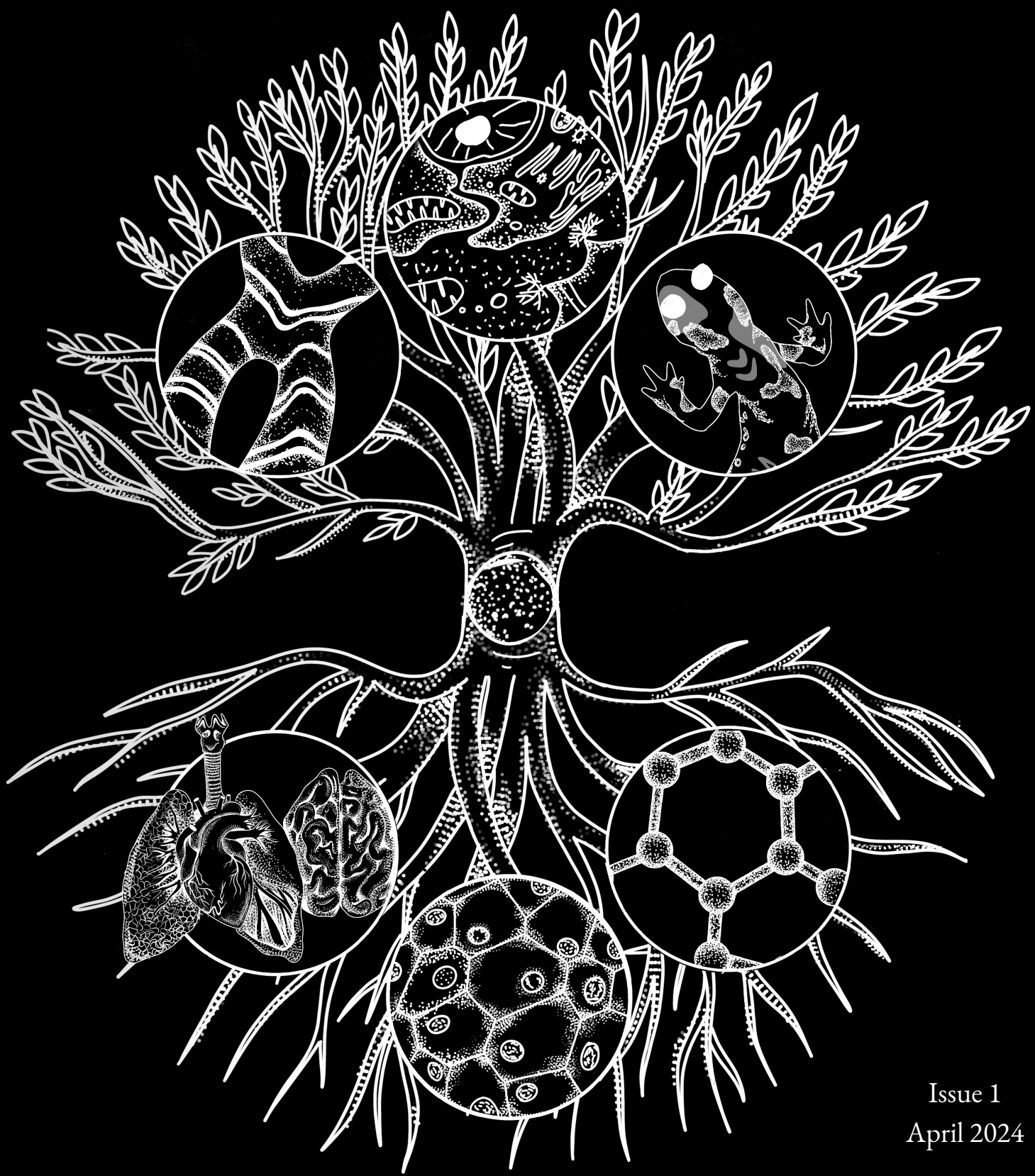
The Ashoka Journal of Biosciences



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Foreword

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A journey of a thousand miles starts with a small step. It is with this optimism that we launch the Ashoka Journal of Biosciences. This endeavour embodies the Ashokan spirit of inquiry, pursuit of knowledge, and commitment to excellence. This also reflects the collective ambition of our student body and their passion about advancing human progress in the century of Biosciences, and towards solving some of the most pressing challenges facing humanity and our planet.

Ashoka University, with its liberal arts philosophy, has always championed interdisciplinary learning and critical thinking. It is this ethos that the journal seeks to reflect by embracing a wide array of bioscience disciplines, bringing together diverse viewpoints from natural and social sciences. We are deeply grateful to the faculty advisors, peer reviewers, and the entire editorial team, whose dedication and expertise have been pivotal in bringing this vision to life. Their guidance and support have been instrumental in establishing a platform that not only celebrates student scholarship but also upholds the highest academic and ethical standards. We envision this journal as a beacon of student-led scientific inquiry, a forum for innovative ideas, and a catalyst for change.

Welcome to the inaugural edition. May it mark the beginning of a journey filled with discovery, innovation, and impact.

With warm regards,
Anurag

Letter from the Editor-in-Chief

The Ashoka Journal of Biosciences, September 2023

This volume marks the inaugural issue of the Ashoka Journal of Biosciences – a peer-reviewed undergraduate journal dedicated to showcasing research and writing produced by students at the outset of their scientific careers. Some may question the significance or calibre of such a publication, over concerns that the areas and methods of enquiry at the undergraduate level are hardly at the cutting edge of the field. While this appraisal of undergraduate research may be valid, the founding team of the AJB finds the importance of our mission to be two-fold. In the present world of scientific academia, not only is publishing an occupational necessity without which it is impossible to establish oneself in the field, but also an area of the field in which students lack both exposure and training. By modelling its peer-review and editorial processes on those established by international scientific journals, the AJB aims to provide student researchers a first-hand insight into the processes of academic publishing. Therefore in the process of showcasing diverse and creative research, the AJB also hopes to familiarise students with a key aspect of their future careers that is often overlooked.

Secondly, undergraduate research is ideally placed to reach a wide audience. Its broader aims and seeming simplicity of approach allow it to be understood by people in varied fields – not only within the scientific community but across disciplines. This highlights the second reason the content of the AJB is of significance – it helps to foster a culture of good communication in the sciences with the potential to open up intersectional dialogue. This issue features articles from microbiology, plant biochemistry, comparative developmental biology, and the ecology of parasitoid wasps. Alongside these diverse articles, the volume includes an in-depth academic essay on the search for the human last common ancestor; a faculty opinion piece on policy in rare disease research, and an interview with an Ashoka faculty about their latest research. Although many of the articles featured are reviews of previous literature or comparative studies of earlier research, they are important for their capacity to reach audiences beyond the narrow fields of technical expertise that often confine the Sciences. Many of them bring forth new insights which may provide new ways of looking at old research – fresh perspectives which may open further avenues for future primary research. This undergraduate research and writing holds many-faceted scientific significance.

As the field of biology grows at an ever-faster rate on both the microscopic and macroscopic frontiers, its boundaries become ever more permeable, broadening the scope for exchanges with diverse other fields. This growth is mirrored by the trajectory of Ashoka university itself, with a rapidly growing community of researchers, professors, and students in the biological sciences. At such a time of unprecedented expansion, it is essential to communicate new advancements, new ideas, and new perspectives in a manner that is open, accessible and stimulating. This role is one that the Ashoka Journal of Biosciences aims to help fulfil, as it rides the same wave of expansion and innovation permeating through the scientific world at the moment. I am deeply grateful to have had the opportunity to work alongside the founding Editorial Board towards the conception and creation of this Journal, and I take pride in presenting the outcome of our efforts to the community at Ashoka, and beyond. It is my hope that this issue serves as a validation of the effort that has gone into its creation, and the nucleus of what will become a thriving journal and community in the years to come.

Soham S Kacker

New Delhi
September 2023

Patients as Drivers of Rare Disease Research: Successes and Challenges

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It may be considered a tribute to modern medicine that for most common ailments, the doctor is able to provide the patient with at least a treatment, if not a cure. From infectious diseases to metabolic disorders and cancers, none has escaped the scrutiny of medical research. It is thus a rude shock to patients of rare diseases when they confront the fact that they have a disease about which little is known, and for which there is no available treatment. There are about 7,000 such diseases, most of which are due to genetic defects. Being relatively rare, doctors and scientists have little knowledge about them, and pharmaceutical companies do not see the profit margins to develop treatments for such few patients.

Upon being handed out the verdict of a debilitating or life-threatening rare disease with no treatment, the initial reaction of most patients/ caregivers is expectedly one of total despair and hopelessness. While many continue in this state for a long time, there are others who gear up to explore the possibilities of a treatment, whatever the odds. Their success stories are a salute to the human spirit. To give an example, two parents of children suffering from Dravet syndrome, (a rare form of severe epilepsy with prolonged seizures), started Tevard Biosciences with the goal of finding treatments for this rare disease. They knew about how to manage a company- so that was the easy part; but they had no clue about the science underlying Dravet. Having taught themselves that the problem was a nonsense mutation in the SCN1A gene, they managed to convince a top molecular biologist, Harvey Lodish, to join their company. They now have a tRNA-based therapy to suppress nonsense mutations that could not only benefit Dravet patients but also many others where the genetic defect is due to nonsense mutations. From being told by doctors that there was no treatment for Dravet, these parents have, indeed, come a long way. And, happily, they are not exceptions. The number of patient-led initiatives to not just fund research in rare diseases, but to keep track of new technologies that could be applied to their disease, and to lobby with regulatory bodies for fast-track approvals, is truly impressive. The single-minded devotion of parents to alleviate the suffering of their loved ones is an inspiration to the doctors and scientists working on the disease, and provides a big boost to their research.

Some of the most active patient advocacy groups (PAGs) like Parent Project Muscular Dystrophy representing Duchenne muscular dystrophy (DMD), have helped the U.S. federal regulatory body, the Food and Drug Administration (FDA) to draft regulatory guidelines for developing drugs for DMD. In reviewing the outcome of new drugs being tested for a rare disease, FDA has recognized the value of incorporating patient opinions. The challenge with rare disease clinical studies is that the number of patients being tested is relatively small, and the endpoints to be measured are not always optimum. In the absence of clear clinical benefits, patients are sometimes better placed to report any positive outcomes of the new drug with respect to ease of daily living, and to weigh the benefits with the potential risks of the new drug. When the disease is life-threatening and a good treatment option is not available, patients have successfully lobbied for accelerated approval of drugs based on initial data. FDA is also stressing the need to include patient-reported outcomes in the assessment of clinical trials.

In India we have a large number of very active PAGs representing a range of rare genetic diseases. Many of them are funding research projects through their own personal resources in the hope that their loved ones would get treatments at affordable costs within the country. Others are lobbying with regulatory agencies to facilitate import of drugs, medical foods, assistive devices etc. After PAGs adopted the legal route, the Government of India formulated the National Policy for Rare Diseases, 2021. Although limited in its scope, the policy does provide up to Rs. 50 lakhs one-time payment to a rare disease patient for treatment purposes. However, given the exorbitant cost of most rare disease drugs, this amount would not even suffice a year's treatment for, say, a patient of lysosomal storage disorders needing life-long enzyme replacement therapy; and it would cover only one-thirtieth the cost of a gene therapy treatment for a child suffering from spinal muscular atrophy.

The big challenge in India is that the policy makers and regulatory bodies are not sufficiently in touch with the ground reality and do not have effective monitoring mechanisms to fill the gap between policy deliverables and patients' needs. They rely primarily on doctors' advice, which in the case of rare diseases, becomes inadequate since the doctor-patient contact itself is rare. PAGs are a huge human resource that policy makers could utilize to help them connect with the ground reality. However, so far, such a sustained, positive interaction has not happened. Much like the US regulatory body, our agencies too must recognize the potential of PAGs to assist in all steps of the drug discovery process. This is not a fanciful idea. Patient groups abroad are pushing research based on latest technologies and discoveries, establishing well-defined cohorts of genotyped pa-

tients, making patient registries, and helping to set up clinical studies by ensuring patient participation.

Boosting rare disease research in India: Scientists teaming up with PAGs

Rare disease, by definition, is one in which the number of known patients is relatively small (tentatively defined in India as affecting <1 in 5,000 persons). The small numbers generate a vicious cycle in which lack of awareness results in little research interest which, in turn, exacerbates the state of ignorance. Breaking this cycle needs a team effort.

Forming a research team

Scientists, once they are made aware of a rare genetic disease, are likely to find it a worthwhile area of research as there are many important, novel questions to explore the mechanism by which disease phenotype emanates from the mutation in patients. In addition, the translational potential of such research is fulfilling to most scientists. To help tilt the decision in favour of initiating rare disease research the scientist can be greatly helped by PAGs. Not only is it motivational to witness the patient's journey, the scientist can benefit from PAGs who are generally storehouses of information about various aspects of their disease, which is not readily available. They are in touch with most of the Doctors actively treating the disease, and are aware of the possible drugs in various stages of development abroad. Teaming up with doctors is essential for the scientist, not only to appreciate the wide spectrum of disease severity and progression seen clinically, but also to obtain patient samples crucial for research.

Initiating the research

Although lately there have been a few 'call for proposals' dedicated to rare diseases, from governmental funding agencies, in general funding for rare disease research requires much more justification as the number of beneficiaries are limited. Funders typically are wary of supporting a research project that is being started from scratch without much background data. Here, the PAGs can be of immense help to get the research started. There are many instances of PAGs generously helping scientists by raising funds through their own personal sources in order to start some aspect of the research and collect preliminary data. For example, funds may be provided to clone the disease gene and express the mutant and wild-type proteins for biochemical analysis; or to design tissue-specific promoters for gene expression; or to obtain knock-in/ knock-out mutants in a cell-line for preliminary investigation of phenotypes, etc. Armed with data from such preliminary studies, and teaming up with doctors, the scientist can greatly improve the chances of getting their proposal funded.

Expanding the team

Most scientific research flourishes in collaborative mode; in the case of rare diseases this becomes a necessity, especially if the research is to reach its translational goals. Expertise is needed in areas like generating and maintaining animal and cellular models of disease; conducting transcriptomic, proteomic and metabolomic studies to identify the pathways leading to disease phenotype and generate biomarkers of disease progression; testing activators/ inhibitors of pathways or molecules implicated in disease and finding their therapeutic potential. In addition, it is important to do a natural history study of the disease in collaboration with doctors to obtain a quantitative measure of disease progression which can be used to determine the efficacy of potential new drugs. If nucleic acid-based therapies like gene or mRNA delivery and gene editing are to be developed and tested, experts in these areas must be on board. The patient will be maximally benefited if scientists with varied expertise could work together towards a common goal of finding a treatment. Once a promising treatment modality is found in model systems, PAGs can help to lobby with government for accelerated approvals for drug testing, and coordinating with patients for participation in clinical trials.

Patient groups have amply demonstrated their capability to act as catalysts. Their own lives, or the lives of their loved ones, depends on the success of rare disease drug discovery. There can be no greater force to propel clinical research. One hopes that in India this energy transfer from patients to other stakeholders can start happening organically.

Biology Across Scales - A Photoessay

Caenorhabditis elegans is a well-known model organism used to study various levels of organisation within the biological system, starting from molecular mechanisms to tissues to more complex behavioural patterns. This organism was established as a model system by Dr. Sydney Brenner due to reasons like their short life span, small body size, transparent body structure, amenability to genetic manipulations etc. Despite their simplicity, this organism exhibits significant similarity to cellular systems and various important molecular mechanisms in higher eukaryotes like mammals. Hence, *C. elegans* offers a simple window into the myriad of complex cellular and molecular processes that we seek to study and also to develop potential therapeutic interventions to a multitude of disorders that plague our lives.

