Exploring the Relationship between Aggression and Spatial-Temporal Learning of Female Swordtails (*Xiphophorus nigrensis)*

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Abstract:

We explored the relationship between aggressive behaviors and learning ability in female *Xiphophorus nigrensis,* commonly known as the swordtail fish. Aggressive behavior was measured by introducing a female into a tank with a mirror and counting the number of times she lunged (bit) at the reflection. Learning ability was measured by a shuttlebox assay, and whether or not a female could accurately learn to predict a pattern. Cross-analyzing this data revealed no statistical significance between the two behaviors.

Swordtail males exhibit complex mating strategies based on body size. Small males sneak copulations (coercive), larger males court (non-coercive) for female mates, and intermediate-sized males are known to use both strategies. Females raised with different-sized males respond differently to each mating tactic. The rearing environment (the size of males the females were raised within a tank) was also tested to see if the environment could explain any variation in learning ability. Tank types were all female (FF), large males only (LL), small males only (SS), a mix of large and small males (LS), and intermediate males (INT). Our data revealed no statistical significance between rearing environment type and learning. Further analysis of female sociability may explain this variation in learning ability.

Introduction:

Aggression is a ubiquitous behavior shared throughout every organism in the animal kingdom, albeit the behavior can manifest through different actions and in different contexts (both social and physiological), depending on the species. Aggressive behaviors can include biting and physical combat with armaments. Aggression can be sexually dimorphic within a species, with females usually exhibiting less aggressive behaviors than their male counterparts, as seen in example species such as rats (Hashikawa *et al.,* 2018). However, it is noted that females and males mice display aggressive behaviors during different stages in their life, with the male spiking in aggression right after puberty and females spiking later in adulthood (Hashikawa *et al.,* 2018). This postulates the idea that males and females practice aggressive behaviors under different circumstances and different hormone concentrations during their lifetime.

Cognitive ability, or the ability to learn, is influenced by both genetic and environmental variables. The cerebellum is an essential brain region for learning, shown in investigating neural circuitry pathways in the zebrafish (Dohaku *et al.,* 2019). Gene expression and epigenetic modifications in the cerebellum have been shown to contribute to variation in learning ability between human monozygotic twins, suggesting that there is a genetic component to learning (Waltes *et al.,* 2015). Looking at fish specifically, learning in some fish species can be determined by analyzing different responses females exhibit to males of different mating styles, proving that females have the capability to learn and recognize different males and respond accordingly (Wang *et al.,* 2014). Fish learning is shaped by experiences (environment), age, and differential gene expression in the brain (Wang *et al.,* 2014).

Learning ability and aggressive tendencies are two behaviors that are often analyzed together for the purpose of determining their correlation with each other. Many studies have shown a positive correlation between aggression and learning. In one such study with mice, it was determined that aggressive male mice are better at learning to avoid being shocked in response to a certain action than non-aggressive male mice (Benus *et al.,* 1990). Conversely, another study focusing on the mangrove rivulus fish, the ability to learn a maze was cross-analyzed aggressive gill display frequency with no significant results, suggesting that aggression and learning were not significantly related at all (Chang *et al.,* 2012).

Due to the inconsistencies between the presence of a correlation between learning and aggression, sexually dimorphic aggression levels, and because both studies focused on males of each species, we decided to focus on learning ability and its possible correlation in female swordtail fish We also analyzed the rearing environment and learning ability as well to see if there was any relationship between the different males females were exposed to and learning ability.

The swordtail fish, *Xiphophorus nigrensis,* is a sexually dimorphic species with males having a distinct extended tail compared to females. Males in this species can exhibit three mating strategies, contingent on body size (Smith and Ryan, 2011). Large males (LL) ‘court’ females, while small males (SS) ‘sneak’ copulations. Males of intermediate size (INT) can either court or sneak, depending on male competition. Females in this study were raised in one of five different tank populations, female-only (FF), small ‘sneaker’ males (SS), large ‘courter’ males (LL), intermediate males, who display both strategies (INT), and a mixture of small and large males (LS). Tanks in which at least some males practice sneaking (SS, LS, INT) were labeled as a coercive environment. Females within those tanks were labeled ‘Experienced’ for having been subjected to forced (sneaked) copulations by males. Tanks in which the only mating strategy was courting (LL) or tanks that only had females (FF) were labeled as ‘Naive’ for coercive experience because females in those environments were not subjected to forced copulations.

