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# Discrimination abilities of 2D pictures of congeners in rainbow trout: the use of an innovative operant conditioning device

Aude Kleiber

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### Mémoire de fin d'études

- d'Ingénieur de l'Institut Supérieur des Sciences agronomiques, agroalimentaires, horticoles et du paysage
- de Master de l'Institut Supérieur des Sciences agronomiques, agroalimentaires, horticoles et du paysage
- d'un autre établissement (étudiant arrivé en M2)

## Discrimination abilities of 2D pictures of congeners in rainbow trout: the use of an innovative operant conditioning device

Par : Aude KLEIBER



**Soutenu à Rennes le 10 septembre 2019**

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*Les analyses et les conclusions de ce travail d'étudiant n'engagent que la responsabilité de son auteur et non celle d'AGROCAMPUS OUEST*

*« Everyone is a genius. But if you judge a fish by its ability to climb a tree, it will live its whole life believing that it is stupid. »*

- Albert Einstein

## REMERCIEMENTS

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## GLOSSARY

**Behaviourism:** a psychological method based on objective observation. For behaviourists, psychology is the study of external behaviour, not the interiority (thoughts, feelings) of the subjects. It is therefore a psychology of behaviour. This current was founded at the end of the 19th century with Thorndike's book "Animal Intelligence" (1898). One of the founders is Watson.

**Chromaticity:** value used to determine a colour without considering its brightness. It gathers information on saturation and tint of colour stimuli.

**Ethology:** science of animal behaviours in their natural environment.

**Livestock:** domestic animals reared for their profitability, i.e. for the production of foodstuffs, wool, skins, milk as well as for other agricultural purposes.

**Operant conditioning:** process by which an animal modifies its natural behaviour for experimental conditions. The animal considers the consequences of its action according to a given reinforcement. This last one encourages it to reproduce the same behaviour in the case of a positive reinforcement, or to reduce and avoid it in the case of a negative reinforcement.

**Paradigm:** the way an animal responds to a problem raised in an experimental condition. It is the set of experiences that determines how an individual perceives reality and reacts to what it perceives.

**RGB code:** English abbreviation that indicates the code of a colour specifying the proportions of red (Red), green (Green) and blue (Blue) that it includes.

**Self-feeder:** a device commonly used in aquaculture, allowing fish to feed themselves by activating a trigger that drops a defined amount of food from the surface.

**Session:** series of successive trials conducted. In the present study, a session refers to only one conducted per day and per individual.

**Trial:** an attempt or effort to do something. In the present study, trials indicate the number of tries an animal is allowed to perform in one session.

**Trigger:** a lever that requires a pressure to be applied to activate it.

**Visual acuity:** eye's ability to perceive details at a more or less significant distance.

**Welfare:** refers to the notion of balance and homeostasis necessary between the animal and its environment but also implies the absence of negative or constraining experiences (diseases, stress) and the possibility of experiencing positive stimuli.

## LIST OF ABBREVIATIONS

**AFC:** Alternative-Forced-Choice test

**2-AFC:** Two-Alternative-Forced-Choice test

**AIC:** Akaike Information Criterion

**CO:** Cut-Off

**CT:** Correction trial

**ERR:** Error

**GLM:** General Linear Model

**ITI:** Inter-Trial time-Interval

**lat:** Latency time to answer

**LM:** Linear model

**MTS:** Matching-To-Sample

**OFS:** Oddity-From-Sample

**RC:** Correct answer

**S+:** Positive stimulus

**S-:** Distracting stimulus

**2D:** Two-Dimensional

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# I. INTRODUCTION

Since Antiquity, humans have been trying to imagine how animals perceive the world, whether they conceive it, and if so, how. We have wondered if they are able to remember past events, what they think about an object, their body or a congener, if they are aware of their activities, etc. (Darmaillacq et al., 2018; Kreutzer & Vauclair, 2004). These questions were for many years addressed to philosophers and sociologists before being also considered by psychologists and naturalists (Darmaillacq et al., 2018). In the 17th century, philosophers relied on beliefs to refute Montaigne's theories according to which animals are close to man and are sentient beings (Boivin et al., 2016). Descartes, for instance, considered the animal as a "machine animal" and as early as the 18th century, Rousseau conceded to the animal a soul and a right to protection, but maintained the hypothesis of the "machine animal". Until the 20th century, the animal kept its status of a mindless assembly of biological mechanisms (Boivin et al., 2016).

One of the first movements in the study of animals' behaviour was the Behaviourism, which was a dominant school in psychology, developed during the first half of the 20th century. It aimed to study the behaviour of an animal in a purely objective way (Veissier et al., 2010). Thus, for Watson, behaviour could be defined as any adaptive response that an organism with a nervous system produces in response to environmental stimuli. Objectively, it is then possible to observe an animal manipulating devices and responding with its body through conditioning tests (Veissier et al., 2010). However, animal behaviour studies have not always been focused on the understanding of how an animal perceives its environment (Veissier et al., 2010). Indeed, after Behaviourism, a new trend opposed it. Ethology emerged, focusing more on animals' natural and spontaneous behaviours rather than on learning abilities (Veissier et al., 2010). The two main pioneers (Lorenz and Tinbergen) of this movement questioned about what the immediate causes of a behaviour were, which value the animal conferred to its survival and how a behaviour was implemented during ontogenesis and phylogenesis. However, these disciplines do not raise questions about the internal and mental states of the animals because these are considered as unobservable (Veissier et al., 2010). Moreover, they do not consider the subjectivity of the animal since they do not confer them the capacity to interpret the stimuli emitted by their environment (Boivin et al., 2016). Another movement was therefore added to the previous ones, under the influence of psychologists such as Tolman, who considered that it is possible to study mental processes in animals and humans, considering an approach focused on the animal in relation with its environment (Veissier et al., 2010). The study of animal cognition therefore appeared, with works often designed to investigate in animals the evidence of known mental processes in

humans (Veissier et al., 2010). Cognition thus covers all psychological structures and activities whose function is to produce and to use knowledge (Le Ny, 2005). Etymologically, this term comes from the Latin *cognitio* which means the action of knowing. The origin of the word is old but its use only dates from the middle of the 20th century (Darmaillacq et al., 2018). It is also defined as information management processes and mental representations, conscious or not, that underlie behaviours (Kreutzer & Vauclair, 2004). In psychology and biology and according to Shettleworth (2009), "cognition refers to the mechanisms by which animals acquire, process, store, and act on information from the environment".

The topics thus covered by cognition studies are memory, language, categorization skills and problem solving, but always considering human's cognition as a reference. From 1970, applied ethology has been developed through the establishment of the International Society for Applied Ethology as well as the launch of the Applied Animal Behaviour Science journal (Veissier et al., 2010). This field is interested in the behaviour of domestic animals (living under the dependence of humans) and was first oriented towards improving animal husbandry (sex, maternal, social...) before today focusing mostly on animal welfare (40% of the journal's articles) (Veissier et al., 2010). Animal welfare is thus an important theme within applied ethology, more than in classical behavioural sciences (classical ethology, behaviourism, animal cognition). The issue of animal welfare requires an exploration of how the animal perceives its environment, considering that it is this factor that will enable welfare to be either good or bad (Veissier et al., 2010). To study animal welfare it is, therefore, necessary to "objectively study the subjectivity of the animal" (Dawkins, 1980).

The interest in animal cognition has grown especially since we know animals can feel pain making people wondering whether animals have consciousness (Le Neindre et al., 2017). The absence of pain is one of the 5 freedoms to be respected to guarantee animal welfare (Farm Animal Welfare Council, 1992). Pain in livestock production is associated with poor zootechnical performance and results in direct or indirect economic losses for the farmer (Fourichon et al., 1999; McDougall et al., 2009). Therefore, in an INRA synthesis report (Le Neindre and al., 2017), it has been concluded that animal consciousness is possible, but is more or less important depending on the species. The notion of welfare targeting farm animals has been emerging for some years under the influence of several awareness and authors. Indeed, the publication of Ruth Harrison's *Animal Machines* in 1964 was the first step in raising collective awareness of the need to respect animal welfare (Veissier et al., 2010). This essay questions the origins of the food supply by shedding light on the abuses of modern livestock farming. Indeed, after the war, since the 1960s, debates have mainly focused on livestock systems designated as intensive. The main criticism concerned the impossibility for the animal to exhibit its natural behaviour, due to confinement, density and mutilating practices (Boivin, 2016). The scientific

community, politicians and consumers have been impacted by Harrison's book and working groups have been set up to review and evaluate livestock conditions (Veissier et al., 2010). Several Conventions concerning the protection of animals were then adopted by the European Council in the 1960s-1970s before the emergence of the first European Directives on the protection of animals in 1980 (Veissier et al., 2010). In 2015, a law modernized animals' legal status by recognizing them as "living beings endowed with sensitivity" in the French Civil Code. This awareness and the recent different scandals concerning intensive livestock (L214, Animal Welfare, "anti-speciesism"), urge the competent authorities to develop welfare monitoring tools for farmed animals in order to take into account an ethological conception in future farming systems (Le Neindre and al., 2017). Assessing animal welfare in the present state of knowledge is to know where it stands between two extremes (harmony or unwellness). This involves understanding the elements that contribute to reach harmony between the individual needs and its environmental conditions. In addition, it is important to evaluate the animal's adaptation to a suboptimal environment. These measures require behavioural studies to better target the specific needs of the animals, passing through the study of animals' cognitive abilities (Le Neindre and al., 2017).

Cognitive ethology is a very recent discipline that focuses on the internal mechanisms underlying animal behaviour, and which considers the possibility that some animals may be capable of constructed representations, memory and emotions (Boivin et al., 2016). A simple search on the Internet shows that there are about 4 times more publications on animal cognition at the international scale in the last decade (2007-2017: 42,532 references, source Bib CNRS, with the terms "Animal Cognition" from Darmaillacq et al., 2018) than in the previous one (1996-2006: 10,751 references). The term "cognitive ethology" was first used by Griffin (1976) in his book *The Question of Animal Awareness* who defined it as "the study of the animals' mental experience when they behave in their natural habitat...". He was one of the first to promote the idea that animal behaviour is guided by complex cognitive processes (Kreutzer & Vauclair, 2004). Behaviour has been described as the specific feature of the animal kingdom (Boissy et al., 2009). Certain types of behaviour are so important to an animal that they become fixed and heritable (Braithwaite and Salvanes, 2008). Other behaviours are flexible and can be adapted to various situations or environment. This ability to behave in a flexible way, especially in changing environment, requires an animal the capacity to learn, remember and update information (cognitive abilities) (Braithwaite and Salvanes, 2008). Moreover, it is through behaviour individuals animate, move, search, avoid, act and react, and face the hazards of their environment. Indeed, in their environment, animals constantly face various situations to be solved. In this way, cognitive abilities play a crucial role in their daily life as they enable certain common key functions such as foraging, predator avoidance, mate-choice, navigation and orientation (Gierszewski

et al., 2013). For these functions, perception of environmental stimuli through the animal's sensory systems is essential. This probably involves the utilisation and assimilation of information belonging to different cognitive domains (for instance, finding food may involve aspects of social, perceptual and physical cognition), implying that cognitive capacity may be closely related to the animal's cognitive environment (Pike, 2019). Thus, it can be presumed that the higher an animal's cognitive abilities are important, the more it may react to variable environments or challenging situations with flexibility (Gierszewski and al., 2013). This is important because high cognitive capacities would lead to increased behavioural plasticity, enhancing survival and reproductive success within novel or changing environments (Dukas, 2004).

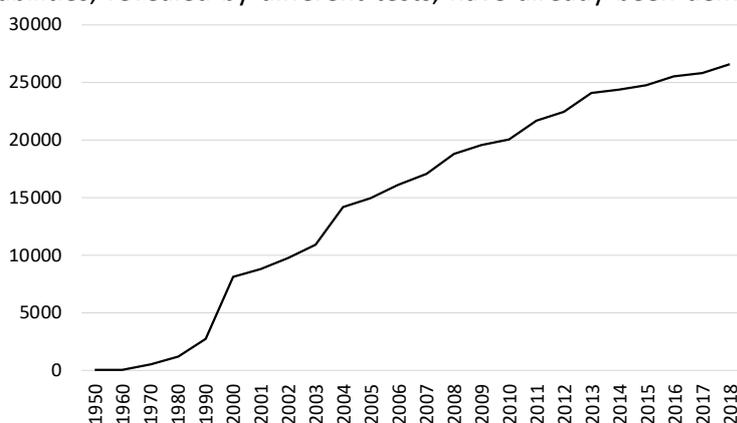
Wolfgang Köhler in his book (1921), for instance, showed that chimpanzees are capable of high cognitive abilities by making them resolve complex problems based on visual perception. Other studies have shown, for example, that primates have spontaneous numerical skills (Brannon and Terrace 1998, Hauser et al. 2000). Based on a series of experiments, Edward Tolman showed in 1948 that rats can learn the structure of a labyrinth by exploring it freely and without any form of reward (Darmaillacq et al., 2018).

To date, most of what we know about cognition has been focused on higher vertebrates (human, monkey, horse, cat, rabbit) to understand the mechanisms underlying human cognition, considering non-human animals as simpler models having a cognitive system. This is called compared cognition (Darmaillacq et al., 2018). This approach enables studying the diversity of solutions that organisms have found to solve similar issues. Comparisons between species are therefore performed in order to identify and explain the general mechanisms implemented in different situations. In this way, the main objectives of comparative cognitive psychology are to discover whether animal species, sometimes phylogenetically distant, structure information from the environment in a similar way. Comparisons are then used to establish the identity of cognitive traits within different species (Vauclair 1998. In: Kreutzer & Vauclair, 2004).

