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Research Article

Regenerative response and molecular adaptation of the planarian osmoregulatory system to pH stress

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Abstract

Planarians, members of the phylum Platyhelminthes, are well-known for their extraordinary regenerative abilities. Although structurally distinct, planarian protonephridia share evolutionary and functional similarities with the mammalian nephron, making them valuable models for studying kidney regeneration. The effects of pH stress on gene expression in planarian renal neoblasts, however, remain poorly understood, as do the molecular mechanisms connecting neoblast pluripotency with mammalian stem cells. In this study, we examined the behavioural changes, regenerative potential, activation levels, and migration patterns of neoblast cells in planarians, particularly in the protonephridial region, under different pH stress conditions. Candidate genes such as EGFR5, DNAHβ3, Rh family, and cysteine-rich proteins, which serve as ammonia transporters in the human kidney, were selected as possible regulators of protonephridial regeneration. The findings show that these genes play a critical role in protonephridial regeneration during pH stress, making them potential candidates for kidney gene therapy in vertebrates. Additionally, key genes identified in stress responses could serve as biomarkers for assessing the health of aquatic species and developing gene-based therapies or interventions to manage environmental stress in farmed species. Notably, after 72 hours of pH stress incubation, planarians attempted to neutralize their surroundings, indicating the secretion of stress-mitigating chemicals. This adaptation underscores their resilience and ability to maintain homeostasis under environmental stress. These insights contribute to understanding sustainable resource management in animal and aquaculture systems, aligning with the "One Health" approach and addressing the interconnected health of humans, animals, and ecosystems amid changing climatic conditions.

Keywords: Kidney gene therapy, Neoblast pluripotency, pH stress response, Planarian regeneration, Protonephridial function

Highlights

- Planarians exhibit regenerative capacity and survival differences under varying pH stress.
- Selected genes, including EGFR5 and DNAHβ3, regulate protonephridial regeneration.
- pH stress significantly affects planarian behaviour, neoblast migration, and gene expression.
- Planarians secrete chemicals to neutralize environmental pH stress after 72 hours.
- Findings offer potential biomarkers for aquatic health and gene therapy in vertebrate kidneys.

INTRODUCTION

Planarians, belonging to the phylum Platyhelminthes, are widely recognized for their extraordinary regenerative abilities, which make them a valuable model organism for studying cellular regeneration and tissue repair. These flatworms possess a primitive excretory system known as protonephridia, which functions analogously to the mammalian kidney in terms of osmoregulation and waste removal. The protonephridial system consists of tubules connected to a highly branched duct network with excretory pores along the sides of the body, facilitating the expulsion of metabolic waste. The cells forming these tubules are called flame cells, named for their clusters of cilia that resemble flickering flames under a microscope. These flame cells play a role similar to that of nephrons in the mammalian kidney, using their cilia to propel waste matter down the tubules and out of the body, while also facilitating filtration by drawing water from the interstitial fluid. After excretion, any useful nutrients are reabsorbed by the cells (Rink *et al.*, 2011).

Regeneration in planarians heavily depends on stem cells known as neoblasts, which are dispersed throughout the body. These undifferentiated cells are crucial for tissue renewal and regeneration; when a part of the planarian is amputated, neoblasts are activated and migrate to the wound site to reform the lost tissues. Studies have shown that these neoblasts are the only mitotically active cells in planarians, and their destruction through radiation leads to a loss of

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regenerative capacity. Research by Baguna *et al.* (1989) demonstrated that neoblasts are totipotent stem cells, capable of regenerating various cell types, and are vital for the regenerative processes in planarians (Baguna *et al.*, 1989; Rink *et al.*, 2011).

The mammalian kidney, composed of millions of nephrons, filters approximately 170 liters of blood daily to remove metabolic waste and maintain fluid balance and acid-base homeostasis (Chrysopoulou and Rinschen, 2024). Similarly, the planarian excretory system, consisting of protonephridia with flame cells, serves as an evolutionary and functional analogue to the mammalian kidney, despite structural differences. Both systems exhibit conserved features, such as the filtration of water and small molecules and the reabsorption of bicarbonates for pH regulation. Studies by Thi-Kim Vu *et al.*, (2015) revealed that mutations in proteins analogous to those in mammalian kidneys lead to similar pathologies in planarians, further supporting the use of planarian protonephridia as an invertebrate model for studying kidney diseases in vertebrates (Thi-Kim Vu *et al.*, 2015).

The maintenance and regeneration of the planarian excretory system are regulated by various signaling pathways, including epidermal growth factor receptor (EGFR) signaling. For example, EGFR-5 is required for flame cell maintenance and branching morphology, and its knockdown leads to edema and impaired regeneration in planarians. Despite these insights, the molecular mechanisms of pH stress on gene expression and regenerative processes in planarian protonephridia remain largely unexplored. Metabolic acidosis and alkalosis, common consequences of chronic kidney diseases and other disorders, alter pH levels in mammals, leading to similar stress responses that may be mirrored in planarians (Rink *et al.*, 2011; Scimone *et al.*, 2011; de Oliveira *et al.*, 2018).