Aggressive behaviors in other studies with fish have been recorded as biting and fast-swimming towards competitors, which can be measured in mirror assays like the aggression assay performed in this experiment (see Materials and Methods) (Michelotti *et al.,* 2018).

Our study focuses on how aggression correlates with learning ability, and if so, is it a direct or inverse relationship? We hypothesized that females who showed more aggressive behaviors would be more likely to learn a pattern than less aggressive females, based on the idea that aggressive females are more likely to be alert and responsive to a changing environment. The changing environment stimulus in our experiment is the shoal video, presented in an alternating pattern in the shuttlebox assay, explained in the Methods section of this article. The ability to learn could also be related to rearing environment, which is another hypothesis we considered in the Results and Discussion sections below.

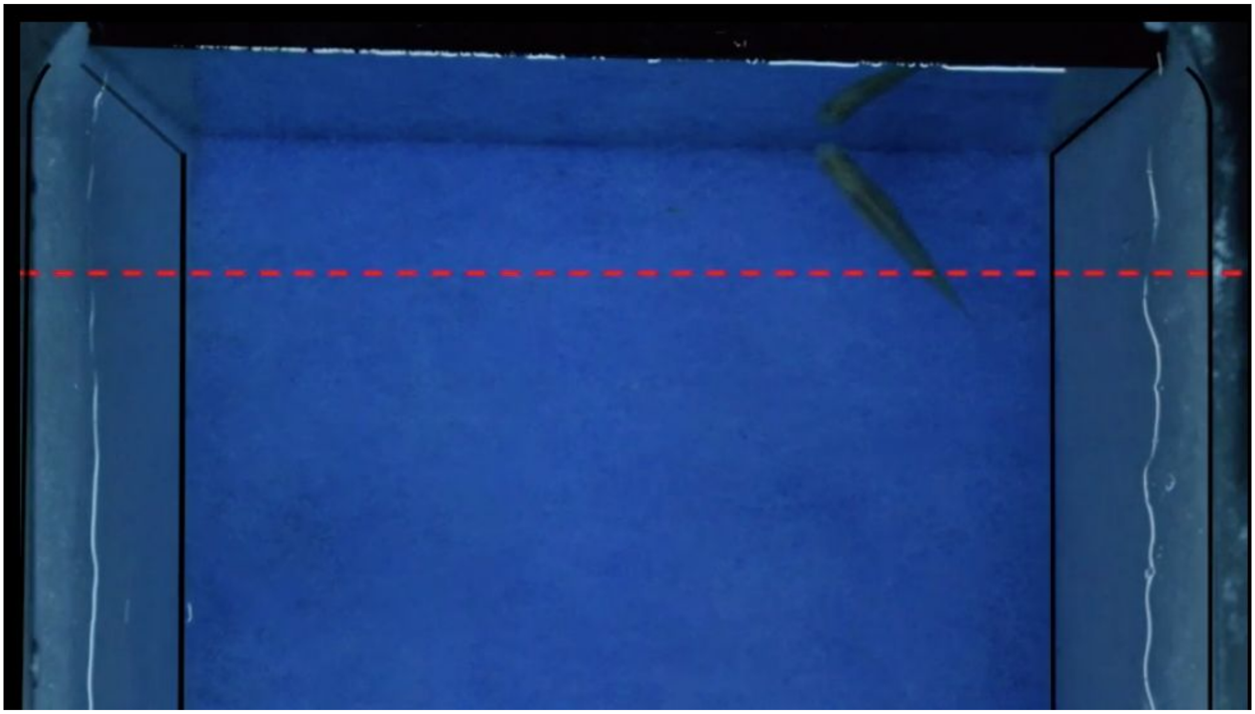
Materials and Methods:

Swordtail fish:

Two separate experiments to measure aggression and learning ability were carried out using a sample size of n=48 of female *Xiphophorus nigrensis*. Females were born and reared in five different environments: all-female (FF), with small males only (SS), with large males only (LL), with both small and large males (LS), and with intermediate males (INT). The rearing environment for each female was not disclosed to us until the after data collection process for the aggression assay and shuttlebox assay was completely finished. Both assays used the same females, and females were subjected to the tests on different days.

