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Invasive Crayfish Faxonius Rusticus Do Not Prefer Pheromones of Conspecifics

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Invasive crayfish *Faxonius rusticus* do not prefer pheromones of conspecifics

By

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Abstract

The invasive crayfish *Faxonius rusticus* has invaded multiple watersheds throughout Pennsylvania and the country. Current trapping methods are time consuming, labor intensive, and ineffective. Because crustaceans produce and utilize pheromones to communicate social status and mating condition, baited pheromone traps might serve as an effective alternative to the current trapping methods. The aim of this study was to examine if *F. rusticus* can distinguish between pheromones produced by conspecifics during breeding and non-breeding seasons. Previous research in our lab showed that during the breeding season females prefer male pheromones, but that preference disappears during the non-breeding season. We collected pheromones from male and female *F. rusticus* during breeding and non-breeding seasons. Collected pheromones were combined to create stocks; female breeding, female non-breeding, male breeding, and male non-breeding. Sexually mature *F. rusticus* were exposed to breeding and non-breeding pheromone stocks of opposite sex by pumping the pheromones through the arms of a Y-maze. The preference of the individual was determined based on which arm of the maze it selected and spent more time in. Findings suggest that during the non-breeding season mature crayfish do not exhibit a preference for pheromone type, breeding or non-breeding. There was no difference in pheromone preference between males and females; neither sex exhibited preference for opposite sex pheromones from the either breeding or non-breeding season. Understanding how pheromones are used by crayfish is essential to developing effective methods for eradication of invasive species.

Introduction

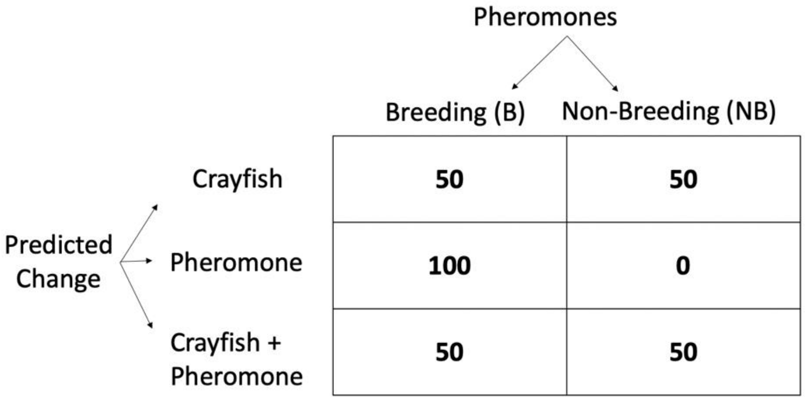
Faxonius rusticus, commonly called rusty crayfish, is an invasive crustacean species originally from the Ohio River that has entered the Susquehanna River Watershed and has continued expansion into New England states (Kuhlmann & Hazelton, 2007: Hill, 1999). This species is commonly identified by reddish spots found on both sides of their carapace. *F. rusticus* outcompetes the native crayfish species, including the Calico (*Faxonius immunis*), Spinycheek (*Faxonius limosus*), and Appalachian brook crayfish (*Cambarus bartonii*) primarily by consuming larger amounts of food typically eaten by the native crustacean and other native species (Wilson et al., 2004). *F. rusticus* also has a crippling effect on the ecosystem by destroying aquatic plant beds and causing bioturbation, which harms the habitat of native organisms like snails, aquatic insects, macroalgae, and diatoms causing destruction of the ecosystem (Kuhlman & Hazelton, 2007; Corkum & Belanger, 2007). The invasiveness of the species has driven scientists to search for ways to remove *F. rusticus* from non-native watersheds in hopes of restoring native ecosystems.

