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Research Article

Epithelial regeneration and association of Innexin-11 and mtMMP-A in Planarian (*Dugesia japonica*), under temperature stress

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Abstract

Planarians, members of the phylum Platyhelminthes, exhibit remarkable regenerative capabilities, making them an ideal model for studying wound healing and tissue regeneration. Unlike mammalian models, which rely on scar tissue formation for wound repair, planarians regenerate lost body parts through the activity of abundant adult stem cells known as neoblasts. However, the underlying mechanisms of epithelial wound healing and their potential application to mammalian systems remain unclear. In this study, we used *Dugesia japonica*, a freshwater planarian species, to investigate the effects of temperature stress on regenerative potential, behavioral changes, and genomic expression of key candidate genes. We exposed 7-day starved planarians to high (31°C) and low (4°C) temperatures and analyzed their mortality rates, regeneration efficiency, and gene expression profiles. Our findings indicate a significant role of Membrane-type matrix metalloproteinase A (mt-MMP-A) and Innexin-11 (Inx-11) in epithelial wound healing, with stress-induced reductions in Inx-11 expression correlating with delayed regeneration and increased degeneration. Additionally, we observed an inverse relationship between Inx-11 and mt-MMP-A expression under stress, highlighting their regulatory role in epithelial tissue maintenance. Lamin-B expression, a marker of epithelial integrity, confirmed the detrimental effects of temperature stress on planarian tissue homeostasis. Moreover, extreme temperatures (3°C and 32°C) resulted in high mortality, emphasizing planarian thermal tolerance limits. This study enhances our understanding of regenerative biology under environmental stress, providing insights into climate change effects on animal health and potential translational applications for improving mammalian wound healing and regenerative medicine.

Keywords: Epithelium, Homeostasis, Regeneration, Temperature stress, Wound healing

Highlights

- Planarians exhibit regenerative capacity and survival differences under varying temperature stress
- There is a remarkable change in terms of planarian behavior under temperature stress
- Candidate genes including Inx-11 and MMP-A are associated with planarian epithelial wound healing
- Finding may contribute to the target identification for regenerative medicine

INTRODUCTION

Planaria, small flatworms from the phylum Platyhelminthes, are renowned for their extraordinary regenerative capabilities, earning them the nickname "immortal under the knife" (Chachain *et al*., 2020). These free-living organisms inhabit diverse freshwater environments and range in size from 3 mm to 15 mm, making them relatively inconspicuous despite their biological significance (Pagan *et al*., 2017). Their soft, ciliated bodies and spade-shaped heads, often accompanied by distinctive eye spots, have led to the moniker "cross-eyed worms" (Love *et al*., 2011). The most striking feature of planarians is their ability to regenerate any lost body part, including the head, tail, and internal organs, even when cut into multiple fragments (Salvetti *et al*., 2021). Each severed portion of a planarian can develop into a fully functional organism, a phenomenon driven by a population of pluripotent adult stem cells known as neoblasts (Zhu *et al*., 2016). Given their robust regenerative potential, planarians have long served as a valuable model for studying stem cell biology, tissue homeostasis, and regenerative medicine (Reddien, 2018). However, while the external aspects of their regeneration are well documented, the molecular mechanisms governing their epithelial wound healing under environmental stress remain poorly understood.

Epithelial wound healing is a fundamental biological process critical for maintaining tissue integrity, yet its efficiency varies across species. In mammals, wounds heal through scar tissue formation, which often leads to incomplete restoration of function and structure (Blanpain *et al*., 2014). In contrast, planarians can regenerate functional tissues rapidly,

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making them an ideal model for studying epithelial repair mechanisms. One crucial component of this process is the role of membrane-type matrix metalloproteinases (mt-MMPs) and Innexin proteins, which regulate tissue remodeling and intercellular communication. Matrix metalloproteinases (MMPs) are enzymes involved in extracellular matrix (ECM) remodeling, and among them, Membrane-type Matrix Metalloproteinase A (mt-MMP-A) plays a pivotal role in epithelial integrity and wound repair (Gotzmann and Foisner, 2006). Similarly, Innexin-11 (Inx-11), a gap junction protein, facilitates intercellular communication necessary for coordinated tissue regeneration. While these molecules are well studied in other biological systems, their function in planarian wound healing, particularly under temperature stress, remains largely unexplored. Immunohistochemical studies have highlighted the presence of these markers, but they provide limited insight into the genetic and epigenetic regulatory mechanisms driving epithelial regeneration. Furthermore, previous research has not fully addressed how environmental stressors such as temperature fluctuations impact the molecular pathways of planarian epithelial healing.

