



**BEHAVIORAL RESPONSE TO ALARM PHEROMONE IN THE MINIATURE FISH,
*DANIONELLA TRANSLUCIDA***

**Jacob Bedke (Adam Douglass, Ariadne Penalva)
Department of Neurobiology and Anatomy**

I. Introduction

Pheromones are chemicals produced by an organism that act as a communication signal from an organism to its conspecifics, members of the same species. While pheromones are most often described and associated within the context of sexual interaction (Darwin, 1889; Gomez-Diaz & Benton, 2013), there are also other situations in which certain pheromones may be exchanged (Chung-Davidson & Huertas, 2010; Morgan, 2008; Yew & Chung, 2015). One such event is when an organism is endangered or threatened by a predator or an environment. During such an event, the organism will release an alarm pheromone that communicates the potential danger to conspecifics to elicit behaviors associated with fear, anxiety, or aggression, depending on the species (Mathis & Smith, 1992; Mizunami, 2010; Vandermoten et. al, 2012). Such a pheromone may be evolutionary advantageous in an altruistic sense as it would allow one organism to alert its kin that are receptive to the pheromone so that they can modify their behavior in a way that may increase their likelihood of survival in the midst of such a threat.

Ostiophysari, a superorder of fish including *Danio rerio* (zebrafish) and minnows, are known to produce and react to an alarm pheromone. Karl von Frisch (1938), a well-known scientist known for his work in honey bee communication, was the first to document this pheromone in minnows, and he gave it the name “schreckstoff” (Stensmyr & Maderspacher, 2012). He noted an acute behavior change in the minnows when he accidentally exposed them to the pheromone when he damaged the skin of a member of the minnow colony, and further research has determined the presence of this alarm pheromone to be present in the skin of fishes (Pfeiffer, 1977). Researchers have continued to characterize this modulation of behavior in response to exposure to this alarm pheromone in a variety of Ostiophysari species including the commonly used biological model, zebrafish. When exposed to a zebrafish-derived alarm pheromone, individual zebrafish exhibit behaviors related to anxiety and fear, including increased periods of motionlessness, an increase in erratic movements, and an increase in dwelling near the bottom of the tank among other behaviors (Faustino et. al, 2017; Speedie & Gerlai, 2008; Waldman, 1982).

The Douglass lab has been working to establish a recently discovered relative of zebrafish, *Danionella translucida*, as a behavioral neuroscience model, in order to better exploit new, optical methods for recording and manipulating neuronal activity. The field of neuroscience has recently seen advancements of *in vivo* methods of imaging and neuronal activity manipulation techniques using transgenic expression of fluorescent biomarkers and light-activated fluorescent proteins, respectively. Among these emerging methods is optogenetics, a method that allows researchers to alter neural activity by stimulating a light-sensitive ion protein channel. Optogenetics allow behavioral neuroscientists to study the relation between behavior and populations of neurons that have been induced to express such a membrane-bound protein channel (Kim et. al, 2017). Larval zebrafish are a common model in neuroscience as they exhibit transparency that allows researchers to examine the contribution of a population of neurons to behavior with minimal invasiveness (Portugues et. al, 2013); however, as zebrafish

develop, they lose this transparent characteristic, and this makes it difficult to study the neural correlates of behaviors that are only present in an adult neural network without unintentionally affecting an individual's behavior with invasive procedures. A close relative of zebrafish, *D. translucida*, maintains its transparency throughout development (Roberts, 1986), making it possible to investigate the contribution of neural populations in its adult behaviors including social behavior using minimally invasive activity readout methods such as calcium imaging (Penalva et al, 2018; Schulze et. al, 2018).

With the future goal of investigating the neural contributions to behavior in *D. translucida* through *in vivo* imaging, this study seeks to establish the presence of a behavioral response to alarm pheromone in this model organism. We hypothesize that *D. translucida* will exhibit a similar, if not identical, behavioral response profile as zebrafish with exposure to this alarm pheromone due to this species' close evolutionary relation with zebrafish.