Aggression Assay:

To measure aggression, a single female was placed in a tank with a mirror at one end. Each female was filmed inside the tank by an overhead camera. The video footage labeled an association zone, about 5 cm away from the end of the tank with the mirror. The first 20 seconds of the video were disregarded to allow each female fish time to be placed in the tank and acclimate to their surroundings. Females were observed for 5 minutes in 20-second intervals and data was collected regarding number of bites/lunges at the mirror(# of bites), time spent within the association zone (time in association zone), time spent outside the association zone(time outside association zone), and whether or not the female swam alongside the mirror during the interval (parallel swimming, stated as either “yes” or “no” for that interval). A total of fifteen 20 second intervals were observed, and data was recorded in an Excel sheet. A total of 50 females were observed with this assay.



Example set up of the mirror aggression assay.

Shuttlebox (Learning) Assay:

To measure learning capabilities, each female X. nigrensis participated in a temporal-spatial associative learning assay referred to as ‘shuttlebox’. The assay provides a reward (a 20sec video of a live shoal group consisting of 5 conspecific females swimming together) in regular time intervals (90 sec) at alternating ends of a fish tank. For example, the 20-second reward video first appeared on the left side of the tank, followed by 90 seconds of blank screens on both ends, followed by 20 seconds of a reward video shown on the right side, followed by 90 seconds of blank screens on both ends, then followed by a 20-second reward video on the left. This alternating series continued for 30 minutes. In order to ‘learn’ the alternating pattern of the rewarding shoal group, we evaluated if the fish was present on the ‘correct side’ in the 20 seconds prior to video turning on (our ‘anticipation window’). If a fish was present in this ‘anticipation zone’ in 2 consecutive intervals, she was categorized as a “shuttlebox learner”. Data was collected for 48 females, and we recorded the data in an Excel sheet.

Example of learning shuttlebox assay

Results:

Aggression and Learning:

To test whether or not learning capabilities were related to aggression, an independent t-test was performed in R between the number of bites (collected in the aggression assay) and whether or not the females learned the shuttlebox pattern (yes = group learned pattern, no = group did not learn pattern). A total of 48 female fish samples were used, 18 learned the shuttlebox pattern, and 30 did not learn. The average number of bites for females who did not learn the shuttlebox assay (shown in red in Figure 1) was 170.06 bites, while the average number of bites in females who did learn the shuttlebox pattern (shown in blue in Figure 1) was 125.22 bites. There is no evidence to suggest that aggression (exhibited by number of bites) is significantly different between the two learning groups ($t = -1.2773$, $df = 45.889$, $p\text{-value} > .05$)