In this study, we aim to investigate the role of mt-MMP-A and Inx-11 in epithelial wound healing and tissue regeneration in *Dugesia japonica* under temperature stress. Our findings will contribute to a deeper understanding of the genetic and molecular basis of epithelial regeneration, with potential implications for wound healing research in mammalian systems. Furthermore, this study provides insights into the impact of environmental stressors, such as global warming, on tissue repair mechanisms, highlighting the broader significance of temperature fluctuations on animal health and homeostasis.

MATERIALS AND METHODS

Planaria maintenance: We used the planarian species *Dugesia japonica* as our model organism for this study. They were kept in a neutral buffer solution (1x planarian buffer) at a population density of not exceeding 150 individuals per beaker (≤150 worms/ 250 mL 1x planarian buffer). The key ingredients of the buffer are 5 M NaCl, 1 M $CaCl_2$, 1 M $MgSO_4$, 1 M $MgCl_2$, 1 M KCl, and 0.540 g of $NaHCO_3$, prepared in double-distilled water. The worms were kept in the 1x planarian buffer at the homeostatic temperature of 19°C in dark conditions. The worms were fed macerated chicken liver for 2 hours each week for their maintenance. The buffer was changed every 2-5 days and after each feeding cycle. For the experiment, all the planarians were starved for 1 week to maintain standardized conditions. Feeding may introduce various foreign molecules into the gut, which could interfere with molecular analyses like gene expression profiling. So, one-week starvation period ensured that the gut was clear, providing cleaner molecular data. By starving planarians for a week, our study ensured greater reproducibility, enhanced regenerative responses, and clearer molecular insights, making it an essential experimental step (Pal *et al*., 2024).

Temperature stress experiment: One-week starved *Dugesia japonica* planarians (n=15 per group) were placed in 50 mL culture media (1x buffer) and subjected to temperature stress at 2°C, 4°C, 19°C, 31°C, and 32°C. 19°C was maintained as the control group, as it is the optimum temperature for healthy planarian growth (Cao *et al*., 2020). Incubation periods were set at 0 hours, 24 hours, 48 hours, 72 hours, and 168 hours (Day 7). Morphological changes, mortality rates, and behavioral responses were recorded at each time point using a stereo-zoom microscope (Olympus, SZX7, Japan) to assess the impact of temperature fluctuations on planarian survival, regeneration, and stress-induced behavioral changes.

Behavioral studies: To assess behavioral changes in *Dugesia japonica* under temperature stress, parameters such as survival, mobility, size, and regeneration were evaluated. Mobility was measured by recording the number of boxes visited by 10 planarians over 5 minutes on centimeter graph paper. Regeneration was assessed by amputating the head, trunk, and tail of 10 planarians and monitoring the process for 30 days, with daily image capture using a stereo zoom microscope (Olympus, SZX7, Japan). Treated and untreated control groups were observed in sterile glass Petri plates, and gross behavior was studied without immobilization under low-intensity stereoscopic light (Pal *et al*., 2024).