This study aims to fill the gaps in our understanding of how environmental pH stress affects the regenerative capacity of planarian protonephridia. Given that pH imbalance due to metabolic acidosis or alkalosis is a significant issue in human kidney diseases, understanding how planarians respond to such stressors could offer insights into potential therapeutic targets and strategies for renal regeneration in mammals. The selection of specific genes related to ammonia transport, acid-base balance, and kidney injury biomarkers- such as Rh family proteins, cysteine-rich proteins, and sodium/hydrogen exchanger isoforms—will allow us to investigate their role in the regeneration of planarian protonephridia under different pH conditions. By studying these processes, this research aims to enhance our understanding of regenerative biology and its potential applications in treating kidney diseases in vertebrates.

MATERIALS AND METHODS

Planaria maintenance: Freshwater planarian species, *Dugesia japonica*, were utilized as the model organism for this study. *D. japonica* is a gold-standard laboratory model due to its small diploid genome (2n = 8, approximately 7 x 10^8 bp), ease of culturing, and propagation under laboratory conditions. Planarians were maintained in detergent-free glass beakers, with a density of fewer than 150 individuals per 250 mL of a neutral buffer solution (1x planarian buffer). The buffer consisted of 5M NaCl, 1M $CaCl_2$, 1M $MgSO_4$, 1M $MgCl_2$, 1M KCl, and 0.540 g $NaHCO_3$, prepared in double-distilled water and maintained at 19°C in a quasi-dark room. The buffer solution was changed every 2-5 days to remove excreted mucus, and always the day following feeding. Planarians were fed weekly with macerated chicken liver for 2-3 hours, but all subsequent experiments were conducted with animals starved for one week to standardize conditions (Dean and Duncan, 2020).

pH stress experiment: Eight tubes, each containing 50 mL of culture media, were prepared, and 30 one-week-starved *D. japonica* were placed in each tube. To study the effects of different pH levels, 1M NaOH and 1M HCl were added gradually to seven of the tubes to achieve basic pH values of 8, 9, and 10, and acidic pH values of 3, 4, 5, and 6, respectively. The eighth tube, maintained at pH 7, served as a control. The morphological changes and behaviour of the planarians were observed under a stereo zoom microscope at 24, 48, and 72 hours post-exposure to pH stress. After 72 hours, the media in each tube were examined for pH changes to indirectly assess osmoregulatory activities reflected by the resultant pH of the media (de Oliveira *et al.*, 2018).

Behavioural studies: Behavioural studies were conducted to assess the survival, mobility, size, and regeneration of planarians under different pH stress conditions. Mobility was measured by recording the number of boxes visited by 10 planarians over 2 minutes on centimetre graph paper. Regeneration was assessed by amputating the head, trunk, and tail of 10 planarians and monitoring the regeneration process over 14 days, with daily picture capture using an Olympus stereo zoom microscope. Both treated and untreated control groups were observed in several sterile glass petri plates, and their gross organismal behavior was studied without

immobilization under low-intensity stereoscopic light (Stevenson and Beane, 2010; Hammoudi *et al.*, 2018).

α-Tubulin staining: To study the cytoskeletal protein α-tubulin and its role in the protonephridial region, 15 planarians from each pH condition were prepared for staining. Planarians were first rinsed in clean 1x planarian buffer to remove mucus and debris, followed by incubation in 0.0625% NAC in 1x planarian buffer for 30 seconds to remove the mucus layer of the epithelium. After rinsing three times in 1x planarian buffer, the animals were fixed in 4% formaldehyde in 1x PBST for 30 minutes. The specimens were then dehydrated through a graded methanol series (50% methanol-PBS for 5 minutes and 100% methanol for 5 minutes) and stored at -20°C overnight. The following day, samples were rehydrated and post-fixed in 4% formaldehyde in 0.5x PBSTx for 10 minutes, then washed three times in PBSTx. Samples were blocked with a Western blocking reagent in PBSTx for 1 hour with gentle agitation, followed by overnight incubation with α-tubulin antibodies (0.1-1 µg/mL) at 4°C. After washing six times with PBSTx over 2 hours, samples were incubated with secondary antibodies (Secondary anti-Rat IgG FITC, 1:1000 diluted in PBSTx) at 4°C overnight. Samples were washed again and observed under a microscope. Relative fluorescence intensity was quantified using ImageJ software (King and Newmark, 2018; Gaetano and King, 2023).