Rusty crayfish typically live in habitats that contain a substantial amount of coverage from predators. Common habitats include fractured concrete, cobble, and woody debris in freshwater streams (Kershner & Lodge, 1995). They become more active during the breeding season which occurs between March and October (Berril & Arsenault, 1984). This means the non-breeding season occurs between November and February for rusty crayfish. During breeding season, females can be identified by the seminal receptacle at the base of their posterior walking legs while the males are identified by the presence of gonopods. These gonopods are only present during the breeding season when males are identified as Form I males (Hamr, 2002). During the non-breeding season males tend to shed their chelae and gonopods (Berril &

Arsenault, 1984). Reproduction occurs when males deposit sperm in a blind-ended receptacle on the female where the sperm resides until the female lays her eggs (Berril & Arsenault, 1984). The increased activity of rusty crayfish during breeding season can increase the chance of catching more of this invasive species.

The most common trapping method for rusty crayfish thus far has been baited, modified minnow traps. Unfortunately, these traps are inefficient for rusty crayfish of all sizes, with most of the small sized crayfish escaping the trap (Ogle & Kret, 2007). Current research has focused on developing methods to use pheromones to trap and remove invasive species. Like most crustaceans, rusty crayfish use chemical signaling for food acquisition, determining conspecific social status, sex recognition (including mating responses), and predator detection (Corkum & Belanger, 2007). These chemical signals, also known as pheromones, are composed of short carbon-based molecules that are released into the water, through urine that is secreted through nephropores and directed anteriorly. This release is controlled by the contraction and relaxation of the nephropore sphincter muscles and currents created by the gills. The gills project the urine forward, while the fan organs allow this current to be pushed in a specific direction (Breithaupt, 2011). Crayfish have multiple appendages that contain chemoreceptors including antennae, antennules, pereopod chelae, telson, and maxillipeds, that are imperative in information acquisition (Moore, 2007). Breithaupt (2011) hypothesized that hair-like appendages called aesthetascs, that reside on the first antennules and major chelae, contain the chemoreceptors for sex pheromones. Because crayfish rely so heavily on chemical signaling for survival, creating traps that incorporate pheromones that attract specific species may be an efficient method for removing invasive crayfish species from watersheds (Corkum and Belanger, 2007).

The goal of this research was to examine preference specificity of *F. rusticus* for conspecific opposite-sex pheromones. Work by a previous lab member has suggested that *F. rusticus* females prefer male pheromones over female pheromones, but only during the breeding season. Male crayfish showed no statistically significant preference toward female or male pheromones during breeding or non-breeding seasons (Luscavage, 2021). Luscavage observed changes in pheromones possibly as a factor of seasons but did not directly test for seasonal effects in her studies. Furthermore, it is not clear whether the preference for the opposite-sex pheromones exhibited by females during the breeding season is caused by the chemical change in the pheromones that the crayfish produce, the change in the sensitivity of the crayfish to these pheromones, or a combination of these factors (i.e. change in the pheromones and change in perceptual threshold of the crayfish). A diagram is seen below that summarizes the expected results (Fig. 1):



	Breeding (B)	Non-Breeding (NB)
Crayfish	**50**	**50**
Pheromone	**100**	**0**
Crayfish + Pheromone	**50**	**50**

Figure 1: Predicted probabilities of arm preference for breeding and non-breeding pheromones for both males and females depending on the predicted change occurring between breeding and non-breeding seasons.

Limited evidence from other crustacean species suggests that physiological changes associated with seasons may impact pheromone preference of the receivers, as well as the actual information carried within the pheromones of the senders. Males within the superorder Peracarid presented with odors of receptive and non-receptive conspecific females, moved towards the odors of receptive females in the breeding season (Breithaupt, 2011). Sexually mature *Pacifastacus leniusculus* males that were exposed to water conditioned by mature females showed a reduced response to the water in the non-breeding season when compared to their responses during the breeding season (Stebbing et al. 2004). These results suggest that the production of sex pheromones that elicit a response from conspecifics may only occur during breeding season in females (Stebbing et al., 2004), as well as possibly affect the receptivity of the males (Breithaupt, 2011).