II. Methods

A. Alarm Substance Acquisition

While there has been research to determine a chemical identity candidate of this alarm pheromone, the claims of this research seem somewhat speculative. The alarm pheromone has been hypothesized to be glycosaminoglycan chondroitin (Mathuru et. al, 2012). While this chemical is present in the skin of members of the Ostiophysari clade, purified glycosaminoglycan chondroitin did not produce a behavioral modification in our lab's attempts to reproduce this finding in zebrafish. We reverted to a more traditional method of alarm substance collection by extracting crude contents of skin tissue in euthanized zebrafish and *D. translucida*. To do this, we euthanized individuals in an ice bath composed of a hypertonic variant of the models' usual tank water (Instant Ocean Sea Salt). Next, we made small incisions in their skin to recreate the physical damage that has been reported to allow the elution of the alarm pheromone. Lastly, we allowed the substance to elute from the skin cells by placing the individuals in a hypotonic solution of their tank water to promote cell lysis and the release of the damaged tissue's content. The extracted solution was stored in aliquots in a -20°C freezer. Freezing and thawing the extracted solution did not affect pheromone's capacity to induce behavioral modification in zebrafish.

B. Environment, Experimental Setup, and Video Acquisition

The experimental assays were performed in a light-tight enclosure in a quiet room. Within the enclosure, the fish subject was situated in a small tank that was placed on an elevated platform. The tank was filled with 500 mL of fresh tank water that had not been exposed to any fish. A solid gray image was projected on the base of the platform to provide visible illumination to help the fish to habituate to its surrounding with visual input. The alarm pheromone delivery apparatus consisted of a syringe attached to silicon tubing. The silicon tubing was then directed into the fish tank just above the water line. The delivery volume and concentration varied by experiment. The control group received a timed substance delivery with a volume and solution identical to the experimental group but containing no alarm pheromone extract. Each subject's trial consisted of three phases lasting 10 minutes each: an environmental habituation phase, a baseline phase, and a pheromone exposure phase.

Videos were recorded using a Point Grey Flea3 USB3.0 camera with an infrared filter lens. Illumination was provided by a custom-built infrared LED fixture. The camera was positioned to capture a horizontal sideview of the tank, while illumination source was positioned on the opposite side. Diffuser paper was positioned between the illumination source and the tank. Video acquisition and motion tracking was performed using an open-source, live visual processing program known as Bonsai (Lopes et. al, 2015). The data generation pipeline used

with this program tracked the fish's motion by subtracting a slowly-updating averaged background from each frame's image, allowing segmentation and determination of the fish's relative position in the tank.

C. Behavioral Analysis

Behavior was quantified by using definitions of how the construct behavior would be characterized according to the fish's position and motion. This construct was then applied to the data to characterize the behaviors in question.

1. Bottom-Dwelling Time and Thigmotaxis Time

Each individual's bottom dwelling time was calculated by determining the proportion of time that the subject fish's position was in the bottom quarter of the tank. Thigmotaxis is defined as 'wall-hugging' and was similarly calculated but this definition included fish positions that were in the bottom quarter of the tank as well as the eighth of the tank on each side of the tank parallel to the focal point of the camera's view.

2. Erratic Movements and Freezing Events

To characterize the motion profiles of an individual, the apparent velocity indicated by the generated two-dimensional position coordinates of the subject fish was calculated. To determine the number of erratic movements made by a subject, a high-pass velocity threshold was applied, and only reasonably long intervals of velocity surpassing the threshold were counted. Freezing events were similarly determined using a low-pass velocity threshold.

