

Understanding the Hox-D3 and Wnt3a expression in different amputation segments of anterior regeneration in *Perionyx excavatus*

Submitted in partial fulfilment of the requirements for the award of
Bachelor of Science in Biotechnology

By

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **SUBHASHREE S (41770035)** who carried out the project entitled "**Understanding the Hox-D3 and Wnt3a expression in different amputation segment of anterior regeneration in *Perionyx excavatus***" under our supervision from November 2023

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ABSTRACT

Regeneration, a captivating and intricate process observed across diverse organisms, stands as a testament to nature's remarkable capacity for self-renewal and repair. The complex process of regeneration is regulated by key regenerative proteins that are involved with stem cells, apoptosis, cell proliferation, differentiation and pattern forming. Although many key regenerative proteins are up regulated upon regeneration, the interlink between Wnt3a and HoxD3 are not studied detailly. This thesis aims to understand the Wnt3a and HoxD3 expression in different segmental position of earthworm, *Perionyx excavatus* upon regeneration. Initially, the posterior segments of the earthworm were amputated into almost four equal segments and observed for the regeneration ability. All the four amputated segments regenerate their anterior head rapidly than the posterior tail segments because the head holds all the vital organs. We also observed the anterior regenerative differences among the all four amputated segments. Interestingly, HoxD3 and Wnt3a are reversibly expressed upon regeneration. HoxD3 over expression is notability observed with lower expression of Wnt-3a and similarly suppression of HoxD3 is combined with the over expression of Wnt-3a. The data clearly concludes, the regenerative bud with more Wnt-3a expression shows more stemness property and which hinders a pattern formation marker Hox-D3.

Keywords: Regeneration, *P. excavatus*, Stem Cell, Hox-D3, Wnt3a.

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CHAPTER-1

INTRODUCTION

1.1 REGENERATION IN A HOMEOSTATIC PROCESS:

Regeneration, a captivating and intricate process observed across diverse organisms, stands as a testament to nature's remarkable capacity for self-renewal and repair. Its significance transcends mere biological curiosity, offering profound insights into the fundamental principles governing life and adaptation. Anchored by the principles of epimorphosis and morphallaxis, as delineated by pioneering researchers such as Morgan (1901, 1910) and Agata et al. (2007), regeneration unfolds as a finely orchestrated symphony of cellular events. Following the clarion call of tissue injury, stem cells emerge from their dormant niches, embarking on a journey of migration towards the site of damage. Here, amidst the milieu of signaling molecules and cytokines orchestrating the healing process, these cells converge, laying the foundation for tissue restoration and renewal.

The intricacies of this phenomenon extend far beyond mere cellular dynamics, reflecting the intricate interplay between genetic predispositions, environmental cues, and evolutionary imperatives. Indeed, regeneration serves as a poignant reminder of life's resilience in the face of adversity, embodying the eternal cycle of growth, decay, and rebirth that underpins the fabric of existence. As we delve deeper into its mysteries, we unravel not only the secrets of regeneration but also the profound interconnectedness of all living beings, bound together by the common threads of life's ceaseless quest for renewal and regeneration. Through the lens of regeneration, we gain a deeper appreciation for the intricate dance of cellular processes and molecular mechanisms that govern the delicate balance between tissue repair and organismal survival. Each instance of regeneration serves as a testament to the resilience and adaptability of life, reminding us of the interconnectedness of all living beings and the universal imperative of growth and renewal. As we navigate the complexities of regeneration, we unlock not only the secrets of tissue repair but also the profound mysteries of life itself, illuminating the path towards a deeper understanding of the fundamental principles that shape the living world.

1.2 *PERIONYX EXCAVATUS*: A MODEL ORGANISM FOR REGENERATION STUDIES

Perionyx excavatus, colloquially known as the Indian blue worm, thrives in the balmy climates of tropical and subtropical regions, finding its niche as a stalwart contributor to the process of vermicomposting (Banik and Chaudhuri, 2017) (*1). Despite being frequently targeted by predators, this topsoil earthworm displays a remarkable resilience, exemplified by its adeptness at posterior regeneration—a trait honed through the crucible of evolution.

Pioneering studies have illuminated the astonishing capacity of *P. excavatus* to regenerate lost or damaged body parts, a feat particularly striking in the case of its head, which can regenerate fully even when severed entirely from the body. Central to this regenerative prowess is the phenomenon of accelerated cell growth observed in the head and tail regions compared to the midsection of the worm's body. Moreover, the species' regenerative potential extends beyond mere tissue repair, with amputated segments situated beyond the post-clitellum (typically the 30th segment) demonstrating the ability to regenerate into complete, functional organisms (Banik and Chaudhuri, 2017).

