Doctoral School in Cognitive and Brain Sciences

PhD Thesis

NUMERICAL COGNITION IN ZEBRAFISH
(*Danio rerio*)

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CONTENTS

ABSTRACT

The use of numerical information is widespread throughout the animal kingdom, providing adaptive benefits in several ecological contexts, including foraging, anti-predatory strategies and mating. Given the importance to possess numerical abilities, it is plausible that similar selective pressures in favor of processing numerical information would have acted in different species, even in those more distantly related to humans, such as fish. The aim of this work was to investigate several aspects of numerical abilities in zebrafish (*Danio rerio*).

In the first part, discrimination of quantity (magnitude) was investigated. Zebrafish were tested in free-choice experiments for their preference for different numerosities of conspecifics, taking advantage of shoaling behavior. Zebrafish chose to approach the location previously occupied by the larger in number between two groups of conspecifics (no longer visible at test) in sets of 1 *versus* 2 items and 2 *versus* 3 items, but failed at 3 *versus* 4 items. Similarly, when tested with larger numbers, zebrafish succeeded with 2 *versus* 4, 4 *versus* 6 and 4 *versus* 8 items, but failed with 6 *versus* 8 items. The results suggest that zebrafish rely on an approximate number system to discriminate memorized sets of conspecifics of different magnitudes and the degree of precision in recall is mainly dependent on the ratio between the sets to be discriminated.

The aim of the second part was to investigate, for the first time, the use of proto-arithmetic addition abilities in zebrafish. Fish were tested in a spontaneous choice-test paradigm in which sets of conspecifics disappeared one-by-one behind one of two opaque screens, forming two groups that differed in number. Fish preferred to inspect the screen occluding the larger group of fish in sets of 1 *versus* 2 items and 2 *versus* 4 items, but failed at 2 *versus* 3 items. When tested under controlled conditions for continuous variables (overall time of the stimuli presentation in the two groups equalized) zebrafish were affected by the motion of the stimuli, showing a preference for the group of conspecifics that moved faster. Although results suggest that fish possess proto-arithmetic addition capacities, further studies seem to be needed to clarify in which circumstances zebrafish use numerical or non-numerical features.

In the third part, ordinal numerical competencies were investigated. Fish learned to identify the second element in a series of five identical elements arranged sequentially. To assess whether zebrafish used ordinal information rather than non-numerical information, such as spatial distances, fish underwent a series of tests. When the length of the apparatus (exp. 1) and the inter-element distance (exp. 2) varied at test, creating a potential conflict with ordinal information, fish selected the

correct ordinal position over spatial distance. Fish showed however difficulty when the set of elements changed, such as when the number of elements almost doubled (from 5 to 9 elements, exp.3).

The aim of the fourth part was to study the possible link between number and space in the mapping of numerosities. Such an ability has been observed in primates and in birds, resembling a human mental number line. Zebrafish learned to associate a target number with a reward and then were tested in a preference choice test between two identical numerosities, but different to the training one, placed on the right and the left side of the experimental apparatus. Results suggested that zebrafish spontaneously associated smaller numbers with the left space and larger numbers with the right space, although a potential limitation of the use of mapping strategies is probably related to the ratio between the numerosities presented during training and testing. Control conditions confirmed that the overall perimeter and the overall area did not strongly influence the orientation of the supposed mental line. However, zebrafish were not completely unaffected by changes in surface areas of the stimuli.

Given its widespread use in the field of genomics, zebrafish may provide a useful model organism in the study of the genetic bases of numerical cognition.

1. INTRODUCTION

Numbers are part of our lives. We constantly experience numerical measures, quantity estimates, arithmetical operations, and it is undisputed that the complexity of numerical competence shown by humans is unique. But is the ability to use and process numerosities unique to our species? For a long time it has been believed that the development of numerical concepts was associated with the use of language, arguing that the origin of the mathematical capacity was an "abstraction" from human language (Chomsky, 2006; Bloom, 1994). However, this hypothesis has been challenged by the evidence suggesting that numerical concepts have origins and neural basis that are independent of language (Gelman & Butterworth, 2005). In fact, evidence from patients with brain damage, showed that severely impaired language can be accompanied by well-preserved numerical skills (Rossor et al., 1995; Varley et al., 2005; Benn et al., 2013).

Humans' knowledge of symbolic number is unique, but it depends on a system that is shared by other animals. It has been hypothesized that basic cognitive mechanisms for the representation of relevant aspects of the environment depend on the presence of a "Core Knowledge" system, a set of basic cognitive tools used to interact with the world (Spelke, 2000). This kit is not therefore the result of a learning process, but it is instead available from birth, forged by the natural selection process and written in our genes. The Core Knowledge kit (Spelke, 2000) includes the main domains of interaction with the world such as: physics, considering a basic concept of object mechanics (like the fact that a solid object can not occupy the same space as another solid object); sociality, that includes all the mechanisms for attending to and recognizing living things; spatial relationship, that allows the subjects to move and orient in the environment using geometry, distances, angles, and sense relations among extended surfaces in the surrounding layout; number representation, related to the ability to use and manipulate numerosities. The idea is that this basic cognitive equipment, by virtue of our common phylogenetic dependence from non-human ancestor, is present among animals and this is the reason why those abilities can be investigated from a comparative perspective, making use of a lot of animal models (Spelke & Kinzler, 2007; Vallortigara, 2012).

1.1. The study of numerical abilities in animals

When scientist describe perception of numerical quantities, they speak of "numerosity", which is substantially different than speaking of "numbers". In fact, with the word number we refer to discrete symbols, and when we count, each item correspond to a move of one step forward in the number

sequence. These differences do not imply that our representation of numbers is different from that of other animals. In fact, we are endowed with mental representations of numerosities/quantities similar to that of other animals, from which we have developed the numbers and our advanced mathematics. Thus, numerosity processing is an evolutionarily preserved cognitive function, distinct from counting and humans' unique symbolic numbers (Dehaene, 1997).

The abilities to deal with numerosities have been studied in a variety of non-human animals in their natural environment and also in the laboratory. Before going into the study of numerical knowledge, one can first wonder whether it is reasonable to assume that animals can have such representative capacities. If an animal possesses a particular ability, this means that it has been essential in the course of the phylogenetic development of the species, only when possessing that particular characteristic allows a greater adaptation to the environment and a greater reproductive capacity. The possession of rudimentary numerical competence can in fact provide advantages for animals in ecological contexts.

In birds, for example, to avoid avian conspecific brood parasitism, a strategy based on a rudimental egg counting is used. Hosts recognize and reject many parasitic eggs, reducing the fitness costs of parasitism (Lyon, 2003). Furthermore, the possibility to estimate the number of a rival group of conspecifics may provide useful information whether to attack or retire. Lions (*Panthera leo*, McComb et al., 1994), chimpanzees (*Pan troglodytes*, Wilson et al., 2002), wild spotted hyenas (*Crocuta crocuta*, Benson-Amram et al., 2011) and feral dogs (*Canis lupus familiaris*, Bonanni et al., 2011) are more willing to attack an opponent group when the number is in a minority compared to that of belonging. Similarly, the ability to identify and join a larger group of conspecifics may reduce the possibility to be preyed upon. Group size is one of the main behavioral mechanisms used by animals to manage their vulnerability to predation risk (Caro, 2005). Redshanks (*Tringa tetanus*) respond to the presence of a predator by flocking in large groups of conspecifics, a strategy that provides an anti-predator benefit (Cresswell, 1994; Cresswell & Quinn, 2011). Finally, in foraging tasks, the ability to identify the larger food patch, may provide a great advantage for an individual's survival and reproduction (Krebs, Ryan & Charnov, 1974).

The examples cited below show how rudimental numerical competence are useful and spontaneously used in natural environments. Given that, it is now clear the reason why the representation of numerosities must be biologically founded, due to selective pressures during the course of development, which provided a benefit to those individuals who made use of numerical representations for the resolution of problems.

1.1.1. Different methods of investigation

The study of cognitive abilities in nature provides great benefits in understanding the ecological importance of using a particular skill and competence. But at the same time it reveals weaknesses as it is not possible to study fully the limits and the potentials of a given skill, and besides, a number of variables could also affect the interpretation of results. To control these problems, a wide range of controlled laboratory studies on a high number of organisms (human and non-human) has been conducted. To do this several research methods have been used to study the presence of numerical abilities, that can be divided only into two main classes: those that are expressed by spontaneous choice procedures and those which instead require training procedures.

A spontaneous discrimination condition that can be found in nature and can easily be reproduced in a laboratory, is the food search behavior. The predictions in these contexts are described by the "optimum foraging theory" (Stephen & Krebs, 1986) according to which animals tend to maximize food intake and energy gain. Typically, in this type of experiment, an animal is placed in front of two different numerical quantities of food that can be presented simultaneously (the two groups are presented at the same time to the subjects) or sequentially, (e.g. a group is presented and then covered and only at that point the other group is shown) and then the animal has the opportunity to approach one of the two groups. A preference to approach the bigger quantity of food has been proved in both mammals (Hanus & Call, 2007; Ward & Smuts, 2007; Panteleeva et al., 2013) and birds (Garland et al., 2012).

Numerical cognition studies have not only explored the ability of discrimination by using different amounts of food as a stimulus, but those same studies have also focused on the importance of group life using social partners as a stimulus. In fact, several animal species live in groups because this provides a number of advantages that increase the chance of survival and reproduce (Alcock, 2001).

Another experimental procedure of spontaneous choice used to investigate numerical cognition, is the "habituation of looking time" technique. This method is mostly used in infants and is based on the assumption that they prefer to look at a novel stimulus if it is compared to a familiar one (Fantz, 1964). The looking time method is also used in paradigms of "violation of the expectation" paradigm, that is based on recording the fixing time of subjects on a particular stimulus or event which is usually longer in case of an unexpected event. For example, in an experiment with primates, rhesus monkeys that observed two groups of four lemons that were placed behind a screen, they looked longer when the screen was lowered to reveal only four lemons (incorrect outcome) than when it revealed the correct outcome of eight lemons (Flombaum et al., 2004).

The study of spontaneous abilities in general provides many interesting aspects and advantages, but at the same time it is also limiting. In fact numerical tasks that are only based on spontaneous preferences have in some cases shown lower performances than those found by following training procedures (Hauser & Spelke, 2004). According to this, it is certainly possible to say that a discriminatory capacity is a symbol of ownership of numerical abilities, but in the same way it is not possible to say that a lack of choices indicates a lack of discrimination. It is also easy to predict how motivation in spontaneous choices decreases as the number of elements presented increases. An animal, for instance, can find advantageous to choose the largest food group when it has to compare for example 2 apples *versus* 3, but the same cannot be done with 11 apples *versus* 12, although the animal may equally have the cognitive structures needed to distinguish such numerosities. Given these assumptions, the use of training procedures can be used to study the potentiality and limits of cognitive abilities linked to the possession of a sense of number. A classical training procedure involves the use of food reinforcement repeatedly associated with a specific numerical quantity. In order to teach an animal the distinction between two exact numbers (e.g. 2 and 3 balls), the choice of only one of the two groups (e.g. the larger one) is reinforced (e.g. with food). Historically, Otto Koehler (1943) was one of the first researchers that demonstrated that various species of birds including pigeons, African gray parrots and crows are able to make judgments of relative numerosities. An example of those abilities of judgment was shown in an experiment in which some pigeons, to get a food reward, had to discriminate between two stimuli that were composed of different numbers of balls, showing that they were able to identify the larger one by pecking at it.

Another widely used learning procedure is the "matching-to-sample task", whereby the animal is supposed to observe a sample number and then choose between two different new stimuli the one associated with that number by comparing it to the sample. Using this paradigm, Koehler proved that crows are able to observe a number of dots drawn on a sheet and then choose between several small covers those associated with that number.

The use of training procedures in general can lead to surprising results in the animal field. Of note are the studies of Irene Pepperberg with the African grey parrot (*Psittacus erithacus*, 2006a), that trained the animal not only to discriminate different number quantities, but also to use a series of verbal numerical labels to call a number of 1-6 items presented simultaneously. The parrot was even able, given a series of heterogeneous objects that varied by shape and color (e.g. blue and red keys and trucks), to report the number of items uniquely defined by the conjunction of one color to one category (e.g., "How many blue keys?").

1.2. Discrimination between numerosities

The ability to attribute judgments of relative numerosities is the most elementary level of numerical knowledge. When two different food distributions are given and an animal needs to evaluate which of these two contains a greater quantity, it must be able to discriminate quantities of the type: "more than ..." or "less than ...", thus making relative comparisons between numerosities. The discrimination of relative numbers is considered to be the most elementary and ancient form of numerical knowledge (Davis & Pérusse, 1988). Such discrimination is made by the subject when the elements are present at the time of choice and also if these elements are not visible during the choice; in this case the discrimination is done by the comparison of the mnestic traces of the quantities observed before. Using the experimental procedures and trainings of spontaneous choice, described in the previous paragraph, it is possible to test humans and animals to numerical tasks with the aim to observe their performance, to compare the results and to understand the cognitive mechanisms that underlie this abilities.

The first evidence of numerical abilities in human infants comes from Starkey and Cooper (1980). Using the "habituation of looking time" technique, 4-8 months old infants were trained to observe an imagine of 2 black dots on a screen when presented. After a series of presentation, when the stimulus fixation time decreased indicating that the infant was already used to the stimulus, a new stimulus, 3 dots, was displayed. From the study emerged that those infants began to look longer at this new and unexpected imagine, proving that in some way they had perceived the numerical variation. The same also happened when the number of objects in the imagine decreased from 3 to 2. Similar results were obtained in other experiments using, instead of dots, photographs of different objects that varied by location and size (Strauss & Curtis, 1981). A few years later, Antell and Keating (1983) noted that even a few days old babies are able to discriminate between sets of two or three elements. In the following years, several studies in preverbal infants had shown that those are able to represent cardinal values of sets of objects (*e.g.* Feigenson et al., 2002a; 2002b; Wynn, 1996; and see review in Cantrell & Smith, 2013 and Carey, 2009), bringing in this way further evidence that rudimental numerical competences do not apparently depend on language.

Despite discriminations of small numerosities, infants showed good performances also during discrimination of large numerosities. A study of Xu and Spelke (2000) showed that infants at 6 months discriminate 8 from 16 items and also 16 from 32, but not 16 from 24, showing to be able to discriminate differences only at a 1:2 ratio. Interestingly, the ratio limit of numerosity discrimination increases in precision during development: infants at 6 months are able to discriminate differences at a 1:2 ratio, while 10 months old babies are able to discriminate numerosities at a 2:3 ratio (e.g. 8 *vs.*

12 elements), both if the stimuli were presented in auditory or visual modalities (Lipton & Spelke, 2003; Xu & Arriaga, 2007). The results obtained with human infants highlight that numerical abilities are already present a few hours after birth, providing in this way innate evidence of a sense of number. A large series of experiments on non-human animals showed performances that are closely related to those obtained in our species.

By using the spontaneous choice task and by knowing the preference of animals for the greater amount of food, Hauser and colleagues (2000) studied on rhesus macaques (*Macaca mulatta*) the discriminatory capacity of the animals by placing two different amounts of apple pieces inside two different opaque containers. Monkeys were able to choose the container with the largest number of apple pieces when the comparison was 1 *vs.* 2, 2 *vs.* 3, 3 *vs.* 4 and 3 *vs.* 5, but they chose randomly when the hidden amounts were 4 *vs.* 5, 4 *vs.* 6, 4 *vs.* 8 and 3 *vs.* 8. It is interesting to note how the performance is similar to those obtained with children by Feigenson and colleagues (2002a; 2002b), where 10-12 month old infants showed a good discrimination in 1 *vs.* 2 and 2 *vs.* 3, but not in 3 *vs.* 4 and 3 *vs.* 6 comparison. The ability to discriminate the largest of two sets of discrete quantities has been studied also in Orangutans (*Pongo pygmaeus*; Call, 2000). The authors found that the performance was related with the ratio between the two numbers (e.g. 2 *vs.* 3 - ratio 0.67 was easier than 5 *vs.* 6 - ratio 0.83). Similar results involving a spontaneous food quantity discrimination were found among primates in chimpanzee, (*Pan troglodytes;* Beeran & Beeran 2004), orangutans (*Pongo pygmaeus*; Shumaker et al., 2001), gorillas *(Gorilla gorilla)* and bonobos *(Pan paniscus*; Hanus & Call, 2007). Using a delayed match-to-sample paradigm, Jordan and Brannon (2006) trained rhesus monkeys to touch on a monitor the set of objects (e.g. 8 and 2 dots) that came closer to the number of a reference sample presented at the beginning of the test (e.g. 8 dots). Both accuracy and reaction time were modulated by the ratio between the correct numerical match and the distracter.
An impressive ability to manipulate numbers has also been shown in an adult female chimpanzee named Ai (Biro & Matsuzawa, 2001). The animal was required to match a number of dots presented with one of two Arabic numbers presented on a touch screen (Figure 1a). In a second task, Ai was required to match an Arabic numeral to one of two alternative sets of dots displayed (Figure 1b). Finally, in a third task, two Arabic numbers appeared on a screen and the subject had to touch the two stimuli from the smallest to largest one, (Figure 1c). With this study it was possible to demonstrate not only that the chimpanzee was able to recognize the cardinal and ordinal aspect of numbers, but that it was also able to associate numerosities to external symbols.
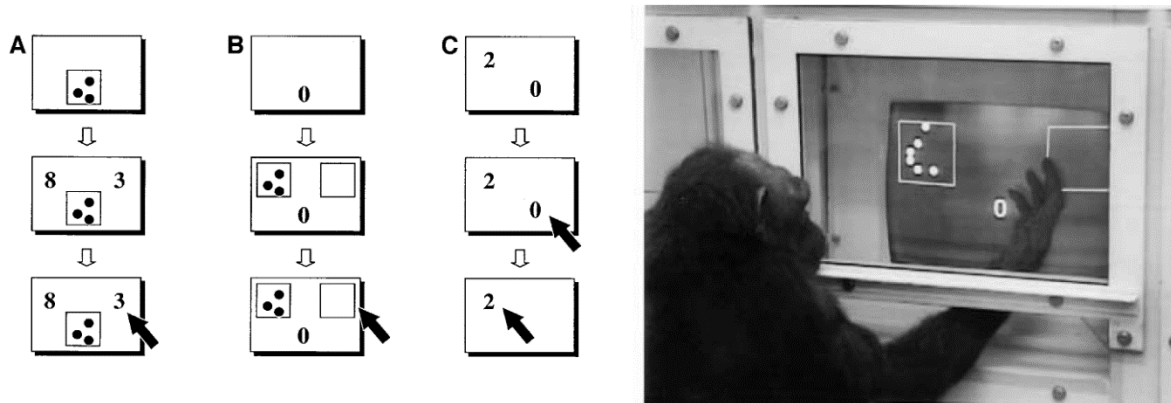
Figure 1. Summary of the three tasks proposed to the subject. (**a**) Dot-to-numerical task, the animal matches the dots with the Arabic number. (**b**) Numeral-to-dot task, reverse version of the first task. (**c**) ordering task of the units in ascending order (Biro & Matzusawa, 2001).