BrdU staining: The BrdU incorporation assay was used to detect DNA synthesis and cell proliferation. Planarians (n = 15 per pH condition) were rinsed and treated similarly to the α-tubulin staining protocol to remove the mucus layer. Subsequently, planarians were incubated in 20 mg/mL BrdU in 3% DMSO in 0.1x planarian buffer in the dark for 1 hour. After rinsing, samples were fixed and dehydrated as described earlier. Following rehydration, samples were treated with 2 N HCl for DNA denaturation at 37°C for 15 minutes, then blocked and incubated with anti-BrdU antibodies (0.1-1 µg/mL) overnight at 4°C. After multiple washes, samples were incubated with secondary antibodies (Secondary goat anti-mouse IgG3-Brilliant Violet, 1:1000 diluted in PBSTx) overnight at 4°C. Samples were then washed and visualized under a microscope, and relative fluorescence intensity was analyzed using ImageJ software (Newmark and Sánchez Alvarado, 2000; Cheng and Alvarado, 2018).

RNA isolation: For RNA isolation, 750 µL of Trizol reagent was added to planarians (n = 15 per pH condition) for cell lysis. Samples were homogenized and incubated at room temperature for 5 minutes, followed by the addition of 200 µL of chloroform, vigorous shaking, and centrifugation at 12,000g for 15 min at 4°C. The aqueous layer was carefully transferred to a new tube, mixed with chilled isopropanol (500 µL per 1 mL of Trizol), and incubated overnight at -20°C. After centrifugation at 12,000g for 10 min at 4°C, the RNA pellet was washed with 75% ethanol, centrifuged, and air-dried (Pal and Banerjee, 2024).

cDNA preparation: cDNA was synthesized using the Thermo Scientific Verso cDNA Synthesis Kit, employing reverse transcriptase to convert RNA into stable complementary DNA (cDNA). This was followed by PCR amplification using the newly synthesized cDNA as a template (Pal and Banerjee, 2024).

Polymerase Chain Reaction (PCR): PCR amplification of cDNA was performed for target genes ACTB, EGFR5, and DNAH-β3 using a thermal cycler. The PCR protocol included initial denaturation at 94°C, followed by cycles of denaturation, annealing (according to primer Tm), and extension. A total of 35-40 cycles were performed, with each cycle doubling the DNA content, resulting in exponential amplification of target DNA sequences (Pal and Banerjee, 2024).

Agarose gel preparation and electrophoresis: A 1% agarose gel was prepared by dissolving 1.3 g of agarose in TAE/TBE buffer (pH adjusted to 8.3), and the solution was microwaved until fully dissolved. 10 µL ethidium bromide (0.3 µg/mL) was added to the cooled solution as a DNA intercalating agent, and the gel was cast in a prepared plate. DNA samples were loaded into the wells, and electrophoresis was conducted at a constant voltage (90V). The separated DNA fragments were visualized using a Thermo Chemidoc imaging system, and band intensity was quantified with ImageJ software (Pal and Banerjee, 2024).

RESULTS

Behaviour study: Our observations indicated that at pH 3, pH 9 and pH 10, the bodies of the planarians incubated for 72 h disintegrated. They could not take the pH stress. Thus, we took the pH 4, pH 5, pH 6, pH 7 (as control), pH 8 stressed planarians and studied their behavioural changes (Fig. 1A). From the observed data, we found that at pH 3, pH 9 and pH 10, the survival potential of the planarian was starting to decrease after 6 h (Fig. 1B). So, from the data we took this pH 4, pH 5, pH 6, pH 7 (control), pH 8 of our further experimental work. We also measured the pH change of the buffer after 72 h.

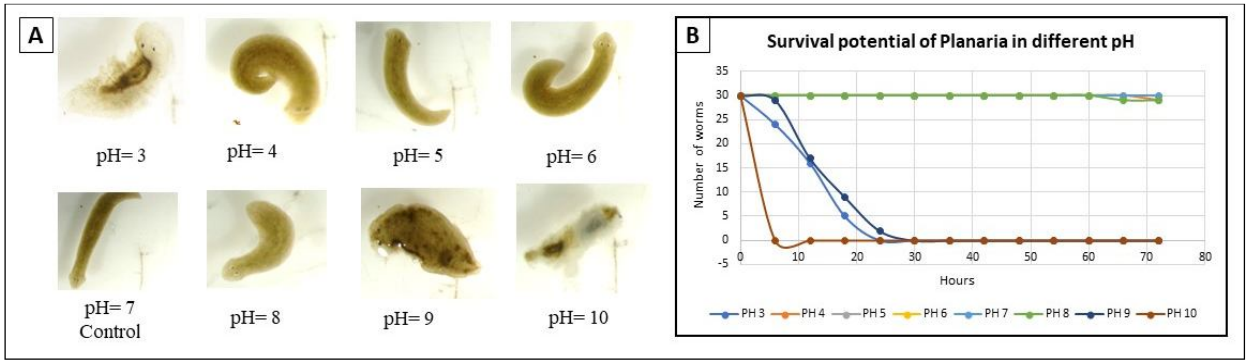

Fig. 1. The above pictures showed the behavioural changes of planarians in different pH [A] and the survival potential of planarians in different pH after 72-hour incubation [B].