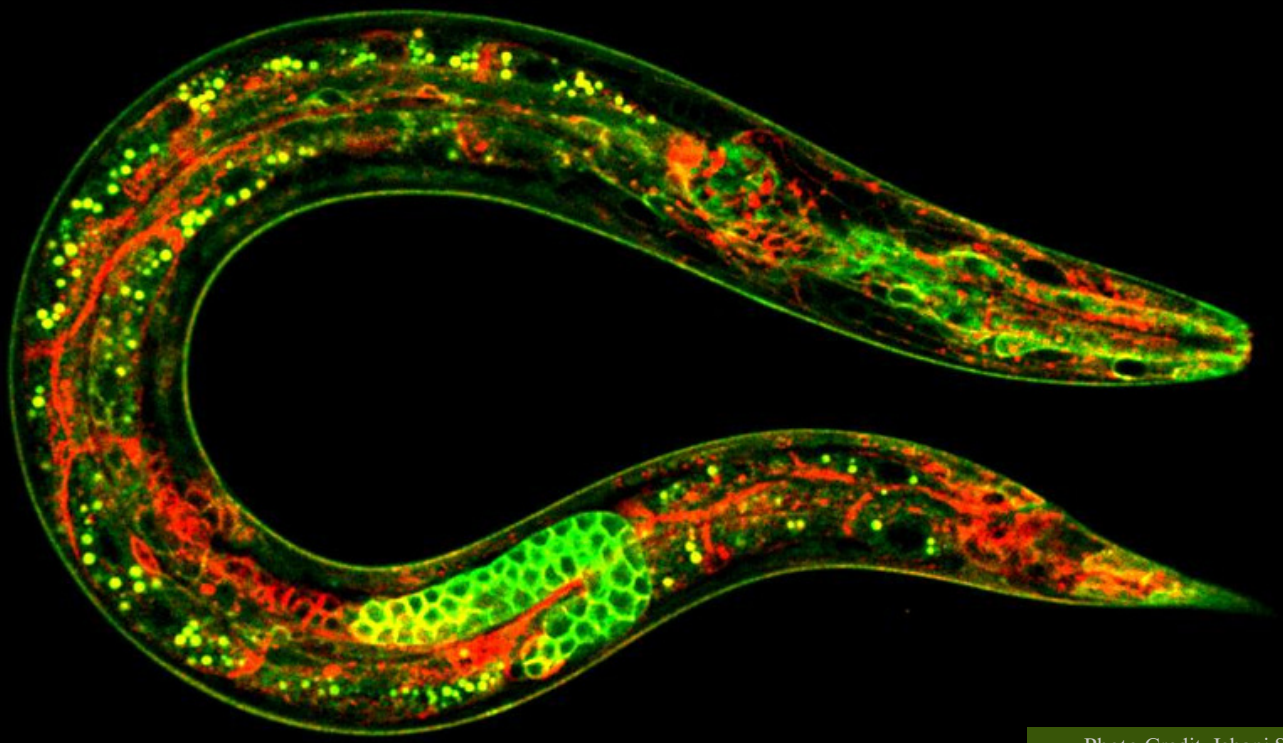

Photo Credit: Ishani Sharma
Submitted by: Dr. Anup Padmanabhan

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Phytoplanktons are a group of unicellular and photosynthetic eukaryotes and prokaryotes that are responsible for half of the global primary production thereby generating valuable oxygen comparable to land plants. Phytoplanktons adapt to their surrounding environment by coordinating different biological tasks with the 24-hour day-night cycle. They can do so with the help of a cell-based clock, termed as the circadian clock. Sougata Roy's laboratory uses unicellular phytoplanktons to a) Study the diversity in clock organization and its components, b) Understand and refine the fundamental features of how cellular clocks regulate physiology and metabolism, c) Study the impact of ocean acidification and sea surface temperature on circadian cycles of carbon and nitrogen and finally d) manipulate circadian systems to engineer algal cells with enhanced metabolic abilities.

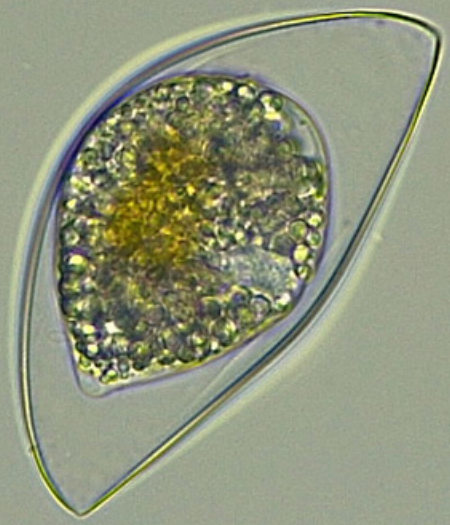

Photo Credit: Dinesh Jadhav
Submitted by: Dr. Sougata Roy

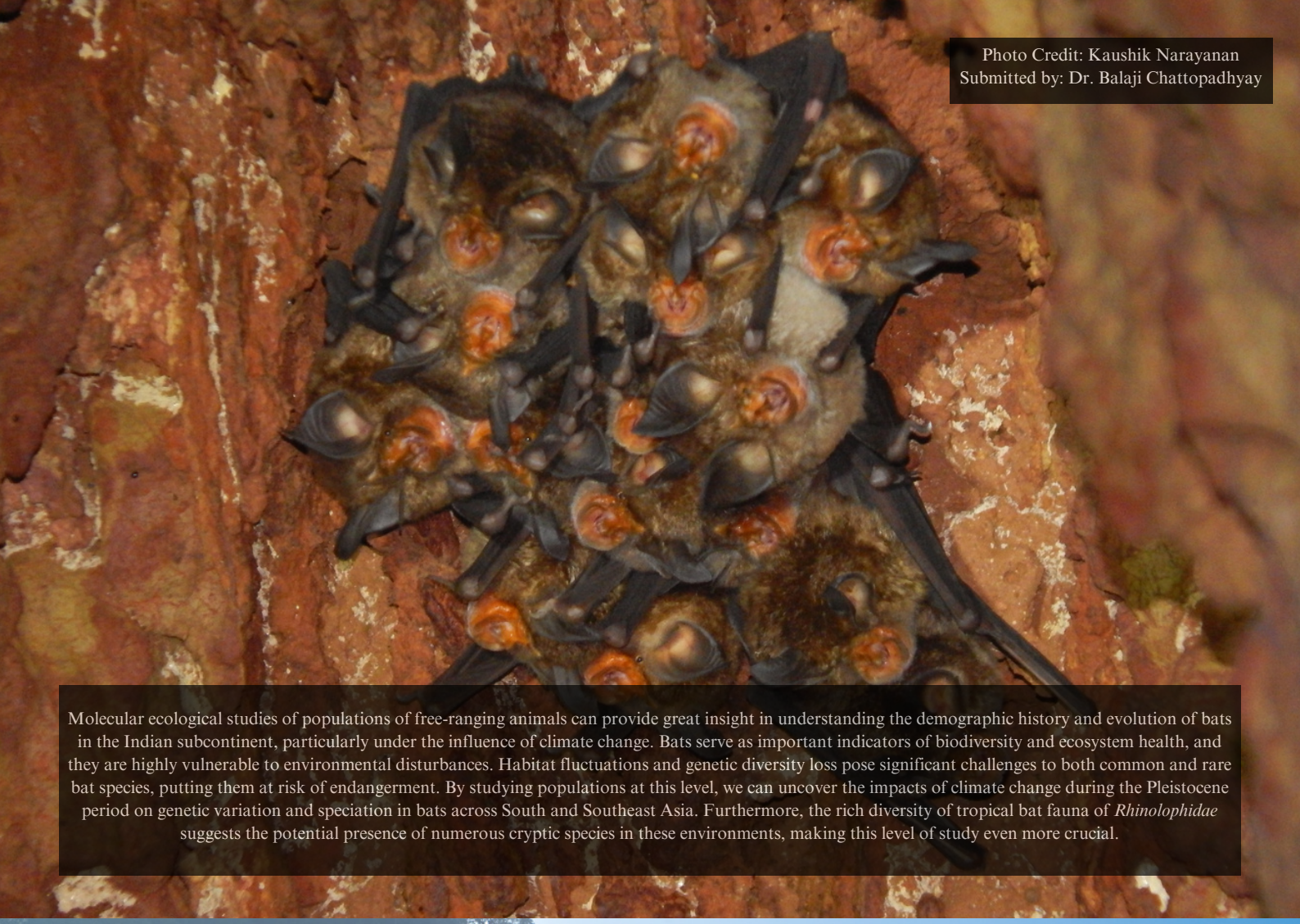

Photo Credit: Kaushik Narayanan
Submitted by: Dr. Balaji Chattopadhyay

Molecular ecological studies of populations of free-ranging animals can provide great insight in understanding the demographic history and evolution of bats in the Indian subcontinent, particularly under the influence of climate change. Bats serve as important indicators of biodiversity and ecosystem health, and they are highly vulnerable to environmental disturbances. Habitat fluctuations and genetic diversity loss pose significant challenges to both common and rare bat species, putting them at risk of endangerment. By studying populations at this level, we can uncover the impacts of climate change during the Pleistocene period on genetic variation and speciation in bats across South and Southeast Asia. Furthermore, the rich diversity of tropical bat fauna of *Rhinolophidae* suggests the potential presence of numerous cryptic species in these environments, making this level of study even more crucial.

The social-ecological landscape of Nagaland is a fascinating one, where lives of people, flora and fauna are intertwined. But it is also one that is seeing rapid change. Local communities, noting the loss of forests and wildlife on their lands, are starting grassroots conservation movement. Our responsibility is to support such movements, to secure the immense biodiversity of the region, an invaluable carbon store and a treasure trove of rich complex people-nature relationships.

Photo Credit: Varun R. Goswami
Submitted by: Dr. Divya Vasudev

All Along the Cellular Watchtower: Reviewing the role of the bacterial flagellar motor & efflux pumps in antibiotic resistance

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Flagella and efflux pumps are two major components of bacterial cells and hotspots of antibiotic activity in drug design via removal of toxins from the interior. These structures play a role in bacterial virulence and their ability to proliferate in their proximal environment through mechanical movement or the removal of harmful substances from their interior respectively. Antibiotic resistance poses a pressing concern in today's time. This makes it necessary to understand the interplay between these bacterial cellular membrane bodies and their effect on the surroundings. As bacteria progress to the proliferative stage of their life cycle, varying down-regulation of flagellar structure is seen combined with the secretion of signalling molecules which create a favourable environment for bacteria. With the danger of antimicrobial resistance, the need to identify new candidates for treatment is also of increasing importance. Understanding the molecular pathways in bacteria that create this resistance internally and externally to the body and their regulatory systems could create a novel paradigm for the study of antibiotic resistance. This review aims to discuss the contributions of flagellar motors and efflux pumps in mediating resistance to antibiotics as well as their involvement in biofilm formation.

1 Introduction

Antibiotics are classified into families by their mechanism of killing or inhibiting bacterial growth [1]. Antibiotic design is done with specific target sites or processes in mind — the cell wall and synthesis pathways in the cytoplasm inhibit DNA synthesis. Combined and controlled antibiotic dosage enables effective bacterial growth control. However, due to misuse and overuse in the public sphere, regulatory standards between different countries/clinical bodies and limited drug development incentive in industry, antibiotic resistance has become a looming concern [2].

Bacterial cell components — efflux pumps (EPs) and bacterial flagellar motor (BFM), play a major role in providing antibiotic resistance. Bacterial cells can respond to antibiotic stress by modifying or destroying antibiotic molecules, altering or bypassing target sites, or removing antibiotic molecules from their interior through EPs. EPs actively limit the internal antibiotic concentration below viable levels so that the cell can continue to survive. They do so by utilising the ATP-binding cassette of proteins that aid in transport of antibiotics or other harmful substances out into the extra-cellular matrix [3]. Resistance to antibiotics is linked with mutations in these pumps and motors, and not the upstream inducer molecules. This is primarily why such resistance arises in the presence of selection agents like a high antibiotic concentration in the proximity of the cell.

Biofilm formation and the associated loss of flagella's migration function are other crucial mechanisms that enable antibiotic resistance via their role in controlling cellular exposure to them within the structure of biofilm. The exposure of planktonic bacterial cells to even minimal antibiotic levels can induce biofilm formation. Biofilm matrices are typically composed of extra-cellular polysaccharides, extra-cellular DNA, lipids, water, extra-cellular vesicles and other extra-cellular structures [4]. The ability to move and remain motile in response to stimuli is key to resisting the effect of antibiotics as bacteria can undergo chemotaxis away from areas of higher concentration of these substances toward less stressful environments. Motility is limited when bacteria become a part of biofilm, leading to loss of function for migration enablers like flagella, which are bacterial cell membrane extensions that provide motility to the organism. They help the cell undergo chemotaxis by the action of BFM rotating an axial body, propagating mechanical and rotational forces that cause movement. The axial body comprises a helical organisation of 11 flagellin protofilaments [5]. Studies have observed multiple different flagellar structures in differing organisms and as research progressed, the roles of the flagella have slowly expanded beyond the initial areas of strictly movement. In this review, I will discuss the contributions of flagellar motors and efflux pumps in mediating resistance to antibiotics as well as their involvement in biofilm formation.

2 Pump the brakes : The bacterial flagellar motor and efflux pumps in the movement of the cell and its contents

Research has shown that flagella are also implicated in virulence, adhesion and immunity due to their primary function of motility [6, 7]. Therefore, inhibiting the assembly or function of flagella is a goal of antibiotic design. Many well-known antibiotic families such as macrolides (e.g.—azithromycin) act by inhibiting protein synthesis.

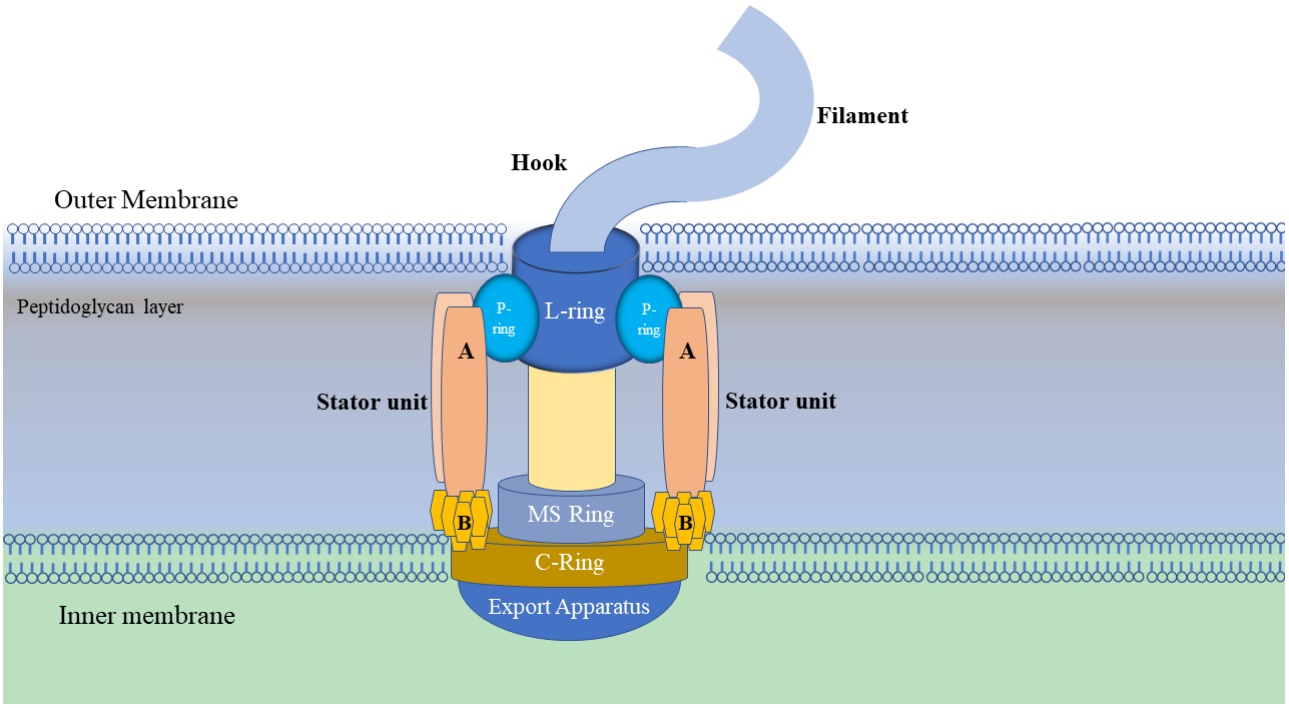

Figure 1: Structure of bacterial flagella in a gram-negative bacterial cell. Stator units are stationary and are associated with the peptidoglycan layer. The flow of protons through stator units leads to the motion of flagella. The assembly process of bacterial flagellum starts from the formation of the basal body's MS ring (FliF ring) in cytoplasmic membrane. It proceeds in all three directions: inward, outward and lateral. The inward assembly involves forming the C ring, export apparatus in cytoplasmic space and preparing to export flagellar proteins through its central rod channel [8].

Their action prevents motility or chemotaxis and reduces virulence via preventing the ability of a cell to move to other areas and proliferate. Developing novel antibiotics, however, also requires equal consideration of the vast diversity in flagellar structures and associations in bacteria. The BFM consists of the MS ring, C ring, rod, and export apparatus that are powered by a proton gradient across the cell membrane [higher outside - lower inside] [9]. In Helicobacter pylori, a known flagellated human gastrointestinal pathogen, flagella exist in polar sheathed clusters of 5 to 7 each. Flagellar motility typically uses cellular energy stored as proton motive force. It makes cells less efficient in pumping out toxic molecules such as antibiotics [10]. Structural integrity of the motor is associated with the expression of the tripartite efflux system genes [11]. Regulating the expression of these genes allows the organism to progress to a new non-motile proliferative life-stage.