Figure 1

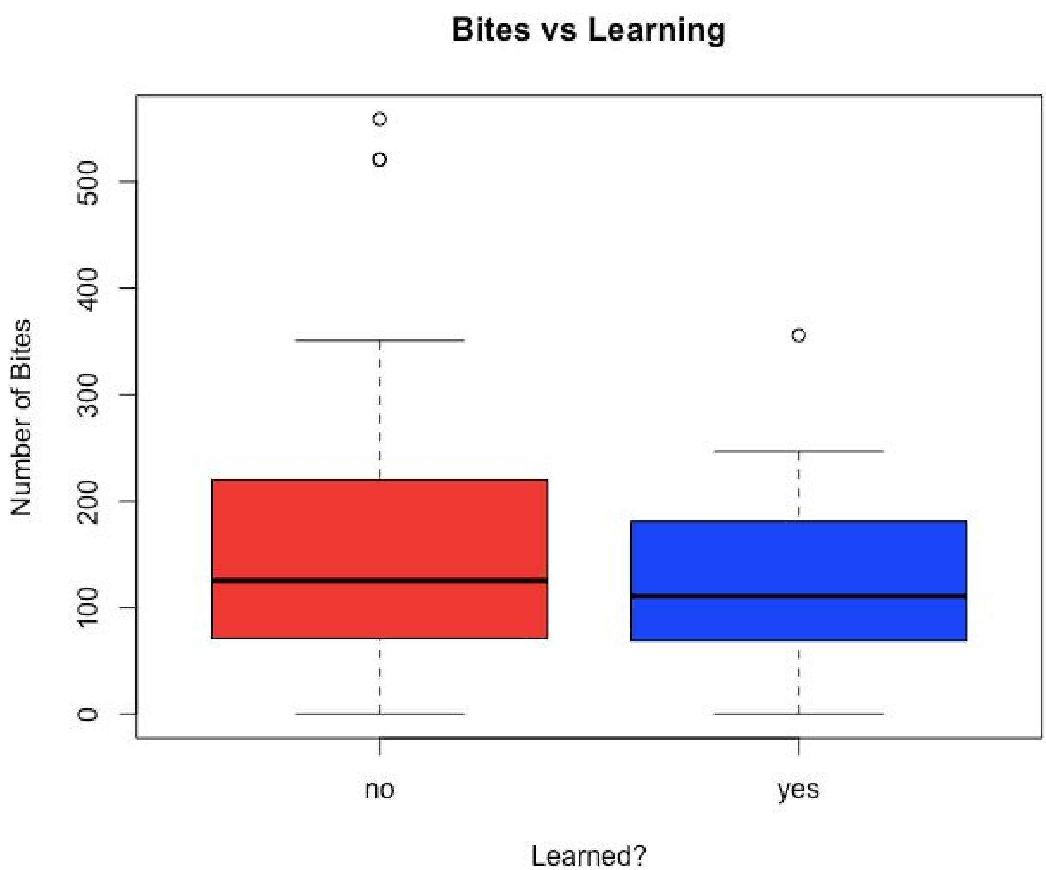


Figure 1 boxplot represents the relationship between the number of bites (y-axis) recorded for a female during the aggression assay and whether or not a female learned (x-axis) in the shuttlebox assay. Those females who did not learn the assay are depicted in red, while those who learned are depicted in blue.

Aggression and Environment:

To see if the rearing environment (FF, SS, LL, LS, INT) had any significant correlation with aggression levels (measured as number of bites), a one-way analysis of variation (ANOVA) was performed. The mean number of bites for each group was FF= 208.00, SS= 158.71, LL= 175.30, LS= 102.21 INT= 170.87. According to the data collected (see materials and methods), there was no significant difference between the number of bites across all rearing environments ($F=.869$, $df=4$, $p\text{-value}>.05$).