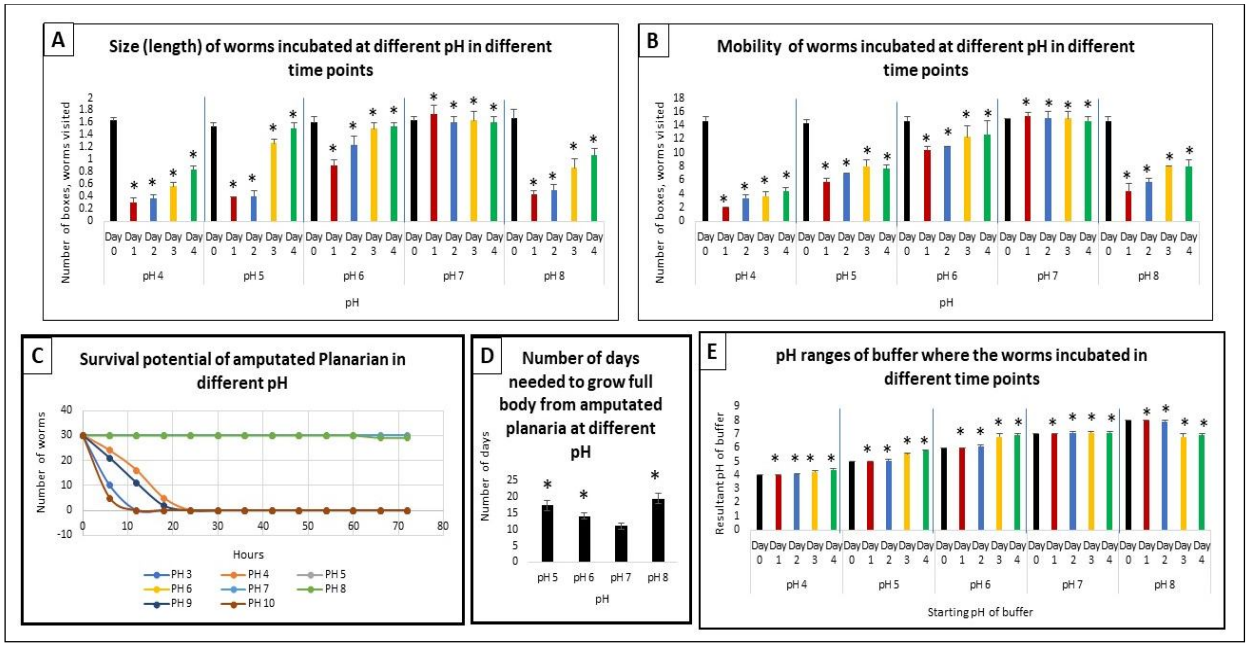

Fig. 2. Different parameters were examined at different pH: [A] Size of the planarians, [B] The mobility of planarians, [C] Survival potential of amputated planaria, [D] After amputation, how many days needed to regrow planarians whole body at different pH, [E] pH ranges of buffer where the worms incubated (*p<0.05, compared to control).

We studied the change in size of the planarians more than 72 h under stress. We observed that the size became decreased as compared to control (pH 7) (Fig. 2A) at pH 3, pH 9 and pH 10 after 72 h incubation. The mobility also decreased in pH 4, pH 5, pH 6, pH 8 as compared to pH 7 (Fig. 2B). When we took 30 amputated planarians and kept them at pH 4, pH 5, pH 6, pH 7 (control), and pH 8, we found that the survival potential of amputated planarians at pH 8, pH 9 and pH 10 decreased (Fig. 2C). We also found that control planaria of pH 7 took 12 to 14 days to grow their whole body, but the planaria kept at pH 5, pH 6, and pH 8 took longer time to grow their whole body (Fig. 2C). We found that in stressed conditions they need more time to regrow lost body part (Fig. 2D) when they were in pH stress. The pH was also affected by them at different time points (Fig. 2E).

Study of the effect of different pH stress on planarian protonephridia by α tubulin staining: α-Tubulin staining assessed the protonephridial stress in where we analyzed that at pH 7 normal distribution of protonephridial cell were found but in stressed conditions (in pH 5, pH 6 and pH 8), the protonephridia cell proliferation decreased significantly as compared

