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TRPA-1 in Planaria: Exploring Regeneration and Avoidance in the Presence of Irritants

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ABSTRACT

Planaria are a genus of freshwater flatworms widely studied for their regenerative properties. Regeneration is possible because planarians have large populations of stem cells, called neoblasts, distributed throughout their bodies. Planaria and humans share a gene that encodes for the transient receptor potential ankyrin 1 (TRPA-1), which is an ion channel that, in humans, activates pain, cold, and itch reception. In planarian cells, TRPA-1 is a receptor for heat and chemical irritation, and it is activated by reactive oxygen species produced in response to subsequent tissue damage. In this study, we investigate TRPA-1 activation in amputated *Dugesia dorotocephala*. In the first experiment, we aim to explore TRPA-1 activation during the planarian regeneration process. We exposed amputated worms to three known activators of TRPA-1 during regeneration and observed regeneration over 7 days. In the second experiment, we explored the relationship between TRPA-1 distribution and irritant avoidance. We placed amputated worms onto plates with irritants and tested the amputated worm pieces' individual avoidance. We observed that while TRPA-1 may not be directly involved in regeneration, activation in response to irritants can slow secondary tissue regeneration. Additionally, we observed that irritant avoidance is stronger in worm heads, where TRPA-1 is primarily localized.

Background

Regeneration in Planarians

Planarians are used as a model organism for humans because of their bilaterally symmetric and simplistic nervous systems. These freshwater flatworms are also widely studied for their regenerative properties because one fifth of their cells are neoblasts, which allow regeneration of virtually any body part after amputation within approximately seven days (Bohr et. al, 2021). These neoblasts are pluripotent stem cells, meaning they differentiate into any of the tissues in the planarian. Planarians are known to activate stem cell division with extracellular signal regulated kinase (ERK), which is a known pathway for cell differentiation and proliferation (Bohr et. al, 2021, Busca et al, 2016). The exact mechanism of stem cell differentiation and proliferation is not well understood in planarians except for the ERK pathway. Bohr et. al hypothesize that planarians have an initial tissue repair response to heal the wound, and after the initial response, planarians have a more specific regenerative response for the damaged organs. Organs span the entirety of the body, so amputation damages many parts of the organism. The initial wound healing response is completed soon after amputation, but the secondary tissue response occurs over the course of a week as many organs need repair. Planarians are simplistic and regeneration is easy to observe, and as such are the objects of current study.

TRPA-1: Chemical and Heat Response

Transient receptor potential cation channel, or TRPA-1, is a pathway shared by many organisms, including humans and planarians. In planarians, TRPA-1 genes are concentrated in the head region of the planarians, and are responsible for heat and chemical avoidance, but this pathway is not activated by those irritants directly. Tissue damage leads to production of reactive oxygen species, which in turn activates TRPA-1 expression (Gallio et. al 2017). In previous studies, mutants where TRPA-1 was knocked out using RNAi showed no avoidance of hot zones, clearly showing that TRPA-1 is a main factor in heat avoidance. TRPA-1 is also involved in chemical avoidance of irritants like allyl isothiocyanate, indicating TRPA-1 is a central gene in the planarian response to irritants. Activation of TRPA-1 leads to morphological responses including body scrunching, wherein planarians curl up to avoid the stimulus. Motility in planarians does not decrease as a result of high heat and TRPA-1 activation, however the worms were killed when exposed for too long to temperatures above 30°C (Cao et. al 2020). TRPA-1 activation is easy to visualize due to the scrunching behavior in planarians, and is activated by easily attainable irritants.

Introduction

Regeneration and irritant avoidance have been studied separately in planarians, but the effect of TRPA-1 on regeneration is not understood. The current study seeks to understand a potential link between TRPA-1 activation and regeneration, and how exposing planarians to irritants during the regeneration process affects the outcome. We investigated planarian regeneration further by disrupting the mechanism with a well understood pathway. We investigated the following questions with two separate experiments: Does the activation of the TRPA-1 pathway change how planarians regenerate? Does TRPA-1 concentration in the worms lead to decreased heat avoidance in amputated worms?

Experiment One: TRPA-1 and Regeneration

TRPA-1 and stem cells are two separate mechanisms in the planarians but may influence each other when activated simultaneously. In Experiment One, we examined regeneration in the presence of capsaicin, hydrogen peroxide and heat, which are all activators of the TRPA-1 pathway (Sabry et. al, 2019). We hypothesized that worms that received more exposures would show defective or slower regeneration.

We designed our exposure conditions to target critical times in regeneration. Since regeneration takes place over 4-7 days, we exposed one group of the worms to irritants immediately after cutting them and every two days afterwards for a week, directly interfering with the 2 day critical period of stem cell proliferation for the pharynx (Bohr et. al, 2021). After targeting pharynx regeneration, we timed the repeated exposures to irritants to target the rest of tissue regeneration, which takes longer to complete.

Experiment Two: Irritant Avoidance in Amputated Planarians

In Experiment Two, we investigated TRPA-1 distribution and heat/chemical avoidance by bisecting worms and testing the individual responses of segments to heat/capsaicin. TRPA-1 genes are concentrated in the head region of the planarians, so we hypothesized that head regions will have a stronger heat and chemical avoidance response than tails.

Materials and Methods

All worms used were Dugesia dorotocephala.

Experiment One

As a negative control group for this experiment, six worms were horizontally bisected to separate their heads and tails. The heads and tails were then placed in well-plates with spring water and monitored over a ten-day period. All individual head and tail regeneration progressions were photographed and compared to regeneration of the irritant groups. Experiment One involved exposing 6 bisected worms to 6 different conditions: 1-time capsaicin exposure, 3-time capsaicin exposure, 1-time heat exposure, 3-time heat exposure, 1-time hydrogen peroxide exposure, and 3-time hydrogen peroxide exposure. All worms were given 7 days to regenerate.

Capsaicin 1-Time Exposure

50ml of 0.015% capsaicin solution was prepared using 48.3 ml spring water and 0.667g cayenne powder. 3 ml of capsaicin solution was pipetted into each well of the 12-well capsaicin 1-time exposure plate. 3 ml of spring water was pipetted into each well of the 12-well capsaicin 1-time exposure regeneration plate. 6 worms were cut by placing a whole worm on top of a small petri dish filled with ice under a dissection microscope. The cool temperature of the petri dish slows worm movement, making them easier to bisect. Worms were segmented into a head and tail using a sterile scalpel. Each segment was pipetted into a well of the capsaicin 1-time exposure plate. During the eight minute exposure time, the wells were stirred gently to resuspend the cayenne in the water. After exposure time, each head and tail was pipetted up and down in spring water to wash off residual cayenne. All worm pieces were placed in respective wells in the 12-well capsaicin 1-time exposure regeneration plate and photographed with a camera attached to the dissection microscope. The worms were left to regenerate for seven days following the immediate exposure, being photographed and checked for motility on Day 1, 2, 3, 5, and 7.