While compared cognition has mainly concerned higher vertebrates, very few studies focused on farmed animals and even fewer on farmed fish species. However, the attribution of cognitive abilities to animals is precisely related to the way in which the animal will be considered and used. The French Institute for Agronomic Research (INRA) therefore initiated a "Cognition project" for this purpose, granted by the INRA-PHASE Department. It aims at adapting a methodology validated in non-human primates to measure whether farmed animals, including the rainbow trout, do also have some elaborated cognitive functions such as working memory, categorization and conceptual learning abilities.

Among farmed animals, rainbow trout is the first French fish continental production with about 30,000 tonnes per year (CIPA; FAO, 2019). However, the well-being of farmed rainbow trout is often neglected, despite the recent findings concerning fish capacity to feel pain (Sneddon et al., 2003). Furthermore, fish is one of the most popular experimental model in the world (Sneddon, 2011). A better knowledge of its mental capacities would make it possible to better understand how this farmed animal perceives its environment and what they may experience when their welfare is compromised (Sneddon, 2011). Such researches would allow fish production evolving towards systems more respectful of the fish needs and welfare. Among vertebrates, fish species are the less concerned by consumers' sensibility because these "cold-blooded" animals lack observable facial expressions linked to positive or negative affective states and do not audibly vocalise (Balcombe, 2016). However, fish are important to consider as they represent more than a half of all vertebrate species including a huge diversity (around 30 000 known species) and inhabit a considerable range of aquatic environments, from mountain springs to the deepest part of the ocean (Nelson et al., 2016. In: Miller, 2017).

Moreover, fish is an interesting experimental model since they lack a cerebral cortex, the part of the brain dedicated to elaborated functions such as learning processes (Seger and Miller, 2010). Thus, studies on fish species would reveal the essential role played or not by neocortex in cognitive functions. Despite a less developed brain and a poor visual acuity (Champ et al., 2014; Hester, 1968; Neumeyer, 2003; Ben-Simon et al., 2012), teleost fish are able to see complex and contrasted patterns. Indeed, fish can discriminate colours, guidelines (*Danio rerio*: Colwill et al., 2005, Arthur and Levin, 2001), two- and three-dimensional shapes and even illusory shape's surface (*Gnathonemus petersii*: Schuster and Amtsfeld, 2002; *Pseudothropheus* sp.: Schluessel et al., 2012, *Pomacentrus amboinensis*: Siebeck et al., 2009, *Carassius auratus*: Wyzisk and Neumeyer, 2007). In this way, simple to complex cognitive abilities, revealed by different tests, have already been demonstrated in some fish species such as



**Figure 1:** Number of publications on fish cognition per year. The graphic indicates the number of results obtained from a search for "fish cognition" on Google Scholar. The search was conducted on August 18, 2019 (© KLEIBER, 2019, INRA-LPGP).

goldfish and cichlids (Goldman and Shapiro, 1979; Zerbolio and Royalty, 1983; Gierszewski et al., 2013). Fish cognition has become one of the fastest growing areas in comparative psychology (Fig.1). Recent works have established that certain fish species can divide attention during foraging (Warburton and Thomson, 2006. In: Miller, 2017), have episodic-like memory (Hamilton et

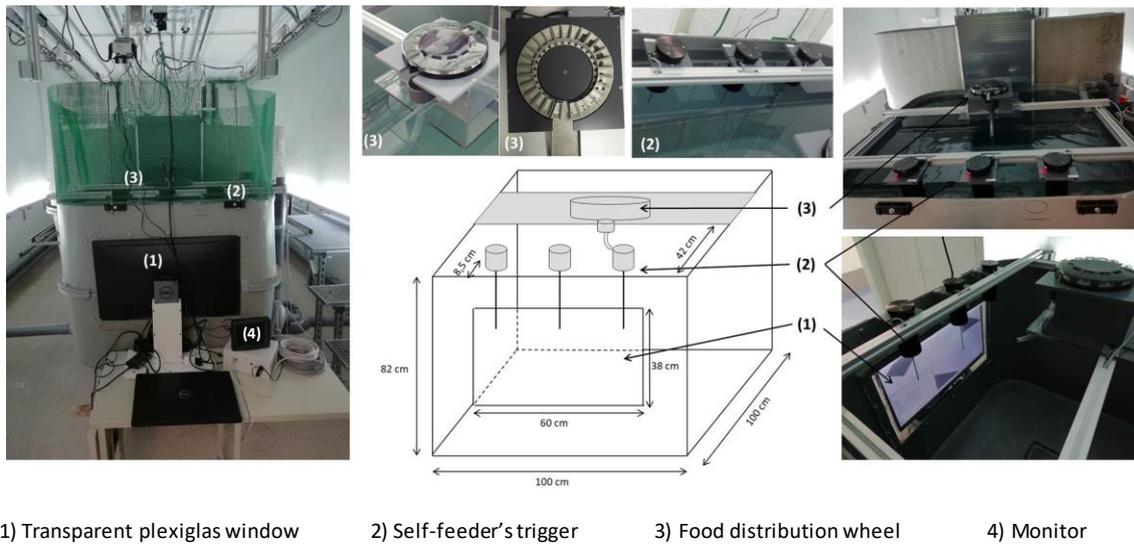
al., 2016, Gerlai, 2017. In: Miller, 2017), make transitive inferences about social relationships (Grosenick et al., 2007. In: Miller, 2017), attend to their own reputation (Bshary & Grutter 2006. In: Miller, 2017), operate (Bshary et al 2006, Vail et al 2013. In: Miller, 2017), use tools (Pasko 2010 Gerullis & Schuster, 2014. In: Miller, 2017), are able to solve numerical rules (Agrillo et al., 2014; Gomez-Laplaza and Gerlai, 2013; DeLong, 2017), to learn concept (Goldman & Shapiro, 1979; Zerbolio & Royalty, 1983; Gierszewski et al., 2013) and even to discriminate themselves from others (Thunken et al., 2009). However, none of these cognitive abilities have ever been demonstrated in rainbow trout.

Most of the tests evaluating fish cognitive abilities are discrimination tests where fish are previously conditioned to associate and recognize a particular stimulus with a positive (S+: food reward) or a negative (S-: electric shocks) reinforcement (Goldman & Shapiro, 1979; Zerbolio & Royalty, 1983). The most commonly used test is the Alternative Forced Choice test (AFC), which consists in presenting simultaneously different stimuli to the subject. Most often, fish are tested for the 2-AFC test, or two-choice discrimination test, where they must choose between two contrasted stimuli, one being rewarded (Schluessel et al., 2012; Sovrano & Bisazza, 2008). Other authors have extended their studies by performing a 4-AFC test where fish are required to choose a S+ stimulus from three other identical (S-) distracting stimuli (Newport and al., 2014). Other tests are based on fish's ability to learn concepts and generalize learned rules such as quantity (Miletto Petrazzini et al., 2015), shape (Gierszewski, 2013; Newport et al., 2014) or colour (Goldman & Shapiro, 1979; Zerbolio & Royalty, 1983) by using Matching-to-Sample (MTS) or Oddity-from-Sample (OFS) tasks. These tests do not require conditioning to particular stimuli since they are based on subject's capacity to learn associative rules in a general concept (Newport et al., 2014). For instance, in a MTS task, a sample stimulus is first presented followed by two stimuli, one being the rewarded comparison (S+) which matches the sample stimulus, and the other being a distractor (S-). The sample can either be presented together with the comparison and the distractor stimuli (simultaneous MTS), or the sample can only be shown prior to the stimuli and then disappears (delayed MTS). In the case of delayed MTS, both working memory and discrimination abilities of the subject are tested. It is also possible to test conceptual learning where the fish is asked to transfer the "same/different" relations between sample and stimuli to any other characteristics (shape, colour). The OFS task follows the same procedure as in the MTS, except that the rewarded stimulus is the one containing different characteristics from those of the sample previously presented (Newport et al., 2014). In most of fish studies, the conditioned response is validated considering the approach or the time spent in the S+ area (Manabe et al, 2012; Ingraham et al, 2016).

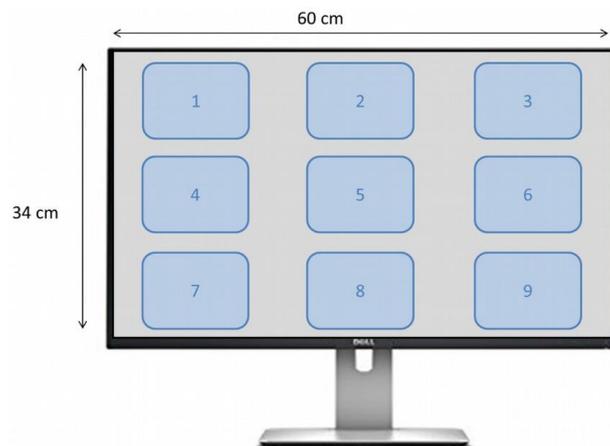
The different reinforcements used in conditioning tasks can also be obtained by the animal when manipulating a lever/rod or pushing a button (operant conditioning: Staddon and Cerutti, 2003).

Indeed, these reinforcements stimulate the fish to perform the requested task by manipulating the device to receive positive (or avoid negative) reinforcements. Rarely used in fish studies, operant (or instrumental) conditioning enable studying the learning and discrimination abilities of an animal ensuring that the answer chosen was an accurate and volunteer choice. Furthermore, adding an operant component to a learning task may improve performance (Heisenberg et al, 2001). Instrumental learning involves a complete panel of cognitive abilities such as spatial orientation, object recognition and temporal association of environmental cues (Jurado-Parras et al., 2013), and thus represents an optimal choice for studying high level cognitive abilities still unknown in the target fish species chosen for the present project.

We therefore designed my internship's subject to contribute to the knowledge on the cognition of a "non-higher-vertebrate" farmed animal species, the rainbow trout, using a 2-AFC task. For this purpose, we developed an innovative operant conditioning device which enables recording fish decisions with accuracy. The aim of this study was to find out if rainbow trout is able to solve a 2-AFC task using an operant conditioning process, the final goal being to highlight more complex cognitive abilities (working memory, categorization and conceptual abilities) by using MTS tasks.



**Figure 2:** Testing-device used for the 2-AFC task. The different units of the experimental device are described and identified by numbers on the diagram and the different photographs. © modified from PEYRAFORT, 2018, INRA-LPGP



**Figure 3:** Positions of the stimuli displayed on the screen. For the different stages, stimuli were displayed in positions 1, 2 or 3 for the 3-trigger training, and only in positions 1 and 3 for the 2-trigger training. © PEYRAFORT, 2018, INRA-LPGP

## **II. MATERIALS AND METHODS**

### **1. Ethics statement**

All experimental procedures used in this study were carried out in strict accordance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes. They have been approved by the Rennes Ethics Committee for Animal Experimentation and have received authorization from the French Ministry of Education, Research and Innovation under the agreement number: 201806050954214-V1-APAFIS#15361.

### **2. Experimental animals**

Females triploids rainbow trout (*Oncorhynchus mykiss*) were obtained from fertilizations performed at INRA-PEIMA facilities (Sizun, France). Eggs were transferred between 1- and 20-hours post-fertilization and the resulting fish were reared at the INRA-LPGP Experimental facilities (Rennes, France). Between 50 and 180 days post-fertilization, fish were reared in two tanks (100L: 55 x 45 x 59 cm) containing 40 fish each. Then 15 fish were kept in each tank in order to keep a density always above 20 kg/m<sup>3</sup>. Tanks were enriched with plants, rocks and PVC pipes since environmental enrichment is known to enhance cognitive capacities in fish (Salvanes et al., 2013; Kotrschal & Taborsky, 2010; Strand et al., 2010). At 5 months, fish (8 cm length) were all identified with a pitag (1,4 x 8 mm Biolog-Tiny) implanted above the dorsal fin under anaesthesia (50mg/L tricaine). Six months after fertilization, the 15 fish used for the learning experiment were housed together in a 336 L tank (102.5 x 102.5 x 32 cm) at 12 ± 0,2°C under an 12:12 h artificial photoperiod. Fish were fed daily at the end of the experiments with extruded and commercial flowing extruded pellets (Le Gouessant, 39% protein and 24% lipid, 0,5 cm) diameter pellets). Feeding ratio, composition and diameter pellets regularly evolved according to fish growth rate. Experiments started after a 2-week acclimation period to the new rearing tank.

### **3. Apparatus**

Subjects were tested individually in a 779 L test tank (104 x 100,5 x 74,5 cm) with dark grey painted walls (RAL code: 7005). A transparent Plexiglas window (63 x 34 cm) included into the tank wall allowed the fish to see the screen placed behind it (Fig.2). The screen used in this study was a LCD Dell REV A00 (17 inches: 60 x 34 cm) and displayed visual stimuli (Fig.3). To avoid any distractions, disruptions and reflections from the screen and to have smooth running tests, a dark sheet was laid behind the screen (Fig.2). From 8.5 cm in the upper part, three (then two) self-feeders (removable if needed) were placed

and the triggers were submerged of around 9 cm water. Fish could reach the pressure needed by the trigger by doing a 5-6° tilt angle with any part of their body (mainly the tail when they were juveniles and the head when they started growing). Once the angle reached, an electronic microchip located into the cylinder validated the answer. Self-feeders were connected to an automatic food distributor containing 28 buckets (IMetronic, France) (Fig.2). In each bucket, were placed two coated appetitive pellets (Diana Aqua). Their diameter evolved according to fish weight and to the pellets granulometric recommendations. When a correct answer was recorded, bucket's content was poured in a funnel connected to a Ø1,5 cm plastic tube and then felt into the water in the middle of the tank at about 42 cm from the screen (Fig.2). A bucket contained approximately 1/6<sup>th</sup> of the fish daily food intake. A camera placed above the experimental device (Samsung SCB-3000PH) and connected to a monitor (Ever-Focus Colour TFT LCD monitor) (Fig.2) permitted to control the entire real-time experiment at distance without disturbing trout behaviour.

