Does the presence of novel vs. familiar companions affect the social buffering response in *Danio rerio*?

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Abstract

Social buffering is a phenomenon observed in social animals where the presence of a member from the same species alleviates the stress response by reducing the amount of stress an animal experiences, helping them to recover faster. Social buffering has been observed across vertebrate taxa, from primates, to birds, to fish. Zebrafish, *Danio rerio,* are a shoaling species – group living fish that form social bonds with other members. To test how familiarity within a group plays a role in social buffering, I exposed 18 zebrafish to a stressor in three treatments: 1) in pairs of new companions; 2) individually; and 3) in pairs where the fish are familiar with each other. After administering the stressor, chasing with a net, I observed the behavior of the fish for 20 minutes and scored four stress-related behaviors: erratic swimming, freezing, reduced exploration, and shoaling cohesion. The isolation treatment exhibited increased erratic movements and overall activity. The novel and familiar treatments did not significantly differ in any of the behaviors. The presence of conspecifics, whether or not they are familiar, seem to alleviate stress-related behaviors.

Introduction

A wide variety of animals exhibit social behaviors and form complex social relationships with members of their own species (conspecifics) and members of different species (allospecifics). The removal of social bonds can cause profound changes in an organism's physiology and behavior. Whenever an animal experiences a perceived harmful event, the stress response is activated. There is considerable evidence that experiencing a stressful event with a group lowers stress levels compared to experiencing it alone. This has been observed in humans, birds, and non-primate mammals (Kikusui et al., 2006). This phenomenon, called social buffering, seems to be a fundamental aspect of all group-living organisms.

Teleost fishes form complex social hierarchies and exhibit many of the same behaviors and stress responses as mammals (Bshary et al., 2014). However, experiments on social buffering in zebrafish have yielded conflicting results. One study found that different types of stress produced different responses in the zebrafish. In response to chasing with a net, individually housed zebrafish had lower plasma cortisol levels. Zebrafish housed in groups, however, had lower cortisol levels when visually exposed to a predator (Forsatkar et al., 2017). Another study found no evidence of social buffering at all. Zebrafish housed in groups had higher levels of cortisol compared to isolated fish when chased with a net and when placed in an unfamiliar environment – a new tank (Giacomini et al., 2015). Yet another study produced the opposite results – zebrafish placed individually in a novel environment had higher and more variable cortisol levels and displayed more erratic behavior (Pagnussat et al., 2013). One study found that zebrafish housed together resumed normal behaviors faster after being handled and fin clipped compared to zebrafish housed individually (White et al., 2017). Another study tested what type of cue matters the most in social buffering. They found that visual cues (being able to see conspecifics) was more

effective at reducing anxiety-related behaviors than olfactory cues (shoal water). They also found that shoal size does not matter – large groups and small groups both had reduced cortisol levels (Faustino et al., 2017).

Because zebrafish are model organisms for developmental biology and genetics, studying how social bonds affect stress will give us a better understanding of how zebrafish should be housed. Stress and the presence of conspecifics could be an unintended variable that affects the outcome of experiments (Pagnussat et al., 2013). No research has been done for zebrafish on whether the presence of novel vs. familiar companions affects the stress response, although it has been done for cichlids and sturgeon. In both studies, the presence of either novel or familiar companions alleviated the stress response (Culbert et al., 2019; Hare et al., 2015). In this project, I tested whether having contact with novel or familiar companions affects the stress response in zebrafish. I exposed the fish to a stressor in three treatments (housed with novel companions, housed in isolation, and housed with familiar companions) and measured their level of stress by analyzing four stress-related behaviors. Because of these results in other species, my hypothesis is that the zebrafish in the isolation treatment will exhibit the most stress-related behaviors, while the novel and familiar treatments will be similar.