Capsaicin 3-Time Exposure

Capsaicin solution, exposure plates, and regeneration plates were prepared according to the procedure listed above. Planaria were cut, exposed, and photographed according to the procedure listed above. On Day 3 and Day 5 of regeneration, an additional exposure plate and regeneration plate were prepared following the same procedure above. The worms were transferred into the exposure plate for eight minutes, then washed and placed into a fresh regeneration plate. The worms were photographed immediately after each exposure on exposure days. The 3-time exposure worms were photographed and monitored for motility levels on Day 1, 2, 3, 5, and 7.

Hydrogen Peroxide 1-Time Exposure

50 mL of 0.015% hydrogen peroxide solution was prepared using 49.75 ml of water and 250 µl of 3% hydrogen peroxide. The procedure was identical to the capsaicin 1-time exposure except for irritant type and exposure time: worms were placed in hydrogen peroxide solution for thirty minutes. Hydrogen peroxide 1-time exposure groups were photographed and monitored for motility levels on Day 1, 2, 4, 6, and 7.

Hydrogen Peroxide 3-Time Exposure

Procedure is the same as the capsaicin 3-time exposure procedure except the worms were exposed to hydrogen peroxide for 30 minutes instead of capsaicin for 8 minutes. Worms were photographed and monitored for motility levels immediately after each exposure and on Day 1, 2, 4, 6, and 7.

Heat 1-Time Exposure

12, 1.5ml microfuge tubes were filled with 500μL of spring water each and placed in a hot water bath at 37°C. 6 worms were horizontally cut and each individual head/tail was transferred into one of the 12 tubes. Worms were heated at 37°C for 10 minutes. One 12-well heat regeneration plate was prepared with 3ml of spring water in each well. Heads and tails were transferred to the well plate and photographed immediately. Worms were monitored for motility levels and photographed on Day 1, 2, 4, 6, and 7.

Heat 3-Time Exposure

The same procedure for heat 1-time exposure was repeated for the 3-time exposure group on Day 1, 2, and 4. The heat experiments and the 1-time hydrogen peroxide group trials began one day after the capsaicin groups, so exposure times for the 3-time exposure heat groups were shifted forward by one day, and monitoring days for all three groups were shifted forward one day.

Wash Control Group

The washing procedure for capsaicin and hydrogen peroxide groups is a possible stress factor that may interfere with results. As such, we prepared a control group to test for washing stress. 3 worms were horizontally bisected, washed by the same procedure as the previous chemical irritant groups, and placed in 5ml spring water in a 6-well regeneration plate. These worms were not exposed to any irritants.

Experiment Two: Heat Avoidance

Experimental Setup

A large petri dish was placed onto a heat block at 60°C. The 60°C portion was underneath the right ⅓ of the dish, and the unheated strip on the edge of the heat block was underneath the middle third of the petri dish. Finally, a petri dish filled with ice was stacked underneath the left hand side of the petri dish, creating three temperature zones (see Fig. 3b). Temperature zones were labeled and lines were drawn to distinguish zones. Fresh 55°C spring water was poured into the hot zone of the plate and room temperature spring water was poured into the cold zone. The dish was left for 15 minutes so that temperature zones were established. During testing, the temperature of the water in the hot zone was between 34-39°C, the neutral zone was between 22-25°C, and the cold zone was between 17-20°C (temperature monitored with a surface reading digital thermometer). Water was left undisturbed for the duration of the experiment to prevent mixing of the temperature zones. Convection of the water would occur if the plate was left to sit for over 15 minutes, therefore worms were added as soon as the desired temperature zones were established.

Control Group

10 uncut worms were gently pipetted into the center of the neutral zone of the plate. Worms were timed until they crossed over into the cold zone of the plate. The cold zone was designed colder than room temperature to combat convection. The neutral zone was slightly hotter than room temperature, so the cold zone was designed to be slightly below room temperature. Room temperature is the ideal temperature for planarians to survive in.

Horizontal Bisection Group

Water in the plate was replaced in the petri dish according to the procedure for heat avoidance experimental set-up. Water was left to sit in the petri dish for 15 minutes until the temperature zones were established. 10 worms were horizontally bisected according to the procedure above. Heads and tails were gently pipetted into the center. The position of heads and tails was noted so the time to cross could be accurately measured.

Vertical Bisection Group

Water in the plate was replaced in the petri dish according to the procedure for heat avoidance experimental set-up. Water was left to sit for 15 minutes until heat zones were established. 10 planarians were vertically bisected and separated into right and left sides (see Fig. 3c). Halves were gently pipetted into the spring water and timed until they crossed over.

Experiment Two: Chemical Avoidance

Experimental Setup

To make agar gel, 3g of 3% agar gel was added to 100ml of water and boiled in the microwave until the powder dissolved. The agar was cooled to 55°C and separated into two 50 ml tubes. 2g ground cayenne powder was added to one of the agar tubes. A foam pad was then used to elevate 3 small petri dishes at a slight angle (see Fig. 3a). 12 ml of the cayenne agar was poured into each of the four plates and allowed to harden for 20-30 minutes. The plate was returned to a flat surface and 12 ml of agar solution without cayenne was poured on top to completely cover the cayenne agar. The plates were left to harden for 20-30 minutes, allowing the agar to solidify and cayenne to diffuse upward into the agar to create a gradient. 4-5 ml of spring water was added on top of each plate after hardening so the worms would be able to swim across the plate during the experiments.

Control Group, Horizontal Bisection, Vertical Bisection

After plate setup, the experimental procedure for chemical gradient was the same as the heat avoidance method. The three worm groups were placed in the middle of their three different plates and timed until they crossed to the side with the lowest concentration of capsaicin.

Results - Experiment 1

Materials

- Fish media
- Sterile scalpels
- 4, 6-well plates
- Stock planaria containers
- Dissection microscope
- Water
- 9, 50mL large tubes
- .15% cayenne powder
- 0.15% capsaicin for arthritis relief
- 3% hydrogen peroxide
- 3% agar powder
- 2 small petri dish filled with ice
- 3 extra large petri dishes
- 1 regular sized petri dish
- Transfer pipettes
- Planarian stock containers
- Incubator
- 36, 1.5 ml microfuge tubes
- Thermometer
- Cayenne powder (.15%)
- Vortex
- 13, 12-well plates
- Hot water bath at 37°C
- Mortar and pestle
- Heat block
- Infrared thermometer
- Microwave
- Hot hands
- Timer

Control Group Worms: Figure 1a

Control group worms demonstrated proper regeneration. By Day 3, both head and tails closed the initial amputation wound with freshly regenerated tissue. By Day 5, signs of eye regeneration were visible in the tail, and by day 7, a new set of eyes had formed. By Day 9, the head had started to properly regrow and elongate the amputated tail, and the tail regenerated some of the missing head tissue. Newly regenerated tissue appears white under the microscope in all worms (control and experimental). All control worms demonstrated high motility for every single day, so no motility charts were recorded.