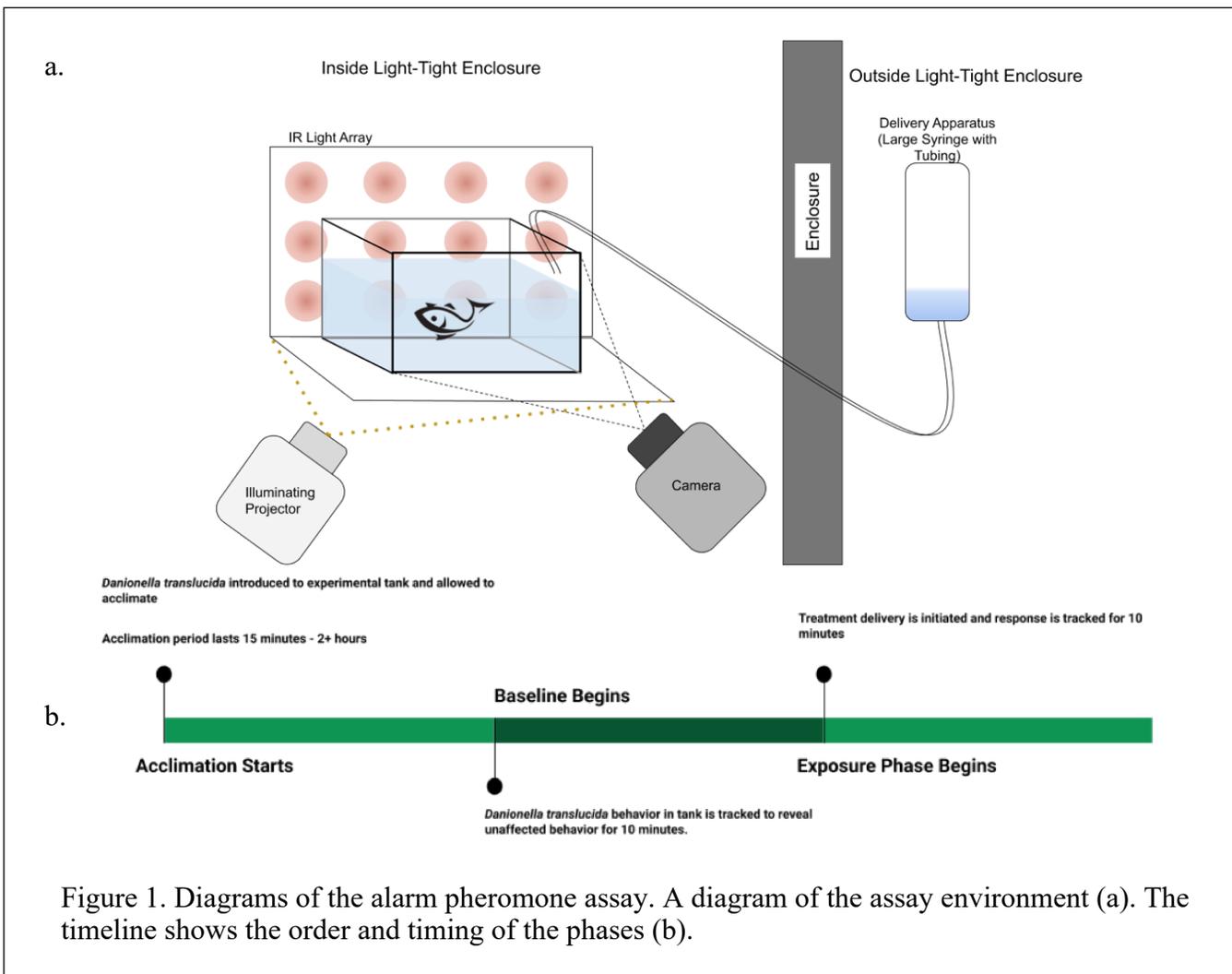


Figure 1. Diagrams of the alarm pheromone assay. A diagram of the assay environment (a). The timeline shows the order and timing of the phases (b).

