IMPACT OF PAIN SENSATION ON DISPLACEMENT PATTERNS IN DROSOPHILA MELANOGASTER LARVAE

Brune Bettler
March 2024

Wednesday Partner: Natalie Jessie

Laboratory in Neurobiology
WINTER 2024

Abstract:

In this study, we investigate the impact of pain sensation on the displacement patterns of Drosophila Melanogaster larvae, exploring the interplay between pain, texture, and odor cues in shaping behavior. Utilizing an optogenetic approach to activate nociceptive PPK neurons, we examine how associative learning based on pain influences larval movement and avoidance behaviors. Our results reveal that larvae exhibit significant alterations in their displacement patterns in response to pain-associated stimuli, demonstrating both immediate and learned avoidance behaviors. Specifically, larvae exposed to painful stimuli together with a ground texture and odor gradient learn to avoid these areas, indicating a capacity for associative learning and memory formation. These findings underscore the complexity of larval behavior in response to environmental cues and highlight the role of pain sensation in mediating adaptive behaviors, paving the way for future research.

Introduction:

Fruit fly larvae exhibit distinctive displacement behaviors when placed in novel environments. Notably, larvae display an elevated level of activity upon arrival in a new environment, characterized by motion with low turn angles predominantly near the environment edge, followed by a stable level of activity (Soibam et al., 2012). In general, if larvae are in favorable conditions, their displacement orientation remains unchanged. These exploratory crawl behaviors are organized and commanded by the abdominal and thoracic networks independently from the larval brain. However, the brain is necessary for modulation of this crawl behavior in response to environmental cues,

such as for avoidance of painful stimuli or for movement toward attractive odorants (chemotaxis) (Berni et al., 2012). The interplay between brain and thoracic networks is notable during reorientation behavior completed as a result of negative phototaxis, in which larvae preferentially move away from areas of light. In fact, a disinhibitory neural mechanism impacts larval head movements to repress reorientation when larvae exit regions of light to prevent their re-entry (Zhao et al., 2019). A similar reflexive form of reorientation occurs as larvae come across a noxious mechanical or thermal sensory stimulus. The resulting “roll” behavior is distinct from typical displacement behavior and causes the animal to bend preferentially towards the side that received the noxious stimulus (Hwang et al., 2007). While reorientation behavior is well characterized during reflexive responses to the environment, less is known about the behavioral changes that occur as a result of learned aversions to stimuli.

Fruit flies are capable of modifying the valence of sensory cues in response to environmental changes through associative learning, wherein larvae can form both positive and negative associations—such as being drawn towards or repelled by specific odors (Felsenberg, 2021). Recent research has shown that for some larvae, only two cycles of training are sufficient to learn these associations (Lesar et al., 2021).

The study of Drosophila larvae, model organisms with approximately 15,000 neurons, offers important insights into the broader neurological circuit mechanisms that enable such adaptive behaviors (Boivin et al., 2023), (Khuong et al., 2019). This is particularly relevant in the context of nociception, a critical survival mechanism enabling the sensation of pain for the avoidance of life-threatening stimuli. In fruit fly larvae, nociceptive PPK neurons are distributed throughout the larval body allowing for

widespread detection of noxious stimuli. By optogenetically activating PPK neurons, fruit fly larvae become ideal organisms through which to investigate the role of learning and adaptation on displacement patterns.

Our investigation seeks to explore the behavioral dynamics of fruit fly larvae in response to pain-texture-odor associations, guided by two primary hypotheses. The first hypothesis posits that despite attraction to a textured region of interest (ROI) due to the presence of an attractive odorant, larvae will avoid this ROI when exposed to red light causing PPK neuron activation. We assume that the larvae will spend more time in the surrounding area outside of the ROI due to odorant attraction rather than wall boundaries. The second hypothesis suggests that larvae will learn to avoid the textured ROI after at least two training sessions even after the removal of the red light. Specifically, we address the question: Can fruit fly larvae learn to associate ground textures with pain, and if so, how does this association influence their movement and avoidance behaviors?

By exploring how pain association impacts larval movement and environmental avoidance, this study aims to shed light on the fundamental processes underlying the ability of organisms to adapt to their environments through learned behaviors.

Method details:

Agar plate ROI texture:

Sterilized 100mm x 15 mm agar-filled petri dishes were provided. Before the placement and recording of each larvae group (both control and experimental), a new agar plate was used and scratched using Personnel's Wide Areas Interdental Brushes

(one per dish). The location of the scratched surface was determined using a paper template containing a drawn ellipse outlining the location of where the red light shone on the dish. Prior to the start of each video recording, the paper template was placed in the behavioral stage below a new dish. The agar was then scratched, as uniformly and replicably as possible and the template was removed before conducting all experiments to avoid video artifacts from the white template surface and red light reflections onto the PPK mutant larva. Following the scratching of the ROI, before the addition of the larvae, a small 4-5 mm square, containing a 10μM solution of attractive odorant (ethyl acetate), was placed in the center of the ROI. To have access to the location of the ROI during post-experiment data analysis, the video recording was started after the agar scratching, and before the removal of the template paper. Before the addition of the larvae into the ROI-textured dish, the template paper below the dish was removed.