Each experiment conducted was managed by the "POLY M2S" software (IMetronic). It allows users to choose the type of experiment but also the number of trials, the inter-trial interval, the different stimuli and their related position on the screen. The software also makes sure stimuli do not appear more than two consecutive times at the same position to prevent any side bias. All stimuli sequences are randomly presented (see General Procedure).

## **4. General Procedure**

The work performed in rainbow trout started in January 2018 in a room entirely reorganized in order to hold and to test individually the same fishes for 3 or 4 years. Fish were tested 5 days a week, with one session per day. Experiments were undertaken between 9:00 and 15:00, resulting in an overnight food deprivation period of 18h to 24h, depending on fish running order.

### **4.1. Trigger activation training**

After having been tested in a spatial learning test in a preliminary experiment (Peyrafort, 2018), 15 trout were selected on the basis of their speed learning performance. They were then trained to get used to the apparatus and to activate the self-feeder triggers. Three triggers were submerged for this habituation phase in order to increase their chance to activate one. During this habituation phase, the monitor displayed a grey screen (RGB code: #EEECEC) to reduce contrast with the tank colour and no matter which trigger was activated, the fish received a food reward. Furthermore, at a fish entrance in the test area, a food reward was given to make them associate netting as a positive event. The first session consisted in 5 trials of 10 minutes each. If a trigger was activated before the 10 min delay, the fish received a reward and the screen turned green (RGB code: #0F790F) during 60 seconds, which acts

as a secondary positive reinforcement (Zerbolio and Royalty, 1983), the green colour having been shown to be appreciated by rainbow trout (Luchiari and Pirhonen, 2008). After this time, a 10-second Inter-Trial-Interval (ITI) with a grey screen occurred and a new trial began. If the fish did not activate the trigger within the 10 minutes delay (Cut-Off), a food reward was still given but the trial stopped. Then a new trial started after the 10s ITI.

In the next sessions, the procedure was the same as in session 1 but they lasted after 10 trials and no more reward was given after a Cut-Off. After each session the trout was netted and joined its congeners. Fish were tested randomly. An individual was considered to have successfully learned to activate the trigger once an activation was significantly different from chance level (33%) in three consecutive sessions ( $\geq 70\%$ ; binomial:  $P\text{-value} = 4.347e-05$ ,  $N = 30$  trials), ensuring the correct acquisition of the operant conditioning device for all subsequent exercises. Nine individuals (out of 15) correctly learned trigger activation in around 2 months (150 trials).

#### 4.2. Training to trigger activation associated to a stimulus

After having correctly learned the operant conditioning device, the 9 remaining trout were trained for the activation of a trigger associated to the presence of a visual stimulus displayed on the screen (Fig.4). This exercise adds a difficulty because a fish is rewarded only if it chooses the trigger upper the stimulus image. One unique stimulus (S+) was displayed on the screen at each trial. Individuals were first given 3 triggers (one being rewarded, the screen behind the 2 others remaining blank). Then, only 2 triggers were still available, one being rewarded (right or left position), the screen behind the other remaining blank. If the incorrect trigger was activated, no food reward was given, and a red screen appeared (RGB code: #E90808) followed by an ITI before turning up to a new trial (Fig.4). Rainbow trout is known to avoid the red colour (Luchiari and Pirohnen, 2008) and so this colour acts as a secondary negative reinforcement. After a Cut-Off, no food reward was given. For this exercise, 10 trials were ran per session. For the 3-trigger training, different kinds of images were displayed as the positive stimulus (4 multi-coloured shapes, black shapes, blue shapes). An individual was considered to have successfully learned the task once a S+ selection was significantly different from chance level (33%) in two consecutive sessions ( $\geq 60\%$ ; binomial:  $P = 0.0119$ ,  $N = 20$  trials). For the 2-trigger training, the S+ represented a photograph of 4 different trout (Fig.4). Indeed, we expected better results with biologically relevant images. An individual was considered to have successfully learned the task once a S+ selection was significantly different from chance (50%) in two consecutive sessions ( $\geq 80\%$ ; binomial:  $P = 0.01182$ ,  $N = 20$  trials).

Training for the activation of a trigger associated to a stimulus lasted 4 months. Fish were trained until a total of 520 trials before starting with the 2-AFC paradigm.

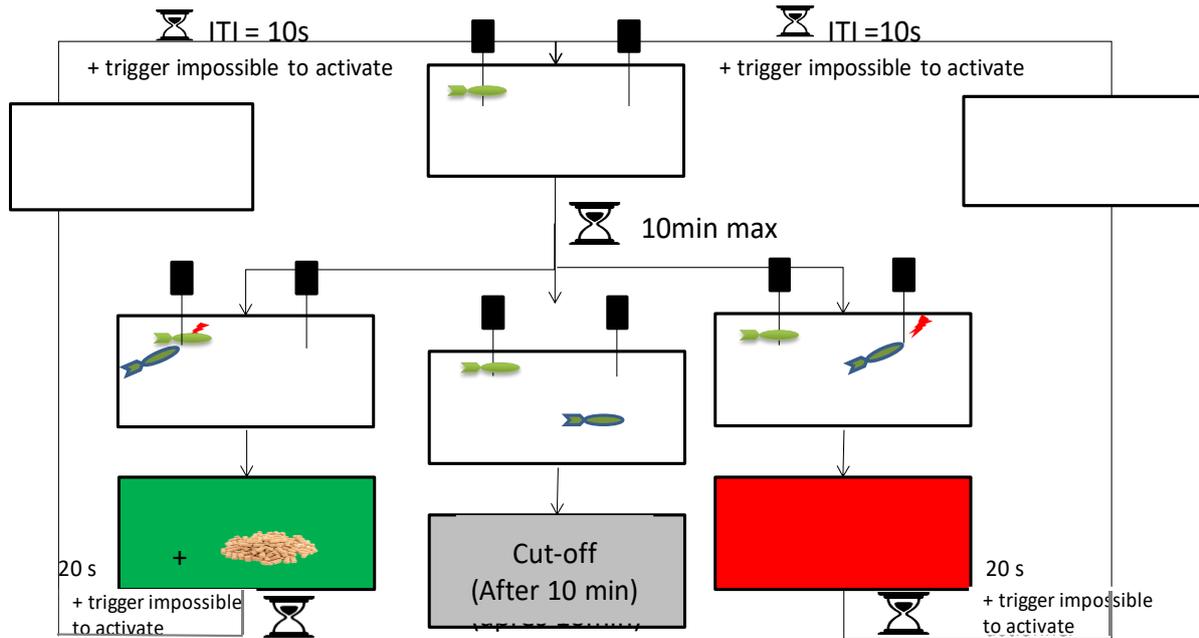


Figure 4: Protocol for the training to trigger activation associated to a photograph of a congener © modified from PEYRAFORT, 2018, INRA-LPGP

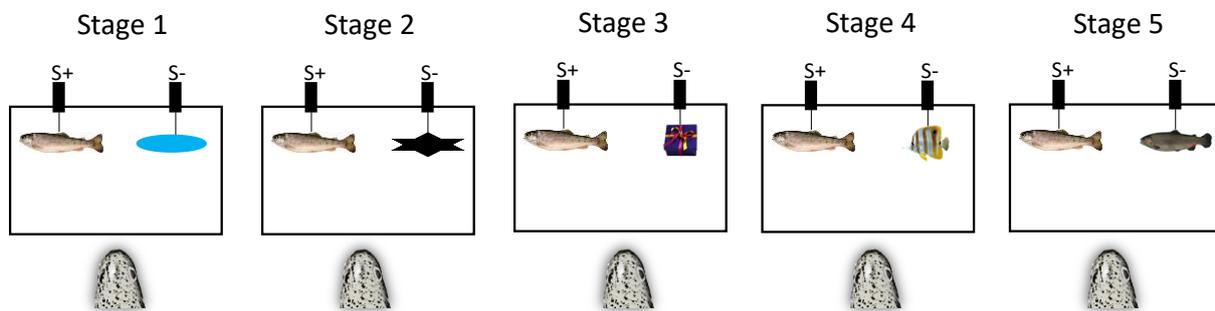


Figure 5: Stages 1 to 5 chosen for the 2-AFC task. Here, the rewarded stimulus (S+) is presented on the left side and different examples of unrewarded stimuli (S-) are presented on the right side for each stage. © KLEIBER, 2019, INRA-LPGP

### 4.3. Two-Alternative Forced Choice test (2-AFC)

Only 5 of the 9 remaining individuals were used for the 2-AFC exercise because two individuals died, and the other stopped being motivated, performing only cut-offs. As the previous exercises were not as successful as expected, a new exercise was proposed to the 5-remaining fish: the 2-AFC test. It consists in presenting two contrasted stimuli simultaneously. Fish must choose between the two stimuli, one being food-rewarded and secondary reinforced by a green screen (S+). For this exercise, S+ represented a trout congener from a pool of 4 recurrent photographs. A distracting stimulus (S-) was presented at 40 cm (horizontally) from the S+. Trout were not rewarded if they activated the

second trigger corresponding to the S- and a red screen appeared. The ITI was still 10 seconds. The distracting stimuli were: (stage 1) blue (RGB code: #1E90FF) abstract shapes (star, triangle, square or circle), (stage 2) a unique black abstract shape (star), (stage 3) the photograph of an object (among a pool of 60 objects), (stage 4) the photograph of another fish species (from a pool of 60 photographs), (stage 5) the photograph of unknown congeners (from a pool of 141 photographs) (Fig.5). Therefore, the discrimination difficulty between S- and S+ increased from stage 2 to stage 5. The first stage (stage 1) was proposed using a blue distracting stimulus since this colour is known to be appreciated by rainbow trout (Luchiari and Pirhonen, 2008). Due to poor results in stage 1, we chose to accentuate the contrast between S+ and S- by using a black coloured shape as S- (stage 2), the black colour being usually avoided by rainbow trout (Luchiari and Pirhonen, 2008). A total of 12 trials per session were run. Fish performed these stages in this order and moved on to the next stage as soon as they validated the learning criterion of 75% correct answers, during two consecutive sessions (different from 50%; binomial:  $P = 0.02266$ ,  $N = 24$  trials). A maximum number of 30 sessions was fixed for stages 1 to 3 (blue shape, black shape and objects as S-), a period where success is supposed to occur (Gierszewski et al., 2013). For stages 4 and 5 (other fish species and unknown trout as S-), the maximum number of sessions was fixed to 10 sessions, since we considered these last instructions too ambiguous and then impossible to learn if the two stimuli were not spontaneously discriminated. If the maximum number of sessions was reached by the subject without validating the learning criteria, the stage 2 (black shape as S-) was re-experimented to control for the initial performance of the trout. Therefore, we registered until 3 steps for stage 2. To prevent any side bias, the S+ and S- never appeared more than twice consecutively on the same side and in the case of the stage 2, a correction trial (CT) was given on the choice of the incorrect response (S-). It allowed re-experimenting the exact same set of stimuli until a correct response was made. However, in subsequent stages, no CT were given since these stages (objects, species and trout as S-) were not training stages (like stage 2) but testing stages with increasing difficulty. Thus, if a side bias was observed, only a forced test was ran to delete it. It consisted in a unique S+ presentation on the screen on the opposite side from the preferred one.

## **5. Stimuli**

All stimuli were created using Microsoft PowerPoint and ImageJ. Images used as negative stimuli were all taken from Google image platform for stages 3 and 4 and from photographs of rainbow trout reared in the PEIMA fish facilities for stage 5. The shapes used in stages 1 and 2 were created using Powerpoint. Stimuli were all stretched according to a PowerPoint slide size (25,4 x 19,05 cm) and were individually saved as png format. Then, a macro from ImageJ was ran to adjust their brightness. On the screen each stimulus appeared at a size of 14.5 x 11 cm.

## 6. Statistical analyses

All graphics and tests were performed using the 1.2.1335 version of RStudio and were plotted using the packages `ggplot2`, `ggthemes`, `ggsignif` and `effects`.

Daily discrimination rates reached by each subject are described graphically as the percent of correct responses per session. Learning criterion were considered as significantly different from chance by using binomial tests. For the 2-AFC paradigm, a probability of 18 correct responses out of 24 (in two consecutive sessions) was significant ( $p = 0.01133$ ), whereas the probability of selecting 9 correct responses out of 12 (in one session) was not significant ( $p = 0.073$ ).

To test a difference of percent of correct responses between two dependent groups (first vs. last session within each exercise), paired Wilcoxon non-parametric tests were used.

Binomial tests were used to compare the average percent of correct responses with chance level (50%) within each exercise.

The preferred side (right or left) chosen by the fish within an exercise were analysed by Wilcoxon and binomial tests. Paired Wilcoxon tests served to check whether there was a global lateralisation (percent of right vs. left activations) within each exercise conducted. Binomial tests were used in order to test for a side bias for each individual within each exercise, the expected selection frequency being 50%.

Preference/aversion for some of the stimuli presented, latency time and differences between trout performance were analysed by using generalized linear models (GLM) with the `MASS` package and linear model (LM) with the `car` package (in R 3.5.3), considering a Gaussian distribution for quantitative discrete variables (latency time to answer, after log-transformation). For proportion of correct responses, GLM were fitted considering a binomial distribution. The linear model used to analyse the variable latency time consisted in testing the effects of the trout, the image used as positive stimulus, trout's running order, the response given (correct or error), the effect of the step (exclusively in the case of the black shape stage) and their interaction. Concerning the binary variable "Response" (0 or 1), only the effect of the image used as positive stimulus was taken into account.