Numerical discrimination abilities are not confined to primates, they have been observed among several other mammal species. For example, it has been discovered that dolphins (*Tursiop truncates*), were able to discriminate 2 from 5 elements that consisted of three-dimensional objects and then were able to successfully transfer this ability to two-dimensional novel numerosities, even in the presence of configurations never seen before (Kilian, 2003).

Spontaneous choice paradigms between two different numerosities of food have been used also in other mammal species such as coyotes (*Canis latrans;* Baker et al., 2011), dogs (*Canis lupus familiaris;* Ward & Smuts, 2007), cats (*Felis silvestris catus*; Bánszegi et al., 2016) and elephants (*Loxodonta africana*; Perdue, 2012) as well as in amphibians such as red-backed salamanders, (*Plethodon cinereus*; Uller et al., 2003) and frogs (*Bombina orientalis;* Stancher et al., 2015).

Exactly like in infants and in primates, results showed a discrimination performance strongly dependent on the ratio between the numerosities, following the Weber's Law. This law establishes that the variability in the estimation of a number increases proportionally with its size. This imprecision is called "scalar variability" and makes the discrimination between two numbers less accurate as their magnitude increases.

The ability to discriminate more from less is a basic ability that can be observed in invertebrates as well. Quantity discrimination has been studied in mealworm beetles too (*Tenebrio molitor*). Exposing males to olfactory stimuli coming from different numbers of females, they were able to discriminate olfactory sources from comparisons with a 1:3 female ratio, but not a 1:2 ratio (Carazo et al., 2009). Another insect evidence comes from bees (*Apis mellifera*). Through a training procedure, bees learned to identify which of the two sets shown had the same number of the set shown before. In this experiment to the bees was initially presented a sample stimuli with a certain number (e.g. 2

balls) and then those had to choose between two comparison stimuli, the same number of sample stimuli elements. Also in this case the increasing of the number made the discrimination more difficult, in fact bees were able to distinguish between 1 *vs*. 2 disks, 2 *vs*. 3 and 3 *vs*. 4, but not 4 *vs*. 5. Control experiments confirmed that the animals were not using other cues such as the color and other visual elements (Gross et al., 2009).

Summarizing, the experimental evidence reported seem to point to the existence of similar discriminative limits among vertebrates and even in invertebrates. Before going into the analysis of cognitive mechanisms that could govern numerical abilities in organisms, we have to face an extremely important aspect related to the study of numerosities, the role of continuous physical variables.

1.3. Numbers and physical variables

It is important to point out that, although several animal species exhibit good competence in tasks involving discrimination of different numerical quantities, to be sure that the abilities refer to "numerosities" and not to something else, it is necessary to control for the physical variables. In fact relative magnitude of continuous variables, such as the cumulative surface of an area or the overall space occupied by items, often co-vary with the numerosity. If an individual is able to identify the larger food patch or the biggest group of conspecifics, this does not give us clues about what kind of information the animal uses. Therefore, it is necessary to control the physical variables in order to verify if the subject relies on numerical or quantitative information.

Xu and Spelke (2000) have shown that 6-month-old infants are able to discriminate between large quantities of objects based on number when other variables are kept under control, provided that the ratio between the sets of objects was sufficiently wide, e.g. 8 *vs*. 16 and not 8 *vs*. 12.

In the spontaneous food choice tasks, most of the experiments have provided a control condition by balancing the variables between the two groups, placing for example the same total volume of food but maintaining the difference of the numerosities between the two groups. Uller and Lewis (2009) demonstrated that horses (*Equus caballus*) selected the greater of two small quantities of artificial apples placed into two buckets, even when the total surface area of the two sets was equalized. In amphibians, frogs (*Bombina orientalis*) were tested in a free-choice experiment between two groups with different numbers of preys. By partially occluding the vision of the preys, it has been possible to equalize the area and the total movement between the two groups excluding non-

numerical variables. Frogs were able to discriminate the bigger numerosity when both volume and movement of the preys were controlled (Figure 2, Stancher et al., 2015).
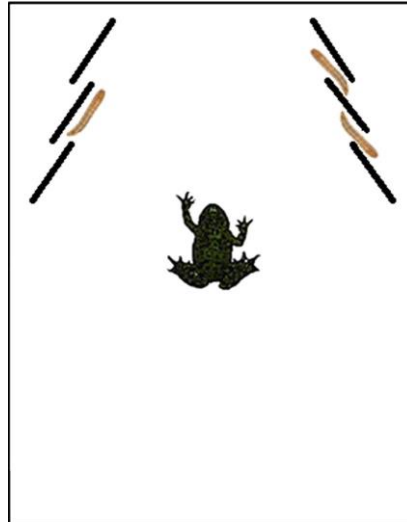
Figure 2. The frog can choose between the two stimuli on the right and on the left. Although the total area and the quantity movement in the stimuli are equivalent, frog prefers the stimulus with two preys (Stancher et al., 2015).

In a study with chicks, Rugani and colleagues (2008) discovered that, once trained to discriminate a small set of two-dimensional identical objects, chicks were able to discriminate successfully 1 *vs*. 2 and 2 *vs*. 3, even when the spatial distribution, the contour length and the overall surface of those elements were balanced.

According to Davis and Pérusse (1988), animals use numerical information as last resource, when all the other variables cannot provide information. This theory has been validated by a series of evidence. In an experiment of Clearfield and Mix (1999), to 6-8 months human infants were proposed a numerical discrimination in which they had to observe a stimulus constant in numerosity (2 or 3), while the total perimeter was constantly maintained. During the test phase to the babies was presented a stimulus which could change in number of elements or in total perimeter. The results obtained indicated that infants were looking longer on the stimulus that changed the total perimeter than on the stimulus that changed in numbers. A study on cats showed similar evidence suggesting that these animals do not spontaneously use numerical information, preferring to use visual clues that covary with the numerosities (Pisa & Agrillo, 2009). The idea that numerical information is used as the last resource, seems therefore to be supported by some empirical evidence. However, an alternative

hypothesis is that animals automatically, spontaneously, and routinely represent the numerical attributes of their environments. Cantlon and Brannon (2007a) investigated this in macaques and checked whether the encoding of information about numbers occurs spontaneously. The monkeys were trained on the "match-to-sample" task in which the correct match stimuli were equal in number and also in non-numerical dimension (shape, color or surface of the area). Each monkey was then tested in trials where the non-numerical dimension and the numerical were in contradiction in order to determine which dimension was spontaneously used by the monkey. The results showed that macaques spontaneously encode the information about numbers, despite the fact that continuous dimensions are available to help them to discriminate the quantities (Figure 3).
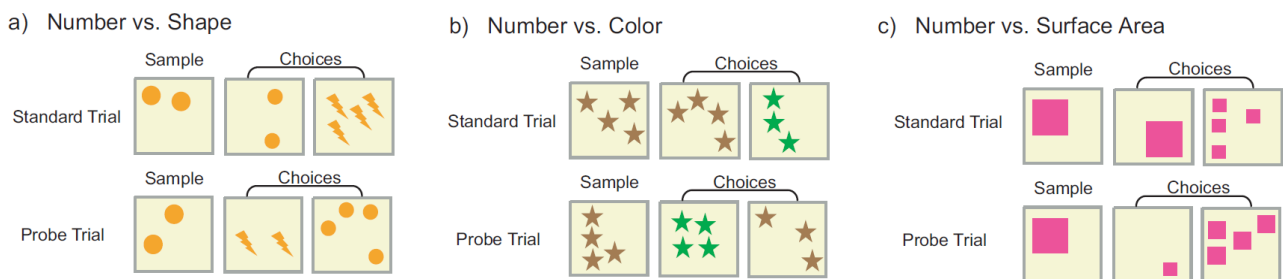
Figure 3. Monkeys were trained to match stimuli. In the standard trial the numerical value was confounded with a second dimension of shape (a), color (b) or surface area (c). Monkeys were then tested with probe trials in which the numerosity was in conflict with the other dimension (Cantlon & Brannon, 2007a).

Other investigations (Feingenson, 2005; Rugani et al., 2010) suggested that the use of numerosities instead of physical variables depends on the nature of the stimulus. In presence of homogeneous stimuli (e.g. food) the process of continuous variable is favored. On the other hand, when the elements are heterogeneous, the subject pays attention on their individual characteristics, leading to a coding based on numerosity.

1.4. Cognitive mechanisms in numerical representation

The observation so far done confirm that the ability to discriminate between two groups seems to depend not so much on the differences between the two numerosities, but on the ratio between them. The ability to discriminate 4 elements from 8 is the same that in the discrimination of 6 elements from 12. In both cases the ratio between the two numbers is of 1:2. Similarly, the inability to discriminate 4 from 5 is also reflected in the 8 *vs.* 10 comparison (ratio = 0.8). This rule is general and governed by Weber's law, which describes the relationship between the physical part of a stimulus

and the perception of the intensity of that stimulus. This rule can be applied to the perception of any physical stimulation. For example, given a weight of 100 g, if a variation of 20 g is needed to perceive the difference (minimum threshold), when the mass is doubled, the differential threshold also doubles to 40 g, so that 240 g can be distinguished from 200 g.

It has been suggested that non-symbolic discrimination of numerical magnitudes (quantities) is based on an approximate number system (ANS; Figure 4). The ANS provides approximate estimates of the numerical magnitude, or numerosity, of a set of objects (see reviews in Butterworth, 1999; Hyde, 2011; Nieder & Dehaene, 2009).
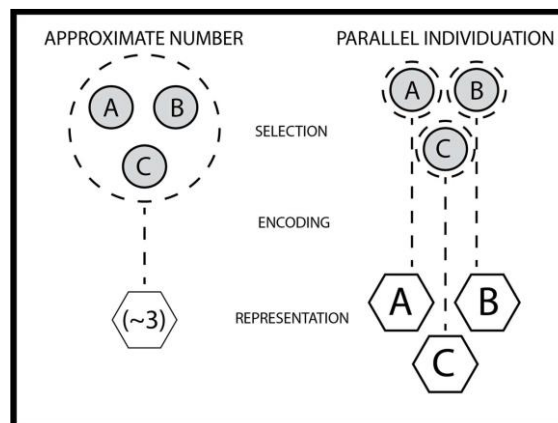
Figure 4. Number representation in the approximate number system and the parallel individuation system (Hyde, 2011).

In the ANS, number discrimination improves as the numerical distance between two values increases ("numerical distance effect"), and, for equal numerical distances, it worsens as their numerical size increases ("numerical magnitude effect"; Dehaene et al., 1998). In other words, the magnitude effect indicates that the discrimination between two numbers is more difficult when the numerical value increases, so distinguishing 4 from 5 is easier than distinguishing 30 from 31. Instead, in the distance effect discrimination is more difficult when the numerical value increases (is more difficult to distinguish 4 from 5 than 5 from 8. The imprecision of ANS increases as a function of numerical magnitudes, and, as a result, discrimination of any two numerical magnitudes depends on the ratio between them, following Weber's Law (Gallistel, 1990).

An ability to simultaneously represent and track objects through a parallel individuation, on the basis of working memory, has been described. This system is dubbed *object file system* (OFS). According to this hypothesis, the OFS system is an attentional mechanism focused on the object. This model hypothesizes that humans and other animals represent every element present in a scene as a single file in their working memory. Each single element contributes to representing the plurality of

the set and the comparison between the groups would then be established through one-to-one correspondence between the different files. Representations through parallel individuation should provide, according to this theory, more fine-grained numerical discrimination than those of ANS, but it is limited to just a few items (usually ≤ three or four; Trick & Pylyshyn 1994; Ross-Sheehy et al., 2003; Le Corre & Carey, 2007).

It has been found that 12- to 14-month-old infants are able to choose the larger set with 1 *vs.* 2, 1 *vs.* 3, and 2 *vs.* 3 elements, but fail in the 1 *vs.* 4, or 2 *vs.* 4, comparison in a quantity discrimination task (Feigenson, Carey, & Hauser, 2002). This suggests an impairment in numerical discrimination when the items to be discriminated may be represented in different systems, that is, the small (parallel individuation system; e.g., two) and the large (ANS, e.g., four) numerical systems. Conflicting results have, however, been recently reported regarding the existence of the OFS in human infants (Cordes & Brannon, 2009; Coubart et al., 2014). It should be noted that for many mammals and birds, and certainly for nearly all primate species tested, the ANS has been shown to account for the observed performances of the animals (e.g., Beran, 2007; Cantlon & Brannon, 2006; Jones & Brannon, 2012; Rugani, Vallortigara, & Regolin, 2014; and see reviews in Brannon, 2006, and Brannon & Merritt, 2011), whereas the OFS has only received limited support (e.g., Hauser, Carey, & Hauser, 2000).

A hypothesis that tries to account for the different experimental results favouring one or other of the two systems stressed that conditions favoring selective attention on individual elements facilitate the activation of the small number system through parallel detection, whereas conditions that do not favor a selective attention on individual elements but rather on sets favour the activation of the approximate number system (Hyde, 2011).

1.5. Animals and Arithmetic

The sense of the number in animals seems to be "transmodal," which means that it is not strictly related to one organ sense (e.g. it is possible to hear three sounds and see three apples and in both cases it is possible to recognize the numerosity "three"). In a study of Izard and colleagues (2009), newborn infants were trained with auditory sequences containing a fixed number of syllables (e.g. 4 or 12 sounds) and then tested with images of the same or a different number of items (4 or 12 elements). When the ratio between the two numbers was 3: 1, infants looked consistently longer on the display that were congruent in number with the auditory sequences presented during the familiarization. Similar results were obtained with rhesus monkeys (Jordan et al., 2005). The animals were shown two screens on which some faces of conspecifics vocalizing were visible; there were

three faces on one screen, and two faces on the other one. At the same time, the subjects listened to a reproduction of a vocalization record of two or three individuals. Monkeys preferred looking at the screen that had a number of faces congruent with the number of vocalizations.

The ability to manipulate numerosities in order to carry out arithmetic operations, such as additions or subtractions, has recently been studied in animals. One of the first experiments that demonstrated this abstract abilities in animals was made by Woodruff and Premack (1981). In this study chimpanzees were faced with two objects, and required to choose which of the two objects was physically identical to a third object. The animal had to correctly associate a half-filled glass, which served as a reference sample, with two others presented objects: one filled for half (correct match) and the other filled for three-quarters. Subsequently, the decision became progressively more abstract. The half-full glass was shown to the chimpanzee and the possible alternatives were half apple or three quarters of an apple. The request was to rely on the similarities of the fractions present in the objects and the chimpanzees were able to associate the half-full-glass to the half-apple, demonstrating that they could grasp the concept of numerical fraction. A subsequent experiment showed how chimpanzees are able to combine two mentally fractured fractions: when the sample stimulus was a quarter of an apple and half a glass and the choice was between a full disk or three quarters of a disc, the animals chose correctly the second option. The chimpanzees were therefore able to add fractional quantities.

The easiest use of addition in proto-arithmetic operation consist in the sequential presentation of objects, an operation that is used in a various number of experiments. For example, in an experiment by Beran and Beran (2004), chimpanzees had to observe an experimenter when inserting bananas into two identical containers: each fruit was sequentially inserted and the subject had no way of seeing how much fruits were contained in the two boxes . This arrangement made it necessary for the subjects to digitize the elements inserted so that, as soon as the experimenter had left, the animals could select the most advantageous banana set. Through this procedure it has shown how chimpanzees can spontaneously sum up small numbers (comparisons 1 *vs.* 2, 2 *vs.* 3 and 3 *vs.* 4), and greater numbers, provided that the relative distance between the groups was increased (as in the case of 5 *vs.* 10 and 6 *vs.* 10).

