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**“BITING INTO THE TRUTH: UNRAVELING PIRANHAS’ COGNITIVE FLEXIBILITY  
THROUGH REVERSAL LEARNING”**

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## **ABSTRACT**

Considering how little research has been done on the cognitive system of the Characiformes family, we were motivated to investigate and have a first look into the cognitive abilities of *Pygocentrus nattereri*. The experiment consisted of an automated colour association reversal learning task divided into two parts: Experiment 1 and 2. In Experiment 1 subjects were required to discriminate between red and green; in Experiment 2, they had to discriminate between white and yellow. We found no evidence of learning capacities after extensive training exceeding one thousand trials overall. We conclude that piranhas may be limited in their capacity to cope with automated operant conditioning devices and call for a further investigation of learning abilities in Characiformes fish.

## **1. Introduction: reversal learning in piranhas**

Reversal learning is considered to be one of the most reliable approaches to test how cognitively flexible both humans and nonhumans can be. Assorted studies have investigated reversal learning in varied species such as passerines (Cauchoix et al., 2017), lizards (Day et al., 1999), and distinct species of fish. My focus in this dissertation will be examining a specific reversal learning task, on a very particular species of fish: piranhas.

In my review of scientific literature on reversal learning, I encountered species such as zebra fish (Kuroda et al., 2017), and freshwater rays (Daniel et al., 2020): not a single article was found on piranhas and their use in experiments regarding the testing of behavioural flexibility.

Piranhas, scientifically known as *Pygocentrus nattereri* of the Serrasalminidae family, are native to the central and southern river systems of South America, where they inhabit tropical rivers and streams. They are often found in murky waters in groups and prefer to hide in dense vegetation. When collected in the United States they have been found in ponds, lakes, rivers, and borrow pits. Animals that populate dynamic habitats and different environments are anticipated to have cognitive flexibility (Day et al., 1999), for this reason, red bellied piranhas appeared to be the perfect fish to experiment with for a reversal learning test. This research could give us precious intel on their ability to learn a rule and reverse it; meaning I was given the opportunity to have a first look inside the cognitive system of the *Serrasalmus nattereri*.

In the first chapter of this thesis, I will on one hand explain how cognitive and behavioural flexibility may be considered beneficial; on the other hand, I will highlight what costs it may have. I will then proceed to clarify how the experiment is structured and what kind of rule we taught our two *Pygocentrus*. I will then partially illustrate their neuroanatomy and visual sensitivities, seeing how

our chosen-rule to reverse pertained to colour association. Seeing also how the first part of reversal learning is based on the acquiring of a rule, I will briefly explain different learning methods in fish.

In the second chapter I will focus on the used methods and the experimental procedure, with special attention to Zantiks, the automated behavioural research system I was lucky to benefit from. Zantiks allowed me to train both of our two specimens efficiently and rapidly. Since we only had two examples of piranhas, to consider our results statistically relevant, it was crucial that we had a high number of trials.

In the third chapter, with the aid of different graphs, I will display the findings of my three-month research and walk you through my data analysis. Examining the graphs, we can determine the absence of any rule learning: for this reason, we never reversed any rule. Since they had never shown any signs of learning to colour associate, what rule could we reverse? Also, the graphs will highlight how we encountered trouble with the piranhas' drive and motivation to work.

For the concluding chapter, I will review my results and argue my conclusions, discussing alternative methods viable to replicate our experiment and offering plausible explanations for our outcome.

### **1.1 Cognitive flexibility: benefits and costs**

Given how our aim for this research was to investigate cognitive flexibility, it is essential to analyse what cognitive flexibility is and why it varies among species.

Cognitive flexibility is the ability of animals to acquire swiftly new information and to switch accordingly their behavioural response. In the context of a laboratory, we refer to an animal as "flexible" when it is able to gain new information while forgetting or retaining the firstly acquired rule. The more flexible the animal, the more adaptable it will be; meaning the animal will be able to learn different rules and reverse multiple times.

Being linked by scientists to reproductive success and an increase in survival, traditionally this ability has been seen only as highly valuable (Snell-Rood & E. C., 2013). In fact, as stated in "Brains,

innovations and evolution in birds and primates “of L Lefebvre, SM Reader and D Sol: “behavioural innovation is one factor enhancing the rate of evolution in animals”.

But is being flexible only beneficial?

In nature we can observe a variety of cognitive flexibility, both inter and intra species, and there is evidence for genetically based individual variation in cognitive traits (Dukas & R., 2004); it is then safe to assume that being cognitively and behaviourally flexible may entail some costs.

These costs may be energy related, and they can also affect other traits like reproduction ability (Mery et al., 2003). Furthermore, studies suggest how there is an actual trade-off between flexibility and memory retention, suggesting how a better ability in one, is associated with a worse ability in the other (Tello-Ramos et al., 2019). For this reason, I would not affirm that not being exceptionally flexible, means necessarily being unintelligent. I would rather state how different species have unique needs and need to sharpen diverse skills for their survival, or more generally there is a trade-off between the energetic costs of a nervous system enable to support cognitive flexibility and the costs of the immune system or other biologically-relevant processes of the organisms.

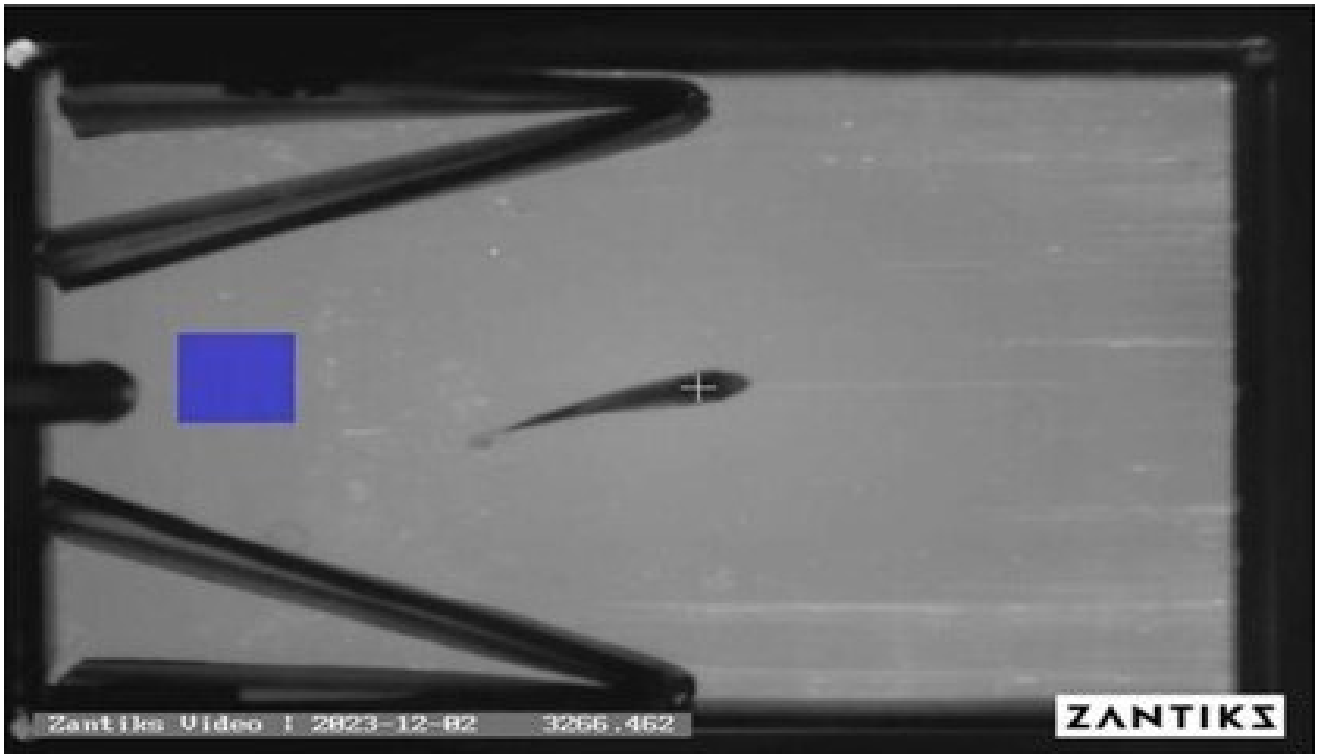
The question now is: in which category do piranhas fall in? Are they cognitively flexible or is it more a cost, than a benefit for them? To answer this question a reversal learning task seemed only logical and fitting. This type of test could give us a deeper understanding of these small carnivores and what is more beneficial for this small type of predator.