For instance, for latency time, data were analysed using the following complete linear model:

```
Model <- lm (log (Latency) ~ Trout * Response * Image.stimulus + Running.order, data=OBJ).
```

For response given by the fish, data were analysed using the following model:

```
Model3 <- glm (Response ~ Image.stimulus, family=binomial (link=logit), OBJ).
```

To test whether there was a preference/aversion for some of the stimuli presented for the objects and other species stages, the following complete general linear model was used:

```
Modeln <-glm (cbind(Nb.successes,Nb.failures) ~ Image, family=binomial (link=logit), OBJn).
```

An additional model was also used to test whether there were any differences in performance between the trout tested. The following complete linear model was used considering the proportion of correct responses per session and per trout as the dependent variable:

```
Modelperf <-lm (Score.RC ~ Trout * step * Running.order, data=FNtRC).
```

The results of the different models were then obtained thanks to the analysis of deviance table with anova (Chi test for binomial family) in case of GLM, and Anova (sum of squares of deviation: type III) or anova in case of LM (Gaussian family). For all models, if there were non-significant effects on factors or interactions, stepwise backward eliminations were performed to sequentially simplify and adjust the full model, with reference to Akaike's information criteria (AIC) (Appendix A) and thanks to a graphical approach evaluating residues' distribution and homogeneity (Appendix B). The final models selected are visible in Appendix A. When significant effects were found, the model was considered for pairwise comparisons between fixed variables to compare them for each condition, using post-hoc HSD-Tukey tests with the R package emmeans.

P-values < 0.05 were considered as statistically significant for all statistical analyses.

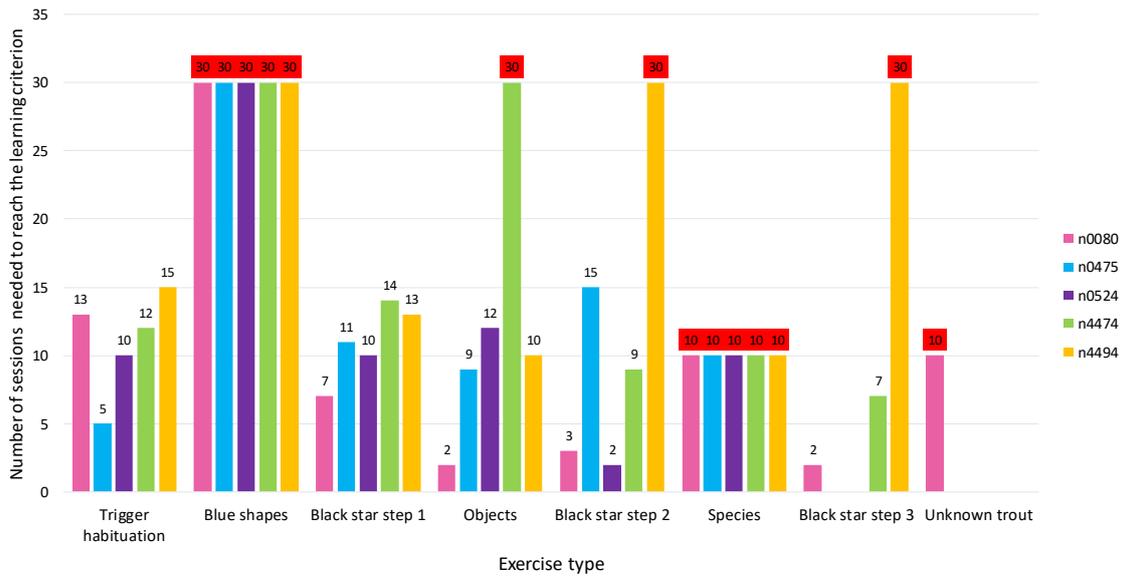
### **III. RESULTS**

#### **1. Trigger activation training**

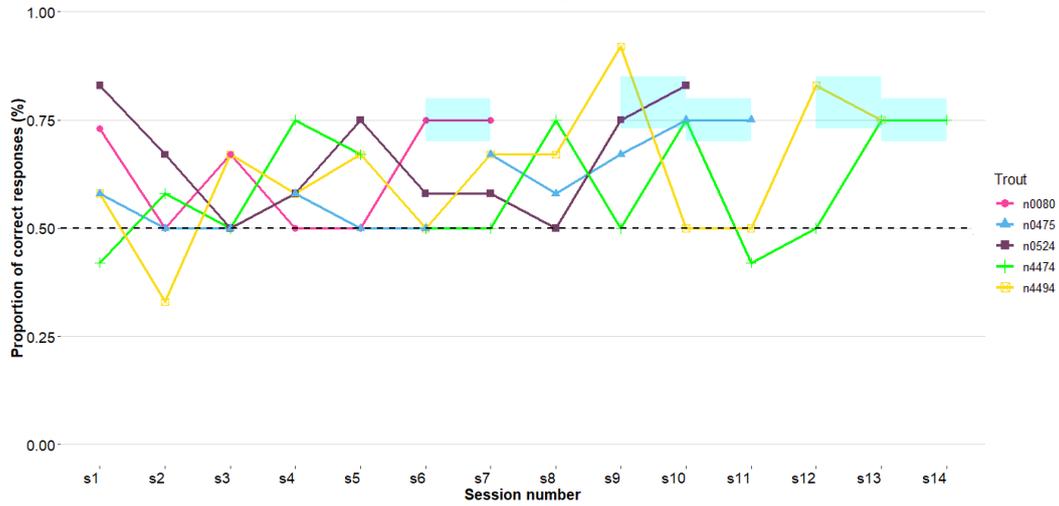
Nine of the fifteen trout initially tested reached the learning criterion of 70 % of correct responses in three consecutive sessions (binomial: P-value = 4.347e-05, N = 30 trials), after 3 (trout N°0331) to 15 sessions (N°4494). One trout (N°0162) has never activated a trigger. The others sometimes activated a trigger, but none were able to reach an accuracy above chance level (33 %). One trout (N°0379) has, however, twice succeeded at 70 % level but in two non-consecutive sessions.

#### **2. Training to trigger activation associated to a stimulus**

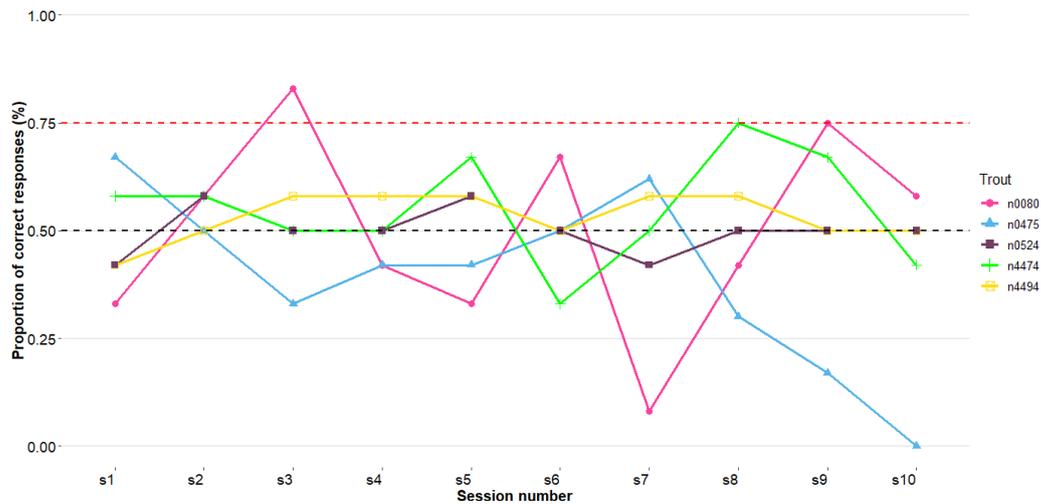
After a total of 520 trials, none of the nine fish reached the learning criterion when using multi-coloured, black or blues shapes as S+ neither in the 3-trigger nor in the 2-trigger training, and even when using pictures of a trout as S+ when no distracting stimulus (S-) was present. As soon as fish were tested with a fish image instead of a shape, scores reached values above chance level but without reaching the learning criterion. A distracting stimulus S- was therefore added in order to improve the results.



**Figure 6:** Number of sessions needed by each of the five trout to complete each exercise. The red labels indicate that the exercise was not completed successfully. © KLEIBER, 2019, INRA-LPGP



**Figure 7:** Proportions of correct responses performed by the five trout during stage 2 of the 2-AFC task. The black dotted line represents chance level. The transparent blue rectangles indicate the two consecutive sessions where an individual reached 75% (or more) of correct responses. © KLEIBER, 2019, INRA-LPGP



**Figure 8:** Proportions of correct responses performed by the five trout during stage 4 (photographs of another fish species as S-) of the 2-AFC task. The black dotted line represents chance level. No individual reached 75% or more (the red dotted line) of correct responses during two consecutive sessions. © KLEIBER, 2019, INRA-LPGP

### 3. Two-Alternative Forced Choice test (2-AFC) with photographs of congeners as S+

#### 3.1. Fish performances within stages

##### 3.1.1 *Stage 1: Blue shapes as S-*

The first stage of the 2-AFC test consisted in displaying 4 blue shapes as S- and 4 photographs of congeners as S+. Even if the scores recorded were mainly at or above chance-level and reached for a few sessions more than 75% of correct responses, none of the five individuals were able to meet the learning criterion (2 consecutive sessions) in 30 sessions completed (Fig.6). A unique black shape (star) instead of blue ones was then proposed as S- (stage 2).

##### 3.1.2 *Stage 2: Black shape as S-*

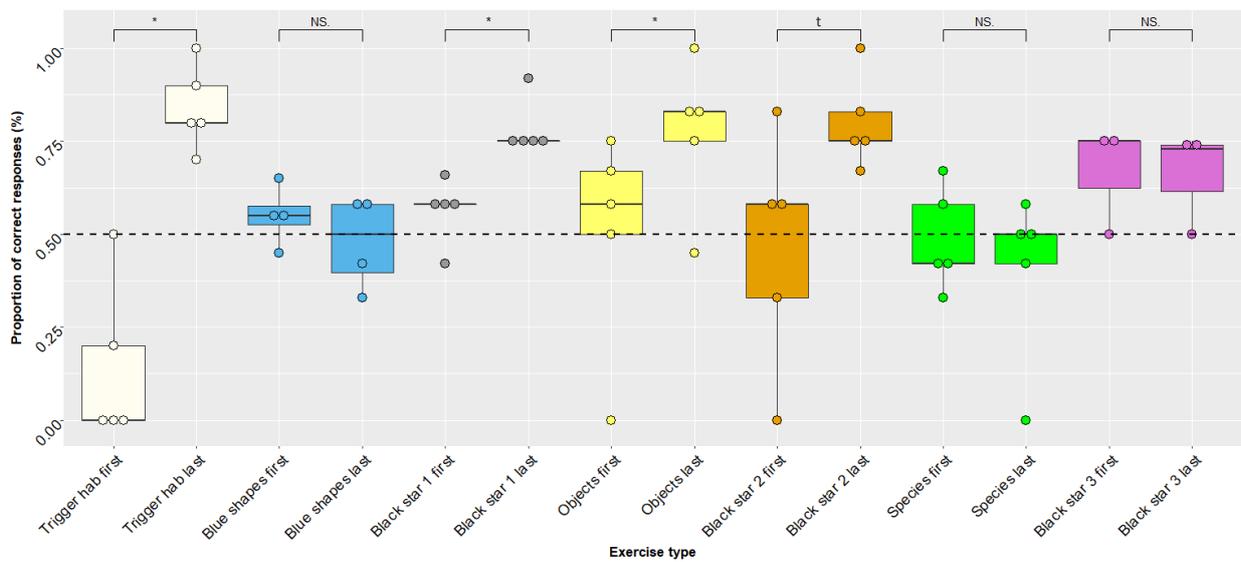
The learning criterion was reached by the five trout when displaying a black shape as S- and the 4 recurrent photographs of congeners as S+ (stage 2-step 1). Trout N°0080 reached the criterion in 7 sessions (binomial:  $P=0,011$   $N=24$  trials), N°0524 in 10 sessions ( $P<0.01$ ,  $N=24$  trials), N°0475 in 11 sessions ( $P=0,011$ ,  $N=24$  trials), N°4494 needed 13 sessions ( $P=0,003$ ,  $N=24$  trials) and N°4474 learned the task in 14 sessions ( $P=0,011$ ,  $N=24$  trials) (Fig.6 and Fig.7).

For step 2, four individuals succeeded in reaching the learning criterion, after only 2 sessions for N°0524 (binomial:  $P=0,003$ ,  $N=24$  trials), 3 sessions for N°0080 ( $P=0,003$ ,  $N=24$  trials), 9 sessions for N°4474 ( $P=0,006$ ,  $N=17$  trials) and 15 sessions for N°0475 ( $P=0,011$ ,  $N=24$  trials) (Fig.6). Trout N°4494 completed 30 sessions without meeting the learning criterion. However, it succeeded 4 times at 75% level, but in non-consecutive sessions.