Figure 2

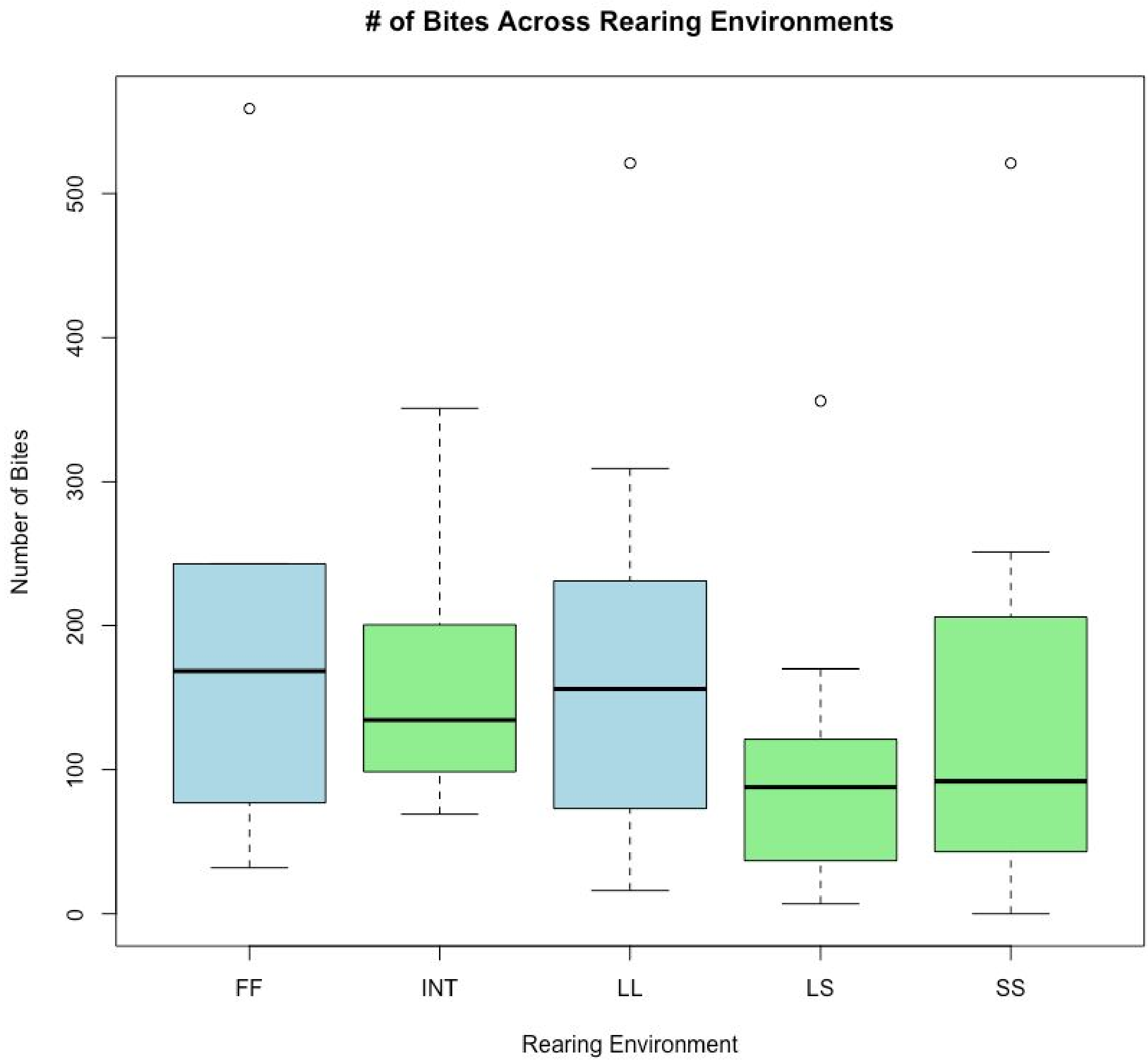


Figure 2 boxplot shows the relationship between rearing environments (x-axis) and aggression (number of bites, y-axis). Color-coding matches coercive experience, which is explained in the section below.

Further analysis of the rearing environment and aggression was performed by labeling coercion experiences for each environment. Females raised in LS, SS, or INT were labeled “Experienced” because they all had been subject to coercive sneaker males. Females raised in FF or LL were labeled “Naive” because they had not been subjected to coercive male behavior. An independent t-test was performed to see if there was a statistically significant relationship between bites and coercive experience. There were 29 Experienced females and 19 Naive females analyzed in this data set. The average number of bites for Experienced females was 130 bites, while the average number of bites for Naive females was 180.8 bites. There is no data to suggest that there is a significant relationship between coercive experience and aggression (see Figure 3) ($t = -1.2901$, $df = 33.544$, p-value = 0.2058).