Such extraordinary regenerative capabilities not only underscore the adaptability of *P. excavatus* to survive in hostile environments but also position it as a prime candidate for investigating the mechanisms underpinning regeneration in multicellular organisms. Through the lens of this humble earthworm, scientists glean insights into the intricate interplay of genetic predispositions, environmental cues, and physiological processes that orchestrate the wondrous phenomenon of regeneration. Thus, *P. excavatus* emerges not merely as a diligent composter but as a beacon of resilience and regeneration in the animal kingdom, offering a glimpse into nature's enduring capacity for renewal and adaptation.

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1.3 STUDY OBJECTIVES

This paper embarks on an ambitious journey to delve into the intricate molecular mechanisms governing segment restoration during both anterior and posterior regeneration in *Perionyx excavatus*, shedding light on a realm of inquiry that remains largely unexplored (reference *6). With a keen focus on elucidating the expression patterns of the Hox-D3 and Wnt3a genes, pivotal players in the orchestration of segment patterning and stem cell maintenance, respectively, this study aims to unravel the molecular intricacies underpinning the remarkable regenerative capabilities of this enigmatic earthworm species.

The specific objectives outlined encompass a comprehensive analysis of the regeneration potential inherent in both anterior and posterior segments, delving into the nuanced nuances of regeneration kinetics across different amputation segments. Additionally, the study endeavors to meticulously assess the dynamic expression profiles of Hox-D3 and Wnt3a throughout the regeneration process, providing invaluable insights into their roles as molecular markers guiding tissue patterning and stem cell activation.

Through a multidisciplinary approach encompassing molecular biology, developmental genetics, and regenerative biology, this research seeks to unravel the enigma of segmental regeneration in *P. excavatus*, thereby contributing to a deeper understanding of the fundamental principles governing tissue renewal and repair in

multicellular organisms. As the scientific community continues to unravel the mysteries of regeneration, this study serves as a beacon illuminating the uncharted territories of molecular biology within the context of tissue regeneration, offering a glimpse into the intricate dance of gene regulation and cellular dynamics underlying the awe-inspiring phenomenon of regeneration.

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Fig:1.1 Perionyx excavatus

CHAPTER 2

LITERATURE SURVEY

In the study by Jie Zhang *et al.*, 2019, Soil macropores are crucial conduits within soil systems, facilitating the movement of water, air, and chemicals. Their significance in soil hydrology and solute transport has garnered considerable attention from researchers (Germann and Beven, 1985; Jarvis, 2007; Luo *et al.*, 2010). Macropores, as preferential flow pathways, expedite the transport of substances through the soil and support root proliferation (Katuwal *et al.*, 2015; Colombi *et al.*, 2017). Moreover, they serve as channels for the downward percolation of surface-applied fertilizers and pesticides, potentially diminishing their efficacy and polluting groundwater (Coquet *et al.*, 2005; Jarvis, 2007). Despite constituting a small portion of total soil porosity (Luxmoore *et al.*, 1990), macropores wield substantial influence over fluid dynamics and solute migration (Luo *et al.*, 2010; Katuwal and Larsbo, 2014).

Earthworms, ubiquitous soil organisms, are exemplary bioturbators, with their burrows significantly shaping macropore-related infiltration (Bottinelli *et al.*, 2015). Macropores comprise two primary pore types—biological and non-biological—differing in their water-conducting capacities owing to their distinct 3D structural attributes (Abou Najm *et al.*, 2010; Zhang *et al.*, 2019; Gackiewicz *et al.*, 2022). Biopores, notably earthworm burrows, exhibit superior efficacy in transporting water, solutes, and air compared to non-biopores (Katuwal and Larsbo, 2014). Additionally, observed that the 3D structural integrity of soil fauna burrows remains largely unaffected by rainfall infiltration and dry-wet cycles, boasting high stability and longevity under natural conditions. Bottinelli *et al.* (2017) further demonstrated that abandoned earthworm burrows endure for months in clay soils and even years in sandy soils, underscoring their enduring significance in soil water dynamics research.

