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Olfactory learning capabilities of Paraphrynus laevifrons

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Abstract:

Amblypygids, a species of nocturnal arachnids from the tropics and subtropics are incredibly intelligent and are able to not only navigate through difficult tropical terrain but also, it is believed, possess olfactory learning capabilities that aid in navigation and recollection of environments. It is hypothesized that through sensitive olfactory receptors on their antenniform legs and highly developed mushroom bodies, Amblypygi can not only learn smells but also learn to associate smells with certain stimuli such as a crevice to take refuge in. To test this hypothesis, *Paraphrynus laevifrons*, a species of amplypygid was subjected to four different learning treatment groups with two distinct odorous compounds (1-Geraniol and 1-Hexanol). Over the course of the three training days and subsequent final test day, it was apparent that *P. laevifrons* was able to correctly associate a certain odor with the correct side at a higher percentage than expected. Although the results demonstrated a statistical significance, a larger sample size and more tests are required to determine to what level olfactory learning takes place within amblypygids.

Key Words: Biological Sciences, Amblypygi, Mushroom Bodies, Olfactory Learning, Arthropods, Navigation

Introduction:

One trait that all animals possess, at least at some level, is the ability to navigate through the environment that it resides. This ability allows animals to not only find shelter, but also food, water and any other necessary material that may be needed for the survival of the animal [2]. The diverse possibilities of habitats that animals reside in is astounding, and it brings up the question as to how do different animals navigate through their environments and to what level could they possibly be learning? And if so, is the learning unique to the individual or do all individuals of a species go through the same process?

Many studies have been done on navigation within the phylum Arthropoda such as with honey bees, fiddler crabs, and dung beetles [9]. However, because the phylum of Arthropoda is so broad and contains a large variety of species, it is difficult to relate navigational preferences between species. Some species rely on line of sight and path integration in less cluttered environments while some species live in a much more complex environment and have other means of navigation and perception [9].

Amblypygids, also called whip spiders, are in the phylum Arthropoda and have been studied often recently due to the ecological, behavioral and sensory perception that these particular animals possess [1]. These animals live in a very diverse range of habitats, often in areas that are extremely cluttered and difficult to move around in [2]. The difficult terrain does not prove to be a challenge to the Amblypygids, in fact these animals prefer this cluttered environment due to their preference for crevices and other hiding spots [2]. Instead of relying on visual clues for path navigation, it has been believed that amblypygids rely on the use of

sensory cues to move through their natural environment and find their way back to their original territory [9]. But how is this accomplished?

Many arachnids along with amblypygids possess an array of filiform hair sensilla that are able to detect air displacement in the environment [7]. Instead of having eight legs for locomotion like normal arachnids, amblypygi possess only six with the anterior two legs being used for sensory perception and termed antenniform legs [1]. These two sensory legs are full of these filiform hairs that the animal uses for sense perception and detecting changes in the environment. The use of these antenniform legs appears to be significant as many amblypgyids first survey an area with these legs to determine the surroundings before moving [7]. Unlike most arthropods however, amblypygi have an enlarged mushroom body located in their brain that is believed to be associated with sensory learning [3]. Mushroom bodies, although present in all arthropods except crustaceans, are subject to evolutionary trends which can be seen in Amblypygi who appear to have repressed other areas of their brains for the development of the mushroom bodies [3][8].

Mushroom bodies have been studied in honey bees, drosophila and other arthropods so their function and implications in olfaction can be understood [4]. These bodies are composed of a changing number of Kenyon cells, depending on the organism, that run parallel down the central complex of the brain [4]. Kenyon cells eventually branch off into multitudes of dendrites that have the possibility of creating an action potential and eventual sense perception [4]. This pathway of olfactory sensation is especially important in arthropods such as honey bees that rely on it to find new sources of food and then finding their way back to the hive [5]. Although olfactory awareness has been a consistency throughout evolutionary history with the

interpretation of pheromones, the understanding and the underlying principles of the actual pathway had not been well researched [10].

In this experiment, the amblypygid *Paraphrynus laevifrons* was placed into one of four treatment options in which a certain odor would either be associated with an open shelter that it could take refuge in, or a closed shelter that it could not enter. Determining which treatment group each subject was in was random, however each trial was nearly identical. The results that were obtained demonstrate that some type of olfactory learning is present in *P. laevifrons*, however the extent to this learning was still unknown.