Figure 3

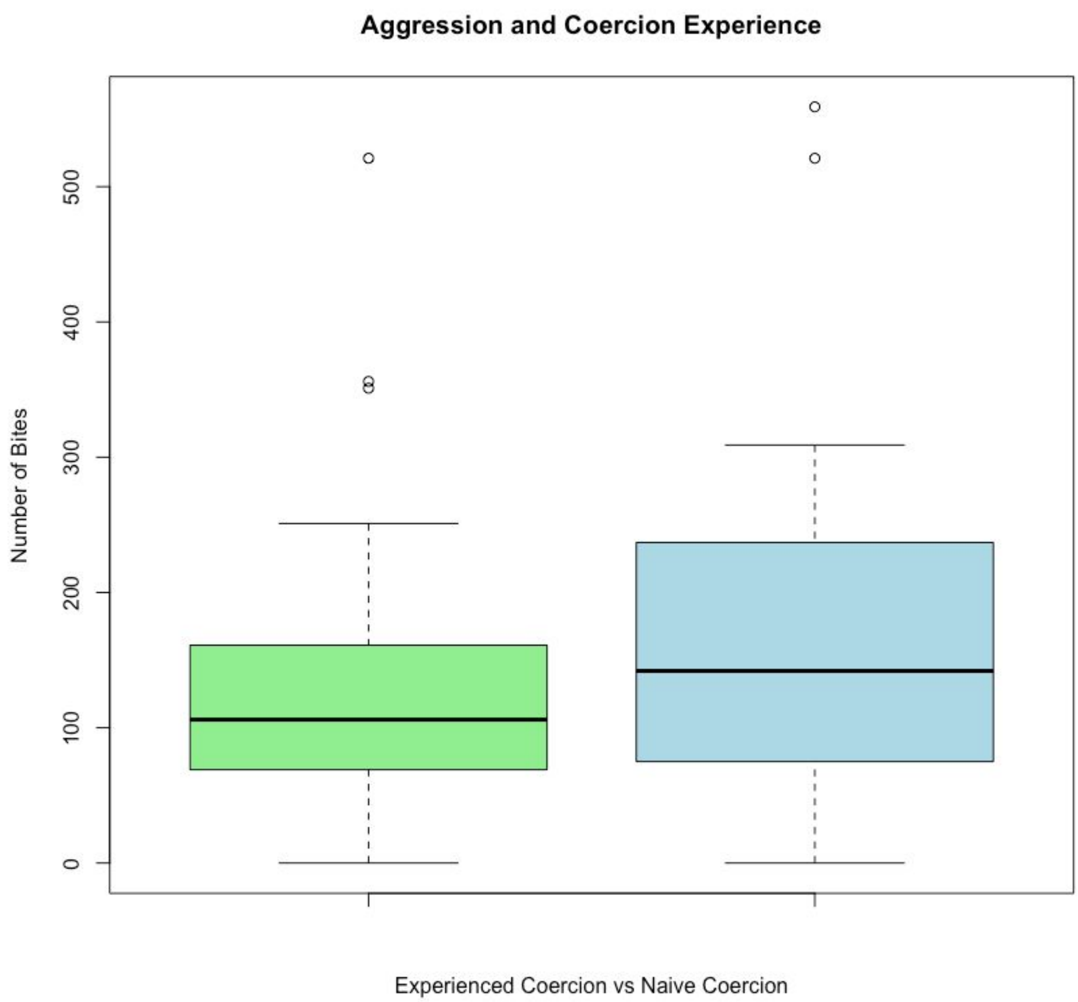


Figure 3 boxplot shows that there is no relationship between coercive experienced females (SS/LS/ INT, light green box) and Naive to coercive behavior females (FF/ LL, light blue) on aggression (number of bites, y-axis).

Learning and Rearing Environment:

To see if there was a relationship between learning ability in the shuttlebox assay and rearing environment of each female, a Chi-squared test of independence was performed. This statistical test did not suggest that there is a significant correlation between the environment each female was raised in and learning ability (yes the female learned, no the female did not learn) (X-squared = 0.55287, $df = 4$, p-value=0.9682). Since our p-value >.05, we can't disprove that learning and environment are independent of each other. Figure 4 depicts this data.