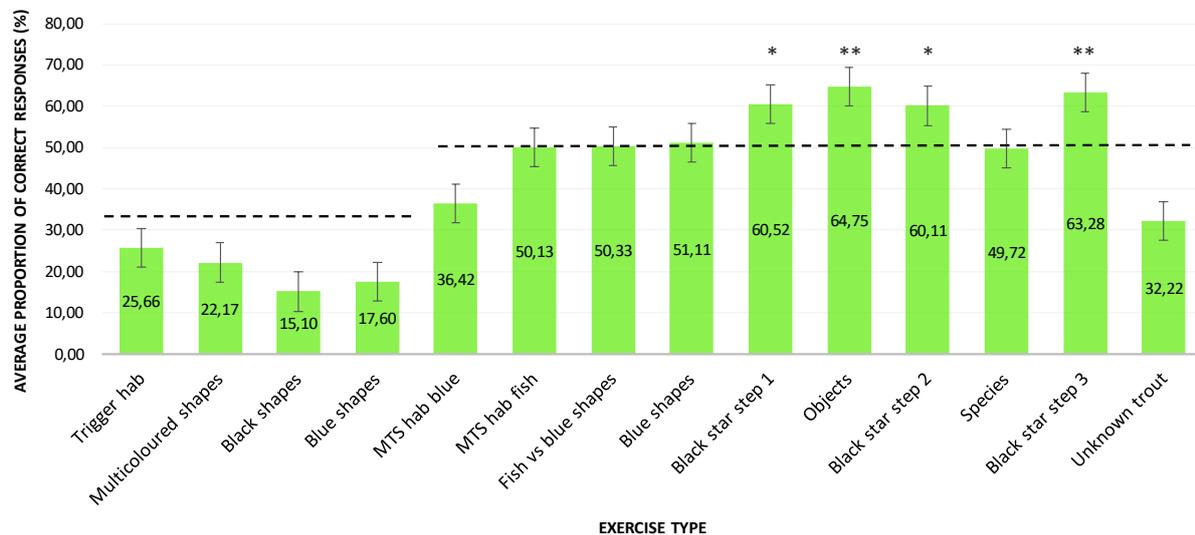
Three trout passed step 3, and two of them met the learning criterion. Trout N°0080 succeeded in 2 sessions (binomial:  $P=0,011$ ,  $N=24$  trials) and N°4474 in 7 sessions ( $P=0,005$ ,  $N=23$  trials) (Fig.6). Trout N°4494 failed a second time to reach the learning criterion in 30 sessions done. This trout reached the 75% correct responses level only once in this step.

##### 3.1.3 *Stage 3: Objects as S-*

When displaying objects as distracting stimuli, four individuals reached a statistically significant S+ selection frequency in 2 sessions for trout N°0080 (binomial:  $P=0,003$ ,  $N=24$  trials), 9 sessions for N°0475 ( $P=0,011$ ,  $N=24$  trials), 10 sessions for N°4494 ( $P=0,003$ ,  $N=24$  trials) and 12 sessions for N°0524 ( $P=0,001$ ,  $N=36$  trials) (Fig.6). Trout N°4474 reached the maximum number of 30 sessions without meeting the learning criterion. This trout reached the 75% correct responses level only once.



**Figure 9:** Median (quartiles: 25, 75%) proportions of correct responses during the first and the last session for each type of exercise. A colour is dedicated to a type of exercise and dots represents the proportion of correct responses performed by the five trout. The black dotted line represents chance level. Medians were compared between first and last session using the Wilcoxon test (t:  $0.05 < p < 0.1$ ; \*:  $p < 0.05$ ; NS: Non-significant). © KLEIBER, 2019, INRA-LPGP



**Figure 10:** Mean ( $\pm$  SEM) proportions of correct responses including all sessions by exercise. The two black dotted lines represent chance levels (33%: 3-trigger training and 50%: 2-trigger training). Proportions were compared to chance levels using a binomial test (\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ) © KLEIBER, 2019, INRA-LPGP

### 3.1.4 Stage 4: Other fish species as S-

In 10 sessions conducted for this stage, none of the five individuals were able to reach the learning criterion (Fig.6 and 8). Most of the scores were at or below chance level. Trout N°0080 reached twice 75 % of correct responses but in non-consecutive sessions and trout N°4474 only once.

### 3.1.5 Stage 5: Unknown congeners as S-

Only trout N°0080 performed stage 5, since it was the only one which succeeded in all previous stages, except in stage 4. At three sessions, it reached a level at or above chance, but remaining scores were below it. From sessions 6 to 10, the trout stopped responding, and performed essentially CO.

## 3.2. Global fish performances: stages comparisons

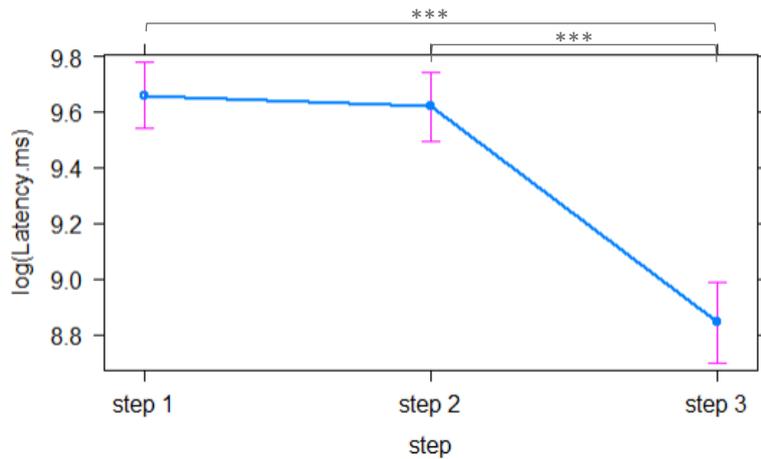
We compared the percent of correct responses between the first session and the last session (when learning criterion was met) for each exercise (Fig.9). The results indicate a significant increase between first and last sessions for the trigger habituation stage (Wilcoxon:  $V=0$ ,  $P=0.031$ ,  $N=5$  individuals), for stage 2 (black shape as S-), step 1 ( $V=0$ ,  $P=0.029$ ,  $N=5$ ) and step 2 (trend:  $V=1$ ,  $P=0.063$ ,  $N=5$ ) and for stage 3 (objects as S-) ( $V=0$ ,  $P=0.029$ ,  $N=5$ ). However, no differences were found for stage 1 (blue shapes as S-) ( $V=9$ ,  $P=0.938$ ,  $N=4$ ), stage 4 (other fish species as S-) ( $V=8$ ,  $P=0.607$ ,  $N=5$ ) and stage 2-step 3 ( $V=1$ ,  $P=0.977$ ,  $N=3$ ). Such Wilcoxon analyses could not be performed within stage 5 since it concerned only one individual (N°0080).

The figure 10 shows that once a black shape was displayed as S- (stage 2-step 1) in the two-trigger exercises, the average percentage of correct responses (60.52%) started to exceed the chance level (binomial:  $P<0.05$ , 50%).

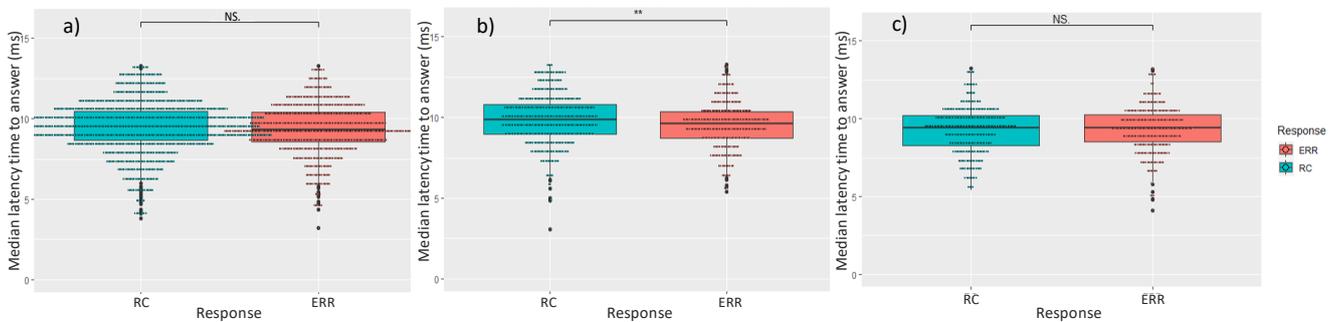
## 3.3. Fish responses analyses

Linear models were used in order to analyse in-depth fish responses.

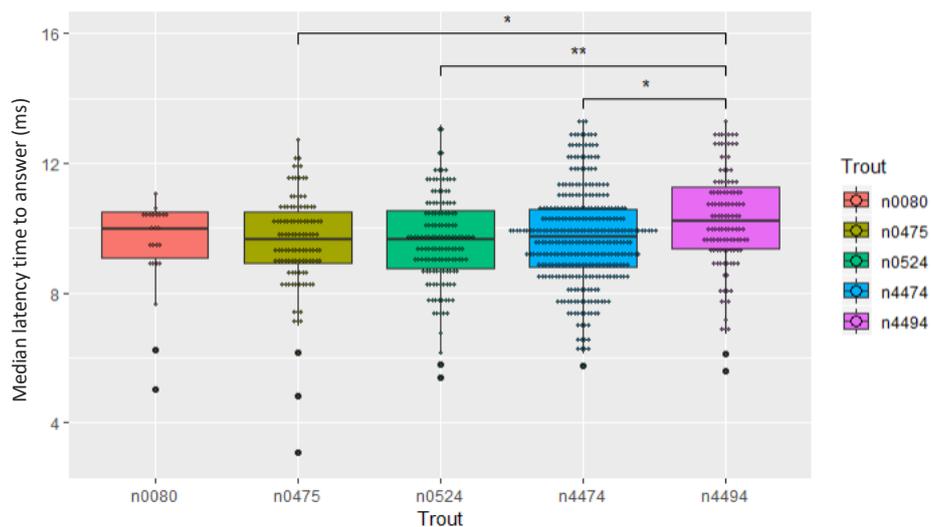
For all stages, no differences in the selection frequencies were found between the four different congener's photographs presented as S+. Likewise, no preference or aversion were found for any objects (stage 3) or any other fish species (stage 4) presented as S- ( $P>0.05$  in both cases). In stage 2 (unique black shape as S-), a significant effect of the fixed factor "step" was found on the latency time to answer ( $F_2 = 41.94$ ,  $P < 0,001$ , Fig.11). Two-by-two comparisons using HSD Tukey's tests showed that fish took significantly less time to answer in step 3 than in steps 1 and 2 ( $t_{1720} = 8.39$ ,  $P < 0.0001$  and  $t_{1720} = 7.86$ ,  $P < 0.0001$ , respectively). There was a significant interaction between the factors step and trout ( $F_6 = 4.80$ ,  $P < 0.001$ ), indicating that N°4494 was the one that contributed the most to this



**Figure 11:** Mean ( $\pm$  SEM) latency time to answer (log-transformed values in milliseconds) during stage 2 (black star as S-) of the 2-AFC task. Steps were two-by-two compared using HSD Tukey's tests (\*\*\*:  $p < 0.001$ ). © KLEIBER, 2019, INRA-LPGP



**Figure 12:** Median (quartiles: 25, 75%) latency time to answer (log-transformed values in milliseconds) as a function of the response given (RC: correct response or ERR: error) during a) stage 2 (black star as S-), b) stage 3 (objects as S-), and c) stage 4 (other fish species as S-). Responses were compared using HSD Tukey's tests (\*\*:  $p < 0.01$ ; NS: Non-significant). © KLEIBER, 2019, INRA-LPGP



**Figure 13:** Median (quartiles: 25, 75%) latency time to answer (log-transformed values in milliseconds) as a function of the trout during stage 3 (objects as S-). Trout were two-by-two compared using HSD Tukey's tests (\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ). © KLEIBER, 2019, INRA-LPGP

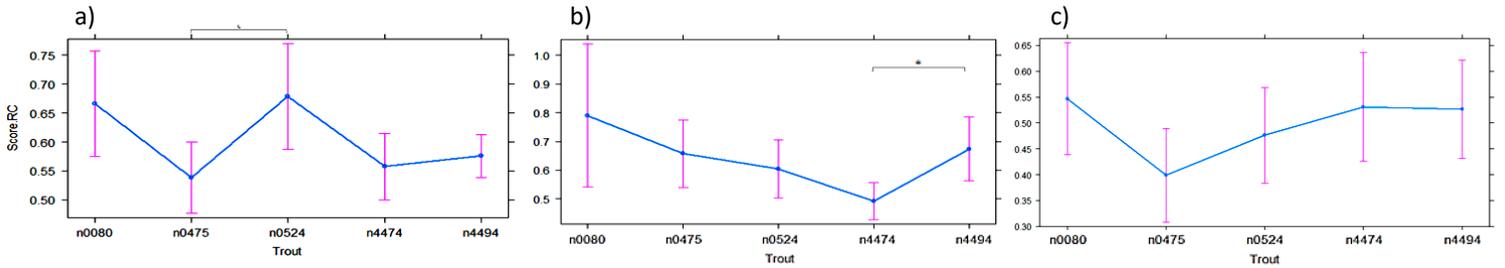
decreased latency time (Tukey: step 1/step 3:  $t_{1710}=8.14$  ,  $P<0.0001$ ; step 2/step 3:  $t_{1710}=7.51$  ,  $P<0.0001$ ). Just a trend was found between steps 1 and 2 for trout N°4494 ( $t_{1710}= 2.29$ ,  $P=0.06$ ). Concerning the remaining trout, N°0475 took longer time to answer in step 2 than in step 1 ( $t_{1710}= -3.10$ ,  $P<0.01$ ) and N°4474 was quicker to answer in step 3 compared to step 1 ( $t_{1710}= 2.47$ ,  $P<0.05$ ).

In stage 3, the linear model showed significant effects of the factors “trout” and “response given” (correct answer (RC) or error (ERR)) on the latency time to answer ( $F_4=3.90$ ,  $P<0.01$  and  $F_1=9.76$ ,  $P<0.01$ , respectively). Indeed, the Tukey’s tests showed that fish took more time to make a correct answer rather than an error (ERR/RC:  $t_{711}=-3.12$ ,  $P<0.01$ ) (Fig.12b). Significant differences were found between 0475/4494, 0524/4494 and 4474/4494 ( $t_{711}=-3.23$ ,  $P<0.05$ ;  $t_{711}=-3.32$ ,  $P<0.01$  and  $t_{711}=-2.95$ ,  $P<0.05$ , respectively) meaning that trout N°4494 took more time to answer than 0475, 0524 and 4474 (Fig.13). We found no effects of the factors trout and response given on latency time in stages 2 and 4 ( $F_1=1.97$ ,  $P>0.1$  and  $F_1=0.06$ ,  $P>0.5$ , respectively) (Fig.12a and 12b).