All experimental groups used ROI-textured plates. In future experiments, it may be interesting to compare the differences between regular non-modified agar plates and ROI-textured plates. The ROI had the following dimensions: 230mm by 130mm ellipse with an area of about 220mm squared.

Experimental Paradigms:

In all experiments, the changing of time was signaled by the appearance of a paper slip that covered the entire video frame. Each section begins on the first frame in which the full behavioral stage is visible following the paper pass.

All larvae were moved from their temporary food-filled plates to the ROI-textured plates with slightly wet paintbrush tips.

Control 15 minutes light off (15O): Red light was kept off during the experiment. Once all 7 larvae were added to the dish, it was signaled in the video frame that the 15-minute period began. The video recording ended when 15 minutes had passed.

Control 15/5 (15R5O): Red light was turned on before larvae addition. Once all 7 larvae were added, it was signaled in the video frame that the 15-minute period began. The paper signal began the 5-minute light-off period in which no red light was shone on the behavior stage. The video recording was stopped after the 5-minute period.

Experimental 5/5: As with the previous condition, the red light was turned on before larvae addition. Once all 7 larvae were added, it was signaled in the video frame that the 5-minute light-on period began. After 5 minutes the paper signal was shone and a 5-minute light-off period occurred. The repetition of 5 minutes light on, 5 minutes light off periods repeated three full times in total. The video recording ended following the last 5 minutes light-off period.

Experimental 5/2: As with the previous conditions, the red light was turned on before larvae addition. Once all 7 larvae were added, it was signaled in the video frame that the 5-minute light-on period began. After 5 minutes the paper signal was shone and a 2-minute light-off period occurred. The repetition of 5 minutes light on, 2 minutes light off periods repeated three full times in total. The final 2-minute light-off period was replaced by a 5-minute light-off period. The video recording ended following this last 5-minute light-off period.

Quantification and statistical analysis:

All larvae ROI entry and exit times as well as larvae entry and exit times from grid squares for two conditions were hand-counted from .avi video files. Entry times began when the tail of the larvae was fully in the region and ended when the full tail exited the region. All further analyses were performed using custom Python code and were graphed using Scipy and Matplotlib. Statistical tests were completed using the Python Mann-Whitney U Test.

Results:

To observe and quantify larvae behavior in both no-light and red-light environmental conditions, we first measured the entry and exit time for all center-scented/scratched region stays (ROI stays) made by each larva in both experimental conditions (total n=24). The first experiment (15O) consisted of two 15-minute sessions for n=7 larvae each in which no red light was shone for the duration of the session (figure 1. A). The second (15R5O) consisted of two 20-minute sessions for n=7 larvae each in which red light was shone on the ROI region only for 15 minutes straight, followed by a 5-minute no-light period (figure 1. B). As expected, in 15R5O, the larvae largely avoided the ROI when the red light was continuously on for 15 minutes and kept their entries short in length on the occasions in which they did enter. There was a significant difference in the mean length of time spent in the ROI per entry between the 15O and 15-minute light-on portion of the 15R5O conditions ($p = 0.0015$) (Figure 1. E,F). In total, when the light was off for 15 straight minutes, the 15O larvae spent an average of 28% of their time in the ROI compared to an average of only 6% for

those in 15R5O during the 15-minute light-on period (Figure 1. C,D top). Interestingly however, in the 15R5O group, once the light was turned off, the number of ROI entries, both in total and per larva, increased (Figure 1. B,E). In fact, during this light-off period, the mean time spent in the ROI averaged over the 14 larvae was significantly higher than that of the 15-minute light-on period that preceded it ($p = 0.02192$) and not significantly different from the control 15-minute light-off period (Figure 1. E). This shows that while the larvae in the 15R5O condition avoided the ROI when the red light was on, they did not continue this avoidance behavior when the light was turned off.