Figure 4

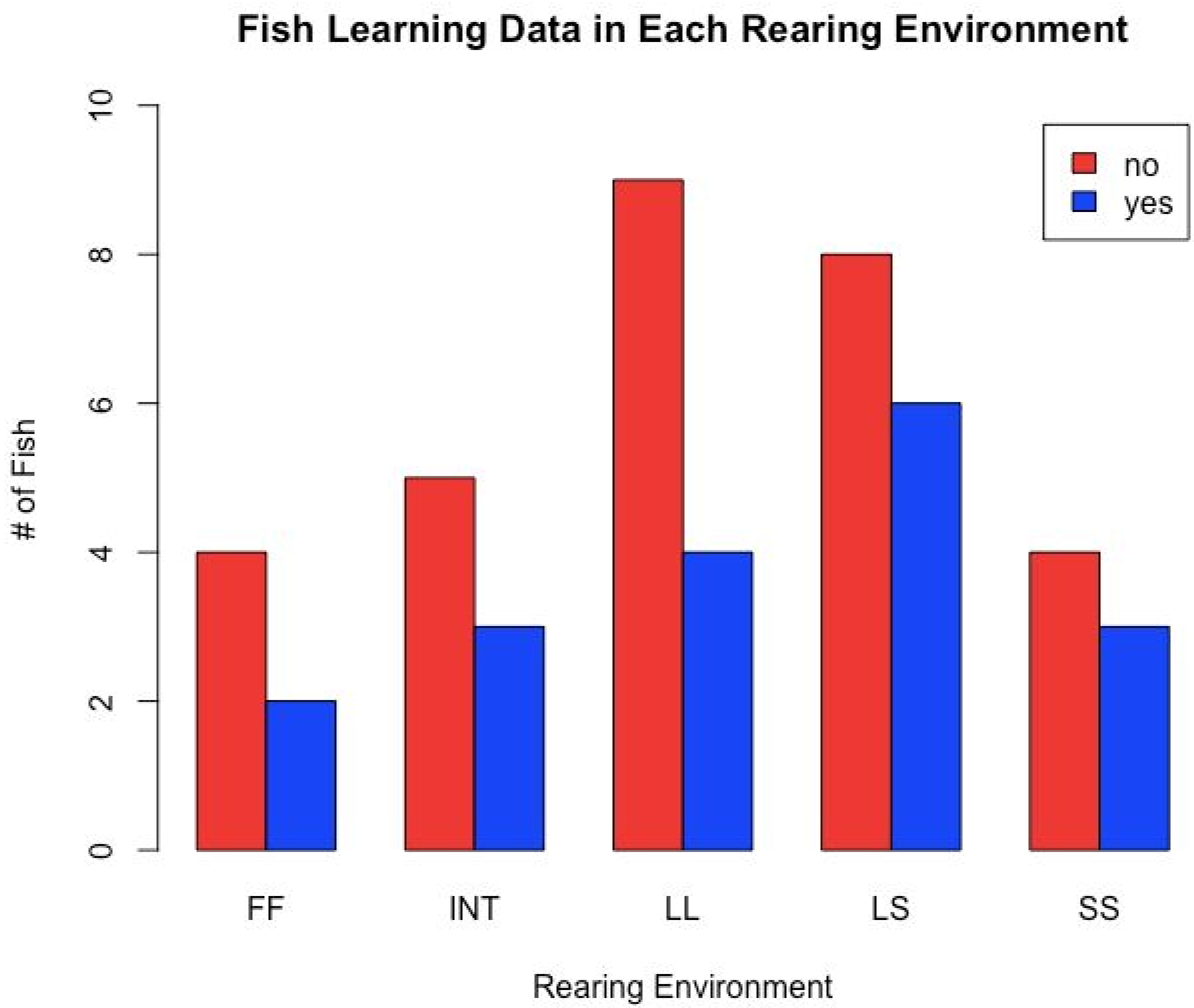


Figure 4 is a grouped bar plot showing the relationship between rearing environment and the number of female fish who learned the shuttlebox assay. Females are grouped by rearing environment (x-axis) and whether or not they learned (blue) or did not learn (red).

Like in the aggression analysis, further analysis of learning was done by organizing females back into their coercive experience groups. Learning ability and Coercion experience females are listed in table 1 below:

Table 1

	Coercion Experience	
Learning ability from Shuttlebox	**Experienced**	**Naive**
Learners	**12**	**6**
Non-learners	**17**	**13**

Table 1 depicts the number of females in each category regarding coercion experience and learning ability. Colors correspond with respective color coding in graphs previously shown throughout this paper.

A Chi-squared test of independence was run on the data to reveal that there is no data to suggest that coercion experience and learning ability are significantly related (X-squared = 0.47042, df = 1, p-value = 0.4928)(seen in figure 5).