In another study, Cantlon and Brannon compared arithmetic abilities (addition) in macaques and human adults. In the experiment, subjects had to look at a screen two sets of dots, separated by a delay (e.g. 3 dots-interval-5dots) and after the presentation subject were required to choose between two new groups: one number was equal to the sum of the two sets previously presented and the other group that was presented had a different number of dots (Figure 5). Both monkeys and humans were able to solve successfully the additions showing the same performance. There was a decreasing

accuracy and an increasing response time as the numerical ratio between the two choice stimuli increased in both species (Cantlon & Brannon, 2007b).
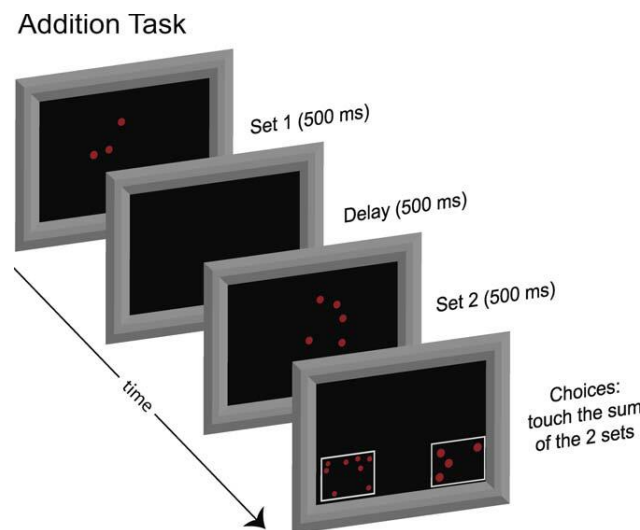
Figure 5. Monkeys and humans were presented one set of dots (set 1). After a delay, a second set of dots was presented (set 2). Then, two new set choices were presented, one correct (the sum of the two set) and one incorrect (distractor) (Cantlon and Brannon, 2007b).

Other evidence of arithmetical abilities that include not only addiction but also subtraction skills were demonstrated in a study of Karen Wynn (1992) with infants, replicated years later by Hauser and colleagues with rhesus monkeys (1996). By exploiting the habituation/dishabituation paradigm and observation time paradigm (which is usually greater for unusual events), 5 months old children and monkeys had to watch a little scene in which two objects (toys for children , eggplant in the case of monkeys) were placed behind an opaque screen. Subsequently, the screen was lowered and the subject could see two possible results: an arithmetically correct result (1 + 1 = 2) or an arithmetically wrong result (1 + 1 = 1). The same paradigm was used for subtractions and the results indicated that both children and macaques looked for longer at the impossible outcome, proving to have rudimentary arithmetic abilities. Similar results were obtained with other species, including tamarins (Uller & Hauser, 2001), lemurs (Santos et al., 2005), dogs (West et al., 2002).

In addition to mammals, spontaneous representation of arithmetic abilities had been found also in birds, employing filial imprinting, to familiarize animals with a certain number of elements (Rugani et al., 2009). The term "imprinting" means an early learning phenomenon, typical of birds in a brief growth phase called 'sensitive period', for which they recognize and follow the mother or other conspicuous or elements introduced to them since their birth (Lorenz, 1935). By exploiting this type

of learning, domestic chicks (*Gallus gallus*) were exposed to five small balls (imprinting objects), then subjected to an arithmetic task. In this experiment, a ball was first hidden behind a screen, then the other 4 balls were hidden behind a second screen. Subsequently, at the end of this addition (1 *vs.* 1 + 1 + 1 + 1 = 4), from the larger group, 1 or 2 items are moved one by one from one screen to the other, adding to the group previously composed of only one element (Figure 6).
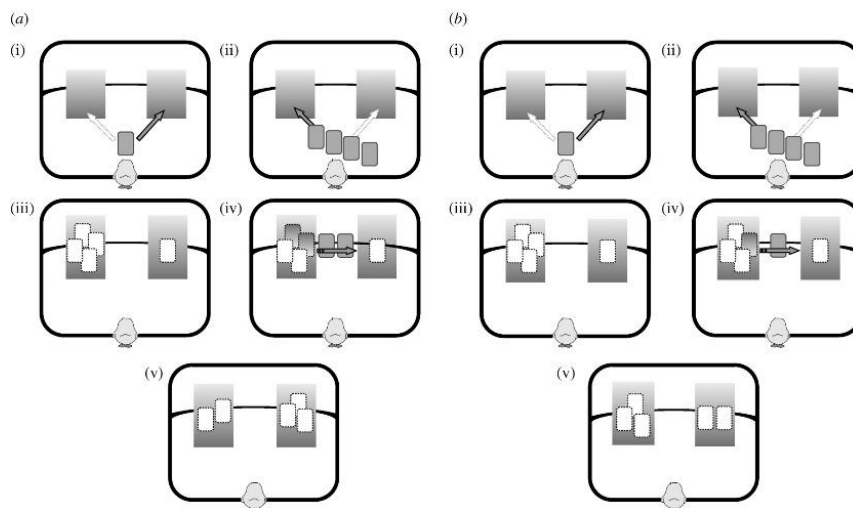
Figure 6. Schematic representation of the two conditions proposed in chicks. After an addition of 1 and 4 elements behind two screens, two elements (a) are moved from the bigger group to the smaller one, then the chicks preference is observed. In order to exclude that the chick bases its choices following the last movement seen, in (b) only one object is moved. (Rugani et al., 2009).

The study demonstrated that chicks were able to identify the panel behind which there are more, demonstrating that they can add and subtract objects in a very precise way.

Finally, another arithmetic competence found in animals involve probabilities and proportion. In foraging behavior, animals can calculate the rate of return, which is the ratio of the total amount of food they obtain to the time it took to procure the food. Great tits bird (*Parus major*), visit feeding sites in direct proportion to the relative abundance of food at that site (Naef-Denzer et al., 2000). Macaques that were trained to associate 2 different lines based on their ratio length (e.g. 1: 4), were able to match the familiar ratio to the proportion of two other stimuli, one with the same ratio and one with a different ratio (e.g. 2: 4, 3: 4 or 4: 4; Vallentin & Nieder, 2008). Recently, a similar ratio abstraction ability has been found in newly hatched chicks, showing that, soon after birth, animals represent abstract proportional information from discrete elements and use this information productively in order to guide adaptive behavior (Rugani et al., 2016).

1.6. Ordinal numerical competence

Besides their cardinal properties, numerosities also possess ordinal properties. These refer to the position or rank of specific elements in relation to the other elements of the same set (*e.g.* the ability to identify the third object in a series of e.g. ten identical objects). In human infants, Brannon (2002) showed that ordinal competences appear at around the 11th month of age. In this study infants were habituated to see ascending or descending sequences of three numerical sets (configurations of black squares; *e.g.* 4-8-16 or 16-8-4) on a computer screen. Subsequently, infants were tested with novel numerical values, where the order of the presentations were maintained or were reversed with respect to those of the habituation phase. As a result, infants looked longer when the ordinal direction was reversed compared to those when it was maintained. Suanda et al. (2008) argued that 9-month-old infants were able to recognize ordinal relations when specified by multiple quantitative cues (*e.g.* number, size of the item, total surface area), but failed when only numerical cues were available. However, further experiments made by Picozzi et al. (2010) showed that 7-month-old infants succeeded at detecting and representing ordinal direction of numerical sequences relying solely on number, even when non-numerical quantitative cues were controlled. The different result obtained by Picozzi and colleagues compared to those obtained from Suanda et al., (2008) is perhaps related to the important methodological manipulation that the authors proposed. They introduced stimuli that varied in color between sequences of different numerosities, and increased the presentation time of each numerical display. Furthermore, another relevant feature that differentiated the two studies was the size of the numbers presented in the ordinal sequences, that was composed only by large numerical values in Picozzi et al. (2010), whereas those used by Suanda et al. were both small (<4) and large (>3).

In primates, Brannon & Terrace (1998) trained rhesus monkeys to order numerosities from 1 to 4 in ascending order (the stimuli were geometrical shapes or clip arts, and each set varied in size, shape or color). Monkeys were able to learn the task and, when tested with novel numerosities such as from 5 to 9, spontaneously ordered them correctly. Interestingly, Brannon and Terrace showed in a subsequent study (2010), that monkeys were also able to learn a descending rule (4→3→2→1) but not a non-monotonic rule (e.g. 3→1→4→2). These findings suggest that ordinal representations are natural and intrinsic for these animals.

Using the task developed by Brannon & Terrace (1998), ordinal abilities have been found in also in hamadryas baboons (*Papio hamadryas*) and in squirrel monkeys (*Saimiri sciureus*) (Smith et al., 2003), as well as in capuchin monkeys (*Cebus paella*; Judge et al., 2005). In non primate-mammals, rats (*Rattus norvegicus*) have been proved able to enter one box of a defined ordinal number among

an array of identical boxes, even when the position or the number of boxes was varied from trial to trial. To control for the possibility of using as cue the cumulative length or size of the boxes, rats were trained with boxes of medium size and tested with others that had a larger or a smaller size. The results confirmed that rats based their choice only on numerical cues (Suzuki & Kobayashi, 2000). Ordinal representations have been also documented in dolphins (Jakkola et al., 2005) and in different species of birds (Emmerton et al., 1997; Pepperberg, 2006b; Scarf et al., 2011). Rugani and colleagues (2007) showed for example that chicks were able to identify and peck at the 3rd, the 4th or the 6th position in a series of 10 identical elements. Moreover, when chicks were trained to peck at the fourth element and then tested with a new sequence in which the absolute distance necessary to reach the reinforced element was changed, chicks chose the fourth serial position even if such position was located much farther away, showing to rely on numerical ordinal information to identify the element rather than on absolute distances.

Even invertebrates seem to be capable of using ordinal numerical information. Dacke and Srinivasan (2008) trained bees to fly into a wide tunnel to receive a food reward in one of five sequential landmarks, whose distances were frequently and randomly varied, keeping the ordinal numerosity constant. The landmark could consist of yellow stripes, yellow disks, or overlapping baffles (Figure 7) making the bee unable to see from one baffle to the next forcing the animal to count the landmarks in a sequential way in order to identify the correct one. This study showed that bees were able to identify the reinforced landmark encountered sequentially during flight counting up to four objects.
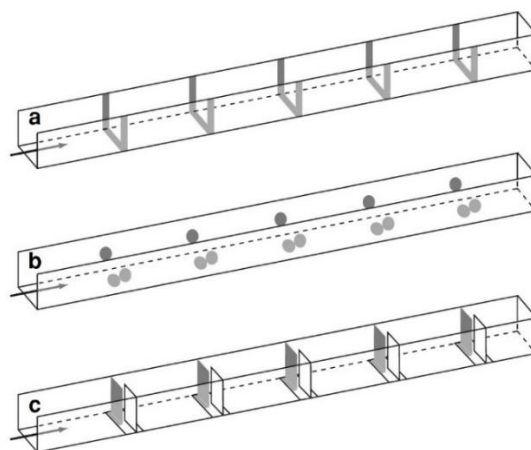

Figure 7. Bees were trained to fly into one tunnel to find a food reward in correspondence of one of the five ordinal landmark consisting of *stripes* (**a**), *circles* (**b**) and *baffles* (**c**) spaced at regular intervals (Dacke & Srinivasan, 2008).

In summary, ordinal representations have been found in several animal species. It is interesting to note that the use of ordinal information is often spontaneously preferred to the use of spatial information. This may be due to the fact that in a natural environment the relative position of stable elements (*e.g.* threes or rocks) could be used as cues for orienting, more reliably than other information such as the shape or other details of objects that could change over time.

1.7. Mapping numerosities into space

A link seems to exist between numerical and spatial representations. The natural expansion of ordinal numbers usually co-vary with the spatial position. Evidence suggest that when representing numbers humans spontaneously arrange them into space. This was first described by Galton (1880), who suggested that humans represent numbers on a left to right oriented continuum, the so called Mental Number Line, MNL (Dehaene et al., 1993).

An experimental demonstration that confirms the presence of a mental number line is the so-called Spatial-Numerical Association of Response Codes effect (SNARC effect). Dehaene, Bossini and Giraux (1993) asked participants to decide, as fast as they could, if a number was odd or even, pressing a button placed on the left or on the right. The results showed that subjects are faster in responding to small numbers with the left hand and to large numbers with the right hand, rather than vice versa. This effect depends only on the left and right side of space and was not affected by whether participants were left- or right-handed or if they crossed their hands on the response buttons.

The left-to-right direction of MNL has been traditionally explained by cultural factors and in particular to our writing and reading habits. Arab populations, with a right-to-left education, show an inverted spatial-numerical association (Zebian,2005), whereas people with mixed reading habits, such as Israelis who read words from right to left but numbers from left to right, do not exhibit reliable spatial-numerical mappings. A further study showed however that, although Israeli people do not make use of a left-to-right or right-to-left oriented mental number line (Shaki et al., 2009), they show a differential spatial mapping for small *versus* larger magnitudes in the vertical response condition, with larger magnitudes located on top of smaller magnitudes (Shaki & Fischer, 2012).

The direction of the MNL is thus clearly influenced by cultural factors. However, this does not imply that its origin is cultural. Indeed there is evidence that suggest a biological origin. One first evidence comes from people blind from birth, that are faster in responding to small acoustically-presented numbers when these are presented on the left ear and faster to big numbers when presented to the right ear (Castronovo & Seron, 2007). The first direct evidence in human infants that

magnitudes are oriented from left to right comes from Bulf, de Hevia and Macchi Cassia (2016). They found that a small numerosity (2 dots) oriented infants' visual attention towards the left hemifield and a large numerosity (9 dots) towards the right one. This suggests that the link between numbers and oriented spatial codes occurs in humans well before any exposure to formal education.

In animals, a disposition to map numbers from left to right into space, has been found in monkeys. Drucker & Brannon (2014) trained rhesus monkeys to select the fourth position from the bottom of a five-element vertical array that changed among the training in shape, location and spacing. When tested with the stimulus array into a horizontal line, monkeys selected the fourth position from the left and not from the right, suggesting that also monkeys map number onto space such as humans do, providing that the number-space association has a biological origin. Similar results had been reported before also in birds. Domestic chicks were trained to peck at the fourth or sixth element in a series of 16 identical elements arranged sagittally in front of the bird. During the test, the series were rotated by 90° in a fronto-parallel position to the birds. The results showed that birds exhibited a bias for identifying the correct element starting from the left and not from the right side (Rugani et al., 2010). However, when inter-element distances were varied during training or testing, chicks were equally likely to choose the correct position from the left or the right, suggesting that left-bias occurred solely when chicks could rely on both spatial and numerical information (Rugani et al., 2011). In a recent study, Rugani and colleagues (2016) investigated the role of the two cerebral hemisphere in MNL in chicks. Taking advantage of the lack of the corpum callosum and a complete crossing of the nerve fibers at the optic chiasm, birds have lateralized visual fields in which the visual information from one side is represented almost entirely in the opposite hemisphere (Rogers et al.,2013). Chicks were trained to identify the fourth element in a sagittal series of 10 elements. During the test the series were rotated by 90° (fronto-parallel) and chicks were tested in three different conditions. Using a temporary monocular occlusion, one group was tested in a left monocular test, one in a right monocular test, and one in a binocular test. Results showed that in all conditions chicks performed successfully the test choosing the 4th element. Interestingly, in the monocular vision condition, when the left eye was in use, chicks chose the 4th element from the left side and when the right was in use the choice was for the 4th from the right end, reflecting that the information could be independently used by each hemisphere. However, chicks tested in binocular condition selected only the element starting from left, showing a left-hemispatial bias. A right hemisphere dominance in processing visuospatial information could be reflected in a bias for attending to the left visual hemifield, resulting in a choice behavior oriented from left to right.

It is important to consider, however, that these results do not prove that birds and monkeys possess a mental number line as humans do. In the human mental number line there is a direct relationship

between the ordinal position and the numerical magnitude, which was absent in the tasks used with birds and rhesus monkeys. In fact, in order to solve the task the sequential ordering of food into containers was enough (*e.g.* the second comes after the first, *etc.*), without implying any judgement of a numerical relative magnitude. Recently, some new paradigms have been developed however with the aim to reveal a spatial-numerical association in non-human species A first evidence comes from one chimpanzee, who was trained to touch in ascending order, from smaller to larger, Arabic numbers (1 to 9) randomly displayed on a computer screen. In the test trials, only two numbers (1 and 9) were presented and horizontally arranged, one on the left side and one on the right side of the screen. Chimpanzee's performances were influenced by the spatial arrangement of the stimuli, with a faster response when 1 was on the left and 9 on the right, than vice versa. The results suggested that chimpanzees map a learned sequence onto space (Adachi, 2014). However, the task required quite an intensive sequential training and thus the results may not be conclusive about the spontaneous mapping of magnitudes onto space. Furthermore, chimpanzees were tested only with numbers "1" and "9", and this will not give us information about the relativity of the mapping, a fundamental characteristic of the mental number line.

Using a different task, Rugani and colleagues (2015) trained chicks to turn around a panel marked with a target number of elements (e.g., 5 elements) for a food reward. During the test, two identical panels were presented to the chicks, one on its left side and one on its right side. Both panels depicted the same number of elements, which was, however, different than in the training phase. The results showed that chicks spontaneously approached the left panel when the numerosity during the test was smaller (2 elements) and the right panel when the numerosity was larger (8 elements). In a second experiment, a group of chicks was presented with the target number "20" and then tested with a smaller test number (8 elements) and a larger test number (32 elements). In this condition, the number "8" constituted the smaller test number and chicks chose the left panel. In the larger test number with 32 elements chicks approached the right panel. Interestingly, chicks associated the number "8" with the right space if during the training they had experienced the number "5", and with the left side if they had experienced the number "20. These results demonstrated that the association of a number on the left or on the right was not absolute, but depended on the relative magnitude of the number referred to the target. In a third experiment, any possible effect of continuous non-numerical cues was ruled out by controlling the shape, color and size of each element. Moreover, the stimulus at training and test were controlled by balancing the overall area, the overall perimeter and density. The control of the continuous physical variables demonstrated that spatial mapping relates to the abstract numerical magnitude (Rugani et al., 2015).