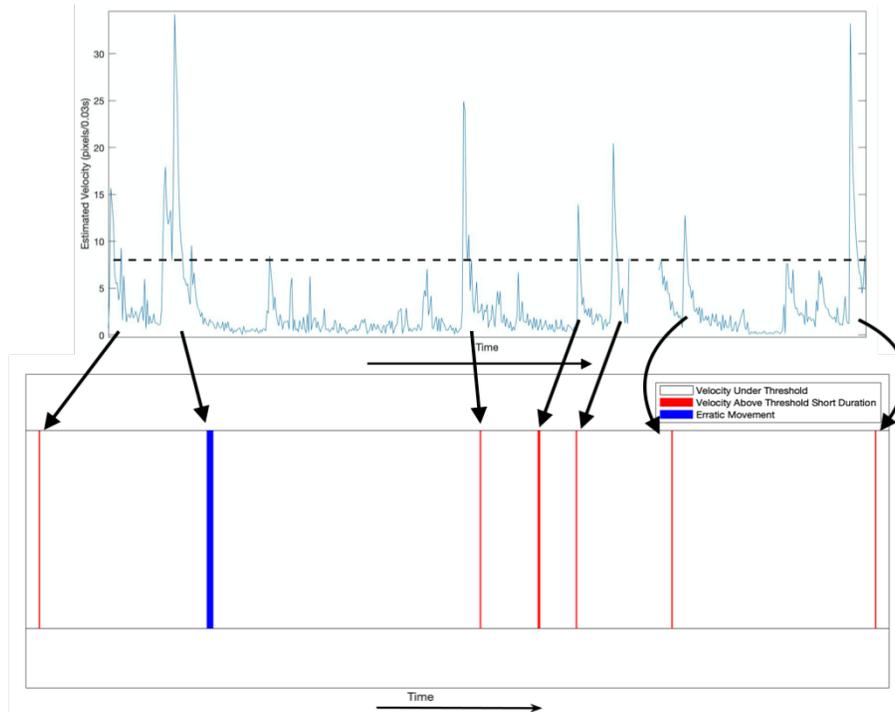


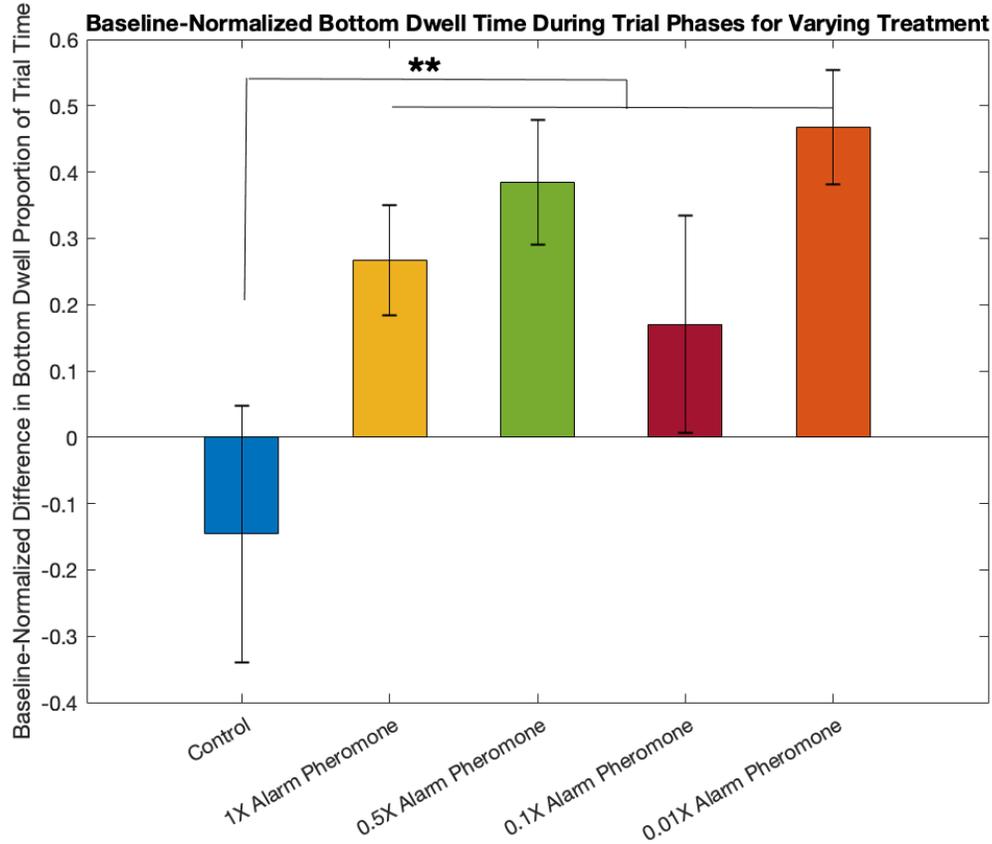
Figure 2. A diagram showing an example of how erratic movements are characterized. A time series of the subject fish's velocity is calculated and passed through a threshold (top graph) such that only velocity values higher than the threshold are considered as being an erratic movement (arrows show considered bouts). Furthermore, the threshold must be surpassed for a given amount of time in order to be considered an erratic bout (blue lines) or else it is not included in the count for erratic bouts (red lines; bottom graph).