Figure 5

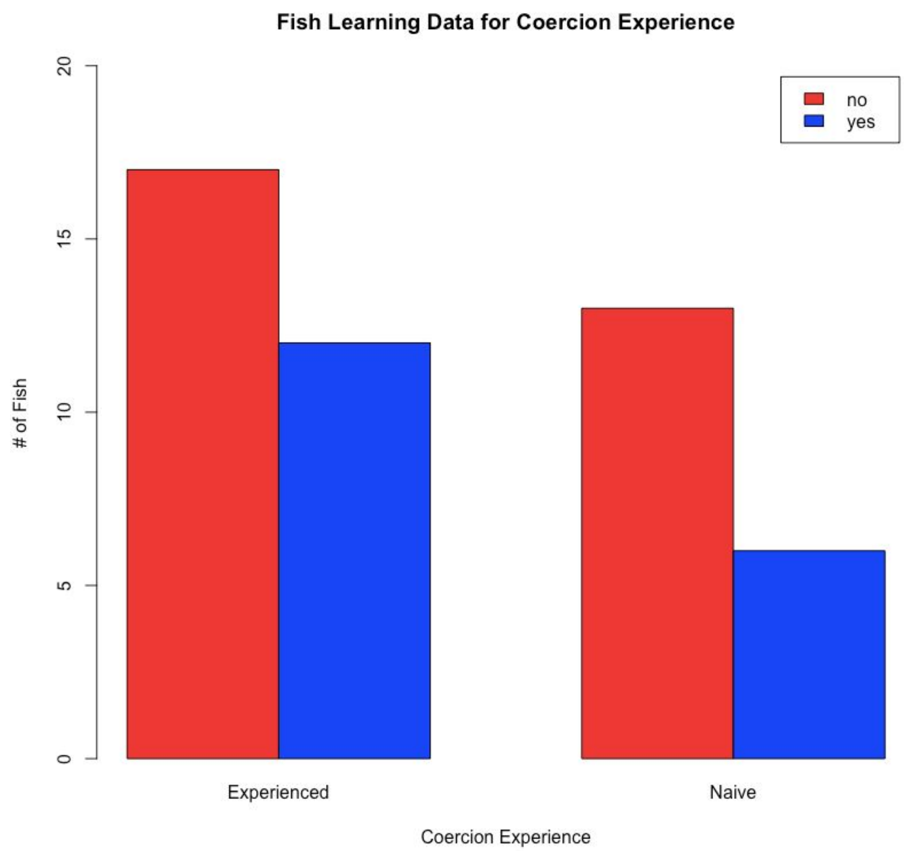


Figure 5 bar plot depicts the results from the chi-square analysis for coercion experience (x-axis) and learning ability (color-coded). The number of fish in each group is shown on the y-axis. Data for Experienced females is on the left, while Naive female data is on the right of the graph. The red bar indicates that the female did not learn, and the blue depicts females who did learn in the shuttlebox assay

Discussion:

The data collected in this study did not show any significant correlations between learning and aggression, aggression and environment, and learning and environment in female swordtails.

Aggression and Learning:

The independent t-test on the data gathered from 48 female swordtail fish from both the shuttlebox assay (learning ability) and mirror assay (aggression) revealed that there is no significant correlation between the two behaviors. Our original hypothesis, that aggression and learning might be correlated due to hormonal behaviors altering brain chemistry that affects learning ability and heightens sensory perception, is not proven to be true by this dataset. Our data seems to match the result from the mangrove fish experiments by Chang, that aggressive displays were not related to learning a maze. Further analysis with a larger sample size of females may help improve the statistical validity of disproving this hypothesis.

Aggression and Environment:

Our additional hypothesis analyzed the possible relationship between aggression levels (number of bites), learning and rearing environment. Specifically looking at aggression and the environment, we proposed that different environments with different coercion experiences may contribute to the variation in aggression between the females. We ran a one-way Analysis of Variance to see if any tank of females (FF, LL, LS, INT, SS) exhibited a mean number of bites that was significantly different from the other tank environments. The analysis did not return any statistically significant results. Another analysis was performed specifically grouping each rearing environment into their respective coercion experience category (Experienced vs Naive). An independent t-test on this data supported the ANOVA in validating there was no significant relationship between environment and aggression. We, therefore, concluded that the variation of aggressive behaviors from female swordtails could not be explained by different rearing environments.

Learning and Environment:

Our last analysis considered the possible variation in learning ability from the females could be due to different rearing environments. A Chi-square test of independence was performed and revealed no statistical relationship between learning and rearing environment. Further analysis between coercion experience environments (Experienced vs Naive) and learning was performed, to once again reveal no statistically significant relationship between the two behaviors.

What could be affecting learning?

Since gene expression of neuroendocrine, dopaminergic, and serotonergic systems can be altered by certain behaviors and environmental stimuli via epigenetic modifications (a phenomenon which was explored in human twin studies) it is possible that aggression and rearing environment may significantly affect the way some organisms think (Waltes *et al.,* 2015). The neuroendocrine system is tightly linked to memory and learning, resulting in different cognitive abilities depending on hormone levels in an individual (Hamson *et al.,* 2016). While this study did not find any significant results between aggression, environment, and behavior, it is possible that other behaviors could contribute to epigenetic changes that alter learning ability, and analyzing these behaviors (one such behavior is proposed in the next paragraph) with learning and ultimately a fish's epigenome in the brain could reveal some explanation for learning ability.

<u>Other Possible Explanations of Variation in Learning Ability:</u>
While aggression and rearing environment yielded no statistically significant results to explain variation in learning ability between females swordtails, other factors, such as sociability, may better explain the variation in learning ability of the females. In the Sumatran and Bornean orangutans, there is a positive correlation between highly social populations and social learning, suggesting there may be a correlation between the two behaviors in our fish (Schuppli, *et al.,* 2017). Further testing of sociability in our female fish could be gathered and cross-analyzed with our learning data to see if there is a relationship between these two behaviors.

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