## **1.2 Colour association reversal learning task**

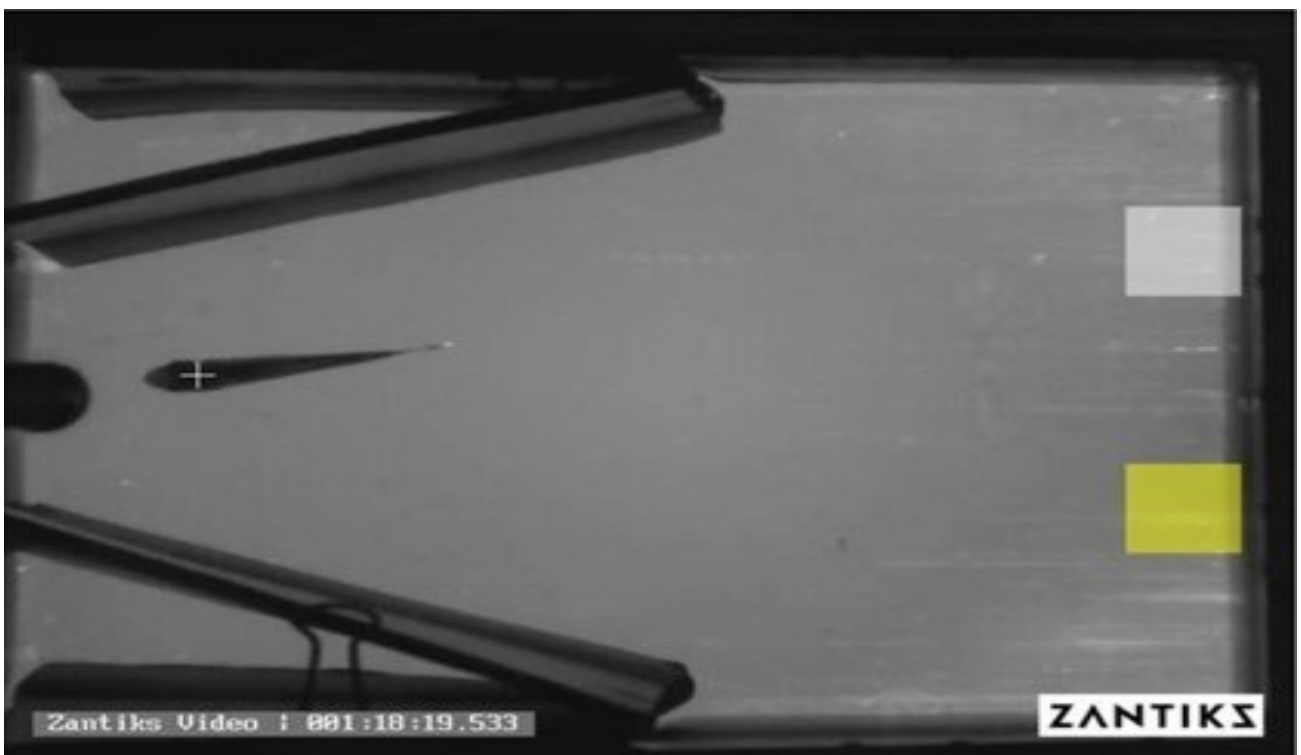
Reversal learning is a cognitive process used to measure an animal cognitive flexibility. In a reversal learning task subjects are trained to discriminate between two visual stimuli, one is rewarded every time it is chosen, while the other one is not. After successful discrimination learning has been demonstrated by reaching a criterion level of performance, the request of the study changes. The subjects are again trained but now they need to choose the other stimulus. If they can reverse the rule,

they will show signs of cognitive flexibility (Izquierdo et al., 2017). The reversal learning task we performed used colours as visual stimuli. The experiment was conducted using two different colours' associations. In Experiment 1, the *Serrasalmus nattereri* were each trained to discriminate between red and green. After this first training, we considered the possibility that the *Pygocentrus* could show a personal preference to specific wavelengths. Therefore, in Experiment 2, we changed colours: they were taught to choose between a yellow square and a white one. Using the comparison between performances and stimuli, we wanted to evaluate if their choices were, (or were not), influenced by their colour perception. We also speculated if, in their choice, they may have been swayed by a natural possible predilection for colours, (such as red or green), more common in their natural habitats.

After the two piranhas were located each in their own tank, where they were trained, the session started. The script provided first a blue square (**Fig.1**) on one end of the tank. When the fish would swim on it, the stimulus was elicited and on the opposite end of the tank, two coloured squares (**Fig.2**) would appear: one red and one green, or for the later trial, one white and one yellow (**Fig. 2**). The correct coloured square could be in the right or left position, taking away the possibility of instilling the preference for a right or left position: the choice had to be based exclusively on colour. To confidently conclude that the learnt rule was colour discrimination, it was necessary that the colour was the only variable manipulated.



**Figure 1.** Piranha in the training tank in front of the blue square stimulus, ready to be elicited.



**Figure 2.** After the blue square is elicited by a piranha, the two coloured-squares appear at the opposite side of the tank.



Having two individuals, we decide to teach each red-bellied piranha a distinct colour. One had to choose the red square first, the other one had to choose the green square. Then, after it was clear how they both had learnt to discriminate between the two colours, we would have reversed the rule.

The colour association training was based on operant conditioning. Also known as Skinnerian conditioning, (named after the famous American psychologist Skinner, who invented it), the instrumental conditioning is a learning method, which through reward and punishment is able to modify someone's behaviour. When the two squares appeared, the piranha was put in front of a choice. If the *Serrasalmus* picked the right stimulus, (meaning the square corresponding to the colour we chose to train it on first), it was rewarded through food, which fell in a white square, the same place where the blue square initially appears (**Fig.1**). The reward was meant as positive reinforcement: it was necessary to build an association with the chosen colour and hopefully it would have led to the internalisation of the rule.

If the fish made the wrong choice, and picked the wrong colour, it was punished by a white light that suddenly appeared and covered the entire tank for a few seconds. As previously mentioned in the introduction, piranhas tend to hide in dense vegetation and live in murky water, therefore, they tend to shy away from the light and feel more comfortable and safer in darker environments (a feature we evaluated for, before starting the reversal learning experiment). The white light seemed to be the perfect candidate as punishment. Taking advantage of their ecological inclination, the bright light was stressful enough to persuade them into not repeating the same choice and at the same time, it was not necessarily cruel for our subjects.

### **1.3 *Pygocentrus nattereri*: how do red bellied piranhas see colours?**

To execute a colour discrimination task, it is vital that we dive into how piranhas see colours.

The *Serrasalmus* belongs to the Characiformes family, an order that includes more than two thousand species: a diverse group of freshwater fishes, living in a wide range of ecosystems. Despite how broad the group is, specific research on the piranhas' vision is exceedingly rare, if almost totally inexistent.

It is in fact important to specify that even though tetras, piranhas, bloodfins, silver dollars, hatchetfishes, headstanders, pencilfishes and their relatives, all belong to the same order, they may differ in spectral sensitivities. Studying the visual system in vertebrate fishes is particularly interesting because of their diverse ecologies and vast phylogenetic history. To better understand the *Pygocentrus* vision, we must keep in mind their natural habitat. Water absorbs and scatters much of the incoming light, and this inevitably causes great variation across aquatic habitats that differ in concentrations of suspended particulates and dissolved compounds. A recent paper on visual pigment evolution (Escobar-Camacho et al., 2020), has in fact highlighted how species living in murkier water will exhibit more red-shifted sensitivities than those living in clear waters, exactly like our red bellied piranhas.

In vertebrates, vision starts when light reaches the retina and is detected by rod (night vision) or cone (diurnal vision) photoreceptors. Photoreceptors are packed with visual pigments. There can be multiple cone types, containing different visual pigments, which absorb light maximally in different parts of the wavelength spectrum. Piranhas had three cone genes expressed: SWS2, a second short-wave class sensitive to violet–blue (410–490 nm), LWS2 and LWS1, sensitive to the green to red spectral region (490–570 nm). In conclusion, although we cannot be sure on the visual sensitivities of those three expressed cone genes, a reasonable guess would be that the colours we had picked were visible to the piranhas' eyes. In light of this conclusion, we may exclude colour blindness as a possible explanation for our failed reversal learning task.

#### **1.4 Learning ability of fish**

As I explained in the previous paragraph (**1.2 Colour association reversal learning task**), a reversal learning task is based on the ability to pick up a new rule. For this reason, I thought it would be relevant to highlight the learning capabilities of aquatic creatures. Fish are smarter than we think, hence why, we supposed that our two piranhas could be able to acquire new information, (such as choosing the right coloured square).

Learning is a key factor in the behavioural development of all vertebrates, fish included. The experimental evidence of learning abilities of fish is very ancient and vast. Through this ample research, with time, researchers were able to deconstruct the narrative that presented fishes as dim-witted, with a small brain and three-seconds memory. They also debunked the idea that a smaller brain equals necessarily a lower level of intelligence (Bshary et al., 2002).

Contrary to people's belief, these animals through the years have developed multiple skills, in multiple areas, deeply relevant for their survival. They have repeatedly shown diverse learning capabilities: the classical trial and error method, avoidance learning (when facing danger like risk of predation), learning through imprinting (to recognize their kin), observational learning, (also known as social transmission of learning), spatial learning (imperative for migration), and lastly classical and operant conditioning (Kieffer et al., 1992).

As we can see from the thorough list of their learning abilities, fish appear to be the ideal subjects to study the phenomenon of gaining new information. Most importantly piranhas and a reversal learning task seemed to be the perfect topic to dive into (Laland et al., 2003).

## 2. Methods and Materials

### 2.1 Subjects

In total we assessed two examples of young *Pygocentrus nattereri* (body size: 5 cm), also known as red bellied piranhas or *Serrasalmus nattereri* (**Fig.3**). We cannot state with certainty their gender seeing how they are still very young (approximately 4-5 months old) and sexually immature. The difference between females and males is clearer later in life, at around one year old. With aging, the prominent red belly, which they are known for, gets brighter, especially in males, while females tend to have a duller red. Another characteristic that can help discerning the sex of this species is that when females reach sexual maturity, they start showing an egg bulge, especially during breeding season, while males look slimmer (Piranhaguide (June 24th in 2019). How to tell if piranhas are male or female? Post in Breed. Read on the 29<sup>th</sup> of February. (<https://piranhaguide.com/how-to-tell-if-piranhas-are-male-or-female/>)). During the first year, while they are still considered fries, they present silvery scales, darker dots all over the body and no visible difference in appearance: making it extremely hard for us to distinguish the sex. It would be premature to affirm their gender now. On the contrary, with time, we will soon be able to make an educated guess as soon as they reach the one-year milestone in the lab.