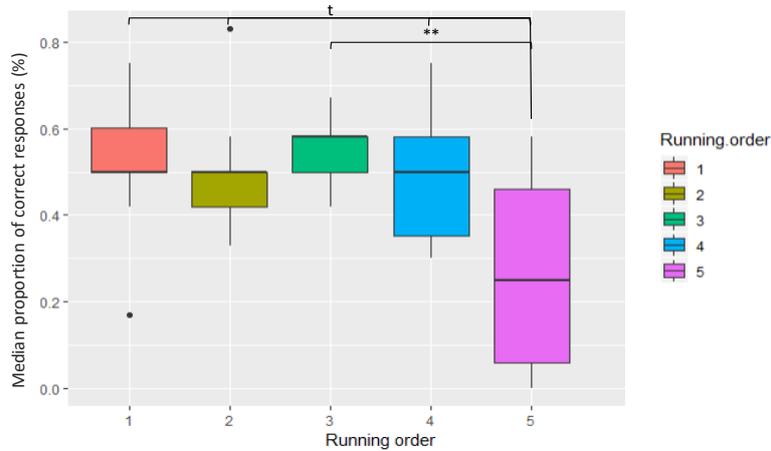
Concerning the percentage of correct responses given by the five fish tested, a global effect of the factor trout was found for stage 2 ( $F_4=2.64$ ,  $P<0.05$ ; Fig.14a) and stage 3 ( $F_4=3.78$ ,  $P<0.01$ ; Fig.14b). However, no differences were found between them for stage 4 ( $F_4= 1.80$ ,  $P = 0.15$ ; Fig.14c). Two-by-two comparisons showed that the trout N°4494 gave more correct responses than trout N°4474 in stage 3 (Tukey:  $t_{58}= -2.83$ ,  $P<0.05$ ; Fig.14b).

Considering the running order at which each fish was netted to perform a session, a significant effect was found on the proportion of correct responses, only for stage 4 ( $F_4=3.15$ ,  $P<0.05$ ). Fish netted in the 5th position had poorer results than those netted in the 3rd position (Tukey:  $t_{41}= 349$ ,  $P<0.01$ ; Fig.15).

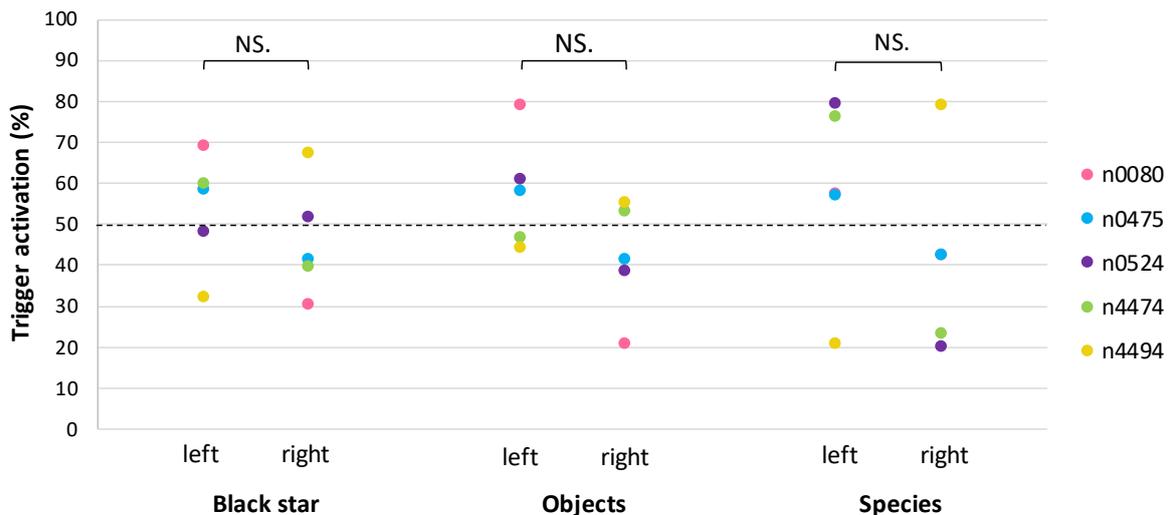
We did not find any global side bias in stage 2 (Wilcoxon:  $V=9$ ,  $P=0.810$ ), stage 3 ( $V=10$ ,  $P=0.630$ ) and stage 4 ( $V=10$ ,  $P=0.630$ ). However, by checking lateralisation on a case-by-case basis, we noticed a left lateralisation for individuals N°0080 (binomial:  $P<0.001$ ,  $N=144$  trials), N°0475 ( $P<0.01$ ,  $N=270$  trials), and N°4474 ( $P<0.001$ ,  $N=302$  trials) and a significant right lateralisation for N°4494 ( $P<0.001$ ,  $N=864$  trials) in the case of stage 2 (Fig.16). Concerning stage 3, N°0080 still preferred the left side ( $P<0.01$ ,  $N=24$  trials), as well as N°0524 ( $P<0.01$ ,  $N=139$  trials) (Fig.16). During stage 4, N°0524 ( $P<0.001$ ,  $N=114$  trials) and N°4474 ( $P<0.001$ ,  $N=119$  trials) were lateralised on the left side, while N°4494 preferred the right side ( $P<0.001$ ,  $N=120$  trials) (Fig.16).



**Figure 14:** Mean ( $\pm$  SEM) proportions of correct responses performed by each trout during a) stage 2 (black star as S-), b) stage 3 (objects as S-), and c) stage 4 (other fish species as S-). Trout were two-by-two compared using HSD Tukey's tests (t:  $0.05 < p < 0.1$ ; \*:  $p < 0.05$ ). © KLEIBER, 2019, INRA-LPGP



**Figure 15:** Median (quartiles: 25, 75%) proportions of correct responses as a function of trout's running order during stage 4 (other fish species as S-) of the 2-AFC task. Running orders were two-by-two compared using HSD Tukey's tests (t:  $0.05 < p < 0.1$ ; \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ). © KLEIBER, 2019, INRA-LPGP



**Figure 16:** Proportions of trigger activation on the left or right side of the screen during stage 2 (black star as S-), stage 3 (objects as S-), and stage 4 (other fish species as S-) of the 2-AFC task, including all sessions. Each coloured dot represents a trout. Left and right sides were compared within each stage using Wilcoxon tests (NS: Non-significant). © KLEIBER, 2019, INRA-LPGP

## **IV. DISCUSSION**

Here, we investigated the capacity of rainbow trout to solve a two-choice discrimination (2-AFC) task, by using an innovative operant conditioning device. Fish used the operant conditioning apparatus successfully and we showed that they were able to discriminate between recurrent 2D-congener photographs displayed on a screen and other distracting visual stimuli on either side, with increasing discrimination difficulty.

### **1. Rainbow trout's cognitive abilities**

Being isolated in a novel tank and manipulating a trigger to obtain a food-reward in the habituation phase was correctly learned by 9 individuals out of 15, and required between 3 and 15 sessions (10 trials each). This performance is impressive considering that with this testing-apparatus, trout faced quite difficult situations regarding its ecology. Indeed, the first challenge might have been to get acclimatized alone in an unknown area, which represents a considerable source of stress. Indeed, trout is a gregarious species known to be neophobic (Sneddon et al., 2003). Time was therefore required for them to associate netting and the testing area with positive events through which they can obtain highly appetitive food items. Moreover, they had to learn how to activate a self-feeder individually, out of its initial context of group use. Trout reared in groups of 100 to 300 individuals required about 25 days to reach a stable level of self-feeding (Anders, 1996). Their successful completion in manipulating a self-feeder's trigger while isolated in the test-tank, therefore already consists in a great achievement for this fish species. Moreover, operate a trigger represents something unnatural towards their ecology. When the trout were juvenile, in the trigger activation training, they first started to operate the trigger by giving it a tail punch to now activate it thanks to their mouth. This change in practice therefore consists in a good indicator of their degree of motivation to receive rewards during the experiments and clearly shows they have appropriated the operant conditioning device. What could have contributed to their fast learning is that experiments began when fish were juveniles, a life stage known to have the most important behavioural plasticity (Kotrschal and Taborsky, 2010). Furthermore, they were reared in enriched tanks containing artificial plants, rocks and pipes, which is known to enhance fish cognitive capacity allowing better responses to environmental challenges and a decrease of stress (Salvanes et al, 2013; Kotrschal and Taborsky, 2010; Strand et al, 2010). Environmental enrichment could have therefore helped them to succeed rapidly in the utilisation of the operant conditioning device.

The major challenge was then to create an association between a visual stimulus positioned behind one trigger (out of two or three), and the behavioural response of activating the respective

trigger, which only then, delivered food. First, the rewarded stimulus represented different shapes of different colours changing between trials. In several consecutive trials the colour remained unchanged either blue or black. After a total of 520 trials, no fish reached the learning criterion in this training phase. Previous studies report that horses required between 840 to 1100 trials (Gabor et al 2012) and goats needed about 1000 trials (Langbein et al., 2004) to learn a similar task, but using only two different black symbols. Whether we should have persisted in this task until at least 1000 trials or we should have presented only the same stimuli (or both options) will remain unknown. As soon as the rewarded stimulus (S+) represented a photograph of a congener, fish started to respond above chance level, but still without reaching the learning criterion. As predicted, they seemed more attracted by a biologically relevant stimulus rather than any kind of abstract shapes.

Fish were therefore already trained with 520 different trials when the 2-AFC test started by adding a distracting stimulus (S-) on either side, keeping four recurrent photographs of congeners as S+. When S- represented four different blue shapes, no fish performed successfully within 30 sessions (12 trials each), but when S- represented a black star (stage 2-step1), the five fish remaining at this stage reached the learning criterion in 7, 10, 11, 13 and 14 sessions respectively. Therefore, rainbow trout required a total of 880 trials using different kind of visual stimuli before starting to respond correctly in a two-choice discrimination task. Then they needed between 84 and 168 more trials to meet the learning criterion. The fact that rainbow trout is able to learn a 2-AFC task as fast as higher order vertebrates tested in a simpler task (Gabor et al, 2012; Langbein et al, 2004) is particularly remarkable. Even in the absence of a neocortex, the cerebral region involved in learning processes in mammals (Seger & Miller, 2010), this finding proves that fish have already good learning skills. Blue has been shown to be one of the preferred colours (with green) when given the choice to rainbow trout (Luchiari and Pirhonen, 2008). This could have caused a conflict of interest between the two stimuli presented on the screen, when S- represented blue shapes. Conversely, as a black shape may be avoided by the fish (Luchiari and Pirhonen, 2008), it was finally chosen as S- (versus photographs of congeners as S+), which constituted a success since all the fish tested with this last combination met the learning criterion in a maximum of 168 trials.

As in other studies, fish decision strategy could have been to avoid S- regarding its characteristics (Peters' elephant-nose fish: Davis and von der Emd, 2003 and 2007). Therefore, discrimination difficulty increased by displaying different objects as S- (stage 3) to test whether trout learned to avoid the black star or conversely to associate the photograph of a congener with a reward. Four individuals performed successfully in this discrimination in 2, 9, 10 and 12 sessions respectively. This demonstrates that fish did not learn to avoid the black star in the previous stage. As the experiment evolved, the four recurrent photographs of congeners (S+) became familiar whereas

objects proposed as S- from a pool of numerous objects were, for most of them, novel at each trial. Trout are known to have a neophobic nature (Sneddon et al., 2003), which would reinforce the hypothesis of avoiding the S- rather than choosing the S+. However, when different photographs of other fish species were displayed as S- (stage 4), performance dropped at the chance threshold, despite the novelty of these new S-. Again, this finding shows that fish were able to choose the S+ because of its positive association with a reward and did not avoid S- because of its novelty.

This is a crucial point for this species since knowing how to discriminate their conspecifics from objects or abstract shapes are essential regarding rainbow trout's ecology. When foraging in their natural habitat, juvenile's trout are usually swimming in shoals of more than 30 fish and appear to be strongly oriented to one another (Tabor and Wurtsbaugh, 1991). When juvenile trout need to forage, they move from complex protected habitats (their nursery where predators cannot forage with efficiency) to exposed area, principally composed of sand and gravel, which has greater prey resources available. Differentiating their congeners from their environment is therefore important to ensure their survival and protection while they are foraging. Moreover, trout were housed together since they were juveniles and environmental enrichment was present but not in important amount. Those two elements combined could also explain why the ability to recognize congeners from objects developed. To support this hypothesis, a study demonstrated that differences between perch's living environment can affect the use of visual stimuli (Balcombe, 2016). Indeed, in strong stream conditions, using visual cues is not necessary for the perch as it is quite possible that cues have moved the next time it came through. On the other hand, perch living in more stable habitat had better results in recognizing its way in a maze task. The perch living under stream conditions used therefore more "egocentric" cues than visual ones. Concerning our experimental subjects, referring to visual cues seems to be the most plausible hypothesis regarding the stability of their artificial habitat. Furthermore, vision is vital for feeding in many near-surface teleosts (Balcombe, 2016). Fish's answers to a behavioural test is thus, certainly dependent on the species and possibly on their particular ecology, as already mentioned in Newport et al. (2014).