RNA isolation: Total RNA was isolated from *Dugesia japonica* (n = 15 per temperature stress condition) using TRIzol reagent (Thermo Fisher Scientific, USA) following the manufacturer's protocol. Briefly, 750 µL of TRIzol was added to the planarians, followed by homogenization and repeated pipetting. The sample was incubated at room temperature for 5 minutes, and 200 µL of chloroform was added per 1 mL of TRIzol. After vigorous shaking for 15 seconds, the mixture was vortexed for 20 - 30 seconds, incubated for 5 minutes, and centrifuged at 12,000 g for 15 minutes

at 4°C. The upper aqueous phase was carefully transferred to a fresh tube, and 500 µL of chilled 100% isopropanol was added per 1 mL of TRIzol. The sample was incubated at -20°C overnight and centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant was discarded, and the RNA pellet was washed with 750 µL of 75% ethanol (chilled), vortexed briefly, and centrifuged at 7,500 g for 5 minutes at 4°C. The supernatant was discarded, and the pellet was air-dried. To ensure RNA integrity, 1 µL of each sample was run on a 1% agarose gel, and bands were visualized under UV light. The RNA purity and concentration were assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) at 260/280 nm. The measured concentration of RNA in each sample was 1.4 µg/µL (control), 1.2 µg/µL (31°C- 24 hr), 1.1 µg/µL (31°C- 48 hr), 1.2 µg/µL (heat stress day 7) respectively and 1.1 µg/µL (4°C - 24 hr), 1.2 µg/µL (4°C- 48), 1.2 µg/µL (cold stress day 7) respectively, for each sample 2 µg RNA was delivered for cDNA preparation. To eliminate genomic DNA contamination, isolated RNA samples were treated with RNase-free DNase I (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The quality of DNase-treated RNA was rechecked by agarose gel (1%) electrophoresis (Pal and Banerjee, 2024).

cDNA Preparation: cDNA was synthesized from 1 µg of total RNA using the Verso cDNA synthesis kit (Thermo Fisher Scientific, USA) following the standard protocol. The reaction mixture included reverse transcriptase, oligo-dT primers, dNTP mix, and reaction buffer. The reaction conditions were set at 42°C for 60 minutes, followed by enzyme inactivation at 85°C for 5 minutes. The synthesized cDNA was stored at -20°C for further analysis (Pal and Banerjee, 2024).

Polymerase Chain Reaction (PCR): PCR amplification of cDNA was performed using gene-specific primers for ACTB (housekeeping gene), Lamin B, INX-11, and MMP-A in a thermal cycler (Applied Biosystems, Veriti 96 Well Thermal Cycler, USA). The primer sequences used were (Table 1): ACTB [Forward- '5 ACACCGTACCAATCTATG 3'; Reverse- 5' GTGAAACTGTAACCTCGT 3'], Lamin B [Forward- '5 TGTGGGTAGCCTTTTCTTCTCCC 3'; Reverse- 5' CGCAAGGTTCAGGTGATCCG 3'], Innexin-11 (INX-11) [Forward- '5 AATGCCTTGCTTC TGATGCG 3'; Reverse- 5' CCCAAAATCCCGTTAC CCGA 3'], Membrane-type Matrix Metalloproteinase-A (MMP-A) [Forward- '5 TGGCTAAACGTTGCGGAA ATC 3'; Reverse- 5' CCAGCTCTGTTGTAGGGAGG 3'] (Yuwen *et al*., 2011). The standard PCR cycling conditions were as follows: Initial denaturation: 94°C for 3 minutes, Denaturation: 94°C for 30 seconds, Annealing: (Tm specific for each primer) for 30 seconds, Extension: 72°C for 30 seconds, Final extension: 72°C for 5 minutes. A total of 35 cycles were performed.

Agarose gel preparation and electrophoresis: PCR products were analyzed on a 1% agarose gel, prepared by dissolving 1.3 g of agarose in TAE/TBE buffer (pH 8.3). Ethidium bromide (10 µL) was added as a DNA intercalating agent, and the gel was cast in a prepared plate. DNA samples were loaded into the wells, and electrophoresis was performed at a constant voltage (90 V for 45 minutes). DNA bands were visualized using a Thermo Chemidoc imaging system (Invitrogen, iBright 1500, USA). Band intensity was quantified using ImageJ software with ACTB as the reference gene. Student's t-test was used for statistical analysis and data were presented as standard deviation (SD), with significance set at $p < 0.05$ (Pal and Banerjee, 2024).