Methods

Experimental Set-up

This experiment was conducted on 18 zebrafish that were housed in two 20-gallon glass tanks with nine fish in each tank. Tank conditions were as follows: temperature: 26-28^{o} C; 14-hour light/10-hour dark cycle; pH 7-7.2; ammonia at less than 0.01 mg/L; hardness at 6 mg/L. These conditions were kept the same for all fish in all tanks during the course of the study. The fish were

fed flake food ad lib and *Artemia salina* (10/mL) twice a day. On days of experiments, they were not fed. The zebrafish had a two-week habituation period to adjust to lab conditions (per IACUC #2022-02).

After the habituation period, I randomly separated the 18 fish into nine pairs. Each pair was composed of fish that had not been housed together previously and were put into a 10-gallon test tank, one pair at a time. After 30 minutes of acclimation time, I subjected each pair to a stress test, chasing with a net for two minutes. Afterwards, I observed their behavior for 20 minutes. The test tank was cleaned between each trial to prevent chemical alarm cues from being a confound.

Next, I randomly chose ten fish from the entire stock, and separated them in two-gallon isolation tanks. The rest of the fish were returned to one of the 20-gallon tanks. The separated fish were isolated for two weeks. At the end of that time, I placed each fish individually into the test tank and allowed it to acclimate for 30 minutes. Afterward, I subjected it to the stress test and observed its behavior for 20 minutes.

For the last treatment, I placed all of the fish back in the two 20-gallon holding tanks with nine fish in each tank, and they were housed together for two weeks. After two weeks, the fish were taken in pairs with their tankmates and placed in the test tank. After 30 minutes of acclimation, I subjected them to the stress test and behavior observation.

Throughout this experiment, I used an ethogram to score the same stress-related behaviors across all treatments: (1) erratic swimming (defined as swimming in a zig-zag manner); (2) freezing (the fish is completely still except for eye and gill movement); (3) reduced exploration (the fish stay near the bottom and the walls of the tank); and (4) shoaling (the fish swim together for 10 or more seconds). Every individual fish was observed for erratic swimming, freezing, and reduced exploration but shoaling cohesion was only scored for fish that were in pairs. To measure

erratic swimming and freezing, I counted every time a fish displayed that behavior. To measure reduced exploration, the tank was divided into three sections vertically, and I marked a rectangle in the center of the tank (**Fig. 1**). This allowed me to track the vertical and horizontal movements of the fish in the tank.

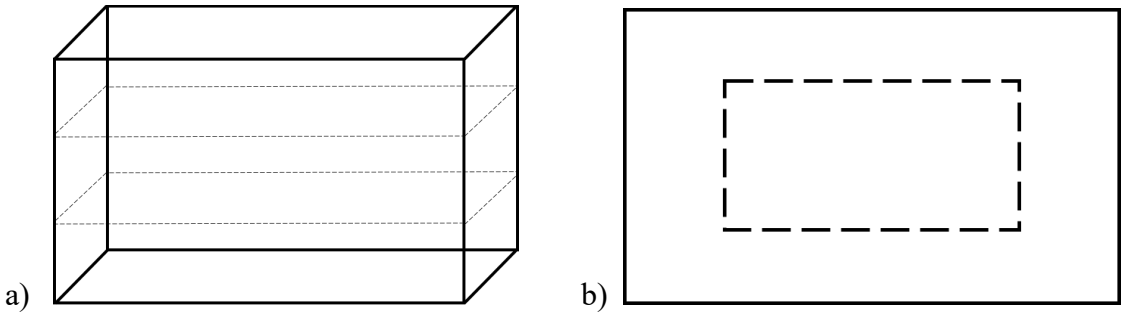


Fig. 1 The set up of the test tank. The tank was divided into (a) three vertical sections of equal size, and (b marked with a rectangle in the center.

To measure shoaling cohesion, I timed how long the fish exhibited shoaling behavior. Behaviors were scored during every trial; however, trials were also video recorded, using an iPhone 8, for further review.