Materials and Methods:

Subjects

16 *Paraphrynus laevifrons* were collected around Las Cruces Biological Station in Coto Brus county, Costa Rica by Tyler Corey [2]. An image of *P. laevifrons* can be seen in Figure 1. Each *P. laevifrons* were housed in separate plastic terrariums (7.1 inches x 4.4 inches x 5.5 inches) with a soil substrate to resemble the natural ground. Each container had a water dish as well as a rectangular cork bark that the individuals could use to hang under. Each were fed either a small, medium, or large sized cricket weekly, depending on the size of the individual at the time. The room they were housed in was separate from the room that experimental trials were conducted in. This room was kept at a humidity range of 20-60% with a temperature ranging from 21-26 ºC.

Experimental Apparatus

The experimental trials were conducted in a single arena with interior dimensions of 28 centimeters (cm) x 14 cm x 5 cm (L x W x H) that was not housed in the same area that the animals were kept when trials were not being conducted. The walls of the arena were constructed of a clear acrylic plastic while the floor was an opaque acrylic with a thin mesh covering overlaying it to provide some form of grip for the animals as they moved throughout the arena during the individual trials (Figure 2). This arena had three 3 cm x 1 cm holes at the base of the arena walls, two holes located at either end of the arena and one being at the center of one of the lengthwise walls.

A 60-watt spiral CFL light bulb (900 lumens) was attached to the center of the lengthwise wall opposite the opening using a clamp. The bulb was placed approximately 15-20 cm from the center of the floor of the arena. The lights were used to provoke movement in the test subjects and increase the likelihood that movement towards the experimental shelters would occur to get out of the bright light.

An additional three shelters were also incorporated into this experiment. Each were constructed of a black acrylic for both the walls, floor, and lid that was placed over them (Figure 1). Like with the arena, a thin mesh covering was placed on the floor of all three shelters. The first shelter was the release shelter that had an interior dimension of 14 cm x 5 cm x 3 cm and was aligned with the opening at the center of the lengthwise wall. The other two shelters were aligned with the openings at either side of the arena and both had an interior dimension of 14 cm x 5 cm x 3 cm.

Within the release shelter was a single 2.5 cm x 2.5 cm x 1 cm clear acrylic cube that had a small well drilled into the top of it. This cube was placed opposite of the opening within the release shelter and would have 15 µL (microliters) of distilled water placed into it for each trial. The cube was separated from the rest of the shelter using a hard-plastic mesh screen to prevent the animals from interacting with it directly. Finally, another hard-plastic mesh screen was placed in front of the opening initially to prevent the animal from entering the arena until the screen was removed. Similar to the release shelter, the two experimental shelters contained the clear acrylic cube that would house either 1-Geraniol (Sigma-Aldrich, Product Number 163333), 1-Hexanol (Sigma-Aldrich, Product Number 471402)**,** or distilled water.

Procedures

The primary goal of this experiment was to determine whether subjects could learn to associate a certain odor (1-Gernaiol or 1-Hexanol) with either an opened shelter or a closed shelter. Four experimental treatments were created with four subjects for each treatment. For the purpose of this experiment, 1-Geraniol was assigned to two experimental groups labeled A, and 1-Hexanol was assigned to the other two experimental groups labeled B. Additionally, a positive (+) symbol was added if the compound was associated with an opening and a negative (-) symbol was added if the compound was associated with a closure. For example, A+ was using 1-Gernaiol and placing it in one of the experimental shelters that was open for the subjects to enter upon starting the trial while the opposite was closed and had distilled water in the acrylic cube, and B- was using 1-Hexanol and placing it in one of the experimental shelters that was closed with a hard plastic mesh screen preventing subjects from entering while the other was open and had distilled water.