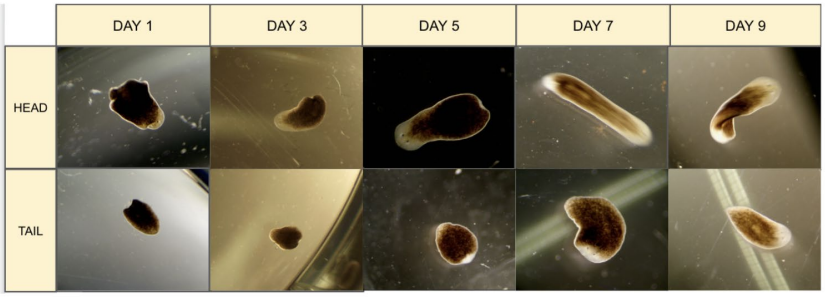


Figure 1a. Dissection microscope photos of horizontal- bisected control planarian regeneration over 9 days.

Capsaicin Worms: Figures 1b and 2a-d

Surviving capsaicin-exposed worms all showed signs of tissue damage. The tissue in the center of the worm pieces was blackened and contained spots, showing burns. The initial amputation wound was largely healed by Day 3 for both exposure times. By Day 7, the 1-time exposure tails showed newly formed eyes. The 3-time exposure heads showed minimal regeneration outside of an initial wound healing response. Many worms died in the process of regeneration, as seen in the 3-time exposure tails. Many worms (not pictured) seemed to disintegrate in the days after exposure, showing extreme tissue damage due to the irritant. 1-time exposure tails had lower mortality rates(50%) than the 1-time exposure heads (67%). In both cases, no worms died after Day 2. In the 3-time exposure groups, worm heads had lower mortality rates (50%) than the worm tails (100%), but neither group had stable motility throughout the week. Motility shifted with each exposure, and many demonstrated weak motility early in the week. In 3-time exposure heads, motility decreased from Day 3 to 5 in between exposures. However, motility of one worm changed from weak to strong between Day 5 and 7 after exposures stopped.

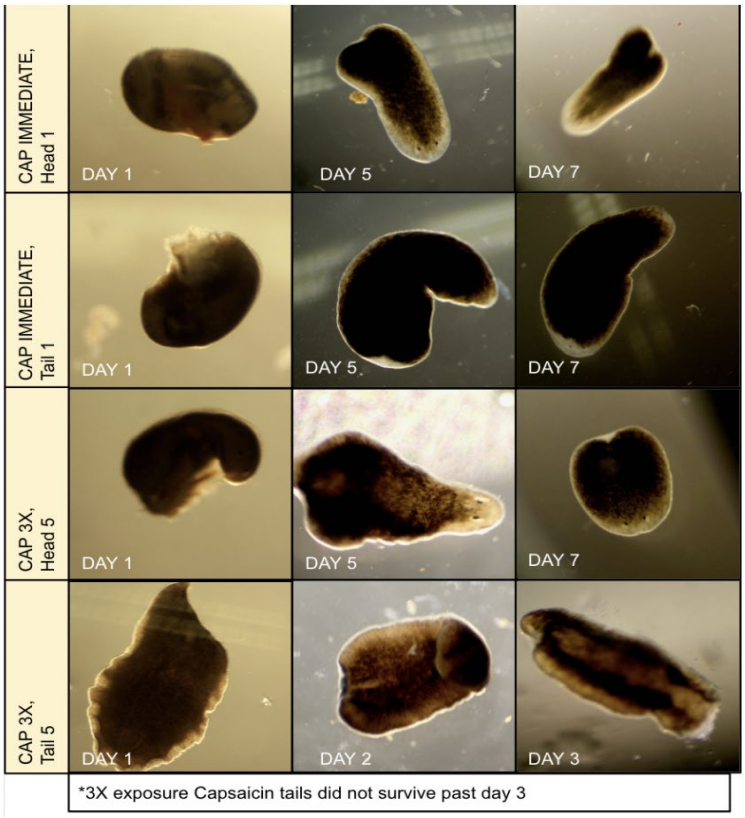


Figure 1b. Dissection microscope photos of regeneration over multiple days of 3-time capsaicin exposure groups (first two columns from the left) and 1-time capsaicin exposure groups (right two columns). 3-time capsaicin exposure tails did not survive past day 3.

Hydrogen Peroxide Worms: Figures 1c and 2e-h

Hydrogen peroxide-exposed groups also showed significant signs of tissue damage. These worms had higher mortality rates in almost all groups than the capsaicin worms. The Day 3 hydrogen peroxide 3-time exposure tail (Fig. 1c) shows what most worms from hydrogen peroxide groups looked like by the end of the week, showing a loss of shape of the worm and destruction of body tissue. The surviving worms show the initial wound healing tissue response, visible in the white regions of the body. The Day 7 3-time exposure head can be seen pooping, visible in its pushed out pharynx and the smears on the plates. By the end of the week in the 1-time exposure groups, worm tissues appear lighter than at the beginning of the week, showing tissue healing. Damaged tissue appears black, so the absence of black tissue shows regeneration. However, in the 3-time hydrogen peroxide exposure groups, the tissue still appears dark, showing the effects of multiple irritant exposures. In both 1-time and 3-time exposure groups, worm heads demonstrated significantly stronger motility than tails (33% vs 17%). Mortality rates were also far higher in hydrogen peroxide worms than capsaicin worms for all groups. Finally, 1-time exposure worms demonstrated either higher or the same motility as the 3-time groups.