Statistical Analyses

I used a Shapiro-Wilks test to determine if the dataset for each behavior was parametric. When the data was not parametric, I used a Friedmann's test to compare between the three treatments and a post-hoc Dunn's test. When the data was parametric, I used a one-way repeated measures ANOVA and a post-hoc Tukey HSD test. For shoaling behavior, since I was only comparing between two groups, I used a paired t-test.

Results

Across all treatments, there was variation in behavior between trials; however, some general trends did emerge. To compare behaviors between the three treatments, I averaged the results from each trial for the familiar and novel treatments, since I scored the behavior of both fish.

I totaled all observations of erratic swimming in the 20 minute observation period for each trial (**Fig. 2**). Although one of the novel trials displayed the most erratic swimming, with 120 counts, overall, the isolated treatment displayed the most erratic swimming and the familiar treatment displayed the least (p-value = 0.0015; Friedmann test, post-hoc Dunn's test).

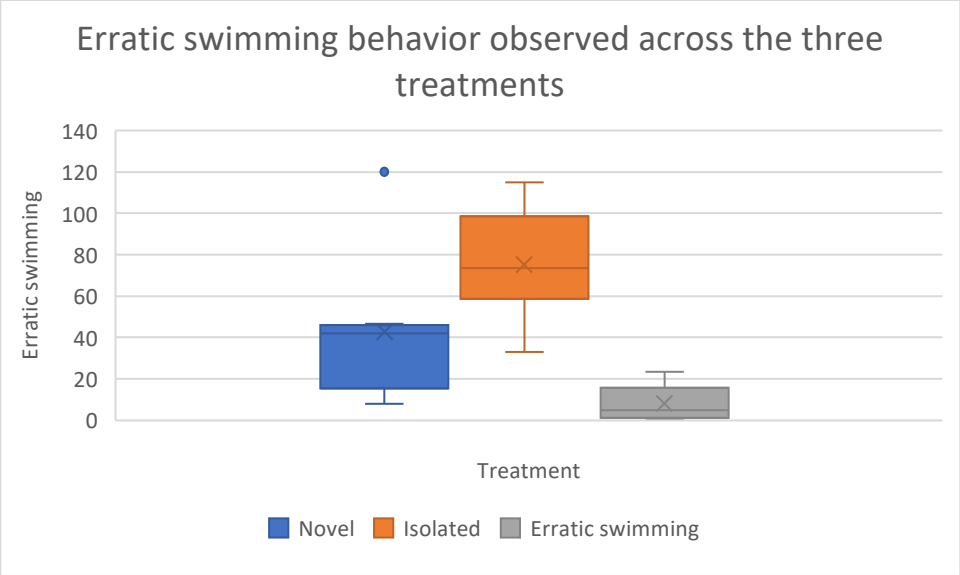


Fig. 2 Total number of erratic swimming behavior after 20 minutes of observation across three treatments: with novel companions, isolated, and with familiar companions. For the novel and familiar treatments, the average of each trial was taken. The isolated and familiar treatments significantly differed from each other (p-value = 0.0015).

Fish in the familiar treatment exhibited the most freezing (**Fig. 3**). In one trial, the fish froze for the whole 20 minutes except for one 10 second burst of movement. However, no significant difference was found between the three treatments. (p-value = 0.2952; Friedmann test).