In a study by Marija Kovacevic *et al.*, 2023, In contemporary agriculture, marked by heightened pesticide usage, the environmental hazards posed by these chemicals are significant, rendering them notable pollutants in agricultural and natural settings. Despite ongoing efforts to address their adverse impacts, global pesticide consumption has continued to escalate, reaching an estimated 4.2 million tons in

2019. This translates to a substantial market worth of nearly USD 84.5 billion, with projections indicating further growth to almost USD 130.7 billion by the conclusion of 2023 . With projections suggesting a requisite 35% to 56% increase in total food consumption between 2010 and 2050, the concomitant rise in pesticide utilization will exacerbate environmental pressures. Tebuconazole (TEB), a triazole fungicide, is extensively employed in agricultural practices to combat soil-borne and foliar fungal pathogens across various crop species. Its fungicidal action hinges on inhibiting C14-demethylase in sterol biosynthesis, thereby impeding cell membrane formation in pathogens. Notably, TEB emerges as one of the most prevalent pesticides detected in agricultural soil samples, often occurring at elevated concentrations. In Central European soils, over 70% of investigations have detected at least one conazole fungicide, with TEB being the second most frequently encountered, present in 36% of soil samples, often surpassing the 0.01 mg/kg threshold. The median TEB concentrations of 0.02 mg/kg, with maximum values ranging from 0.16 to 0.31 mg/kg in European agricultural soils. Due to its characteristics, TEB is classified as a pesticide with high-risk properties, including significant bioaccumulation potential, strong soil adsorption, low-to-moderate water solubility, and prolonged persistence . Given reported half-lives (DT₅₀) ranging from 120 days to over a year , TEB poses a considerable threat to various non-target soil organisms.

In a research by L.Hallatt *et al.*, 2017, mature earthworms reached sexual maturity and were introduced into organic soil to initiate mating. After an approximate period of 28 days, the diligent worms deposited their cocoons into the fertile soil, marking the beginning of a crucial phase in the earthworm life cycle. These cocoons, each a harbinger of potential life, were delicately transferred to a controlled environment within Petri dishes, where they underwent a 14-day period of embryonic development. Remarkably, around 70% of the embryos within these cocoons displayed robust viability, a testament to the species' resilience. Upon hatching, each cocoon yielded one or two juveniles, each representing a promise of continuity for the species. These young earthworms were then reintroduced into their natural habitat, the organic soil, where they embarked on a journey of growth and maturation. After an additional 30 days, keen observers were rewarded with the sight of adult earthworms, their mature status indicated by the presence of a clitellum. These observations not only underscore the intricacies of the earthworm life cycle but also

reaffirm the consistency of *P. excavatus* reproductive patterns with existing knowledge in the field.

According to the study by Jie Wang *et al* 2023, The earthworm species *Perionyx excavatus* exhibits a remarkable anatomical complexity, characterized by a meticulously segmented body housing a plethora of vital structures essential for its survival and functionality. Spanning over a hundred repetitive body segments, the earthworm's anatomy delineates distinct regions with specialized functions crucial for its existence. At the anterior portion, encompassing segments 1 to 12, lies the head region, serving as the epicenter of essential physiological processes. Within this domain, a myriad of vital organs find their abode, each playing a pivotal role in sustaining the organism's life functions. am, In contemporary agriculture, marked by heightened pesticide usage, the environmental hazards posed by these chemicals are significant, rendering them notable pollutants in agricultural and natural settings. Despite ongoing efforts to address their adverse impacts, global pesticide consumption has continued to escalate, reaching an estimated 4.2 million tons in 2019. This translates to a substantial market worth of nearly USD 84.5 billion, with projections indicating further growth to almost USD 130.7 billion by the conclusion of 2023 . With projections suggesting a requisite 35% to 56% increase in total food consumption between 2010 and 2050, the concomitant rise in pesticide utilization will exacerbate environmental pressures. Tebuconazole (TEB), a triazole fungicide, is extensively employed in agricultural practices to combat soil-borne and foliar fungal pathogens across various crop species. Its fungicidal action hinges on inhibiting C14-demethylase in sterol biosynthesis, thereby impeding cell membrane formation in pathogens. Notably, TEB emerges as one of the most prevalent pesticides detected in agricultural soil samples, often occurring at elevated concentrations. In Central European soils, over 70% of investigations have detected at least one conazole fungicide, with TEB being the second most frequently encountered, present in 36% of soil samples, often surpassing the 0.01 mg/kg threshold. The median TEB concentrations of 0.02 mg/kg, with maximum values ranging from 0.16 to 0.31 mg/kg in European agricultural soils. Due to its characteristics, TEB is classified as a pesticide with high-risk properties, including significant bioaccumulation potential, strong soil adsorption, low-to-moderate water solubility, and prolonged persistence .