In stage 3, S- represented objects, visually very different from a photograph of a congener. For stage 4, when S- represented other fish species, the difference between S- and S+ was not so obvious and all fish failed in the two-discrimination task. However, it was shown that teleost fish are able to see complex and contrasted patterns (Champ et al., 2014; Hester, 1968; Neumeyer, 2003; Ben-Simon et al., 2012). Here, we consider that fish were able to recognize the outline of a conspecific as a fish, but similarly recognized the shape of any other fish species. However, 3 scores reached the 75 % level of correct responses but in non-consecutive sessions. Perhaps few more sessions could have been necessary to know with accuracy if rainbow trout can distinguish between its congeners and

other fish species in this experimental set up. A lack of motivation to complete the task and a side bias strategy could also explain this result. Indeed, fish may have chosen a simpler decision strategy consisting in always going to the same side as further discussed, which was the case for three out of the five tested trout. Moreover, an effect of trout's running order was noticed in this stage. This can occur when there is over-training or if testing-schedules from one day to another drastically change, which induce a plausible change in discrimination strategy (Mandler and Hooper 1967).

To control for the fish initial performance between stages, the black star was again presented as S- and four individuals (out of five) regained criterion level in stage 2-step 2 and two of the three tested individuals succeeded again in stage 2-step 3. Most of them were therefore still able to perform correctly even after a failure in the preceding stage.

Only one trout was subjected to the most difficult stage, namely different photographs of unknown rainbow trout as S- (stage 5), since it was the only one which succeeded in all previous stages, except in stage 4. As it failed to discriminate between recurrent learned photographs of congeners and photographs of other fish species, this individual also failed to discriminate between photographs of learned congeners and unknown congeners. After successive errors, this fish finally stopped being motivated and stopped responding. The task was certainly too complex to understand, the two stimuli being visually very similar. A misunderstanding of the initial directive could have induced a decrease in its motivation to participate. Indeed, complex directions inducing a lack of motivation has been shown in humans (Simons et al., 2004) and fish (Newport et al., 2014). Further experiments need to be conducted using more individuals to elucidate whether rainbow trout can discriminate between different conspecifics in this experimental set up. It was already demonstrated that other fish species are able to recognize conspecifics individually using visual cues (Clark and Stephenson 1999; Culumber 2015; Goncalves et al. 2000; Johnston and Dixson 2017; Karenina et al. 2013; Knight and Turner 1999; Roux et al. 2016; Satoh et al. 2016) and that guppies prefer to school with familiar individuals (Griffiths and Magurran, 1997). Moreover, recognizing individually their congeners may help to avoid costly physical fighting associated with social conflicts frequently observed in rainbow trout under farming conditions (for a review: Martins et al. 2012), and may then represent an advantageous ability.

In training procedures, a few individuals succeeding is enough to generalise proven cognitive abilities to the whole species. In some cases, even one individual completing a task is enough, meaning that its species' brain is endowed with neuro-cognitive systems that probably support the resolution of the task (Agrillo and Bisazza, 2014). As in the present experiment, animal cognitive studies generally include few experimental subjects (archerfish: 6 individuals tested: Newport et al., 2015; sheep: 8

individuals tested: Knolle et al., 2018; horses: 7 individuals tested: Gabor and Gerken, 2012). We can therefore consider that with four individuals succeeding in the 2-AFC task, rainbow trout is capable to discriminate between a 2-D photograph of congeners and a black shape and also between congeners and any other object, but not between congeners and other fish species. This performance is highly remarkable for this species since it has been demonstrated using an operant conditioning device which requires a complete panel of complex cognitive functions such as spatial orientation, visual stimuli recognition and temporal association of environmental cues (Jurado-Parras et al., 2013).

## **2. Individuals' learning strategies**

We observed an important intra-species variability in performances. Trout N°4474 required more sessions to learn the task proposed in stage 2 than the other fish. It is possible that this individual did not understand the task as well as the others or adopted a different decision strategy than the others. Different learning strategies can be adopted by the fish when responding to a 2-AFC task, which has been mentioned in previous studies in the case of archerfish, such as develop a strong preference for stimuli on a particular side or a strategy that requires more sessions to learn (Newport et al., 2013, 2014 and 2016). In stage 3 also, N°4474 did not reached the learning criterion and obtained fewer good results than N°4494. Perhaps, with additional sessions, this individual could have succeeded in the object's stage, but we noticed that this trout was easily stressed compared to the others. Any perturbation (noise, new exercise) was enough to induce a behavioural inhibition and the trout stopped responding during the session, motionless and close to the walls of the tank, as commonly observed in fish subjected to a novel-tank test (Sharma et al, 2009; Champagne et al, 2010; Colson et al., 2015). In rainbow trout, it is well known that individuals exhibit different stress-coping styles (Overli et al, 2002), which may influence the results obtained in learning tests performed individually. Trout N°4474 was probably a high-reactive individual and stress-coping styles should be previously taken into account when selecting trout for a learning experiment. Possible other issues could have influenced trout's behaviour. Indeed, some fishes had injuries, due to aggressive behaviour occurring occasionally in their rearing tank. This required few days of isolation in salty water to treat the sore, resulting in a break in the experiment for the fish concerned. Gender and age effects could also had interfered with different learning strategies. However, only same age triploids females were used for the experiments in order to overcome any sexual maturation problems (increased aggressiveness in males and ovulating females), which could have affected behavioural results.

We did not observe any global side preference when comparing the percent of trigger activation on the left and on the right side, taking into account the five individuals together. Indeed, the test-tank was built to be symmetrical and uniform in its entirety, to avoid any side preference. However, side

bias was trout dependent, some of them activating preferentially the left trigger rather than the right one and inversely. Position bias is a phenomenon commonly observed in fish (archerfish: Newport et al., 2014 from Northmore and Yager, 1975) and in other animals (dogs: Huber, 2013) in this type of protocol. In a goat's study, similar issue of side bias was found for a maze task, when animals had not yet understand the rule (Langbein 2012), meaning that it could be the case for our subjects. The more a task is complex to resolve, the more the subject can change of learning/decision strategy for one easier such as always going to the same side. To control for side bias, S+ did not appear more than twice on the same side, correction trials (CT) were implemented when S- was chosen in the stage 2 training and forced tests were ran for other stages. Therefore, even if some of the trout were lateralised, side bias was controlled and did not prevent them to succeed. As we saw in the results, those corrections enabled to avoid a global side bias in all stages.

In cognitive studies it is usually found that animals take more time answering when doing an error rather than a correct response (Knolle et al., 2018). Interestingly, we recorded latency time to answer significantly longer when the trout did a correct response in stage 3. This indicates that they took more time analysing the situation before making the good choice, while doing an error (selecting the object) would suggest they were not concentrated when answering. Trout N°4494 is the one which contributes the most to this observation since it took more time to answer compared to the others and it is also this one which had the highest percentage of correct responses in stage 3. Stage 3 was probably the task that required the most attention (distinguish differences between trout and objects photographs), stage 2 being the training stage using an easily discriminable S- (black star), and the two subsequent stages being the ones where the trout were too confused to keep motivated.

## **V. CONCLUSIONS**

This study was part of an INRA project, which aims to highlight cognitive abilities in farmed animals, here rainbow trout, in order to better understand and adapt present and future rearing systems to their mental needs.

Rainbow trout have rapidly learned how to operate a self-feeder individually in a condition of social isolation despite their neophobic and gregarious nature. We also showed that rainbow trout is able to discriminate a pool of four recurrent congeners from a black shape and from different objects, which is not trivial given the ecology of the species. The results clearly show that object recognition tasks can be learnt, even in the absence of a neocortex, cerebral region involved in learning processes in mammals. Moreover, during the experiments, trout faced various

challenges (neophobic nature, injuries, lateralisation) which have not prevented them from succeeding.

As far as we know, this is the first study demonstrating such performance in rainbow trout, which represents however the first French fish continental production. This study shed a new light on the mental capacities of this captive animal often evinced from animal cause, and might inform the societal debate concerning fish consciousness.

From a methodological point of view, these promising findings reveal the possibility to test isolated rainbow trout in an experimental device very different from their rearing conditions and represent an interesting opportunity for further fish behavioural studies. In addition, rainbow trout have been able to distinguish 2D-images on a computer-controlled screen within an innovative operant conditioning context. The 2-AFC paradigm and the operant conditioning device used in this study can therefore be added to the existing repertoire of behavioural paradigms and testing devices suitable for the investigation of cognitive abilities in fish.

## **Perspectives**

In the given experimental setup, trout were unable to discriminate between different conspecifics in 10 sessions, which raises several questions. Further experiments using more individuals might be interesting to elucidate whether rainbow trout are truly unable to make this discrimination. Few more sessions are also needed in response to intra-species discriminative strategies (more time required depending on individuals) and variability in performance. Known congeners as S+ rather than unknown ones could also represent a better way to study it. Indeed, it was already demonstrated that other fish species are able to recognize conspecifics individually using visual cues (Clark and Stephenson 1999; Culumber 2015; Goncalves et al. 2000; Johnston and Dixon 2017; Karenina et al. 2013; Knight and Turner 1999; Roux et al. 2016; Satoh et al. 2016) and that guppies prefer to school with familiar individuals (Griffiths and Magurran, 1997). As mentioned above, rainbow trout are used to school to avoid predators or when encountering stressful situations. Knowing whether known congeners (S+) would be more frequently chosen by the trout than unknown ones (S-) could represent a good insight of the ecology of the species. On the one hand, it could show if, as guppies, rainbow trout prefer to school with familiar individuals (Griffiths and Magurran, 1997) or if whatever the congener presented on the screen and selected, the trout ensures itself enough protection from predators (Agrillo and Bisazza, 2014).

Although the AFC test has been proven to provide reliable results, it is limited in the fact that subjects must be conditioned to a particular stimulus (Newport et al., 2014). Further investigations

reflecting more complex cognitive abilities (working memory, conceptual learning) are therefore necessary. A MTS task will, for instance, allow to test a variety of conceptual learning such as categorisation (colour, shape, size) and quantity discrimination. The five experimental individuals already began a habituation phase to the MTS test during the last 2 months, but no results are currently available since they do not understand yet the MTS guideline.

The idea is, once trout will have understood and acquired individually the habituation step of the MTS (the sample presented is the same as the S+), they will perform conceptual tests to assess their elaborated cognitive abilities. A first application could be based on a colour discrimination criterion (the colour of the sample is the same as the colour of the S+, despite a different shape), as it was demonstrated that when visual acuity is not affected, fish prefer to choose a high chromaticity rather than a specific pattern (goldfish: Ohnishi, 1991). Moreover, a concept based on a colour criterion should be possible since Luchiari and Pirohnen (2008) have already shown that rainbow trout can easily discriminate between different colours.

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## APPENDIX A

### Models used for statistical analyses

Stage of the 2-AFC	Model name	Type	Code	AIC
2	modellatFNtot	Linear model	lm(log(Latency.ms)~Trout*step,data=FNtot)	6407.20
2	modF	Linear model	lm(Score.RC~Trout,data=FNtRC)	-120.13
3	modellatOBJ1	Linear model	lm(log(Latence.ms)~Trout+Response,data=OBJj)	2600.70
3	Modeln	General linear model	glm(cbind(Nb.successes,Nb.failures)~Object.image, family=binomial(link = "logit"),data=OBJn)	281.59
3	modelperfOBJ	Linear model	lm(Score.RC~Trout,data=OBJtRC)	-33.54
4	modellatESPj1	Linear model	lm(log(latence.ms)~Trout*Response,data=ESPj)	2046.98
4	ModelESPn	General linear model	glm(cbind(Nb.successes,Nb.failures)~Image.species, family=binomial(link = "logit"),data=ESPn)	267.26
4	modESPtRC	Linear model	modESPtRC<- lm(Score.RC~Trout+Running.order,data=ESPtRC)	77.40

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**modellatFNtot**: model designed to analyse the latency time to answer function of the interaction between the variables trout and step for the stage 2 of the 2-AFC task

**modF**: model designed to analyse the proportion of correct responses function of the trout for the stage 2 of the 2-AFC task (performance between trout)

**modellatOBJ1**: model designed to analyse the latency time to answer function of the trout and the response for the stage 3 of the 2-AFC task

**Modeln**: model designed to analyse if there were any preference or aversion towards the pictures of objects chose as S- in the stage 3 of the 2-AFC task

**modelperfOBJ**: model designed to analyse the proportion of correct responses function of the trout (performance between trout in stage 3)

**modellatESPj1**: model designed to analyse the latency time to answer function of interaction between the variables trout and response for the stage 4 of the 2-AFC task

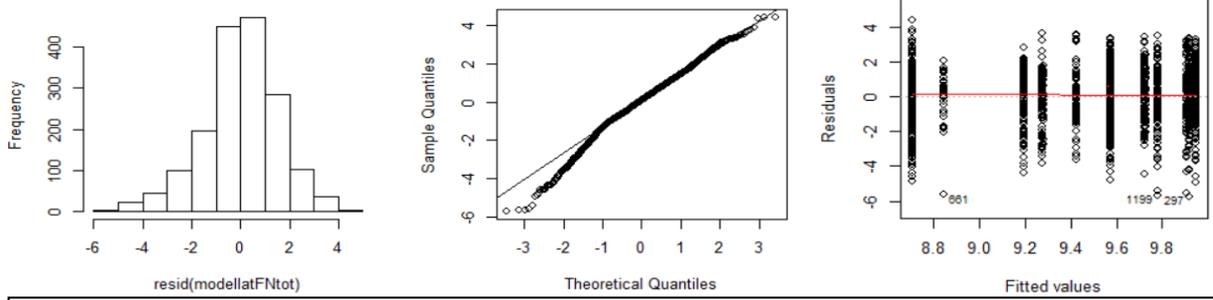
**ModelESPn**: model designed to analyse if there were any preference or aversion towards the pictures of other fish species chose as S- in the stage 4 of the 2-AFC task