RESULTS

Behavior study: Extreme temperature stress significantly affected the survival and physiological integrity of planarians (Fig. 1). At 3°C and 32°C, planarians exhibited severe degeneration, ultimately leading to complete body disintegration within 72 hours (Fig. 1A). Consequently, experimental observations focused on three groups maintained at 4°C (low temperature), 19°C (control), and 31°C (high temperature). Behavioral analysis demonstrated that planarians at 3°C and 32°C showed significantly (*p<0.05,

Table 1. List of primers used for determination of gene expression by reverse transcriptase PCR

Genes		Primer sequence	Tm(°C)
ACTB	F	5'-ACACCGTACCAATCTATG-3'	51.41
	R	5'-GTGAAACTGTAACCTCGT-3'	51.41
Lamin B	F	5'-TGTGGGTAGCCTTTTCTTCTCCC-3'	62.43
	R	5'-CGCAAGGTTCAGGTGATCCG-3'	61.40
Innexin-11	F	5'-AATGCCTTGCTTCTGATGCG-3'	57.30
	R	5'-CCCAAAATCCCGTTACCCGA-3'	59.35
mt MMP-A	F	5'-TGGCTAAACGTTGCGGAAATC-3'	57.87
	R	5'-CCAGCTCTGTTGTAGGGAGG-3'	61.40

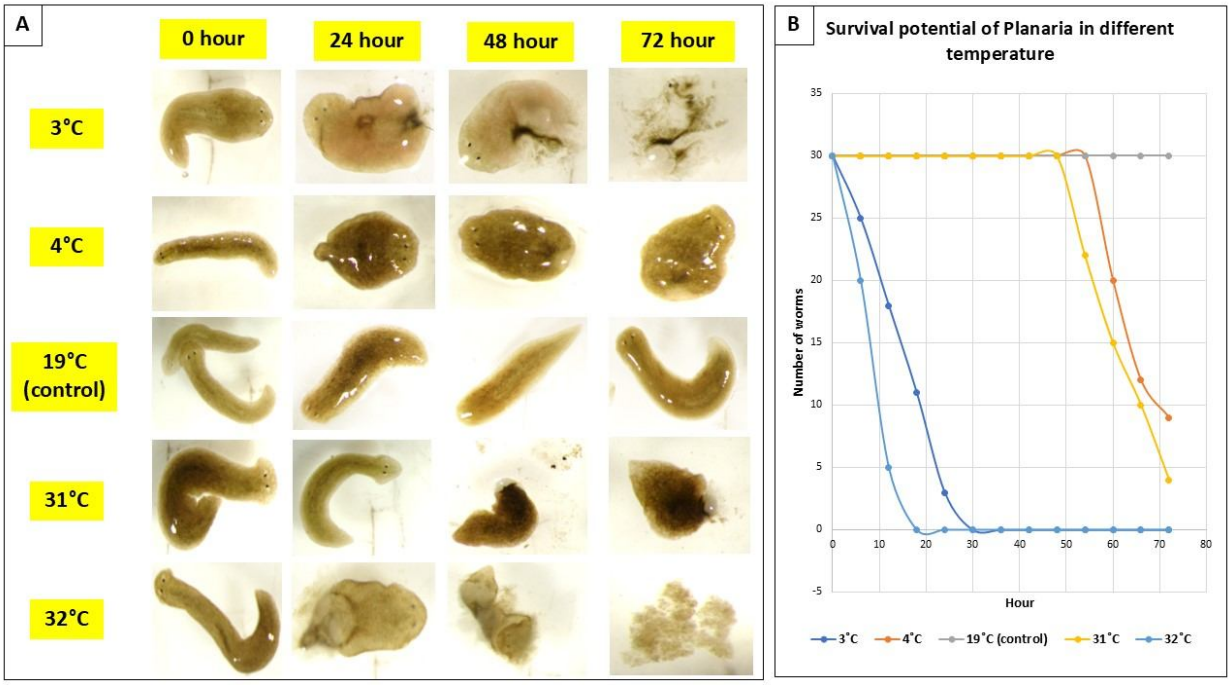

Fig. 1. The above pictures showed the behavioral changes of planarians in different temperatures [A] and the survival potential of planarians in different temperatures after 72-hour incubation [B].

