CHAPTER 4

TEMPORAL EXPRESSION PATTERN OF MMP2 AND MMP9 DURING APPENDAGE REGENERATION IN *HEMIDACTYLUS FLAVIVIRIDIS AND POECILIA LATIPINNA*

INTRODUCTION

Epimorphic regeneration involves restoration of the lost body part through dedifferentiation, proliferation and subsequent re-differentiation of the existing tissue (Sicard, 1985). Three distinct stages identified during epimorphosis are wound epithelium, blastema formation, and differentiation. In some vertebrates, once the tail/limb is amputated, it initiates a series of post embryonic developmental events. Among them, one of the very critical events during regeneration is the formation of wound epithelium (Stocum, 1995; Tsonis, 1996; Brockes, 1997) followed by extensive remodelling of the extracellular matrix (ECM) (Daley *et al*., 2008; Govindan and Iovine, 2015). During wound epithelium stage, epidermal cells from the existing part of the epithelium, start migrating to cover the open wound (Bryant *et al*., 2002). Upon covering the wound, blastema formation occurs beneath the epidermal layer which later on culminates into the reconstruction of tissues with muscles, bones, and nerves (Hay and Fischman, 1961; Iten and Bryant, 1973; Carlson, 2007). Regeneration cannot be achieved until and unless the amputation wound is healed (Wallace, 1981). Hence, for the successful restoration of the lost tissue by epimorphic regeneration, wound healing is very important and crucial. Most of the signals for the formation of the blastema and subsequent structures are secreted by the newly formed wound epithelium (Campbell and Crews, 2008).

Wound epithelium is considered as one of the important signalling centres that govern a myriad of subsequent events of regeneration and hence understanding the process of wound healing is indispensable in a study of effect. Wound healing during the regenerative process involves the proliferation of keratinocytes from the living layer of epithelium, vasodilation of broken blood vessels, secretion of the intrinsic

cytokines and release of growth factors for immune response, which prevent local parasitic infection (Godwin and Brockes, 2006). Macrophages also accumulate at the amputation site and generate an immune response (Godwin *et al*., 2013). They are responsible for removal of the parasites through phagocytosis and for programmed cell death of the damaged local cells. However, prolonged activation of macrophages inhibits the regeneration process. Several reports favour tissue regeneration in the presence of a relatively low and brief inflammatory response (Tuchunduva *et al*., 2001; Mescher and Neff, 2005; Wynn, 2008; McCusker *et al*., 2015), while intense immune response does not allow establishment of contact between the wound epithelium and underlying mesenchyme, thus resulting in scar formation (Ferguson and O'Kane, 2004; Alibardi, 2010). Along with numerous inflammatory modulators, some of the growth factors like FGFs are also released by macrophages which are necessary during wound covering phase (Godwin *et al*., 2013). FGFs are reported to be regulated by proteinases which are involved in ECM remodelling (Flaumenhaft and Rifkin, 1991).

Following amputation, the macrophage mediated response is initiated; synthesis of proteinases for degradation of the matrix occurs, and the proliferative phase commences (Stocum, 1995; Broughton *et al*., 2006). Accumulation of proliferating cells takes place beneath the epithelial layer. There are ample of evidence which shows that generation of these mononucleated proliferating cells occurs from the existing tissues (Hay, 1959; Namenwirth, 1974). These existing tissues have been formed through dedifferentiation process with the help of hydrolases and proteinases (Nagase and Woessner, 1999). All the newly formed capillaries and fibroblast cells are embedded in the loose extra cellular matrix (ECM) (Metcalfe and Ferguson, 2005). Loose ECM contains fibronectin and fibrin which creates a conditional matrix to promote the migration of wound keratinocytes (Schultz and Wysocki, 2009).

Moreover, ECM provides a dynamic environment for all the factors to facilitate proper proliferation and migration of different cell types (Mathew *et al*., 2006). ECM is composed of many components which include fibrous structural proteins, specialised proteins and proteoglycans (Okita *et al*., 2004; Grounds, 2008). Recent studies have reported that these components collectively provide major information to the cells as well as a matrix for the functional aspects in addition to the structural support (Kim *et*

al., 2011; Hynes, 2014). The matrix has also been recognized as a crucial factor for the maintenance of adult stem cells, stem cell niche, and is supposed to be involved in their differentiation, activation, and release (William *et al*., 2007; Chen, 2010; Reilly and Engler, 2010). Other than regenerative process, ECM remodelling occurs during major processes of development, morphogenesis, wound healing and even in the case of regenerative medication of certain diseases (Daley and Yamada, 2013).

In order to facilitate the expansion of proliferating cells, controlled degradation of extra cellular matrix (ECM) is inevitable during epimorphic regeneration. Also, all the important cellular processes like cell movement, cell-cell adhesion, cell-matrix interactions, and dedifferentiation can occur only if ECM is altered or degraded (Tsonis *et al*., 1996). ECM remodelling has been reported in case of several animal models of regeneration including zebrafish, *Xenopus*, newt and axolotl (Toole and Gross, 1971; Gulati *et al*., 1983; Tassava *et al*., 1996; Calve *et al*., 2010; Mercer *et al*., 2013, Govindan and Iovine, 2015). For ECM remodelling, the first important step is tissue degradation which is achieved by acid hydrolases and matrix metalloproteinases (MMPs). Several hydrolases including cathepsin D, acid phosphatase, β-glucuronidase, carboxyl ester hydrolases and N-acetyl-glucosaminidase have been identified during urodele limb regeneration (Schmidt, 1966; 1968; Rivera *et al*., 1981; Ju and Kim, 1998; Park and Kim, 1999). Other than these proteases, it has been widely accepted that MMPs - the zinc dependent endopeptidases play a significant role during the degradation and remodelling of ECM (Nagase and Woessner 1999). They constitute a family of more than 20 members that are involved in degrading distinct components of ECM and are classified according to their substrate specificity (Birkedal-Hansen *et al*., 1993). The subgroup of MMPs includes collagenases which degrade collagen type I, II and III, gelatinases which breakdown basement membrane collagens and denatured collagens (gelatins), stromelysins, matrilysins as well as membrane-type MMPs. MMPs possess a wide range of substrate specificity including proteoglycans, laminins, fibronectins, and gelatins (Song *et al*., 2006). In the matrix, peptide side chains with the hydrophobic interaction of leucine, isoleucine, methionine, phenylalanine, or tyrosine are cleaved by MMPs (Visse and Nagase, 2003).

All MMPs are made up of three domains, pre, pro or propeptide and a catalytic domain. All the MMPs are produced as pre proenzymes, and the propeptide domain needs to be cleaved to activate an enzyme (Vincenti *et al.*, 1996; Hulboy *et al.*, 1997). The catalytic domain consists of two Zn^{2+} ions (structural and catalytic) and two or three Ca^{2+} ions, required for stability and activity of the enzyme. Amongst all, MMP-2 and MMP-9 are unique wherein they contain three repeats of fibronectin-type II domain, inserted within the catalytic domain which enhances their substrate binding ability (Ganea *et al.*, 2007). All MMPs except MMP-7, MMP-23, and MMP-26 contain a C-terminal hemopexin-like domain which is responsible for their substrate specificity (Hulboy *et al.*, 1997; Hillegass *et al.*, 2007; Ganea *et al.*, 2007).