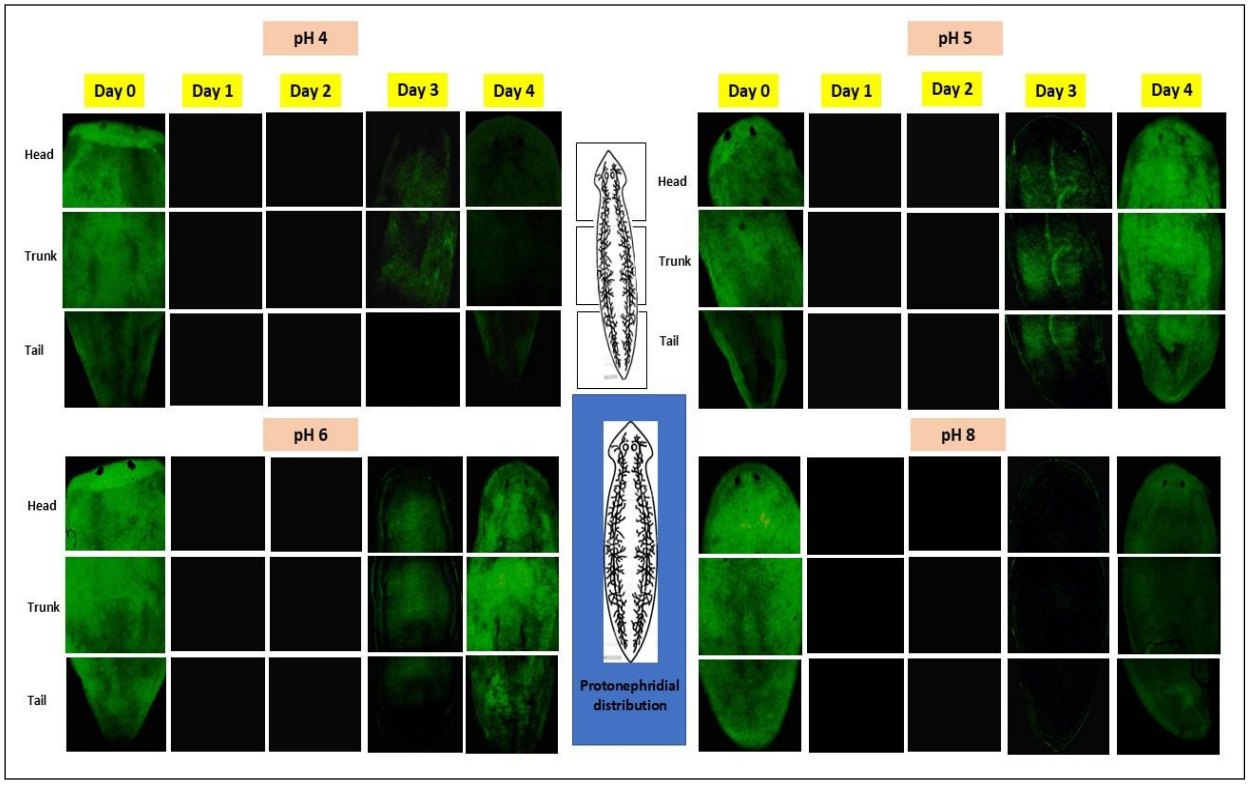

Fig. 3. Study of the effect of different pH stress on planarian protonephridia by α tubulin staining

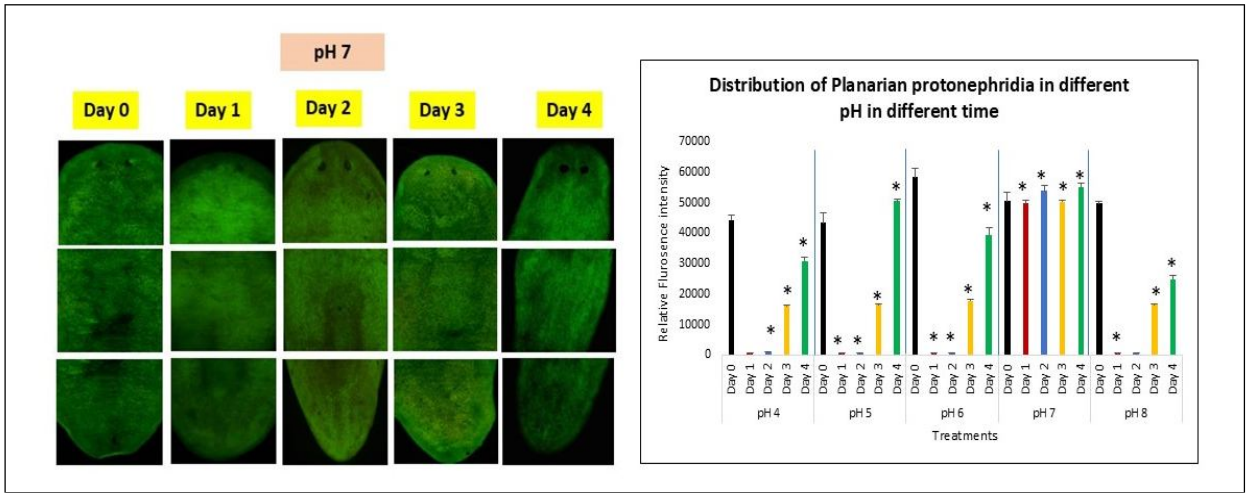

Fig. 4. Distribution of planarian protonephridia in different pH in different time

to control (Fig. 3, 4).