In this study, the same behavioral paradigm used by Luscavage was utilized to expose *F. rusticus* to conspecific, opposite-sex pheromones of breeding (late August-mid-October) and non-breeding seasons (late October-March). Specifically, I examined if male and female rusty crayfish had a preference between breeding and non-breeding pheromones during the non-breeding season. If the behavioral response to pheromones is due to seasonal changes in crayfish sensitivity to the pheromones, then females would be expected to only show preference for the breeding pheromones when they are tested during the breeding season and show no preference outside of the breeding season. If it is the chemical composition of the pheromone that is driving changes in the behavioral response of the receiving crayfish, then the females should show the same preference for the breeding pheromones regardless of what season they are in. Similar results should be seen in males as well. This preference would be seen in the crayfish spending significantly more time in the non-breeding arm while in non-breeding season and vice versa. I decided to restrict my trials to the non-breeding season, when individuals should be in non-breeding condition and therefore not receptive to pheromones collected during the breeding season. This set-up allowed me to tease apart the potential change in receptive physiology of the crayfish from the change in the chemical composition of the pheromones. Understanding how pheromones are used by the crayfish is essential to developing effective eradication of invasive species, specifically *F. rusticus.*

Methods and Materials

1. General Maintenance of Crayfish

Faxonius rusticus were collected from Conoy Creek, Middletown Reservoir, and Conewago Creek in the Elizabethtown and Middletown area of Pennsylvania in the years 2017-2021. Males and females were housed together in 150 cm X 72 cm x 30 cm troughs filled with room temperature well water. Troughs contained air stones, rocks, wood, PVC pipe, and shells to mimic natural habitats. These troughs were half-emptied and refilled with water once every two weeks and fully cleaned (water emptied, habitats cleaned, rocks/trough rinsed) once every six weeks. Animals were fed half an algae pellet twice a week. Any individuals found dead were removed as soon as possible from the trough. All changes made to the troughs were documented on the log sheet found in the aquarium room. The aquarium room is light controlled in a 12-hour light and 12-hour dark cycle. A total number of 18 crayfish were isolated and used in Y-maze experiments. A total number of 24 crayfish were isolated for pheromone stock collection.

2. Setting Up Isolation Tanks

Prior to experiments, animals were transferred to an isolation tank measuring 29.85 cm x 19.68 cm x 20.32 cm. Each isolation tank had a mesh net elevated by suction cups approximately 2 cm from the bottom, air stone, air tube, six suction cups, and a half pvc pipe as a shelter. Two suction cups were placed at the lowest point possible on two opposing sides of the tank. The air tube was fed through the lid and mesh net and attached to the air stone. This apparatus was then placed upon the two suction cups at the bottom of the tank ensuring the mesh net was not touching the bottom of the tank. The remaining four suction cups were added to each side of the tank to reinforce the placement of the mesh net and the air tube was secured to one of the suction cups. The elevated mesh netting allowed fecal matter to settle to the bottom of the tank, helping maintain water quality. All equipment was cleaned with soap and water then rinsed thoroughly with water and air dried before each use. Each isolation tank was filled with well water to about 7/8ths of the tank.

3. Isolating Individuals

Individuals were taken from the aquarium room troughs and placed in an isolation tank 7-9 days prior to experiments. Prior to placement in tank the total length (mm), carapace length (mm), weight (g), sex, and any unique identifiers were recorded for each individual. (Note: total length is calculated from the end of the telson to the tip of the acumen of rostrum). The isolation room was maintained at a 12h dark :12h light cycle and maintained at a constant temperature. Visual isolation was maintained by placing isolation tanks on different shelves. Following isolation all individuals were placed into the same trough to ensure they are not used for pheromone stock (see below). Isolation tanks were cleaned with soap and water after each use and allowed to dry.

4. Pheromone collection

Pheromones were obtained by collecting the water from the isolation tanks in which individuals were kept. Water was collected and filtered using a Buchner funnel fitted onto a 2000 mL filter flask and attached to a vacuum. A 150 nm filter plus paper was placed on the Buchner funnel. Filtered water was labeled and stored in plastic bags until combined with the filtered water of three other same-sex isolated individuals (300 mL per individual total). Filtered water was placed into bags that were labelled with the sex, season (breeding or non-breeding) of the individuals' water, total amount, initials, and the date. Breeding season was considered samples collected between June-mid-October and non-breeding water was collected between late October-March. Bags were then frozen and then thawed two days prior to experiment.