**ModESPtRC**: model designed to analyse the proportion of correct responses function of the trout and the trout's running order for the stage 4 of the 2-AFC task

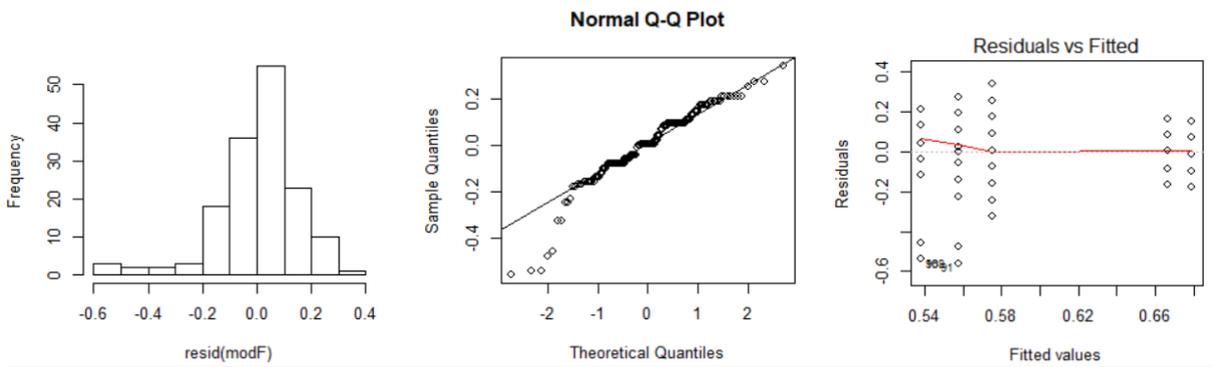
# APPENDIX B

## Normality and homoscedasticity of the models used for statistical analyses

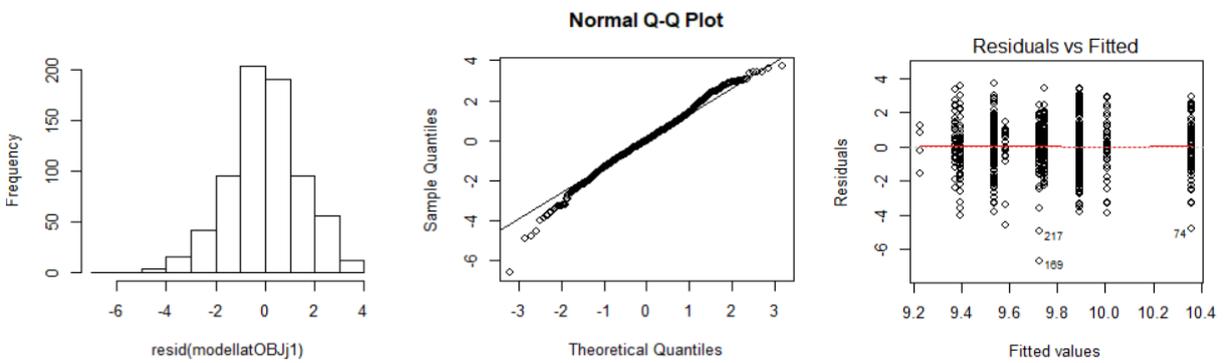
### modellatFNtot



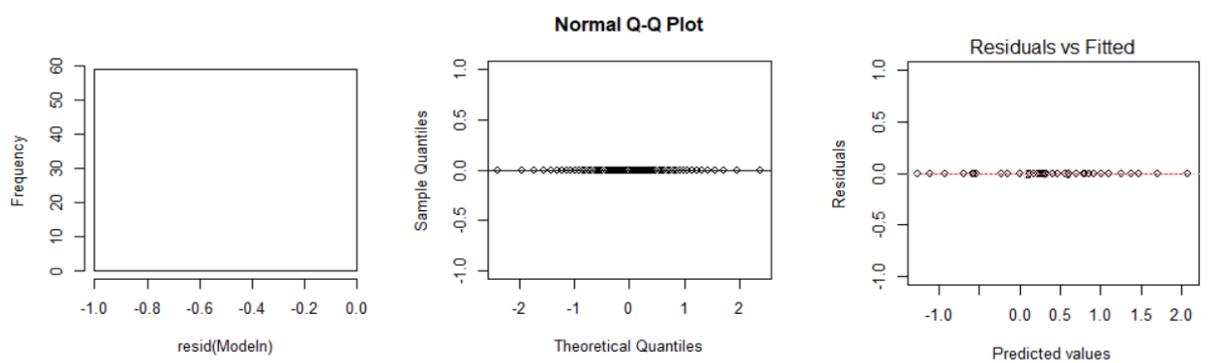
### modF



### modellatOBJ1



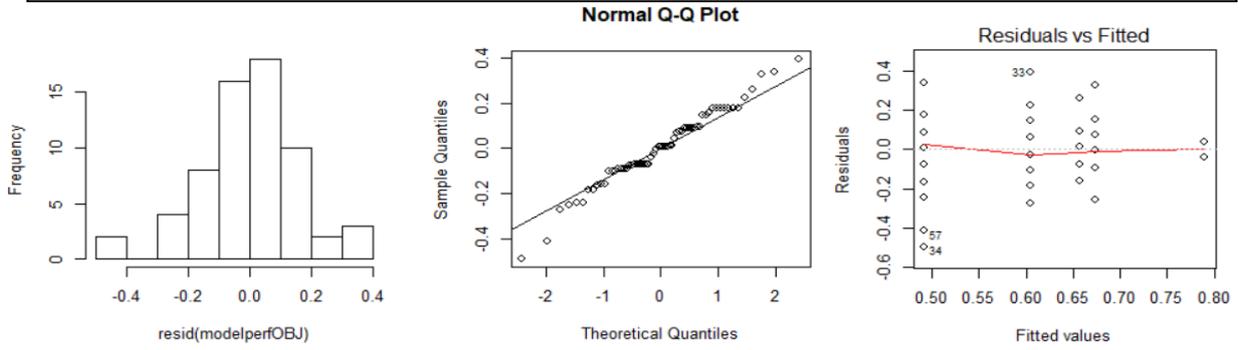
### Modeln



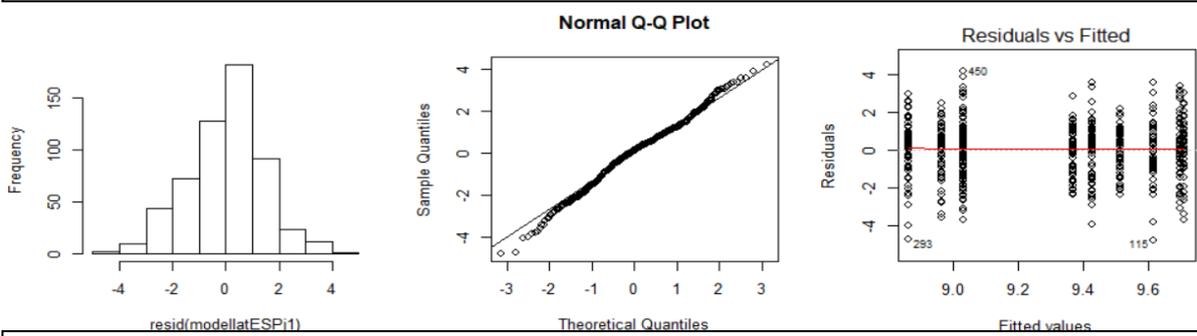
# APPENDIX B

## Normality and homoscedasticity of the models used for statistical analyses

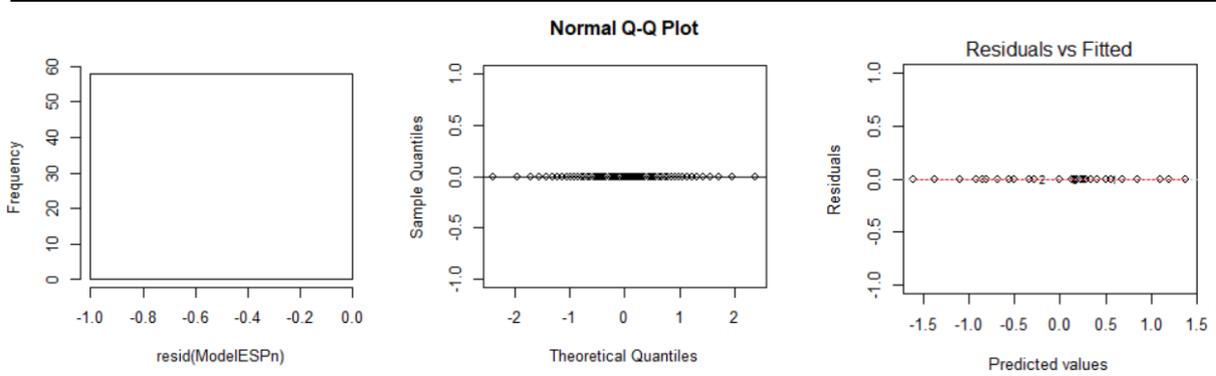
### modelperfOBJ



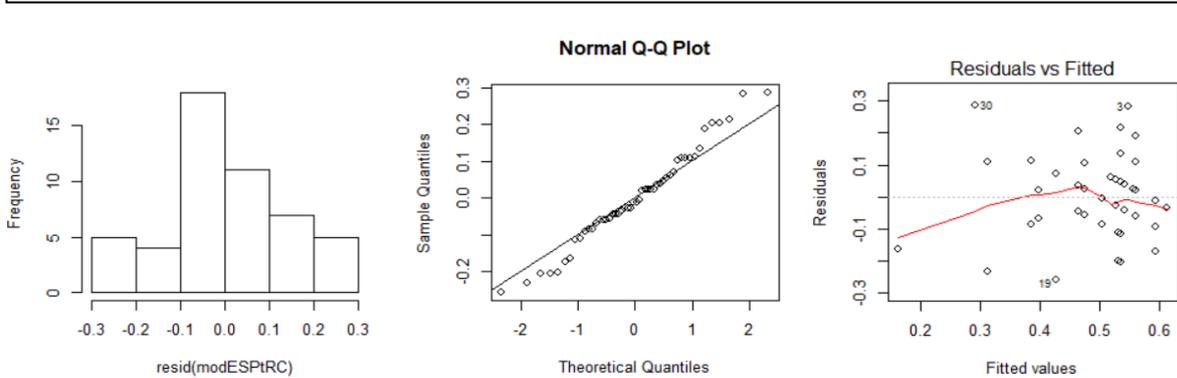
### modellatESPj1



### ModelESPn



### modESPtRC





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**Spécialité** : Sciences Halieutiques et Aquacoles

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**Titre français** : Capacités de discrimination d'images en 2D de congénères chez la truite arc-en-ciel : utilisation d'un dispositif innovant de conditionnement opérant

**Titre anglais**: Discrimination abilities of 2D pictures of congeners in rainbow trout: the use of an innovative operant conditioning device

**Résumé.** Cette étude visait à étudier les capacités cognitives d'une espèce couramment utilisée par l'homme dans la chaîne alimentaire : la truite arc-en-ciel. Une meilleure connaissance de ses capacités mentales permettrait de mieux comprendre la façon dont cet animal de rente perçoit son environnement et ce qu'il peut éprouver lorsque son bien-être est compromis. Pour ce faire, un dispositif de conditionnement opérant innovant a été conçu. Celui-ci a été couplé à un paradigme de 2-AFC, un test puissant et fréquemment utilisé dans le cas d'études comportementales, afin de tester si cette espèce peut distinguer ses congénères représentées en 2D sur des photographies, de différents stimuli visuels. Nous avons montré que la truite arc-en-ciel est capable de différencier ses congénères de formes abstraites ou d'objets, ce qui coïncide avec l'écologie de l'espèce. De plus, elles ont appris à activer un self-feeder individuellement dans un environnement inconnu, ce qui est particulièrement remarquable pour une espèce grégaire et néophobe. A notre connaissance, il s'agit de la première étude montrant de telles performances cognitives chez la truite arc-en-ciel. Des recherches complémentaires viseront à mettre en évidence d'autres capacités cognitives encore plus complexes (mémoire de travail, capacités de catégorisation et apprentissage de concepts). Ainsi, l'apport de connaissances sur les capacités cognitives des animaux de rente, encore trop peu documentées se poursuivront, et alimenteront le débat sociétal concernant la conscience animale.

**Abstract.** This study aimed to study the cognitive abilities of a species widely used by humans for food production: the rainbow trout. A better knowledge of its mental abilities could aim to better understand how this farmed animal perceives its environment and what it may experience when its welfare is compromised. To do so, an innovative operant conditioning device was designed. This one was coupled with a 2-AFC paradigm, a powerful and frequently used test for behavioural studies, to test whether this species can discriminate its conspecifics represented on 2D photographs, from different visual stimuli. We showed that rainbow trout is able to differentiate its congeners from abstract shapes or objects, which is consistent with the ecology of the species. Moreover, they managed to activate a self-feeder individually in an unknown environment, which is particularly noteworthy for a gregarious and neophobic species. As far as we know, this is the first study demonstrating such cognitive performance in rainbow trout. Further investigations will aim to highlight other, even more complex cognitive abilities (working memory, categorization abilities and concept learning). In this way, the contribution to the knowledge of the still under-documented farmed animals' cognitive abilities will continue and will inform the societal debate concerning animal consciousness.

**Mots-clefs** : truite arc-en-ciel, comportement, capacités cognitives, conditionnement opérant, 2-AFC, discrimination, stimuli visuels.

**Keywords:** rainbow trout, behaviour, cognitive abilities, operant conditioning, 2-AFC, discrimination, visual stimuli.