A subject was placed within the release shelter that already had 15 µL of distilled water placed in the acrylic cube that was opposite the opening. Once in the release shelter, the lid was placed on and the subject was allowed to acclimate to the shelter for five minutes before being release to the main shelter. Depending on the assigned treatment group, either 15 µL of 1-Gernaiol or 15 µL of 1-Hexanol would be used in one acrylic cube and 15 µL of distilled water would be placed in the other. A coin was flipped to determine which side the odor would be applied to prevent the subject from continually going to the same side, a head would place the compound in the left experimental shelter and a tail would place the compound in the right experimental shelter. If the subject was in a positive treatment group, the acrylic cube would be

placed away from the opening of the experimental shelter with a hard-plastic mesh surrounding it allowing the subject to enter, while the experimental shelter with distilled water was blocked off with the hard-plastic mesh. However, if the subject was in a negative treatment group, the acrylic cube would be placed near the opening with a hard-plastic mesh over the opening preventing the subject from entering but allowing the fragrance of the compound to enter the arena and the opposite experimental shelter with distilled water was open.

After the five minute acclimation time, the subject was gently coaxed into the arena using a blunt probe, closing off the release shelter once the subject entered the testing arena. 15 minutes were given for each trial for the subject to move around on their own and find the correct opening, after this time if the subject still had not entered the correct experimental shelter, it was again softly coaxed with a blunt probe. Once in the experimental shelter, another five minutes were allowed for the subject to sit in the shelter before being returned back to their containers. Once the subject was place in the container, all parts of the arena and experimental shelters were cleaned and dried with 95% ethanol and reset for the next trial.

A total of three days was allocated for each subject with a total of 14 training trials and one test trial. Five training trials were conducted on day one and day two. On day three there were only four training trials followed by the final test trial. In the test trial, both experimental shelters were closed with 1-Geraniol on one side and 1-Hexanol on the other and the amount of time the subject spent near the correct side (same side as the compound in positive treatments and opposite side of the compound in negative treatments) was recorded. The arena was divided into four equal 7 cm x 14 cm (L X W) sections. The subject was only considered to be near either experimental shelter and thus time recorded when the entire body

was within either of the outer fourths of the arena. Whenever the subject entered the middle half of the arena the time was stopped until it moved to one of the outer fourth sections. The test trial lasted for a total of 15 minutes before the subject was returned to its container.

Statistical Analysis

Three questions were analyzed throughout this experiment. The first question was whether or not there was an effective treatment that the test subjects learned better or worse. The next question was whether there was a statistically significant difference between time spent near the correct side (θ) and the predetermined θ value of .5. The final question was whether there was a difference in the learning within different individuals during the training and test trials. Because the data was not normally distributed, a Kruskal-Wallis analysis was used to determine whether an effective treatment existed between 1-Gernaiol and 1-Hexanol. Continuing with this data, a Wilcox test was utilized to determine the statistical significance of the θ value among each treatment groups. Finally, a repeated measure anova test was used to compare individuals within training days to determine if there was the difference in training.

Results:

Effective Treatment

The mean theta value for all treatment options was .681 ± .173. The mean theta value for 1-Geraniol (A) was .593 ± .177 and the mean theta value for 1-Hexanol (B) was .796 ± .189. Figure 3 shows the distribution of values for all four treatment options tested.

Is theta statistically higher than expected, and if so what is the learning scale

Using a mean test of distribution for all theta values across all treatments, a statistical value was found ($\theta = .681 \pm .321$, $df = 12$, $p = .0322$). However, upon further analysis within each day, only day one produced a significant value (latency mean = 828.46 ± 544.82, $p = .0178$) while day 2 (latency mean = 548.3 ± 433.74, $p = .293$) and day 3 (543.94 ± 455.89, $p = .919$) were statistically insignificant. Figure 4 shows the average latency times for each day along with each time from every subject for all three days. Additionally, Figure 5 shows the trend, which was slightly significant, of the decreasing latency times during the day which it was the most pronounced.

Discussion:

In this experiment, three key questions were analyzed in order to better understand the olfactory capabilities of *P. laevifrons.* The first question was whether or not there was an effective treatment during these experiments. The fact that neither 1-Gernaiol or 1-Hexanol had a statistical difference from one another is great and supports the multitude of chemicals that were able to be identified by amblypygids [3]. If there had been a statistical difference, the learning difference could simply have been that the subjects were more receptive to one compound over the other, but due to the fact that this statistical significance was not present, we could further the analysis of olfactory learning.