5. Running the Y-Maze

All experiments were conducted in the non-breeding season from late October to April. A Y-maze was used to assess preference of opposite-sex chemical signals of *Faxonius rusticus* (Figure 2). This Y-maze design and experimental paradigm was developed and validated by Henry (2017) and Luscavage (2021). The Y-maze had a total length of 1.5 m with 1.1m and 0.3m wide long arms. The maze was covered with dark paper towels so that an individual within the maze could not see movement outside. The maze was then filled with 50 L of room temperature water. Two separate containers were filled with 1200 mL of combined pheromone stocks as described above. Treatments options included opposite sex breeding versus non-breeding pheromone stocks. Containers were connected to the Y-maze through MasterFlex® I/P Pump Drives Model 77410 with I/P 88 tubing into a respective arm of the Y-maze. The placement of breeding and non-breeding pheromones in the containers was randomized such that no arm bias would exist during the trial. A removable mesh netting was placed where the arms converge to hold the focal crayfish in a neutral location until the experiment began (Fig. 2).

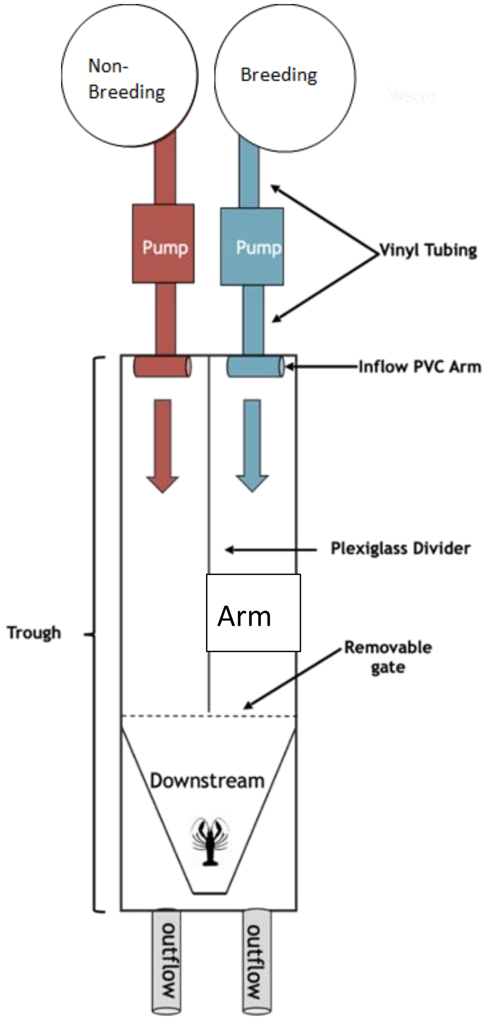


Figure 2: The experimental design as described above. Breeding and non-breeding pheromones, as well as filtered water, are pumped through the respective arms until convergence at the base of the Y. The crayfish was able to move around each arm for 15 minutes after the pumps were started and the gate was removed.

The crayfish was placed into the bottom of the Y-maze behind the mesh netting, into the “downstream” section. A camera began recording from above the Y-maze and the individual was allowed to acclimate to the conditions for 10 minutes. At the end of the acclimation period, the pumps were turned on with an inflow rate of 2.4 L/min for 3.33 minutes. A validation trial conducted earlier, using food dye to observe and quantify flow rate showed that 3.33 minutes is sufficient for the dye to travel through the arms of the maze. The mesh netting was then removed, and the individual's movement was recorded for the next 15 minutes. At the end of the 15 minutes crayfish was removed and recording was stopped. Video was used later for data analysis. Once an experiment has concluded the containers and the Y-maze were emptied of all remaining water and the maze was cleaned. Each container was then filled with 50 mL of bleach and filled with water. The pumps were turned on until the water had emptied from the containers into the Y-maze. The containers were filled again with water and the pumps were run a second time. The containers were then dried. The Y-maze was emptied of all remaining water and dried. The cleaning protocol described above was used after every trial.