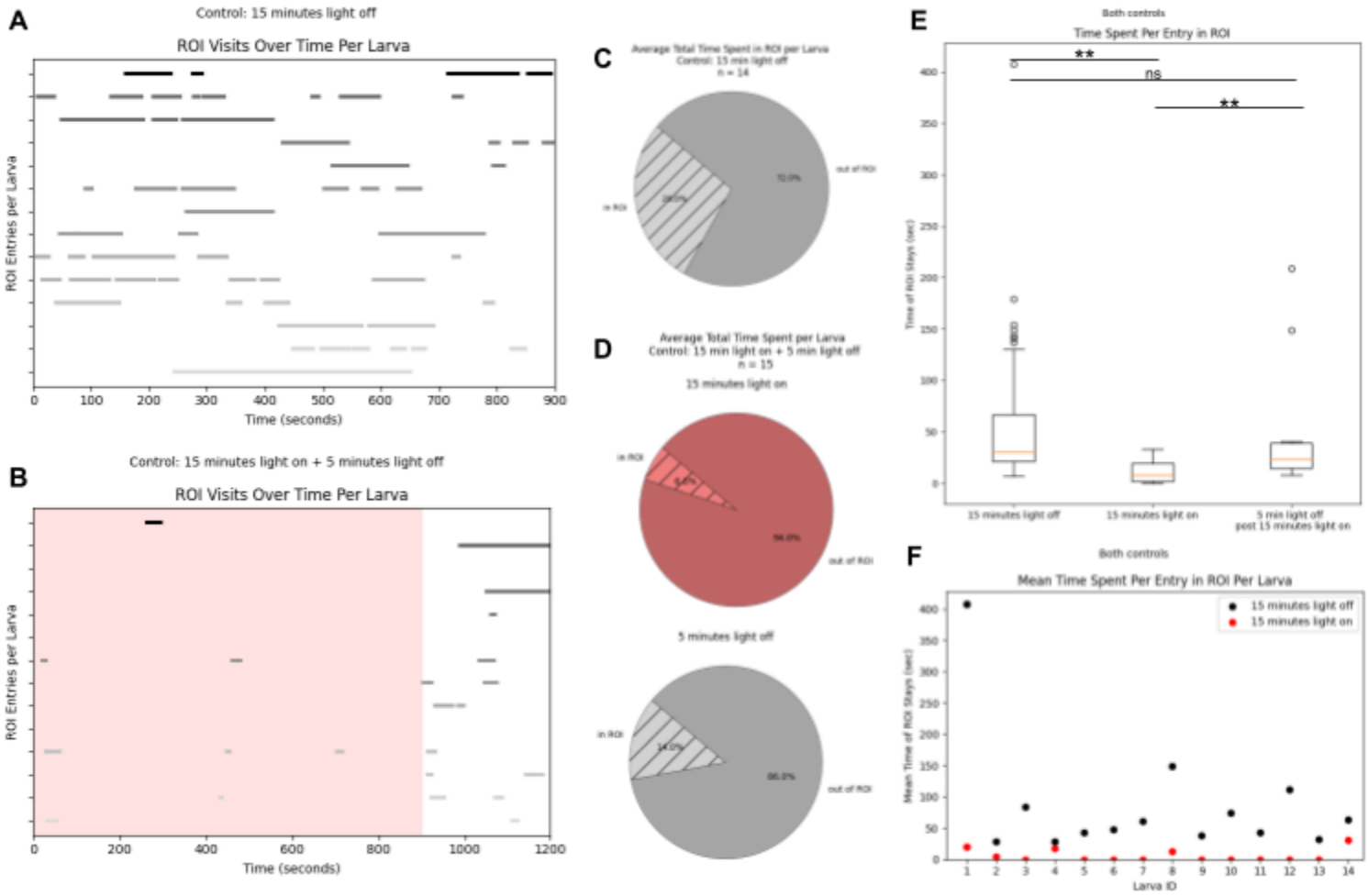

Figure 1. Overview of behavioral control experiments

(A) Full view of all ROI entries made by each of the n=14 larvae during a 15 minute no-light on period (15O). Each row corresponds to the entries over time for a single larva.
(B) Full view of all ROI entries made by each of the n=14 larvae during a 15 minute light on period followed by a 5 minute no light period (15R5O). The red portion of the graph corresponds to the time during which the red-light was on. Each row corresponds to the entries over time for a single larva.
(C-D) Pie plots showing total time spent in vs out of the ROI averaged across the 14 larvae per experimental condition. Lighter and striped regions correspond to in-ROI while darker uniform regions correspond to out-of ROI. (C) corresponds to the 15O condition in (A). (D) corresponds to the 15R5O condition with the top plot showing the percentages during the 15 minutes light on period and the bottom showing the percentages during the 5 minute light off period.
(E) Box and whisker plot of time spent per ROI entry in each experimental condition. The first shows a plot for the 15O condition while the second two show the 15R5O condition split into the 15-minute light on period and 5-minute light off period respectively. A Mann Whitney U Test gave significant differences between the first two plots and between the second and third plot (ns: $p >= 0.05$, **: $0.001 <= p < 0.01$).
(F) Scatter plot of mean entry length per larva in both conditions. Larva were not the same between the two experimental conditions (total n = 24).