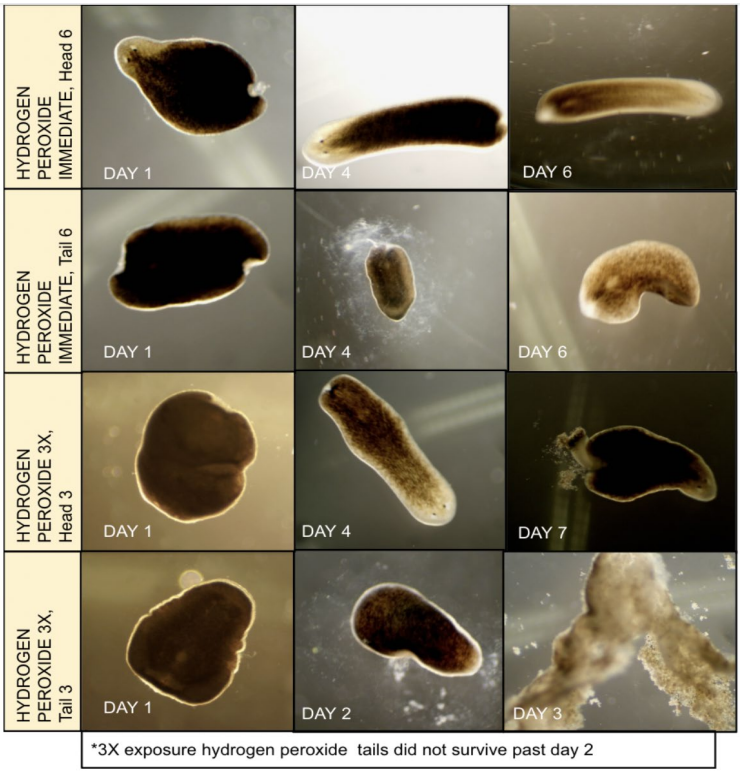
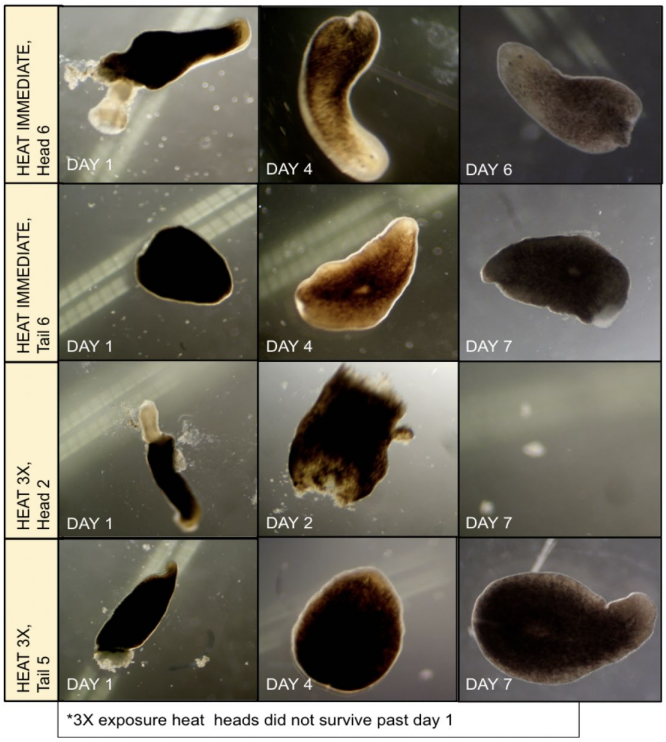


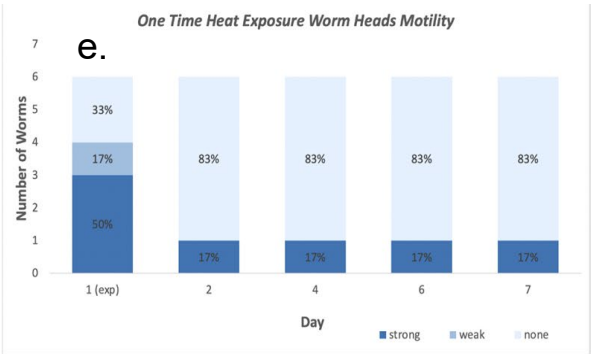
Figure 1c. Dissection microscope photos of regeneration over multiple days of 3-time hydrogen peroxide exposure groups (first two columns from the left) and 1-time hydrogen peroxide exposure groups (right two columns). 3-time hydrogen peroxide exposure tails did not survive past day 2.

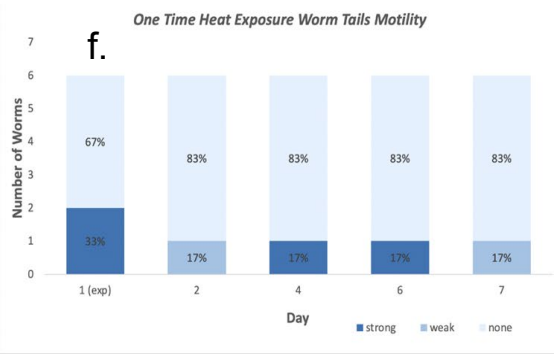
Heat Worms: Figures 1d and 2i-l

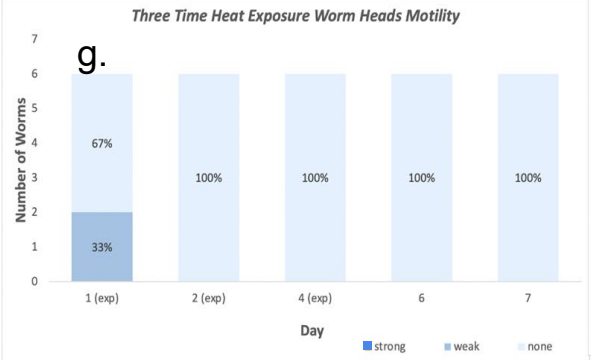
Almost all worms died during the heat exposure trials, so the images in Fig. 1d are of the sole surviving pieces. Like the hydrogen peroxide groups, many worms appeared to disintegrate in the water following exposures. As such, dead worm tissue was not even present in the water by Day 7, such as the Day 7 3-time heat exposure head. Pharynx ejection was a common phenomenon after the first exposure on day 1.These worms had blackened tissue through day 4, and for the 3 time exposure group, day 7. Initial wound healing was once again visible in all surviving worms, but secondary tissue does not appear to be regrown. The worms appear short and unhealed by Day 7, not demonstrating tail elongation or proper head regrowth. Neither of the two surviving tails regenerated eyes by Day 7. 1-time heads demonstrated the same mortality rates as one time tails (83%). 3-time exposure tails had lower mortality (83%) than the 3-time heads (100%). Heat worms had the highest mortality rates out of all three irritants, as well as weaker overall motility. Most other groups showed strong motility

by Day 7 in surviving worms, but both 1- and 3-time exposure tails showed only weak motility.