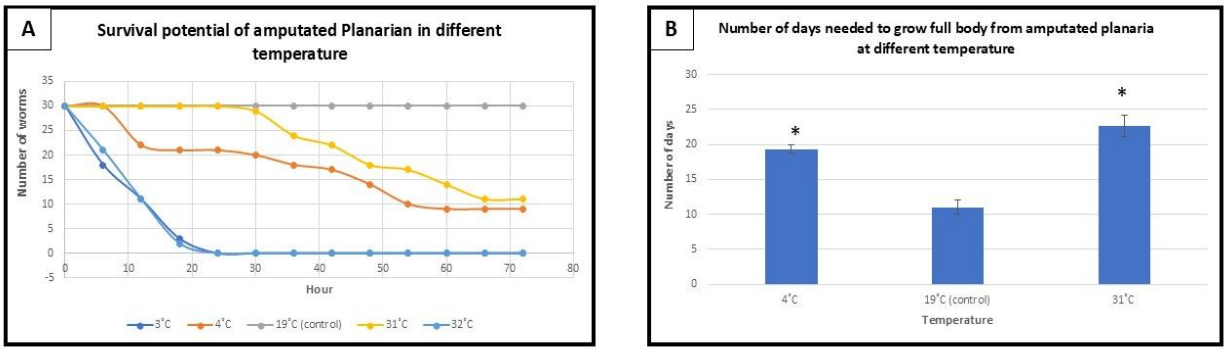

Fig. 2. Different parameters were examined at low and high temperature after 72 hours of incubations: [A] Survival potential of amputated planaria, [B] After amputation how many days were needed to regrow planarian whole body at low and high temperatures and 19°C was considered as control (*p<0.05, compared to control).

compared to control) reduced survival rates, with marked degeneration observed between 24 to 48 hours of incubation. Planarians in the 4°C and 31°C groups exhibited sluggish movement and morphological changes, including body curling and decreased locomotion compared to the control group at 19°C.

We investigated the survival potential of amputated planarians under different temperature stresses and we found that stressed amputated planarians failed to survive and regenerate under extreme temperature stress (3°C and 32°C) compared to tolerable stress conditions (4°C and 31°C) (Fig. 2A).

We also found that control planaria of 19°C took 12 to 14 days to grow their whole body but the planaria kept at 4°C and 31°C, took longer time to grow their whole body (Fig. 2B).

We evaluated the body size of planaria under temperature stress (Fig. 3A). Under heat stress (31°C), the body size of the worms decreased by 1.27- and 1.94- fold, respectively. However, upon returning to optimal conditions, body size gradually recovered, increasing by 1.04-, 1.06-, 1.09-, 1.17-, and 1.33- fold over seven days. Similarly, under cold stress (4°C), body size decreased by 1.83- and 2.55-fold (Fig. 3D)