We then sought to understand the underlying distribution of movement both as a function of location in the circular dish and over time for both conditions. Analysis was completed by dividing the circular Petri dish into a 10x10 grid resulting in 1cm x 1cm grid squares (Figure 2. E). Analysis of the first 15-minute no-light experiment (n=7) is shown in Figure 2. Overall, the larvae entered the ROI region the most compared to other locations, with the two highest total entry values belonging to the two main locations within the ROI (figure 2. A). These two inner-ROI locations also had larvae within them for over 50% of the 15-minute session as opposed to an average of 22% for other locations (figure 2. B). Over all locations, the larvae spent an average of 8.13 seconds per grid square. Interestingly, over the 15-minute session, the larvae visited the ROI region less as time elapsed as opposed to boundary regions that showed higher frequency as time elapsed (figure 2. D). In most locations around the ROI, the larvae most often moved directly toward the ROI while boundary locations most often led to movement continuing along the dish edge (figure 2. F). While the ROI region attracted the larvae, our analysis shows that another region in the top right region of the dish also resulted in a greater and longer number of entries (especially over time) than other non-ROI grid locations (Figure 2. A,B,C,D,F). We suspect this may be due to a residual food residue that remained in our dish following the placement of larvae from their storing dish (containing food) to our clean agar dish. This residue was small enough to

be displaced between grid spaces while the larvae moved across it (but remained in the top right region). It can be seen in Figure 2. E as a bright spot in the top right region of the dish.