In addition to flagella, the role of EPs in antibiotic resistance highlights the need for a deeper understanding of their functionality. EPs have many different functions within cells. However, they are most well-known for their work as active multi-drug transporters. EPs' functionality also depends on the organisation of the cellular periphery; Gram-negative or Gram-positive. Gram-negative bacteria often possess an outer membrane rich in lipopolysaccharide which is strongly hydrophobic. Common antibiotics such as vancomycin typically cannot penetrate the double membrane of Gram-negative bacteria due to the physical and chemical barrier presented by the same [12]. There are six families of motor efflux proteins: Small Multidrug Resistance (SMR) family, Major Facilitator Superfamily (MFS), Multidrug And Toxic compound Extrusion (MATE) superfamily, ATP Binding Cassette (ABC) superfamily and Resistance Nodulation Division (RND) family [13]. The RND EPs in Acinetobacter baumannii, a Gram-negative bacterium, span both membranes and function in tandem with the outer membrane's low permeability barrier [14].

The major function of multi-drug transportation in these cells is possible due to the tripartite assembly exhibited by EPs. RND pumps in Escherichia coli comprise an outer membrane channel protein and membrane adaptor protein that span both membranes and function by proton motive force. They exhibit a tripartite assembly along with an inner membrane RND transporter protein; an outer membrane protein called TolC; and membrane adapter proteins; forming a duct to transport antibiotics and other compounds. This tripartite assembly facilitates the expulsion of molecules to exoplasmic regions. It accomplishes this by a successive mechanism of pumping mechanisms through each membrane and the periplasm; or by using efflux motor transporter molecules that have crossed the non-permeable inner membrane. It then expels molecules to the exterior by channel proteins [15].

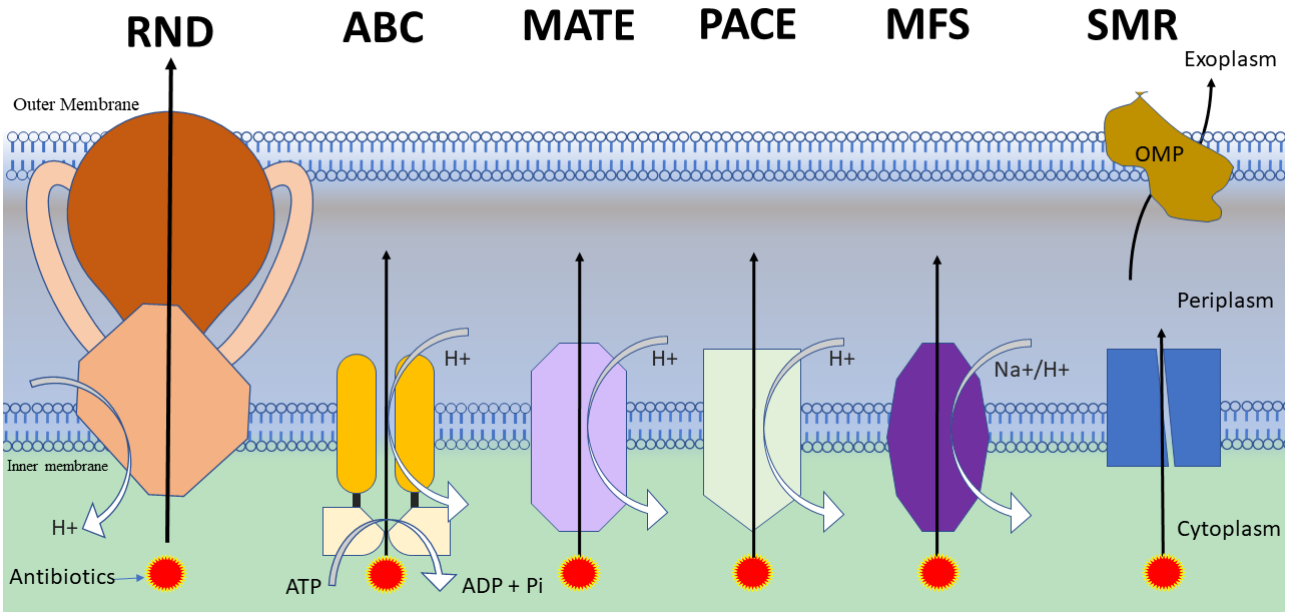

Figure 2: The 6 major families of efflux pumps in bacteria. Their primary function involves efflux of toxic substances such as heavy metals, toxins and antibiotics. Gram-negative bacteria typically showcase more antibiotic resistance. This is due to their low permeability of the outer membrane and their RND transporters [16]. All major superfamilies of efflux pumps, except ABC function, utilise proton motive force. The ABC family uses ATP/ADP to move substances across cell membrane into the exoplasm.

The level of antibiotic resistance, however, is not uniform across all bacterial cells. The specificity of substrate binding and pump expression level determine the spectrum of resistance present in the cell against antibiotic treatment. Efflux binding occurs due to physiochemical linkages between hydrophobic or hydrophilic antibiotics subunits and complementary transporter binding sites. The expression of EPs is typically controlled by transcriptional repressors located upstream of the pump operon. Consequently, antibiotic resistance can be developed as a phenotype at different levels based on the expression level of pumps. Particular MDR (Multi Drug Resistant) pumps, such as Escherichia coli AcrAB-TolC, can present a base expression that is enough to contribute to innate antibiotic resistance [17]. Reverting the expression of repressed EPs can be achieved by mutating these same regulatory proteins, granting stable resistance. In this manner, EPs provide a chemical pathway to expel substances from within the protoplasm, among other antibiotic-resistant enabling actions.

3 Breaking siege : Biofilm, bacterial flagellar motor and efflux pumps

As bacteria grow, they come under the effect of their environment and strive to remain in a favourable environment for their growth (neutral/slightly acidic pH, sufficient nutrition etc). However, when presented with environmental or chemical stresses such as extreme pH or temperature, there are varying chemical and temporal responses by groups to constrain the effect of these stresses through different means One such related example of the same would be the up-regulation of particular efflux pumps activity when undergoing higher antibiotic stress. Bacteria respond to such stresses by secreting extra-cellular polymeric substances such as polysaccharides and proteins. Along with this secretion, they form large symbiotic aggregations to develop structures known as biofilms. Biofilms serve as hotspots for bacterial mutations and horizontal gene transfer due to their structure, composition and regulated gene expression in the colonies [18]. The initial stage of biofilm development occurs via a reversible attachment period where motile bacteria adhere to a suitable surface. Studies have shown that by a series of successive attachments and detachments, cells become surface-adapted [19]. A form of adaptation such as this requires a phenotypic change in surface-bonding bacteria. Such change is communicated by intra-cellular cascade signal systems.

The relation between biofilms and flagellar motility is elaborated through the example of an integral communicating molecule. In numerous Gram-negative species, cyclic di-GMP or c-di-GMP is an integral communicating molecule that promotes biofilm matrix secretion and flagellar motility repression [20]. It does so by binding and causing a conformational change in the structure of flagellar C-terminal extension that affects motility [21]. C-di-GMP signalling events cause MotCD stator proteins, which are the stable component of the flagellar motor of Pseudomonas aeruginosa, to disengage from the channel, halting movement.

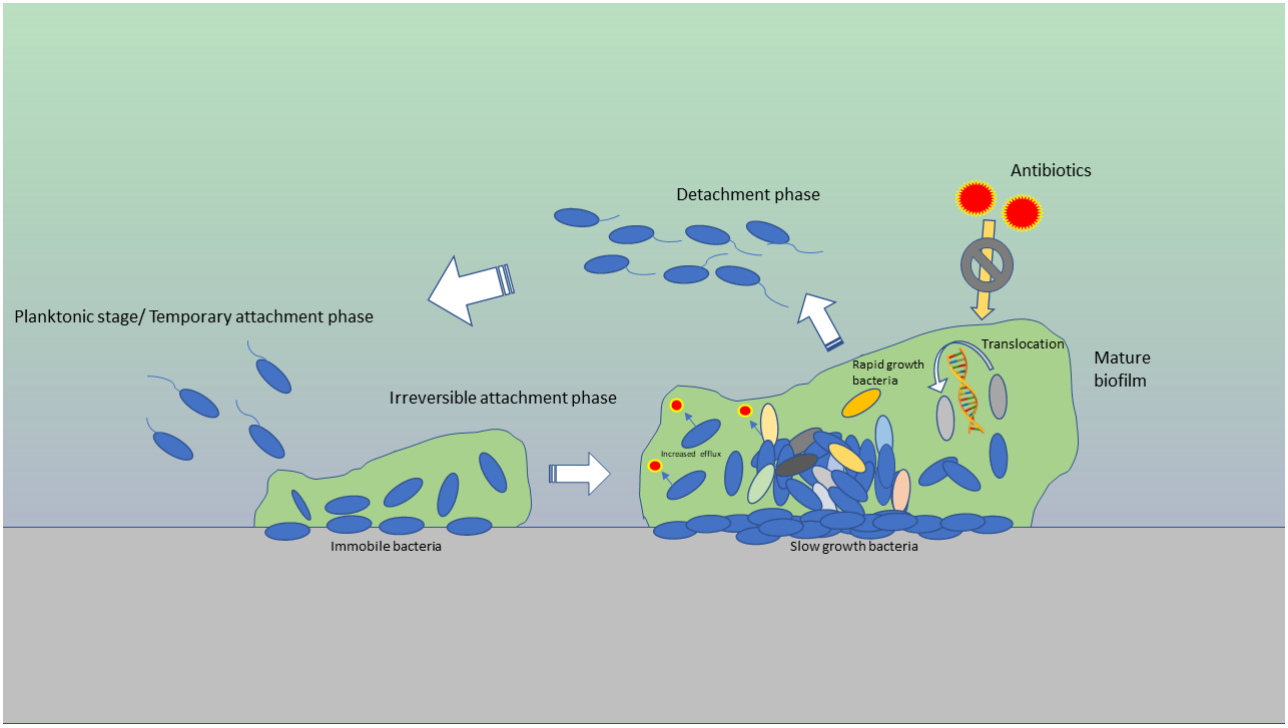

Figure 3: Biofilm formation and its key characteristics. Planktonic bacteria form polar structures to temporarily bond to the nutrient surface. During this process, they rapidly gyrate or rotate; and allow their flagella and adhesions to interact with the substrate. Permanent bonding to substrate surface depends on numerous upstream cellular processes that promote polysaccharide secretion. E.g. - Bonding of flagella to substrate has been found to be related to polysaccharide secretion in biofilm matrix. [22]

This is followed by disengaged stators stimulating c-di-GMP production to reinforce a biofilm mode of growth [23]. Attacking flagellar motility to inhibit biofilm formation is a possible method of preventing antibiotic resistance.

Besides flagellar motility, prevention of antibiotic resistance is also possible by attacking the BFM. This can be seen through an example of Vibrio cholerae which undergoes a definitive change in morphology when entering the biofilm stage of its life cycle. A loss of flagellar structure and function is exhibited in Vibrio cholerae at the beginning stage of biofilm formation. This loss can be associated with a reduction in biofilm formation on the administration of a sub-inhibitory level of antibiotic polymixin-B [24]. The flagellar hook protein, flgE, has been involved in the biofilm tolerance of Pseudomonas aeruginosa towards various antibiotics. Cells lacking flgE proceed to self-assemble into a denser biofilm matrix due to enhanced growth yield and compacted biofilm growth [25]. Therefore, perturbing the BFM and its assembly can arrest possible antibiotic resistance arising from biofilm development.

Furthermore, the expression of EPs is related to biofilm formation and thus to antibiotic resistance through biofilm. The heterogeneity of biofilm composition is integral to its inhabitants' antibiotic resistance. Individual bacteria rely on quorum sensing to effectively communicate between cells. Quorum sensing is a method of cell-cell communication which enables an individual cell to detect and respond to population density in its environment. Through the extracellular production/secretion of inducer molecules that cause signalling cascades, they organise gene expression to trigger outcomes such as chemotactic movement or bioluminescence. EPs assist with the movement of these chemicals across membranes [26]. In Escherichia Coli, it was shown that positive expression of emrD, emrE, emrK, acrD, acrE and mdtE EP encoding genes were directly linked to better biofilm formation. This may be attributed to the ability of these pumps to export or import substances that are necessary or harmful for biofilm growth [27]. In this manner, the ability of pumps and the flagella and their temporal regulation in biofilm can work in tandem to create the optimal environment for antibiotic resistance to emerge.

4 Future prospects

The current understanding of BFM, EPs, biofilm and antibiotic resistance hints at unknown functional and structural connections that could reveal themselves in the future. Studies into the construction of the BFM and its connection to quorum sensing could provide a model of study to explore biofilm dynamics. Novel antibiotics that perturb the structural foundation of BFM without necessarily destroying its assembly should be considered viable alternatives to current medications for infections. There should be a renewed focus on molecular and chemical pathways within the motor. The ultimate goal should be developing a means of inhibiting chemical receptors in flagella for chemotaxis and making them immobile without halting the construction of flagella. Studies of EP inhibitors or EPIs require further understanding of the structural aspects of EPs such as their epigenetic regulation, substrate profiling and

molecular composition. Understanding the basis of initial biofilm attachment can help prevent biofilm growth on internal stents and implants. This can decrease the frequency of infections that arise from the same. Further research should also take place into the possible role of flagellar proteins in translocation and horizontal gene transfer within biofilms. Drugs that can permeate the biofilm matrix could function as treatment by making the biofilm unfavourable for translocation. The area of carrier molecules, molecules which aid in signalling between the levels of the biofilm, connected to the motility of cells should be explored as an alternative novel treatment for biofilm eradication.

On an evolutionary scale, it becomes clear that efflux and flagellar motility are functions that have been conserved over several species [28]. Contextualising their relations to bacterial virulence and the mechanisms behind the same could unveil novel regulation pathways and unveil new study paradigms for further analysis. By studying the correlations between these integral aspects of the bacterial life cycle, we may extrapolate other key theories that allow us to develop novel antibiotics to bypass the threat of antibiotic resistance.

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Medicinal applications of the hemostatic & anti-inflammatory potential of plant latex proteases from *Euphorbiaceae* & *Moraceae*

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The literature review examines the medicinal applications of plant latex proteases from the *Euphorbiaceae* and *Moraceae* families, specifically in the context of their hemostatic and anti-inflammatory potential for the treatment of acute wounds. The review delves into the existing scientific literature to provide a thorough analysis of the properties and activities exhibited by certain plant species within these families, both through in-vivo and in-vitro studies. Additionally, the extraction, processing, and delivery techniques involved in obtaining plant latex are briefly examined. By highlighting the current knowledge and advancements in this field, the review aims to shed light on the valuable therapeutic properties of plant latex proteases and their potential as alternative treatments for acute wounds. Furthermore, this study emphasizes the necessity for future research to further explore and optimize the application of these proteases in the avenue of wound healing.

1 Introduction

Historically, plants have played a major role in the development of traditional Asian medicine with the oldest written evidence of medicinal plants' usage for preparation of drugs dating as far back as 5000 years [1]. Medicinal plants continue to be a major source of vital organic compounds which have also enabled several developments in modern medicine and pharmacology.

Amongst the many components of a plant which have been explored for drug development, plant latex has been identified as one which has significantly high medicinal potential. Latex is a complex, milky emulsion of alkaloids, cardenolides, phenolics, starch, furanocoumarins, sugars, and amino acids which is produced and stored in the laciferous system of certain plant species (Apocynaceae, Lactuceae, Asteraceae, Euphorbiaceae, Moraceae, etc) [2].

Plant latices naturally serve a variety of purposes such as mechanical and biochemical defence against pathogens & predators. Today, plant latices are also processed for various industrial applications. The Brazilian rubber tree (Hevea brasiliensis) latex is used for the generation of nearly 90% of the world's non-synthetic rubber and is the only natural, commercial source of rubber [3]. Important narcotic analgesics like Codeine & Morphine are also retrieved from the plant latex of Papaver somniferum, commonly known as Opium Poppy [4]. However, in the past few decades, wound healing capabilities of plant latex have been demonstrated which drastically increases its range of applications in medicine [5]. Plant latices contain proteases which can aid at various stages of wound healing such as haemostasis & inflammation. This specifically suggests the potential implications of plant latex proteases on acute wound healing.

Acute wounds generally refer to any trauma caused to bodily tissues that heal in an orderly manner. These include surgical wounds (excision, incision), burn- wounds, ulcers, etc. Furthermore, acute wounds heal in a coherent manner, where the process is divided into 4 distinct yet typically overlapping stages: Haemostasis, Inflammation, Proliferation, and Remodelling [6].