The next question that was asked was whether or not the subjects spent a longer amount of time near the target side than expected. A theta value of .5 was designated for this because if there was no sensory learning, the subject should spend equal time on both sides of the enclosure. However, the data showed that in fact there was a statistical significance. The subjects on average, spent more time near the correct side than would be expected under normal conditions. This supports the hypothesis that with the enlarged mushroom bodies and antenniform legs that possessed a multitude of sensory villi, that amblypygids in fact can learn smells. During all of the trials, before the subject would move, they would test the area with the antenniform leg first before moving to either side which correlates with what was found with whip spiders by Santer and Hebets in 2011.

Another study done with ghost spiders (*Hibana futilis*), it was determined that with the addition of a honey odor would allow the spiders to find a nectar source much quicker than was recorded without the honey odor [6]. Although the two species are different, they are all in the

Arthropoda phylum and presumably possess similar processing pathways in their mushroom bodies structures that allow for olfactory stimulation. However, because of the larger mushroom bodies found in amblypygi, it would be assumed that their olfactory perception would be much greater. However, there are currently no studies focusing on this and could be a point of future studies for differences within a phylum.

The last question asked was to what extend do individuals learn an odor. Although there was no significant value recorded through these experiments within days, a general trend can be seen. Figure 4 shows the trends for Day 1 through Day 3 for all individuals. On day one, a steep decline can be seen as the training trials proceed. But on days two and three, the decline starts to plateau, possibly alluding to the fact that the amblypygids learn the most on the first day and then slowly plateau as the days proceed. Future experiments should be run with a larger sample size as well as a longer time scale to really determine the effective learning time for these animals. Now that we know that amblypygids can indeed learn to differentiate smells, the next goal should be to further expand upon this to fully understand the extent to which they can learn and how they incorporate this in their natural environment and what other factors could potentially be at work.

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Figure 1. *Paraphrynus laevifrons*. There are three walking appendages with an anterior antenniform legs on each side that are not used for locomotion but rather sensing the environment. The body is divided into the anterior prosoma and the posterior opisthosoma.