Given reported half-lives (DT50) ranging from 120 days to over a year , TEB poses a considerable threat to various non-target soil organisms.

According to the study by Ray Sabarna *et al.*,2020, Earthworms possess a remarkable ability to regenerate lost segments, a process governed by intricate molecular mechanisms that operate at the cellular level. This regeneration mechanism is notably influenced by the specific site of amputation, exhibiting a remarkable degree of site specificity. For instance, if a worm is bisected, the anterior region demonstrates the capacity to regenerate the entire posterior part, while the posterior part lacks the ability to develop an anterior segment. (However, the earthworm species *Perionyx excavatus* presents a fascinating exception to this rule, as both the anterior and posterior segments exhibit the capability to regenerate. This unique characteristic highlights the species' exceptional adaptability and resilience in the face of injury and loss. Moreover, the process of segment restoration in earthworms is intricately intertwined with environmental factors, such as the availability of food, the developmental stage of the worm, the number of lost segments, and the precise position of the cut parts. These external influences play a pivotal role in modulating the pace and extent of regeneration, underscoring the dynamic interplay between genetic predispositions and environmental cues. At the heart of this remarkable phenomenon lies the process of epimorphosis, a complex biological process that orchestrates tissue regeneration and restoration. Through the intricate dance of cellular signaling pathways and gene expression patterns, epimorphosis guides the earthworm's segmental restoration, ensuring the seamless integration of newly formed tissues with existing structures. As scientists delve deeper into the mysteries of earthworm regeneration, they uncover not only the remarkable adaptive capabilities of these humble creatures but also invaluable insights into the fundamental principles governing tissue repair and renewal in multicellular organisms. Thus, the study of earthworm regeneration serves as a poignant reminder of nature's boundless resilience and the intricate beauty of biological adaptation.

The chiral acaricide Cyflumetofen (CYF) has gained registration and distribution in various countries and regions, including Japan, Brazil, the United States, and China, primarily for preventive mite control on citrus, apple, and other fruit trees (Feng *et al.*, 2019; Li *et al.*, 2013). Its principal mode of action involves inhibiting mite

mitochondrial respiration (Feng *et al.*, 2020; Feng *et al.*, 2019). CYF's molecular structure features a central carbon atom allowing separation into its enantiomers, (+)-CYF and (–)-CYF, using NP-HPLC analysis (Shi *et al.*, 2021b). While existing research on CYF enantiomers primarily focuses on their degradation in soils and effects on soil microbial functions, there remains a gap in understanding their toxicity to non-target soil organisms (Shi *et al.*, 2021b; Sun *et al.*, 2016). Initial observations on the chronic toxicity of CYF racemates in rats, fed a CYF-containing diet for 90 days, revealed renal and hepatic effects, including vacuolization of adrenal cortical cells (Yoshida *et al.*, 2012). Additionally, studies highlighted the stereoselective degradation of CYF in citrus, apple, and soil. In citrus, the half-lives of (–)-CYF and (+)-CYF were approximately 16.5 and 20.0 days, respectively, while in soil, they were about 6.4 and 7.0 days, respectively (Sun *et al.*, 2016). Furthermore, the enantiomeric fraction (EF) values of CYF varied between 0.49–0.48 in soil and 0.42–0.50 in citrus, indicating some degree of enantioselectivity in the degradation process (Sun *et al.*, 2016). Generally, the (+)-CYF residue exhibited greater persistence, with the two enantiomers displaying interconversion (Guo *et al.*, 2018). Against this backdrop of previous findings, evaluating CYF's selective toxicity at the enantiomeric level becomes crucial to complement the risk assessment of chiral CYF in the environment.

Similarly, the study by Hao Chen *et al.*, Pre-B-cell leukemia transcription factor 1 (PBX1) belongs to the triple amino acid loop extension (TALE) family of homologous transcription factors. Its pivotal role in lymphocytic leukemia and various cancers is well-documented. Beyond oncogenesis, PBX1 also exerts significant influence on stem cell developmental gene expression, fostering stem cell stemness, self-renewal, and regulating cellular oxidative stress and apoptosis during development. Moreover, PBX1 contributes to anti-aging processes, tissue and organ homeostasis, as well as tissue and organ regeneration. Given its multifaceted functions, PBX1 emerges as a promising therapeutic target and biomarker in developmental biology and regenerative medicine. In this comprehensive review, we delineate the structural characteristics of PBX1 and delve into its regulatory mechanisms governing the development of diverse tissues and organs. Additionally, we highlight PBX1's potential as a research target in the realm of regenerative medicine. By elucidating the potential interplay between PBX1 and these two fields, we aim to offer a novel

perspective for exploring cell homeostasis and facilitating tissue and organ reconstruction in the future.