The activation level and the migration pattern of neoblast cells in planarian whole body in different pH stress by BrdU staining: Fluorescence microscopic observations revealed the typical morphological distribution of neoblasts. BrdU staining showed the migration of neoblast cells in the following pictures (Fig. 5). The results showed that at pH 7 the neoblast arrangements are normal as the planaria always have neoblast distribution in their body. But the neoblast cells migrated to protonephridial region (Fig. 5) when they were getting stressed at pH 5, pH 6 and pH 8. The graphical representation (Fig. 5) showed that the distribution or

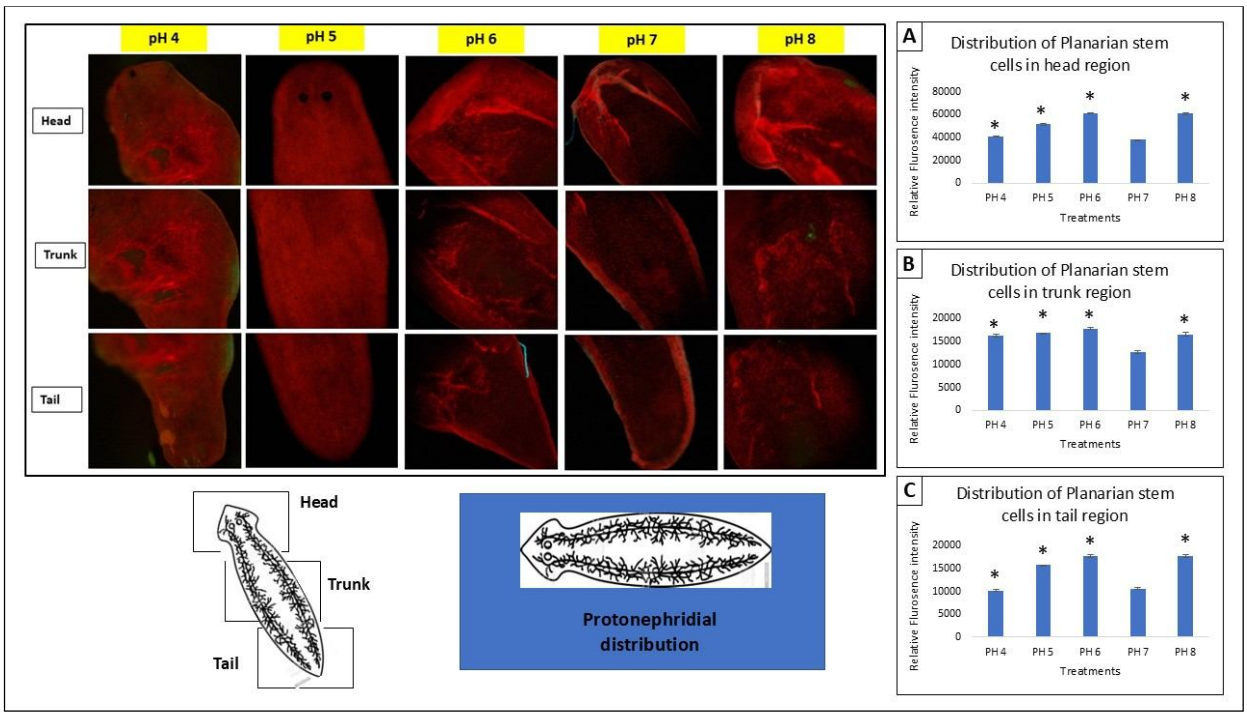

Fig. 5. The activation level and the migration pattern of neoblast cells in planarian whole body in different pH stress by BrdU staining. Distribution of planarian stem cells in different regions of the body incubated at different pH after 72 hours- In the head region [A], the band intensity increased significantly by 1.71 folds and increased significantly by 1.21 folds at pH 5 as compared to control. In the trunk region [B], the distribution of planarian stem cells increased significantly by 1.5 folds at pH 6 as compared to pH 7. At pH 5 and pH 8 the distribution of planarian stem cells increased significantly by 1.33 folds as compared to pH 7. In the tail region [C], at pH 6 and pH 8, the distribution of planarian stem cells increased significantly by 1.6 folds and at pH 5 the band intensity increased significantly by 1.4 folds as compared to pH 7 (*p<0.05, compared to control).

proliferation of neoblast, increased significantly at pH 5, pH 6, and pH 8 as compared to control.

Study of the gene expression of planarians in different pH stress by RT PCR: Gene expression level was studied after 72 h (day 3) in Fig. 6. The EGFR 5 expression [B] increased significantly around 2.14 folds at pH- 4,5 and 6 as compared to control. The band intensity increased significantly 1.8 folds at pH- 8 as compared to control. The DNAH-β3 expression [C] increased significantly around 2.5 folds at pH 6, 8 as compared to control and increased by 1.16 fold at pH 5 as compared to control. In Fig. [D], the Rh protein expression increased significantly around 1.4 folds at pH- 4, 5, 6, 8 as compared to control. In Fig. [E], the V-ATPase expression increased significantly around 2.5 folds at pH- 4, 5, 6 and around 12 folds at pH 8 as compared to control. In Fig. [F], the Na+/K+-ATPase expression increased significantly around 4 folds at pH- 4, 5, 6, 8 as compared to control. In Fig. [G], the Cysteine protease expression increased significantly around 6 folds at pH- 5, 6, 8 as compared to control. In Fig. [A], Band intensities of the genes, ACTB were observed in gel electrophoresis where we took ACTB as housekeeping gene.