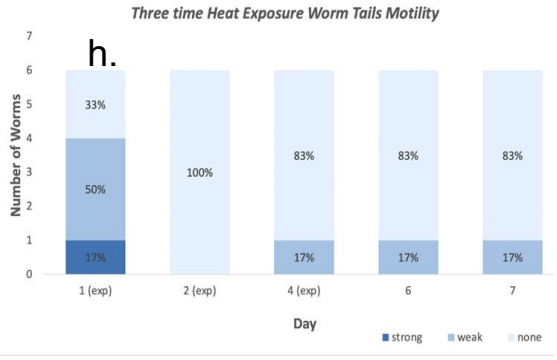
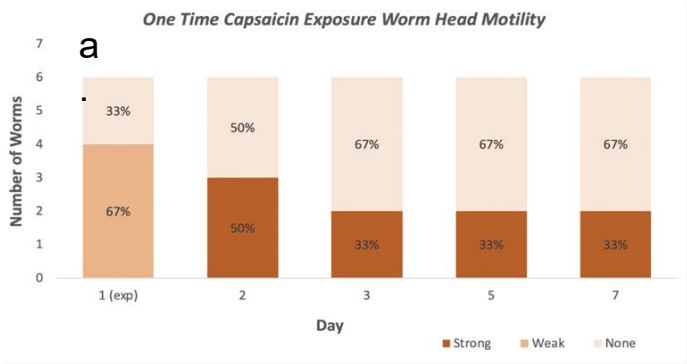
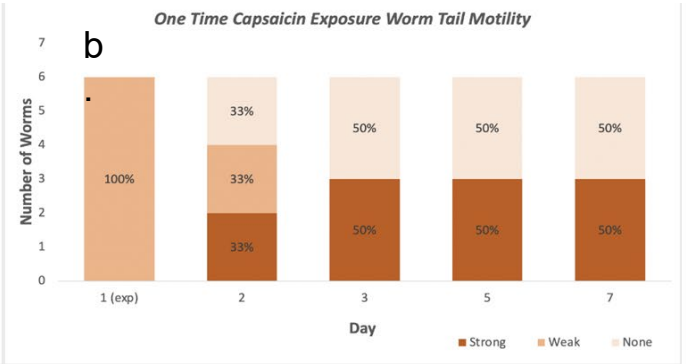
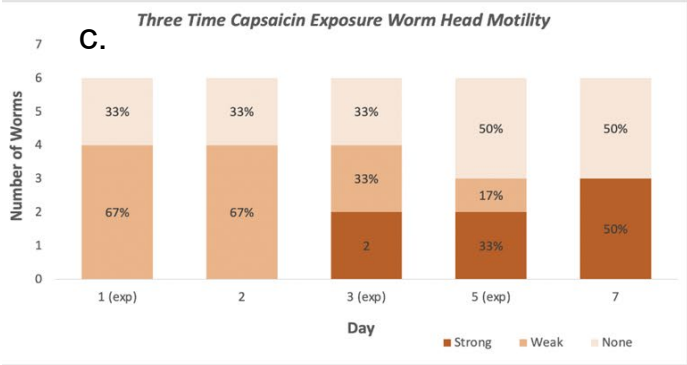
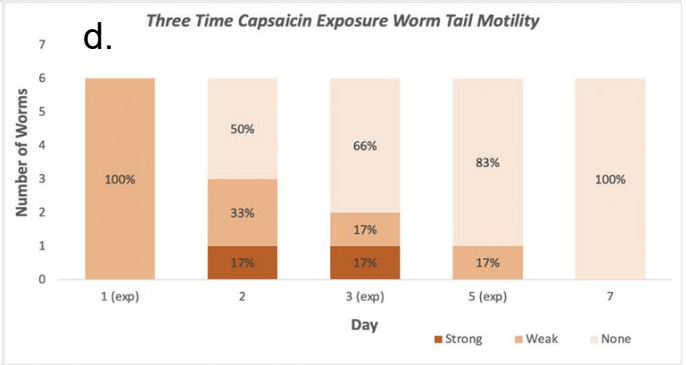


Figure 1d. Dissection microscope photos of regeneration over multiple days of 3-time heat exposure groups (first two columns from the left) and 1-time heat exposure groups (right two columns). 3-time heat exposure tails did not survive past day 1.


One Time Capsaicin Exposure Worm Head Motility
a.
Number of Worms
Day
1 (exp)
2
3
5
7
33%
67%
50%
50%
67%
33%
67%
33%
67%
33%
Strong
Weak
None


One Time Capsaicin Exposure Worm Tail Motility
b.
Number of Worms
Day
1 (exp)
2
3
5
7
100%
33%
33%
33%
50%
50%
50%
50%
50%
Strong
Weak
None


Three Time Capsaicin Exposure Worm Head Motility
c.
Number of Worms
Day
1 (exp)
2
3 (exp)
5 (exp)
7
33%
67%
33%
67%
33%
2
33%
33%
17%
50%
50%
50%
Strong
Weak
None


Three Time Capsaicin Exposure Worm Tail Motility
d.
Number of Worms
Day
1 (exp)
2
3 (exp)
5 (exp)
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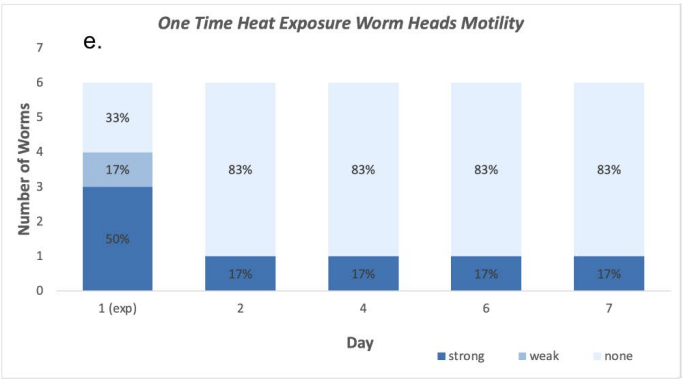
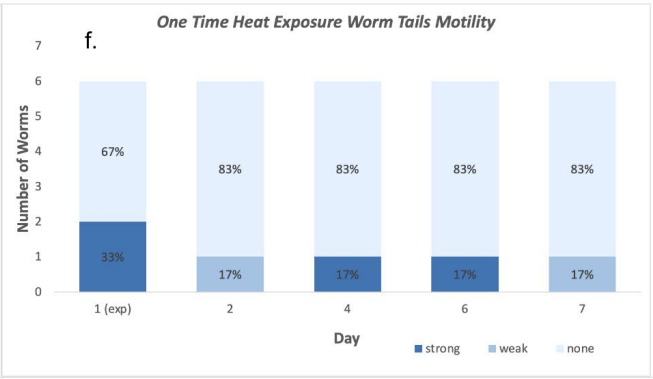
100%
50%
33%
17%
66%
17%
17%
83%
17%
100%
Strong
Weak
None


One Time Heat Exposure Worm Heads Motility
e.
Number of Worms
Day
1 (exp)
2
4
6
7
33%
17%
50%
83%
83%
83%
83%
17%
17%
17%
17%
strong
weak
none


One Time Heat Exposure Worm Tails Motility
f.
Number of Worms
Day
1 (exp)
2
4
6
7
67%
33%
83%
83%
83%
83%
17%
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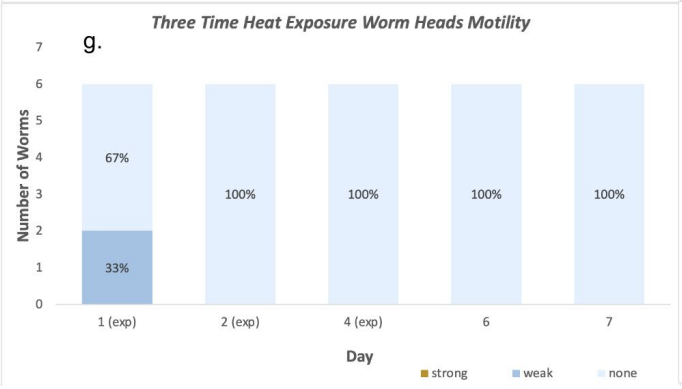
strong
weak
none


Three Time Heat Exposure Worm Heads Motility
g.
Number of Worms
Day
1 (exp)
2 (exp)
4 (exp)
6
7
67%
33%
100%
100%
100%
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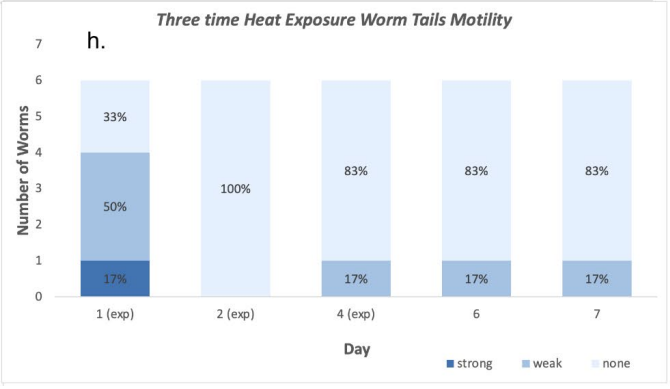
strong
weak
none