For the sake of our research, we named the two specimens to distinguish the two distinct colour associations of the reversal learning task. We called them Leo and Little José: despite what one would expect, Little José is the slightly bigger one, with a more prominent belly (**Fig. 3**). They are both housed together in the laboratory of Comparative Psychology of the University of Padua, kept captive in an aquarium of 150-litre (size: 40 × 60 × 40 cm (width × length × height)). An 18-W fluorescent light was provided above each stock tank (photoperiod was 14:10 h light: dark). The tank also has air filters, natural gravel, and live plants. The water temperature was set to 26 ± 1°C. We purposely placed both piranhas in the same tank considering how in nature piranhas live in shoals and do not thrive while separated: against the rhetoric perpetrated by the media and movies, piranhas are more fearful than feared. A 2005 article suggested that an important function of shoaling behaviour in the species

is defence against predation. Indeed, they are regularly predated by river dolphins, caiman, aquatic birds and large piscivorous fishes (Queiroz et al., 2005).



**Figure 3.** Here one of the subjects tested (Little José).

In the introduction of this dissertation, we briefly touched upon how during the end of our research, both the two subjects' drive had drastically plummeted. One of our hypotheses had to do with their innate preference to be in group: the different behaviour displayed in their everyday aquarium and in the training tank, where they were located alone during the experiment, seemed to be proof enough. Apart from stating how piranhas are a social species, a good explanation of their behaviour is how a fish's perception of risk is affected by shoal size: bigger shoals showcase a lower preparedness to flee (Queiroz et al., 2005). Although the *Pygocentri* did show some sign of intraspecific aggression as expected, while placed in their aquarium together, they appeared rather comfortable together and actively swam (Oldfield et al., 2023). When they had to be evaluated and separated for the sake of

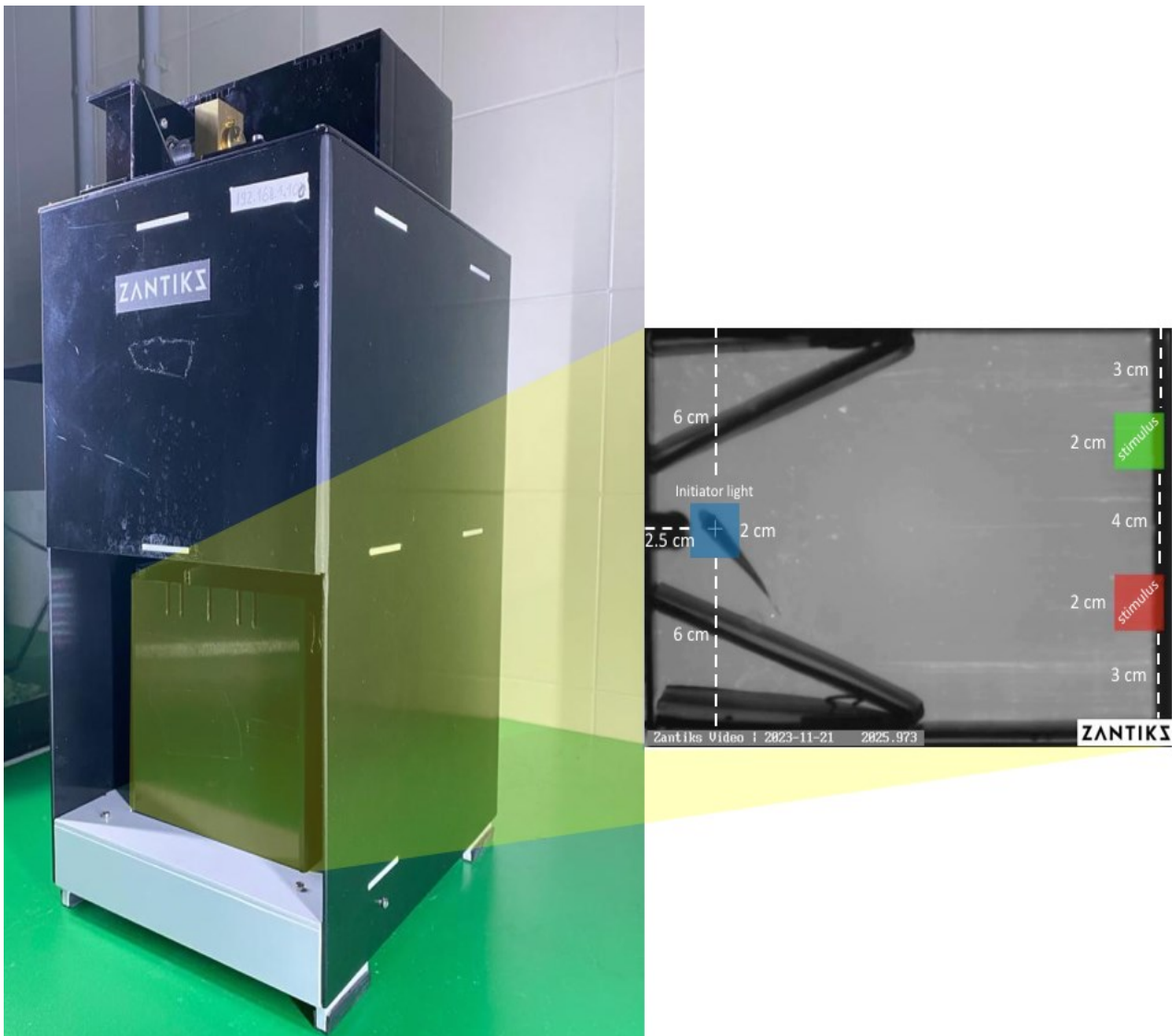
the experiment, they froze for a period that ranged between 10 to 30 minutes, even more in the last training days. Freezing behaviour in fish is an “energy-saving” strategy indicating a stressful situation or the presence of a threat: facing a stressful situation without their companion worsen the threat perceived (Shi et al., 2024). The difference in behaviours we observed, proved how important companionship is for our predators.

The subjects followed the same diet and were fed by me once or twice a day according to their training. Food was a positive reinforcement essential to teach the needed rule. During training days, they were not fed in the morning, but only in the afternoon after they finished the daily trials. After the end of our research, they were normally fed twice a day, in the morning with commercial food flakes while in the afternoon with live prey: brine shrimp (*Artemia salina*).

## **2.2 Apparatus and stimuli.**

The apparatus used for the reversal learning task in this experiment is called Zantiks©. More specifically we used Zantiks AD, ideal for measuring the behaviour of small fish like our two young Serrasalminidae (**Fig. 4**). All Zantiks systems are built along the same principles, enabling high throughput and easy-to-run experiments. This automated system gave us a controlled and isolated testing environment, thanks to the tanks included in the Zantiks kit for fish. It granted me complete supervision: Zantiks units are supplied with a router and controlled via a web browser, allowing me to operate from my own laptop. I oversaw when the experiment started, I was able to monitor live our two subjects throughout the entirety of the experiment, and I could also end the experiment whenever I wanted. When the script ran its course, the machine gave back the outputs in .csv format, stating clearly all the information needed such as total of trials, total of both correct and incorrect trials, total of feeder visits and total of stimuli appeared on the right/left. This automated system sped up consistently the research with its efficiency and accessibility.

The apparatus is suitable to train animals in operant tasks with food as a reward due to a tracking system, predefined scripts, and built-in stimuli. The visual stimuli are supplied on a screen from underneath the tank that has a clear bottom and black sides. The chosen reward, (small amount of dry food in this case), is delivered through a brass feeder mechanism that attaches to the motor.



**Figure 4.** Zantiks and the colour discrimination script.

### **2.3 Preliminary Assessment of colour preference.**

Before starting the experiment, we needed to assess whether the fish had a spontaneous preference for the colours used in the operant conditioning tasks, a fact that could have biased the learning curves.

We inserted fish into an experimental chamber of an automated apparatus. The whole unit (Zantiks ©) (**Fig.4**) included the experimental chamber (22 × 30 × 50 cm), a computer, and the software with

C++ language. The chamber ( $14 \times 20 \times 15$  cm) was made up of black plastic walls and a transparent plastic floor and it was filled with 7 cm of water. A computer screen ( $16 \times 10$  cm) was placed beneath the tank and was used to present two different colours on the floor. A wireless router enabled the use of a laptop to run the program and collect the data. The system automatically detected the subject's position through an infrared camera placed above the chamber and an infrared source placed below the chamber itself. The floor of the tank was split into two halves: in Test 1, half of the floor was red (RGB: 255, 0, 0) and half of it was green (RGB: 0, 255, 0). In Test 2, the discrimination was between white (RGB: 255, 255, 255) and yellow (RGB: 255, 255, 0). Lastly, as the two pairs of colour could have been discriminated thanks to the brightness of the stimuli (if seen on a greyscale, red and yellow appeared darker than green and white), we also assessed (Test 3) whether subjects had a preference for light (RGB: 255, 255, 255)/dark (RGB: 0, 0, 0) colour (a classical scototaxis test, Maximino et al., 2010; Miletto Petrazzini et al., 2021).

After two minutes of acclimation, during which the screen remained blank, we recorded the proportion of time spent in the red area (Test 1), in the yellow area (Test 2) and in the darker area (Test 3). The observation lasted 10 minutes. After 5 minutes, the position of the two colours was automatically swiped by the system to ensure that any potential preference of the fish for one side of the tank was ascribed to the colour/brightness of the background only.

Before starting the actual reversal learning experiment, we also performed a scototaxis test. The light/dark preference test was necessary to confirm both fish preferred darker shades (in line with the darker environments they inhabit) and that the white light could have been used as a potential punishment in the operant conditioning part of the reversal. In a scototaxis test a fish is placed in a central compartment of a half-black, half-white tank; following acclimation, the fish is allowed to explore the tank for fifteen minutes; the number and duration of entries in each compartment (white or black) are recorded by the observer for the whole session (Maximino et al., 2010).