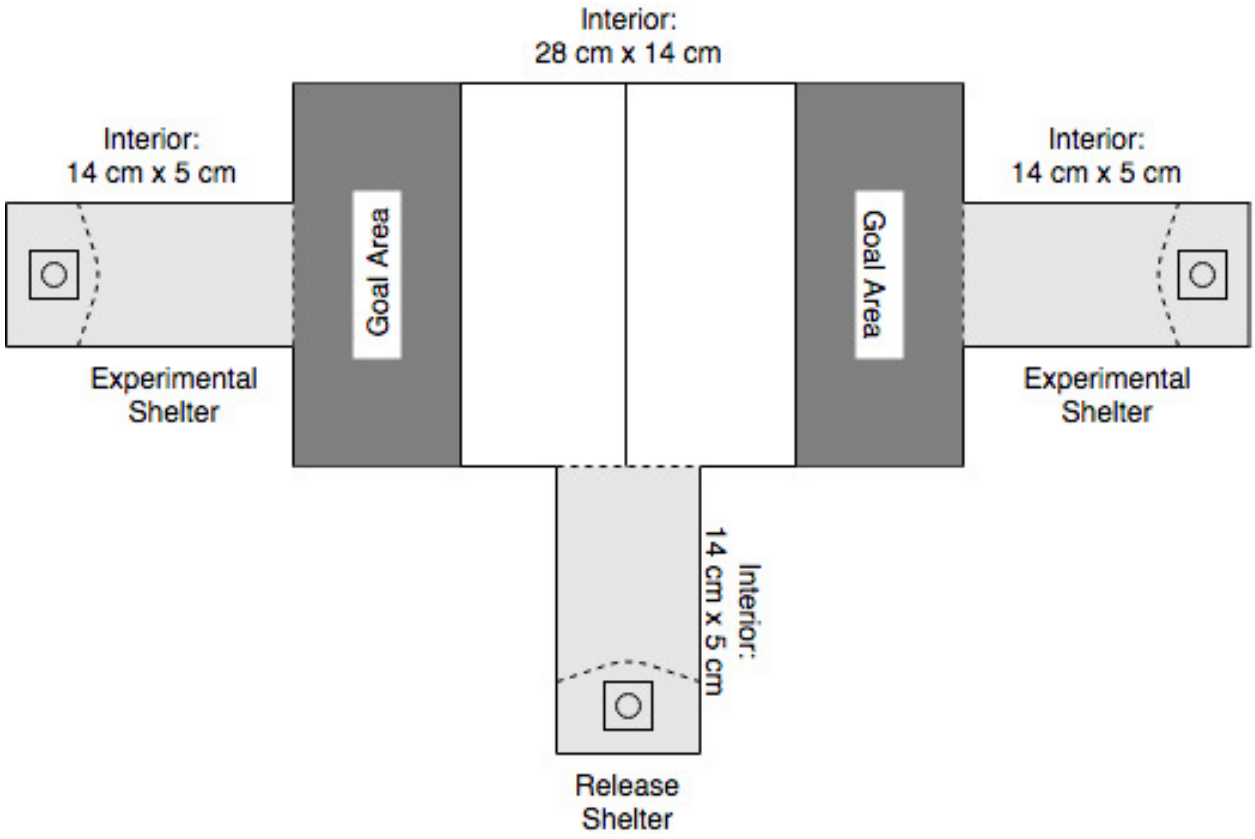

Figure 2. Arena and shelters used throughout the experiment. Dashed lines are locations where hard plastic mesh would have been located and each small square within the shelters would hold either distilled water, 1-Geraniol or 1-Hexanol depending on the treatment.