Although the activity of MMPs is very much essential for ECM remodelling during regeneration, their expression level needs to be controlled since elevated level of MMPs is always detrimental for cells. Therefore, they are controlled at transcription level as well as at the proteolytic activation of zymogens and with activity inhibition by natural inhibitors (Nagase and Woessner 1999; Chakraborti *et al.,* 2003; Nagase *et al.,* 2006). Hence, to regulate the activity of MMPs some endogenous inhibitors; α2-macroglobulin and tissue inhibitors of MMPs (TIMPs) are expressed (Barrett, 1981; Stevenson and Vinarsky, 2006). They have been reported for their potential action of reducing proliferation, invasion, and metastasis by inhibiting MMP activity (Sternlicht and Werb, 2001; Baker *et al*., 2002).

In a biological system, MMPs are produced by mesenchymal cells as well as by hematopoietic cells (Birkedal-Hansen *et al*., 1993). Also, during the immune response, neutrophils and macrophages infiltrated through the blood vessels are responsible for the production of MMPs during wound healing (Schwartz *et al*., 1998; Yong *et al*., 1998). In epimorphic regeneration, a few MMP family members have been investigated for wound healing of skin and cornea (Azar *et al*., 1996; Fini *et al*., 1998; Ye and Azar, 1998) during Zebrafish heart and fin regeneration (Poss *et al*., 2000; Bai *et al*., 2005; Lien *et al*., 2006), for appendage regeneration of newts, axolotl, lizard (Grillo *et al*., 1968; Yang and Bryant, 1994; Miyazaki *et al*., 1996; Yang *et al*., 1999; Vinarsky *et al*., 2005; Delorme *et al*., 2012) and in the tissue of mouse muscle (Kherif *et al*., 1999). MMPs contribute in several physiological events like angiogenesis, cell migration, and aggregation, and apoptosis (Stamenkovic, 2003;

Lemaitre and D'Armiento, 2006; Hynes, 2009; De Franceschi *et al*., 2015). Also, different MMPs' expression levels were analysed using antibody array at different time-points and the expression patterns of MMP-2, 3, 8, 9, 10, and 13 have been examined in wild type axolotl and regeneration-deficient model of *Xenopus* froglets during blastema formation, which showed the distinct pattern of MMPs (Santosh *et al*., 2011). Simultaneously, regulated expression of these proteinases is necessary because irregular expression levels may lead to several pathological conditions such as arthritis, cancer, atherosclerosis, aneurysms, tissue ulcers, and fibrosis (Visse and Nagase, 2003; Lu *et al.*, 2011).

Amongst the MMPs, gelatinases namely, MMP2 and MMP9 are reported during appendage regeneration in various vertebrate models (Yang and Bryant, 1994; Miyazaki *et al*., 1996, Carinato *et al*., 2000; Poss *et al*., 2000a). It has been documented that gelatinases act on type IV collagen and on other components of ECM such as fibronectin, laminin, aggrecan, elastin, large tenasin-C and collagen I, V, VII, and X (Nagase and Woessner, 1999; Davis *et al*., 2000; Zhang *et al*., 2003). In addition, MMPs have been reported to clear the cellular debris surrounding the epithelium (Baker and Leaper, 2000). Following digestion of matrix by MMPs, keratinocytes and fibroblasts can migrate through the matrix easily. For the success of regeneration epithelial/mesenchymal interaction is inevitable. Basement membrane formation should be prevented for this epithelial/mesenchymal interaction to occur. MMPs are reported to promote such interactions (Yang *et al*., 1999). Thus, MMPs support the blastemal growth during vertebrate appendage regeneration (Tsonis, 1996). Likewise, MMPs may also indirectly give the signal for secretion of the growth factors from the matrix and make it available to the proliferating cells for further growth (Boilly *et al*., 1991; Hondermarck and Boilly, 1992). Upon ablation of these vital MMPs via MMP inhibitor GM6001, a scar was formed in the newt limb (Vinarsky *et al*., 2005). Inhibition of gelatinases by using MMP inhibitor, GM6001, lead to impaired growth and development in newts and hence proved that gelatinase was required for the proper progression of regeneration. During *Xenopus* limb regeneration, a surge in the MMP9 activity was observed six hours post-amputation (hpa) (Carinato *et al*., 2000). Thus, it can be stated that a controlled MMP activity promotes regeneration.

To understand tissue remodelling events involved during tail regeneration in lizard *Hemidactylus flaviviridis*, MMP2, and MMP9 activities were checked in our lab through inhibition studies (Pillai, 2012). Zymographic analyses showed that MMP2 and MMP9 activities were affected and altered in SU5402 treated animals in comparison to the control ones.) In order to reaffirm this result, expression pattern of MMP2 and MMP9 at transcriptional and translational level during all the stages of regeneration *viz*., wound epithelium formation, proliferation of blastemal cells and at the fully regenerated state of *H. flaviviridis* was studied

Another model of regeneration, teleost fish, having an excellent regenerative capability has evoked immense interest among the developmental biologists and has resulted in bringing to fore the nuances of mechanisms that govern regeneration (Poss *et al*., 2003). Caudal fin regeneration in teleost fish is a well-established study model to unearth the mechanism of regeneration. Immediately post amputation, the epithelial cells at the distal margin migrate to cover the wound surface to form an apical epithelial cap (AEC). The AEC then interacts with the mesenchyme beneath to recruit a pool of multipotent cells which are subsequently re-specified to sculpt the lost structure (Grandel and Schulte-Merker, 1998; Poss *et al*., 2000a; 2000b; 2003). However, in order to achieve the above milestones, constant tissue remodelling is imperative and as mentioned earlier, gelatinases *viz.,* MMP2 and 9 are suspected to play a critical role. Though the presence of gelatinases was reported during certain stages of regeneration, a timed expression pattern of MMP2 and 9 has not been undertaken during epimorphosis of teleost fish, *Poecilia latipinna* caudal fin. Hence, the expression and activity pattern of MMP2 and MMP9 during tail fin regeneration in Sailfin Molly, *P. latipinna* was chosen as a research question.

MATERIAL AND METHODS

(A) Evaluate protein expression pattern of MMP2 and MMP9 during *H. flaviviridis* tail regeneration.

Northern House Geckos, *Hemidactylus flaviviridis*, of both sexes with normal intact tail were collected, acclimatised and amputated at the third segment from the stump by inducing autotomy. All the animals were maintained in the animal house as per conditions described in the chapter Material and Methods.

Tissues were collected for the stages; resting (Original tail segment), wound healing (4dpa), blastema (6dpa) and differentiation or regenerate (40dpa). 10% homogenate from each sample was prepared using PBS lysis buffer with a protease inhibitor. Supernatant from all the samples were collected after centrifugation at 8000 rpm at 4°C for 15 minutes and stored at ≤ -20 °C until used. All the samples were estimated for protein content using Bradford method (Bradford, 1976).