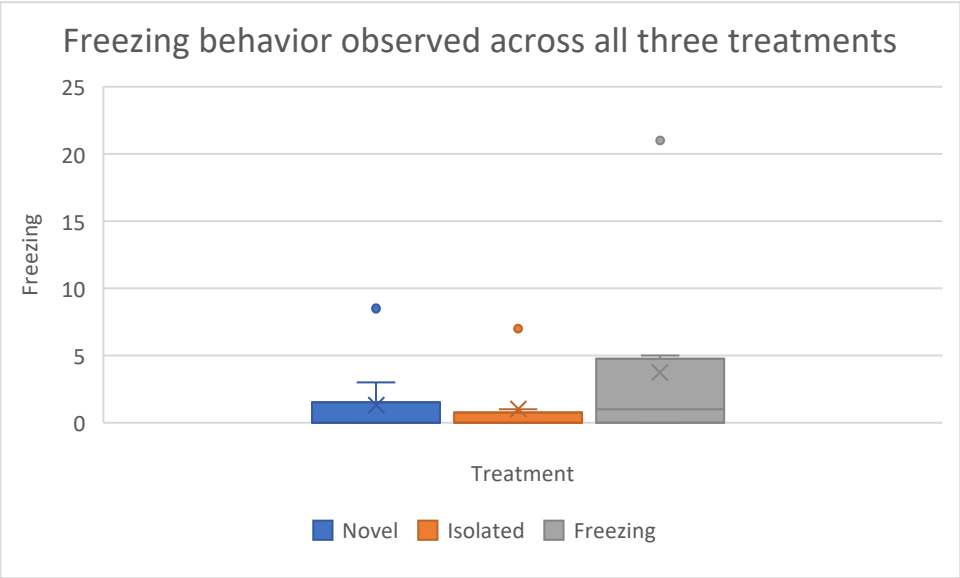


Fig. 3 Total number of freezing behavior after 20 minutes of observation across three treatments: with novel companions, isolated, and with familiar companions. For the novel and familiar treatments, the average of each trial was taken. No significant difference between treatments was found (p-value = 0.2952).

Shoaling behavior, scored in the novel and familiar treatments, was analyzed as the total amount of time the zebrafish spent shoaling in each trial (**Fig. 4**). There was no significant difference found between these two treatments (p-value = 0.8331; paired t-test).

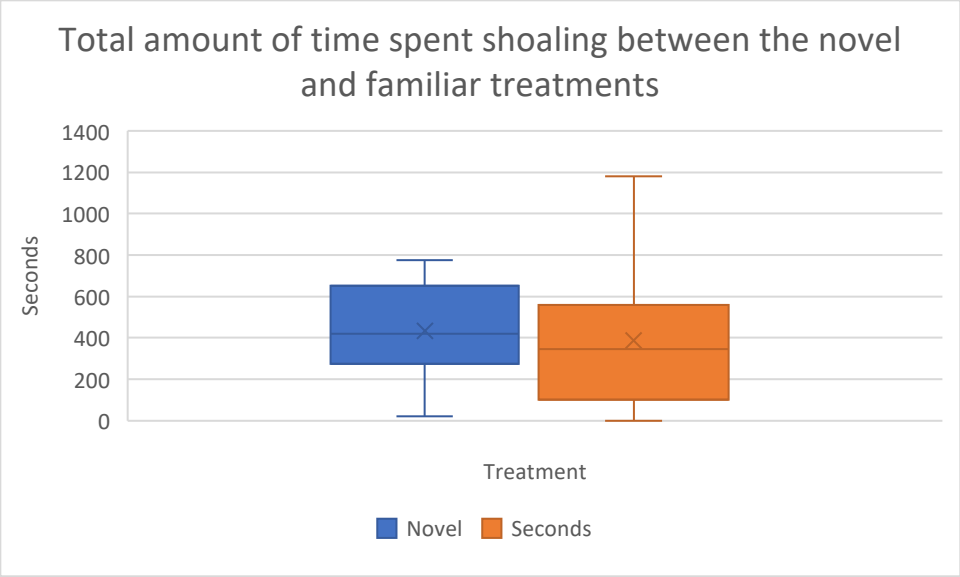
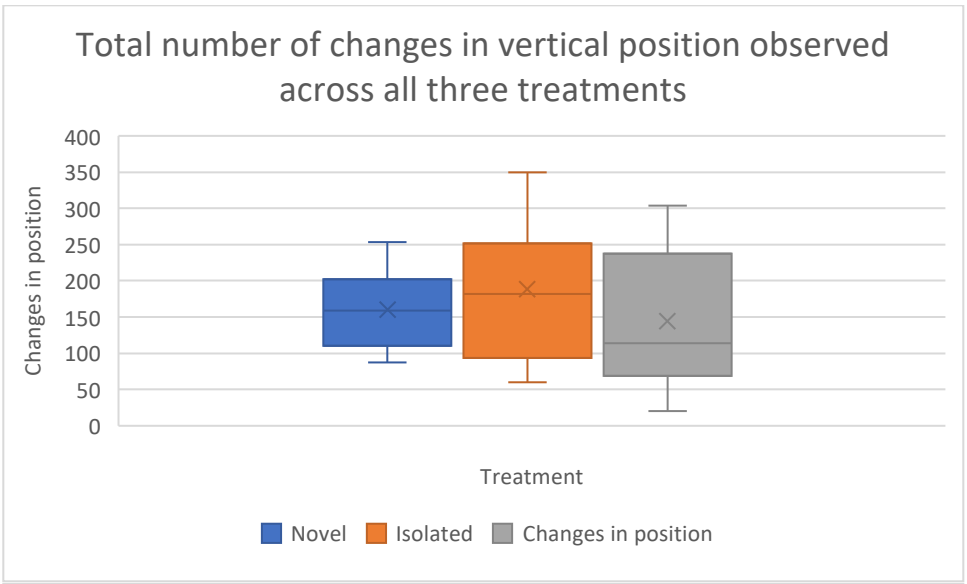