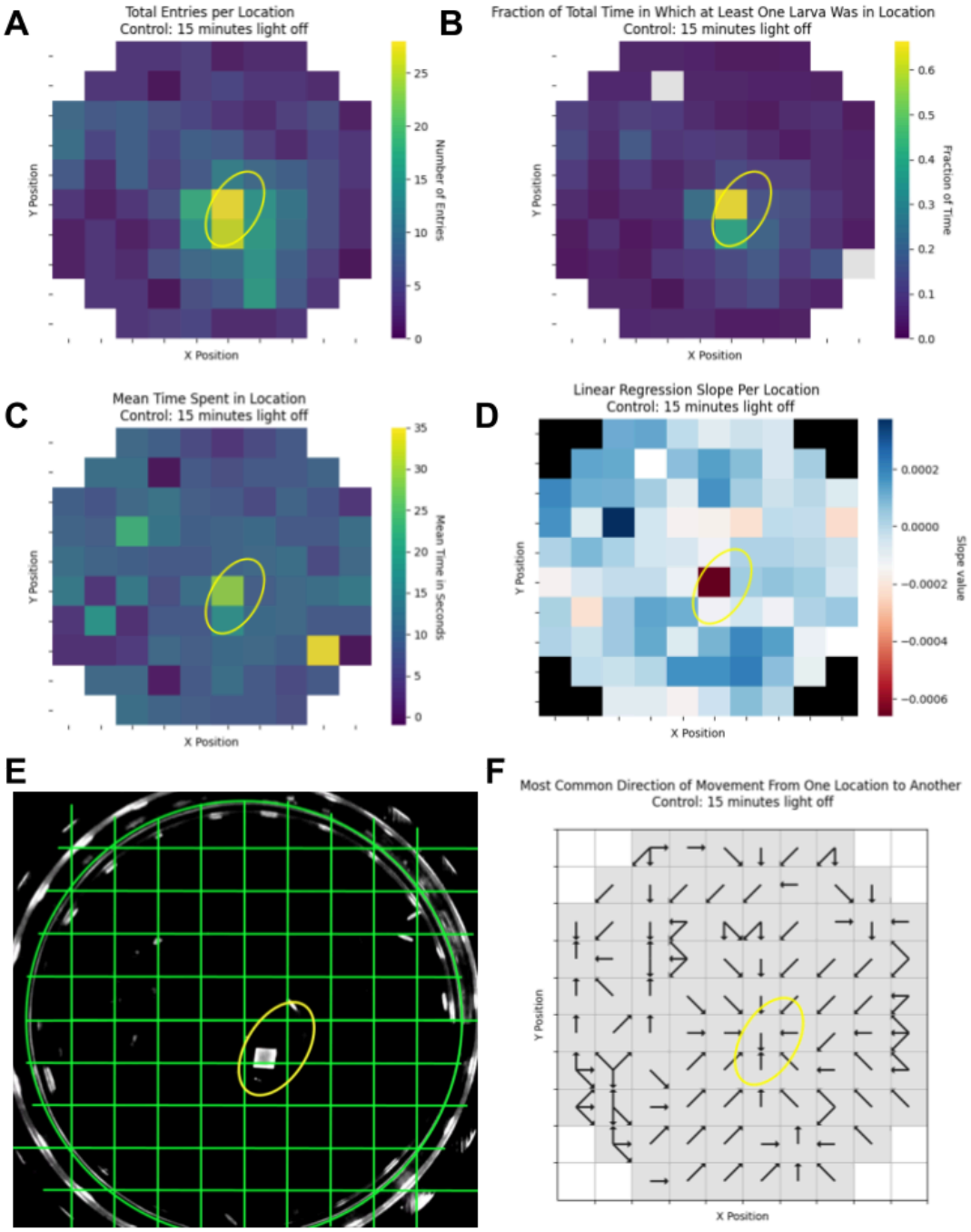

Figure 2. Movement patterns during the 15-minute no-light condition n = 7

The yellow ellipse represents the scratched ROI region which also contained a 10uL amount of ethyl acetate.
(A) Sum of total larva entries per location over time.
(B) Fraction of time in which at least one larva was in the grid square.
(C) Average length of time in which at least one larva was in each grid square.
(D) Linear Regression Slope per location. A positive slope indicates an increase in larval visits over time. A negative slope indicates decreased larval visits over time.
(E) Image frame of the .avi files from which the entry and exit times were drawn.
(F) Most common direction of movement from one location to another. Arrows indicate the most common next location during larval displacement. Multiple arrows indicate an equal number of displacements in the indicated directions.

The same analyses were then done on the 15-minute light-on period of the 15R5O session (figure 3.). Overall, the larvae largely avoided the bottom region of the dish and did not enter the center ROI grid square. Because the bottom region of the dish was not often visited, most of the top half of the dish grid squares had over 15 larval visits compared to about 7 in the top half of the 15-minute light-off condition (figure 3. A, figure 2. A). Surprisingly, nearly all boundary grid squares of the light-on condition resulted in little to no larval visits (figure 2. B). This may be due to possible reflections of the red light on the dish walls resulting in increased pain sensation in these edge locations. Overall, the average time spent around the ROI was higher than both the ROI region and the other dish locations in the light-on condition than the ROI surround in the light-off condition. However, the time spent per grid location was similar to that of the light-off condition with an average of 9.25 seconds. Interestingly, the range of average stay lengths was greater in the light-on condition (max 48.97 seconds) than that of the light-off condition (max 35.0 seconds). Over time, the grid squares surrounding the ROI saw an increase in larval entries (figure 2. D). Overall, the larvae avoided the ROI region and lower half of the dish while moving toward a grid square adjacent to the ROI (figure 2. F).