Three time Heat Exposure Worm Tails Motility
h.
Number of Worms
Day
1 (exp)
2 (exp)
4 (exp)
6
7
33%
50%
17%
100%
83%
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17%
strong
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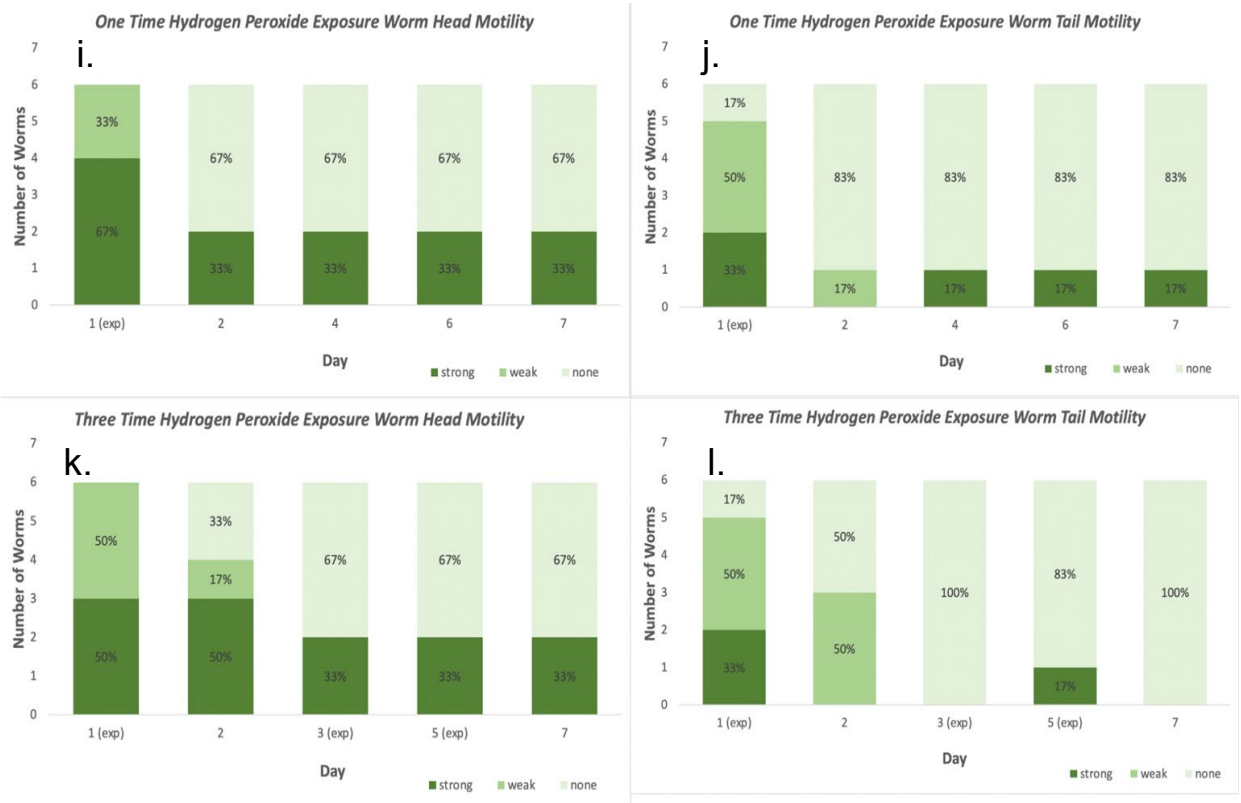

Figure 2. Motility and Survival of Cut Planarians After Exposure to Irritants. a-d represents capsaicin exposures, the e-h represents heat exposures, and the i-l represents hydrogen peroxide exposures. Each condition had 6 worms cut into heads and tails that were monitored over a 7-day period. Top panels depict results of one-time exposure and bottom panels depict three time exposures.

Results - Experiment 2

Heat Avoidance - Figure 3 and 4b

The whole uncut planaria demonstrated a much quicker avoidance response to the heat than heads/tails or right/left worms. The time listed in fig. 3 for heads/tails is for heads to cross into the cold zone as tails largely remained in the neutral zone. The right/left sides did not move away from the neutral zone; instead, they exhibited scrunching behavior and stayed in the neutral zone. Whole worms showed the strongest response to irritants out of the groups, but heads still showed a response to the irritants. (see fig. 4c for cutting scheme of the worms).

Chemical Avoidance - Figure 3 and 4a

The worms in capsaicin agar infusion plates demonstrated the same behavior as the heat plate worms. The capsaicin worms took longer overall to cross out of the irritant zones (9 minutes vs 2 minutes for whole worms). The heads, in total, took 11 minutes to move into the low concentration capsaicin zone, with only some tails moving over. However, almost all tails remained along the meridian of the plate, unable to detect the irritant. The right/left bisection demonstrated the same scrunching behavior as the heat worms. Instead of moving to the lower capsaicin concentration, they curled up to protect their internal organs. (see fig. 4c for cutting scheme of the worms)

WORM TYPE	*Time to Cross into Cold Zone*	*Time to Cross onto Agar only gel*
Whole	2 minutes	9 minutes
Heads/Tails	6 minutes	11 minutes
Right/Left	N/A.	N/A.

Figure 3. Three groups of vertically cut, horizontally cut, and whole worms were tested in plates with a gradient of temperatures and chemical concentrations to observe their avoidance of irritants. The right/left vertical bi-section groups did not move out of the neutral zone and therefore do not have time data.

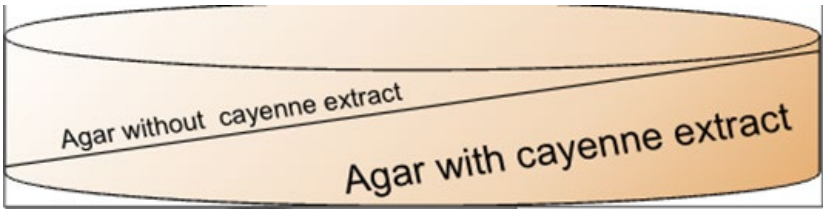

Figure 4a. Capsaicin Experimental Set-Up: Capsaicin gradient plate for the chemical avoidance experiment made to test the ability of different worm pieces to detect and avoid capsaicin.