6. Statistical Analysis

Variables identified from the recording included arm selected, total time spent in each arm, total distance walked in each arm, speed, and total numbers of turns. Within each sex, a binomial test was conducted to determine if there was an overall preference for one pheromone type compared to the other. One tailed t-tests were completed comparing the time crayfish spent in each arm and the number of turns in each arm. A significant difference was assumed when the resulting p-value was less than 0.05. Side bias was assessed by comparing the amount of time spent in arm 1 versus arm 2 for breeding pheromones followed by the same comparison looking at the non-breeding pheromones. Similarly, a one-tailed t-test with a p-value greater than 0.05 indicated no side bias. The significance criterion for all tests was $\alpha=0.05$.

Results

Females:

Side bias was assessed first to ensure any choices made by individuals were not influenced by the physical structure of the Y-maze. The average time spent with opposite-sex breeding pheromones in arm 1 compared to arm 2 showed no significant difference ($p=0.257$).

Similarly, there was no significant difference in the time spent in arm 1 and arm 2 when looking only at the non-breeding pheromones (p=0.202), indicating that preference to one pheromone type over the other was not influenced by the layout of the Y-maze. There was also no significant preference toward one pheromone type over the other. One female did not make a selection and so was removed from statistical analyses. Within the female crayfish 3 individuals chose the breeding pheromone and 4 chose the non-breeding pheromone (p=0.273; Figure 3). The average time spent in the non-breeding pheromone and breeding pheromone arms were 303.71and 341.41 seconds, respectively (t-test, p=0.425; Figure 4). There was no difference in the total number of turns made within each arm between breeding and non-breeding arms (t-test, p=0.249; Figure 5).

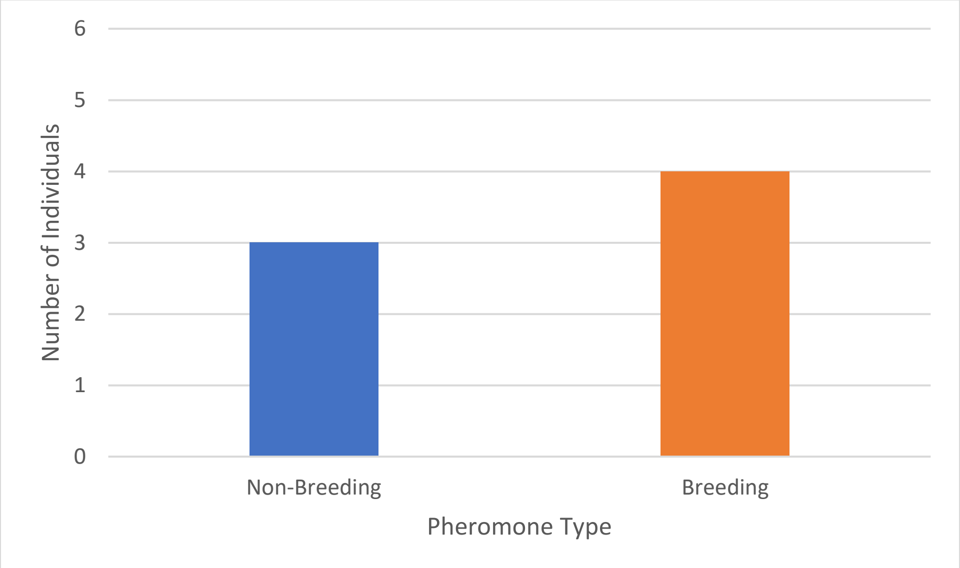


Figure 3: Total number of females that preferred breeding and non-breeding pheromones (p=0.273).