The research by Riga Wu *et al.*, 2015, OX genes have been extensively studied in a variety of animals, not only for their importance during development, but also for their genomic organization, such as number of genes, number of clusters and elements in the non-coding regions. Often, the genomic organization of Hox clusters provides insights about the animal's genome. For example, a pattern from one single Hox cluster in the cephalochordate amphioxus to four paralogous Hox clusters (termed HoxA, HoxB, HoxC and HoxD) in tetrapods was one of the empirical findings that support the two round genome duplication hypothesis. The subsequent discovery of extra Hox clusters (up to 7 or more clusters) in teleost fishes was the first indication of an additional teleost-specific genome duplication. The comparatively slow evolution of the Hox clusters in coelacanth was among some of the earliest molecular evidence supporting the slow evolution of its genome . In anole lizard, a massive enrichment of transposable elements at the Hox loci was revealed, reflecting a genomic character that a large number of transposable elements accumulated in the development-related-gene-containing regions . Thus, information on Hox clusters is valuable, especially for animals of which the genome has not been sequenced.

CHAPTER 3

AIM AND OBJECTIVES

3.1 AIM

- ❖ To understanding the Hox-D3 and Wnt3a expression indifferent amputation segment of anterior and posterior regeneration in *Perionyx excavatus*

3.2 OBJECTIVES

- ❖ To analyse the regeneration potential of anterior and posterior segments in earthworm, *Perionyx excavatus*.
- ❖ To determine the anterior and posterior regeneration kinetics in different amputation segments.
- ❖ To analyse the HoxD3 (segment patterning marker) expression in different anterior and posterior regeneration segments.
- ❖ To analyse the Wnt3a (Stem cell marker) in different anterior and posterior regeneration segments

CHAPTER 4

MATERIALS AND METHODS

4.1 SAMPLE COLLECTION AND MAINTAINENCE

For this experiment, commercially available earthworm samples were procured from the market and cultivated under controlled conditions. The earthworms were carefully maintained and provided with a suitable environment conducive to their growth and development. This included feeding them a diet consisting of soil, leaf litter, and cow dung, which are commonly recognized as appropriate food sources for various earthworm species. The maintenance regimen also ensured that the earthworms were kept in a humid environment with a controlled temperature, facilitating optimal conditions for their growth and well-being. Upon establishing a suitable environment for the earthworms, the next step involved segmenting the earthworms at specific sites along their bodies. Both anterior and posterior segments of the earthworms were precisely amputated at designated intervals, spanning from the 1st to the 16th segments, the 17th to the 36th segments, the 37th to the 50th segments, the 51st to the 75th segments, and finally, the 76th to the 98th segments. Each amputation event was meticulously executed to ensure consistency and accuracy across all samples, with the timing of the procedure marked as the 0th hour for reference and standardization purposes. This comprehensive approach aimed to investigate the regenerative capabilities of different segments of the earthworms and to analyze any variations in regeneration patterns across distinct anatomical regions. Through these meticulously controlled experimental procedures, researchers sought to uncover insights of earthworm regeneration

4.2 EXPERIMENTAL DESIGN

To analyse the growth of the each segments SDS and Western blotting tests were done by using the segments of earthworm.

4.3 SDS-PAGE

(i) SAMPLE PREPARATION

- ❖ Homogenize *Perionyx excavatus* tissues in an appropriate lysis buffer (e.g., RIPA buffer) containing protease inhibitors.
- ❖ Centrifuge the homogenate at high speed to pellet cellular debris.
- ❖ Collect the supernatant containing soluble proteins.
- ❖ Determine protein concentration using a protein assay kit (e.g., Bradford assay or BCA assay).

(ii) PREPARING THE GEL

- ❖ Prepare a resolving gel and a stacking gel with appropriate concentrations of acrylamide (e.g., 12% resolving gel and 5% stacking gel).
- ❖ Assemble the gel cassette and insert a comb to create wells.

(iii) SAMPLE LOADING

- ❖ Mix protein samples with SDS sample buffer (containing β -mercaptoethanol) and heat at 95°C for 5 minutes to denature proteins.
- ❖ Load equal amounts of protein samples along with a protein molecular weight marker into the wells.