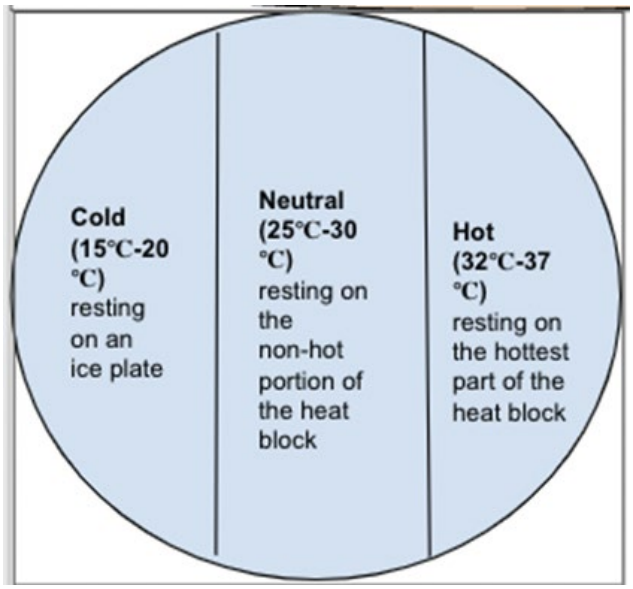

Figure 4b. Heat Experimental Set-Up: Heat avoidance plate with different temperature zones on 150mm petri dish used to test different worm pieces ability to detect and avoid heat.

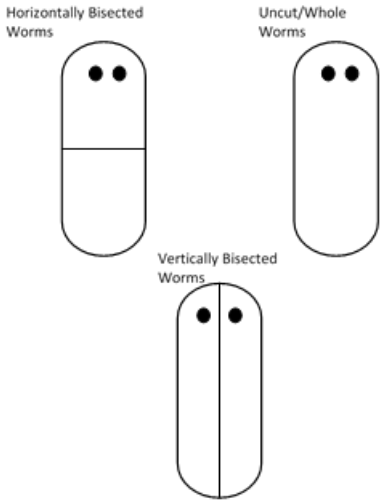

Figure 4c. Cutting Scheme: Three groups of worms were tested, two of which were bisected. This was done in order to create different experimental and control groups for experiments 1 and 2.

Discussion

Experiment One

Regeneration Was Not Affected In Surviving 1-Time Capsaicin Exposure Worms

As seen in Figure 2a-b, after 1 week, the following results were observed for 1-time exposure worms: 33% of heads survived and 50% of tails survived. Surviving worms showed strong motility and signs of regeneration. By Day 3, the initial cut had been healed with new tissue, and by Day 7, the planarians had regenerated their eyes. The control groups demonstrated the same regeneration timeline as the surviving capsaicin 1-time exposure worms. While many worms died from the capsaicin exposure, regeneration in those surviving worms remained less affected. After Day 3, for both heads and tails, no additional worms died, demonstrating that one capsaicin exposure did not have long term effects on worm survival. Small sample size interfered with each of our experiments, so if this were conducted on a larger scale, more conclusive results may be observed. 1-time capsaicin exposure worms did not support our hypothesis of altered regeneration.

High Head Survival Rates and Low Tail Survival Rates Observed In 3-Time Capsaicin Exposure Worms

As seen in Figure 2c-d, By the end of seven days, 50% of heads and 0% of tails survived. The high head survival and low tail survival for this condition is unexpected as survival rate of the 3-time exposure heads should be lower than for 1-time exposure heads. One contributing factor to the high mortality rates could be the washing process after capsaicin exposure. The washing process for these worms was particularly rigorous as there was particulate in the cayenne solution. The cayenne had a tendency to stick to the mucus on the worms so the washing process was quite vigorous. We observed the washing control group 1 day after the procedure and found that 2 out of the 6 worms showed tissue scarring and had died. This washing process likely contributed to the high death rates. The high death rate could also be because the sample size is small enough that random variations could be the difference between survival numbers.

Time in Between Exposures Allowed for Survival of 3-Time Capsaicin Exposure Worms

In capsaicin 3-time exposure worms, motility was not greatly affected after the first exposure. The worms were given a full two days to recover after each exposure, which may explain why the worm heads did not continue dying with each new exposure. The recovery period allowed for further tissue regeneration before a new exposure shock, so mortality rates did not drastically increase with each new exposure. Immediately after the 2nd and 3rd exposures to irritants, the worms displayed both weak and strong motility. Most worms exhibited scrunching behavior following exposure, which reflects TRPA-1 activation, during regeneration. The scrunching was recorded as weak motility, which was visible immediately after each new exposure. However, in between exposures, the worms returned to high motility, showing temporary activation of TRPA-1. By Day 7, the worms had fully recovered to strong motility, demonstrating that the worms needed time to recover in between exposures.

Secondary Tissue Regeneration Was Stunted in 3-Time Capsaicin Exposure Worms

By Day 3 in both control and 3-time exposure capsaicin worms, the initial location of amputation had been covered with new tissue. However, on Day 7, regeneration was no longer aligned in the two groups. By Day 7, the control heads showed elongation of the tail region, demonstrating successful secondary specific tissue regeneration (Figure 1a). By Day 7 in the 3-time capsaicin worms, the wound had healed but the tail had not been extended (Figure 1b). This suggests that the initial tissue healing response had not been interrupted by TRPA-1 activation, but later tissue regeneration may have been stunted. Repeated activation of TRPA-1 during regeneration may have consumed energy needed to regenerate the secondary tissue of the planarians. The second and third exposures were timed to interfere with longer specific tissue response by activating TRPA-1, and it appears that their activation stunted the longer regenerative process. The capsaicin 3-time exposure worms support our hypothesis of altered regeneration.

1-Time Hydrogen Peroxide Exposure Worms Demonstrated Slowed Regeneration

As seen in Figure 2i, the 1-time exposure hydrogen peroxide worm heads had a mortality rate of 67% on Day 2, which remained stable throughout the week. Although all the worms survived the initial exposure to hydrogen peroxide solution, many died overnight. Hydrogen peroxide was found to be a stronger irritant for planarians than capsaicin, so higher mortality rates in these trials are consistent with our expectations (Sabry et. al, 2019). Hydrogen peroxide does not prevent regeneration in planarians; instead, in the worms that survived the exposure, regeneration was slowed by at least one day. The remaining 33% alive heads exhibited strong motility throughout the week, indicating that the worms have recovered from the initial stress of capsaicin irritant exposure. These worms healed their wounds by the end of the 7-day period, though they progressed *at least* 1 day slower than the control group. Worm tails began regenerating heads on Day 5 for the control group and on Day 6 for the one time hydrogen peroxide exposure group. The control tails had visibly regenerated their eyes on Day 6, but the tails exposed to the hydrogen peroxide solution did not regrow eyes within the 7 day observation period.

3-Time Hydrogen Peroxide Exposure Showed Similar Results to Capsaicin 3-Time Exposure Worms

After the initial exposure to the hydrogen peroxide solution, 33% of the 3-time exposure worm heads died by Day 2 (Figure 2k). Additional exposures did not affect survival rate, as survival and motility remained constant throughout the 7 day period. Similar to the capsaicin 3-time exposure worms, recovery time in between exposures to irritants may have allowed for greater regeneration and survival rates. Between the second and third exposures and between the third exposure and the last day of observation, there was a one-day period where no

data was collected. This period potentially served as a time of recovery before the next hydrogen peroxide exposure, accounting for the consistency in survival and motility between Day 3, 5, and 7 (Figs. 2k and 2l).