Haemostasis takes place instantly after any physical injury as tissue breakage results in loss of blood from the wound. This exposes subendothelial collagen (Type-1) to platelet cells, allowing for activation. The endothelial cells also secrete the Von Willebrand factor into the bloodstream, an adhesive plasma glycoprotein, with a crucial role in primary haemostasis, which binds to fibrillar collagen and the Gp1b-9 receptors on the platelet to mediate platelet adhesion. The activated platelets undergo degranulation, releasing multiple growth factors, clotting factors, fibrinogen, serotonin, ADP, thromboxane A2, and calcium ions into the extracellular matrix. The process is accelerated by PAF (platelet activation factor) and helps in the aggregation of activated platelets to form a hemostatic platelet plug which halts bleeding. Following the formation of a temporary plug, an enzyme regulated coagulation cascade occurs, which is important for secondary haemostasis. The endothelial collagen exposure and the thromboplastin from injured tissues, trigger intrinsic & extrinsic pathways responsible for the formation of prothrombin activator. The prothrombin activator proteolytically cleaves prothrombin to thrombin in the presence of calcium ions. As the chain reaction proceeds, thrombin breaks down the polypeptide bond of inactive fibrinogen to produce 2 fibrin monomers, which are used as the constituent material for the thread-based meshwork of the stable blood clot. Haemostasis is not finished at this point, because plasmin is later formed to help in clot retraction via fibrinolysis. [7–10]

Following the coagulation cascade, the inflammatory stage occurs as blood clots trap leukocytes in the fibrin meshwork. Monocytes, macrophages, neutrophils, and other leukocytes rush to the site of injury to kill any possi-

ble infection. Along with that, mastocytes release enzyme filled granules, alongside active amines like histamine and others, that cause the characteristic signs of inflammation. The vascular permeability (caused by the inflammation) along with high levels of chemotactic substances and cytokines stimulates neutrophil movement that helps in the phagocytosis of pathogens and debris.

The proliferation and remodelling phases include the gradual replacement of the temporary fibrin mesh with collagen fibres, proteoglycans, and fibronectin by fibroblasts. These then migrate to the wound in response to the initial platelet cell signalling. The wound site also undergoes angiogenesis as new capillaries form to restore blood circulation.

During the final stage of healing, the wound matures into scar tissue and capillaries gradually combine to form larger blood vessels. Though proliferation and remodelling are vital for wound healing, the stages are not directly relevant to plant latex proteases as those are not proteolytically regulated processes.

Plant latex proteases from *Apocynaceae* have been extensively studied whereas, other families such as *Euphorbiaceae* & *Moraceae* have been less explored in this regard. Novel plant-latex proteases in species belonging to these families may hold immense potential in changing how pharmacological treatments currently work, in regard to wound-healing and other forms of skin trauma. Hence, this review aims to explore latex proteases from *Euphorbiaceae* & *Moraceae* that exhibit haemostatic & anti-inflammatory properties, with a view to acquaint ourselves of what can be done to investigate and develop these avenues further.

2 Wound healing activity of *Euphorbiaceae* & *Moraceae*

2.1 *Euphorbiaceae*

2.1.1 *Synadenium grantii*

Plant latex proteases from *Synadenium grantii* latex have proven to be highly effective for primary & secondary haemostasis. Though there is no evidence of anti-inflammatory activity, the *Synadenium grantii* latex proteases display significant advantages for secondary & tertiary haemostasis. Latex proteases from *Synadenium grantii* have shown to reduce clotting time to 20 seconds at a concentration of 40 µg/300ml. This was relative to the $\approx$ 55 second and $\approx$ 80 second of clotting time of *Apocynaceae* species: *Calotropis gigantea* & *Wrightia tinctoria*. Furthermore, the haemorrhagic activity of *Synadenium grantii* was also evaluated relative to latex proteases from the *Apocynaceae* species. Though the *Calotropis gigantea* latex extract induced haemorrhage when injected into the dorsal surface of anesthetized mice, *Synadenium grantii* displayed no such activity, indicating the safe nature of its latex. Along with this, *Synadenium grantii* also showed considerable clot hydrolysis activity which suggests plasmin like nature of serine proteases. At 100 µg concentration in Tris-HCL buffer, SGL displayed $\approx$ 4 Units/h activity for blood clot hydrolysis. This level of activity is moderate and confirms the potential of SGL for coagulation and wound contraction. [11–13]

Species	Protease Type(s)	In-vivo & In-vitro methods
Synadenium grantii	Serine	Whole blood clotting time, Plasma clotting time, Bleeding time, Human fibrinogenolytic activity, Fibrin clot hydrolysing activity.
Euphorbia tithymaloides	N/A	Whole blood clotting time, Bleeding time.
Euphorbia nivulia	Cysteine	Excision wound healing model, Coagulation time of whole blood, Bleeding/Clotting time test, Antimicrobial activity.
Euphorbia caducifolia	N/A	Clotting of platelet free plasma, Angiogenesis in chick chorioallantoic membrane, Excision wound healing model, Incision wound healing model, Hydroxyproline estimation, DNA estimation.
Euphorbia antiquorum	Serine	Platelet aggregation, PT, APTT, & Von-Clauss assays, Plasma clotting time, Excision wound healing model.

Table 1: Overview of *Euphorbiaceae* species

2.1.2 *Euphorbia nivulia*

Crude Cysteine Proteases (CCP) from *Euphorbia nivulia* latex displayed a large reduction in bleeding time of mice wounds from 57-60 seconds (Control) to 8-14 seconds. Along with this, CCPs also showed a notable increase of percentage of wound contraction until 12 post-wounding days, which indicates accelerated wound healing in the short term. The crude proteases also displayed antimicrobial activity as the zone of inhibition for multiple bacterial species was comparable to Gentamicin, a broad-spectrum antibiotic. CCP at 100 µg/ml concentration, could form a zone of inhibition of 12.59 ± 0.57 mm for *Pseudomonas aeruginosa* and 15.28 ± 2.45 mm for *Staphylococcus aureus*. Though CCPs do not demonstrate the same degree of antimicrobial activity as conventional antibiotics, CCPs could have huge implications in wound treatment because of their nature [14].

2.1.3 *Euphorbia tithymaloides*

Euphorbia tithymaloides displayed high procoagulant activity, relative to *Synadenium grantii* & *Euphorbia nivulia*. This was evident as the whole blood clotting time of multiple mammal species was significantly reduced when introduced to latex. At the peak level of activity, *Euphorbia tithymaloides* latex reduced whole blood clotting of *Ovibos moschatus* by 97%. However, the same degree of blood clotting was not evident for human blood samples. Upon comparative analysis with other *Euphorbia* species, it is evident that *Euphorbia tithymaloides* has one of the lowest bleeding times which directly correlates to haemostatic activity [11].

Extract	**Protein content (µg)**	**Bleeding time (seconds)**
Euphorbia tithymaloides	100	14.68 ± 0.22
Euphorbia nivulia	100	26.83 ± 1.16
Synadenium grantii	100	30.38 ± 1.64
Saline	-	59.28 ± 0.58
Control	-	57.17 ± 0.98

Table 2: Comparative Analysis: Effect of latex proteases from *Synadenium grantii*, *Euphorbia nivulia* & *Euphorbia tithymaloides* on bleeding time of mice

2.1.4 *Euphorbia caducifolia*

Euphorbia caducifolia latex (ECL) demonstrated a statistically significant decrease in clotting of time of platelet free plasma, as 30, 40 and 50 mg/ml concentrations respectively took 90, 82 and 68% time for clotting, relative to the control. This indicates the presence of fibrinogen-cleaving proteases that can aid in secondary haemostasis. ECL also demonstrated a notable increase in the area of angiogenesis of chick chorioallantoic membranes as 2.5, 5.0 and 10 mg/ml led to a 56.77, 74.48 and 78.09% increase, respectively. ECL at the lowest experimental concentration showed a slight increase in hydroxyproline content, which is a vital component of collagen fibres and crucial for the proliferation stage. However, compared to Silver (10mg/g of excised wound), a topical antibiotic ointment, ECL does not suggest significant advances in advancing wound healing activity. At equal concentrations of ECL and Silver sulfadiazine, the latter demonstrated a 100% excised wound contraction in 12 days, compared to 14 days with ECL. In addition to that, silver sulfadiazine exhibited a 15% increase in skin tensile strength, relative to ECL. Therefore, through pharmacological evidence, it can be indicated that *Euphorbia caducifolia* latex has considerable implications for haemostasis and is a contributor to pro-healing activity. [15]

2.1.5 *Euphorbia antiquorum*

Antiquorin is the thrombin-like protease from *Euphorbia antiquorum* latex which displays significant haemostatic activity. Antiquorin induces platelet aggregation which has been evidently shown through the quantification of TXA2 levels. TXA2 is a thromboxane produced during platelet activation and serves an indicator of procoagulant activity. At 50µg concentration, antiquorin reported a TXA2 of nearly 150000 pg/ml which is approximately equal to the levels induced by 200ng thrombin. Furthermore, antiquorin also demonstrated significantly high levels of clotting activity as it reduced fibrinogen clotting time by 99.8% when evaluated at 10µg concentration relative to a saline control. Along with this, antiquorin displayed significant progress in excision wound healing as wound size contracted to <25 mm2 at post-wounding day 9. This was supported as evidence of cellular signalling was observed when antiquorin showed greater p38 activation than thrombin. Euphorbia antiquorum latex has high potential for haemostatic activity and use, which is well substantiated from empirical data. [16]

2.2 *Moraceae*

2.2.1 *Ficus carica*

Ficin is the primary latex protease of *Ficus carica*. Ficin has evidently displayed the activation of human factor X, as it was evaluated by examining the effect of ficin on the generation of FXa in typical, defibrinated plasma and FX-deficient plasma. Ficin helped in producing 43 nmol/l FXa in 6 minutes, which is approximately 64% of the available FX. However, further research has also suggested that *Ficus carica* latex is a mixture of procoagulant and anticoagulant chemical when contradictory action was discovered by serine proteases. From empirical data, it can be observed

that *Ficus carica* latex proteins induces procoagulant activity at low protein concentrations and vice versa at high protein concentrations. However, it is not entirely known how this dual property of *Ficus carica* latex occurs. It is currently speculated that the presence of protease inhibitors or the gradual decrease in coagulation due to the complete digestion of fibrinogen may be responsible for this phenomenon. However, although dual-contradictory properties are not extremely uncommon in plants, the observance of this could possibly also occur due to errors during enzyme purification or assay preparation. [17, 18]

2.2.2 *Ficus drupacea*

Drupin is a latex protease in *Ficus drupacea*, that has greater implications in tertiary haemostasis & tissue proliferation than primary haemostasis. It has potent fibrinolytic properties and displays various properties which indicate wound healing effect. Drupin down-modulates levels of MMP-9 an antagonistic wound healing enzyme that prolongs the deposition of collagen. Furthermore, drupin demonstrated the highest arginase-1 activity, peaking at greater than 1.5 units at 3 post-wounding days, when studied in comparison to papain, Neosporin, saline, and untreated skin control. Arginase-1 is an important macrophage marker and has crucial anti-inflammatory roles which also participates in the production of proline, a precursor of collagen. However, level of arginase-1 activity drastically reduced between post wounding day 3 & post wounding day 6. At post wounding day 9, drupin exhibited the greatest excised wound contraction in mice. This latex protease has the potential to be instrumental in wound healing and is highly effective for midterm treatment of wounds [19].

Species	**Protease Type(s)**	**In-vivo & In-vitro methods**
Ficus carica	Cysteine, Serine	PT & APTT assays, Measurement of factor Xa (FXa) activity, Fibrinogenolytic activity.
Ficus drupacea	Cysteine	Plasma clot hydrolysing activity, Excision wound healing model, Arginase activity, Mouse peritoneal macrophage culture, In vitro scratch cell migration assay.
Artocarpus altilis	Cysteine	Plasma clotting time, Fibrinogen agarose plate assay, Blood clot lysis assay.
Macular spinosa	Serine	Fibrinogenolytic activity, Excision wound healing model, Hydroxyproline estimation.
Jatropha curcas	N/A	PT & APTT, assays, Blood clotting time, Histopathological and immunohistochemical expression model through wound healing.

Table 3: Overview of *Moraceae* species

2.2.3 *Artocarpus altilis*

Artocarpus altilis is another plant latex source with an abundance of proteases that can aid in wound healing. Latex proteases from *Artocarpus altilis* display considerable wound healing potential. At 100µg/300ml concentration, latex proteases reduced clotting time from 190 seconds to 71 seconds. This indicates statistically significant thrombin-like activity. Furthermore, the procoagulant potential was also observed with a fibrinogen agarose plate assay. 10µg of Artocarpus altilis latex protease formed a zone of precipitation spanning a diameter of 0.7 cm. This is comparable to thrombin, which formed a zone of precipitation spanning a diameter of 0.9 cm. Along with this, latex proteases also demonstrated significant fibrinolytic activity as 100µg concentration of protein led to a 70.24 ± 1.52% clot lysis. This suggests a dual nature in *Artocarpus altilis* latex which is similar to the observations in Ficus carica. The same observation is also visible for species from other plant families like *Tabernaemontana divaricata*. *Artocarpus altilis* shows considerable procoagulant potential which is accompanied by high fibrinolytic activity [20].

2.2.4 *Maclura spinosa*

Maclura spinosa is a plant species which has widely been speculated to contain haemostatically potent proteases in its latex. The haemostatic potential is mainly due to the fibrinogen-cleaving activity of crude-enzyme extracts. A dose-dependent fibrinogen-cleaving assay showed that

10µg of crude-enzyme extracts of *Maclura spinosa* resulted in a 0% band- density when analysed by 10% SDS-polyacrylamide gel electrophoresis, suggesting high levels of fibrinogen cleavage. Furthermore, *Maclura spinosa* latex proteases have been proven to show 75% excision-wound closure at 12 post-wounding days. At the same point of time, the hydroxyproline content of the excision wounds was also estimated to be 2100µg/mg tissue. As mentioned earlier, hydroxyproline is vital for tissue proliferation and its increased presence relative to the control suggests that the wound healing potential of *Maclura spinosa* expands beyond haemostasis. [21, 22]

2.2.5 *Jatropha curcas*

Jatropha curcas latex has shown potency for contribution towards all stages on wound healing. Like a few of the other plant species which belong to *Moraceae*, *Jatropha curcas* latex has a dual, procoagulant & anticoagulant nature. This is evident as the anticoagulant activity occurs at low crude enzyme concentrations & procoagulant activity at relatively high crude enzyme concentrations. At 1 in 8 dilutions of plant latex, there was a visibly prolonged blood clotting time. This trend further continued as dilutions were made, until lower concentrations begun displaying anticoagulant activity. Aside from coagulation, *Jatropha curcas* also has high anti-inflammatory potential. Immunohistochemical examination shows that 15% latex-cream of *Jatropha curcas* can increase the expression of CD68. CD68 is an important transmembrane glycoprotein which serves as a biomarker for macrophages. The increased expression of CD68 can catalyse the inflammation stage and the overall wound healing process [23-25]. Furthermore, it has also been suggested via empirical evidence that *Jatropha curcas* latex can contribute to tissue proliferation & remodelling by aiding in angiogenesis, showing the wide range of possible applications.

3 Plant Latex

Though, multiple plant species from *Euphorbiaceae* & *Moraceae* demonstrate potential for acute wound healing, it is crucial to understand the processes behind the extraction and purification of plant latex proteases along with the safety concerns which lie around its usage.

3.1 Extraction and Purification

Plant latex proteases were extracted & processed in a multitude of ways; however, most research studies followed the same principle. The first step of the process is purification of crude enzymes from the freshly collected plant latex which can obtained by injuring tender parts of the plant or excising the petioles, leaves, or stems. The fresh plant latex, depending on its viscosity, is typically diluted with a phosphate/Tris-HCL buffer of a relatively neutral 7.0–7.6 pH level as extreme pH levels can denature proteases. The diluted plant latex then undergoes a repeated freeze and thaw treatment and is then centrifuged at 4ºC. After centrifugation, the resulting supernatant may be further dialysed against a buffer forming a clear solution which is the crude enzyme source. However, there is no conventional method for latex purification, and many other methodologies exist to achieve the same outcome. Freshly collected plant latex can also be filtered through a muslin cloth prior to dilution & centrifugation, allowing for the filtration of large particles Furthermore, dialysis of the supernatant helps in the removal of metallic ions such as Ca2+, Mg2+ and other heavy ions which exert influence over enzymatic reactions & generally serve as enzyme inhibitors.

3.2 Delivery Mechanisms

The next stage in the process, is latex protease formulation and finding the appropriate delivery mechanism. Most research methodologies utilize an excision wound healing model; however, such in-vivo studies usually result in the death of mice. The process begins as mice are sacrificed using high dosages of anaesthesia. The dorsal skin of most mice is removed to form a wound of typically 1cm diameter. The injury initiates the coagulation process which can be catalysed using crude enzymes from plant latex. These crude enzymes are delivered using an injection taken prior to anaesthesia or applied directly to the wound site. This is not feasible for use in a human setting and raises multiple ethical concerns.

Furthermore, there is a more feasible way for delivery of plant latex proteases which is the use of ointment bases. The topical application of ointment bases can greatly influence the potential & accessibility of the plant latex proteases. Ointment bases can further dilute the crude enzyme extract, reducing the risk of protein allergy from latices. One of the most commonly found gel bases, which can be effective for the use for crude enzymes is petroleum jelly. Petroleum jelly is a chemically inert compound and is therefore ideal in this scenario. Another promising delivery mechanism is the formulation of a plant latex spray. Within an in-vitro study [26], 50% plant latex extract (with butanol) was dissolved in ethanol & acetone as a co-solvent. Furthermore, a lubricating was also mixed, and the mixture was stirred until the formation of a translucent solution. A hydrofluoroalkane propellant was also added to solution, which was then sealed in a canister, making a functional spray which retained the pH, weight, colour, and homogeneity of the latex formulation. Though these methods show a lot of prospects in research studies, much greater clinical research is required to find the appropriate delivery mechanism of plant latex proteases.