(iv) ELECTROPHORESIS:

- ❖ Place the gel cassette in the electrophoresis tank and fill it with running buffer.
- ❖ Run the gel at a constant voltage (e.g., 120 V) until the dye front reaches the bottom of the gel.
- ❖ SDS-PAGE separates proteins based on their molecular weight.

(v) STAINING:

- ❖ After electrophoresis, remove the gel from the cassette and fix it in a fixing solution (e.g., methanol/acetic acid).
- ❖ Stain the gel with Coomassie Brilliant Blue or Silver stain to visualize protein bands.
- ❖ Destain the gel to remove excess stain.

4.4 WESTERN BLOTTING:

(i) TRANSFER:

- ❖ Cut a PVDF or nitrocellulose membrane to the size of the gel.
- ❖ Activate the membrane by soaking it in methanol for a few seconds, then transfer to transfer buffer.
- ❖ Assemble the transfer sandwich: gel, membrane, filter paper, and sponge.
- ❖ Perform semi-dry or wet transfer at a constant voltage (e.g., 15 V for semi-dry transfer) for 1-2 hours.

(ii) BLOCKING:

Block the membrane by incubating it in blocking buffer (e.g., 5% non-fat dry milk in TBST) for 1 hour at room temperature with gentle agitation.

(iii) PRIMARY ANTIBODY INCUBATION:

- ❖ Dilute the primary antibody (specific to the protein of interest) in blocking buffer.
- ❖ Incubate the membrane with the primary antibody overnight at 4°C or for 2 hours at room temperature with gentle agitation.

(iv) WASHING:

Wash the membrane several times with TBST to remove unbound primary antibody (3-5 washes, 5 minutes each).

(v) SECONDARY ANTIBODY INCUBATION:

- ❖ Dilute the secondary antibody (conjugated to HRP or fluorophore) in blocking buffer.
- ❖ Incubate the membrane with the secondary antibody for 1 hour at room temperature with gentle agitation.

(vi) DETECTION:

- ❖ Wash the membrane with TBST to remove unbound secondary antibody.
- ❖ Detect protein bands using chemiluminescence substrate (e.g., ECL) for HRP-conjugated antibodies.
- ❖ Image the membrane using a chemiluminescence imaging system.

(vii) ANALYSIS:

- ❖ Analyze band intensity using image analysis software.
- ❖ Compare protein expression levels between samples.

CHAPTER-5

RESULT AND DISCUSSION

5.1 MORPHOLOGICAL CHANGES DURING ANTERIOR AND POSTERIOR REGENERATION:

To comprehensively explore the regenerative potential among different segments, a systematic series of amputations was meticulously conducted on earthworms, segmenting them into five distinct regions: segments 1 to 17, segments 18 to 36, segments 37 to 50, segments 51 to 75, and segments 76 to 98 (as visually depicted in Figure 2A). Following a five-day period post-amputation, the regenerative capacity of each amputated segment was systematically assessed. The findings starkly illuminated a profound dichotomy in regenerative abilities between the anterior and posterior segments. Specifically, segments 1 to 17, encompassing the head to clitellum region, exhibited a mere semblance of wound healing without instigating substantial regeneration, ultimately succumbing to demise by the sixth day. In stark contrast, all other amputated segments displayed robust regenerative activity at both ends (as vividly illustrated in Figure 2B). Surviving segments that effectively initiated regeneration were vigilantly monitored until the twelfth day post-amputation. The accumulated data unequivocally showcased a significantly higher rate of regeneration in the anterior region, particularly evident in the regrowth of the head region, compared to the posterior region (as visually depicted in Figure 2C), a phenomenon discernible as early as the fifth day post-amputation. This conspicuous disparity underscored the pronounced inclination of *Perionyx excavatus* towards head regeneration, vividly evidenced by the conspicuous development of the prostomium and mouth segments (as strikingly depicted in Figure 2D). In contrast, regenerative processes in the intestines and tail exhibited a comparatively slower pace. Notably, on the twelfth day post-amputation, an incomplete regeneration of the 17th segment was observed, with only the first fourteen segments being regenerated (as distinctly depicted in Figure 2D). This noteworthy observation underscores the critical significance of head regeneration, encompassing numerous essential organs,

and further underscores the remarkable adaptive capacity of *P. excavatus* in response to injury and loss.