1.8. The process of numerical information in the brain

Burr and Ross (2008) first showed that perceived numerosity is susceptible to adaptation, similarly to others primary visual properties of a scene such as color or size. Adaptation occurs even for sequentially presented items, across modalities (from auditory to visual and vice versa) and across spatio-temporal presentation formats (adapting to sequential of flashes affects the perceived numerosity of spatial arrays; Ross & Burr 2010; Arrighi et al., 2014). This provides indirect psychophysical evidence for the existence of neural mechanisms sensitive to numerosities.

The first experimental evidence for a direct connection between neural activity and numerosities derives from studies on the association cortex of anesthetized cat (Thompson et al., 1970). By recording neurons in the brain after the presentation of sound sequences or light flashes, the authors found that some cells were activated only after a number of events. One of the cells, for example, responded after 5 events regardless of the sensory modality (sound or light flash). This neuron began to discharge at number 4, reached the peak at 5 and then declined.

Studies with rhesus monkeys, by using a delayed match-to-numerosity task, revealed the presence of neurons which selectively encode the numerosity of visual items (Nieder et al., 2002, Nieder & Miller, 2004). In these tasks, to the monkey was shown on a screen a sample stimulus comprising a number of dots (which varies between trials) and the animal had to memorize this stimulus during a delay period. During the test the monkey had to respond whenever the numerosity displayed was the same as in the sample phase. During the initial stimulus presentation, neurons tuned to numerosity were active in the lateral prefrontal cortex (L-PFC), in the ventral intraparietal area (VIP) and in the Intraparietal Sulcus (IPS) (Figure 8). There were also some specific neurons that displayed a maximum activity in presence of a particular number of the presented quantities, showing a progressive decrease as the quantity deviates from that particular number (Nieder & Merten, 2007).
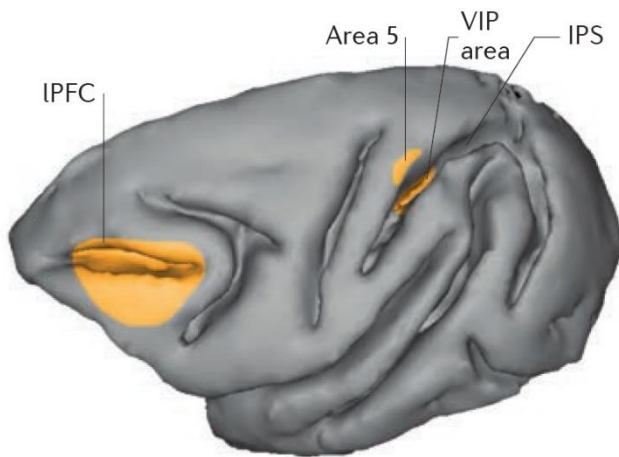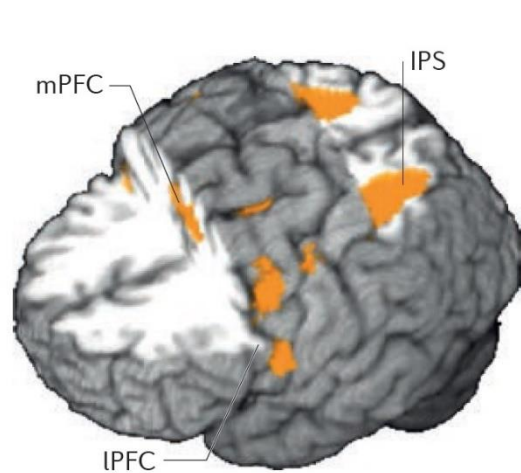
Figure 8. Frontolateral view of a monkey brain shows the areas of the number network and a frontolateral view of a human brain with areas that are consistently activated by numbers in functional imaging studies (Nieder, 2016).

In humans, a series of functional imaging studies suggests that frontal lobe areas and posterior parietal areas are involved in the nonverbal and symbolic representation of quantities (Piazza et al., 2007). Using quantitative meta-analyses of fMRI studies, Arsalidou and Taylor (2011) identified brain regions among studies that used number and calculation tasks, showing that numerical processing is repeatedly associated with a mainly activation in the inferior and superior parietal lobe and in the IPS, as well as in frontal lobe areas such as the inferior and middle frontal gyrus of the lPFC and the cingulate gyrus of the medial PFC (mPFC).

Harvey and colleagues (2013) reported that the posterior parietal areas shows a spatially organized numerosity map. Different numbers activate different contiguous areas in the parietal lobe in a topographical representation, where numerosities that are nearby to each other (e.g. 4 and 5) activate also brain areas that are positioned next to the other (Harvey et al., 2013).

1.9. Numerical discrimination in fish

Fish have recently become a well investigated model species in the study of cognition under many aspects, including the study of numerical abilities. The use of animal species phylogenetically very distant from human and without any kind of language can be useful to increase our knowledge on the underlying biological mechanisms and can provide evidence on the origin of numerical abilities in vertebrates.

1.9.1. Spontaneous numerical discrimination

Several fish species manifest social behaviors and live in groups of conspecifics. Pitcher and Parrish (1993) distinguish two types of aggregations: "shoal" and "school". "Shoal" is defined as any generic group of fish tied to social attraction (Magurran, 1990), while "school" means a group that has synchronized and polarized swimming. Living in a large shoal provides a protection against predators, the so-called "dilution effect" (Foster & Treherne, 1981), and at the same time provides a greater opportunity to detect predators (Pulliam, 1973). These advantages increase as the number of the shoal increases, so when two groups of conspecifics are available, the single subject is strongly motivated to join the bigger one, especially if the environment is potentially dangerous.

One of the first experiments that investigated spontaneous discrimination in fish has been conducted by Hager and Helfman (1991) in fathead minnows (*Phimephales promelas*). Individual minnows were presented with a series of choices between two shoal sizes, ranging from 1 to 28 fish, both with and without a predator present. Although responses were highly variable, minnows displayed an ability to choose between shoal sizes even when size differences were small, preferring the larger one. In the presence of a predator, fish made quicker shoaling decisions and showed a strong tendency to avoid very small shoals.

Most of the studies in fish that aim to assess the quantity discrimination ability have taken advantage of the shoaling behavior. In this task, a subject is typically inserted into an unfamiliar tank where it sees two groups of social companions, differing in number. The approach and the time spend near the two shoals is taken as a measure of discrimination (Figure 9).
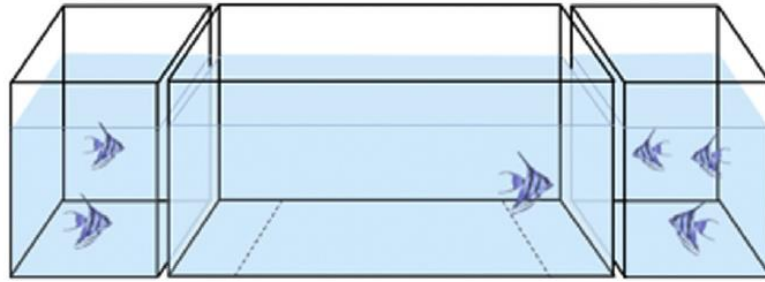
Figura 9. Schematic representation of the experimental apparatus used in a spontaneous choice between two different shoals of social companion (Agrillo et al., 2017).

Using this procedure, Gómez-Laplaza and Gerlai (2011b) have examined whether angelfish (*Pterophyllum scalare*) are able to discriminate between shoals of small numbers of conspecifics. The results demonstrate that fish reliably chose 1 *vs.* 4, 1 *vs.* 3, 1 *vs.* 2 and 2 *vs.* 3 individuals, but were at chance performance level when having to choose between 3 *vs.* 4. Similar results have been obtained in goldbelly topminnow (*Girardinus falcatus;* Agrillo & Dadda, 2007). Other fish species tested in similar condition show a better discrimination, mosquitofish (*Gambusia affinis*; (Agrillo et al., 2008a) and guppies (*Poecilia retriculata*; Agrillo et al., 2012a; Piffer, Agrillo & Hyde, 2012) can discriminate groups of 1 *vs.* 2, 2 *vs.* 3 and 3 *vs.* 4, but not 4 *vs.* 5. Recently, Lucon-Xiccato and colleagues (2016), using a similar method to investigate shoal preference, had extended to guppies discrimination up to 4 *vs.* 5 elements.

Besides small quantities comparison, fish show abilities even in large shoal discrimination. Swordtails fish (*Xiphoporus ellerm*; Buckingham et al., 2007), mosquitofish (Agrillo 2008a) and guppies (Agrillo 2012a) were able to discriminate large numerosities such as 4 *vs.* 8 or 8 *vs.* 16, providing that the bigger group was at least twice that of the smaller one (ratio up to 0,5). Other evidence ratio related has been found in angelfish (Gómez-Laplaza 2011a), with a limit at 0.56 ratio. In three-spined sticklebacks (*Gasterosteus aculeatus*) it has been found a preference to shoal with the larger groups with contrast discrimination up to 6 *vs.* 7, equaling a numerical ratio of 0.86. Such a large ratio has not been shown to be discriminated by any other fish species (Mehlis et al., 2015).

As previously described, the choice for the bigger group is the most effective strategy in order to receive protection, but is equally useful for predators when they have to locate and attack a prey. Blue acara fish (*Aequidens pulcher*) prefer to attack first, given two shoals of guppies, which differed in size (2 *vs.* 5 fish, 5 *vs.* 10, 2 *vs.* 10), the larger one. Interestingly, wherein predators were given an opportunity to attack free-swimming shoals, predator hunting success decreased with increasing shoal size (the so called "confusion effect". Landeau & Terborgh, 1986; Krause & Godin 1995). The same

behavior to attack the larger group has been obtained in wolf fish (*Hoplias malabaricus*; Botham & Krause 2005).

Shoaling behavior towards the bigger group is a good strategy, but is not always the preferred choice for the individual, because sometimes it is overwhelmed by the effect of hunger. Golden shiners fish (*Notemigonus crysoleucas*) deprived of food have, for example, a significantly weaker preference for the larger shoal compared to well fed fish (Reebs & Saulnier, 1997). Hoare and colleagues (2004) have shown that Banded killifish (*Fundulus diaphanus*) prefer to join larger shoals when the risk of predation is high. On the other hand, when there is the possibility to get food they tend to move towards a smaller group. Furthermore, when both food and alarm cues are presented together, fish formed groups that are larger than control groups but smaller than those seen with only alarm cues. Results support the hypothesis that hungry fish tend to prefer food instead of protection and this must be taken into account when numerical abilities are investigated, because in this scenario a lack of preference could not reflect an inability to discriminate, but a strategy to maximize the food intake.

In addition to the evidence described above, shoaling preference for the bigger group has been reported in several other fish species such as in red-bellied piranha (*Pygocentrus nattereri*, Queiroz & Magurran, 2005), siamese fighting fish (*Betta splendens*; Snekser et al., 2006) sailfin molly (Poec*ilia latipinna*; Bradner & McRobert, 2001), two-spotted goby (*Gobiusculus flavescens*; Svensson et al., 2000). In contrast, in some species, shoaling is not a strategy as strong as for other fish. Central mudminnow fish (*Umbra limi*) prefer to spend time near the aggregate of conspecific rather than an empty aquarium, but does not between a small and a big shoal of conspecifics (3 *vs.* 7 and 3 *vs.* 12) suggesting that the shoaling decisions are not affected by the shoal size (Jenkins & Miller, 2007). Sailfin molly has a shoaling behavior that is strongly related to the color of conspecifics. Although there is a preference for the larger shoal when both groups are similar or dissimilar in color to themselves, when the two groups differ in color, fish prefer the similarly colored to themselves, even if the group is smaller, suggesting that color has a strong influence in the shoal decision (Bradner & McRobert 2001).

Despite the bunch of evidence described, the lack of control of the non-numerical physical variables does not allow us to consider it as a proof of numerical competence. In fact, in all these studies, the stimuli were visible at the moment of the choice and the fish could have used non-numerical continuous information, such as the cumulative area, the density, or the total activity, that are bigger in the larger group.

Gómez-Laplaza and Gerlai (2013) studied the possible influence of continuous variables in angelfish manipulating density (Figure 10a), inter-fish distance (Figure 10b) and overall space

occupied by the shoals (Figure 10c), one factor at a time. Proposing shoals contrast of 2 *vs.* 3 and 5 *vs.* 10 conspecifics, the fish did not show any interference in discrimination when the distance between the fish and the size of space occupied by the two shoals was manipulated, preferring the larger group in both conditions. However, when the density of the two shoals was varied, a preference for the denser shoal has been found even if it was smaller in number, suggesting that this non-numerical cue could be used by angelfish to discriminate shoal size.
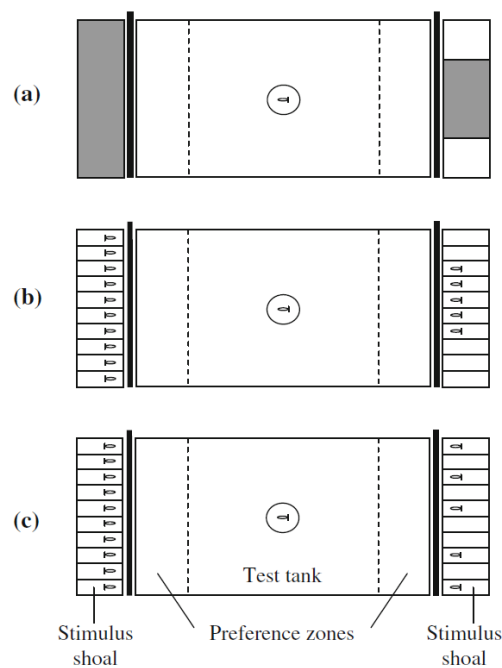
Figure 10. Schematic overview of the experimental apparatus. **(a)** The grey area on the sides indicate the stimulus shoal, density of the stimulus shoals in was balanced. **(b)** The distance among fish shoal was keep constant placing individual fish into single partitions. **(c)** To control for the overall spatial area occupied by the shoals the distance between the outermost fish was equated (Gómez-Laplaza & Gerlai (2013).

A strategy used in several studies to control the movement and the activity of the shoal is to modify the temperature of the water. Fish usually live in a range of optimal temperature and the activity increases as water temperature increases. Given that, it is possible to control for the different activity of the two shoals placing the bigger group in a lower temperature water and the smaller one in warmer water. Zebrafish (*Danio rerio*) tested in a shoaling discrimination with normal temperature prefer the larger shoal in 1 *vs.* 4 and 2 *vs.* 4 comparison, but not in 3 *vs.* 4 and 4 *vs.*4. However, when the water temperature differed in the two groups (warmer for the smaller and colder for the bigger), zebrafish spent more time with the group that was more active overall, even if it was smaller (Pritchard et al., 2001). Water temperature affects the performance of other fish species such as mosquitofish, that did not discriminate in this condition 2 *vs.* 3 conspecifics, but discriminate large

quantity discrimination such as 4 *vs*.8 (Agrillo et al., 2008a). In the same condition, Angelfish did not show preference both for the small and larger set (Gómez-Laplaza & Gerlai, 2012). A critical observation made on these studies that used the temperature water to control for the shoal activity is that the behavior of the stimulus and the attractiveness is lower. In some species, immobility is a strategy for avoiding predator detection, called "freezing" (Chivers & Smith, 1994, 1995), and the fish could then interpret the lower movement as an alarm cue and avoid the shoal.

A technique that prevent access to continuous variable is the item-by-item presentation, where the subject can see each element, one at time, without having the entire view of the global set of stimuli. An adaptation of this method has been studied in mosquitofish using a modified version of the shoal choice test in which each fish was isolated (Dadda et al., 2009). In this test, subjects could choose between one large and one small group of companions. However, the subject could see only one fish at a time, because a series of screen did not allow to have an overview of the entire group of conspecifics (Figure 11). Results showed that fish were able to identify the bigger shoal both in small (3 *vs.* 2) and large numbers (8 *vs.* 4) discrimination, showing that the performance was not affected by the overall space occupied by the group and reflecting a pure numerical ability, since all the potential physical variables was controlled for.
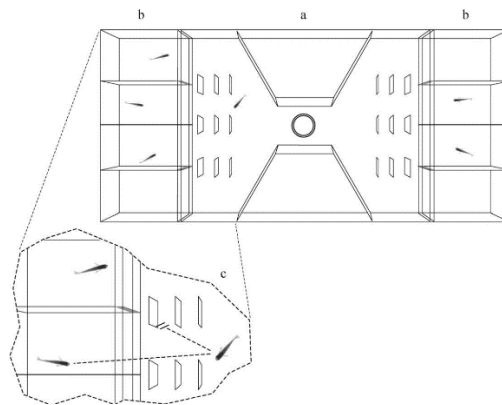
Figure 11. Schematic representation of the apparatus. Subject were placed in the central sector (a) and two shoals of conspecific were placed on opposite sides (b)In each choice area were placed 9 vertical plastic screens aligned in a grid of 3 by 3, so that the subject could only see one stimulus fish at time from any position of its sector (c) (Dadda et al., 2009).

Another experimental strategy that could reduce the use of continuous variables has been proposed by Stancher and colleagues (2013) in redtail splitfin (*Xenotoca eiseni*) fish. In this experiment, two numerically different shoals were presented at the opposite ends of the test aquarium, directly visible to the test fish during an observation period. At test, some fish from the bigger shoal were hidden by the experimenter behind an opaque area, so that an equal number of fish in each shoal became visible to the subjects (for example, for the contrast 1 *vs.* 2, one fish were visible in each

shoal, whereas for the 2 *vs.* 3 contrast, two fish were visible). Doing this, stimuli shoals were not fully visible at the moment of the choice preference by the test fish. Fish prefer to approach the location previously occupied by the larger number between two groups of conspecifics in sets of 2 *vs.* 3 items, but failed at 3 *vs.* 4 items. The same limit of discrimination has been found using this method in angelfish (Gómez-Laplaza et al., 2015) and zebrafish (Potrich et al., 2015).