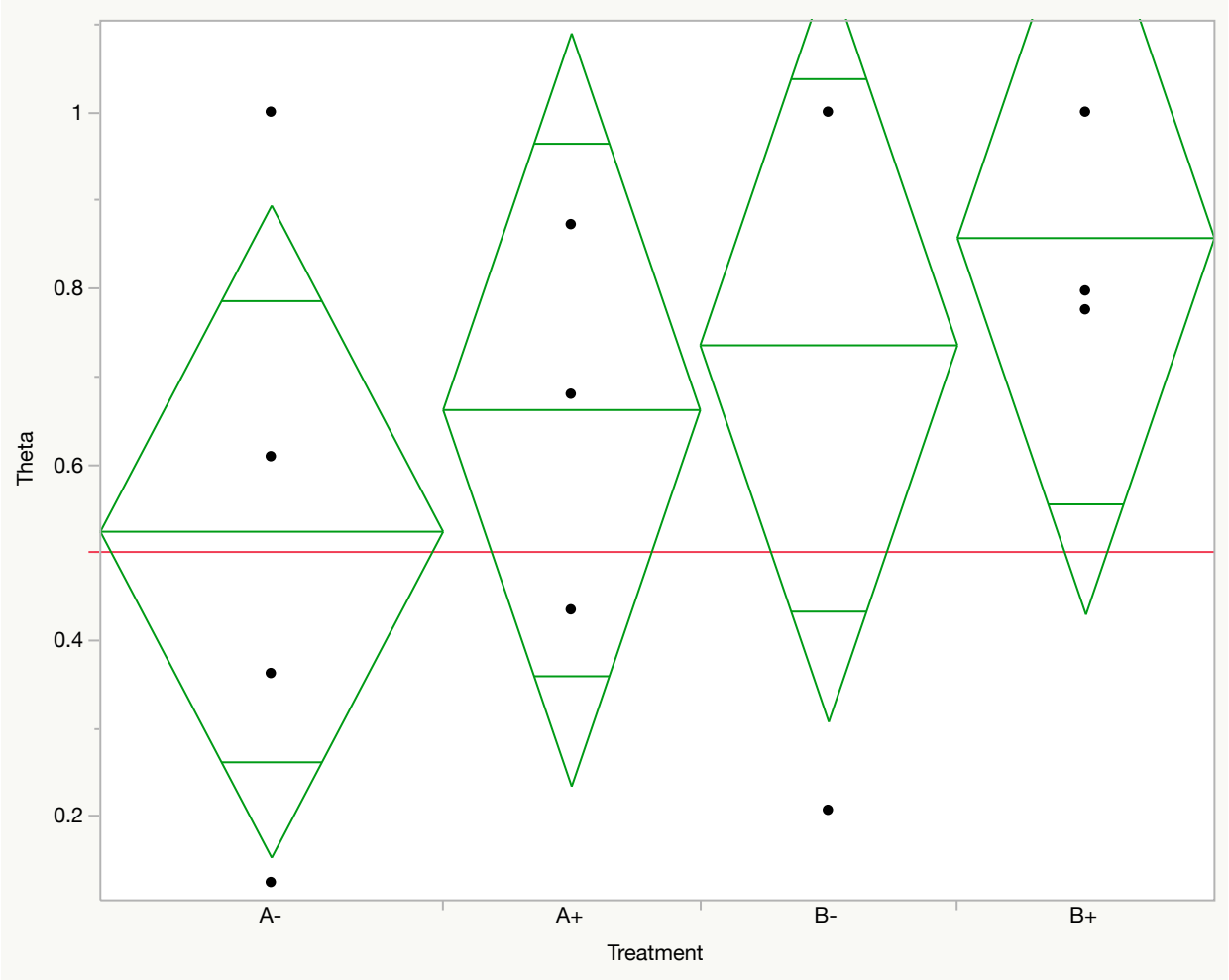

Figure 3. Theta scores for each treatment. There was no significant value within the various treatments. However, in general there was a statistical significance in treatment and the theta values were higher than expected (θ = .681 ± .312, df = 12, p = .0322).