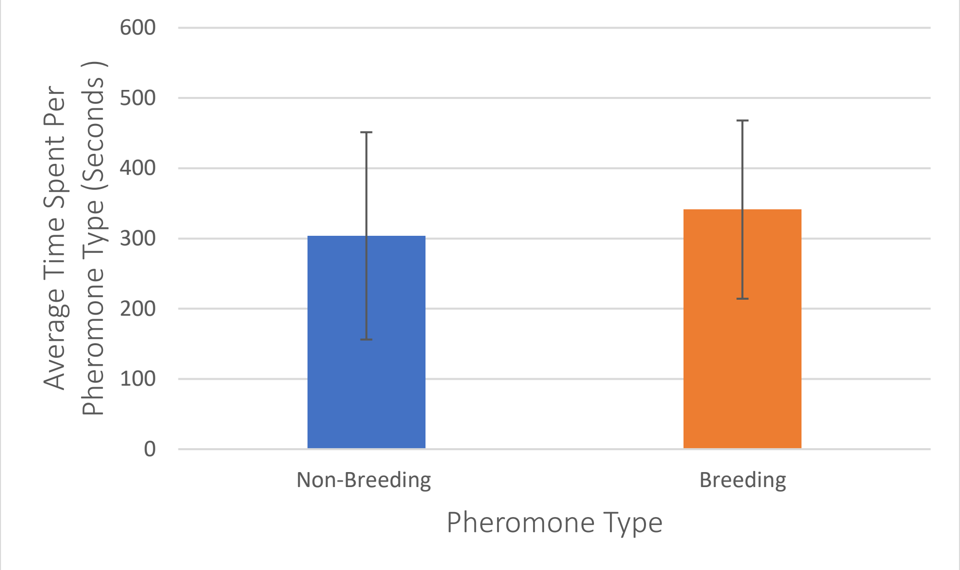


Figure 4: Average time for female *F. rusticus* in each arm, breeding pheromones and non-breeding pheromones, of Y-maze during the non-breeding season (p=0.425).

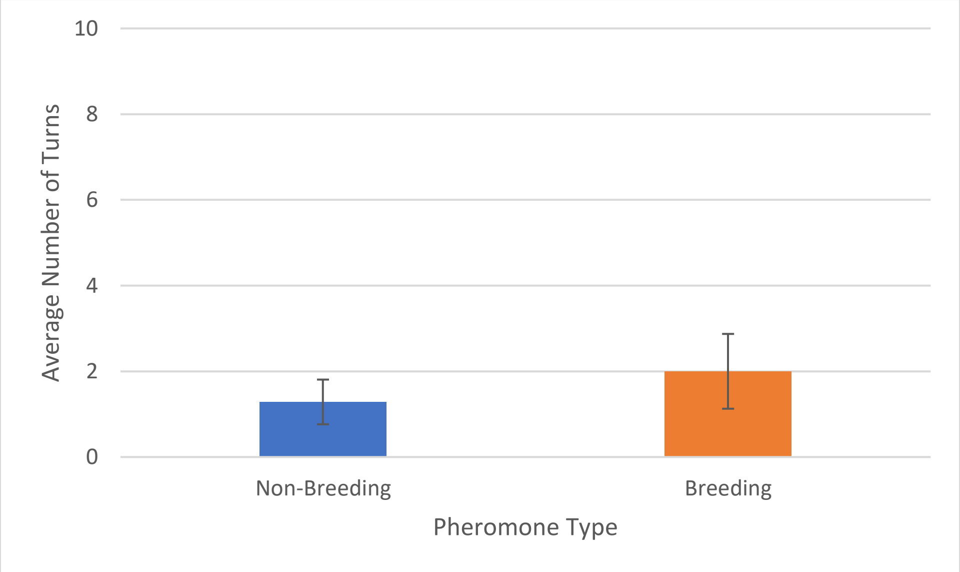


Figure 5: Average number of turns for female *F. rusticus* in each arm, breeding and non-breeding pheromones, of Y-maze during non-breeding season(p=0.249).

Males:

Side bias was assessed first to ensure any choices made by individuals were not influenced by the physical structure of the Y-maze. There was no difference between time spent with opposite-sex breeding pheromones in arm 1 compared to arm 2 (p=0.100). Similarly, there was no significant difference in the time spent in arm 1 and arm 2 when looking only at the non-breeding pheromones (p=0.219), indicating that preference to one pheromone type over the other was not influenced by the layout of the Y-maze. There was also no significant preference toward one pheromone type over the other. One male did not make a selection and so was removed from statistical analyses. Within the male crayfish 2 individuals chose the breeding pheromone and 5 chose the non-breeding pheromone (p=0.164; Figure 6). When comparing time spent in the breeding pheromone arm versus non-breeding pheromone arm, the p-value is not significant. The average time spent in the non-breeding pheromone and breeding pheromone arms were 410.43 and 293.29 seconds, respectively (t-test, p=0.265; Figure 7). While this test was not significant, these two averages appear much further apart from each other compared to the averages reported above for the females. There was no difference in the total number of turns made within each arm between breeding and non-breeding arms (t-test, p=0.441; Figure 8).