Spontaneous numerical discrimination in fish has used not only shoaling techniques, there is also evidence of food quantity discrimination. Guppies discriminate and prefer the bigger quantity of small pieces of food flakes glued on plastic cards in 1 *vs.* 4 and 2 *vs.* 4 comparison, but not in 2 *vs.* 3 (Lucon-Xiccato, 2015). Guppies discrimination accuracy using this food choice technique was lower than that reported in shoal choice experiments, in which guppies discriminated up to 3 *vs.* 4 conspecifics (Agrillo et al., 2012a). Furthermore, other conditions pointed out that guppies prioritize the selection of the largest food item over the total number of items and the total amount of food. The reason of this preference is probably justified by a competition for food in shoal, favoring a strategy that maximize the chance of consuming larger food items before their companions.

1.9.2. Training procedure in fish

Studies that involve a training procedure are recently increasing, due to the need to have more precise control over the stimuli and to study in detail specific numerical abilities that are not easily identifiable using a spontaneous procedure.

Using a training procedure with social reward, mosquitofish have been trained to discriminate between different numbers of two-dimensional figures. Fish were placed into an unfamiliar square environment and were allowed to join a group of social conspecifics by passing through one of two identical tunnels at the opposite corners. Each tunnel was associated with two or three geometric figures and only the one with number of elements reinforced allowed the fish to go out of the apparatus and rejoin the companions. Results showed that fish were able to discriminate 2 *vs.* 3 elements, even when the total perimeter length or the brightness of the stimuli was equalized. However, when the stimuli were matched for the cumulative surface area fish did not discriminate, suggesting that area is primarily used during the learning process (Agrillo et al., 2009).

With the attempt to directly compare numerical abilities in different fish species using the same methodology, Agrillo and colleagues (2012c) proposed an identical task to redtail splitfin, guppies, zebrafish, siamese fighting fish and angelfish. The fish were trained to discriminate between two sets of geometrical figures on an easy numerical ratio (5 *vs.* 10 and 6 *vs.* 12) using a food reward. Once

they reached the learning criterion, they were observed in non-reinforced probe trials in which the numerical ratios or total set size varied. Fish trained with the larger or smaller numerosities as positive showed equal accuracy in all species. When the ratio increased, all the species except angelfish were able to discriminate the 0.67 ratio (8 *vs*. 12), and no species being able to discriminate the 0.75 ratio (9 *vs*. 12). When the ratio was constant, but the total set size was increased (25 *vs*. 50) or decreased (2 *vs*. 4), fish generalized the learned discrimination to a smaller set size, but not to a larger one. Only minor differences were observed among the five species and these data support the existence of similar numerical discrimination among fish.

In mammals and birds, numerical competence has been tested using a variety of stimuli such as visual and auditory, but in fish the evidence reported until recently was limited to visual stimuli. To overcome this, numerical abilities have been investigated in a blind cavefish species (*Phreatichthys andruzzii*) in a non-visual sensory modality (Bisazza et al., 2014). Fish were trained to receive a food reward to discriminate between two groups of objects placed in opposite positions of their home tank. Cavefish showed ability to discriminate a 0.5 ratio (2 *vs*. 4) even when continuous quantities were not available from the beginning of the training, showing evidence of non-visual numerical discrimination in fish.

Finally, aside from the great number of evidence that investigate the cardinal aspect of the number, recently use of ordinal information has been found in guppies (Miletto Petrazzini, 2015). Guppies, in fact, learned to recognize the 3rd feeder in a row of 8 identical feeders, with distances between feeders that changed among trials. To assess whether guppies spontaneously use ordinal or spatial information, fish were trained with a fixed spatial configuration of the feeders and tested with a different position and inter-feeder distance. At test, fish selected the correct ordinal position more often than the original spatial disposition, showing a spontaneous use of ordinal information.

The study of numerical abilities in fish have begun to be intensively investigated. Several fish species show an use of numerical/quantity information, suggesting a similarity in numerical abilities between fish and other vertebrate animals.

1.10. Aim of the thesis

The aim of this work was to investigate several aspects of numerical abilities in zebrafish (*Danio rerio*). This species was chosen because, given its widespread use in the field of genomics, it may provide a useful model organism linking research from behavior and genetics to the study of numerical representations. The experimental part is divided in four main sections, each of which analyzes a different aspect of numerical cognition.

In the first part the spontaneous discrimination of quantity (magnitude) between two shoal groups with different number of social companions was investigated. Using a shoal quantity discrimination with stimuli not visible at the time of choice, I tested a wide range of discrimination conditions both with small and large numerosities. Besides assessing the range of number discrimination, this experiment aimed to provide evidence on which representational systems (ANS or OFS) could be engaged in the discrimination of small and large numerosities.

The aim of the second part was to establish if, in addition to evidence of a discrimination of numerosities simultaneously presented, zebrafish can track one-by-one additions of single elements. The stimuli proposed were single conspecifics that one at time appeared and then disappeared behind one of two opaque screens, forming two groups that differ in numbers. By observing the spontaneous approach towards one of the two groups, I assessed if zebrafish are able to do mental transformations over numerical values, highlighting the possess of proto-arithmetic addition abilities. In order to exclude the interference of potential non-numerical variables, such as the different overall time of the stimuli presentation in the two groups, we provided control condition equalizing the temporal variable.

In the third part I investigated whether zebrafish are able to identify a particular element in a series of identical object on the basis of its ordinal position. Afterwards, the fish underwent tests that helped us to understand if ordinal information rather than other non-numerical information such as spatial distances among the set of objects was spontaneously used by the fish.

The aim of the fourth part was to study the possible link between number and space in the mapping of numerosity in zebrafish. I trained zebrafish to associate a target number with a reward, and then tested them in a preference choice test between two identical numerosities, but different to the training one, placed on the right and the left side of the experimental apparatus. By observing the approach towards one of the two stimuli, I checked whether zebrafish spontaneously associated smaller numbers with the left space and larger number with the right space, as in the case of the mental number line observed in other species.

EXPERIMENTAL PART

THE MODEL SPECIES: ZEBRAFISH (*Danio rerio*)

2.1. Ecology and morphology

Danio Rerio, commonly known as "zebrafish", is a small and gregarious tropical freshwater fish belonging to the order *Cypriniformes*. It is a small cyprinid fish native to the streams of south-eastern Himalayan region (Talwar & Jhingran, 1991) and it is widely distributed over the Indian subcontinent. In nature, zebrafish usually lives in stagnant or shallow basins (e.g. canal, brooks and rice fields) rich in vegetation and from the silty substrate. The typical monsoon climate of the Indian subcontinent also creates significant seasonal variations, which are reflected on the temperature range tolerated by the species: about 6 ° C in winter and 38 ° C in summer (Spence et al., 2008). Zebrafish lives in large shoals, feeds on zooplankton and insects, and has an extremely vibrant reproductive life, sensitive to photoperiod and food cycles.

From the morphological point of view, zebrafish is a bony fish with a fusiform and laterally compressed body shape, with a terminal oblique mouth directed upwards and a lower jaw that protrudes further than upper. The fish has two pairs of barbels and five to seven dark blue longitudinal stripes alternate to gold stripes, extending from behind the operculum into the caudal fin (Barman, 1991). Adult rarely exceeds 40 mm in length (from the tip of the snout to the origin of the caudal fin). In this species there is a small sexual dimorphism between males and females. However, it is possible to distinguish between them because the male tends to have a bigger and deep colored anal fin, while females have a less thin body and a bigger size (Figure 12).

Figure 12. The image shows a male (on the top) and a female (on the bottom) zebrafish. Although the color is almost similar, females and males differ in body size and shape: females, in fact, have larger size and a more prominent abdomen than males.

Reproduction, ovulation and egg laying occurs only if the male is present. Embryonic development begins with the release of eggs. A very useful feature for genetic studies is the transparency of fertilized eggs during embryonic development, making this species a convenient model for research. All the major organs begin to develop from 12 to 36 hours after the laying of the eggs, depending on the outside temperature and internal conditions of the embryo, while the development process lasts 3-4 months. The fish can survive for up to 3-5 years (Spence et al., 2008).

The extreme versatility of zebrafish in experimentation is partly due to the fact that, compared to other animal models (e.g. *Drosophila melanogaster* and small mammals), it can be easily manipulated from a genetic and embryological point of view, and with lesser costs (Spence et al., 2008). The ability to visualize in vivo embryos during the fertilization phases has enabled scientists to accurately determine the development stages in zebrafish. Incubated at a temperature of about 28 °C, zygote begins to differentiate within the first hour; after 10 hours the first movements and tail (segmentation period and primary organogenesis) appear; after 24 hours the pigmentation and the circulatory system are visible, the fins begin to develop; after 48 hours the morphogenesis of the primary organs is complete, the cartilage develops on the pectoral fins and on the head (Kimmel et al., 1995). Larvae hatch at 72 hours post-fertilization and reach maturity at around 3 months in laboratory, while in the wild they need 6 months (Spence et al., 2008).

Zebrafish is an animal model widely used in genetic, pharmacological and biological research. Zebrafish can be used in the study of human genetic disorders because about 70% of human genes have at least one obvious zebrafish orthologue, a homologous gene present in different species but that encodes for proteins with similar structure and features (Howe et al., 2013).

2.2. Social Behavior

Numerous species of fish, including zebrafish, tend to aggregate into groups of social companions, a behavior called "shoaling". Shoaling guarantees each member of the group some important evolutionary benefits. For example, shoaling provides anti-predatory advantages such as less time spent on vigilance and more time for foraging (Magurran & Pitcher, 1983) and it facilitates the localization of food (Krause & Ruxton, 2002). However, shoaling could also have a negative effect on foraging success because there is more competition to food access. Zebrafish that are deprived of food are less likely to create a compact shoal (Miller & Gerlai, 2007).

In zebrafish, shoaling behavior is related to selective pressures, and it occurs early after the hatch. Zebrafish larvae begin to display a preference for conspecifics approximately 12 days post-fertilization. Nevertheless, larvae do not discriminate between stimulus shoal, such as the reared stimulus phenotype, until it arrives at the juvenile stage (Engeszer et al., 2007a)

In general, zebrafish always prefer to stay with conspecifics, even if they have a different phenotype to the individual (Sneckeser et al., 2006), though whenever possible they prefer to shoal with conspecifics of a similar phenotype (Rosenthal & Ryan, 2005; Sneckser et al., 2010).

Zebrafish show a high degree of social cohesion and exhibit particular preferences for some distinctive traits of the species. A study by Saverino and Gerlai (2008) has shown that zebrafish respond to some visual features during discrimination of conspecifics from other species. The absence or the orientation of the stripes in a virtual stimulus companion did not affect the attraction by the fish, whereas a yellow coloration elicited a strong attraction (due probably to the fact that yellow is a signal of health or reproductive maturity). Elongated body silhouettes created an avoidance response, probably associated with the one of a predator.

Zebrafish, like many other fish species, prefer to join large shoals of conspecifics (Pritchard et al., 2001). The sex of the fish could influence choice. Male zebrafish prefer to shoal with females over males, while females do not show preferences (Ruhl & McRobert, 2005).

2.3. Vision perception: shape and color discrimination

Zebrafish is a fish that make strong use of the vision and, considering the fact that the studies proposed in this thesis made use of visual stimuli, it is important to review some of the visual abilities in zebrafish, such as the discrimination of form and the natural preference to certain colors.

Oliveira and colleagues (2015) studied the ability to discriminate three-dimensional objects on the basis of shape, color and size cues. Zebrafish were able to discriminate objects according to the shape (for example, cube *vs.* cylinder), color (for example, blue *vs.* yellow) and the combination of shape and color (for example, blue cube *vs.* yellow cylinder). However, the size did not seem to be a salient cue: two objects of the same shape and color but different in size, were not distinguished. The ability to identify, recognize and categorize objects in the environment allows the animal, in nature, to identify food, shelter, conspecifics and predators.

Colors are an important feature used by zebrafish, and the natural preference for particular color pattern has been investigated. Compared to humans, vision in zebrafish is tetrachromatic, showing a color vision with peak absorbance in ultraviolet (362nm), blue (415nm), green (480nm)

and red (570nm) (Fleisch & Neuhauss, 2006; Spence, 2008). Testing zebrafish in a circular tank divided into four lateral compartments with different environmental colors (respectively blue, green, yellow, red), revealed a preference for the blue and green sectors (Oliveira et al., 2005). In contrast, Avdesh and colleagues (2010) found that zebrafish manifest an avoidance reactions to the blue, compared to green, red and yellow. In another task, a *T-maze test* with different color choice, red and green were equally preferred, and both were preferred over yellow (Avdesh et al., 2012). Zebrafish show also color biases preference both in context of foraging and mate choice. Conspecifics with a high yellow coloration, trigger a strong approach response (Saverino & Gerlai, 2008). Similarly, in a color preference task in a foraging context, zebrafish respond more strongly to red (Spence & Smith, 2008). The natural color preference in zebrafish seems therefore to be related to the characteristics of the object; a neutral object, a conspecific or the environment could be associated with different color preferences.

STUDY 1

Quantity discrimination by Zebrafish

The present study has been published in a peer-reviewed journal: Potrich D., Sovrano V.A., Stancher G. and Vallortigara G. (2015). Quantity Discrimination by Zebrafish (*Danio rerio*). *Journal of Comparative Psychology* 129(4):388-93. Part of the original manuscript has been reported in the present study.

The aim of this study was to test the numerical/quantities discrimination in zebrafish (*Danio rerio*) by using a spontaneous choice preference task between two shoals with different numbers of social companion.

The discrimination ability is a widespread competence well documented throughout the animal kingdom. Despite the evidence, it is unclear and currently under discussion which are the mechanisms underlying numerical discrimination. Discrimination of quantity has been suggested to rely on an "*Approximate Number System*" (ANS). This system provides approximate estimates of numerical magnitude of a set of objects, and the accuracy of the response depends on the ratio between the two numerical magnitudes that must be discriminated, following the Weber's Law (Gallistel, 1990). There is now evidence for the existence of ANS in a variety of non-human species (for a review, see Vallortigara, 2014, see Introduction 1.2). It has been hypothesized that a second system would exist, that allows to simultaneously represent and track objects through parallel individuation, dubbed "*Object File System*" (OFS) (see Carey, 2009, for a review). Representations through parallel individuation is therefore more accurate and precise than those of the ANS, but it is limited to only a few items (usually about three).

Some evidence has been reported that the two systems may operate independently. For instance, when 12- to 14-month-old infants were required to choose between two quantities of crackers, visibly placed, one cracker after the other, in one of two opaque buckets, they succeeded and chose the larger set with 1 *vs.* 2, 1 *vs.* 3, and 2 *vs.* 3 elements, but failed with 1 *vs.* 4 or 2 *vs.* 4. The failure with 1 *vs.* 4 or 2 *vs.* 4 was interpreted as due to activation of representations in the two separate systems (Feigenson et al. 2002).

Research using phylogenetically distant species may provide insights about the origins and the mechanisms for quantity discrimination. In fish, some evidence for a two-system mechanism has

been reported. Piffer, Agrillo, and Hyde (2012) confined *Poecilia reticulata* fish singly in the center of a tank, where, on the two opposite sides, two numerically different groups of conspecifics were located. After an observation period, the test fish were left free to move toward one or the other of the two groups, choosing the larger one. The results showed that, like human infants, fish were able to discriminate between two large numerosities (e.g. 5 *vs.* 10), as well as two small numerosities (e.g. 3 *vs.* 4), but they failed in the comparison between two groups with numerosities that crossed the boundary of the two systems (3 *vs.* 5). However, these results were not confirmed in a different species of fish (angelfish, *Pterophyllum scalare*) by Gómez-Laplaza and Gerlai (2011). These authors found that angelfish succeeded when had to compare groups with two numerosities that belonged, respectively, one to the small system and one to the large system (such as, e.g., a discrimination between 1 *vs.* 4 elements).

In all these studies with fish, the elements to be discriminated (conspecifics) were fully visible to the test animals, making the role of continuous physical variables that co-vary with number (area, contour length, amount of motion and so on) very prominent. Stancher, Sovrano, Potrich, and Vallortigara (2013) developed a novel procedure that made use of social stimuli as attractors, as in studies of Piffer et al. (2012) and Gómez-Laplaza and Gerlai (2011), that were, however, not visible to the fish during the test, similar to experiments with infants (Feingenson et al., 2002). In this task, fish should compare what they see at the moment of choice (an equal number of conspecifics in two different locations) with memory of the location previously occupied by the larger in number between two groups of conspecifics. The results with redtail splitfin fish (*Xenotoca eiseni*) showed that they chose the location previously occupied by the larger in number between two groups of conspecifics in sets of 1 *vs.* 2 items, and 2 *vs.* 3 items, but failed at 3 *vs.* 4 items, showing the same set-size limit as infants and other species of fish for discrimination of small quantities. However, unlike infants, fish succeeded when they were tested for performance at discriminations between two numerosities that spanned outside the boundary of the two presumed numerical systems (1 *vs.* 4 and 2 *vs.* 4). This would suggest that the set-size limit at around three items would reflect a ratio limit (i.e., discrimination of three *vs.* four) rather than the signature of a different system (the OFS).

In the present study, we used the same method in zebrafish, comparing different numerical discriminations, with the aim to provide an extension to the range of number ratios and fish species studied.