Experiment I

Western blot

For western blot analysis, an equal amount of proteins were loaded on to 10% SDS-PAGE and electrophoresed at 100V for 2-3hours. Proteins were transferred onto nitrocellulose membrane with 0.45µm pore size at 100V for 100minutes with transfer buffer containing methanol-tris-glycine. The membrane was developed for the presence of the band for MMP2 and MMP9 using anti-MMP2 Mouse IgG (Sigma-Aldrich, USA) and anti-MMP9 Goat IgG (Sigma-Aldrich, USA) and anti-β-actin mouse IgG. β-actin was used as internal control.

Experiment II

quantitative Real Time Reverse Transcriptase PCR

Tissues of each stage were collected in TRIzol reagent (Applied Biosystems, USA). RNA was isolated from the tissues and cDNA was prepared using cDNA synthesis kit (Applied Biosystems, USA). For quantitative expression analysis, cDNAs were subjected to real time quantitative reverse transcriptase PCR using SYBR green ready mix (Applied Biosystems, USA). 18S rRNA was taken as endogenous control. The fold change was calculated using Livak and Schmittgen method (Livak and Schmittgen, 2001).

Sequences used for qRT-PCR are as follows:

mmp2 Forward: 5'GTCTCCTGGCTCATGCCTTT-3',

mmp2 Reverse: 5'-TTTCACCACTTGGCCCTCTC-3',

mmp9 Forward: 5'-GCCTTCAAGGTGTGGAGTGA-3',

mmp9 Reverse: 5'-ATCCCCGTGGTCAGCTTTTC-3',

18SrRNA Forward: 5'-GGCCGTTCTTAGTTGGTGGA-3'

18SrRNA Reverse: 5'-TCAATCTCGGGTGGCTGAAC-3'

(B) Study of temporal expression patterns of MMP2 and MMP9 during caudal fin regeneration in *P. latipinna*

Maintenance and amputation of fish fin

Poecilia latipinna (Lesueur, 1821) of both the sexes of the same age with an average size of 4-5 cm and weighing about 4-5 g were purchased from a commercial supplier. They were acclimatised for a week and maintained within specific temperature range and as per the conditions described in the material and methods. During each experiment, a set of six fishes per group were used, and from the distal tip of the caudal fin, 1/3 part was amputated under hypothermic anaesthesia. Upon achieving the particular time point of interest, fin tissues were collected and processed as per the requirement of the experiment. In the time scale study of MMPs activity, tissues were collected at 0hpa, 1hpa, 2hpa, 3hpa, 4hpa, 5hpa, 6hpa, 12hpa, 24hpa, 60hpa and 5dpa.

Experiment III

Gelatin Zymography

7.5% gelatin zymogram gel was prepared containing 0.5% gelatin and allowed to run at 4˚C on constant voltage with an equal amount of proteins from all the time-point tissue extracts of collected caudal fin tissues. After electrophoresis, gels were washed with Triton wash buffer and then incubated in incubation buffer containing 50mM Tris-HCl, 5mM $CaCl_2$, 0.2M NaCl and 1%triton X-100 overnight for activation of the MMPs and their relative activities were measured. Gels were stained with 0.5% Coomassie Brilliant Blue R-250, where bands appeared as clear area against the dark background of stained gelatin. Relative intensities were determined using Doc-ItLs software (GeNei, Merck, USA).

Experiment IV

qRT-PCR and Western blot

These methods are described in brief earlier in this chapter in section (A) as well as in detail in chapter materials and methods.

Statistical analysis

All data were tested for homogeneity of variance and then subjected to one-way ANOVA followed by Bonferroni's multiple comparisons tests using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). The values are expressed as mean ± SEM. A 'p' value of 0.05 or less was accepted as being statistically significant.

RESULTS

In the current study, translation and transcription levels of gelatinases, MMP2 and MMP9 were checked with well-established methods like gelatin zymography, western blot, and qRT-PCR. Gelatinases activity pattern during tail regeneration in lizard *H. flaviviridis* has already been determined for tissue remodelling (Pillai, 2012). To reaffirm these results, western blot analyses of MMP2 and MMP9 in lizard tail tissues for resting wound epithelium, blastema and regenerated stages were carried out. Band intensities were measured with Doc-ItLS software (GeNei, India) and expressed in arbitrary unit.

Western blot analysis of MMP2 during *H. flaviviridis* tail regeneration for WE and BL stages showed significantly elevated levels when compared with resting and regenerated stage (Table 1; Figure 1 and 2). Transcript levels of *mmp2* were also found notably increased during WE stage with respect to the resting stage. Whereas, in the blastema and regenerated stage *mmp2* gene expression was found declined (Table 2; Figure 4).

Other than MMP2, another gelatinase named MMP9 was also looked upon for its gene and protein levels analysis across the stages of lizard tail regeneration. During the wound epithelium stage, the intensity value of MMP9 was higher compared to the resting stage stating its requirement for wound epithelium formation following lizard tail amputation. However, its level was found to be decreased during blastema and regenerated stages (Table 1; Figure 1 and 3) which is contrary to the transcript level of *mmp9* in which the mRNA levels of *mmp9* was up-regulated for all the stages of regeneration with respect to the resting stage (Table 2; Figure 5).

Also, in the 2D gels of lizard regeneration stages, upon their molecular weight analysis it was evaluated that MMP2 (Red circle annotated in the gel in Figure 4 of chapter 3) intensity was observed higher in the proliferative stage (*viz*. wound epithelium stage and blastema stage; where remodelling of the tissue takes place, and undifferentiated cells accumulate) in comparison to resting and regenerated stage while MMP9 spot (Blue triangle annotated in the gel in Figure 4 of chapter 3) was noted with increased intensity at BL stage.

Upon achieving the stage specific expression of MMP2 and MMP9 during lizard tail regeneration, the temporal expression profile of MMP2 and MMP9 was studied during caudal fin regeneration in *P. latipinna*.

The study involved certain time points which are 0hpa, 1hpa, 2hpa, 3hpa, 4hpa, 5 hpa, 6hpa, 12hpa, 18hpa, 24hpa, 60hpa and 5dpa. Initially, gelatinase activity during each time point was determined using gelatin zymography (Figure 6). The zymograms obtained were digitised, and intensities of bands were computed. Intensity values of both pro- and active forms of MMP2 and MMP9 were recorded and analysed.

For pro-MMP2, activity was found to be at basal level till 3hpa and then onwards it started increasing gradually. Its maximum activity was recorded at 24hpa, and again it was decreased at 60hpa and 5dpa (Table 3; Figure 6 and 7). Active MMP2 was found to be changed till 5hpa with the exception at 3hpa where it showed some activity. Thereafter, active MMP2 had been expressed in all the subsequent time points suggesting its involvement in the continuous remodelling of ECM.

It is apparent from the intensities of zymogram that the activity of active-MMP2 was more pronounced during the wound epithelium and late blastema stages of regeneration i.e. in 24hpa and 5dpa. Analysis of zymogram showed a steady increase in the activity of both pro as well as active-MMP2 from 6hpa to 24hpa. Thereafter, a slight but significant decline in pro-MMP2 at 60hpa and 5dpa was observed marking its conversion into active MMP2 and hence justifying the increment in intensities of active MMP2 during the stages of regeneration.

Following zymography, protein expression of MMP2 was undertaken by western blot. Densitometric analysis of western blot for MMP2 showed the basal level expression at 1hpa. 2hpa onwards the MMP2 protein expression was increased in comparison to resting and the expression remained constant till 5hpa. From 6hpa till 24hpa, there was a gradual increase in MMP2 levels which was further elevated during 60hpa and 5dpa (Table 4; Figure 9 and 10).