3.3 Bio-safety Concerns

Latex allergy is a widespread medical issue, and most cases occur due to exposure to rubber latex or natural rubber-based products [27]. In contrast to rubber, the processing of plant latex proteases for wound healing purposes is very different. The latex extract is diluted and purified multiple times, prior to use. Furthermore, multiple plant species from *Euphorbiaceae* & *Moraceae* have demonstrated some degree of anti- inflammatory activity, which can potentially contribute to dampening any possible allergic response.

Currently, only few allergenic proteins from Hevea latex extracts have been identified to trigger IgE antibody mediated immune responses. However, appropriate clinical research is required to advance understanding about the potential hazards of using plant latices for wound treatment.

4 Conclusion

Although plant latex proteases demonstrate the ability to completely change the way acute wounds are treated, further research on delivery mechanisms & latex allergies is required, as mentioned before. This raises the need of phytochemical characterization which needs to be undertaken for *Euphorbia* & *Moraceae* plant species. Furthermore, protease characterisation is also needed for certain plant species, including *Euphorbia tithymaloides*, *Euphorbia caducifolia*, & *Jatropha curcas*.

In addition to the need of phytochemical characterization, the investigation of the effect plant latex proteases on acute wound healing can be expanded to other plant families. For example, plant species like *Manilkara zapota* of *Sapotaceae* have been widely used in traditional medicine for the treatment of wounds and its haemostatic activity has been demonstrated in scientific literature [28]. The investigation can also be expanded to different parts of a plant like the roots, leaves, flower, fruit & seeds which yield alcoholic/acetone extracts that could contribute to wound contraction & anti-inflammatory activity. Though this greatly differs from latex proteases, it shows implications for wound healing.

There is another area of exploration within this topic which is likely to influence future research. Plant species such as *Euphorbia palusrtis* typically undergo significant metabolic changes during fungal infections as the composition of *Euphorbia palusrtis* latex changes and exhibits stronger antifungal activity than an uninfected plant of the same species [2]. Although the change in antifungal activity has not been directly attributed to latex proteases, the idea can form the basis for a new type of research. Similarly, infections can be induced in plants prior to latex extraction, which can therefore produce an extract that has greater anti-inflammatory activity.

There are currently no direct clinical applications of plant-latex proteases in facilitating wound-care, however, formulations of these could be utilised in multiple scenarios. These include the use of plant-latex proteases as topical applications involved in the care of multiple-wound types such as burns, boils, cracks, cuts, blisters, excisions, also extending to ulcers, warts, and scabies [29]. Currently, there have been research studies of latex-cream formulations of certain species such as *Jatropha gaumeri* which evaluate the wound-healing action in a pre-clinical model. The results of such studies show promising activity by PLPs which are more effective than the typical positive control – a commercial skincare ointment [30].

However, there are still many other stages of testing and development which need to be conducted, including clinical human trials, before plant latex proteases can find a safe form of use in western medicine, and gain relevancy in both medical realms. Regardless, plant latex proteases remain an untapped source of novel enzymes which hold promising potential when it comes to the pharmacological treatment of wounds in the future.

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Shooting in the Dark: Our Search for the Last Common Ancestor

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The origins of humanity have always been shrouded in mystery. Our past has been up for speculation since time immemorial, with theological philosophers and naturalists preaching divine creation, and others postulating that we have always been here. Today, we know that the key to unravelling the story of our beginning lies in untangling the evolutionary relationships in the group known as the 'great apes'. In the 21st century, with the advent of genetics, we have made enormous progress in our pursuit of this goal.

For millennia, naturalists have proposed that there lies a connection between humans and apes. In pre-modern times, despite the obvious similarities between humans and apes, the influence of the Catholic Church stifled any serious questioning of our relationship. Centuries' worth of work on anatomical similarities was shelved away as being antithetical to Biblical ideas of creation which asserted man's spiritual and thereby biological superiority. Taxonomical classifications placing humans and apes in a similar group were heavily criticised for diminishing the position of man in the hierarchy of creation. However, things changed drastically in the 19th century when a man named Charles Darwin shook up the biological world with his theory of natural selection. Species were no longer set in stone – they could be born and die; and could evolve and transmute from one to the other. The origins of humanity were suddenly opened for review, and legions of theories would follow.

The theory of evolution maintains that every species is related, and that all life on Earth shares common ancestry. Under this scheme, the same applies to humans. As naturalists have earlier observed, we share anatomical features with many of the great apes, clearly indicating some sort of relation. How, then, do we then find our closest relative among them? The answer is written within us, in the code that makes us who we are, biologically speaking – the genome. Aside from containing the instruction manual for life, the genome of an organism also possesses clues about our origin. Mutations, the drivers of evolution, accumulate over time in the genome. In order to be inherited from one generation to the next, the genome is copied, and as more time passes, the more likely it is that a mistake will be made in copying. These mistakes are also passed down the generations, and hence over evolutionary time, differences accumulate in a genome. By comparing the number of differences in the genome of two species, one could estimate their degree of relatedness. The fewer the differences, the closer any two species are, and by inference the lesser time has passed since the separation of the two species. So, which ape shows the least differences to us? The answer: chimpanzees [1]

The chimpanzee lineage (Pan), along with the human lineage (Homo), together constitute the group Hominini, or the hominins [2]. What does that tell us? It means that chimpanzees, along with bonobos, are our closest living relatives, with our lineages separating from a common ancestor. Ever since this relationship was proposed (initially with morphological analyses, with later DNA analysis further confirming the hunch), a question has emerged: who was the Last Common Ancestor (LCA)? This animal would have lived millions of years in the past, and over evolutionary time its descendants went on to form the two lineages that we know today. Unmasking the identity of this organism could be thought of as the holy grail of palaeoanthropology. However, it remains as elusive as ever; despite the numerous breakthroughs in the fields of palaeontology, palaeoecology and genetics. How do we find this ancestor?

Several methods have been adopted in the past, with varying degrees of success. The most effective method to gather information about the LCA is to collect physical evidence of it. Fossils remain the primary source of such evidence, and provide a source of direct material from the LCA from which we can glean many textbooks worth of knowledge. A fossil isn't just an old bone, but a node at the confluence of ecological, morphological and sexual pressures at a specific moment in time; all of which co-occurred to create its form. The information contained in a single mineralised bone is enormous. Without fossils, our ancestor remains a ghost: a dark silhouette we find at the base of our evolutionary tree. Just a single fossil would illuminate a vast region of that tree. So where have we reached in our search?

In 1856, some odd fossils were found in a quarry in Germany. These became the first fossils to be recognised as belonging to another species of human. This species was given the name of Homo neanderthalensis, commonly known today as the Neanderthals [3]. The religious uproar against this classification held up Neanderthal research for decades. We have come a long way from those cautious, unsteady initial days. Today we hold in our repositories the knowledge of numerous extinct human species: from often studied classical species like the Neanderthals and Homo erectus, to newer discoveries reshaping our view like Homo naledi and the Denisovans, to curious offshoots like Homo floresiensis and Homo antecessor. These species evolved concurrently, coexisted, and even interbred in some cases where their populations overlapped; and made sure that biologists would have a very difficult time putting the full puzzle together. Beyond all the messiness of the last few million years however, we see distinct clues of our simian origins.

For this, we have to travel back over 3 million years to

what we now call the Afar region of Ethiopia. What we know today as a dry, arid desert was once a wooded grassland with occasional floodplains [4] [5]. It was an open field, dominated by tall grasses, and a few trees dotting the horizon. It is here that we meet our first, and perhaps our most well-known ancestor: Australopithecus afarensis [6] . It stands at 4 feet tall [7], with long arms by its side, providing balance. Analysis of its lower limbs and pelvis has revealed significant weight bearing ability, hinting at a bipedal organism [8]. Moreover, we have preserved footprints from A. afarensis which show an animal walking on two feet [9].

This bipedal habit isn't entirely surprising for a human ancestor. However, Australopithecus shows some other puzzling traits. Despite the relatively straightforward analysis of the lower body of A. afarensis, the upper body suggests something completely different. The upper limbs, along with the face and jawbone, resemble other great apes. The jawbone is reminiscent of that of gorillas, while the limbs look like they would be adapted to an arboreal environment, similar to those of some apes today [10]. This is especially telling, seeing as there were not many trees in the environment it inhabited. Why would an animal need tree climbing adaptations in a relatively treeless environment?

One hypothesis suggests that these long, powerful arms are the remnants of a much older ancestor – the LCA [11]. In the absence of major selective pressures, natural selection may leave a trait unchanged. Perhaps tree climbing adaptations in earlier ancestors were not enough of an evolutionary cost to be selected against. Further, it is possible that the few trees that persisted in Australopithecus's environment were enough to justify their retention once the environment changed to a more open habitat. Maybe the change in environment from forest to grassland was a relatively sudden one, which did not allow selection enough time to act. Whatever the reason, if proven true, this would mean that the LCA was a tree dwelling animal which inhabited a forested environment. We are able to make hypotheses about the LCA's habitat, just by examining fossils millions of years after its time. However, this theory is highly contested, with no accepted resolution so far. But why try to make extrapolations from A. afarensis, which lived millions of years later, when we are interested in the LCA itself? Why not directly study the LCA's environment? In order to gather clues about the LCA's habitat, we first need to determine the time period during which the LCA lived.

It is difficult to date the LCA using the fossils that are currently available. A lack of convincing material leaves us unsure about the age of the divergence between the LCA and the oldest human ancestor. Could genetics be of help here? As already discussed, the fewer the differences in the genome of two species, the more closely related they are. By taking the number of differences, along with the mutation rate (the rate at which mutations occur in the genome), one can estimate the age of the split between the two. Some assumptions regarding the mutation rate and generation times are used in these calculations, which makes for a slightly uncertain figure. Using this technique, most genetic studies settle on a number between 6 and 12 million years ago (mya) for the time period of the LCA's existence, with the range between 7 and 10 mya being likely [12] [13]. Now that we have a rough estimate for the age of the LCA, we can now ask what environments were present in the LCA's geographic location during this time.

Unfortunately, our inability to narrow down to the exact location of the LCA makes this question difficult to answer. One theory is that the LCA lived in Africa, and more specifically, in Eastern Africa. Palaeoecological studies of this time indicate that Eastern Africa was dominated by open woodlands, with patches of forests [14]. Analysis of carbon isotopes from sediment soil samples of some flood plains from this time show signs of wooded grasslands, with varying vegetation. Does this contradict our earlier hypothesis of the LCA being arboreal? Not exactly. Grasslands may differ in tree density. The Cerrado, a wooded savanna in Brazil today, approaches forests in terms of density [15]. Moreover, there are numerous examples even today of transitional areas between tropical forests and open grasslands, such as The Victoria Basin forest – grassland mosaic. The interpretations from fossil evidence and palaeoecological studies would both hold true if the LCA inhabited one of these transitional landscapes.

Another alternative is that the LCA did not originate in Africa at all. A 7-million-year-old fossil found in Southeast Europe, called Graecopithecus, was claimed to be a hominin; which meant that it was extremely close to the LCA in evolutionary time [16] (Fuss et al., 2017). This finding would make it likely that the LCA was of European origin. However, the specimen's classification as a hominin is highly controversial, with too little material found to reach a definite conclusion. For what it is worth, Southern Europe was dominated by temperate woodland forests at the time [14]. These forests were evergreen and would have provided ample cover. Despite this, Africa is the likelier option, due to the fact that most, if not all human and chimpanzee ancestors, have been found in the vicinity of Eastern Africa. What other sources of evidence can we use to gather information about the LCA?

A very popular early method was to observe chimpanzee behaviour in order to infer possible behavioural traits of the LCA. Chimpanzees are often used as 'proxies' for the LCA, and social and behavioural characteristics are then extrapolated from chimpanzee studies. This approach assumes the LCA to be a primitive chimpanzee, and bases its evaluation of the LCA's traits on what chimpanzees do in similar environments. For example, some compare the LCA to a savannah chimpanzee, i.e., modern day groups of chimpanzees that live on savannahs [17]. Studies in chimpanzees have also been used to infer the origins of certain human traits. The intergroup relations of these modern-day chimpanzee groups are used to speculate upon the origins of violence in humans. Tool use, hunting behaviours, division of labour, and other social and cultural practices, are also hypothesised to have originated due to the occurrence of a chimpanzee-like ancestor in a savannah environment. This approach is tempting due to the simple and satisfying narrative it provides us with. However, it is deeply flawed.

To understand the problem with this method, we have to examine the assumptions the theory makes. The first, and

most important assumption is that the LCA was physically similar to chimpanzees, and that chimpanzees have not undergone much change morphologically as compared to the LCA. This is categorically wrong. The chimpanzee line has been around for as long as ours and hence, there is no reason to believe that it was not subject to enough selection to change it markedly – in fact, as drastically as humans. Genetic evidence actually suggests that chimpanzee genes have undergone more selection than humans [18]. Further, the proponents of this theory tend to ignore bonobos as a reference, despite bonobos and chimpanzees both emerging from our LCA. The separation between chimpanzees and bonobos is much more recent [19], and so there is no reason to not consider them. The second assumption this theory makes is that the selective forces shaping the LCA would be similar to the forces at play for chimpanzees. Once again, this is not a valid claim. We do not know enough about the morphological features of the LCA, or of the environment and ecology in which it lived to justify making such an equivalence with chimpanzees. How then can we be sure that the LCA was not just a primitive chimpanzee?

In 1994, a fossilised hominin was found in Ethiopia. This specimen was dated at around 4.5 million years, and was called Ardipithecus ramidus. It likely lived in a wooded environment, with open woodlands and dense patches of forests [20]. The features that this organism displays completely revolutionise our understanding of our origins. For one, Ardipithecus shows characteristics that differ significantly from extant African apes. For example, Ar. ramidus shows a lack of exaggerated canines, which is a feature seen in many apes today [21]. Exaggerated canines are a sign of social aggression and competition, and so the lack of such characters implies reduced sexual dimorphism. The pelvis seems to have both hominin and ape characters, with features suited to both tree climbing and bipedalism; but its thorax and femur are completely different, and suggest an entirely different model of bipedalism [22]. Apes today engage in knuckle walking and vertical climbing, both of which were thought to be present in our ancestors, and were thought to be the origin of bipedalism. However, Ardipithecus does not show signs of either. It could neither climb vertically, nor could it knuckle walk; however, it could probably still walk on two feet for short periods of time.

Moreover, the foot of Ar. ramidus lacks many features that extant African apes use for everyday activities. It has a grasping big toe that would be helpful in tree climbing, and yet it contains features that would aid in bipedalism [23]. The foot is especially curious, as it contains a bone called the Os peroneum [24]. The Os peroneum is completely absent in all great apes except in some humans; but is present in the Old World monkeys, from whom the great apes diverged from around 30 mya [25]. This suggests that our LCA had this bone, and all our great ape cousins evolved to remove this bone independently. This means that the model of bipedalism they all share today could be one they developed separately, and converged upon. This is completely contrary to previous ideas of apes being "primitive" in morphology, and suggests that their features are highly derived from earlier primitive characters. Ardipithecus completely revamps our understanding of the LCA; and firmly provides evidence against a rudimentary, chimpanzee-like ancestor. In fact, the chimpanzee itself wasn't rudimentary anymore, but a specialized animal in its own right.

Thus, our ancestors were not just primitive chimpanzees. Is there anything else we can learn about our LCA from chimpanzees?

Whiten (2011)[26] compares chimpanzee and human cultures to get an idea of the social traits of the LCA. Similar to how shared morphological traits between two closely related species can be used to hypothesise the trait's existence in their shared ancestor, the same may be done with shared social traits. The author makes a list of shared population-wide traits (existence of different traditions, basic ideas that cultures form around, cultural evolution); individual "social learning" skills (copying, teaching, conformity); and socio-cultural content (social customs, dialects). Through this, Whiten is able to sketch a basic idea of what traits our ancestor may have had. This interpretation envisions our ancestor as a social animal, in which the foundations for the evolution of cultural complexity have been laid down. Copying is an especially distinct feature that is well documented in both cultures (and other great ape cultures too), suggesting that it may have played an important role.

One perceived drawback in this research is the lack of attention directed towards bonobo cultures, which the author acknowledges. If bonobos showed a departure from some of these shared characteristics, we would be forced to rethink our idea of what the LCA was like. A possible method to resolve this could be to compare chimpanzee and bonobo cultures first, before then comparing them to human cultures to gain a more comprehensive understanding of the LCA.

So where exactly does current research leave us in our pursuit?

In 1846, a French astronomer called Urbain Le Verrier predicted that an eighth planet was orbiting the Sun, beyond Uranus. He did this not by guesswork, but with math, declaring that the discrepancies between our predictions of Uranus' orbit and real observations could be solved if another planet was gravitationally influencing it. He calculated this hypothetical planet's mass and position using nothing but data from Uranus' orbit, and when astronomers looked at where he pointed, they found a blue dot – what we now call Neptune [27]. We are now at a similar stage regarding the LCA.