3.1. EXPERIMENT 1

The aim of the first experiment was to check whether zebrafish are able to discriminate between small quantities of conspecifics by testing the comparisons 1 *vs.* 2, 2 *vs.* 3, 3 *vs.* 4. We tested then the performance in discriminations of the fish between two numerosities spanning outside the boundary of the two presumed numerical systems by proposing the comparison 2 *vs.* 4. Finally, the condition 4 *vs.* 6 tested zebrafish's ability between large quantities discrimination.

3.1.1. Subjects

Subjects were reared in a 20-L plastic aquarium (23 x 38 x 25 cm) in isolated sex groups, in order to make the discrimination of sex easier. The ground was covered with gravel and vegetation mimicking a natural environment. The water temperature was maintained at 25 °C and each aquarium contained a pump and filter system (Micro Jet Filter MCF 40). The aquariums were illuminated by an 18-W white fluorescent lamp following a light cycle of 12 hr. Fish were fed twice a day with dry food (Sera GVG-Mix). In all the experiments, males were used as test subjects, whereas females were used as target (sexual) stimuli. The range of fish total lengths was between 4 cm and 5 cm for both males and females.

3.1.2. Apparatus

The experimental apparatus (see Figure 13a) consisted of a plastic rectangular test tank (33 x 23 x 20 cm) with white-colored walls. On the shorter sides of the tank, two isolated sectors (20 x 5 x 17 cm) were located, each housing one of the groups of females of different numerical size used as target stimuli. The visible and the opaque sectors were separated by a white plastic (Poliplak) panel, which could slide vertically, dividing each sector into two environments: a transparent environment, fully visible from the center of the tank, and an opaque environment, not visible from the outside (Figure 13b).
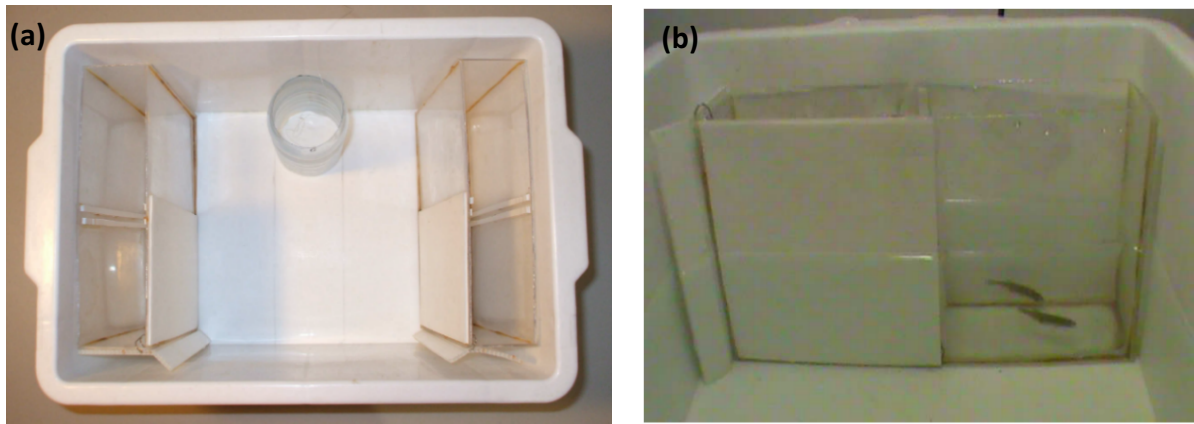
Figure 13. (a) photograph of the apparatus used for the experiment; **(b)** Photograph of sector with two female stimulus visible.

At the center of the tank, the male subjects could freely swim during the test trial. This central part has been divided in three different areas, indicated by two thin lines on the floor of the tank. The lines delimitated a neutral central area (6,5x23 cm), and two choice area (23x8 cm) close to the two sectors housing the female shoals. This allowed us to have an objective assessment of the choices made by the subject during the test.

During each test, a transparent plastic cylinder (6.5 cm in diameter and 14.5 cm in height) was placed in the center of the apparatus, where the experimental subject was confined. At the top of the cylinder, a transparent nylon wire allowed to lift up the cylinder vertically using a pulley system. In this way, the fish was not disturbed by the experimenter's presence while he was releasing it. To avoid interference from the outside, a white Poliplak screen (23 x 23) was placed over the tank during the experiment. The apparatus was lit by a fluorescent 60-W lamp, and a video camera (SONY Handycam DCR-SR58) recorded fish behavior from above (80 cm) the setup.

3.1.3. Procedure

Before starting the experiment, both male and females were fed in order to reduce the possible competition for food and a less strong shoaling behavior (Reebs & Saulnier, 1997). At the beginning of each trial, female stimuli were first introduced into the transparent part of the sectors (e.g. in the case of a comparison of one *vs.* two, two females were introduced into the sector to the right, and one female into the sector to the left). Subsequently, the test male was introduced in the middle of the tank in the transparent plastic cylinder. At this stage, defined "observation period", the male had the opportunity to see both groups of females for 5 minutes (Figure 14a).

At the end of this observation phase, one or more of the females, depending on the discrimination to be carried out (in the example of 1 *vs.* 2 discrimination, one female), of the sector housing the larger females group were gently moved, using a transparent plastic stick (without touching the animals), into the opaque part of the sector so that they were no longer visible to the test male, leaving exactly the same number of females visible in the transparent parts of each of the two sectors (one female in each sector in the example; see Figure 14b).
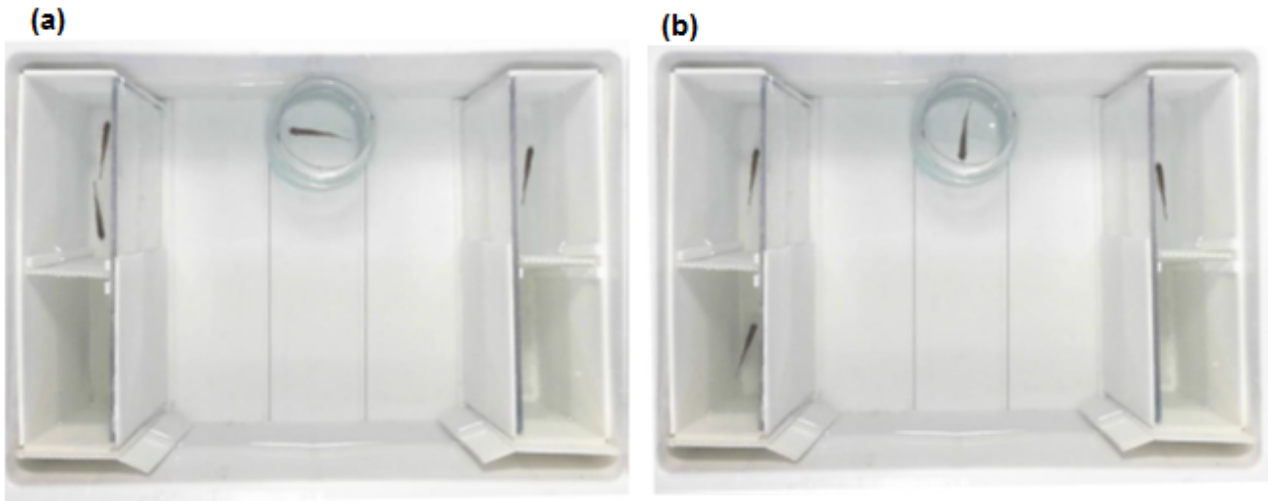

Figure 14. Photographs of a trial during the 1 *vs.* 2 discrimination **(a)** "Observation phase": the fish can see one group with two females and one group with one female. **(b)** At test, an identical number of fish is visible to the test fish at the opposite ends of the set up through the transparent sectors.

After a delay of 5 or 30 seconds ("latency period"), the cylinder housing the test male was gently lifted up, allowing the test fish to approach one or the other of the two sectors. Male fish were tested in two daily blocks of 15 trials, one half of the animals first with a delay of 5 s, and then with a delay of 30 s, after disappearance of some of the females, and vice versa for the other half. The interval between trials was 2 minutes and the male subject was confined in the cylinder and placed in correspondence of the opaque sectors of the apparatus.

The first choices of the test males were computed from the video recording. A choice was considered when the fish entered with the entire body in one of the two areas close to the two sectors identified by the black lines drawn on the floor. The position (sector to the left or to the right) of the larger group of females was changed at every test trial, following a semi-random sequence (Fellows, 1967). The use of a semi-random procedure is extremely important because it excludes possible results due to a lateralized behavior or an effect due to learning among the trials.

We paid particular attention to select females of the same size and as close as possible to the size of the test male.

At the end of the experiment, before starting a new subject, the water of the apparatus was completely substituted with clean water, deleting any possible olfactory cue.

3.1.4. Discrimination 1 *versus* 2

Eight males (experimental subjects) and three females (sexual attractors) of *Danio rerio* were used in this experiment. One female was placed in one sector, and two females in the other sector. At the end of the observation phase, one of the females of the larger group was moved into the opaque part of the sector, so that at test, after a delay of 5 s or 30 s, exactly the same number of females ($n = 1$) were visible to the test male subject in each of the two sectors.

3.1.5. Discrimination 2 *versus* 3

Eight males and five females of *Danio rerio* were used in this experiment. Two females were placed in one sector, and three females in the other sector. At the end of the observation phase, one of the females of the larger group was moved into the opaque part of the sector, so that at test, after a delay of 5 s or 30 s, exactly the same number of females ($n = 2$) were visible to the test male subject in each of the two sectors.

3.1.6. Discrimination 3 *versus* 4

Eight males and seven females of *Danio rerio* were used in this experiment. Three females were placed in one sector, and four females in the other sector. At the end of the observation phase, one of the females of the larger group was moved into the opaque part of the sector, so that at test, after a delay of 5 s or 30 s, exactly the same number of females ($n = 3$) were visible to the test male subject in each of the two sectors.

3.1.7. Discrimination 2 *versus* 4

Eight males and six females of *Danio rerio* were used in this experiment. Two females were placed in one sector, and four females in the other sector. At the end of the observation phase, two of the females of the bigger group was moved into the opaque part of the sector, so that at test, after a delay of 5 s or 30 s, exactly the same number of females ($n = 2$) were visible to the test male subject in each of the two sectors.

3.1.8. Discrimination 4 *versus* 6

Eight males and 10 females of *Danio rerio* were used in this experiment. Four females were placed in one sector, and six females in the other sector. At the end of the observation phase, two of the females of the bigger group was moved into the opaque part of the sector, so that at test, after a delay of 5 s or 30 s, exactly the same number of females ($n = 4$) were visible to the test male subject in each of the two sectors.

3.1.9. Results

The first choice made by the test fish in either of the two end areas of the tank, close to one or the other of the two groups, was computed from the video recordings. Percentages of choices for the sector with the group with large numerosities were computed for each fish (for each of the two latency times) and entered an analysis of variance with type of test (e.g., one *vs.* two, two *vs.* three, three *vs.* four, and so on) and latency (time elapsed after disappearance of some of the female fish, i.e., 5 s or 30 s). One-sample *t* tests (two-tailed) were then used to estimate significant departures from chance level (50%) in percentages of choices for each type of test. To estimate the effect sizes, we used partial eta-squared (η_p^2) as the index for ANOVA, and Cohen's *d* as the index for *t* tests. Cohen's kappa (κ) was used to evaluate the interobserver reliability based on 20% of the trials, which revealed a strong interobserver agreement ($\kappa = 0.99$).

An ANOVA was performed with Discrimination (one *vs.* two, two *vs.* three, three *vs.* four, two *vs.* four, four *vs.* six) as a between subjects factor, and Latency (5 s, 30 s) as a within-subjects factor. The ANOVA revealed only a significant main effect of Discrimination, $F(4, 35) = 4.501, p = $

0.005, $\eta_p^2 = 0.34$. There were no other statistically significant effects (Latency, $F[1, 35] = 0.268$, $p = 0.608$; Discrimination x Latency, $F[4, 35] = 0.489$, $p = 0.744$). The results are shown in Figure 15.

The Least Significant Difference (LSD) post hoc test revealed significant differences between Conditions 1 *versus* 2 and 3 *versus* 4 ($p = 0.001$, Cohen's $d = 1.53$), Conditions 2 *versus* 3 and 3 *versus* 4 ($p = 0.012$, Cohen's $d = 1.21$), but not between Conditions 1 *versus* 2 and 2 *versus* 3 ($p = 0.357$), or Conditions 2 *versus* 4 and 4 *versus* 6 ($p = 0.156$). There were other significant differences between Conditions 1 *versus* 2 and 4 *versus* 6 ($p = 0.003$, Cohen's $d = 1.70$), and Conditions 2 versus 3 and 4 *versus* 6 ($p = 0.031$, Cohen's $d = 1.33$).

One-sample two-tailed *t*-tests revealed significant choices for the larger numerosity in Discriminations 1 *versus* 2, $t(7) = 5.974$, $p = 0.001$, 2 *versus* 3, $t(7) = 5.916$, $p = 0.001$, and 2 *versus* 4, $t(7) = 2.816$, $p = 0.026$, but not Discriminations 3 *versus* 4, $t(7) = 0.007$, $p = 0.995$, and 4 *versus* 6, $t(7) = 0.552$, $p = 0.598$.
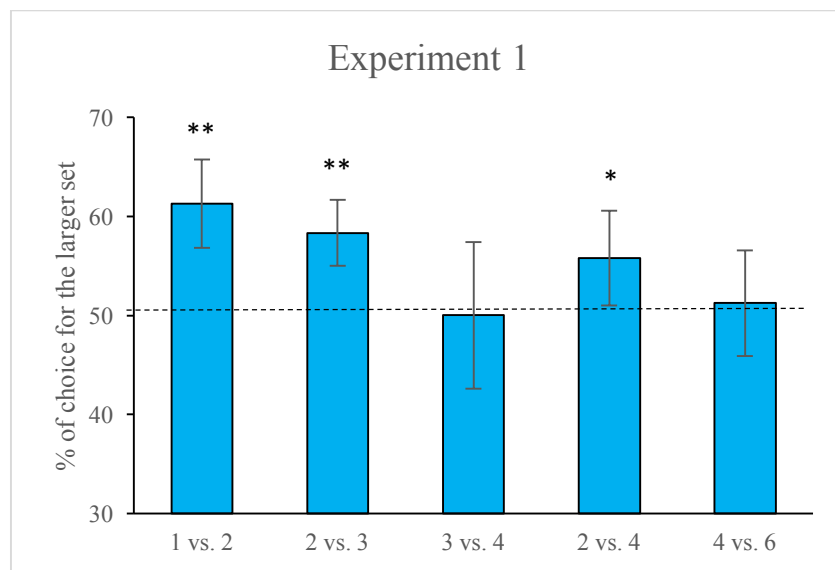
Figure 15. The graph shows the percentages of choice for the larger set in discriminations with different numerousness (group means with 95% confidence intervals are shown). Significant departures from chance level (50%) are shown by asterisks (two-tailed one-sample *t* tests; * $p < .05$, ** $p < .01$).

The results apparently confirmed those obtained with redtail splitfin (Stancher et al., 2013) and angel fish (Gómez-Laplaza & Gerlai, 2011), with a drop-off of discrimination with a ratio of 3:4, which seemed to occur irrespective of the largeness of numerical magnitudes, because significant discrimination was apparent with two *versus* four items (i.e., with a 1:2 ratio, and one number set

exceeding any alleged small number system). One problem with this interpretation, however, is that zebrafish failed the 4 *versus* 6 discrimination, which has the same ratio (2:3) as the 2 *versus* 3 discrimination that zebrafish succeeded to perform.

We wondered whether this unusual outcome, which would be incompatible with either the ANS and OFS, can be accounted for by a simple hypothesis, namely, that when the number of females is very large, their visibility by the test fish would be difficult in the small tank because of mutual visual occlusion among females. This hypothesis was tested in Experiment 2.

3.2. EXPERIMENT 2

In the second experiment, four discriminations were investigated. Two aimed to verify the lack of discrimination with a 3:4 ratio in the range of both small (3 *vs.* 4) and large (6 *vs.* 8) numerosities, and two aimed to verify the presence of discrimination with higher ratios (1:2 and 2:3), even with large numerosities (4 *vs.* 8, 4 *vs.* 6).

3.2.1. Subjects, apparatus, and rearing conditions

Rearing conditions were the same as in Experiment 1. Naïve zebrafish were used. In this experiment we used a slightly different version of the apparatus, in which only the sizes of the parts of the two sectors housing the females were different, the transparent part was now 13 cm and the opaque part being 7 cm (Figure 16). The procedure was the same as the one used in the first experiment.

Figure 16. Details of the sector parts (transparent and opaque), visible from the experimental fish, in the original (Experiment 1) and modified (Experiment 2) apparatus.

3.2.2. Discrimination 3 *versus* 4

Six males and seven females of *Danio rerio* were used in this experiment. Three females were placed in one sector, and four females in the other sector. At the end of the observation phase, one of the females of the larger group was moved into the opaque part of the sector, so that at test, after a delay of 5 s or 30 s, exactly the same number of females ($n = 3$) were visible to the test male subject in each of the two sectors.

3.2.3 Discrimination 4 *versus* 6

Eight males and 10 females of *Danio rerio* were used in this experiment. Four females were placed in one sector, and six females in the other sector. At the end of the observation phase, two of the females of the bigger group was moved into the opaque part of the sector, so that at test, after a delay of 5 s or 30 s, exactly the same number of females ($n = 4$) were visible to the test male subject in each of the two sectors.

3.2.4. Discrimination 4 *versus* 8

Eight males and 12 females of *Danio rerio* were used in this experiment. Four females were placed in one sector, and eight females in the other sector. At the end of the observation phase, four of the females of the bigger group was moved into the opaque part of the sector, so that at test, after

a delay of 5 s or 30 s, exactly the same number of females ($n = 4$) were visible to the test male subject in each of the two sectors.