Upon transcript level analysis of mmp2, the mRNA level of mmp2 was up-regulated from 1hpa to 5hpa when compared to resting stage except at 3hpa in which it remained unaffected. Further, from 4hpa till 5dpa the mmp2 transcript levels were increased except for at 6hpa and 12hpa where there was a steep decline in gene expression was noticed (Table 5; Figure 12). Transcript level study illustrated requirement of mmp2 during initial time points following amputation as well as across the stages of regeneration MMP2 showed a significant increase in expression.

Upon analysis, the activity of pro-MMP9 was found to be increased from 4hpa till 24hpa, significantly compared to 0hpa. At 60hpa and 5dpa, its activity decreased. It was noticed that active-MMP9 level increased significantly 6hpa onwards till the early differentiation stage (5dpa) (Table 3). As the regeneration progressed and reached wound epithelium stage (24hpa), the active form of MMP9 was more prominently expressed perhaps to facilitate heightened extra cellular matrix digestion and remodelling required at this stage of regeneration (Table 3; Figure 6 and 8). Active MMP9 levels remained induced ever after for the subsequent stages of regeneration.

Concomitant western blot analysis of MMP9 showed an elevated protein expression of MMP9 during regeneration stages in tail fin (Figure 9) and the presence of MMP9 in all stages signifying its role during the regeneration process. Computed analysis of the band intensities for western blot of MMP9 for the selected time points can be found in Table 4; Figure 11. All the differences in the intensity values were significant when compared to 0hpa except 4hpa which showed the almost same abundance of MMP9 as of the resting stage.

In order to further consolidate the earlier observations based on zymogram and western blot, relative quantitative analysis of *mmp9* transcript was also performed.

The values of fold change showed the significantly increased level of *mmp9* expression for all the time points with respect to the resting stage (0hpa) (Table 5; Figure 13). The trend of increasing expression of *mmp9* with the progression in regeneration process suggests that MMP9 was utilised for extracellular digestion throughout regeneration process in caudal fin of the teleost.

DISCUSSION

It has been documented that ECM is remodeled constantly during development and wound healing (Stamenkovic, 2003). Major proteolytic activities for ECM remodelling are achieved by matrix metalloproteinases (Werb, 1991; Page-McCaw *et al*., 2007). Among all matrix metalloproteinases, MMP2 and MMP9 have been studied extensively for their role in ECM remodelling (Matrisian, 1990; Stamenkovic, 2003; Bonnans *et al.*, 2014). It is therefore presumed that an understanding of the expression pattern of MMP2 and MMP9 might help us getting a holistic picture of proteolytic activity, which is not only unique for each stage of regeneration but is also necessary for the successful progression of regeneration. It is now accepted that regeneration succeeds between the fine line of scar formation and scarless wound healing through the strict regulation of signaling pathways that control matrix turnover at the site of amputation (Godwin *et al*., 2014; Takeo *et al*., 2015). Hence, in the current study, generating an expression profile of two matrix metalloproteinases, MMP2 and MMP9 during *H. flaviviridis* tail regeneration was aimed.

Increased protein expression of both MMP2 and MMP9 at wound epithelium stage signifies their involvement for migration of epithelial cells to cover the stump. The continued expression of MMP2 signifies its requirement for further growth of the regenerating tail during blastema stage. These results are in accordance with the observations made during zebrafish fin regeneration by Bai *et al*. (2005).

Data obtained from transcript analysis for *mmp2* and *mmp9* also coincided with western blot results for wound epithelium stage justifying their role in regeneration. However, there was a significant decrease in mRNA levels of both *mmp2* and *mmp9* during blastema and regenerated stages. Decreased levels of these genes suggest transcriptional regulation of these gelatinases during lizard tail regeneration.

After achieving the stage-wise expression pattern of MMP2 and MMP9 during lizard tail regeneration, their temporal expression profile during regeneration was pursued with a relatively rapid regenerating system, caudal fin of *P. latipinna*. Our lab has been working in this field extensively, and MMPs activity pattern across the stages of regeneration in caudal fin of teleost has been obtained (Saradamba *et al*., 2013). Hence, this study was further broadened up by checking the timed expression pattern of MMP2 and MMP9 at transcriptional, translational and activity level during caudal fin regeneration in *P. latipinna*.

When the activities of pro-MMP2 and active MMP2 were checked by gelatin zymography, both started showing a tremendous increase in their activities 6hpa onwards till the early differentiation. Their activities were almost comparable for the very initial time points following amputation when compared to resting stage. Similar results were obtained in the case of rat liver regeneration where pro-MMP2 levels were elevated at 6 to 12 hours following hepatectomy, and later on, active MMP2 levels went up (Kim *et al*., 2000). Further upon western blot analysis, it was observed that MMP2 protein levels were significantly increased from 2hpa and remained high thereafter till 5dpa. This reflects the importance of MMP2 in ECM degradation near amputation site and hence allowing migration of epithelial cells to cover the wound. Such significance of MMP2 has been reported during salamander limb regeneration (Park and Kim, 1999), rat skeletal muscle regeneration (Zimowska *et al*., 2003) and zebrafish caudal fin regeneration (Bai *et al*., 2005). Following protein expression analysis of MMP2, transcript level analysis of *mmp2* was carried out. *mmp2* levels were up-regulated significantly from 1hpa to 5hpa except at 3hpa stating its upcoming requirement for progression of regeneration. However, its level went down at 6hpa and 12hpa and then again it increased for the remaining time points. These fluctuations in the transcript levels of *mmp2* might be explained by the tight regulation of transcription and translation of MMP2. As dysregulation of mmp2 can lead to the cancerous growth of tissue, its activity has to be controlled in a growing tissue (Hojilla *et al*., 2003; Chan *et al*., 2016).

The result here in this study explains an indirect role of MMP2 in the proliferative stage but not in the initial remodelling. Also, it suggests the involvement of MMP2 during osteogenesis in the growing lepidotrichia of fish or during chondrogenesis in

the growing tail of a lizard. Studies with the MMP2 null mice displayed bone abnormality, decreased bone mineral density, as well as article cartilage destruction, were reported in such cases (Itoh *et al.*, 1997; Mosig *et al.*, 2007). It has been documented that osteoblast differentiation requires BMP signaling and inhibition of this signaling hampers caudal fin regeneration in zebrafish (Smith *et al*., 2006). Moreover, studies have shown that inhibition of BMP signaling negatively influences the MMP2 activity in cancer cells (Laulan and St-Pierre, 2015) and also in the regenerating caudal fin of *P. latipinna* (Rajaram *et al*., 2016). Mosig and co-workers in 2007 have reported that MMP2 plays an important role in the early skeletal growth and development. Thus, collectively, it could be inferred that MMP2 is essential for inducing skeletal differentiation and hence, its activity was found high during early differentiation stage of tail/fin in lizard/teleost fish.