## 2.4 Operant conditioning task: colour discrimination.

Before the start of the experiment, the red bellied piranhas, were given 10 minutes of acclimation in the new environment to cope with the stress of being fished out of their aquarium, separated from their companion and lastly to cope with the new exposed tank that had no vegetation or place to hide.

The actual reversal learning task starts with operant conditioning: training each piranha to choose a specific colour. Leo was trained to pick the red coloured square while Piccolo José had to choose the green coloured square. In Experiment 2, where we used different wavelengths, Leo had to choose the yellow square while Piccolo José had to choose the white one. Inside the Zantiks tanks, on one side of the aquarium we put two black plastic walls ( $0.5 \times 11 \times 15$  cm) in correspondence with one of the two short ends in a funnel-like way to maximize the chance of triggering the initiator light, creating the starting point of the experiment for both subjects (**Fig.2**). In the v part of the tank, the blue rectangle (RGB: 0, 0, 255), which signalled the start of the experiment, appeared. If they swam on it within 30 seconds, the stimulus was elicited and on the other side of the tank, where there was no v shape structure, the two-coloured squares would appear ( $2 \times 2$  cm). The inter-stimulus distance was equal to 4 cm. The position of the stimuli on the left-right axis was switched across trials according to a random sequence. If they chose correctly, a white square ( $2 \times 2$  cm) appeared in the same position of the initiator light, inside the v structure, and they were rewarded by Zantiks 2 mg portion of commercial flake food, GVG Sera ©. If they chose incorrectly the bottom of the tank became white until the beginning of the next trial (inter-trial interval: 10 seconds). If subjects did not select the initiator light within 30 seconds, or they did not select either stimulus within 30 seconds, all stimuli disappeared, and the trial was considered null.

The subjects were tested every day, once a day, during the afternoon. They had to complete a total of 100 trials per day, (except for session 13 of Leo, Experiment 1, interrupted after 90 trials for a technical problem) after which the script ended automatically. Each trial could be classified as a valid trial (either correct or incorrect) or an invalid trial (none of the stimuli was chosen or the initiator blue

light was not triggered). After the acclimation, the training started; the duration of the experiment is based on how fast the fish executed the mandatory number of trials. The more the stimuli were elicited by the fish, the more time the experiment lasted.

Experiment 1 ended when the fish reached 600 valid trials; Experiment 2 ended when the fish reached 430 valid trials. We needed to interrupt Experiment 2 earlier than Experiment 1, as the number of invalid trials largely increased, thus making it difficult to continue the training. Overall, piranhas underwent more than one thousand trials in the Skinner box.

The difference between the two experiments is due to the drastic plummeting of trials executed by the subjects during the training. In the red-green colour discrimination, Leo executed an average of 41.53 of valid trials per day; Piccolo José executed an average of 43.72 of valid trials per day. In the yellow-white colour discrimination, Leo executed an average of 14.9 trials per day; Piccolo José executed an average of 17.4 per day.

For both experiments the learning criterion was fixed to 75% of correct choices in the last 50 trials, corresponding to a statistically significant choice in the binomial test. In addition, as a secondary criterion, we analysed the proportion of correct choices for each fish in the first 50 trials and in the last 50 trials to assess whether there was any sign of learning beyond the primary criterion. In the cases of fish reaching either learning criterion, we would have switched the rewarded colour and studied reversal learning.

Considering how quickly they grew in a month, we hypothesized that the set reward of the Zantiks was too little of a prize; meaning that working for that little quantity of food was not considered energy well spent by the two small predators. We proceeded to reward both piranhas manually with brine shrimp; although they seemed more engaged in the experiment, they did not reach the number of trials completed in the red-green training.

At the end of each colour discrimination task, we investigated a right/left bias in both Leo and José that could influence the responses in the colour discrimination test. Lateralization plays a significant role in fish behaviour and information processing, and it is often displayed as a behavioural bias like handedness (Berlinghieri et al., 2021). In this case to evaluate laterality, we examined the tendency to choose and swim more on the right or left square.

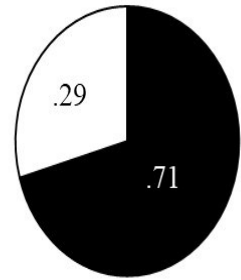
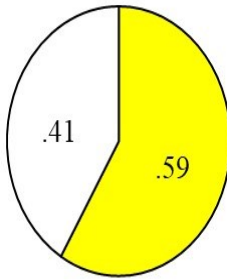
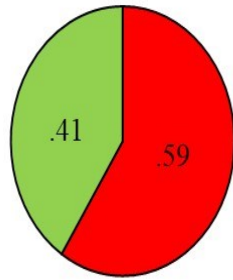
### 3. Results

#### 3.1 Preliminary assessment of colour preference and Scototaxis results.

In the preliminary assessment of colour preference Test 1 (red vs. green), Both Leo and Little José spent 59% of the time swimming in the red area (**Fig. 5**). In Test 2 (yellow vs. white), Leo and Little José spent respectively 59% and 58% of the time swimming in the yellow area (**Fig. 6**). Although no inference can be drawn due to the limited data, the small proportion of time spent in correspondence with either colour encouraged us to start the operant conditioning tasks without the risk that a spontaneous fish preference might facilitate or interfere with their learning curves. However, a tiny preference for red (Test 1) and yellow (Test 2) can be traced.

The results of our scototaxis test confirmed our hypothesis: in the light/dark preference test (Test 3, scototaxis response) both Leo and Little José showed a marked preference for the darker area (71% and 73%), which is in line with a robust literature of scototaxis in fish (Maximino et al. 2007, 2010; Miletto Petrazzini et al., 2021) (**Fig.7**). Considering how the piranhas' preference is more marked for black in the scototaxis test (Test 3), we can assume that the small preference for red and yellow might be related to the brightness of these stimuli (darker than the alternative colours) rather than being based on a specific preference for the hue.

Leo



Little Josè

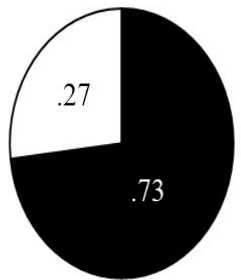
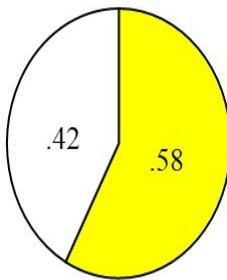
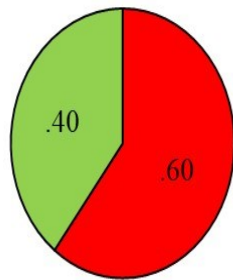


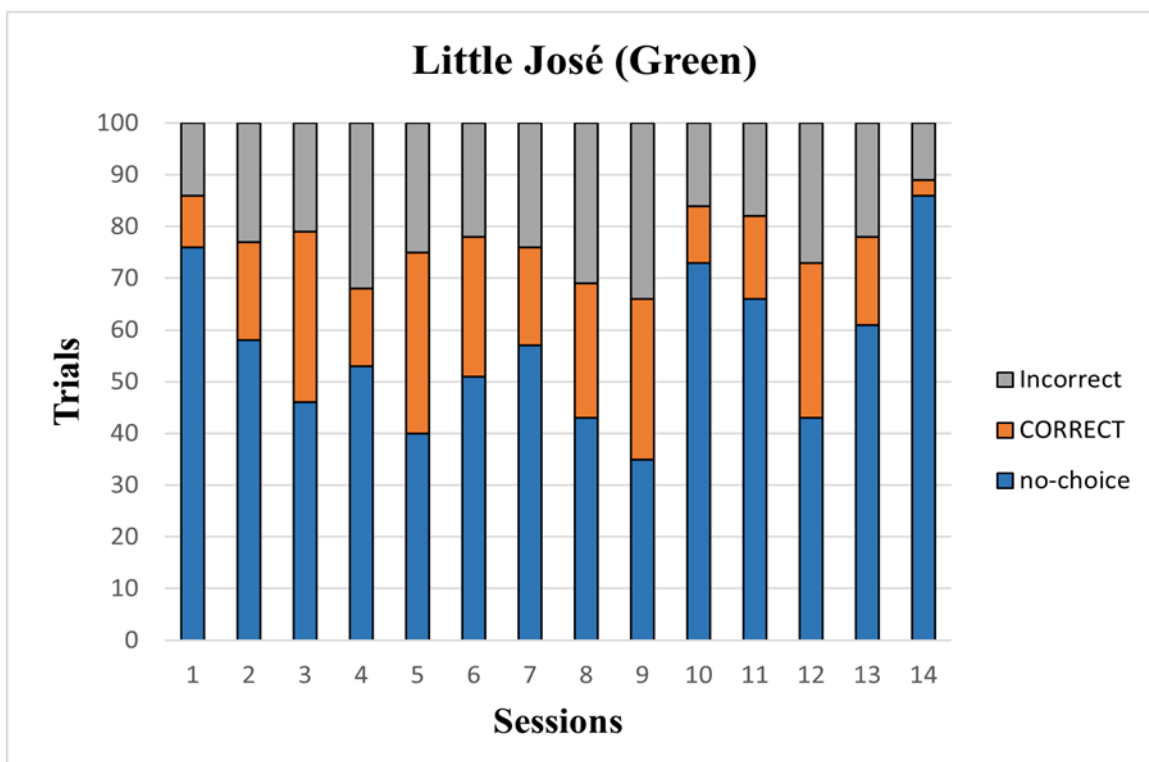
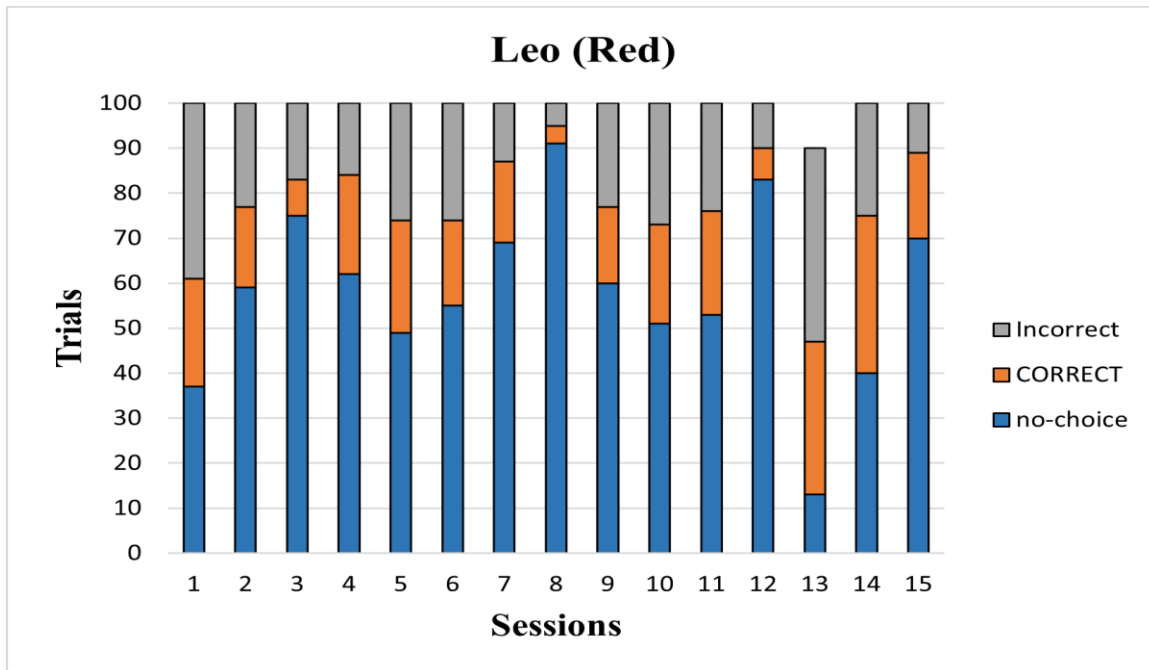
Figure 5. Test 1 results