3.2.5. Discrimination 6 *versus* 8

Seven males and 14 females of *Danio rerio* were used in this experiment. Six females were placed in one sector, and eight females in the other sector. At the end of the observation phase, two of the females of the bigger group was moved into the opaque part of the sector, so that at test, after a delay of 5 s or 30 s, exactly the same number of females ($n = 6$) were visible to the test male subject in each of the two sectors.

3.2.6. Results

Results are shown in Figure 17. The ANOVA revealed only a significant main effect of Discrimination, $F(3, 25) = 6.142$, $p = 0.003$, $\eta_p^2 = 0.424$. Latency, $F(1, 25) = 0.073$, $p = 0.790$, and Discrimination x Latency, $F(3, 25) = 1.170$, $p = 0.341$, were not significant. The LSD post hoc test revealed significant differences between Conditions 3 *vs.* 4 and 4 *vs.* 6 ($p = 0.003$, Cohen's $d = 1.60$), Conditions 3 *vs.* 4 and 4 *vs.* 8 ($p = 0.001$, Cohen's $d = 2.05$), but not between Conditions 3 *vs.* 4 and 6 *vs.* 8 ($p = 0.294$). There were other significant differences between Conditions 4 *vs.* 8 and 6 *vs.* 8 ($p = 0.013$, Cohen's $d = 1.44$), and Conditions 4 *vs.* 6 and 6 *vs.* 8 ($p = 0.035$, Cohen's $d = 1.05$).

One sample two-tailed t tests revealed significant choices for the larger numerosity in discriminations of four *vs.* six, $t(7) = 4.470$, $p = 0.003$, and four *vs.* eight, $t(7) = 9.510$, $p = 0.0001$), but not three *vs.* four, $t(5) = 0.791$, $p = 0.465$, and six *vs.* eight, $t(6) = 0.367$, $p = 0.726$.
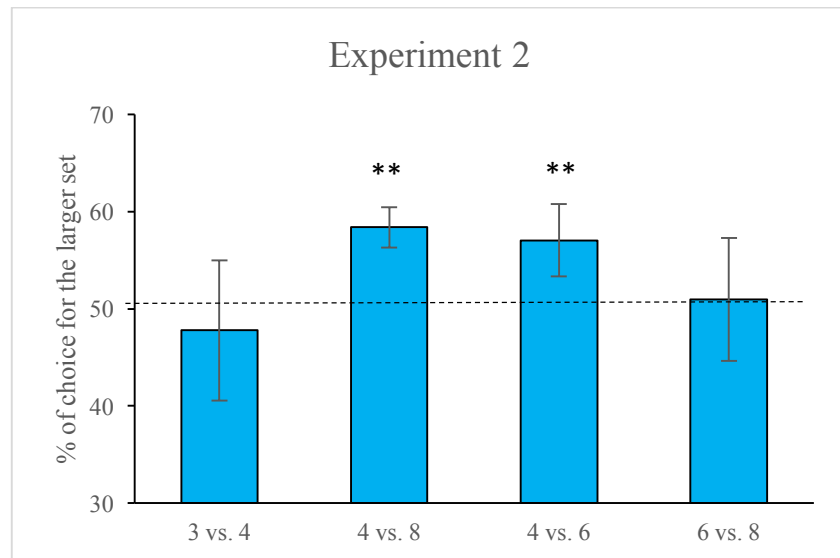
Figure 17. The graph shows the percentages of choice for the larger set in discriminations with different numerousness (group means with 95% confidence intervals are shown). Significant departures from chance level (50%) are shown by asterisks (two-tailed one-sample *t* tests; * *p* < .05, ** *p* < .01).

Results using a larger tank, which prevented the fish to overlap too much and made it possible that all individuals could be seen clearly, showed that quantity discrimination fitted nicely with the prediction from the ANS mechanism, that is, the ratio between the sets to be discriminated was the main determinant of fish performance.

3.3 Discussion

In this study, fish were tested in a spontaneous discrimination task between groups of different numerical quantities. The results reported here with zebrafish appear to be in agreement with the idea of a single mechanism for dealing with approximate estimation of the magnitude of numerosity of sets of social objects, both small and large, that is, the ANS or some equivalent. Zebrafish accuracy in discrimination depended mainly on the ratio between the numerosities, showing that zebrafish discriminate very well ratio of 0.5 (discrimination 1 *vs*. 2, 2 vs. 4 and 3 *vs*. 6) and 0.67 (2 *vs*. 3 and 3 *vs*. 6), failing when the ratio was at 0.75 (3 *vs*.4 and 6 *vs*.8). This ratio limit has been found in other fish species such as angelfish (*P. scalare*) (Gómez-Laplaza et al., 2012), siamese fighting fish (*Betta splendens*; Agrillo et al. 2012a) and redtail splitfin (*Xenotoca eiseni*; Stancher et al. 2013).

Results of Experiment 2 revealed that when visibility of relatively large numbers of conspecifics is guaranteed via a large tank in the exposure phase, zebrafish could deal with discrimination of large numbers, and the degree of imprecision depends mainly on the ratio of the numbers, not the fact that the two items are above or below a threshold of about three elements.

It should be noted, however, that although most of the evidence available suggest continuity in the processing of small and large numerosity (e.g., non-human primates, see Brannon & Terrace, 1998, Cantlon & Brannon, 2007, Judge et al., 2005, and Smith et al., 2003; birds, see Rugani et al., 2009; 2011; 2013; fish, see Gómez-Laplaza & Gerlai, 2011), there are also reports suggesting that the existence of some equivalent of an OFS with a set-size limit at around three to four items (e.g., chimpanzees, see Tomonaga & Matsuzawa, 2002; birds, see Rugani et al., 2008; 2010; honeybees, see Dacke & Srinivasan, 2008). It is also important to emphasize that in at least one case, a nonhuman species, an African grey parrot (*Psittacus erithacus*) named Alex, has shown an ability to use exact, and not approximate, number representations (Pepperberg, 2012; Pepperberg & Carey, 2012).

It should also be stressed that although fish in these experiments make their judgments on the basis of memory, rather than by direct sensory stimulation with different number of conspecifics, the issue of the control of continuous physical variables remains unaddressed (in principle, fish could have memorized continuous physical variables, as in most animals studies, rather than number per se). Thus, quantity discrimination rather than number discrimination appears to be the most appropriate labeling for these sort of studies.

STUDY 2

Proto-Arithmetic abilities in fish

The possession of arithmetic abilities is a complex numerical competence that requires a form of numerical mental manipulation. To manipulate over numerical values, it is necessary to operate a mental transformation of numerical information. When we do an arithmetical addition of elements, this operation involves a combine of mental quantitative representations, the "addends", that form a new representation, what we call "sum".

The ability to manipulate numerosities in order to carry out arithmetic operations, such as additions or subtractions, has been studied in human infants and animals (see Introduction 1.5). Spontaneous arithmetic abilities have been described in 5-month-old human infants using the habituation/dishabituation paradigm (Wynn, 1992). Infants were presented with arithmetic operations over small numbers (e.g. $1 + 1 = 2$) where two toys were added behind an opaque screen. Subsequently, the screen was lowered showing the toys behind. Infants looked longer when they were presented with an arithmetically wrong result (e.g. $1 + 1 = 1$). The same paradigm was used with rhesus monkeys both for addition and subtraction, with the same results as in infants (Hauser et al., 1996). Similar results were obtained in other mammalian species (tamarins, Uller & Hauser, 2001; lemurs, Santos et al., 2005; dogs, West et al., 2002) and birds (Rugani et al., 2009).

The aim of this study was to provide, for the first time, evidence in zebrafish of proto-arithmetic addition ability. Taking advantage of the shoaling behavior, we tested the fish preference between two groups of social companions, added one-by-one behind two opaque screens. This task did not require a training procedure, so it was possible to observe the spontaneous ability to deal with simple addition tasks. The use of fish allowed us to observe how such a complex competence, albeit in its simplest form (addition), is widespread among vertebrates.

4.1. EXPERIMENT 1

Fish were tested in a spontaneous discrimination task between two groups of conspecifics differing in number, by presenting each conspecific one at a time. The fish did not have the possibility to see and compare simultaneously the numerosity of the two groups. Fish were tested in a 1 *vs.* 2, 2 *vs.* 3 and 2 *vs.*4 discrimination condition.

4.1.1. Subjects and rearing conditions

Subjects were reared in an automated aquarium system (ZebTEC Benchtop, Tecniplast) specific for zebrafish housing. Fish were reared in 3.5-L plastic tanks in isolated sex groups of 10 individuals. A couple of days before the experiment, fish were transferred in groups of 10-15 subjects in 20-L glass aquariums (23x38x25 cm) with gravel and plant. The water in each aquarium was maintained at the temperature of 26 °C and was kept clean by a pump and filter system (Micro Jet Filter MCF 40). The aquariums were illuminated by a 2.4-W LED lamp (Orion Led River Wave) following a light cycle of 12 hr. The reason of this change of housing before test has been introduced because with this procedure fish showed a more confident and natural approach in the experimental apparatus, probably due to the possibility to familiarize with an environment aquarium more similar to the one used at test in terms of sizes of the tanks. Fish were fed twice a day with dry food (Sera GVG-Mix). In all the experiments, males were used as test subjects, whereas females were used as target (sexual) stimuli (we selected females of the same size). The range of fish total lengths was between 4 cm and 5 cm for both males and females.

4.1.2. Apparatus

The experimental apparatus consisted of a plastic rectangular test tank (36 x 27 x 20 cm) with white-colored walls (Figure 18a). Near the shorter sides of the tank, two white vertical panels (11 x 18.5 cm) were located. Each panel was distant 4.5 cm from the shorter side of the tank, and was perpendicularly adjacent to one of the two long sides of the apparatus. These panels had the function to occlude two sectors of the apparatus, making it impossible to see behind them from the center.

In order to present single conspecifics one at a time and to modulate their movement, single females were gently introduced into small plastic transparent boxes (4.5 x 8.5 x 2 cm; Figure 18b). Each box was covered with a top, in order to prevent the animal from coming out. On the back of

each box, a long transparent stick (28 cm) was fixed, with the aim to move easily the box into the apparatus. Each female was constantly monitored during the experiment and immediately replaced in case it showed stress related behavior. Moreover, the water in each box was partially changed among trials maintaining an optimum quality level.
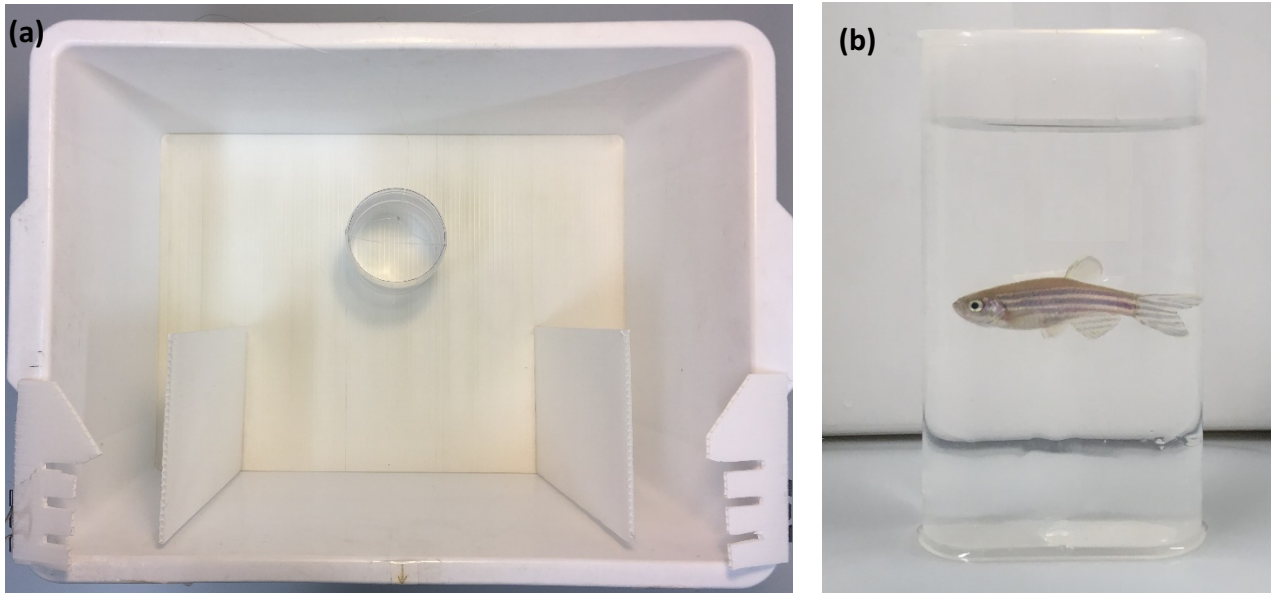

Figure 18. **(a)** A photograph of the apparatus used. **(b)** Female stimulus into a transparent box.

During the test, the male subject could swim freely in the tank. The central area of the apparatus has been divided in three different areas, indicated by two black thin lines on the floor. These lines delimitated a neutral central area (7 x 27 cm), and two choice areas (14.5 x 23 cm) close to the two panels. This allowed us to have an objective assessment of the choices made by the subject during the test.

At the beginning of each trial, the test male was inserted into a transparent plastic cylinder (6.5 cm in diameter and 14.5 cm in height) at the center of the apparatus into the neutral area. At the top of the cylinder, a transparent nylon wire allowed to lift it up vertically using a pulley system. In this way, the fish was not disturbed by the experimenter's presence during the cylinder lifting. The apparatus was lit by a fluorescent 60-W lamp, and a webcam (Microsoft LifeCam Studio) recorded the fish behavior from above (70 cm) the setup.

4.1.3. Procedure

Before starting the experiment, the fish was fed in order to maximize the shoaling response, avoiding food competition behavior. Then, a group of females identical in size to the test male subject were selected and singly gently introduced into the transparent boxes. Once the operation was concluded, the test male was initially introduced into the apparatus for two minutes leaving it free to explore and familiarize with the new environment.

At the beginning of each trial, the test fish was introduced in the middle of the tank into the transparent cylinder and left for two minutes. Subsequently, one box (containing the female) was gently introduced in the apparatus in front of the subject into the neutral area and then moved first toward it in order to catch its attention, and then towards one of the two panels on the left or on the right side, hiding behind it (see Figure 19).
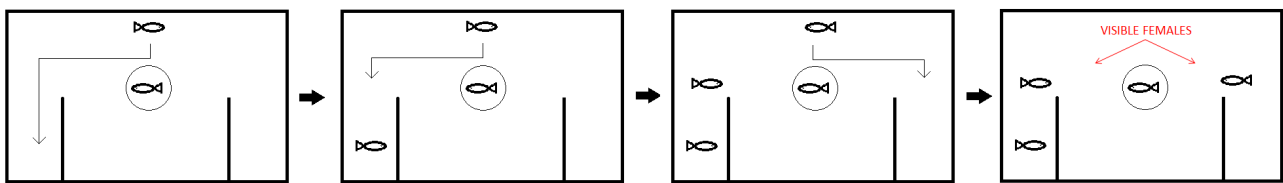

Figure 19. Schematic trial example of the stimuli presentation in a 2 *vs.* 1 discrimination: one conspecific is hidden behind the left panel; a second fish moves along the same trajectory close to the left panel, remaining visible; a third fish appears and moves close to the right panel with a specular path, remaining visible too; then the test fish is released and the first approach response is recorded. Note that the position and the sequence presentation are randomized among trials.

The same sequence was repeated for the other female conspecifics, creating two groups with different numerosities, one on the right side and one on the left side. It is important to note that not all the females were hidden behind the panel, in fact, the last female of each series, instead of hiding, remained visible next to the panels. For example, in the 2 *vs.* 1 discrimination, from the bigger group, the first females is hidden behind the panel and the second remain visible close to it; on the other group, one female moves close to the panel, remaining visible. This procedure was used because in a previous pilot experiment, where all the females were hidden, the test fish did not show any preference for the two groups. We interpreted this result not as an impairment of discrimination, but rather as a loss of motivation. With this hypothesis, leaving an element visible, the male fish should show more interest to look for the bigger shoal.

When all the conspecifics had completed the movement, after a delay of 5 seconds, the cylinder housing the test male was gently lifted up, allowing it to approach one or the other of the two

sectors. The interval between trials was 2 minutes and each subject was tested in a single daily session of 16 trials.

The first choices of the test male were computed from the video recording. A choice was considered valid when the fish entered with the entire body in one of the two areas close to the two sectors identified by the black rows drawn on the floor. The position of the larger group of females was changed among trials and balanced between the right and left side, following a semi-random sequence. Moreover, also the order presentation of the two groups was balanced in the session (in half of the trials it was presented first the bigger group and then the smaller group; for the other half vice-versa) and among the subjects (in the trials 1-8, half of the fish saw the bigger group as last movement and in the trials 9-16 the smaller one, for the other half vice versa).

At the end of the experiment, before starting a new subject, the water of the apparatus was completely substituted with clean water, deleting any possible olfactory cue.

4.1.4. Discrimination 1 *versus* 2

Twenty-two males of *Danio rerio* were used in this discrimination condition. Female conspecifics were sequentially moved one at a time forming two groups with different numerosities: one group with one hidden female and one visible female (1+1= 2), and one group with one visible female (1) (see Figure 20 for a trial example).
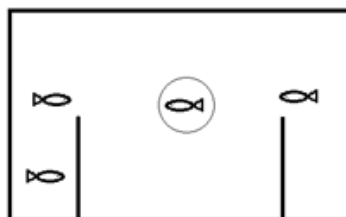

Figure 20. Schematic representation of a 1 *vs.* 2 discrimination.