Nevertheless, unlike pro-MMP2, pro-MMP9 was present throughout the span of regeneration immediately after amputation suggesting the requirement of MMP9 during the process of ECM remodelling. Vinarsky and co-workers (2005) have studied the effect of gelatinase inhibitor on regeneration process and concluded that gelatinases are required all through regeneration which supports our result of pro-MMP9. It has been documented that pro-MMPs are largely inactive and are converted into active-MMPs by the removal of the prodomain when required (Nagase, 1997). The gelatinases activity has been reported to be regulated by several factors that include serine protease inhibitor, tissue factor pathway inhibitor-2 (Herman *et al.*, 2001), a C-terminal fragment of the procollagen, C-terminal proteinase enhancer protein (Mott *et al.*, 2000), the GPI anchored glycoprotein reversion-inducing-cysteine-rich protein with kazal motifs (Oh *et al.,* 2001), α2-macroglobulin and TIMPs (Barrett, 1981).

With these regulated activities of pro-MMP9, active MMP9 showed heightened expression at any early time point of 3hpa signifying the role of MMP9 in the potential regulation of inflammation process as regeneration always finds its way away from prolonged inflammation and scar formation by modulating various inflammatory molecules. Spiegel and team (1996) had reported that inflammatory cytokine interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) stimulate MMP gene expression at the site of inflammation through the ceramide signaling pathway.

Stimulated MMPs inactivate some of the inflammatory mediators and regulate the inflammation process. MMP9 inactivates IL-1β (Ito *et al*., 1996), CXCL5 (Van Den Steen *et al*., 2003) and chemokines (McQuibban *et al*., 2001) all of them important mediators of the inflammatory process. Therefore, increased level of MMP9 in our study at the transcription and translation levels in the initiation phase (1-4hpa) suggests its role in the regulation of inflammatory process before the initiation of tissue remodelling.

Nonetheless, the level of active-MMP9 was found elevated at 6hpa and 12hpa; probably to digest basement membrane of the epithelial lining at the site of amputation to facilitate the migration of epithelial cells from the edges of the cut surface of the tail to cover the amputation site. It is well known that MMPs contribute to the formation of wound epithelium by degrading basement membrane (Yang and Bryant, 1994; Miyazaki *et al*., 1996; Nagase and Woessner, 1999; Vinarsky *et al*., 2005) which gives credence to the present notion. MMP2 and MMP9 have also been reported to initiate TGF-β signaling pathway by activating TGF-β from its latent LTBP-LAP complex. The activated TGF-β signaling, in turn, promotes cell invasiveness and tumor growth (Yu and Stamenkovic, 2000) as well as fibroblast contraction (Kobayashi *et al*., 2014) depicting the involvement of MMPs in cell proliferation and wound covering, albeit indirectly. Our personal observation in the selected animal model suggests elevated activity of MMP9 in the wound epithelium stage of *H. flaviviridis* regeneration study (Pillai *et al*., 2013). Therefore, in the light of the current observations, it could be construed that the MMP9 activity peaked a few hours before the wound epithelium formation so as to digest the basement membrane of the epithelial layer and hence paved the way for the formation of wound epithelium. However, MMP9 activity and expression during late blastema stage in the caudal fin regeneration might involve its role during chondrogenesis as well. Earlier studies with MMP9 knockout mice model had shown an abnormal pattern of skeletal growth plate vascularization and ossification (Vu *et al.*,1998) as well as impaired migration in the smooth muscle cells isolated from the MMP9 deficient mice (Cho and Reidy 2002).

Thus, based on the present result it is prudent to presume that MMP2 and MMP9 actively participate in the ECM digestion, tissue remodelling, and skeletogenesis

during the regeneration process in *H. flaviviridis* as well as *P. latipinna*. Looking closely at the timed expression of these gelatinases during caudal fin regeneration in teleost suggests their roles in a very structured manner whereas MMP9 activity kicked off early on to facilitate the migration of epithelial cells at the site of amputation so as to form the wound epithelium, MMP2 activity peaked post wound healing perhaps to recruit blastemal cells by digesting the ECM of the mesenchyme beneath the wound epithelium.

Stage of Regeneration	Mean intensity ± SEM	
	MMP2	MMP9
Resting	8.62 ± 0.62	6.75 ± 0.67
Wound Epithelium	18.21 ± 0.56^{c}	14.60 ± 0.67^{c}
Blastema	32.45 ± 0.82^{c}	10.38 ± 0.71^{a}
Regenerated	10.77± 0.49^{c}	7.78 ± 0.70

Table 1: Densitometry analysis of Western blot of MMP2 and MMP9 during *H. flaviviridis* tail regeneration. For statistical significance, all the stages were compared to resting stage. SEM standard error of mean, where superscripts a and c represent $p \leq 0.05$ and $p \leq 0.001$ respectively, (n=3).

Stage of Regeneration	Mean fold change ± SEM	
	mmp2	*mmp9*
Wound Epithelium	5.24 ± 0.659^{c}	62.21 ± 1.08^{c}
Blastema	0.0477 ± 0.005^{c}	4.03 ± 0.52^{c}
Regenerated	0.512 ± 0.007^{c}	1.823 ± 0.17^{c}

Table 2: Fold change expression of *mmp2* and *mmp9* during *H. flaviviridis* tail regeneration. SEM standard error of mean, where superscripts c represents $p \leq 0.001$, (n=3).

Time points	Mean intensity ± SEM			
	Pro-MMP2	Active-MMP2	Pro-MMP9	Active-MMP9
0hpa	2.13 ± 0.19	2.45 ± 0.27	6.36 ± 0.78	3.75 ± 0.46
1hpa	2.71 ± 0.07	2.96 ± 0.34	18.08 ± 4.14^{c}	5.23±0.59
2hpa	3.53 ± 0.38	2.87 ± 0.29	16.7 ± 1.84^{c}	5.10 ± 0.66
3hpa	3.82 ± 0.41	4.55 ± 0.47^{a}	1.10 ± 0.16^{b}	12.25 ± 1.37^{b}
4hpa	3.91 ± 0.45^{a}	3.10 ± 0.34	1.23 ± 0.13^{b}	25.58 ± 2.61^{c}
5hpa	4.08 ± 0.51^{a}	3.15 ± 0.35	1.11 ± 0.18^{b}	30.58 ± 3.24^{c}
6hpa	8.93 ± 0.93^{b}	15.78 ± 1.64^{c}	1.54 ± 0.32^{b}	30.77 ± 3.14^{c}
12hpa	9.54 ± 0.98^{b}	17.89 ± 1.89^{c}	4.58 ± 0.48	40.12 ± 5.16^{c}
18hpa	15.25 ± 1.82^{c}	25.23 ± 2.67^{c}	15.87 ± 1.60^{c}	21.25 ±2.54^{c}
24hpa	35.89 ± 3.97^{c}	40.28 ± 4.12^{c}	15.41 ± 1.78^{c}	20.78 ± 2.25^{c}
60hpa	21.32 ± 2.57^{c}	42.63 ± 4.35^{c}	4.08 ± 0.52	70.69 ± 7.22^{c}
5dpa	18.02 ±1.97^{c}	45.41 ± 4.52^{c}	4.35 ± 0.54	72.23 ± 7.53^{c}

Table 3: Densitometry analysis of zymogram for relative activities of both pro and active forms of MMP2 and MMP9 at the selected time points during *P. latipinna* fin regeneration (intensities in arbitrary unit). Values are expressed as mean ± SEM; where superscripts a, b and c represents $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively, (n=6).

