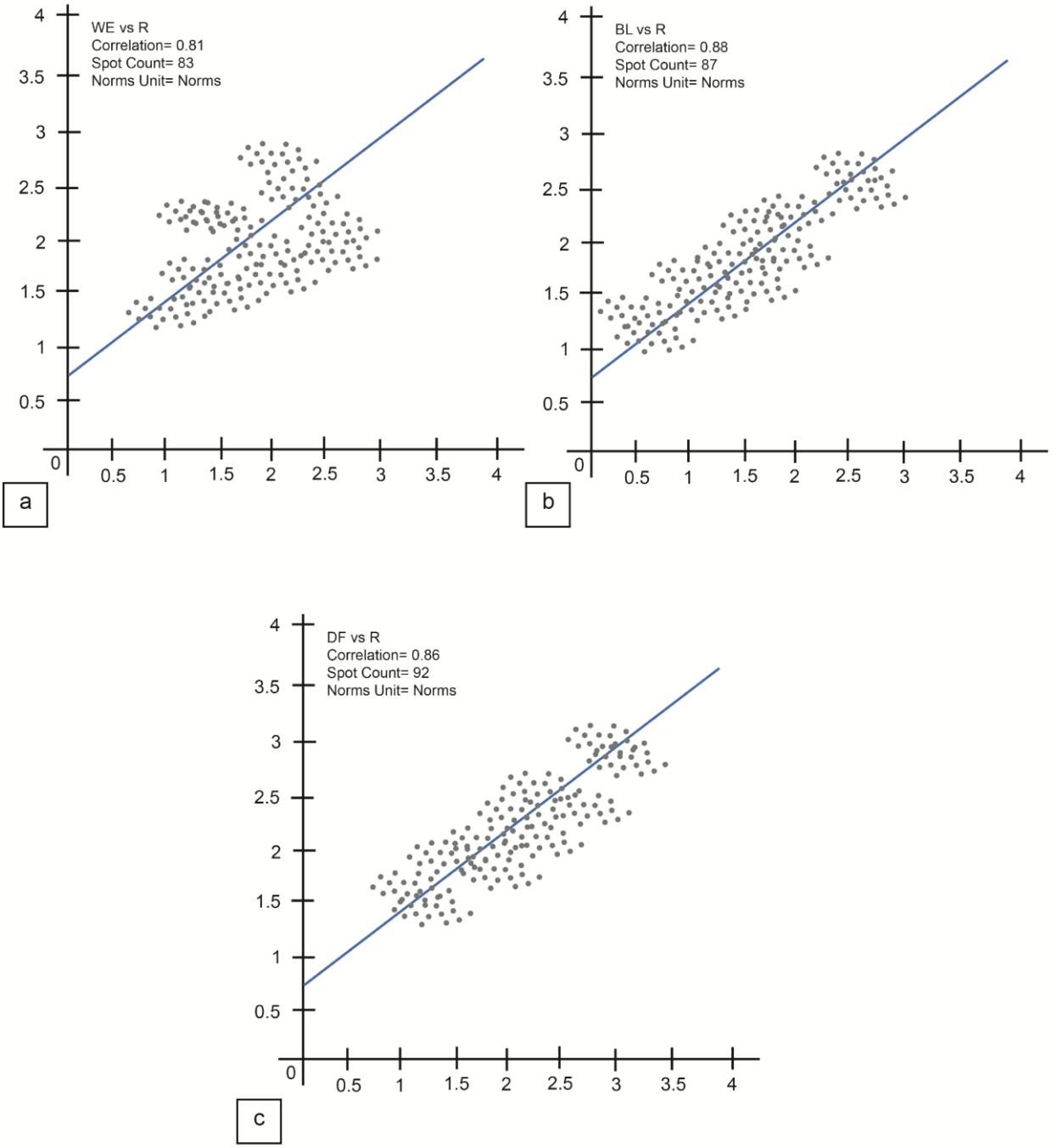
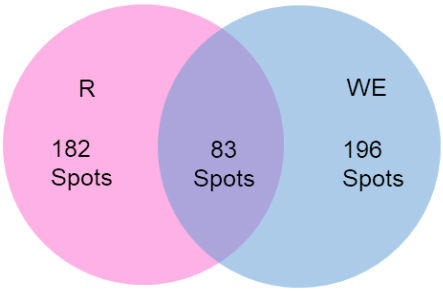
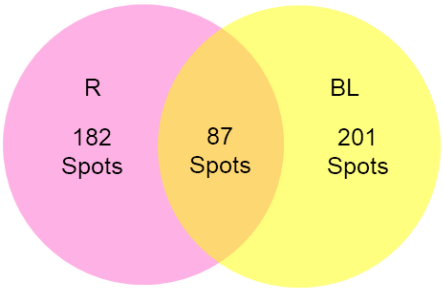


Figure 9B:Correlation graphs of 2D-gel electrophoresis analysis: a) correlation graph of wound epithelium stage vs. resting, b) correlation graph of blastema stage vs. resting and c) correlation graph of regenerated stage vs. resting in *Poecilia latipinna*.