3-Time Hydrogen Peroxide Exposure Tails Demonstrated High Mortality Rates, Heads Show Delayed Regeneration

The heads demonstrated similar regeneration to the capsaicin worms. The heads completed the initial wound healing response but did not re-extend their tails any further. More complete extension of the tail is visible in the 1-time exposure hydrogen peroxide group than in the 3-time group (Fig. 1c). However, no conclusions can be drawn for tail regeneration as the tails had a 0% survival rate. The irritant was likely too strong for the tails, leading to the 100% mortality rate. As such, no observations can be made about regeneration rates in the 3-time hydrogen peroxide exposure worm tails (fig. 2l). Overall, the regeneration rates and survival rates were lower than that of capsaicin. Although their concentrations were equal, this suggests that planaria are generally more sensitive to hydrogen peroxide than capsaicin.

Disproportionately High Mortality Rates Observed in Both 1- And 3-Time Heat Exposure Worms

By Day 2 after each initial 10-minute exposure to the heat bath at 37°C, the mortality rate was 83% or higher in all heat exposure groups (figs. 2e-h). The temperature used was likely too high or the exposure time too long to facilitate irritation only. Experiments on planaria survival in various temperature conditions have shown that the survival threshold for planaria in hot water is 1 hour at 30℃. While our exposure time was only 10 minutes, the high death rate of the worms demonstrates that 37°C was too hot for planarian survival. The survival rates were higher in the 1-time exposure groups, although no groups demonstrated a survival rate higher than 17%. All planaria heads of the 3-time exposure group were dead by Day 2, post second exposure. However, the heat exposure groups follow the general trend of greater survival rates in worm heads.

Experimental Inconsistencies Reduced Viability of Heat Exposure Groups

Both heat exposure trials were performed with slight differences to the capsaicin and hydrogen peroxide exposure groups. As opposed to the capsaicin and hydrogen peroxide trials, the planaria exposed to heat were not washed after each exposure and did not undergo additional washing stress. For the 3-time exposure groups, the first two exposures were also performed on consecutive days (fig. 2g and 2h). There was no 1 day regenerative period between the first and second exposures, which was present in the other 3-time exposure trials with capsaicin and hydrogen peroxide. The high temperature and inconsistent exposure times likely contributed to the high death rate, making the results harder to interpret.

Heat Exposure Slows the Progress of Regeneration, But Not Enough Data to Draw Full Conclusions

Comparing the images on Day 7 of each heat exposure group against the control, the zones of regeneration are considerably smaller. In the surviving worms, the tail extension and eye formation visible in the Day 7 control images are not present in any of the heat-exposed planaria. Before specific tissue regeneration could begin, the worms underwent further heat stress and regeneration was stunted. However, there is not enough data from the heat irritation trial to attribute changes in motility to TRPA-1 expression. Although we cannot draw definitive conclusions due to experimental error resulting in discrepancies in survival rates compared to the chemical irritant trials, the 3-time heat exposure mortality trend follows the general pattern of having a lower survival rate than the 1 time exposure of the same irritant in both heads and tails.

Scrunching as A Reaction to Exposure to Chemical Irritants Was Visible in All Groups of Worms When They Were Monitored for Motility.

Scrunching is a direct result of TRPA-1 activation, demonstrating successful TRPA-1 activation in the worms during regeneration.

Overall, We Observed That Repeated TRPA-1 Activation During Regeneration Slows Specific Regenerative Tissue Responses.

TRPA-1 is necessary for worm survival when exposed to irritants. Scrunching assists in protecting internal organs from further damage following irritant exposures. However, TRPA-1 activation leads to energy consumption, which may interfere with the overall regenerative process. Initial wound healing is vital to worm survival, but secondary tissue regeneration does not appear to be favored over TRPA-1 activation. Planarian regeneration is known to involve both a wound healing/tissue regeneration step and an elongation step which allows regenerating worms to grow to full size. Over the 7 days, our data showed that exposure across all irritant groups allowed worms to progress to the healing and tissue regeneration step, however none of the groups elongated to the extent of the control on Day 7.

Experiment Two

Whole Worms and Worm Heads in The Heat Exposure Plates Rapidly Moved Away from Heat, But The Tails Did Not

When placed in a petri dish containing water with different temperature zones, the whole uncut worms moved away from the heat into the cold zone within 2 minutes of being placed in the neutral zone (fig. 3). The uncut planaria reapproached the hot zone but immediately returned to the cold zone, demonstrating successful avoidance of the irritant. The heads, taking 6 minutes to move into the cold zone to avoid the hot water, recognized these high stress conditions and moved away from the heat towards the cold zone. Most of the tails did not move from the neutral zone, with few recognizing the heat and moving into the cold zone. The worm heads likely had a much stronger response to the irritants due to the higher concentration of TRPA-1 in the heads. Higher TRPA-1 concentration leads to stronger avoidance responses. The heads responded slower than the whole worms. This is due to the fact that whole worms have the highest TRPA-1 concentration. Whole worms do not have to heal a fresh wound as the other worm pieces did, so they likely moved quickly because they could function at full capacity.

Vertical Bisection Led to Scrunching but Not Avoidance

TRPA-1 concentration does not differ between right and left sides of planaria, as vertically bisected worm segments displayed the same curling response to heat exposure. Both left and right worm pieces curled when placed on the meridian of the neutral zone of the plate. We suspect vertical bisection may have disrupted a pathway that affects planarian motility, causing them to curl up to protect exposed internal structures rather than actively move away from the irritant.

Conclusion/Analysis

The current study suggests that planarian stem cell regeneration and the TRPA-1 pathway activation by irritants directly affect one another. Initially, we hypothesized that the irritants would prevent regeneration in planarians, and areas with low TRPA-1 cannot survive to avoid the irritants. Although regeneration was not prevented by exposure to the chemical irritants and activation of TRPA-1, we did observe slower regeneration in worms

exposed to hydrogen peroxide and capsaicin in comparison to the control group. Regeneration after heat exposure was also slowed, but low survival rates and experimental errors make it difficult to draw full conclusions. We observed initial wound healing in all worms, but in the hydrogen peroxide and heat worms, more specific tissue regeneration was not visible. Areas with lower TRPA-1 concentration also seemed to be considerably less sensitive and responsive to these irritants. During all trials of Experiment Two when testing planarian chemical and heat avoidance, the heads, which have a higher concentration of TRPA-1, reflected a stronger ability to detect irritants compared to tails. The heads avoided capsaicin and heat by moving more rapidly to areas of lower irritation.

Larger sample sizes are required for future investigation in order to examine the conditions that yielded high mortality rates and to further observe the slowed regeneration of worms placed in hydrogen peroxide. Reducing heat and decreasing exposure time may also increase survival rates over several exposures, allowing for clearer observation of the effects of heat exposures.

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