Figure 6. Test 2 results

Figure 7. Scototaxis results

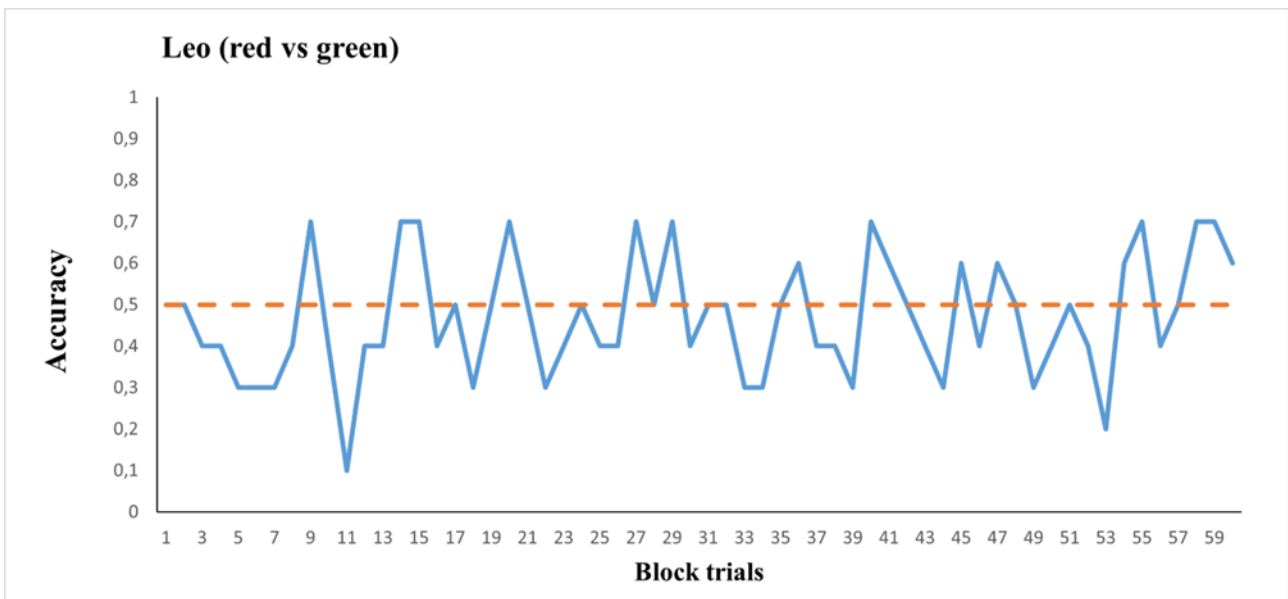
### 3.2 Experiment 1: red vs green results.

Figure 8 shows the number of correct, incorrect and invalid (no-choice) trials.

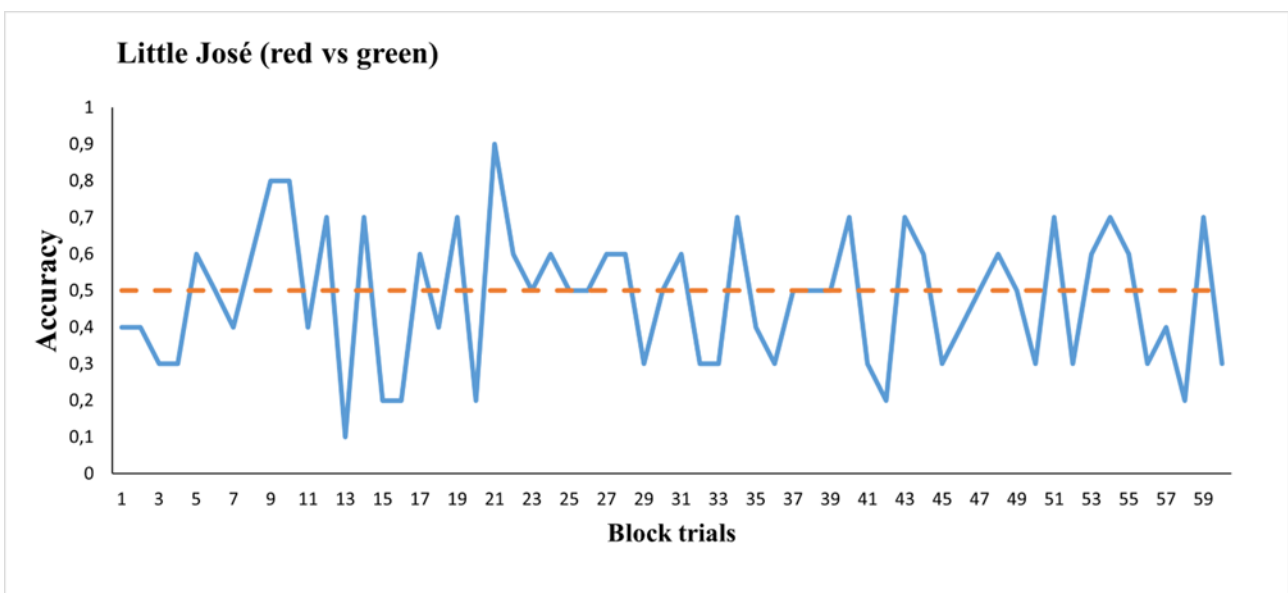


**Figure 8.** Number of valid (correct/incorrect) and invalid (no-choice) trials for both Leo and Little José in the red vs green discrimination test.

Regarding the learning curves, neither Leo nor Little José reached the learning criterion, nor were they close to reaching it at any stage (**Fig.9 & Fig.10**).



**Figure 9.** Experiment 1: Learning curves. Proportion of correct choices (accuracy) as a function of blocks of 10 trials for Leo. Fish did not reach either learning criterion.