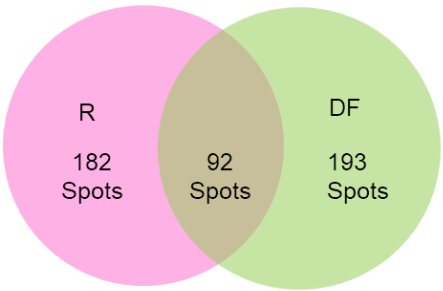
Stages	Spots
R	182
WE	196
BL	201
DF	193



WE vs RES	Spots
1. New	113
2. Shared	83
2a. Up	40
2b. Down	43



BL vs RES	Spots
1. New	114
2. Shared	87
2a. Up	57
2b. Down	30



REG vs RES	Spots
1. New	101
2. Shared	92
2a. Up	30
2b. Down	62

Figure 10: Venn diagram representing the overlapping spots in the gel when resting stage is compared with other stages. The table shows the newly expressed proteins and from the shared proteins the ones that are up-regulated and the ones which are down-regulated. All the stages are compared with the resting stage of the caudal fin of *P. latipinna* (R: resting stage, WE: wound epithelium stage: BL: blastema stage; DF: Differentiation stage).

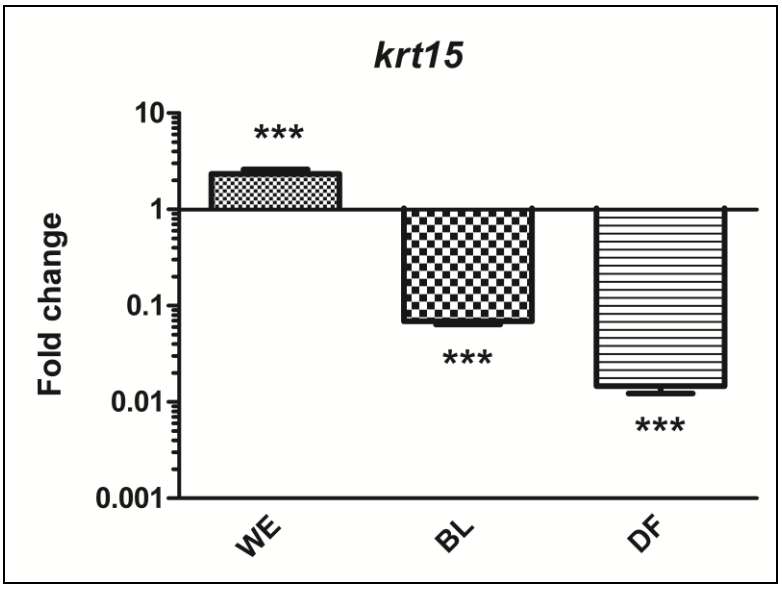


Figure 11: *Keratin type 1 cytoskeletal 15 like (krt15)* transcript fold change expression during *P. latipinna* fin regeneration. Error bars represent standard errors of the mean. **p<0.01, ***p<0.001. n=6.

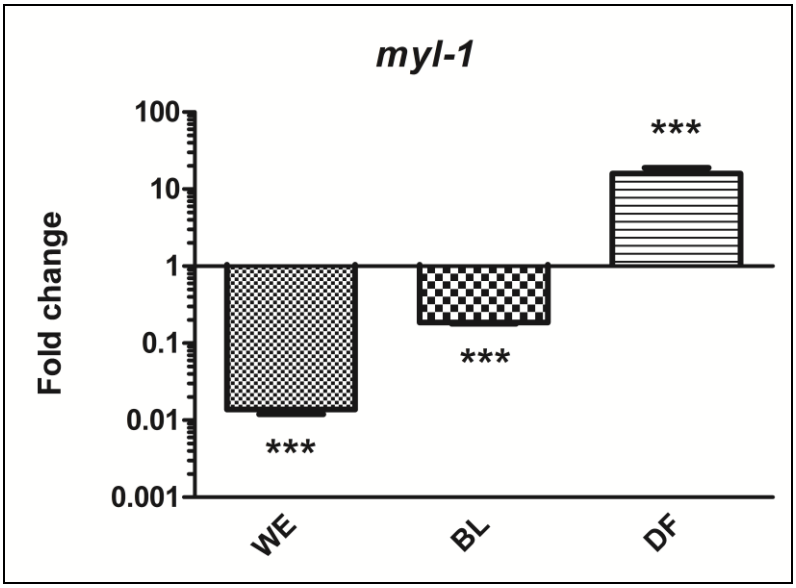


Figure 12: *Myosin light chain-1(myl-1)* transcript fold change expression during *P. latipinna* fin regeneration. Error bars represent standard errors of the mean. **p<0.01, ***p<0.001. n=6.