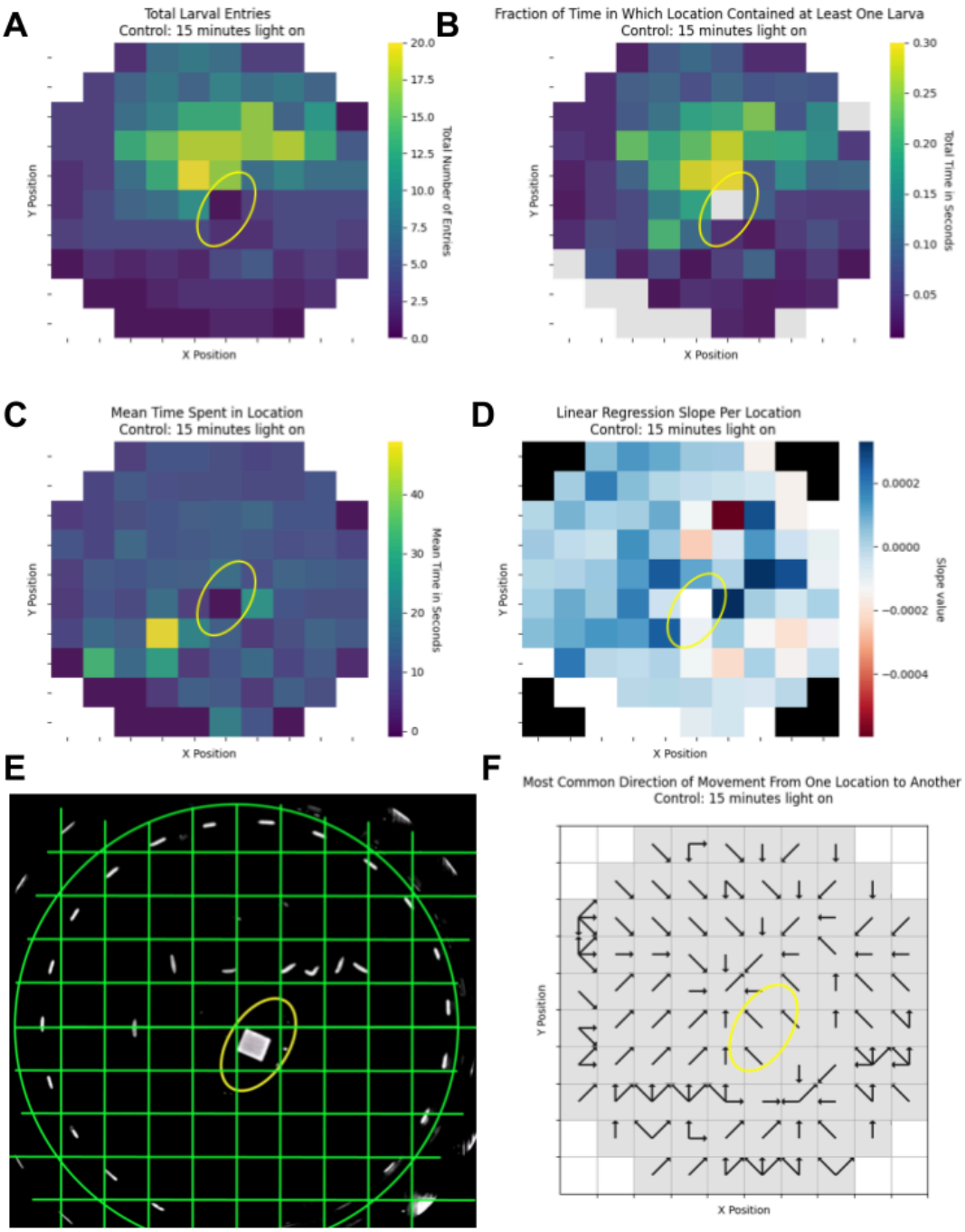

Figure 3. Movement patterns during the 15-minute light-on condition n = 7

The yellow ellipse represents the scratched ROI region which also contained a 10uL amount of ethyl acetate.
(A) Sum of total larva entries per location over time.
(B) Fraction of time in which at least one larva was in the grid square.
(C) Average length of time in which at least one larva was in each grid square.
(D) Linear Regression Slope per location. A positive slope indicates an increase in larval visits over time. A negative slope indicates decreased larval visits over time.
(E) Image frame of the .avi files from which the entry and exit times were drawn.
(F) Most common direction of movement from one location to another. Arrows indicate the most common next location during larval displacement. Multiple arrows indicate an equal number of displacements in the indicated directions.

In preparation for future analysis to quantify the movement patterns of larvae as a result of conditional learning, two further experiments were run. During these experiments, red light was turned on over the ROI at 3 repeating periods of either 5on-5off or 5on-2off alternations (n=15 and n=14 total for each experimental condition respectively). As previously done, the entry number and length over time were plotted as shown in Figure 4. A and 4. B and summarized, with the two control experiments mentioned previously, in Figure 4. C. In the 5 on 2 off condition (5R2O), 17 ROI entries occurred during the red light period with an average entry length of 33.3 seconds. During the no-light periods, a total of 17 ROI entries occurred though with an average length of 55.7 seconds (no significant difference between the two condition means). During the three 5-minute red light on periods, 8, 2, and 7 ROI entries occurred with an average stay length of 38.8, 31.2, and 27.7 seconds respectively. When normalized for the 14 total worms and length of periods, the red light periods had a normalized total-time-spent-in-ROI of 0.045 compared to 0.125 for the no light period. For the 5 minutes light on 5 minutes light off condition, two larvae dug underneath the scented paper within the ROI and remained there for the duration of the experiment (larva number 8 and 14 (see Figure 4. B)). These two larvae were excluded from further calculations. For this experiment, the red light on periods together saw 12 ROI entries while the light off saw only 6. The mean ROI stay length for these two total periods was 38.9 seconds for the light-on and 88.8 seconds for the light-off period (no significant difference in means). Their normalized sums were 0.040 and 0.046 respectively. The first red-light-on period saw 8 ROI entries with a mean entry time of 40.2 seconds while the second red-light-on period saw no entries. The third red-light-on period saw 4 ROI

entries with an average ROI stay length of 36.5 seconds. The first and third red-light-on periods had significantly different mean length-of-stay values. Over time, the light-off periods also saw a significant decrease in ROI entries and corresponding lengths of stay values.