III. Results

A. Zebrafish Replication Assays

In order to assess the validity of our alarm pheromone extraction method as well as to determine a valid way to assess behavior through our defined constructs, we replicated findings of adult zebrafish's response to alarm substance. In these experiments, a solution of the zebrafish-derived alarm pheromone was delivered to the subject in a 20 mL volume with 5 varying concentrations (1X, 0.5X, 0.1X, 0.01X, 0X) of the crude extract per $n = 4$ subjects. Delivery at the onset of the exposure phase was rather quick (mean time: 18s, range: 15-22s) and did not vary across concentration conditions. In this assay, bottom-dwelling increased relative to the baseline in those exposed to the alarm pheromone relative those who were not (2-sample t-test, d.f. = 18, $p < 0.01$). There was also a significant interaction in the concentration of the alarm substance on the amount of bottom-dwelling increase (1-way ANOVA, $F = 3.2371$, d.f. = 4, $p = 0.042$). The subjects exposed to the alarm pheromone increased the frequency of erratic movements relative to the baseline when compared to the control group (2-sample t-test, d.f. = 18, $p = 0.02$). With the current assay, however, fish from all groups tended to reduce their freezing in all groups with no significant difference between the pheromone-exposed and control groups. These results generally replicate past findings with zebrafish as well as validate both our experimental setup as well as our alarm pheromone extraction process.

a.



b.

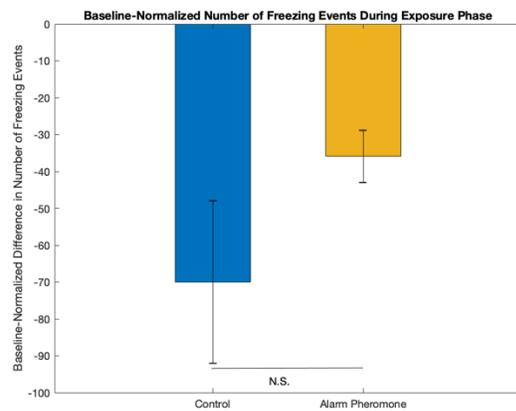
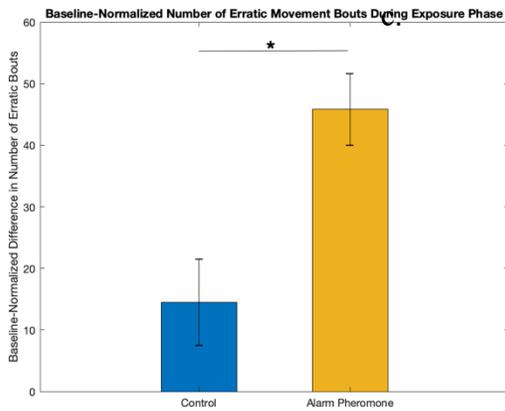
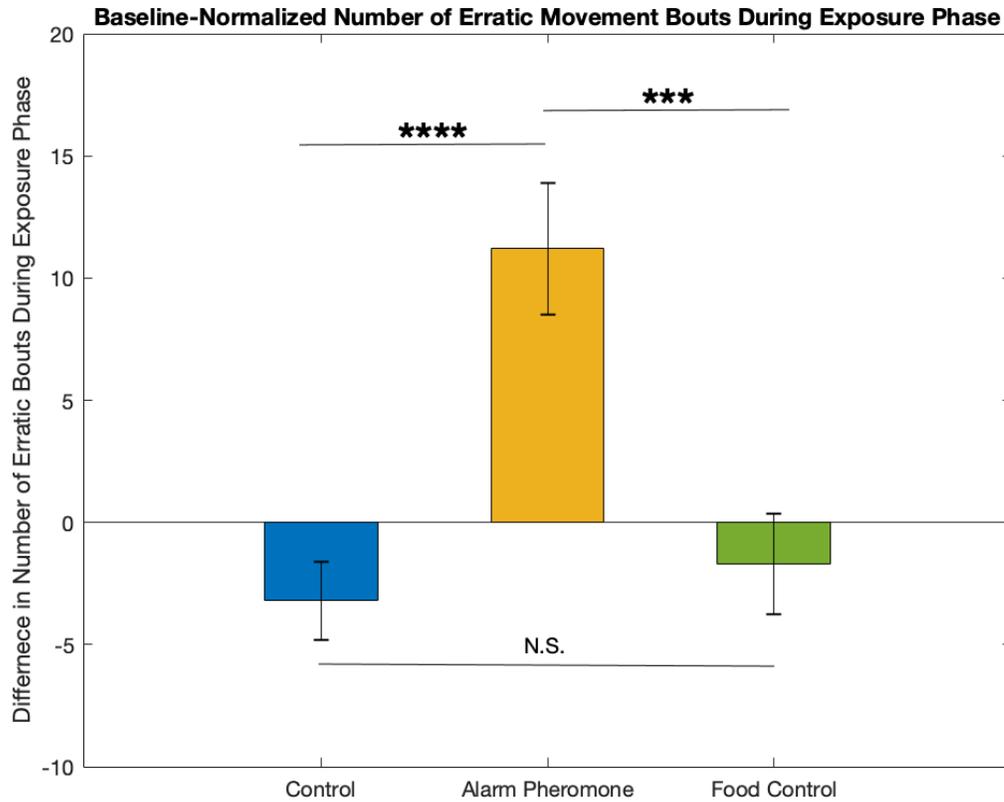