Fig. 4 Total amount of time spent shoaling after 20 minutes of observation between two treatments: with novel companions and with familiar companions. No significant difference between treatments was found (p-value = 0.8331).

To quantify reduced exploration, I measured vertical and horizontal position of the fish. I totaled all of the instances where a fish crossed into another section of the test tank in each trial (**Fig. 5**). Overall, the fish in the isolation treatment displayed the most movement, and the fish in the novel treatment displayed the least. For the vertical position data, there was no significant difference between the three treatments (p-value = 0.6590; ANOVA), but for horizontal position, the isolation treatment differed significantly from the other two (p-value = 0.0025; ANOVA, post-hoc Tukey HSD).

(a)



(b)

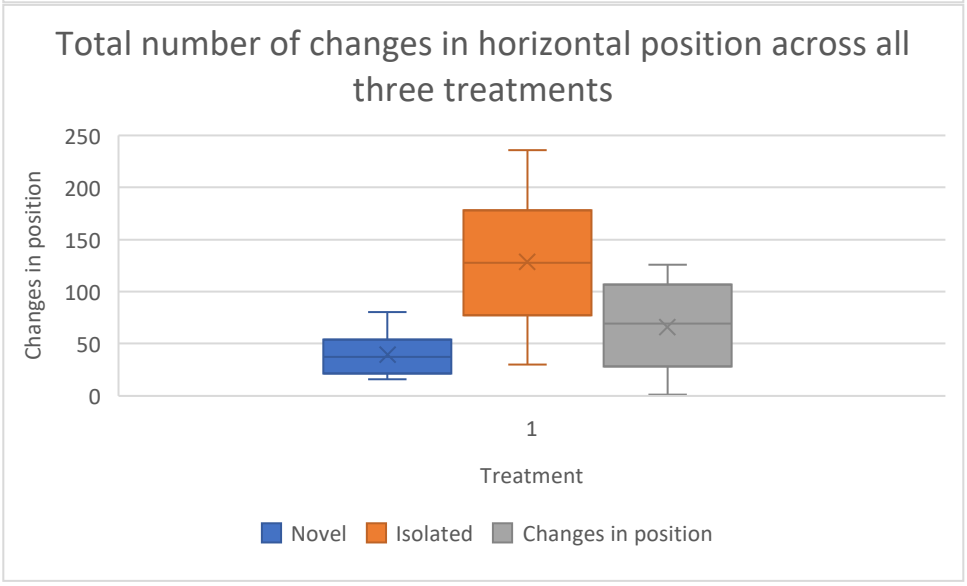


Fig. 5 Total number of changes in (a) vertical and (b) horizontal position after 20 minutes of observation across three treatments: with novel companions, isolated, and with familiar companions. The test tank was marked into top, middle, bottom, and center, and it was recorded whenever a fish crossed into another section. For the novel and familiar treatments, the average of each trial was taken**.** No significant difference between treatments was found for vertical position (p-value = 0.6590). For horizontal position, the isolated treatment significantly differed from the other two (p-value = 0.0025).

Discussion

My hypothesis that the isolation treatment would show the most stress-related behaviors and the novel and familiar treatments would be similar was partially supported. As I expected, there were no significant differences between the novel and familiar treatments in any of the behaviors. However, there was also no significant difference between the three treatments in vertical movement, whereas I had predicted there would be. Zebrafish exhibit thigmotaxis – wall hugging behavior – a very common measure of stress in animal behavior studies, which is determined by how much time fish spend in the bottom vs. top of the tank. It is well documented that fish spend more time in the bottom of a tank when stressed, and they take longer to venture into the top (Cachat et al., 2010). In my experiment, all of the trials started with the fish in the bottom section of the tank, so by scoring whenever they crossed into another section, I measured how much they explored upwards. I expected the novel and familiar treatments to exhibit the most exploration, however, this behavior did not differ between the treatments. I did not record latency to enter the top; previous research indicates that latency increases for fish in more stressful conditions (Cachat et al., 2010). This metric might be a good thing to observe in future studies; it would be an interesting comparison point with overall vertical position data. Other research on social buffering has also had mixed results when measuring reduced exploration. One study found reduced exploratory behavior in isolated fish, while another study did not (Pagnussat et al., 2013; White et al., 2017).