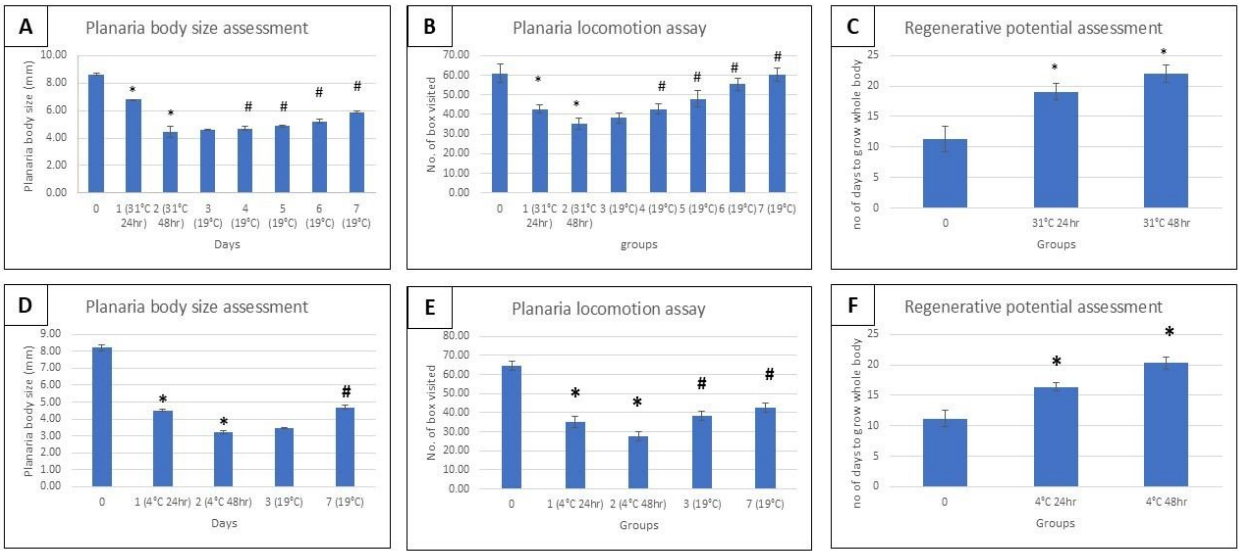

Fig. 3. Different parameters were investigated under temperature stress: [A] Size of the planarians under heat stress (31°C), [B] The mobility of planaria under heat stress (31°C), [C] Survival potential of amputated planaria under heat stress (31°C), [D] Size of the planarians under cold stress (4°C), [E] The mobility of planaria under cold stress (4°C), [F] Survival potential of amputated planaria under cold stress (4°C) (*p<0.05, compared to control, #p<0.05, compared to 48 hours of temperature stress).

but was restored under ideal conditions, increasing by 1.07- and 1.46- fold over seven days.

Locomotion was also affected by temperature stress. Under heat stress (31°C), locomotor activity decreased by 1.43- and 1.73-fold (Fig. 3B), but upon returning to optimal conditions, locomotion improved, increasing by 1.08-, 1.21-, 1.36-, 1.57-, and 1.71-fold over seven days. Under cold stress (4°C), locomotion decreased by 1.85- and 2.34-fold (Fig. 3E) but was restored upon returning to normal conditions, increasing by 1.39- and 1.54- fold over seven days.

To assess the regenerative potential of amputated planaria under stress, 30 transversely amputated planaria were subjected to temperature stress for 72 hours. The time required for complete body regeneration was recorded. The average number of days taken for full regeneration increased by approximately 1.81-fold and 1.99-fold (Fig. 3C) in the cold stress (4°C) (Fig. 3F) and heat stress (31°C) (Fig. 3C) groups, respectively (*p<0.05, compared to control, #p<0.05, compared to 48 hours of temperature stress).

Study of the gene expression of planarians in different temperature stress by RT PCR: Gene expression analysis via RT-PCR further elucidated the molecular effects of temperature stress on key genes (Fig. 4). The Inx-11 gene, which plays a crucial role in intercellular communication, exhibited a significant (P<0.05) downregulation at 31°C, with a 3.55-fold decrease at 24 hours and a 3.06-fold decrease at 48 hours, followed by an upregulation of 2.66-fold at day 7, suggesting a compensatory response during regeneration (Fig. 4C). Similarly, the Lamin B gene, essential for nuclear envelope integrity, showed significant downregulation at 31°C, with expression levels decreasing by 1.74- fold at 24 hours and 2.97-fold at 48 hours, followed by further suppression at day 7 (2.06-fold reduction) (P<0.05) (Fig. 4B). mtMMP-A expression declined at 24 hours (2.64-fold decrease) and 48 hours (2.85-fold decrease) but exhibited a 4.59-fold increase at day 7, indicating differential stress responses based on temperature variations (Fig. 4D). In contrast, at 4°C, Lamin B expression declined at 24 hours (1.74-fold decrease) and 48 hours (2.21-fold decrease) but exhibited a 1.46-fold increase (P<0.05) at day 7, indicating differential stress responses based on temperature variations(P<0.05) (Fig. 4E).