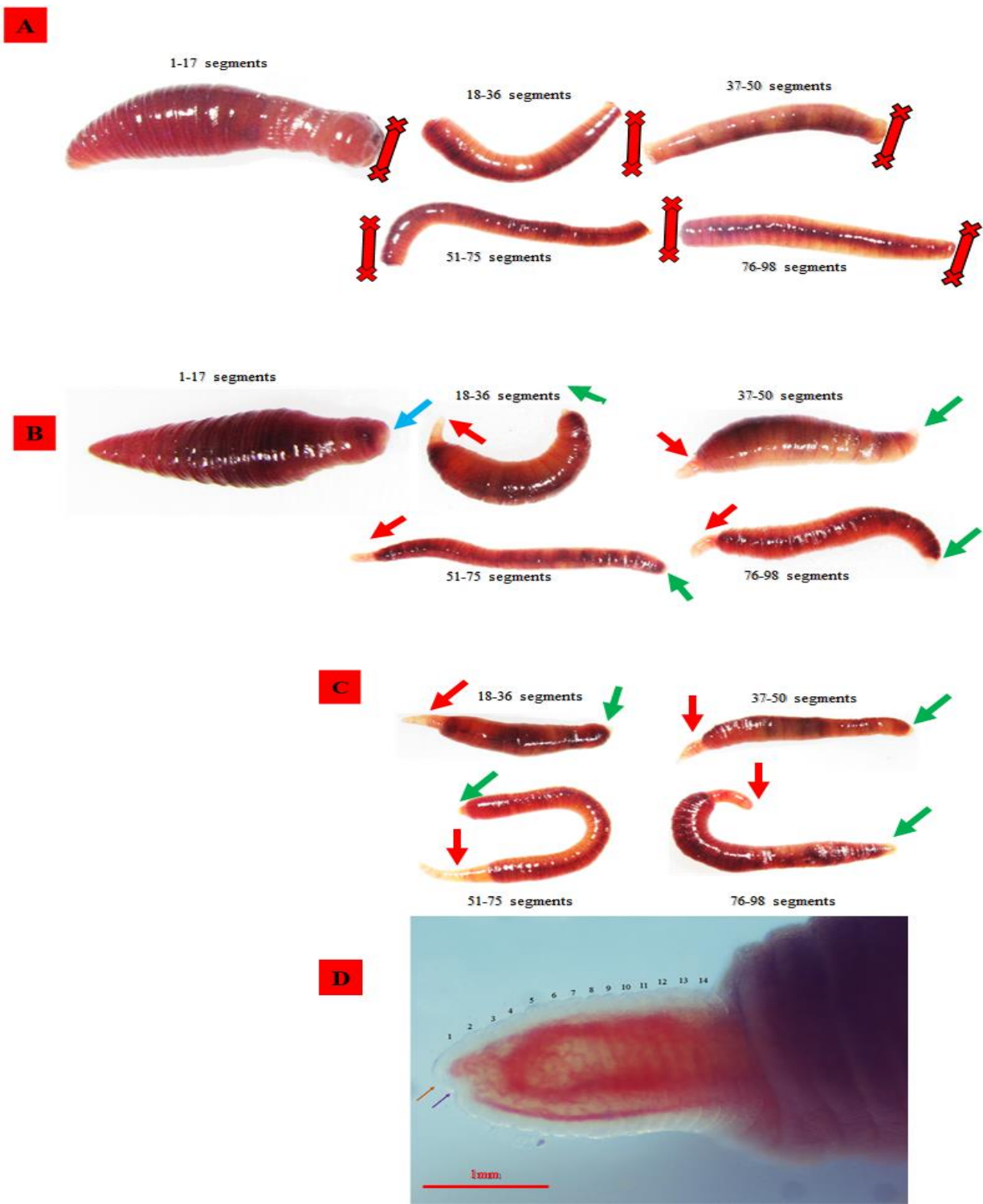


Fig: 5.1 :potential of *P. excavatus* worm

potential of *P. excavatus* worm: A. Both anterior and posterior segments of earthworms were amputated at equally as 1st to 16th segments, 17th to 36th segments, 37th to 50th segments, 51st to 75th segments, 76th to 98th segments and represented as on 0th hour. B. Wound healing was observed in anterior region (1-16 segments) of *P. excavatus* worm but in all amputated posterior segments the formation of blastema was observed in both ends of 17th to 36th segments, 37th to 50th segments, 51st to 75th segments, 76th to 98th segments on 5th day of post amputation. C. Anterior region (1st to 16 segments) was dead on 6th day but in all amputated posterior segments blastema were elongated and form the differentiated segments which are visible along with blood vessel formation as on 9th day of post amputation. In all the amputated posterior segments, the anterior regeneration kinetics is higher than the posterior regeneration. D. Following 12 days of post amputation of posterior segments it forms well developed prostomium and mouth while regenerating the anterior segments. Careful observation of regeneration represents that it forms only the first 14 segments instead of restoring the complete lost structure of 16 segments. Sky blue arrow (wound healing), Red arrow - anterior directional regeneration of posterior body segments, green arrow – posterior directional regeneration of posterior body segments, Violet arrow - mouth of anterior directional regeneration of blastema of posterior body segments, Brown arrow - depicts the prostomium region.