DISCUSSION

This study underscored the profound effects of pH stress on planarian survival, behaviour, cellular responses, and gene expression, providing insights into their extraordinary regenerative capabilities and potential medical applications. The observed stress-induced responses align with findings in higher organisms where environmental stress impacted cellular communication and metabolic pathways, particularly in the kidney. Chrysopoulou and Rinschen (2024) described how metabolic rewiring and signalling adaptations in the proximal tubule of mammalian kidneys support homeostasis under stress, paralleling the adaptive mechanisms seen in planarian protonephridia (Chrysopoulou and Rinschen, 2024). Similarly, studies on freshwater planarians have demonstrated that stressors influence epidermal

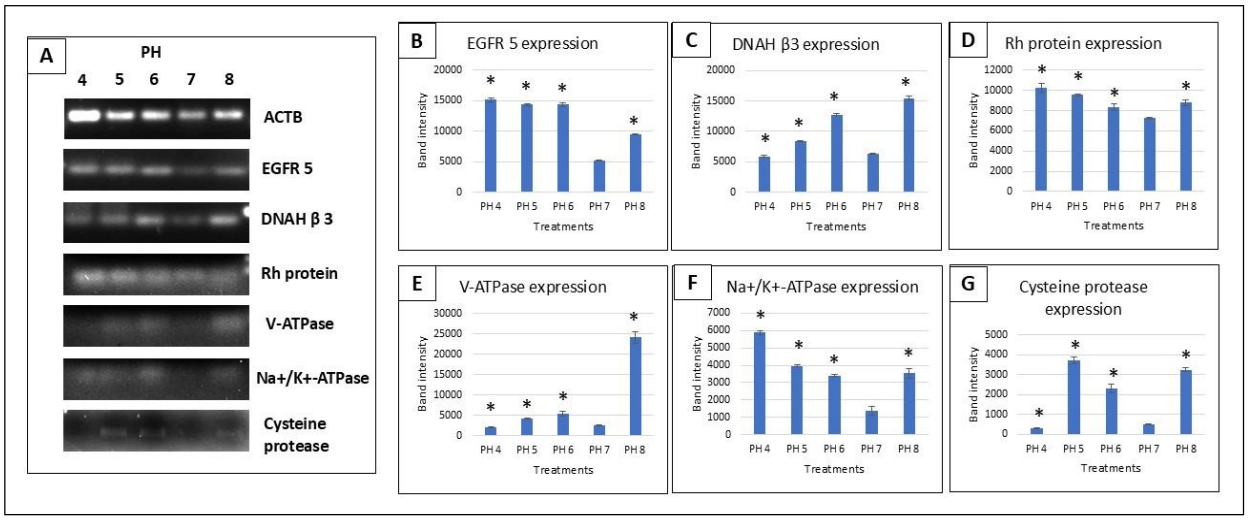

Fig. 6. Study of the gene expression of planarians in different pH stress by RT PCR (*p<0.05, compared to control)

behaviour and regeneration, with EGFR signalling playing a pivotal role in maintaining and regenerating the excretory system (Rink *et al.*, 2011). This was consistent with earlier findings that EGFR-mediated pathways regulate stem cell activity during regeneration in both planarians and higher organisms. These studies collectively supported the idea that the mechanisms identified in planarians could serve as models for understanding stress responses and regeneration in vertebrates, including humans. The observed secretion of stress-mitigating chemicals after 72 h of pH exposure suggested parallels to adaptive excretory mechanisms in mammals, which are critical under conditions like metabolic acidosis. Integrating these findings with previous research highlighted the translational potential of planarian models in understanding kidney disease and regenerative therapies.

Planarians demonstrated an inability to survive in extreme acidic (pH 3) and basic (pH 9 and pH 10) environments, suggesting a higher tolerance for acidic conditions than for alkaline ones. This intolerance is evidenced by the disintegration of planarian bodies under these pH extremes, indicating a narrow range of pH within which planarians can maintain homeostasis and survive. When exposed to milder pH stress (pH 4 to pH 8), planarians showed a decrease in survival potential, particularly when amputated, highlighting their vulnerability to pH-induced stress.