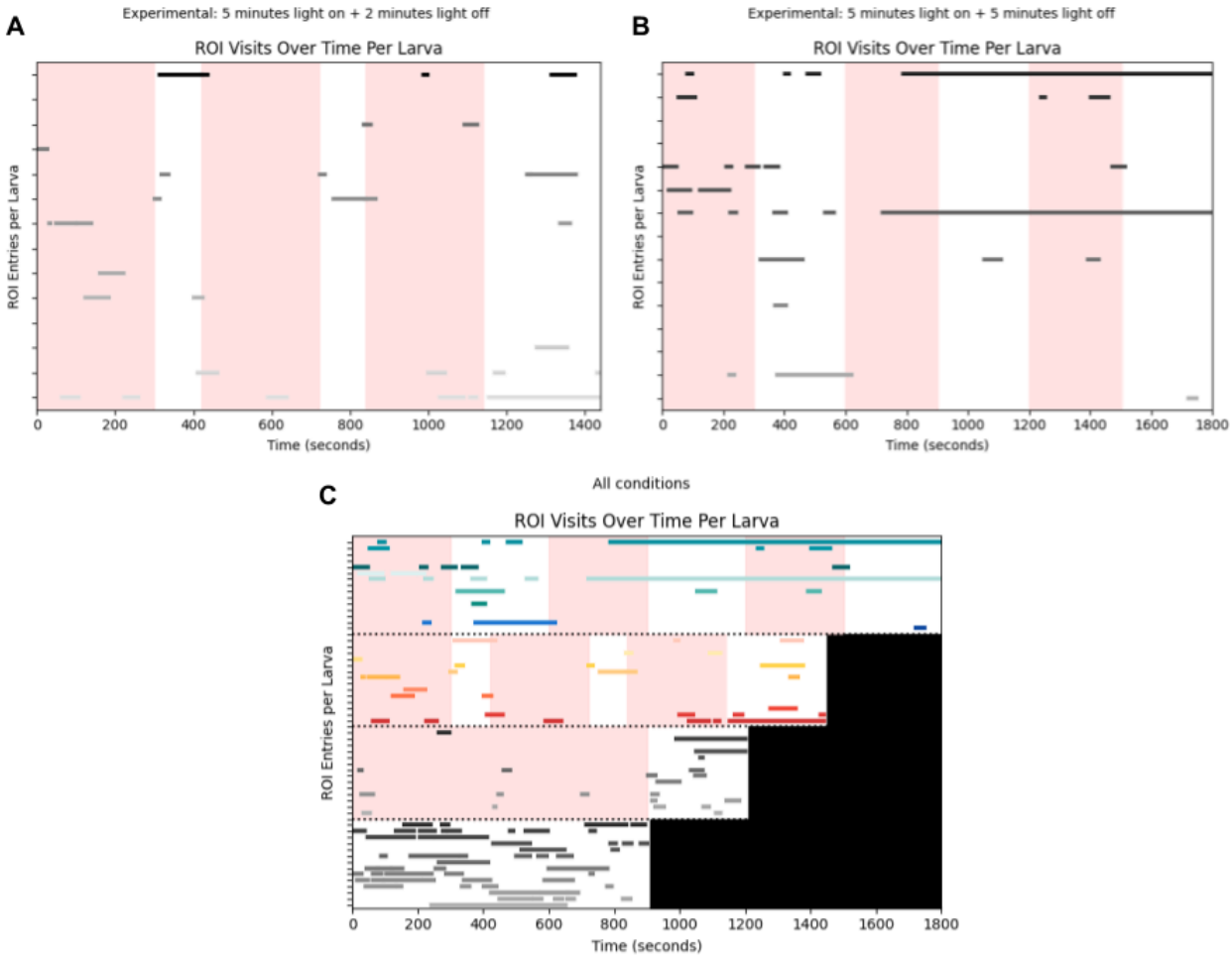

Figure 4. Overview of ROI entries during alternating light on-off periods

Red regions on the graphs indicate periods of light shone on the ROI, white regions indicate light-off periods and black regions indicate periods of no experimental recordings.
(A) Full view of all ROI entries made by each of the n=14 larvae during 3 repeated periods of 5-minute light on and 2-minute light off periods. The last light-off period was extended to 5 minutes to better observe larval behavior. Each row corresponds to the entries over time for a single larva.
(B) Full view of all ROI entries made by each of the n=15 larvae during 3 repeated periods of 5-minute light on and 5-minute light off periods. Each row corresponds to the entries over time for a single larva.
(C) Overview of all ROI entries made by each of n = 57 larvae throughout all experimental and control conditions. The top two rows are the 5 minutes on 5 minutes off and 5 minutes on 2 minutes off conditions respectively while the bottom two rows are the 15 minutes light on and 5 minutes light off and 15 minutes light off control conditions respectively.

Discussion:

Our results demonstrate that fly larvae have characteristic movement patterns and may be able to learn to avoid a certain texture through conditional learning. We first showed that when placed in a novel environment containing an attractive odorant, larvae are attracted to the location of highest odorant concentration (HSC). Despite the lack of food at this location, the larvae visit and return to this location multiple times. This suggests that while the larvae leave the HSC location (presumably to continue to explore their environment for food), they return due to their biological attraction to the scent. Because of this attraction, the larvae did not spend as much time on the dish boundaries, as suggested in previous literature (Soibam et al., 2012). Our results from the 15-minute light-on condition demonstrate that while the larvae are attracted to the HSC location, they remain outside of the ROI due to the pain felt upon entry. As such, the sensation of pain is a stronger deterrent than the attraction of the positive odorant. The results also confirmed our hypothesis that the larvae would spend the majority of their time in a region around the ROI. However, further testing is needed as it is not clear whether the larvae avoided the boundary locations due to an attraction to the positive odorant or due to repulsion from higher sensations of pain felt by reflections of the red light on the petri dish walls.