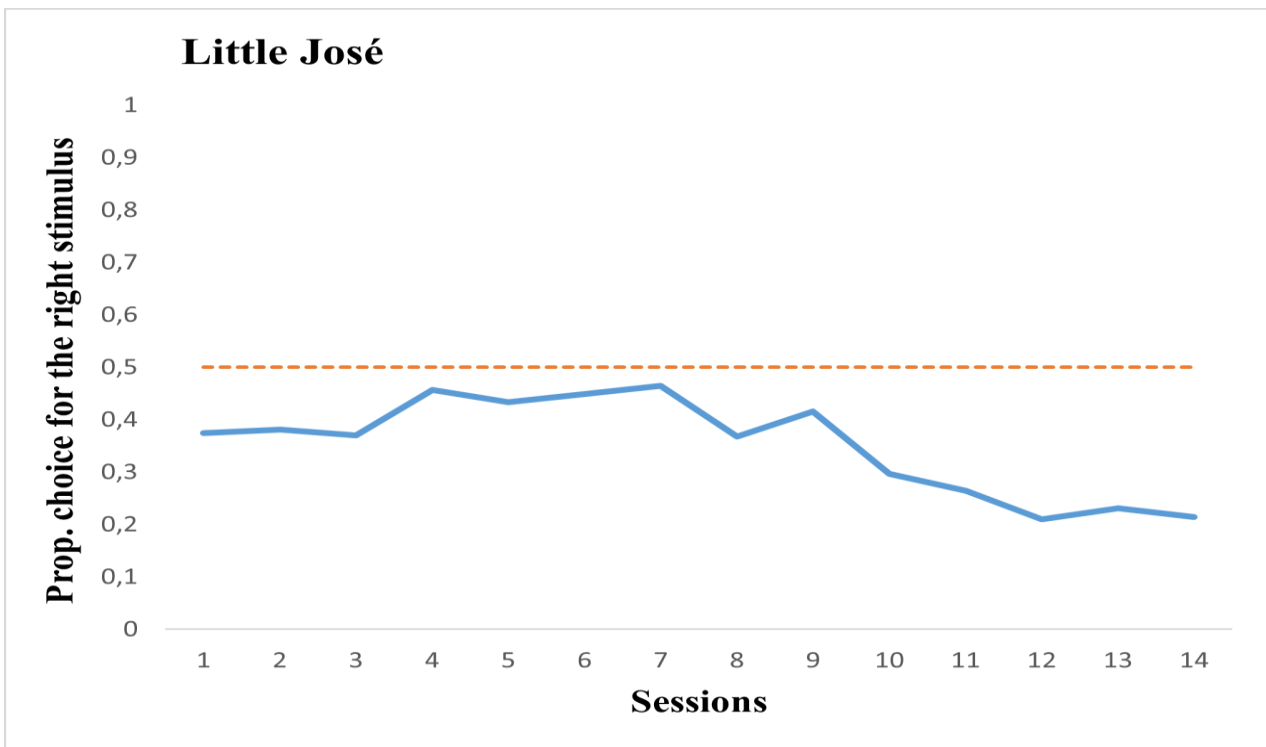
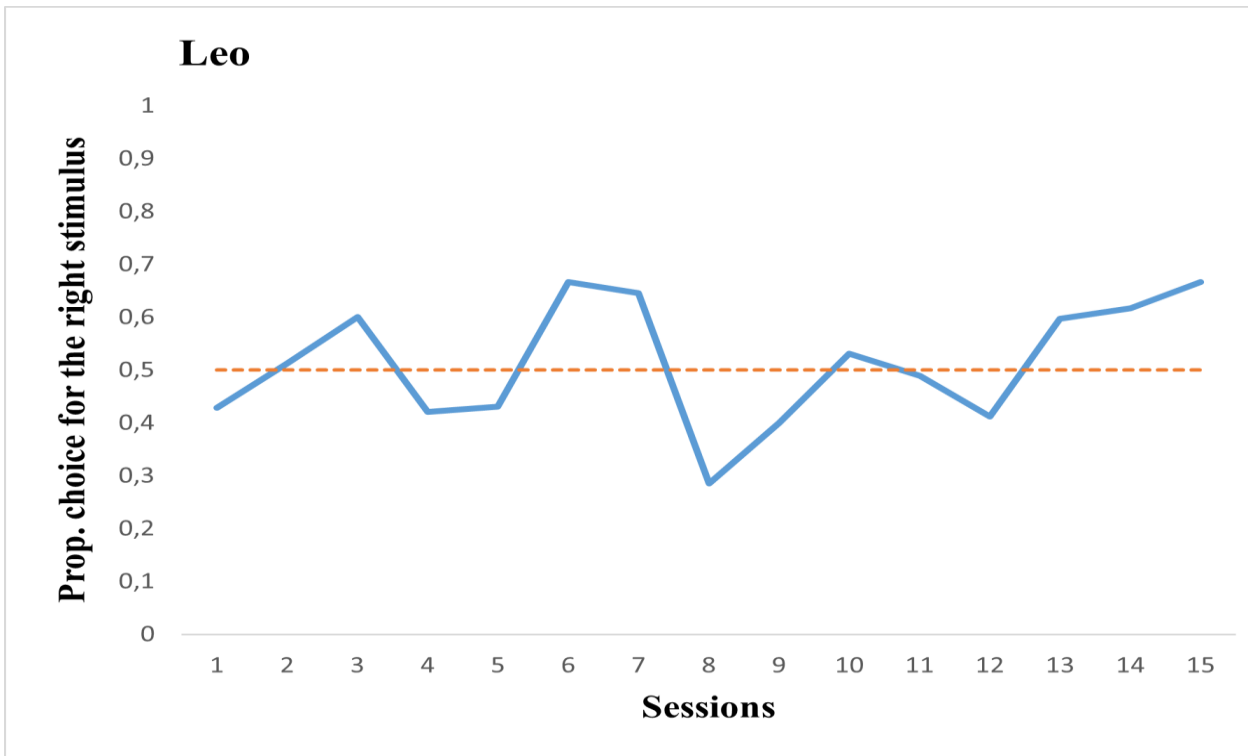


**Figure 10.** Experiment 1: Learning curves. Proportion of correct choices (accuracy) as a function of blocks of 10 trials for Little José. Fish did not reach either learning criterion.

When we compared the first 50 trials vs. the last 50 trials (secondary criterion) we did not find a significant difference for either subject (Leo: first 50 trials: 21 correct choices, last 50 trials: 29 correct choices; chi-square test  $\chi^2 = 2.560$ ,  $p = .110$ ; Little José: first 50 trials: 20 correct choices, last 50 trials: 19 correct choices;  $\chi^2 = .042$ ,  $p = .838$ ).

The lack of significant choice toward the reinforced stimulus might have been affected by the side bias that typically emerges in binary tests (Miletto Petrazzini et al., 2020). To control for this, we performed a binomial test on the frequency of choices for the stimulus presented on the left and the frequency of choices for the stimulus presented on the right side. Leo did not show any side bias (binomial test,  $p = .172$ ). On the contrary, Little José showed a significant bias in selecting the stimulus on the left (binomial test,  $p < .001$ ). This bias seemed to be consistent since the beginning of the training (**Fig.10**).

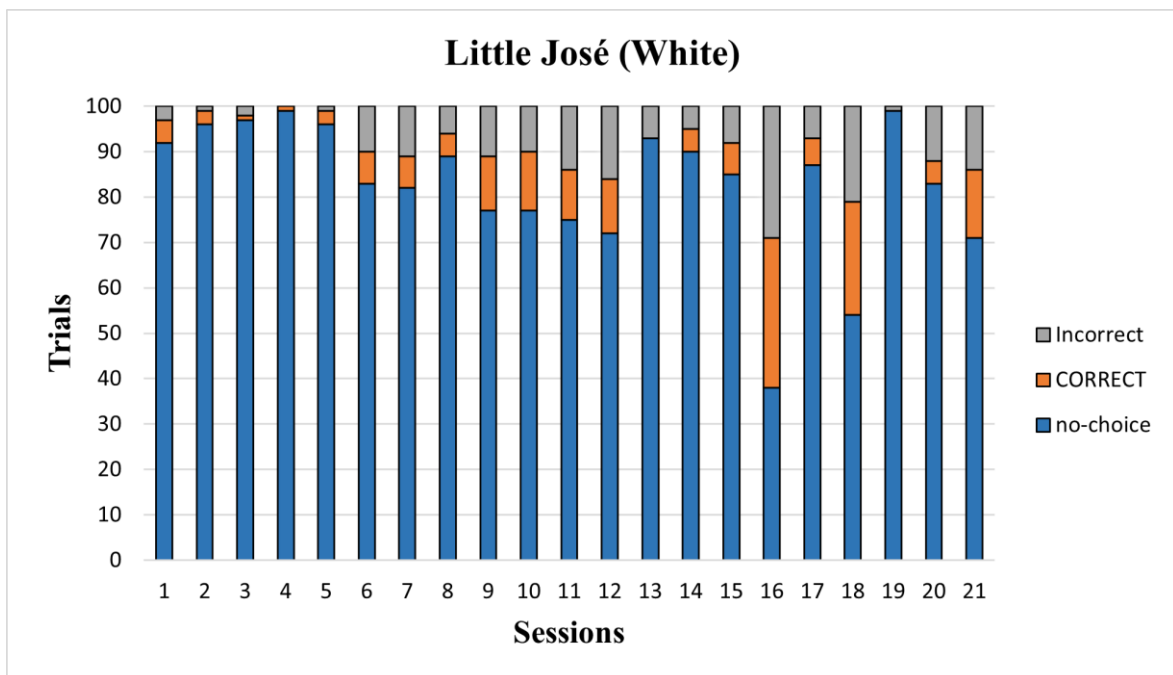
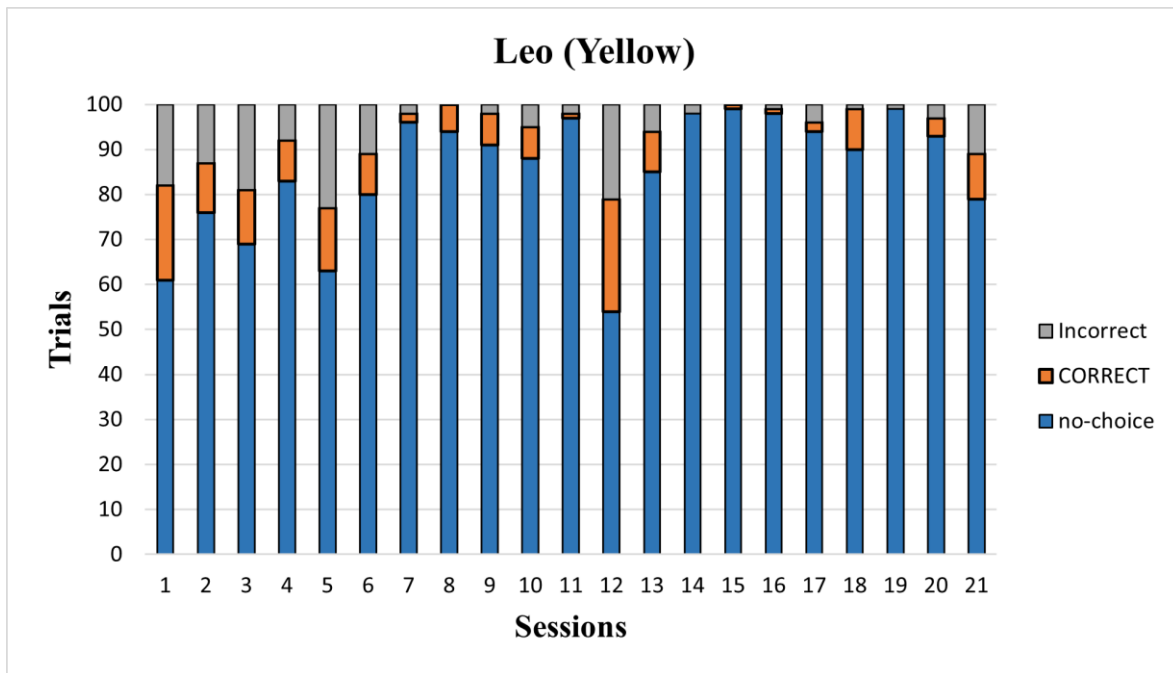




**Figure 10.** Experiment 1: Side bias. The proportion of choices for the stimuli presented on the right as a function of the experimental sessions. While Leo did not show a significant spatial bias, Little José consistently selected the stimulus presented on the left.