Time points	Intensity (Mean) ± SEM	
	MMP2	MMP9
0hpa	11.23 ± 1.42	10.24 ± 1.13
1hpa	12.43 ± 1.46	30.96 ± 3.62^{c}
2hpa	24.20 ± 2.32^{c}	40.95 ± 4.84^{c}
3hpa	25.98 ± 3.57^{c}	40.98 ± 4.53^{c}
4hpa	19.85 ± 1.76^{c}	30.01 ± 3.78^{c}
5hpa	20.12 ± 1.943^{c}	68.45 ± 7.72^{c}
6hpa	50.28 ± 6.265^{c}	68.13 ± 6.76^{c}
12hpa	52.41 ± 5.065^{c}	72.98 ± 7.89^{c}
18hpa	65.12± 6.42^{c}	65.19 ± 6.47^{c}
24hpa	60.21± 6.12^{c}	65.46 ± 6.91^{c}
60hpa	89.26 ± 9.09^{c}	70.46 ± 7.35^{c}
5dpa	89.19 ± 9.55^{c}	72.46 ± 7.21^{c}

Table 4: Densitometry analysis of western blot images for relative intensities of MMP2 and MMP9 at the selected time points during *P. latipinna* fin regeneration (intensities in arbitrary unit). Values are expressed as mean ± SEM, where superscript c represents $p \leq 0.001$; (n=6).

Time points	Mean fold change ± SEM	
	mmp2	*mmp9*
1hpa	1.79 ± 0.07^{b}	2.18 ± 0.098^{b}
2hpa	2.75 ± 0.08^{c}	3.34 ± 0.11^{b}
3hpa	0.99 ± 0.013	2.75 ± 0.37^{b}
4hpa	2.83 ± 0.23^{c}	3.57 ± 0.41^{b}
5hpa	1.80 ± 0.14^{b}	2.699 ± 0.24^{b}
6hpa	0.34 ± 0.0018^{c}	10.15 ± 1.31^{c}
12hpa	0.30 ± 0.0088^{c}	22.04 ± 2.54^{c}
18hpa	4.79 ± 0.52^{c}	32.07 ± 3.89^{c}
24hpa	2.10 ± 0.26^{b}	43.25 ± 4.57^{c}
60hpa	1.93 ± 0.10^{b}	48.27 ± 4.25^{c}
5dpa	2.20 ± 0.08^{b}	35.33 ± 2.89^{c}

Table 5: Fold change expression of *mmp2* and *mmp9* at the selected time points during *P. latipinna* fin regeneration. Values are expressed as mean ± SEM; where superscripts b and c represent $p \leq 0.01$ and $p \leq 0.001$ respectively; (n=6).

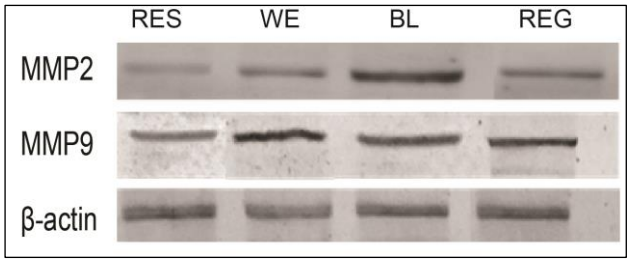

Figure1: Western blot of MMP2 and MMP9 during *H. flaviviridis* tail regeneration, (n=3).

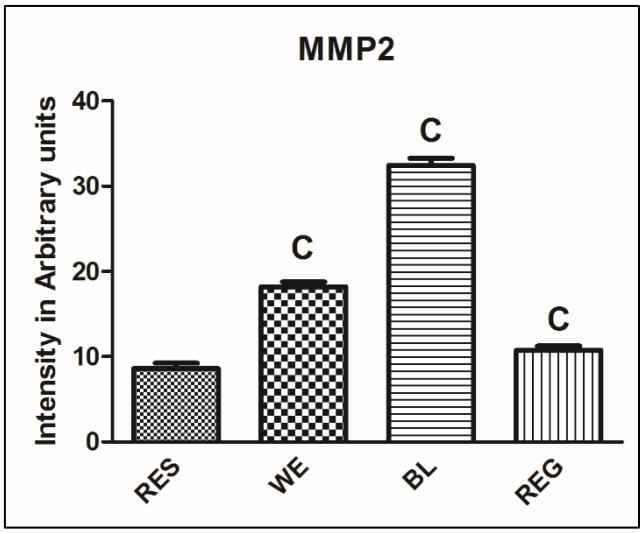

Figure 2: Analysis of western blot of MMP2 in stages during *H. flaviviridis* tail regeneration. The standard error of mean, where alphabet over error bar c represents $p \leq 0.001$, (n=3).

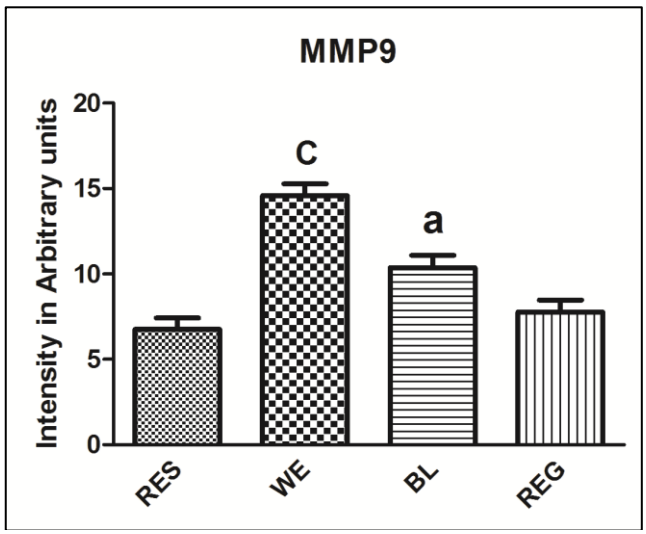

Figure 3: MMP9 expression with western blot analysis in stages during *H. flaviviridis* tail regeneration. The standard error of mean, where alphabets over error bar a and c represent $p \leq 0.05$ and $p \leq 0.001$ respectively, (n=3).

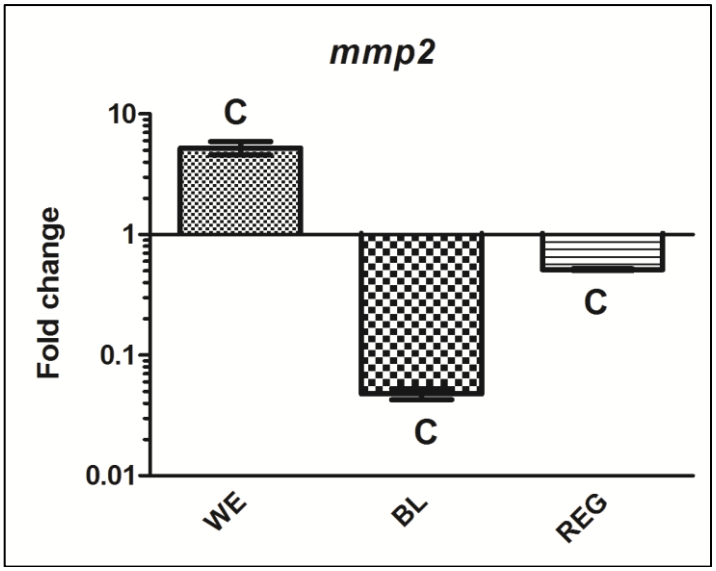

Figure 4: *mmp2* transcript fold change expression during *H. flaviviridis* tail regeneration. The standard error of mean, where alphabet over error bar c represents p ≤ 0.001, (n=3).