Our second consideration in this study was the larvae's ability to retain information about pain location. Because we were interested in the larvae's displacement characteristics, we paired both a texture and increased odorant concentration to the sensation of pain in hopes that the larvae would quickly learn to avoid the ROI. While this resulted in more naturalistic learning, this approach introduces

complexities in isolating the exact cues that drive avoidance behavior. In previous conditional learning experiments, larvae were able to learn and retain pairing information after a minimum of two training periods (Lesar et al., 2021). However, our experiment does not confirm the number of training periods but rather the interval of training periods. As shown in Figure 4. A more consistent interval period of light on and light off (5 and 5 vs 5 and 2) resulted in a greater avoidance of the ROI region over time. However, this condition also resulted in the curious case of two larvae that dug under the scented paper slip during the session. This behavior could suggest an alternative strategy for avoiding the conditioned stimulus or a variation in sensitivity to the pain stimulus. It would be interesting to study whether larvae are more prone to this behavior following regular light-on/light-off intervals or irregular (test also with more training periods).

While we chose to complete our experiment using continuous light-on/light-off periods for ease of testing larger numbers of larvae, this method may not be the most efficient or replicable way in which to train the larvae to avoid the ROI. As opposed to isolated individual bouts of targeted light flashes when a single larva enters the ROI, our method does not allow for controlling the amount of time or the number of times each larva associates the pain with the texture or odor gradient. Because some larvae may have spent greater time roaming outside the ROI, they may only have entered the ROI during times in which the light was not on resulting in no learning or association.

As such, future studies should aim to solidify these findings by expanding the experimental design in several ways. First, completing the analyses done for the displacement characteristics for all sessions and over all conditions would enhance the

robustness of our conclusions and allow for better generalization of the larvae's displacement. Ideally, this would also involve recording from at least two more n=7 larvae groups per experimental and control conditions for a total of 28 larvae per experiment. It could also be worthwhile to vary the number of larvae in the dish to control for the effects of larval concentration and scent in the dish. Additionally, introducing and analyzing a control condition in which no texture is present in the dish (only the odor gradient on a smooth surface) could help disentangle the specific effects of texture and odorant concentration on avoidance behavior.

In conclusion, our findings suggest that fly larvae may be able to associate ground textures with pain sensation and modify their displacement characteristics accordingly.

Resource availability:

Data and code availability: All original code and data are in GitHub and publicly available as of March 2024 and can be accessed through the following link: https://github.com/BruneBettler/BIOL-389-Optogenetics

Experimental model and subject details:
57 Crimson-PPK mutant Drosophila melanogaster larvae raised in standard laboratory environments were used in these experiments. Larvae were housed in large groups with adequate food and sheltered from light before and after experiments.

Hardware and behavior setup:
- Arduino
- Logitech Webcam
- Tristar LEDs
- Assembled as described in the biol 389 overview document

Software:
- Bonsai: https://bonsai-rx.org/
- Arduino IDE: https://www.arduino.cc/en/software

References:

Berni, J., Pulver, S. R., Griffith, L. C., & Bate, M. (2012). Autonomous circuitry for substrate exploration in freely moving Drosophila larvae. *Current Biology: CB*, *22*(20), 1861–1870. https://doi.org/10.1016/j.cub.2012.07.048

Boivin, J.-C., Zhu, J., & Ohyama, T. (2023). Nociception in fruit fly larvae. *Frontiers in Pain Research*, *4*. https://doi.org/10.3389/fpain.2023.1076017

Felsenberg, J. (2021). Changing memories on the fly: The neural circuits of memory re-evaluation in *Drosophila melanogaster*. *Current Opinion in Neurobiology*, *67*, 190–198. https://doi.org/10.1016/j.conb.2020.12.003

Hwang, R. Y., Zhong, L., Xu, Y., Johnson, T., Zhang, F., Deisseroth, K., & Tracey, W. D. (2007). Nociceptive Neurons Protect *Drosophila* Larvae from Parasitoid Wasps. *Current Biology*, *17*(24), 2105–2116. https://doi.org/10.1016/j.cub.2007.11.029

Khuong, T. M., Wang, Q.-P., Manion, J., Oyston, L. J., Lau, M.-T., Towler, H., Lin, Y. Q., & Neely, G. G. (2019). Nerve injury drives a heightened state of vigilance and neuropathic sensitization in Drosophila. *Science Advances*, *5*(7), eaaw4099. https://doi.org/10.1126/sciadv.aaw4099

Lesar, A., Tahir, J., Wolk, J., & Gershow, M. (2021). Switch-like and persistent memory formation in individual Drosophila larvae. *eLife*, *10*, e70317. https://doi.org/10.7554/eLife.70317

Soibam, B., Mann, M., Liu, L., Tran, J., Lobaina, M., Kang, Y. Y., Gunaratne, G. H., Pletcher, S., & Roman, G. (2012). Open-field arena boundary is a primary

object of exploration for Drosophila. *Brain and Behavior*, *2*(2), 97–108. https://doi.org/10.1002/brb3.36

Zhao, W., Zhou, P., Gong, C., Ouyang, Z., Wang, J., Zheng, N., & Gong, Z. (2019). A disinhibitory mechanism biases Drosophila innate light preference. *Nature Communications*, *10*(1), 124. https://doi.org/10.1038/s41467-018-07929-w