Interestingly, after 72 h of incubation under pH stress, planarians attempted to neutralize their environment, suggesting that they may secrete certain chemicals to mitigate external stress conditions. This adaptive response underscores the planarians' resilience and capacity for physiological adjustments to maintain internal balance. Additionally, planarian protonephridia were significantly affected by pH stress, with evidence of structural and functional impairment. However, regeneration of the protonephridia was observed to initiate by day 3, alongside increased proliferation and migration of neoblast cells, which began to divide and migrate towards damaged areas to facilitate repair.

Gene expression analysis revealed that the selected genes were upregulated during regeneration under different pH stress conditions. This suggests that these genes play a crucial role in initiating and supporting protonephridial regeneration. Given these findings, the upregulated genes identified in this study could serve as potential markers for protonephridial regeneration in planarians and may have broader applications in other organisms. Notably, these genes could be explored in vertebrate models of kidney injury, where metabolic acidosis and alkalosis are common, to develop gene therapies aimed at enhancing kidney regeneration. Such therapeutic strategies could revolutionize treatment approaches for kidney injuries in mammals by promoting cellular repair and regeneration.

Our study of the effects of pH stress on planarian behaviour, regeneration, and gene expression, particularly in the context of protonephridia, aligns with previous research exploring the mechanisms behind excretory system regeneration in planarians. The role of yorkie (Yki), a downstream effector in the Hippo signaling pathway, has been identified as crucial for maintaining the homeostasis of the excretory system and regulating stem cell proliferation (Lin and Pearson, 2014). This aligns with our findings, where pH stress appears to impact stem cell dynamics and, consequently, the ability of planarians to regenerate their excretory system. In addition, EGFR signalling has been shown to be essential for both the maintenance and regeneration

of the planarian excretory system (Rink *et al.*, 2011). This mechanism likely plays a role in the differential gene expression patterns we observed under varying pH conditions. Disruption of EGFR signaling could be linked to the impaired protonephridial function we detected, which may explain the observed behavioural and regeneration deficits. Further, stress exposure in planarians has been demonstrated to affect not only their physiological responses but also their epidermal integrity (Adler *et al.*, 2014; de Oliveira *et al.*, 2018). Our data corroborate these findings, suggesting that the epidermis' protective role is compromised under pH stress, potentially leading to the behavioural changes observed in our study. Thus, the collective insights from these studies support our hypothesis that pH stress disrupts both the structural integrity and the regenerative capacity of planarian excretory systems, mediated through key signaling pathways.

Research on planarian regeneration has significant potential to advance the field of regenerative medicine. By understanding the genetic and cellular mechanisms underlying planarian regeneration, we can gain valuable insights into similar regenerative processes in humans. This knowledge could pave the way for new treatments and therapies for a range of diseases and injuries, particularly those involving tissue damage and cellular loss.

Studying planarians provides a unique opportunity to explore fundamental aspects of regenerative biology that may be conserved across species, including humans. By deciphering how planarians activate and control their regenerative processes, we can enhance our understanding of human biology and improve our capacity to stimulate similar regenerative responses in human tissues.

As research techniques and technologies continue to evolve, planarian-based studies are poised to yield new and exciting discoveries. Future research could focus on the identification of novel genes and pathways involved in regeneration, the development of advanced gene-editing tools to manipulate these pathways, and the exploration of planarian models to simulate human disease conditions. Such efforts will not only deepen our understanding of regenerative biology but also open new avenues for the development of innovative therapies and treatments in regenerative medicine.

This study provides new insights into the effects of pH stress on planarian survival, behaviour, and gene expression, particularly focusing on their protonephridia, which shares functional similarities with the mammalian nephron. We demonstrated that planarians could not survive at extreme pH levels (pH 3, 9, and 10), but they tolerated acidic conditions better than alkaline ones. Interestingly, they showed an adaptive response by attempting to neutralize their environment after 72 h, suggesting the secretion of chemicals to mitigate external stress. This resilience underscores the potential for using planarians as models for environmental stress response and regenerative biology.

Our findings revealed that planarian neoblasts, particularly those responsible for protonephridial regeneration, were activated under pH stress, with key genes such as EGFR5, DNAH-β3, and cysteine-rich proteins showing significant upregulation. These genes may play a critical role in initiating regeneration processes and can serve as potential biomarkers for monitoring the health of aquatic species in changing climatic conditions.

This research aligns with the One Health approach, emphasizing the interconnectedness of human, animal, and environmental health. By understanding the molecular mechanisms of regeneration under environmental stress, we can explore gene-based therapies for kidney injuries in vertebrates, offering solutions for sustainable resource management in aquaculture. These insights contribute to the development of technological interventions that support biodiversity and ecosystem stability, which are essential in the context of climate change and achieving Sustainable Development Goals.

Conflict of interest: The authors have declared that no conflict of interest exists in the study.

Author's contribution: PP: Responsible for the culture maintenance of planarians, conducted behavioural studies, performed gene expression analyses, analyzed the data, and drafted the manuscript; BB: Contributed to the culture maintenance of planarians; ERB: Initiated the project, designed the experiments, analyzed the data, and wrote the manuscript.

Data availability statement: The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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