DAY 5



Fig: 5.2 : segment 1



Fig: 5.3 : segment 2



Fig: 5.4 : segment 3

On day 5, the separation of segments was conducted, and the segmented earthworms were then allowed to grow under monitored conditions.

Day 7



Fig: 5.5 : all 3 segmented samples on day 7

The segmented earthworms, which were allowed to grow following the separation of segments, were carefully observed after a period of 2 days to

monitor their progress. Subsequently, on day 12, the development of segments was meticulously observed and documented to assess the regenerative potential and growth patterns of the earthworms' segments over time as



Fig: 5.6 :anterior (16 segments)



Fig: 5.7 :middle (3 segments)



Fig: 5.8 :posterior (19 segments)

Then the protein sample is collected and prepared from segment 1,2,3 as shown in fig



Fig: 5.9 : Protein Sample preparation from segment 1,2 and 3

5.2 EXPRESSION PATTERNS OF WNT3A GENE & HOX-D3 IN DIFFERENT AMPUTATION SEGMENTS:

Key Regenerative protein expression studies in *P. excavatus* posterior regeneration.

To unravel the intricate molecular underpinnings of posterior regeneration in *Perionyx excavatus*, protein samples were meticulously prepared from three distinct sets of segments. The focus was on two key proteins: Wnt3a, recognized for its pivotal role in stem cell activation, proliferation, and differentiation, and HoxD3, responsible for orchestrating organ patterning. Remarkably, the analysis unveiled a striking pattern of protein expression across the segmented regions. Specifically, Hox-D3 expression exhibited a marked increase in the first set of posterior segments (17th to 36th segments), suggesting its crucial involvement in guiding tissue patterning and organ development in these segments. Intriguingly, Wnt3a expression was detected in all three sets of posterior segments, indicating a ubiquitous activation of stem cells throughout the regenerative process. This collective evidence points towards a common activation of stem cells across regenerating earthworm segments, while highlighting a distinct preference for organ patterning in the first set of posterior

segments (17th to 36th segments). These findings offer invaluable insights into the molecular orchestration of posterior regeneration in *P. excavatus*, shedding light on the dynamic interplay between stem cell activation and tissue patterning mechanisms underlying the remarkable regenerative capacity of this enigmatic earthworm species.

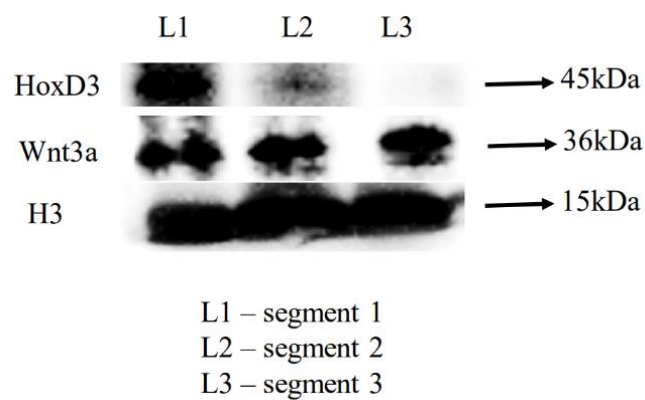


Fig: 5.2.1 : Wnt3a and Hox-D3 protein expression in three set of posterior segments. L1 – segment 1 (17th to 36th segments), L2 – segment 2 (37th to 50th segments), L3 – segment 3 (50th segment to 78th segments). For attaining the statistical significance, the experiments were conducted three times, and their results are presented as mean $\pm$ SD. Statistical data was consider as significant consider as significant whether p-value is <0.05. The western blotting data have represented in Figure 3

CHAPTER 6

SUMMARY AND CONCLUSION

The regeneration studies concludes the earthworm, *P. excavatus* give more preference for anterior regeneration than the posterior regeneration. Also, among anterior regeneration in different amputated segments regeneration varies. Wnt3a expression is enriched in initial blastemal development and after that HoxD3, a pattern forming protein dominate in the expression. Taken together, Wnt3a and HoxD3 are coordinately expressed for the successful regeneration of anterior segments.

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