Figure 3. Graphs showing various behavioral differences between the control and zebrafish-derived alarm pheromone-exposed groups in zebrafish. Each graph shows the population means and S.E.M. of each subject's difference between the exposure and baseline phase scores (exposure phase score – baseline phase score) for bottom-dwell time proportion (a), number of erratic bouts (b), and freezing events (c).

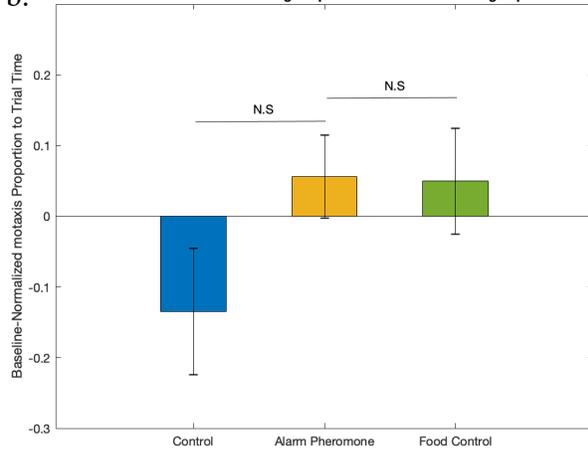
B. *D. translucida* Assays

For these assays, we reduced the delivery volume to 5 mL and kept the delivery time the same relative to the previous assay as control treated *D. translucida* seemed to respond with stress if the delivery was too intense and not subtle enough. Furthermore, we only delivered one concentration (0.1X) of the alarm pheromone derived from other *D. translucida*. Additionally, subject fish were allowed to habituate to their tank for at least two hours prior to the habituation phase to prevent a pre-stimulus stress response. Subjects exposed to the alarm pheromone during the exposure phase increased their erratic movement frequency relative to the baseline phase when compared with the control group (Figure ;2-sample t-test, d.f. =12, $p < 0.001$). On the other hand, bottom-dwelling time did not significantly differ between the pheromone-exposed group and the control group (2-sample t-test, d.f. = 12, $p > 0.05$); however, there did seem to be a qualitative difference in how the subjects positioned themselves in the tank if they were exposed to alarm substance. This led us to consider a post-hoc measurement of thigmotaxis. Thigmotaxis time differed with marginal significance between the alarm pheromone-exposed group relative to the control group such that the pheromone-exposed group increased their time spent near the edges of the arena, away from open space (2-sample t-test, d.f.=12, $p = 0.064$). However, another control was performed with a control delivery treatment containing food-conditioned water in order to distinguish a fear response from a feeding response as the crude pheromone extract may possibly be perceived as food. In this control, the frequency of the erratic movement difference between the baseline to exposure phases differed between the food exposure and alarm pheromone exposure (2-sample t-test, d.f.=9, $p < 0.01$), whereas exposure-related thigmotaxis change did not differ between treatments (2-sample t-test, d.f.=9, $p > 0.05$). These results suggest that changes in thigmotaxis is not an indicative measure of the alarm pheromone response, while changes in the frequency of erratic movements does seem to be.

a.



b. **Baseline-Normalized Bottom-Dwelling Proportion of Trial Time During Exposure Phase**



c.