We have made predictions about the age, environment, characteristics and even behavioural traits of the LCA, without any direct evidence from the organism itself. However, unlike Le Verrier's planet, there is no guarantee that we will ever find it, however thoroughly we may look. Fossilisation is a chance event, and to find fossils requires a great deal of luck both in terms of the formation of a fossil and its eventual intact discovery. There are vast gaps in the fossil record, and an immense number of species we will never know. Perhaps our LCA never fossilised, and we will never have a complete picture of our origin. Or maybe it is waiting for us out there. All we can do is to keep looking and continue filling in the gaps in our understanding, in the hope that our shots in the dark land on something.

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Investigating the parallels between budding in *Hydra* and limb development in vertebrates

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Budding and limb development are key morphogenetic processes in the development of *Hydra* and vertebrates respectively. While budding serves as the main mode of asexual reproduction, the development of limbs aids in locomotion. Further, these processes also greatly vary in terms of complexity and time scales. Given these differences, it seems somewhat improbable that these processes would have any parallels. However, both processes have striking visual similarities - they start with a protrusion or outgrowth on the body surface, which grows and develops to culminate in a fingered/tentacled structure. This review analyses existing literature to understand whether these similarities are purely physical, or whether they are a result of similarities in the genes and molecules involved. The findings show that there are indeed strong genetic parallels that underlie what is seen physically.

1 Introduction

Budding and limb development are key morphogenetic processes in the development of *Hydra* and vertebrates, respectively. Interestingly, both these processes have strong visual similarities (Fig.1). These similarities suggest that there is some homology in the proteins, genes and pathways governing these processes. This review aims to systematically summarise the various molecules involved in the two processes, thereby teasing out the parallels between them. Here, it is important to note that studies on *Hydra* budding are restricted to a single genus, while in vertebrates, the studies span the entire subphylum, with the main model organisms being Zebrafish (*Danio rerio*), frogs (*Xenopus sp.*), chicks (*Gallus gallus*) and mice (*Mus musculus*).

2 Budding in *Hydra*

The freshwater Cnidarian, *Hydra* (Fig.2), reproduces asexually through budding. The offspring emerges as a bud or protrusion on the body column of the parent, closer to the basal disc. The body axis of the bud develops such that it is perpendicular to that of the parent [1]. This bud grows and develops into an adult *Hydra*, complete with its basal disc and tentacles, after which it detaches from the body column of the parent (Fig.3).

Budding in *Hydra* occurs in 8 stages. Sanyal and colleagues classified and demarcated these stages based on morphological differences in the bud (Fig.4.) [2]. The first stage is the growth and development of the parent *Hydra* itself, where there is no bud formed on the parent. During the second stage, the bud emerges as a small conical protrusion on the body column of the parent. This stage also involves the growth of the protrusion. Stage 3 is characterized by the development of a secondary axis, i.e., the axis of the bud. Following this, the development of the secondary axis is completed in stage 4. Stage 5 corresponds to the beginning of tentacle development, where small out growths are seen around the tip of the bud. In stage 6, the hypostome (mouth) and tentacles are fully formed, with no appreciable development of the basal disc. In stage 7, a constriction begins to develop at the base of the bud, between the bud and parent. Finally, in the last stage, the constriction progresses until the bud is completely separated from the parent [2].

As the bud grows, it begins to descend down the body axis of the parent [3]. When subsequent buds emerge, they occupy regions higher up on the parent's body column, such that they form a spiral arrangement along the parent [4].

Since the bud develops as a protrusion on the parent, it is majorly composed of parental cells. Thereafter, as the bud grows and develops, there are two sources from which it may derive its cells: (a) parental cells that have been integrated into the bud and undergo division to give rise to new cells (b) cells from the parent's body column that continuously get added to the bud. Experiments conducted by Shostak and Kankel (1967) illustrate that the later scenario is more accurate [3]. These experiments used *Hydra* viridis, which is green in colour due to the presence of an endosymbiotic algae, Chlorella sp. They also used white individuals of the same species that were created by exposure to glycerine. They inserted grafts from white individuals into the green ones (and vice versa), and were able to show that as the bud descends along the parent's body column, it grows in size by incorporating pre-existing cells from the parent's body column, rather than by undergoing cell division itself [3].

Apart from the growth and incorporation of cells, another key aspect of budding is the formation of tentacles in the offspring. As the bud begins to evaginate, HYM-301 (a novel peptide) begins to get expressed in what will become the hypostome of the bud. The role of this peptide in tentacle formation was elucidated by Takahashi and colleagues [5]. A key feature of this peptide is that it is expressed only in ectodermal cells, and not the endodermal cells. As the bud grows in size, there is a progressive increase in Hym-301 expression. When the bud reaches a stage when the head is clearly visible, the expression of the peptide is restricted to the head alone.

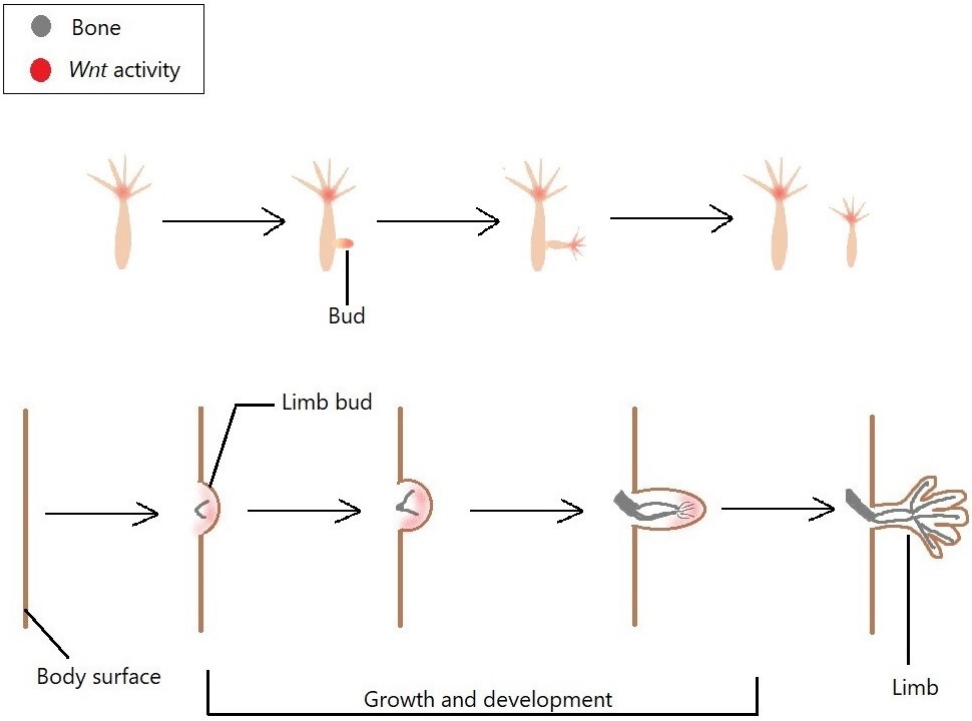

Figure 1: Visual similarities between budding in *Hydra* and limb development in vertebrates. Here, the red colour highlights the activity of the Wnt signalling pathway at the extremity of the bud and the developing limb

Notably, the activity of Hym-301 is determined by the head organiser (which is a key player in defining the body axis of the organism). An important thing to note is that while Hym-301 determines the number of tentacles formed, it does not impact the rate of tentacle formation [5].

Another protein that plays a role in tentacle development in *Hydra* is HyBMP5-8b, which is an orthologue of BMP5-8 (Bone Morphogenetic Protein). Reinhardt et.al. carried out a series of molecular analyses on *Hydra vulgaris* to understand the expression of this protein in tentacle development [6]. Northern blot as well as in situ hybridisation showed a high expression of this protein in the tentacle regions of the hypostome in the adult animals. In developing buds, expression of HyBMP5-8b was not seen until the tentacles began to form. Further, the expression of the protein was seen at the exact locations where the tentacles develop. Here, it is interesting to note that HyBMP5-8b is expressed in the endoderm, as compared to Hym-301 which is expressed in the ectoderm [6]. This shows that the genetic controls on this process show a strong spatio-temporal variation.

The last key protein involved in tentacle formation is the aristaless HyALX. Smith, Gee and Bode studied the expression and role of this protein in *Hydra* development using RNAi, in situ hybridisation and tissue manipulation [7]. They found that both in adult *Hydra* and during budding, the expression of HyALX is similar to that of HYM-301- it is expressed only in the ectoderm, and is expressed even when the bud is just a growing protrusion, rather than only after the tentacles begin to form. Next, when HyALX expression was observed using in situ hybridisation, the size of the region in which it was expressed corresponded to the size of the base of the tentacles, indicating that it had a direct impact on tentacle development. This was further proved using RNAi based experiments against HyALX, where treated organisms showed a significant delay in HyALX expression during budding [7].

While the above-mentioned proteins are indeed essential to budding in *Hydra*, the process also depends on some more common morphogens, which are seen in developmental processes of other organisms too. These morphogens include the canonical and noncanonical Wnt pathways, variations of the Hedgehog protein (Hh), the Notch-signalling pathway, some growth factors and the Hox gene family. The role of these morphogens in the process of budding in *Hydra* is detailed below.

The role of Wnt proteins was determined by Philip et.al. and Braun et.al. When *Hydra* begins to initiate the process of budding, the budding area is demarcated by the activity of two non-canonical Wnt proteins: HyWNT5, and HyWNT88. These proteins also determine the location of tentacles on the head. Therefore, these proteins, in combination with Hym-301 control the formation of tentacles in the *Hydra* [8]. Most importantly, the canonical Wnt pathway is an important component of the head organiser. As the organism grows, a concentration gradient is set up along the body, where the head shows a high concentration/ activity of canonical Wnt proteins while the activity at the basal disc is significantly lower [9]. Specifically, HyWNT3 is responsible for setting up the body axis while HyWNT2 is expressed at the tip of the bud during early stages of budding [9].

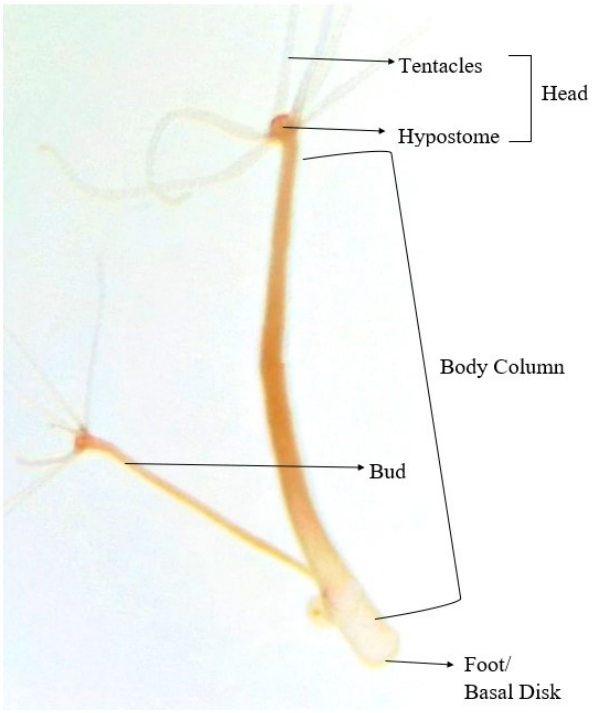

Figure 2: Morphology of *Hydra*

The next key morphogen is the Hedgehog (HH protein. The expression and role of this protein were studied by Kaloulis in 2001. In his study, Kaloulis used *Hydra magnipapillata*, *Hydra viridis* and *Hydra vulgaris* to study the expression of the desired genes using whole mount RNA in situ hybridisation and grafting experiments [10]. In adult *Hydra*, Hh expression was localised to endodermal cells, and was absent in regions where the body was composed primarily of stem cells and precursor cells (for ex. the mid gastric region). In effect, Hh expression was localised to the endoderm of the head and foot. This localisation could be seen as a clear demarcation between regions of expression and non-expression. This was particularly interesting as there is no known cellular or anatomical demarcation between these regions. This indicates that the borders of the Hh transcript domain could control the budding region of the *Hydra*. To probe this further, Kaloulis performed grafting experiments between stage 4 budding *Hydra* and non-budding *Hydra* (hosts). Here, grafts of tissue from the peduncular region (expressing HH) were harvested and transferred to an apical position along the body axis. Grafts from non-expressing tissues acted as a negative control. The grafts were placed in an apical position, to avoid any interactions with the natural budding process of the host. Grafts at the mid gastric level acted as a control. Within 3 days, the *Hydra* with Hh expressing tissue grafts showed the formation of ectopic feet, followed by the development of buds facing the foot within 10 days of grafting. In the case of mid gastric grafts, there was no ectopic budding observed. This further presented a strong case for the role of HH in the establishment of the budding zone as well as in the initiation of budding. To conclusively prove this, Kaloulis performed RNA in situ hybridisation with an HH antisense riboprobe on the grafted *Hydra*. The expression of HH in the grafted *Hydra* corresponded to the formation of ectopic feet as well as ectopic buds, thus establishing the role of Hh in budding zone determination as well as bud initiation [10].

In all animals, the Notch signalling pathway is a transmembrane pathway that plays a key role in determining cell fate [11]. Münder et.al. further ascertained the role of this pathway in *Hydra vulgaris* by inhibiting it using the inhibitor DAPT [12]. *Hydra* were subjected to DAPT treatment for 48 hours, after which they were transferred to fresh medium and observed for 2 more weeks. These experiments were done on fully grown as well as head regenerating *Hydra*. For the head regenerating *Hydra*, the animals were also pre-exposed to DAPT, before being excised. During the initial 48 hours of exposure, the *Hydra* expressed shortened tentacles, followed by the formation of a constriction of at the base of the tentacles. Once they were transferred to fresh medium, they showed further abnormalities in their head structures: branched tentacles, dislocated tentacles, and irregular arrangement. Münder et.al. also noted that Notch activity was important to the expression of HyALX, but it did not have much of an effect on HyWNT3, indicating that Notch was essential to the formation of tentacles but was not essential in setting up the body axis of the bud. Inhibiting Notch using DAPT resulted in abnormal expression of HyALX. The outcomes of this were deformed tentacles and ectopic tentacles. Essentially, in *Hydra*, Notch is required for head pattern formation as well as tentacle development [12].

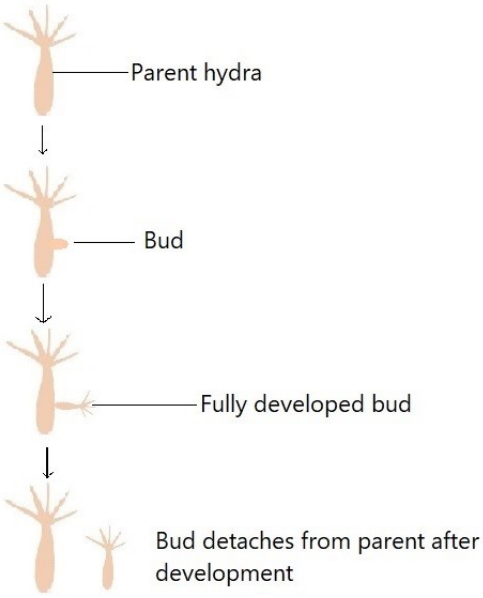

Figure 3: Budding in *Hydra*

Another important group of proteins involved in budding are the growth factors, specifically the vascular endothelial growth factors (VEGF) and the family of fibroblast growth factors (FGF). The role of these proteins in *Hydra* budding was characterised by Surekha and Ghaskadbi in 2013. The duo used techniques like whole mount in situ hybridisation, RT-PCR and exposure to protein inhibitors to study the expression and function of VEGF and FGF in *Hydra magnipapillata* and *Hydra vulgaris* (Ind-Pune) [13]. They found that VEGF were primarily localised to the endoderm of the peduncle. FGFs were observed in both layers of the body column, but were higher in the ectoderm of the budding region. Further, it was found that FGFs were primarily expressed in interstitial stem cells — when the *Hydra* were maintained at a higher temperature than usual, they showed a down-regulation of FGF. This down-regulation corresponded to the loss of interstitial stem cells at this temperature, indicating that FGF was mainly expressed in these cells. To understand the role of these proteins in budding, the inhibitor SU5416 was used against them. When non-budding *Hydra* were cultured with the inhibitor, there were no significant morphological changes. However, when budding adults were exposed to the inhibitor, there was a dosage-dependent delay in bud growth and elongation, indicating that VEGF were involved in bud development. Another interesting finding was the interplay between VEGF/FGF and Wnt —it was found that activation of the canonical Wnt pathway led to a significant decrease in the expression of VEGF/FGF. This was confirmed by subjecting the *Hydra* to Alsterpaullone, which is known to highly upregulate the Wnt pathway. Using RT-PCR, Surekha and Ghaskadbi were able to show that as Wnt expression increased, VEGF/FGF expression decreased [13].