The mtMMP-A gene, a metalloproteinase involved in extracellular matrix remodeling, exhibited a drastic downregulation under cold stress, with a 2.86-fold reduction (P<0.05) at 24 hours and a 4.13-fold decrease (P<0.05) at 48 hours, but a subsequent 9.14-fold increase (P<0.05) at day 7, indicating activation during regeneration (Fig. 4G). Similarly, at 4°C, Inx-11 expression decreased by 2.39-fold (P<0.05) at 24 hours and 2.57-fold (P<0.05) at 48 hours, followed by a compensatory 2.98-fold increase (P<0.05) at day 7, suggesting temperature-dependent modulation of

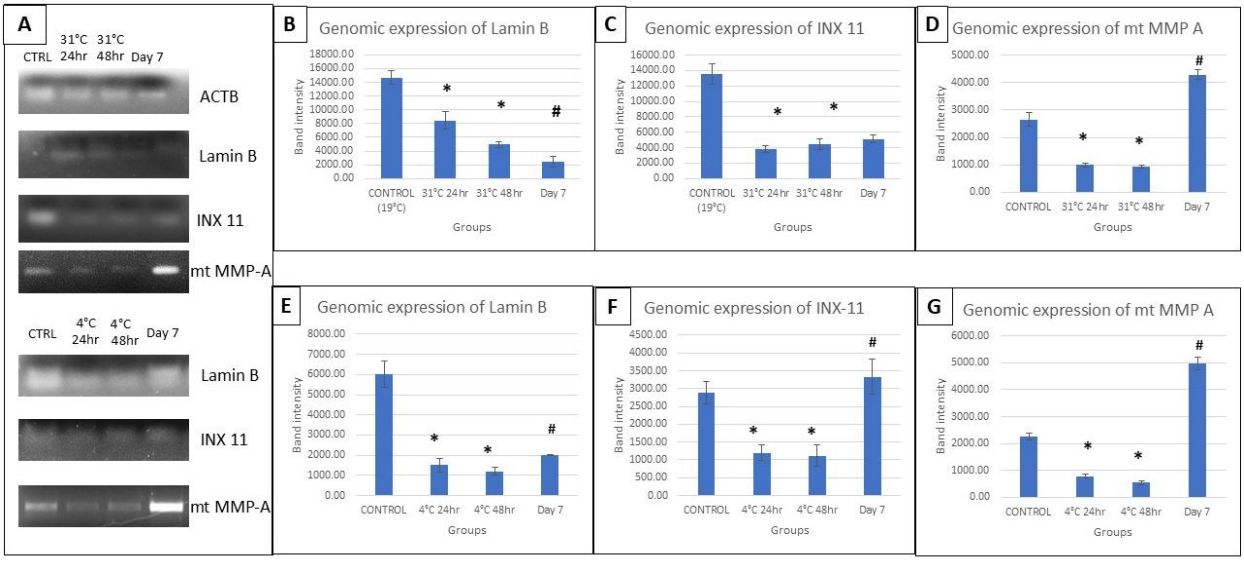

Fig. 4. Study of the gene expression of planarians in different temperature stress by RT PCR (*p<0.05, compared to control, #p<0.05, compared to 48 hours of temperature stress).

cellular and molecular responses (Fig. 4F). ACTB was used as the housekeeping gene for normalization across all expression studies.

DISCUSSION

The present study demonstrated that elevated temperatures significantly influenced planarian physiology, impacting body size, mobility, and regenerative capabilities. The findings highlighted the critical role of Inx-11 and mtMMP-A in planarian epithelial regeneration under thermal stress conditions. We exposed 7-day starved planarians to high (31°C) and low (4°C) temperatures to assess the impact of thermal stress on their regenerative potential, behavioral responses, and gene expression profiles. We hypothesized that temperature stress would modulate the expression of mt-MMP-A and Inx-11, influencing wound healing efficiency and overall tissue integrity. By analyzing mortality rates, regeneration speed, and gene expression levels, we seek to elucidate the molecular pathways involved in stress-induced epithelial repair. Additionally, we assessed Lamin-B expression as an epithelial marker to determine the structural effects of temperature stress on planarian tissues (Molina *et al*., 2021). Given that many wound-induced genes are conserved across metazoans, understanding these genes and their underlying mechanisms has been crucial in elucidating wound signaling and the initiation of regeneration (Poss *et al*., 2010).