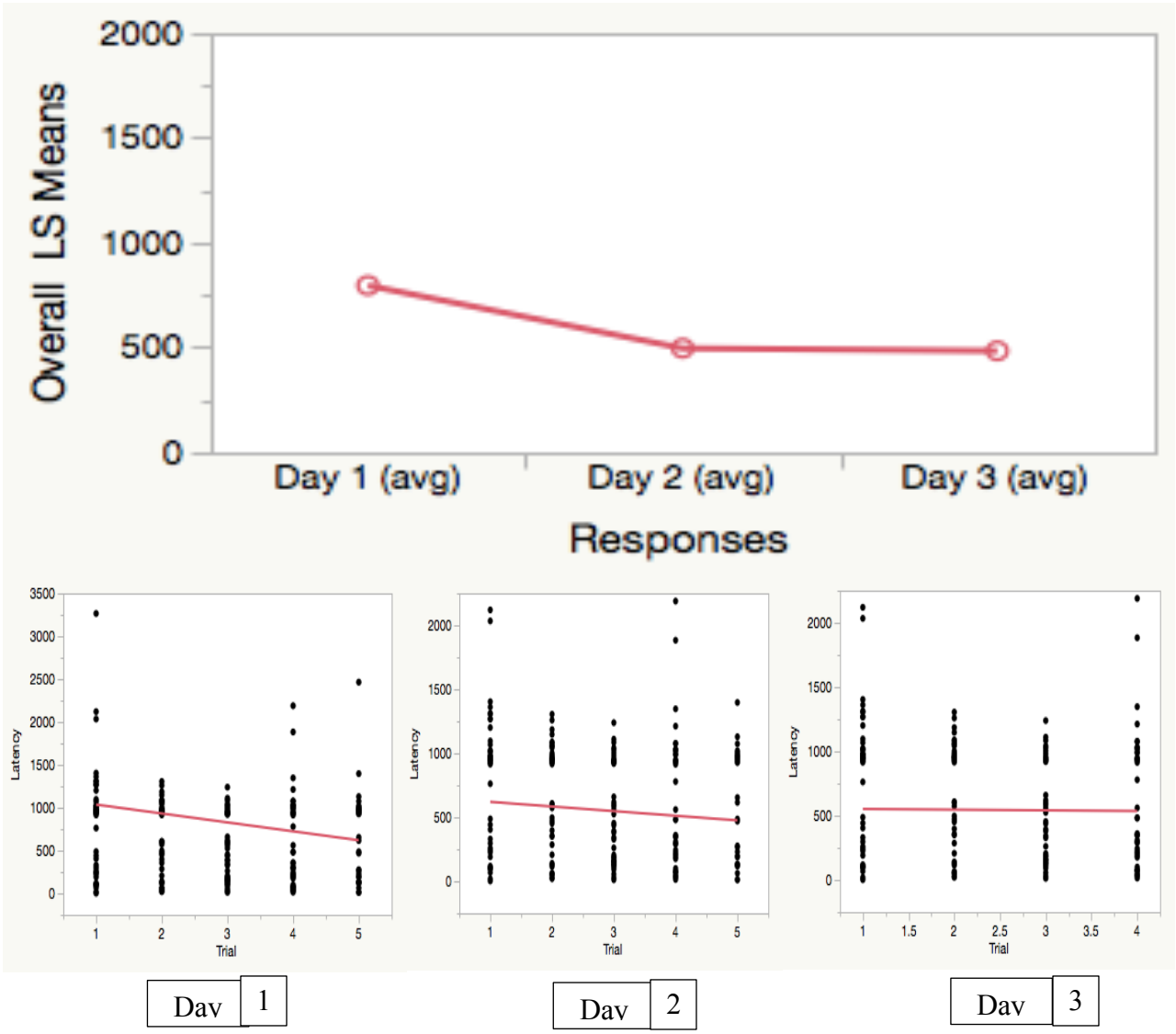

Figure 4. Learning curve for *P. laevifrons* noted in the three days. Although the data was not large enough to create a statistical significance, a decreasing trend can still be seen.

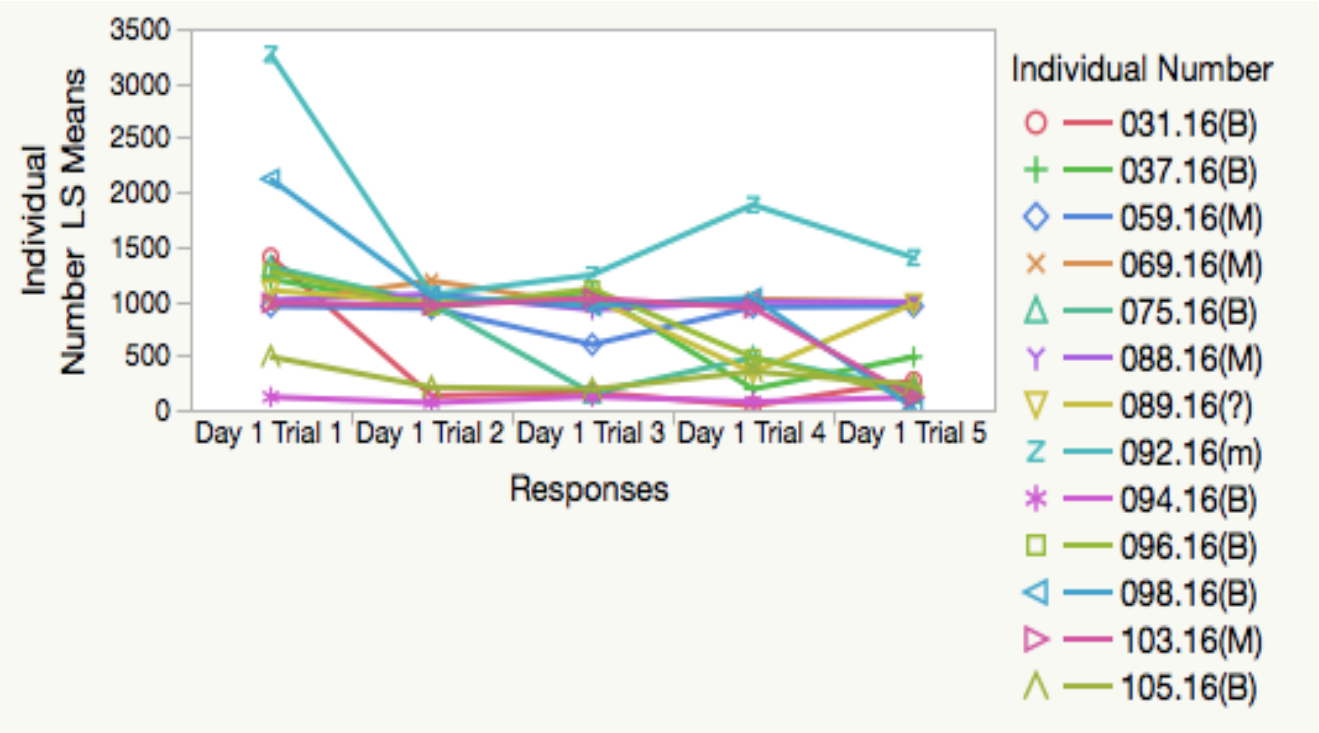

Figure 5. The learning curve for *P. laevifrons* was very pronounced on the first day of training. This can be seen with the significant drop-off in times for almost every subject.