The last set of key morphogens in *Hydra* budding is the Hox and Hox-like gene cluster. In 2001, using PCR, Bode was successfully able to isolate 5 homeobox or hox genes [14]. Two of the main ones were CNOX-3 and CNOX-2. Whole mount in situ hybridisation was performed to track the expression of these genes. The results showed that CNOX-3 was localised to the ectodermal epithelium of the head region, specifically around the tentacle zone, thereby implying that it is somehow involved in pattern formation. Further, since the expression of CNOX-3 in the head region is quite consistent, Bode inferred that there is no gradient involved (with respect to CNOX-3) in the initiation of tentacle formation. On the other hand, CNOX-2 was expressed in both the ectoderm and endoderm of the body column, and it was virtually absent from the head region. Further, CNOX-2 expression dropped during head formation in both budding and regeneration. During budding, as the bud evaginates and begins to form its head and tentacles, CNOX-2, which was initially expressed uniformly, begins to disappear from the distal end and becomes more concentrated towards the lower 2/3 of the bud [14].

The process of budding in *Hydra* ends with the detachment of a fully developed bud from the parent *Hydra*.

3 Limb development in vertebrates

Limb development in vertebrates is initiated by the demarcation of the limb field through the action of hox genes, FGF genes and the SHH pathway, followed by the proliferation of mesenchymal stem cells in the region, which leads to the formation of a protrusion [15]. These mesenchymal cells are derived from the lateral plate mesoderm. The position of the growing limb bud is specified by the expression of Hox genes with respect to the anterior-posterior axis. Specifically, the limb buds are formed at the anterior-most region along the thoracic vertebrae, where HOXc6 is expressed [15]. This is in contrast to the process of budding in *Hydra* where the bud initially grows by incorporating pre-existing parental cells rather than by cell division3. The limb bud, on the other hand, grows through the proliferation of mesenchymal cells.

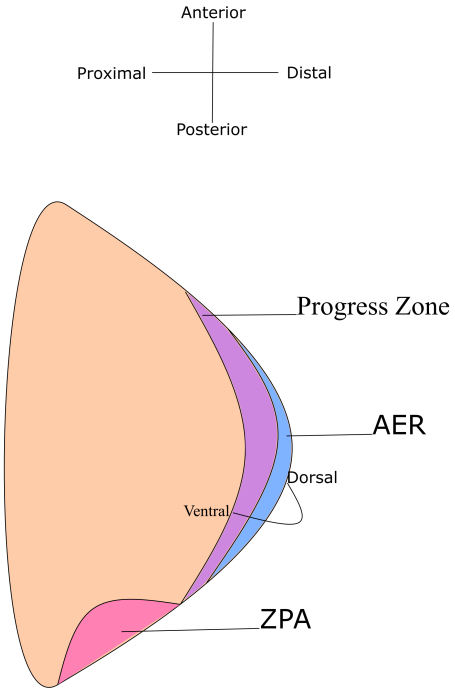

Figure 4: Zones and axes of a developing limb bud

Further, the process involves the formation of 3 axes (dorsal-ventral (DV), anterior-posterior (AP) and proximal-distal (PD)) as compared to the single secondary body axis needed for a *Hydra* bud. These distinctions primarily stem from the fact that *Hydra* show radial symmetry, while vertebrates show bilateral symmetry.

Once the limb fields and axes have been demarcated, limb development is initiated by the action of growth factors and Wnt proteins. Mesoderm cells in the limb field secrete fibroblast growth factors (FGFs) to initiate the process. FGF10 acts as a signalling molecule between the ectoderm and mesoderm. FGF8, on the other hand, acts before FGF10 and demarcates the AP axis. Additionally, FGF8 drives cell movement by forming a gradient along the PD axis with a high concentration at the distal end [16]. Once the AP axis has been demarcated, FGF8 is eliminated from the limb fields by the action of retinoic acid. This is followed by the action of FGF10. Initially, FGF10 acts throughout the lateral plate. However, as soon as limb development is initiated, it is restricted to the limb field. In developing chicks, this restriction of FGF10 results from the action of WNT2b in the forelimbs and WNT8c in the hind limbs [17].

The proliferating mesenchymal cells accumulate below the ectoderm, leading to the formation of a protrusion known as the limb bud [15]. At this stage, the limb bud shows one striking similarity to *Hydra* buds —it has the ability to regrow, repair and recover from any form of damage. However, as it develops, the limb bud slowly loses the ability to regrow. The growing limb bud has 3 main zones: the apical ectodermal ridge (AER), the progress zone and the zone of polarising activity (ZPA). The AER, which is located at the distal end of the bud, is responsible for forming the ectodermal cells and acts as a major signalling centre to ensure proper development. The progress zone, located below the AER, comprises mesodermal cells which differentiate to form the various cells of the limb. The ZPA, located along the AP axis, is responsible for signalling, specifically at the time of digit formation (Fig.5.) [15, 17].

As mentioned previously, a key morphogen involved in limb organogenesis is the Wnt pathway. A study by Gros et.al. characterised the role of the WNT5a protein in mouse and chick limb development [16]. The expression and localisation of the protein was observed using in situ hybridisation. The protein is expressed in the ectoderm of the developing limb and is localised to the distal end of the limb bud. This bears resemblance to the budding process in *Hydra* where the Wnt proteins are expressed in the head of the developing bud with the expression slowly decreasing along the body column, thereby leading to the formation of the gradient which plays a key role in axis formation. To understand the role of WNT5a in mice, Gros and colleagues compared limb development in wild type (WT) and mutant mice (wnt5a$^{-/-}$). They found that, in the mutant mice, the proportions of the limb were affected — there was a significant decrease in the PD axis, a slight decrease in the AP axis and an increase in the DV axis, indicating that the WNT5a protein is responsible for regulating the size of the limbs along the 3 axes, particularly along the PD axis. Further, the team investigated the impact of this protein on the orientation of cell division. This was done by GFP-tagging the cells of the WT and mutant mice and performing time-lapse experiments. These experiments showed that the orientation in WT mice was towards the overlying ectoderm, while the orientation in the mutant mice is more haphazard. Specifically, cells on the distal surface orient themselves towards the ectoderm while the ones along the DV axis are disoriented. This indicates that WNT5a is also responsible for the orientation and alignment of cells during division in a growing limb bud [16].

The next key morphogen is the Sonic Hedgehog protein (SHH), belonging to the Hedgehog (HH) family. The role of SHH in limb development was characterised by Laufer et.al. in 1993 and Kimura and Ide in 1998. Both groups used quantitative RT-PCR and in situ hybridisation [18, 19]. Laufer's team's work focused on characterising the role of SHH as a regulatory and signalling molecule between the AER and ZPA. Using in situ hybridisation, Laufer and colleagues compared the relative expression of HOXd9 to HOXd13. HOXd9 and Hoxd10 were found to be expressed before SHH. Hoxd11 was expressed around the same time as SHH, and HOXd12 and HOXd13 were expressed shortly after this. These results indicate that SHH is in some way involved in the initiation of HOXd11, HOXd12 and HOXd13 expression in the developing limb bud. However, as the bud begins to grow, SHH expression is restricted to the posterior margin while the hox cluster is expressed more dynamically, indicating that SHH is not involved in the maintenance of hox expression. SHH injections into the developing limb bud did not result in ectopic expression of the hox cluster This showed that the initiation of hox expressions was controlled by other factors as well. Next, the team tested the relation between SHH and FGF4, and found that SHH influences the expression of FGF4 in the posterior mesoderm. Lastly, it was also found that SHH affected only the mesodermal cells that were present directly below the AER. Essentially, SHH induces a polarised expression of FGF4 [18]. Kimura and Ide's study picks up from here, and further investigates the interaction between SHH and FGF4. They found that in the posterior cell cultures, expression of SHH rapidly decreased till there was no expression with or without the addition of FGF4 to the cultures. In the anterior cell cultures, there was never any SHH expression with or without FGF4. However, when the expression of SHH was studied in cultures that were still attached to the developing bud, Kimura and Ide found that FGF4 could recover the lost SHH expression, indicating that FGF4 maintains the competence of SHH expression, rather than maintaining the actual expression itself. Lastly, Kimura and Ide reinforced Laufer's inference that FGF4 is involved in the expression of HOXd13. They found that FGF4 was able to maintain in vitro expression of HOXd13 in posterior cell cultures [19].

A review conducted by Tickle and Towers characterised and compiled the importance of SHH in limb development. This review, which also draws from the two studies mentioned above, highlights the role of SHH in controlling the width of the limb bud by impacting the proliferation of mesenchymal cells. Furthermore, its signalling activity between the ZPA and AER regulates the anterior-posterior length as well as the growth of limb structures along the proximal-distal axis [20].

As in *Hydra*, another important component involved in the process of development is the Notch signalling pathway. As mentioned earlier, the Notch pathway is a key morphogen in the development of numerous organisms. It fulfils various roles including the maintenance of stem cells, initiation of cell differentiation, decisions on cell fate, proliferation and apoptosis. Souihoul et.al. conducted a study in 2006 to visualise and understand the activity of the Notch pathway in vivo [21]. The team used methods like whole mount staining, RT-PCR and in situ hybridisation as a part of their study. Further, the team also used transgenic mice. These mice were genetically modified such that the expression of the E. coli lacZ reporter gene (codes for β-galactosidase) was used as a proxy for Notch expression. Souihoul et.al. found that, during limb development, expression of β-galactosidase (β-gal) was initially scattered but it began to get concentrated towards the apical tip and AER as development progressed.

BMP-2	**BMP-4**	**BMP-7**	**Effects**
Mutant	WT	WT	Soft tissue syndactyly, Normal skeletal development
WT	Mutant	WT	Pre and post axial polydactyly, Normal skeletal development
WT	WT	Mutant	No defects, Normal skeletal development
Mutant	WT	Mutant	Variety of skeletal defects - While the differentiation of skeletal cells proceeded as normal, their final structures were deformed-some had missing phalanges, some had abnormal fibulae (in the hind limbs) and some had defects in their scapula
Mutant	Mutant	WT	Complete syndactyly in both sets of limbs, which could be caused by the lack of apoptosis of a part of the AER

Table 1: Effects of various combinations of BMP mutations on developing mice

Further, when jagged [2] of the Notch pathway was targeted, mutant mice showed a significantly thicker AER, indicating that the pathway is important for the maintenance of the AER. This pathway is also one of the key pathways that feeds into the signalling role of the AER [21].

The last key morphogen is the BMP. Bandyopadhyay et.al. studied the role of various BMPs in vertebrate limb development [22]. The study was primarily conducted using in situ hybridisation to visualise the proteins in mutant mice to ascertain the role of different proteins. The team primarily focused on BMP-2, BMP-4 and BMP-7. Mice with homozygous mutant alleles showed defects as detailed in Table 1.

Essentially, the study showed that these proteins were essential for the positional identification of digits, as well as for structural development. However, they did not impact skeletal cell differentiation or integrity [22]. This is very similar to the BMP found in *Hydra* (HyBMP5-8b), which plays a role in tentacle zone demarcation as well as in the normal development of tentacles. Both sets of proteins control the positional specification and normal development of the "outgrowths" on the developing buds.

The last group of proteins, though not necessarily morphogens, are important to the process of sculpting limb digits. These proteins, such as APAF-1, are responsible for apoptosis. The role of this protein in limb development was investigated by Cecconi et.al. in 1998[23]. To understand the role of APAF-1 in the development of mice, Cecconi and colleagues compared limb development in wild type (WT) and mutant mice (apaf-$1^{-/-}$).While the team found that the mutants had a lot of abnormalities in their development, the most relevant observation was that the mice showed interdigital webs in their limbs. Further, Cecconi and colleagues performed in situ hybridisation to visualise the DNA of the cells, since DNA fragmentation is one of the key markers of apoptosis. The screens showed that the wild type cells showed fragmented DNA, indicating that they were undergoing apoptosis. On the contrary, the cells of the mutant mice did not show any fragmentation [23].

The process of limb development ends with the formation of a functional limb, with a unique morphology depending on the organism.

4 Conclusion

This review highlights that there exist some clear parallels between the two processes. These parallels have been summarised in the tables below. Table 2 provides a summary of the key morphogens discussed in the review, along with their role in *Hydra* budding as well as vertebrate limb development.

Morphogen	***Hydra* Budding**	**Vertebrate Limb Development**
Hox family	CNOX -3 - Pattern formation in the head and CNOX-2 - Forms a gradient which plays a role in head and tentacle formation	HOXc6 - Specification of limb fields and HOX 8 - 13 - Pattern formation, signalling molecules between AER and ZPA
Hedgehog Family	Hedgehog (HH) - Determination of budding zone, initiation of bud formation	Sonic Hedgehog (SHH) - Signalling between AER and ZPA, bud width, anterior-posterior length, limb structures along proximal-distal axis, expression of BMPs
Wnt	HvWNT5 - Part of head organiser, specifies location of tentacles on the head and HvWNT8 - Part of head organiser, specifies location of tentacles on the head and HyWNT2 - Expressed at the apex during the initial stages of head formation and HyWNT3 - Setting up the body axis	WNT2b & WNT8c - Restrict FGF10 activity to limb field and WNT5a - Growth along PD axis, orientation of cells towards ectoderm during division and WNT7a - Maintenance of SHH expression
Growth Factors	VEGF/FGF - Bud development and growth	FGF4 - Maintenance of competent SHH expression; maintenance of ZPA activity FGF8 - Demarcation of AP axis
Bone Morphogenetic Proteins (BMP)	HyBMP5-8b - Tentacle development	BMP-2, BMP-4 & BMP-7 - Skeletal development, interdigital apoptosis, interdigital pattern formation, positional information of digits
Notch Signalling Pathway	Expression of HyAlx, head and tentacle formation	Maintenance of AER, signalling by AER

Table 2: Summary of proteins involved in budding in *Hydra* and vertebrate limb development

Table 3: Parallels between budding in *Hydra* and vertebrate limb development

Parameter	Parallel/Description	
	Hydra	**Vertebrates**
Symmetry	Radial	Bilateral
Axes of Development	A single axis	3 axes: anterior-posterior, dorsal-ventral, proximal-distal
Growth of the bud	Incorporation of parental cells, followed by cell division	Proliferation of mesenchymal cells
End Structure	Fully grown *Hydra* that detaches from the parent	Functional limbs that are attached to the organism's body
Number of buds developing simultaneously	Usually only 1, but even if the parent Hydra has multiple buds, they are usually at different stages of development.	Since most vertebrates are tetrapods, all 4 limb buds develop simultaneously
Movement relative to the main/parent organism	Bud descends along the body column of the parent *Hydra*	No motion of the limb bud or the fully developed limb along the organism
Apoptosis	No requirement of apoptosis	Necessary for sculpting of digits
Homologous proteins/protein families	HOX, HH, WNT, BMP, GFs, Notch (refer Table 3.1.2. for details)	
Unique proteins	HyALX, HYM-301	APAF-1

To conclude, this review shows that the process of budding in *Hydra* and limb development have numerous parallels. These parallels exist both in terms of the genes and proteins, and in how both processes proceed physically through protein interactions. While there are numerous similarities, there are also some differences. For example, budding in *Hydra* happens along a single axis, while limb development in vertebrates is bound by 3 axes. However, it appears that these differences primarily arise as a result of the difference in complexity of the body plan of both groups. Understanding these parallels and building on this knowledge would give us a better understanding of the evolutionary relationship between the two groups and allow us to extrapolate our knowledge from one group to another.

5 Glossary

Head organiser	A group of cells located in the head of the organism, which have the ability to instruct and determine cell fate by secreting morphogens and other signalling molecules
Morphogens	Proteins and other signalling molecules that determine cell fate and direct tissue pattering. The fate of the cells depends on the concentration of morphogen they are exposed to, which is a function of their distance from the source of morphogen
Canonical Wnt pathway	Driven by the accumulation of β-catenin and its translocation into the nucleus
Non canonical	Does not depend on the intracellular accumulation and translocation of β-catenin
Ectopic	Expression in an abnormal location or position
Alsterpaullone	Cyclin-dependent kinase inhibitor, that has been shown to upregulate the Wnt pathway by inhibiting the cytosolic degradation of β-catenin
Homeobox/hox genes	Genes that are responsible for determining the body plan of the organism
Mesenchyme	Tissue that develops into connective tissues in animals
Lateral plate mesoderm	Group of cells that later develop into the heart and cardiovascular system, blood, kidneys, smooth muscle lineage and limb skeleton
Syndactyly	Condition where some of the digits of the limb are either partially or completely fused together. This could be natural or the result of a malformation
Polydactyly	Presence of one or more extra digits on the limb

Table 4: Glossary

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Candid Conversations: An Interview With Professor Bittu

By Tista Bhattacharya

July 2023

"Whenever I have spoken to other biology students about their interests in the field or what they might like for their work to encompass, I have most often been met with a similar response: confusion. You can hardly blame them – I, myself have no idea where to start. How do you choose a topic? What model organism would be best to use?"