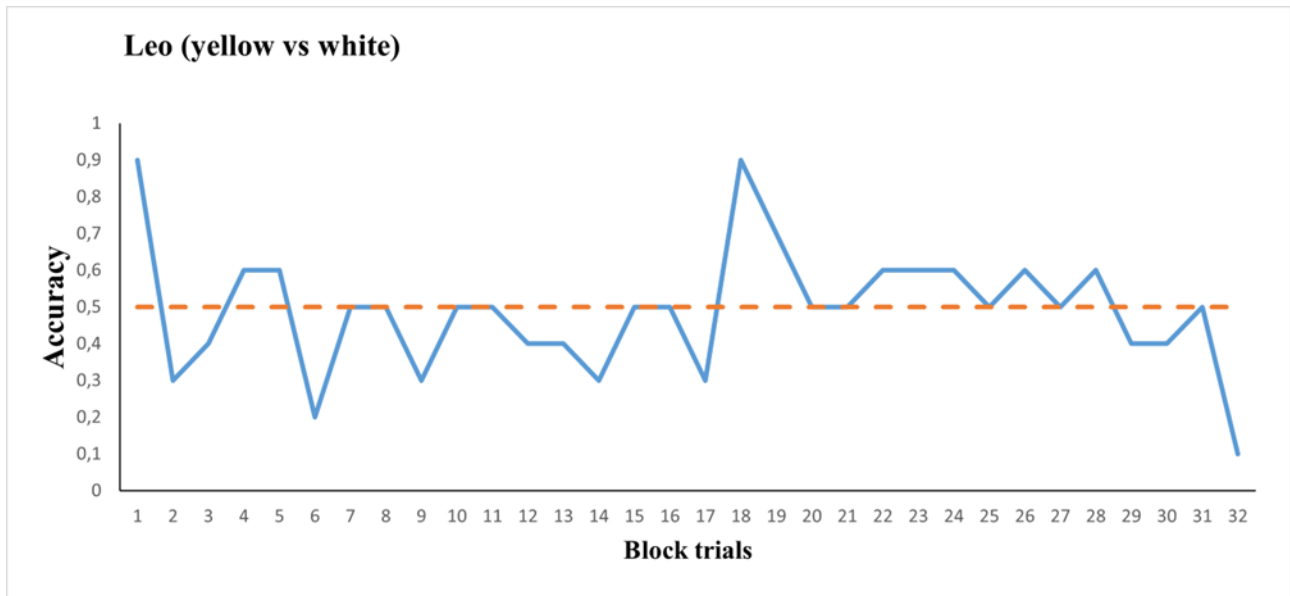
### 3.3 Experiment 2: yellow vs white results.

Figure 11 shows the number of correct, incorrect and invalid (no-choice) trials.



**Figure 11.** Number of valid (correct/incorrect) and invalid (no-choice) trials for both Leo and Little José in the yellow vs white discrimination test.

Again, neither Leo nor Little José reached the learning criterion, nor were they close to reaching it at any stage (Fig.12 & 13).



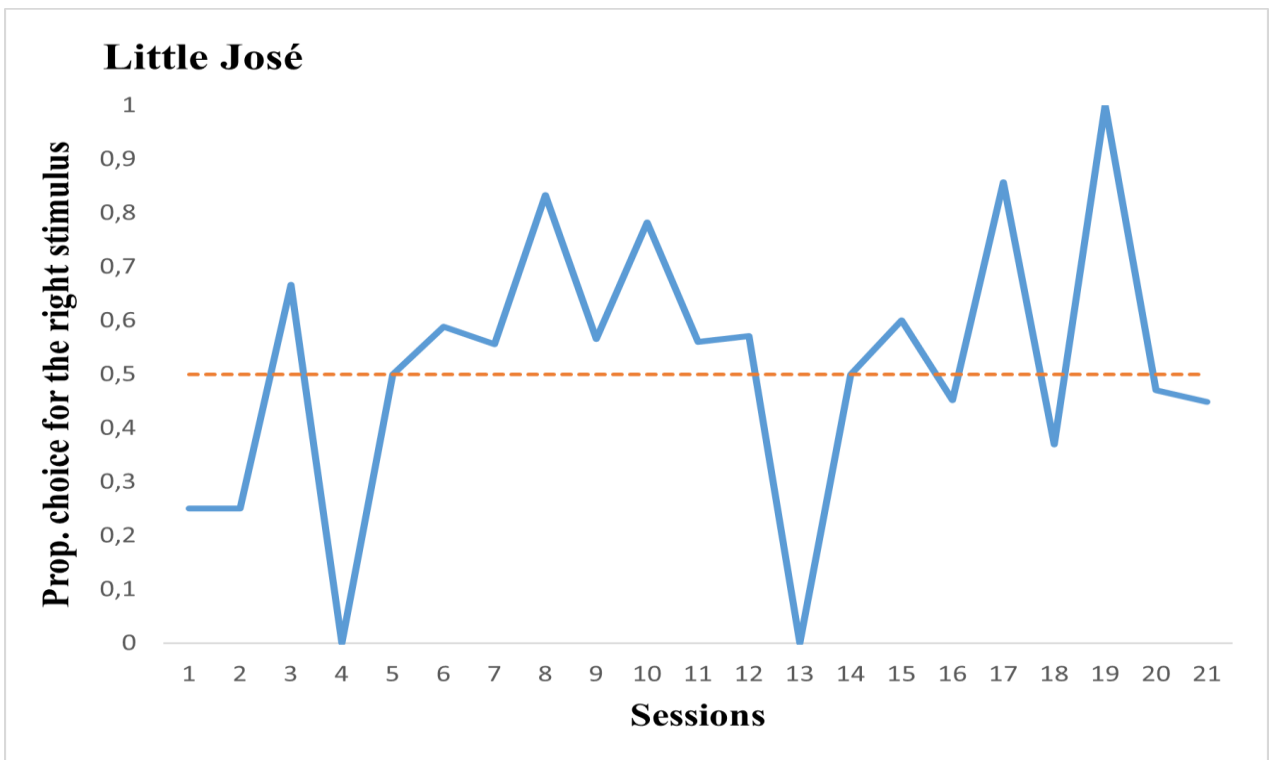
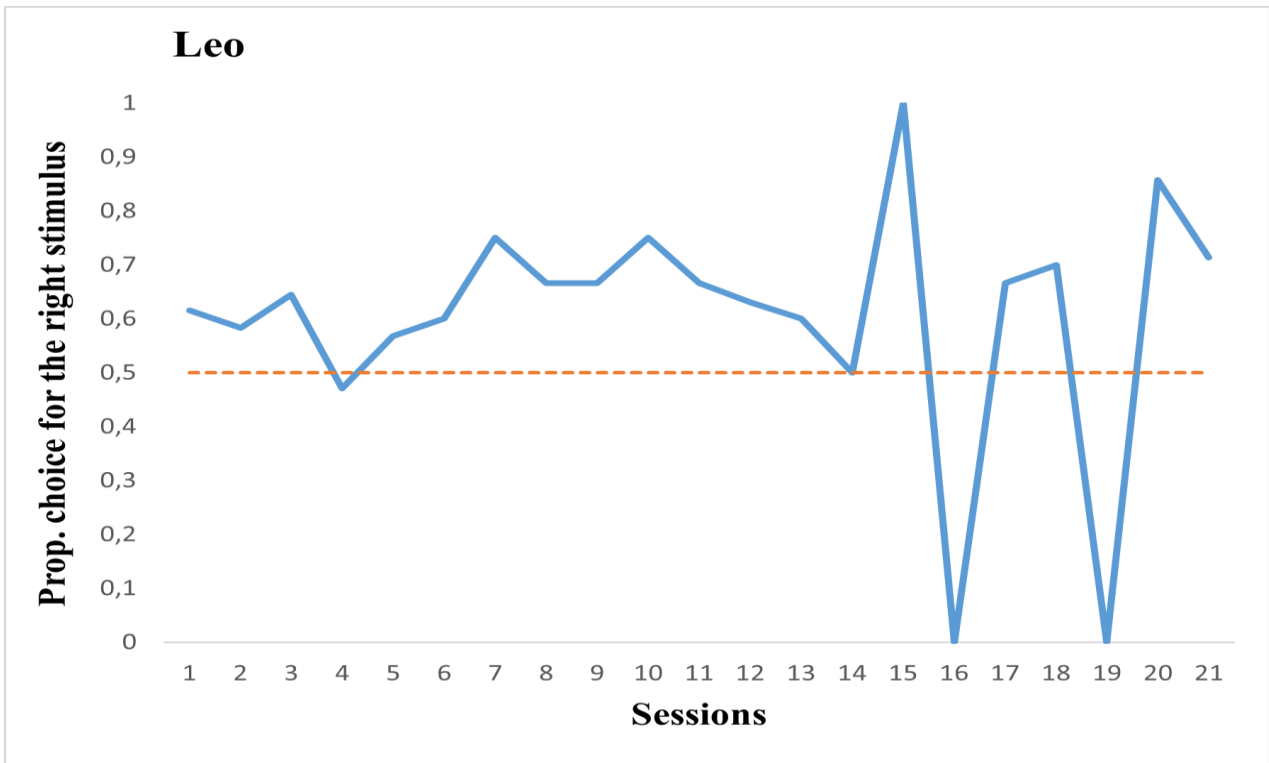
**Figure 12.** Experiment 2: Learning curves. Proportion of correct choices (accuracy) as a function of blocks of 10 trials for Leo. Fish did not reach either learning criterion.