The isolation treatment exhibited a significant increase in erratic swimming, which I expected; however, I did not expect the isolation treatment to also have the most horizontal movement. I expected a decrease as part of reduced exploration. This could possibly be due to the overall increase of erratic movement in the isolation treatment. Previous research has also found increased erratic behavior and general activity level in isolated fish that are exposed to a stressor

(White et al., 2017). To reduce any confounding variables between activity level and exploration, in future studies, I could use only vertical position data to measure reduced exploration. To measure overall activity level or movement I could mark 4 quadrants on the bottom of the tank and score whenever the fish entered another quadrant. This combined with the vertical position data would give me a clearer picture as to where the fish are moving.

Future studies could also include behavioral test batteries, where animals are tested in multiple behavioral tasks, and the results are triangulated for more robust results. Behavioral test batteries are more common in rodent research, but relatively rare in fish. The novel tank test and light-dark test are two common tests to measure stress in fish that I did not use. The novel tank test measures stress by exposing fish to a new environment and the light-dark test measures their affinity for light (Fontana et al., 2022). My experiment used a small sample size, so my results did not have high statistical power. Using a larger sample size is a simple answer to get more robust, statistically accurate results, but it's not always practical. A behavioral test battery could help get a clearer picture of the fishes' behavior by exposing them to multiple stressors.

Another line of future study: is social buffering still effective when an animal is in a group of allospecifics? The term "social buffering" is used almost exclusively to refer to conspecifics alleviating stress. Very little research has been done on how the presence of allospecifics affects stress. One interesting aspect could be studying whether social buffering differs in the presence of species that are closely related vs. distantly related. For example, comparing *Danio rerio* with another *Danio* species vs. a species of tetra.

Zebrafish are a model organism for many kinds of research, and the presence or absence of conspecifics could be a confounding factor that impacts the outcome of experiments. My research demonstrated that when exposed to stress away from a group, zebrafish increase erratic

movements and overall activity. In addition, the presence of conspecifics, whether or not they are familiar, alleviate stress-related behaviors.

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