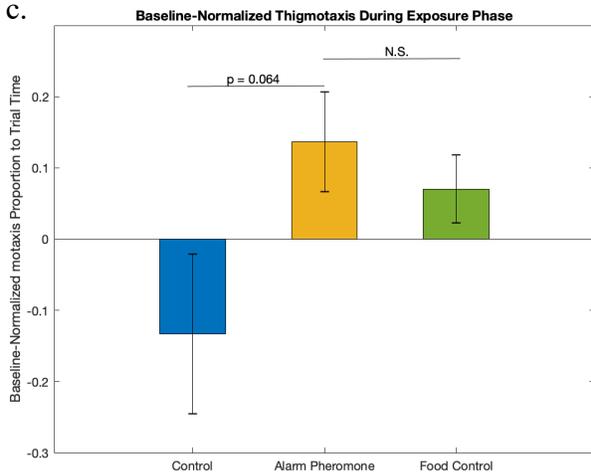


Figure 4. . Graphs showing various behavioral differences between the control treatments (blank and food-treated delivery) and *D. translucida*-derived alarm pheromone-exposed groups in *D. translucida*. Each graph shows the population means and S.E.M. of each subject's difference between the exposure and baseline phase scores (exposure phase score – baseline phase score) for the number erratic bouts (a), bottom-dwell time proportion (b), and thigmotaxis time proportion (c).

IV. Discussion

In these studies, we performed exploratory work on an alarm pheromone response in *Danionella translucida*. We found that these fish have a characterizable response with exposure to the ‘schreckstoff’ alarm pheromone. Furthermore, this species’ response to an alarm pheromone differ to some degree from that observed in zebrafish. Specifically, *D. translucida* do not seem to respond by increasing the amount of time that they spend on the bottom of their tank whereas zebrafish do in order to evade a predator, and this may be inductively explained in terms of the species’ ecological origin within turbid waters as well as its small size. Overall, more experiments are necessary to further characterize the fear response of *D. translucida* so that a stronger association of the fear response may be made in relation to exposure to the alarm pheromone.

If this work proves to be fruitful, we could look at an effect of social buffering in response to the alarm pheromone. It has been found in adult zebrafish that in the presence of visual and/or olfactory conspecific cues, an individual that receives exposure to the alarm pheromone has a diminished response (Faustino et. al, 2017; Speedie & Gerlai, 2008). Extending this research to *D. translucida* may open a line of investigation to determine the neural basis of such social buffering.

Additionally, the *D. translucida* in our sample did not seem to exhibit an alarm response to the alarm pheromone derived from zebrafish. Because our sample have never experienced prolonged exposure to zebrafish, it may be that these fish may are not sensitive to olfactory cues from zebrafish. This may suggest that the reaction to some pheromones may be in part due to previous experience of the pheromone and an association of coinciding events. With properly controlled experiments, there may be another line of work to investigate the potentiality of experience-dependence in the alarm pheromone response.