**Figure 13.** Experiment 2: Learning curves. Proportion of correct choices (accuracy) as a function of blocks of 10 trials for Little José. Fish did not reach either learning criterion.

When we compared the first 50 trials vs. the last 50 trials (secondary criterion) we did not find a significant difference for either subject (Leo: first 50 trials: 28 correct choices, last 50 trials: 19 correct choices;  $\chi^2 = 3.252$ ,  $p = .071$ ; Little José: first 50 trials: 25 correct choices, last 50 trials: 31 correct choices,  $\chi^2 = 1.461$ ,  $p = .227$ ).

To assess whether there were side biases, we performed a binomial test on the frequency of choices toward the stimulus presented on the left/right side. This time, Leo showed a right-side bias (binomial test,  $p < .001$ , **Fig. 14**). The bias has been quite consistent since the beginning of the training. Unlike Experiment 1, Little José did not show any side bias (binomial test,  $p = .405$ ). When we compared the first 50 trials vs. the last 50 trials (secondary criterion) we did not find a significant difference for either subject (Leo: first 50 trials: 28 correct choices, last 50 trials: 19 correct choices;  $\chi^2 = 3.252$ ,  $p = .071$ ; Little José: first 50 trials: 25 correct choices, last 50 trials: 31 correct choices,  $\chi^2 = 1.461$ ,  $p = .227$ ).



**Figure 14.** Experiment 2: Side bias. The proportion of choices for the stimuli presented on the right as a function of the experimental sessions. While Little José did not show a significant spatial bias, Leo consistently selected more than chance the stimulus presented on the right.

## **4. Discussion & Conclusion**

In the preceding chapter, a meticulous examination of the outcomes of Experiment 1 (red vs. green) and Experiment 2 (yellow vs. white) was conducted through comprehensive graphical representations. These charts effectively demonstrated that neither subject, Leo nor Little José, attained the learning criteria for colour discrimination. Consequently, the absence of learning rendered it impossible for us to advance to a reversal learning task. In the forthcoming paragraphs, I will delve into potential causes and elucidate the rationale behind our findings, shedding light on the intriguing behaviour exhibited by these fascinating predatory subjects.

### **4.1 Interpretation of findings**

#### **4.1.1 Cognitive impairment**

A possible explanation for the lack of rule learning (previously displayed through our graphs) in the red-bellied piranha may be connected to a cognitive limit of the species: the cognitive component plays a key role in the success of operant conditioning experiments. However, experimentation within the Characiformes family is scarce and our research represents only a preliminary investigation inside their cognitive system. To grasp what element prevented them from acquiring the taught rule, it is necessary further knowledge in the *Pygocentrus* cognitive system. Techniques such as lesioning, tract tracing and quantifying changes in gene expression could be used to discover more of the neuroanatomy of the red-bellied piranha (Ebbesson et al., 2012). The cognitive capacity of animals is deeply connected and dependent on the environment they inhabit and the social interactions they take part in (Fernö et al., 2020). From an ecological point of view, animals specifically develop abilities needed to adapt to their ever-changing environment. In light of the connection between cognition and natural environment, the ability to colour discriminate may not be a skill present ecologically nor useful for the fitness of this species; meaning piranhas might not have the cognitive

flexibility to face this task considering how they have no practical use to colour discriminate in their habitat.

Consider the context of the experiments and how both (Experiment 1 and Experiment 2) were conducted on young piranhas. It is important to note that their cognitive abilities might not have fully developed at that stage. While certain skills are innate, others develop over time through maturation and social interactions (Bisazza et al., 2010). Replicating the experiment after the piranhas have reached sexual maturity could provide valuable insights into the timeline of cognitive development in piranhas.

#### 4.1.2 Perception of colours

Since no successful learning was made by the subjects, one may argue that our results may be connected to the way *Pygocentrus nattereri* perceive colours and not the ability to learn a rule. I was able to find almost no scientific literature on what wavelengths piranhas do see, aside from this paper: “Visual pigment evolution in Characiformes: the dynamic interplay of teleost whole-genome duplication, surviving opsins and spectral tuning” (Escobar-Camacho et al., 2020). The author discovered three different cone genes in the red-bellied piranha, corresponding to wavelengths of the colours used in our experiment. Even though the subjects do possess the genes to be able to see red, green, yellow, and white, we do not know their actual visual sensitivities and how they perceive what we call “red”, “green”, “yellow” or “white”. No microspectrophotometry was done on this specific species, so we can only make a calculated guess by considering the visual sensitivities of other members of the Characiformes family. Even though the research paper gave us enough information to perform in theory a successful colour discrimination test, it would be wise, before proceeding with colour discrimination tasks in the Characiformes family, to keep doing research on their colour perception.

### 4.1.3 Lateralization and colour preferences

An interesting topic to delve into while researching the coloured vision of *Pygocentrus nattereri*, would be how warm colours can affect the emotive state of animals. Experimental evidence suggest that fish have emotional states that are functional in many of the same aspects as in other higher vertebrates. Quoting directly a 2022 study on a group of emperor tamarins” Colours could be associated with emotions and the process of emotions could be related to hemispheric specialization, with different brain hemispheres dealing with different emotional states” (Spiezio et al., 2022). The article highlighted how specific warm hues activate one hemisphere over the other, leading to behavioural lateralization. Lateralized functioning, during emotional contexts, has been reported not only in mammals but also in fish (Forrester & Todd, 2018; Leliveld et al., 2013). It would be fascinating to investigate in vertebrate fish how distinct colours may activate different cerebral hemispheres. Through the fish swimming behaviour, we can assess whether a connection is present between a colour and a specific cerebral hemisphere. Evaluating side biases and colour preference together would allow a better understanding of both brain asymmetries and colour perception (Crowley, 1993; Elliot, 2015). This kind of research could lead to a different interpretation of our findings: a connection between colour and right/left hemisphere could prevent a possible colour association (and learning) from happening (Spiezio et al., 2022).

### 4.2 Study's limitations

We concluded that piranhas may have limited capabilities in an automated colour discrimination task, but several factors may have influenced the course and outcomes of our trials. Most of the research on fish has been done in a laboratory setting, here an experiment can be carried out not only through an automated device, but also in a manual way. A different method can have a relevant impact on the results. Studies show how fish performance in a laboratory setting is worse with automated devices than with the traditional manual procedure (Gatto et al., 2021). The 2021 article “Automated operant



conditioning devices for fish. Do they work?” highlights how different species perform differently in specific tasks (such as colour or shape discrimination) depending on the chosen procedure (manual or automated). Quoting the said article: “It is worth noting that other studies using automated devices have reported unexpectedly low performance in other species and with different tasks, which calls into question the reliability of this approach of operant conditioning in fish”. Why did we choose an automated device then? First, automated devices appear to be very efficient in some experiments of colour association: “guppies showed excellent performance, reaching 90–95% accuracy in two–three sessions”. Secondly, discrimination learning requires thousands of training trials to consider the results statistically relevant, meaning our two subjects needed to complete a high number of trials in a short period of time (Cantlon et al., 2007; Jaakkola et al., 2005). The automated device Zantiks was ideal; it reduced considerably the time needed for training and the related human labour required. In only three months of training, we were able to gain from each subject more than a thousand valid trials. Lastly, automated equipment allows for the control of every detail of the experiment, minimizing human error and the possibility to influence (consciously or not) the fish performance through the experimenter’s expectations (Rosenthal et al., 2007).

It is not easy to point out what causes the efficiency gap between automated devices and traditional methods. It is possible that stimuli introduced manually into the aquarium are much more salient and focus the subject’s attention on the task, rather than the stimuli generated on a computer screen. But there is research that proves how Computer-generated stimuli have already been used successfully in fish for visual discrimination tasks (Gori et al., 2014; Lucon-Xiccato et al., 2019) and there are papers that show how fish react to stimuli presented on a monitor as they react to the real objects in the tank (Müller et al., 2017; Zeil & J, 2000). For future research it may be interesting exploring a manual method for a reversal learning colour association task with piranhas and then proceed to compare performances.

Additionally, a limitation to consider for this study is sample size (Marszalek et al.,2011). The number of subjects used in an experiment is an essential component for the design of a research including animals because low sample size can lower the statistical accuracy of a research (Charan et al., 2013; Button et al., 2013). In our study we trained only two piranhas and concluded how both did not reach the learning criteria; this conclusion, although scientifically valid, needs to recognize possible individual differences of Leo and Little José. Our findings, although representative of this species, need to be confirmed on a bigger scale to clarify if the outcome is depictive of all members of this species or if it is connected only to our two examples.

### **4.3 Conclusion**

The present study aimed to investigate the cognitive abilities of the *Pygocentrus nattereri*, member of the Characiformes family. As highlighted in the graphs of the previous chapter, both piranhas never reached the learning criteria, meaning we were not successful in the reversal learning colour association task. In conclusion both piranhas did not show signs of cognitive flexibility. I hope that this subject can be further explored and examined again for future investigations within the realm of cognitive science and comparative psychology.

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