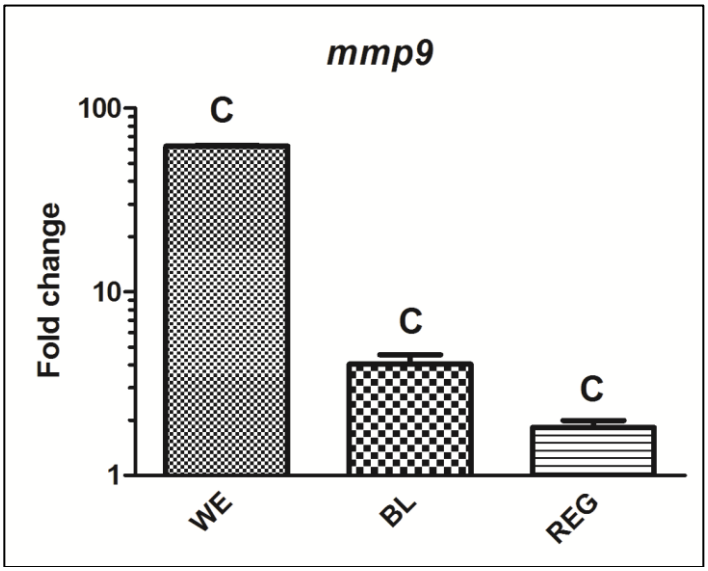

Figure 5: *mmp9* transcript fold change expression during *H. flaviviridis* tail regeneration. Error bars represent standard error of mean, where alphabet over error bar c represents p ≤ 0.001, (n=3).

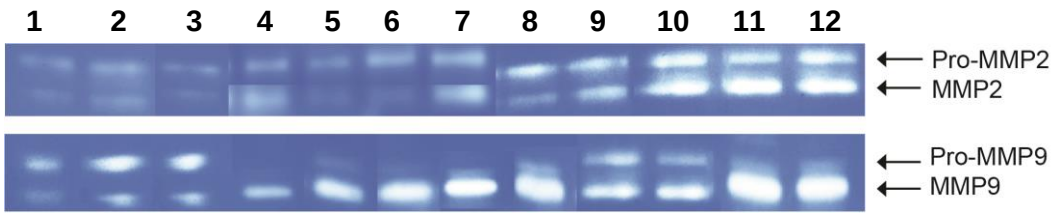

Figure 6: Zymogram of pro and active forms of gelatinases, MMP2 and MMP9 during various time points during *P. latipinna* fin regeneration, (n=6).
Lane 1: 0hpa, Lane 2: 1hpa, Lane 3: 2hpa, Lane 4: 3hpa, Lane 5: 4hpa, Lane 6: 5hpa, Lane 7: 6hpa, Lane 8: 12hpa, Lane 9: 18hpa, Lane 10: 24hpa, Lane 11: 60hpa, Lane 12: 5dpa

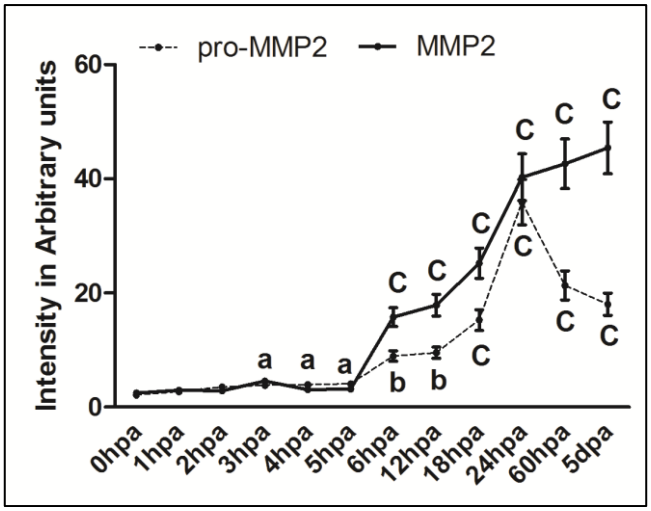

Figure7: Densitometry analysis of gelatinase activity of MMP2 at the selected time points during *P. latipinna* caudal fin regeneration. Error bars represent standard error of mean where alphabets over error bar a, b and c represent $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, (n=6)

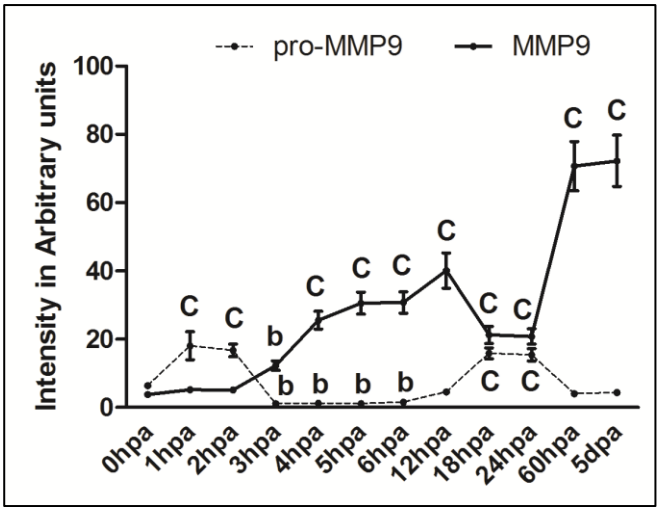

Figure 8: Densitometry analysis of gelatinase activity of MMP9 at the selected time points during *P. latipinna* fin regeneration. Error bars represent standard error of mean where alphabets over error bar b and c represent $p \leq 0.01$ and $p \leq 0.001$, (n=6).

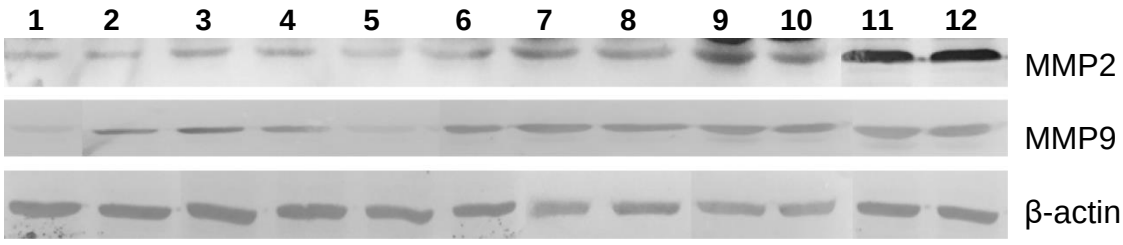

Figure 9: Western blot image of MMP2 and MMP9 of the selected stages of regeneration of *Poecilia latipinna.* β-actin was taken as a loading control.
Lane 1: 1hpa, Lane 2: 2hpa, Lane 3: 3hpa, Lane 4: 4hpa, Lane 5: 5hpa, Lane 6: 0hpa, Lane 7: 6hpa, Lane 8: 12hpa, Lane 9: 18hpa, Lane 10: 24hpa, Lane 11: 60hpa, Lane 12: 5dpa