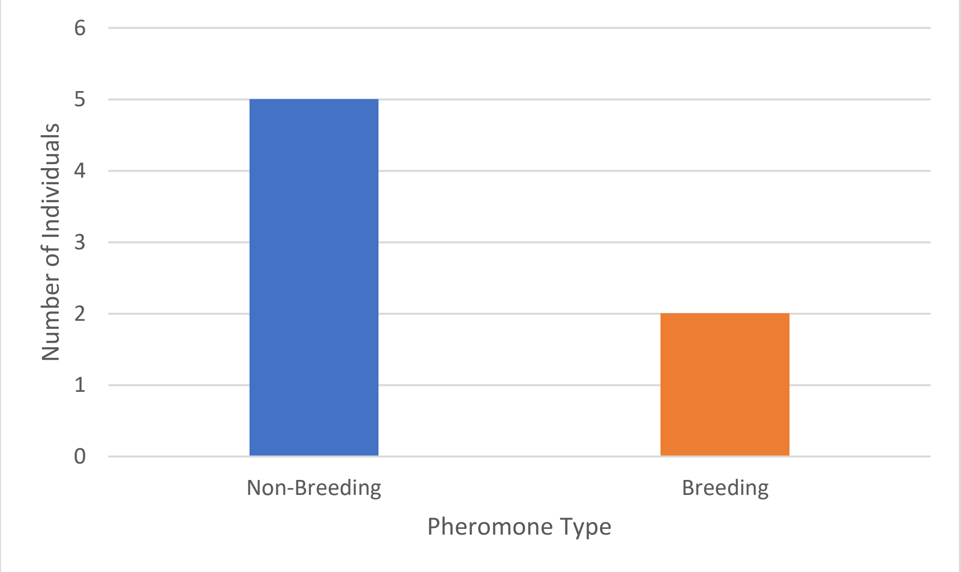


Figure 6: Total number of males that preferred breeding and non-breeding pheromones (p=0.164).

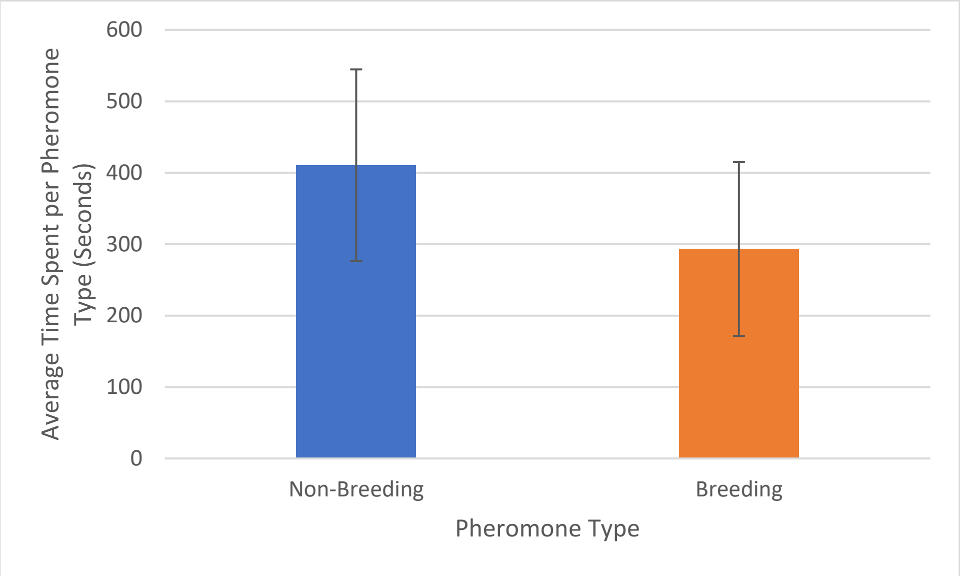


Figure 7: Average time for male *F. rusticus* in each arm, breeding pheromones and non-breeding pheromones, of Y-maze during the non-breeding season (p=0.265).