This study provided insights into temperature-dependent behavioral changes in planarians. The mortality rates observed at extreme temperatures of 3°C and 32°C suggested that these temperatures exceeded the tolerance limit of planarians, leading to significant physiological stress. These findings were consistent with previous studies indicating that extreme temperatures compromised planarian survival by disrupting cellular homeostasis. Furthermore, the regenerative potential of planarians was adversely affected under stress conditions compared to homeostatic conditions. The delayed regeneration of lost body parts under thermal stress indicated a reduction in regenerative efficiency, aligning with earlier observations on the effects of environmental stressors on planarian regeneration (Rink, 2012).

Locomotion and body size analysis also revealed temperature-dependent changes in the planarian population. Temperature stress led to a decline in locomotor activity and body size, suggesting injury and degeneration. These findings corroborated previous research indicating that environmental stressors influenced planarian behavior and physiology by affecting neoblast proliferation and differentiation (Reddien *et al*., 2005).

Genomic expression analysis further substantiated the role of Inx-11 and MMP-A in epithelial regeneration. The results indicated a decrease in Inx-11 expression under heightened stress and degenerative conditions. Given that Inx-11 is a junction protein involved in cellular communication, its downregulation under stress suggested impaired intercellular signaling essential for epithelial maintenance and regeneration (Oviedo *et al*., 2010).

Conversely, the expression levels of MMP-A, a matrix metalloproteinase associated with extracellular matrix remodeling, were differentially regulated under both heat and cold stress conditions. This finding was consistent with previous reports demonstrating the involvement of MMPs in tissue remodeling and wound healing (Molina *et al*., 2021). The inverse relationship between Inx-11 and MMP-A expression levels under stress indicated their crucial role in planarian epithelial regeneration.

Additionally, the study validated the effectiveness of temperature stress in inducing epithelial injury in planarians, as evidenced by the expression of Lamin-B, an established epithelial marker. Lamin-B has been widely recognized as an indicator of epithelial integrity and its expression pattern under stress conditions reinforced its reliability in assessing temperature-induced epithelial damage (Molina *et al*., 2021). Being the epithelial marker of planarian epithelial cells, the observed changes in Lamin-B expression suggested that epidermal integrity was compromised under temperature stress as the genomic expression level of Lamin-B was downregulated under stress, leading to behavioral and genomic alterations.

Overall, the findings supported the hypothesis that Inx-11 and MMP-A played essential roles in epithelial degeneration and regeneration. Furthermore, the results suggested that these genes may have been involved in neoblast regulation and maturation, which are critical for the regeneration process. Given that neoblasts serve as the primary stem cell population responsible for regeneration in planarians, the association between neoblast activity and the expression of Inx-11 and MMP-A highlighted the mechanistic link between cellular stress responses and regenerative capacity (Reddien *et al*., 2005).

In conclusion, this study underscored the impact of temperature stress on planarian body size, mobility, and regeneration. The results provided substantial evidence that Inx-11 played a pivotal role in planarian epithelial regeneration, while MMP-A exhibited differential expression under heat and cold stress conditions. The findings also suggested that the neoblast population, essential for planarian regeneration, was closely associated with the expression of Inx-11 and MMP-A, indicating their potential role in neoblast regulation. Given that these genes are also present in mammalian organisms, including humans, they may represent promising targets for regenerative medicine and gene therapy. Future research should explore the translational potential of these genes in human regenerative processes, which could contribute to advancements in tissue engineering and wound healing therapies.

Conflict of interest: There is no conflict of interest.

Author's contribution: BB: Responsible for the culture maintenance of planarians, conducted behavioral studies, performed gene expression analyses, analyzed the data, and drafted the manuscript; PP: Contributed to the culture maintenance of planarians and drafted the manuscript; 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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