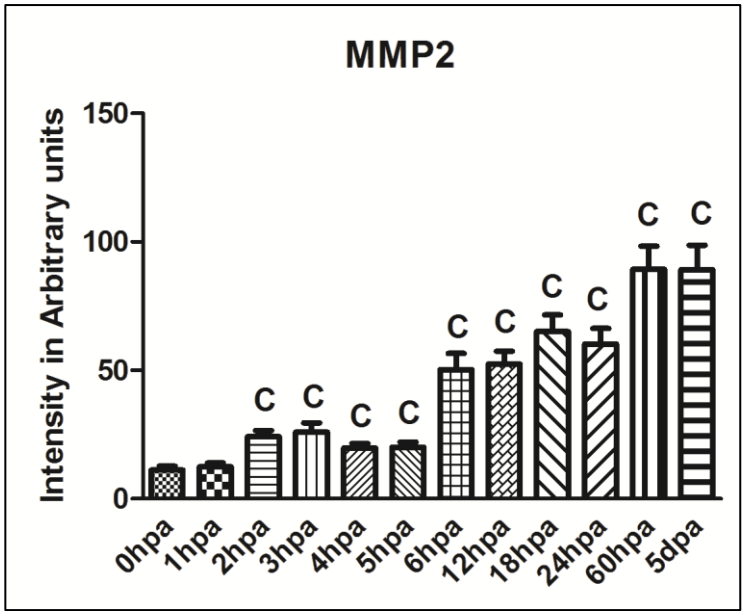

Figure 10: MMP2 western blot expression at selected time points during fish fin regeneration in arbitrary unit. Error bars represent standard error of mean where alphabet over error bar c represents $p \leq 0.001$, (n=6).

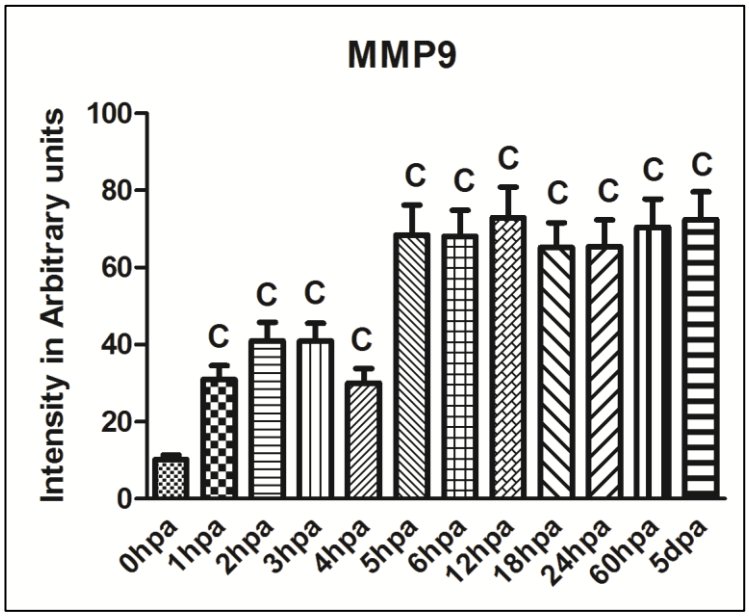

Figure 11: MMP9 western blot expression at each time point from 1hpa to 5dpa of fish fin regeneration in arbitrary unit. Error bars represent standard error of mean where alphabet over error bar c represents $p \leq 0.001$, (n=6).

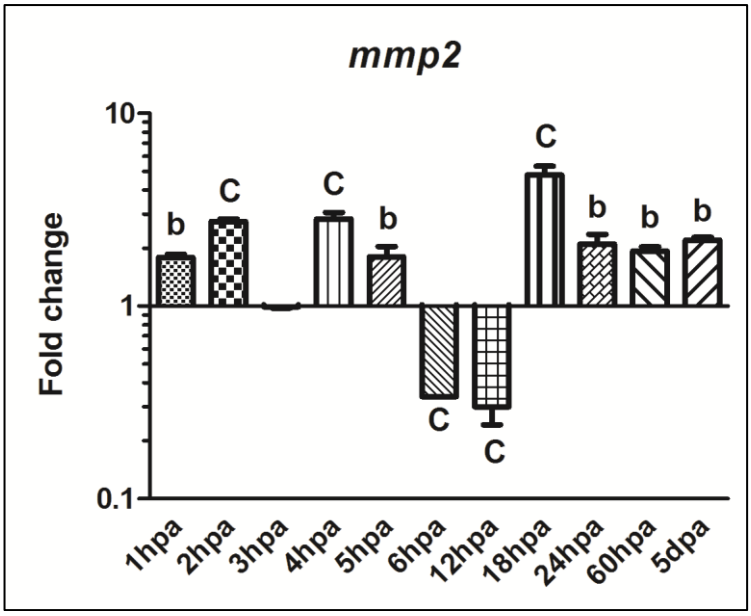

Figure 12: *mmp2* relative transcript levels expression at each time point from 1hpa to 5dpa of fish fin regeneration. Error bars represent standard error of mean where alphabet beyond error bar b and c represent $p \leq 0.01$ and $p \leq 0.001$, (n=6).

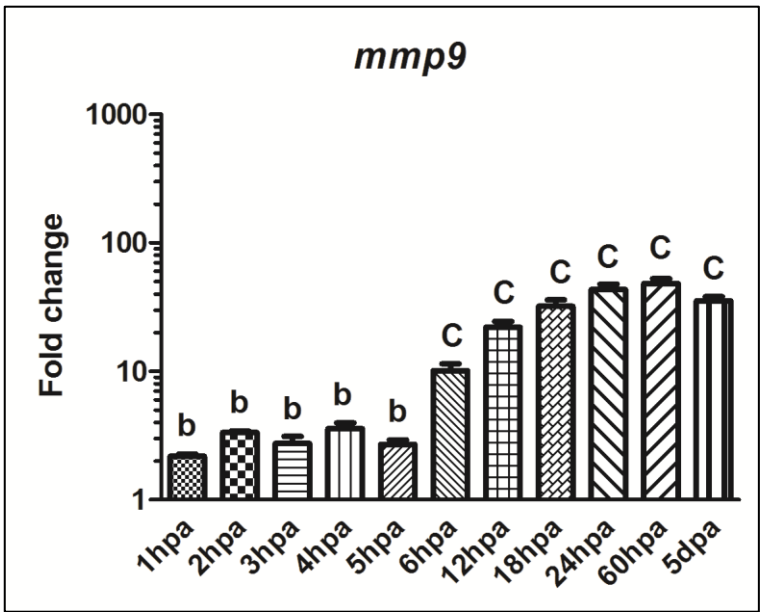

Figure 13: *mmp9* relative transcript levels expression at each time point from 1hpa to 5dpa of fish fin regeneration. Error bars represent standard error of mean where alphabet beyond error bar b and c represent $p \leq 0.01$ and $p \leq 0.001$, (n=6).