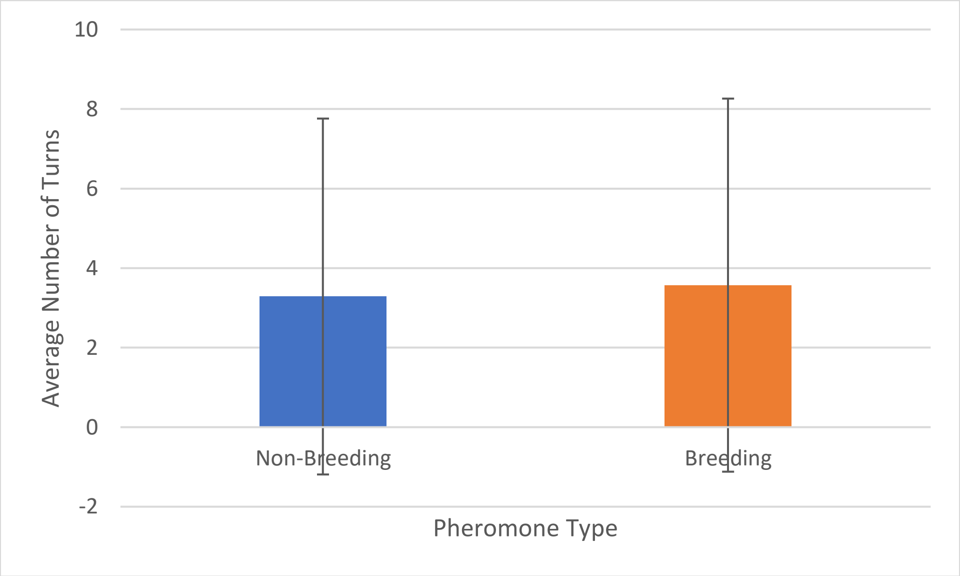


Figure 8: Average number of turns for male *F. rusticus* in each arm, breeding and non-breeding pheromones, of Y-maze during non-breeding season (p=0.441).

Discussion

The results indicate that neither male nor female *F. rusticus* conspecifics shows a preference toward breeding or non-breeding pheromones during the non-breeding season. Males tended to spend more time in the non-breeding pheromone type arm (Fig. 7). The opposite can be said for the females, they tended to spend more time in the arm with the breeding pheromones (Fig. 4). In the context of my initial predictions (Figure 1), these results suggest that neither sex can sense a difference between the pheromones during the non-breeding season. This could mean that they themselves are physiologically changing between breeding and non-breeding seasons where they are unable to detect sex pheromones during the non-breeding season. Alternatively, they can sense and differentiate both breeding and non-breeding pheromones, however, since they are in the non-breeding season, they show no preference. With this rationale there could be no preference in that in the non-breeding season individuals would have no need to trend toward the breeding pheromone because they do not need to breed. This second hypothesis would need to be tested by running the same experiments in the breeding season and seeing a preference toward the breeding pheromone arm.

In many crustaceans there is an obvious change in behavior in the breeding season compared to the non-breeding season. Males and females are known to rely on chemical signaling to find a mate. Additionally, males are seen to increase in aggression and fighting while competing for a female followed by mate guarding after (Subramoniam, 2017). With a definitive change in behavior, it would not be presumptuous to assume that other changes are occurring within the individuals, including physiological changes. From there, it would make sense that a physical change could lead to a change in pheromone reception, specifically during the breeding season.

In future studies an increase in replicates would be helpful in gaining more accurate estimations of time spent in each pheromone type arm. Additionally, conducting these experiments in both breeding and non-breeding timeframes would allow a greater understanding of potential differences in time spent in each pheromone arm during breeding and non-breeding seasons. Very little research has been conducted evaluating the chemical composition of crayfish pheromones which could prove to be useful in the future in determining changes between breeding and non-breeding seasons. More research will have to be conducted to determine if utilizing breeding pheromones in traps during breeding season and non-breeding pheromones during the non-breeding season would be more efficient in capturing the invasive crayfish *F. rusticus*.

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