References

- Chung-Davidson, Y.-W., Huertas, M., & Li, W. (2010). A Review of Research in Fish Pheromones. *Chemical Communication in Crustaceans*, 467–482. doi: 10.1007/978-0-387-77101-4_24
- Darwin, C. (1889). *The descent of man and selection in relation to sex*. doi: 10.5962/bhl.title.106468
- Frisch, K. V. 1938. Zur Psychologie des Fisch- Schwarmes. *Naturwissenschaften* 26:601-606. . 1941a. Die Bedeutung des Geruchssinnes im Leben der Fische. *Ibid.* 29:321-333. 1941b. tYber einen Schrecksto'f der Fisch- haut und seine biologische Bedeutung. *Z. vergl. Physiol.* 29:46-145
- Faustino, A. I., Tacão-Monteiro, A., & Oliveira, R. F. (2017). Mechanisms of social buffering of fear in zebrafish. *Scientific Reports*, 7(1).
- Gomez-Diaz, C., & Benton, R. (2013). The joy of sex pheromones. *EMBO Reports*, 14(10), 874–883. doi: 10.1038/embor.2013.140
- Kim, C. K., Adhikari, A., & Deisseroth, K. (2017). Integration of optogenetics with complementary methodologies in systems neuroscience. *Nature Reviews Neuroscience*, 18(4), 222–235. doi: 10.1038/nrn.2017.15
- Lopes, G. A., Bonacchi, N., Frazão, J. O., Neto, J. P., Atallah, B. V., Soares, S., ... Kampff, A. R. (2015). Bonsai: an event-based framework for processing and controlling data streams. *Frontiers in Neuroinformatics*, 9. doi: 10.3389/fninf.2015.00007
- Mathis, A., & Smith, R. J. F. (1992). Avoidance of areas marked with a chemical alarm substance by fathead minnows (*Pimephales promelas*) in a natural habitat. *Canadian Journal of Zoology*, 70(8), 1473–1476. doi: 10.1139/z92-203
- Mathuru, A. S., Kibat, C., Cheong, W. F., Shui, G., Wenk, M. R., Friedrich, R. W., & Jesuthasan, S. (2012). Chondroitin Fragments Are Odorants that Trigger Fear Behavior in Fish. *Current Biology*, 22(6), 538–544. doi: 10.1016/j.cub.2012.01.061
- Mizunami, M. (2010). Alarm pheromone processing in the ant brain: an evolutionary perspective. *Frontiers in Behavioral Neuroscience*, 4. doi: 10.3389/fnbeh.2010.00028
- Morgan, E. D. (2008). Trail pheromones of ants. *Physiological Entomology*, 34(1), 1–17. doi: 10.1111/j.1365-3032.2008.00658.x
- Penalva, A., Bedke, J., Cook, E. S., Barrios, J. P., Bertram, E. P., & Douglass, A. D. (2018). Establishment of the miniature fish species *Danionella translucida* as a genetically and optically tractable neuroscience model.
- Pfeiffer, W. (1977). The Distribution of Fright Reaction and Alarm Substance Cells in Fishes. *Copeia*, 1977(4), 653.

- Portugues, R., Severi, K. E., Wyart, C., & Ahrens, M. B. (2013). Optogenetics in a transparent animal: circuit function in the larval zebrafish. *Current Opinion in Neurobiology*, 23(1), 119–126. doi: 10.1016/j.conb.2012.11.001
- Roberts, T. R. (1986). *Danionella translucida*, a new genus and species of cyprinid fish from Burma, one of the smallest living vertebrates. *Environmental Biology of Fishes*, 16(4), 231–241. doi: 10.1007/bf00842977
- Schulze, L., Henninger, J., Kadobianskyi, M., Chaigne, T., Faustino, A. I., Hakiy, N., ... Judkewitz, B. (2018). Transparent *Danionella translucida* as a genetically tractable vertebrate brain model. *Nature Methods*, 15(11), 977–983. doi: 10.1038/s41592-018-0144-6
- Speedie, N., & Gerlai, R. (2008). Alarm substance induced behavioral responses in zebrafish (*Danio rerio*). *Behavioural Brain Research*, 188(1), 168–177.
- Stensmyr, M. C., & Maderspacher, F. (2012). Pheromones: Fish Fear Factor. *Current Biology*, 22(6).
- Vandermoten, S., Mescher, M. C., Francis, F., Haubruge, E., & Verheggen, F. J. (2012). Aphid alarm pheromone: An overview of current knowledge on biosynthesis and functions. *Insect Biochemistry and Molecular Biology*, 42(3), 155–163. doi: 10.1016/j.ibmb.2011.11.008
- Waldman, B. (1982). Quantitative and Developmental Analyses of the Alarm Reaction in the Zebra Danio, *Brachydanio rerio*. *Copeia*, 1982(1), 1.
- Yew, J. Y., & Chung, H. (2015). Insect pheromones: An overview of function, form, and discovery. *Progress in Lipid Research*, 59, 88–105. doi: 10.1016/j.plipres.2015.06.001