It is from this conversation that my first interest in Dr. Bittu Rajaraman's laboratory rose. His work was in Neuroscience and, well, I'd never really heard of people doing research on crickets before. As I researched the lab more, I learned a lot more about the work that happens there. For starters, it's called the neuroethology lab. The lab's work primarily focuses on "various puzzles in animal communication, sensation and cognition, and the evolution and ecology of neural systems using neuroethological tools", words which have been taken directly from his website.

Earlier last year, the cricket team in this lab put out a paper describing three new species of the predatory genus *Hexacentrus* crickets (one of which was also named *Hexacentrus ashoka*). So, we sat down for an interview with Professor Bittu to learn more about the work that went behind describing these three new species as well as some of the other work that goes on in his lab.

BioJournal (J): *So, Professor, why crickets? What drew you towards picking them up as a model organism?*

Professor Bittu (B): I was looking for organisms that presented interesting sensory questions, which was when I came across bush crickets. I spoke to Rohini (my PostDoc mentor) and that was it!

J: *Can you tell us a little bit more about crickets– some of the species familiar to us perhaps?*

B: So it isn't trivial to morphologically identify these crickets at the species level; ground crickets tend to look similar to one another. We recently started working *Gryllus bimaculatus* though, it's a somewhat distinct ground cricket; and we mostly work with, bush crickets like *Mecopoda* and *Hexacentrus* and they tend to be much more unique.

J: *Okay, so this question is more by popular demand because whenever I bring up the crickets, the question that almost always comes up is: what exactly does work with crickets entail?*

B: Haha – there's a lot of glamorous feeding and poop cleaning, of course. To start with behaviour, we record and play back certain calls to them; study aspects of the call - whether they're single or multi-syllable and what each specific syllable elicits in terms of behavioural responses from the listener. We also look at other aspects of behaviour like examining the behavioural basis of sexual selection in the crickets. For our neural/electrophysiological experiments, we're looking at the neural basis of call production and reception. If a system shows strong behavioural responses traits we tend to spend a lot of time on just completing the behavioural story, and it saves the animal from dissections for electrophysiology!

J: *Haha – but it is great that you brought up calls and songs in crickets because it gives me a really good segue to my next question. How does one go about exploring this "song diversity and reception" in crickets?*

B: Well, we start out with psychoacoustic training, which essentially means getting used to listening to and distinguishing their calls. Cricket calls are typically produced for sexual advertisement and seem to be more tightly genetically controlled than birdsong or mammalian vocalisations, and so they lend themselves to answering the question of how mutations might affect the song landscape. Call evolution can then be understood more thoroughly through looking at call diversity in terms of listener preference, sexual selection, and other ecological factors such as predator eavesdropping.

J: *So, you recently published a paper where you describe Hexacentrus ashoka. Can you walk us through the entire process of – essentially – naming a new species?* https://www.biotaxa.org/Zootaxa/article/view/zootaxa.5249.3.2

B: First you use the classical morphological taxonomy-based method of classification, such as what characters delineate one's specimen from other known species. We also did some genetic work and sequenced a standard marker.

J: *Something pretty much all researchers have in common is having to face roadblocks. Did you have to face any during this whole process? If so, how did you deal with them?*

B: I would say, working with social justice movements is good training for science, where one gets used to encountering so many roadblocks! Really, the question is to enjoy the process of saying 'aha!' this is a roadblock and I have to find a new way to work around it.

J: *Ha! So we're at the tail end of the conversation here, and*

I wanted to leave the conversation with this question. We have all of this information with the crickets, but the question is what is the importance of sustaining these species levels in the surrounding ecosystem?

B: Understanding ecological relevance can be done through fieldwork - the abundance of these species is a good sign of the situation of the broader health of the ecosystem. These insects in turn constitute food for birds and mammals and help sustain a more diverse ecosystem.

J: Well, that was all we had for today's questions– thank you so much for agreeing to do this, Professor!

The Cockroach And The Wasp: Exploring Parasitoid And Host Interaction

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The parasitoid emerald jewel wasp turns its host, the American cockroach into a 'compliant zombie' by injecting venom into its brain. This puts the cockroach into a state of lethargy with decreased will to self-initiate locomotion (hypokinesia), rather than paralysing it. A new family of ampulexins were discovered in the venom, which might be responsible for the hijack of the host's cognition and behaviour. For the venom to be most effective, it must be deposited in the cockroach's brain, especially the subesophageal and the supraesophageal ganglia. The stinger of the wasp uses mechanosensory input to differentiate between the brain and the other tissues in the head capsule of the cockroach. After the second sting, the cockroach starts grooming itself. This is most likely caused due to a dopamine-like substance in the venom. The immature wasp offspring secretes a mixture of antimicrobials to defend itself against harmful microbes prevalent in the cockroach. Additionally there are some possible hypotheses for the bizarre behaviour of the cockroach—grooming, the immature wasp's conversion from ectoparasitoid to endoparasitoid and some future directions the research of this interaction can take.

1 Introduction

Whether emerald jewel wasps can ever become bartenders remains to be seen, but they sure do make a mean cocktail of neurotoxins to hijack the nervous system of their host- *Periplaneta americana*, the common American cockroach. The emerald jewel wasp, *Ampulex compressa*, is a parasitoid, which lays an egg on its host. Parasitoids are a group of insects that lay eggs on (ectoparasitoid) or in (endoparasitoid) the bodies of other hosts, which they end up killing [1]. When the emerald jewel wasp's larva is ready to pupate, it pierces through the cockroach's cuticle to feed on its internal organs, and later pupates inside the abdominal cavity of the cockroach [2]. The larvae feed on the cockroach's body first as an ectoparasitoid and then as an endoparasitoid [3]. However, it is important to bear in mind that the cockroach is alive and complying throughout the whole process.

To understand how the jewel wasp can achieve such a feat, we need to understand the intricacies of wasp venom, its cognitive behavioural manipulation, its precise sting which leads to the zombification of the cockroach, the larval defence mechanism, and the possible evolutionary theories for this interaction.

2 The Beautiful Mind And Body Of The Cockroach And How The Wasp Hijacks It

The emerald jewel wasp induces a state of hypokinesia — a lethargic state of decreased will to self-initiate locomotion — in the cockroach as it injects a variety of neurotoxins into the central nervous system of its prey. It stings the host's thorax by penetrating the cuticle with its modified stinger (ovipositor). This piercing disables the central motor circuit outputs in the cockroach's forelegs, allowing a second, longer sting through the neck into the subesophageal ganglion (SOG) and supraesophageal ganglion (SupOG) of the cockroach [4].

2.1 Intricacies of Emerald Jewel Wasp Venom - Grooming

After the second sting, the wasp leaves for roughly 30 minutes to search for a nest to lay its egg. This is a perfect opportunity for the cockroach to flee and save itself from the terror ahead. But instead, it starts grooming itself excessively with its legs and mandibles [2]. This strange urge for vanity is induced by a monoamine (or its agonist) present in the venom, injected by the wasp [4]. In a study conducted by Weisel-Eichler *et al.*, direct injection of a dopamine agonist into the SOG and the SupOG of unstung cockroaches induced prolonged grooming, whereas flupenthixol, a dopamine antagonist, greatly reduced venom-induced grooming [5]. Thus, it found evidence of a dopamine-like substance (either dopamine or octopamine) in the emerald jewel wasp's venom [5].

2.2 Cognitive Behaviour Manipulation Due To The Wasp Venom

Manipulation refers to the changes in host behaviour that are driven by the parasitoid to increase its own fitness. When the wasp returns from its search for a nest, we see the full extent of the behavioural manipulation as the wasp venom inhibits the drive to self-initiate locomotion and escape behaviour. American cockroaches are known for their highly effective escape responses, due to the wind-sensitive hairs on their cerci, which detect changes in air pressure and convey them to the ganglia [2]. The venom manipulates the central ganglia, that are responsible for the initiation of

Figure 1: Emerald Jewel Wasp [6]

walking by decreasing inputs from the ganglion to the thoracic central pattern generators. This causes a low propensity for walking-related behaviours without interfering with other non-related behaviours [2].

It is important to note that the cockroach is not paralyzed. *Periplaneta americana* being a terrestrial insect, will produce rigorous motor patterns in stressful environments like immersion in water [2]. An experiment conducted by Gal and Libersat, showed that stung cockroaches initiated walking-like motor movements, albeit slower than those not stung, when immersed in water [2]. A 'paralysed cockroach' could not have produced any such motor movements. This suggests that the stung cockroaches are not paralysed; rather the venom has increased the threshold for initiating walking-related behaviours and did not inhibit them completely [4]. That is why the cockroach endures some horrifying torture by the wasp, without fleeing or fighting, such as breaking the cockroach's antenna, and eerie escort to the pre-selected nest [2]. In experimental conditions the stung cockroach, without an egg on its body (experimentally removed), regains normal behavioural activity in three to seven days [2]. But unfortunately, the cockroach, with an egg on its body, is killed by the larvae 6 days after the sting to pupate in the abdomen [2].

Furthermore, a new family of peptides called ampulexins was found in emerald jewel wasp's venom, which are amongst the most abundant venom components [7]. They greatly affect the escape behaviour of the cockroach by increasing its escape threshold [8, 9]. The analysis of these peptides in the venom reveals a 'multi-pronged attack' on the central nervous system (CNS) of the cockroach to usurp control of its mobility, targeting the endogenous signalling systems, and likely structural alterations of the synapse [7]. The novelty of ampulexins and their structural similarity with cell-penetrating class of peptides could provide further insights into the interaction between the host and the parasitoid [9]. Moreover, there is evidence that indicates dopamine receptor dysfunction as a possible underlying reason for hypokinesia too [9].

Upon the wasp's return to the cockroach, it drags the compliant cockroach to the chosen nest, lays an egg, and sticks it to the leg-cuticle of the host. Following this, it seals the entrance and leaves the nest. The willing slave of a cockroach is served on a silver platter to the developing larva, which emerges as an adult a month later, thereby continuing this vicious life cycle [2].

2.3 Neuronal Tissue Recognition By The Wasp Due To The Sensory Cues From Its Stinger

Affecting behaviour to this extent requires precision in delivery of venom into the subesophageal ganglion and the supraesophageal ganglion of the cockroach; how does the emerald jewel wasp achieve it? The stinger, which is only found in female jewel wasps, is long enough to reach the host's brain through the neck. The wasp uses mechanosensory input from its stinger to differentiate between the brain and other tissues inside the head capsule of the cockroach [10]. The emerald jewel wasp's stinger has evolved to identify the neuronal tissue in the head capsule of the cockroach through two morphologically-distinct, sensilla-like cuticular structures [10]. An experiment by Gal *et al.*, showed that the firing rate of the sensory neurons significantly increased when an agarose pellet (representing neuronal tissue) was pushed towards the stinger, compared to when pushed away from it [10]. Furthermore, the higher firing rate is also seen when denser agarose (representing the brain of the cockroach) is pushed against the stinger rather than softer agarose (representing other tissues in the head of the cockroach) [10]. This shows that the stinger can differentiate between the brain and other tissues inside the head of the cockroach based on their mechanical properties. Moreover, when the jewel wasp was introduced to surgically removed brain-less cockroaches, the headsting duration was prolonged 10 times, as was the case in soft agarose-replaced

brain ones. Additionally, softer agarose showed no traces of venom after the sting [10]. But the sting was normal when the brain was replaced by hard agarose pellets [10]. All these findings reiterate that the wasp can differentiate between the different head-borne tissues of the cockroach and that the sting contains venom only when it is in the brain of the cockroach.

2.4 Defence Mechanism Against Microbes in the Cockroach

The cockroach, Periplaneta americana, is known to have the bacterium *Serratia marcescens* in abundance, which is a potent entomopathogen that can rapidly kill insect larvae through sepsis [11]. This causes the immature wasp offspring to defend itself against virulent microbes. A study found that the larvae use a mixture of antimicrobials such as (R)-(-)-mellein and micromolide, as well as some minor compounds, to fight off the pathogens. Both of the microbials showed antibacterial activity against *Serratia marcescens* and *Staphylococcus hyicus*, another harmful microbe in the cockroach. Particularly micromolide displayed clear growth-inhibiting properties against *Staphylococcus hyicus* but not *Serratia marcescens* [11]. They also demonstrated that such composition of antimicrobials were found only in parasitised cockroaches and not in non parasitized ones [11]. This suggests that the wasp larvae secrete antimicrobial compounds and sanitise the hosts from inside, providing an effective defence against the spectrum of microbes they might encounter in the host [11].

3 Possible Evolutionary Reasons For The Persisting Wasp Behaviour

For the venom to be most effective it has to be injected in the SOG and the SupOG of the cockroach's brain. This is because the aforementioned structures are responsible for regulation of locomotion in insects [12]. By doing so, the wasp can ensure manipulation of walking and escape related behaviours without interfering with other non walking related behaviours [12]. Therefore the structures and mechanisms to identify the specific parts of the brain of the cockroach are unique to *Ampulex compressa*, which suggests that it might be a specialised host specific adaptation. Such evolution can also be witnessed in the successful parasitisation of the cockroach when the wasp takes over its body, brain and defence mechanisms to benefit its own grisly motives. Let us now look into the possible evolutionary reasons for such persisting wasp behaviour and abilities.

3.1 Evolutionary reasons for grooming

After the second sting, the cockroach starts grooming itself with its legs and mandibles [2]. We have explored the cause of such behaviour being dopamine-like substances (either dopamine or octopamine) in the venom. But the evolutionary reasons for grooming are yet to be known. Here are a few hypotheses of the evolutionary reasons for grooming. A study done in 2021 postulates that grooming is not an evolutionary adaptive strategy to remove microbial infections from the middle legs of the cockroach [13]. In laboratory settings, the microbial prevalence in stung cockroaches, experimentally prevented from self grooming, was not higher than in self-groomed ones [13]. Rather, they suggested that it could be a cue for the returning wasp to locate the cockroach and take it to the burrow. However, the microbial hypothesis can still be supported under relevant ecological conditions or if more information regarding the fungal infections in the cockroach is available. It is still not certain whether grooming increases parasite fitness or not. An experiment comparing the successful completion of wasp life cycle in groomed and ungroomed hosts can be conducted to determine if there are any evolutionary reasons for grooming [13].

3.2 Evolution from Ectoparasitoid to Endoparasitoid

There is always a predation risk between oviposition and adult emergence, which is a strong determinant of the evolution of parasitoids. Immature wasps are prone to a wide range of natural enemies when developing in exposed hosts [14]. But jewel wasps seem to have successfully found ways around this hindrance. As an ectoparasitoid, they face a lot of threats such as higher exposure to predators and microbes in the open environment. Just as the larva is ready to pupate, it penetrates into the abdomen of the cockroach, becoming endoparasitic, safe from the external threats. Additionally, the female adult wasp covers the entrance of the place with leaves and stems, camouflaging it from the outside world. As mentioned before, the cockroach is killed by the larva 6 days after the sting, but the wasp emerges as an adult only after 4-6 weeks after the sting. Here, there is an avenue for further research into whether the immature emerald jewel wasp secretes any substance which keeps the host's carcass from decomposing. Perhaps, the venom has some substance that can ensure this too. By studying these possibilities in detail, we can better understand the mechanism at play in this interaction.

3.3 A Model Interaction

The wasp-cockroach interaction is an ideal model to study parasite-induced alterations of host behaviour. It can be used to study the alterations in host behaviour in terms of biochemical reactions, neurotransmitters, neurotoxins, and how they manifest as physiological behaviour. Further study can lead to the identification of the chemical components (monoamines, amino acids, peptides, and proteins) and corresponding molecular targets responsible for determining the "motivation", specifically for walking [2, 4]. Understanding these components will shed some more light on the unknown signalling systems and pathways present in the adult insect brain [7]. The neuropharmacology of this wasp's venom can provide us with an effective long term yet reversible suppression of locomotion without paralysis [7]. Each protein, peptide and other components in the venom have a role to play and each call for further study and analysis.

4 Conclusion

In this article, we have explored how the emerald jewel wasp successfully hijacks its host to provide its progeny with a suitable environment to develop and grow. It achieves this through various proteins in its venom which increase the escape threshold for walking related behaviours and result in a state of hypokinesia. Mechanisms, and small molecules and proteins in the venom that are the basis for hypokinesia can be further explored to understand the nuances of this peculiar interaction. Hypokinesia also bears some resemblance to Parkinson's disease, which is caused due to bradykinesia (slow movements and reflexes) [9]. Furthering the research to better understand the cockroach's hypokinesia and wasp's ability to do so, can give us interesting insights into the treatment of Parkinson's disease.

Through a mechanosensory input from the specialised stinger, the wasp can detect the neuronal tissue in the head capsule of the cockroach, to precisely deliver the venom. The immature parasitoids are also hypothesised to secrete antimicrobial compounds to fight off the detrimental microbes in the cockroach. Furthermore, we have looked into the possible evolutionary reasons for the various wasp and cockroach behaviours such as grooming, and the shift from ectoparasitoid to endoparasitoid. These various studies and lines of investigations have hopefully provided you with interesting insights into the bizarre interaction of the wasp and the cockroach, its baffling ability to zombify the cockroach to serve its vicious motives and above all, an appreciation for the complexity of nature.

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