4.1.5. Discrimination 2 *versus* 3

Ten males of *Danio rerio* were used in this experiment. Female conspecifics were moved one at a time forming two groups with different numerosities: one group with two hidden females and one visible female (1+1+1= 3), and one group with one hidden and one visible female (1+1= 2) (see Figure 21 for a trial example).

Figure 21. Schematic representation of a 2 *vs.* 3 discrimination.

4.1.6. Discrimination 2 *versus* 4

Ten males of *Danio rerio* were used in this experiment. Female conspecifics were moved one at a time forming two groups with different numerosities: one group with 3 hidden females and one visible female (1+1+1+1= 4), and one group with one hidden female and one visible female (1+1= 2) (see Figure 22 for a trial example).
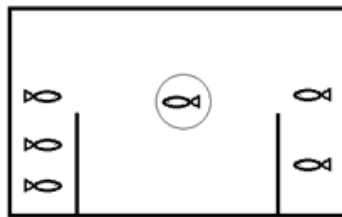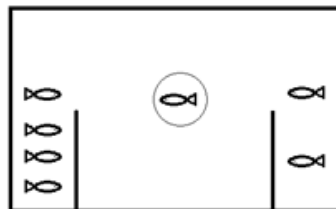

Figure 22. Schematic representation of a 2 *vs.* 4 discrimination.

4.1.7. Results

In each condition, for each fish, an index of choice for the largest group was calculated according to the formula:

$$index = \frac{choices\ for\ the\ larger\ group}{choices\ for\ the\ larger\ group + choices\ for\ the\ smaller\ group}$$

The data were analyzed using one-sample t-tests, with the aim to analyze whether the number of choices towards the largest group was greater than the chance level (50%). Subsequently, two comparison within subjects has been done. Using a paired sample t-test, we analyzed if the choice preference for the bigger set was influenced by the order of presentation.

Results are reported in Figure 23 and show the percentage of choice for the larger group in each discrimination condition. One-sample two-tailed t-tests revealed that zebrafish choices were significantly greater for the larger numerousness in the condition 1 $vs.$ 2 ($t(21) = 2.595$ $p = 0.017$) and 2 $vs.$ 4 ($t(9)=3.280$, $p=0.010$), but not in the condition 2 $vs.3$ ($t(9) = -0.227$ $p = 0.825$). No significant difference has been found analyzing the choices for the larger group comparing the order of presentation (when as last event it was moved the bigger or the smaller group) (1 $vs.$ 2: $t(21) = -0.157$, $p = 0.877$; 2 $vs.$ 3: $t(9) = 0.958$, $p = 0.363$; 2 $vs.$ 4: $t(9) = 0.943$, $p = 0.370$).
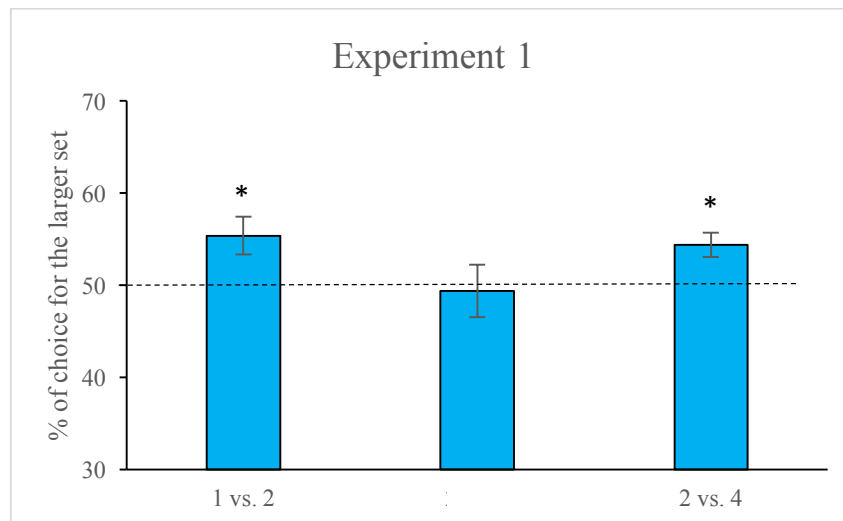

Figure 23. The index of choice for the larger group (mean ± SEM) in each numerical comparison is shown. The dotted line represents chance level (50 %). Asterisks indicate significant departures from chance level (*p < 0.05)

The results obtained showed that zebrafish are able to track sequential stimuli presented one at a time, indicating a preference for the group that added the major number of conspecifics in the condition 1 *vs.* 2 and 2 *vs.* 4, but not in 2 *vs.* 3. This suggests that fish are able to discriminate sequential addition of elements, providing that the numerical difference between the two group differed at least twice as much (ratio = 0.5). In fact, when the ratio become less advantageous (such as in 2 *vs.* 3, ratio =0.67) fish fail in the discrimination.

This experiment suggest that zebrafish could operate on simple addition among elements. However, potential non-numerical variables could have helped the fish to localize the bigger shoal, such as the different amount of total time of presentation between the groups, which is longer for the bigger one (for example, moving four elements requires twice the time compared to two elements). This is what we have controlled for in the second experiment, proposing two control conditions where the total time of presentation was equalized in the two sets.

4.3. EXPERIMENT 2

4.3.1. Materials and procedure

Naïve zebrafish were used in this experiment. Subjects, apparatus, and procedure were the same as described in the ''Experiment 1'' section. The only difference was reported in the procedure, where the time stimulus presentation of female conspecifics has been equalized. During the sequential presentation, the speed of motion of the conspecifics for each set was manipulated and balanced, so that it took the same total time to disappear both for the larger and the smaller set. In this second experiment we used a discrimination between 2 *vs.* 3 and a 2 *vs.* 4 elements.

4.3.2. Discrimination 2 *versus* 3

Sixteen males of *Danio rerio* were used in this experiment. Female conspecifics were moved one at a time forming two groups with different numerosity: one group with two hidden females and one visible female (1+1+1= 3) and one group with one hidden and one visible female (1+1= 2). The disappearance time of each whole group took approximately 30 seconds, spending 10 seconds for each element in the set of three and 15 seconds for each element in the set of two.

4.3.3. Discrimination 2 *versus* 4

Twenty-three males of *Danio rerio* were used in this experiment. Female conspecifics were moved one at a time forming two groups with different numerosity: one group with 3 hidden females and one visible female (1+1+1+1= 4), and one group with one hidden female and one visible female (1+1= 2). Disappearance of each whole group took approximately 40 seconds, spending 10 seconds for each element in the set of four and 20 seconds for each element in the set of two.

4.3.4. Results

Results are reported in Figure 24 and show the percentage of choice for the larger group in each different discrimination condition. One-sample two-tailed t-tests revealed that zebrafish choices were significantly greater for the larger numerousness in both control condition 2 *vs.* 3 ($t(15) = 2.651$ $p = 0.018$) and 2 *vs.* 4 ($t(22) = 2.509$, $p = 0.020$). A paired sample t-test analyzing the choices for the bigger group in relation to the last movement did show a significant effect in the condition 2 *vs.* 3 ($t(15) = 2.353$, $p = 0.033$) revealing that zebrafish preferred to choose the big group when it was presented as last movement. The same trend has not been found in the condition 2 *vs.* 4 ($t(22) = 0.898$, $p = 0.379$).
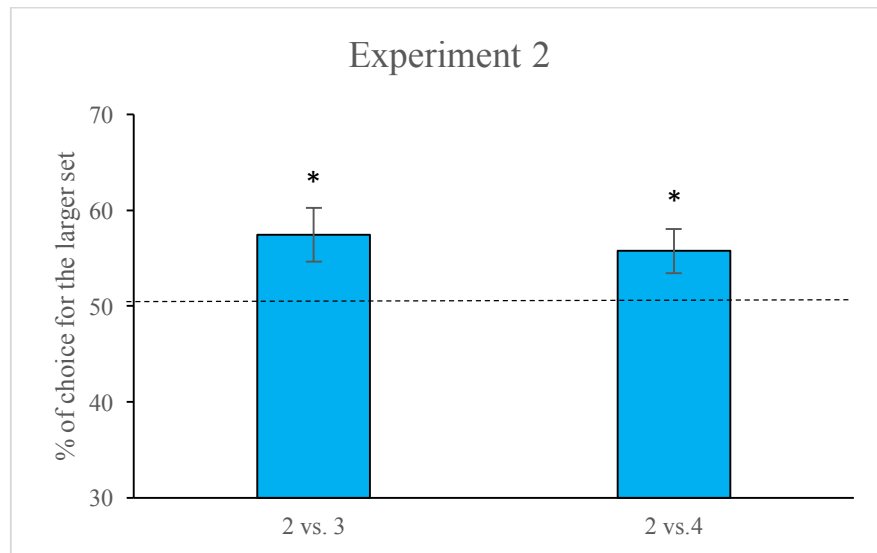
Figure 24. The index of choice for the larger group (mean ± SEM) in each numerical comparison is shown. The dotted line represents chance level (50 %). Asterisks indicate significant departures from chance level (*p < 0.05)

The control conditions had revealed a preference for the larger group of conspecifics comparison both in 2 *vs.* 3 and 2 *vs.* 4. The results showed that, when time was equalized, fish were facilitated in the identification of the bigger group. The possible explanation is connected to a direct consequence of the time controlling that caused a change of speed in the sequential presentation. Moreover, in the condition 2 *vs.* 3 it has been found that fish had a preference for the larger group when this is presented as last event, that could be emphasized by the faster speed of the group.

4.4 Discussion

In this study, fish were tested in a spontaneous discrimination task between sets with different number of conspecifics sequentially presented, in order to investigate their proto-arithmetical addition abilities. Results showed that fish were able to distinguish small numerositites of females companion that added one at a time behind two opaque screens. A similar procedure has been used in several other vertebrate species (infants: Wynn; 1992; primates: Beran and Beran, 2004; Hauser et al., 1996; Uller et al., 2001; Santos et al., 2005; dogs: West et al., 2002; birds: Rugani et al., 2009). This is the first report in a fish species.

When exposed to two numerical different series of sequential stimuli, zebrafish showed a preference for the larger group in the condition 1 *vs.* 2 and 2 *vs.* 4, but not 2 *vs.*3. The ratio between the two groups was clearly an important factor. Zebrafish discriminated comparison with a 0.5 ratio (1 *vs.* 2 and 2 *vs.* 4), but not with a 0.67 ratio (2 *vs.* 3), confirming that the ability to discriminate

between two groups seems to depend not so much on the differences between the two numerosities, but on the ratio between them (Gallistel, 1990).

Fish however may have used non-numerical information such as the different time of disappearance during the stimuli presentation. The aim of the second experiment was to equalize the total time presentation in the two sets. Fish showed a strong preference for the larger group, even in a discrimination condition where they previously failed. In fact, fish were not able to distinguish 2 *vs.* 3 conspecifics in the experiment 1 when time presentation was not balanced, but showed a good preference for the larger set when time was equalized. This result could be explained by the change of speed in the sequential presentation of the two group stimuli in the control condition. The elements of the smaller one needed to decrease their speed in order to equalize the time as the other more numerous group. A possible explanation connected to the preference for the faster group may be related to the fact that, joining a more active fish group provide advantages like the ability to find food patches more rapidly and creating more confusion to predators. On the other hand, a slower moving shoal increased predation risk from the oddity effect (Theodokrakis, 1989). This hypothesis is supported by other evidence in fish that aimed to control for the movement and the activity of conspecific shoals by decreasing the water temperature. In these experiments, several fish species, included zebrafish, preferred to spend more time with the group that was more active overall, avoiding the one with a lower activity (Pritchard et al., 2011; Agrillo et al., 2008a).

In conclusion, the results obtained in this study provided the first evidence in zebrafish of ability to discriminate groups of elements that are presented sequentially instead of simultaneously. The movement and activity of the fish is a key factor in the social attraction behavior. Further studies controlling for non-numerical information would help to understand better what is the role of continuous physical variables in arithmetic abilities and numerousness estimation.

STUDY 3

Use of ordinal information by zebrafish

Numbers have different properties that refer to different aspects of them. When we evaluate the numerosity of elements in a set, calculate a quantity or estimate a size, we refer to the cardinal aspect of the numbers, responding to the question "How many?". Besides the cardinal aspect, another important property of numbers is the ordinality, which answer to the question "Which position/rank?". The ordinal information is used to identify a position, or rank of a particular element in a series of other elements. Human infants are able to detect and represent ordinal direction of numerical sequences on the basis of their ordinal position (Brannon, 2002; Picozzi et al., 2010). Ordinal competence investigation has been extended in non-human animals showing to be present in primates (Brannon & Terrace, 1998; Smith et al., 2003; Judge et al., 2005), other mammals (Suzuki & Kobayashi, 2000; Jakkola et al., 2005) birds (Rugani et al., 2007) and even invertebrates (Dacke & Srinivasan, 2008; and see introduction 1.6).

Recently, the use of ordinal information has been documented for the first time in a fish species, the guppy (*Poecilia reticulata*) (Miletto Petrazzini et al., 2015a). Guppies can learn to recognize the 3rd feeder in a row of 8 identical feeders, with distances between elements that changed among trials. Moreover, if trained with a fixed spatial configuration of the feeders and tested with a different position and inter-feeder distance, guppies showed a spontaneously use of ordinal information over the spatial one. Lastly, the authors have observed that guppies decrease their precision as the ordinal position increases, making more errors in selecting for example the 5th position.

The aim of this study was to investigate whether zebrafish could learn to identify an element in a series of identical elements placed at the same distance. Fish were then tested in order to establish whether the spontaneous strategy used was based on the ordinal numerical information rather than other non-numerical information, such as spatial cues.

5.1. EXPERIMENT 1

Zebrafish ability to identify a certain target element on the basis of its position in a series of identical elements was assessed by training them to identify the second corridor in a series of five. The fish were then tested in a condition where the total length of the apparatus was reduced, creating a conflict between ordinal positon and spatial distance. The aim of the test was to check whether the fish would choose a corridor using the spatial distances provided by the length of the apparatus rather than the ordinal numerical information.

5.1.1. Subjects

In this first experiment we used 7 adult males (ranging 4-5 cm in length), whereas females were used as social reinforcement. Subjects were reared in an automated aquarium system (ZebTEC Benchtop, Tecniplast) in 3.5-L plastic tanks in isolated sex groups of 10 individuals. The water temperature was maintained at 26°C and the system were illuminated following a light cycle of 12 hours. Fish were fed three times a day with dry food (Sera GVG-Mix). During the entire experiment, the subjects were kept separated one to each other into a 20-L plastic aquarium (23 x 38 x 25 cm) set with gravel and vegetation, mimicking a natural environment. Despite each animal was isolated, it could see the conspecifics from a net divider, in order to avoid potential negative effect due to the social deprivation.

5.1.2. Apparatus

The experimental apparatus consisted of a rectangular arena (50 x 9 x 11 cm) made of white plastic material (Poliplak), where along one of the two long walls there were 5 corridors (9 cm in length, 3 cm in width, 11 cm in height; see Figure 25a). The corridors series was placed at a distance of 10.5 cm from the apparatus shorter sides, and the inter-distance between corridors was 3.5 cm. The corridors acted as a passage, allowing the fish to leave the closed arena. Each corridor was marked, at a depth of 3.5 cm, by a blue plastic frame (thickness; Figure 25b). All the corridors, except one, had a plastic transparent panel placed in correspondence of the blue frame, not allowing the fish to pass through it. As result, all the doors looked the same, but just one was actually open,

leaving the passage in the outer area. The experimental arena was in turn inserted into a large black tank (110 x 45 x 52 cm). To give the animal a comfortable and rewarding environment, the outside tank was set with gravel, plants, and two female conspecifics, thus giving the animals a strong motivation to go out from the apparatus arena.

Figura 25. (a) A photograph of the apparatus used in the experiment 1. **(b)** Particular of a corridor.

The water in the experimental apparatus was maintained at a constant temperature of 25°C and kept clean by a pump and a filter system (Micro Jet Filter MCF 40). The apparatus was lit by a fluorescent 18-W neon lamp, and a webcam (Microsoft LifeCam Studio) recorded fish behavior from above (50 cm) the setup.

5.1.3. Procedure

The "experimental" procedure was divided in two parts: a *training* phase and a *test* phase.

Training

During the training procedure the fish could leave the experimental apparatus by entering the only open corridor, as the other were blocked. . In a frontal view of the five corridors, the reinforced one was always the second starting from the left side.

Before starting each session, the fish was taken from the housing aquarium and left for 5 minutes in the comfortable environment outside the apparatus. At the beginning of each trial, the

fish was confined into a transparent plastic cylinder in the center of the apparatus. After a short wait of about 10 seconds, the cylinder was lifted up, allowing the fish to move freely within the apparatus. As described above, only the second corridor from left remained open, while the other four were closed. The behavior of the animal was observed for a maximum of 15 minutes, recording the number of attempt for each individual corridor, until the correct one was identified, allowing the subject to reach the comfortable environment with females and shelters. An attempt choice was considered done when the fish entered the corridor with the entire body. If the fish approached the corridor only partially, it was not considered as a choice. The reward obtained at the end of every trial depended on the fish performance: when the correct corridor was chosen as first attempt, it received 6 minutes of interval, a small quantity of food, and it was allowed to reach two female conspecifics; when the animal did not chose directly the correct door, approaching at least once one of the closed corridors, it received 3 minutes and no food and females were provided. . The fish were given daily sessions of 10 trials, and, at the end of each, the number of total choices frequencies in each corridor was calculated. Learning was considered complete when the subject presented, for two consecutive daily sessions, a frequency of the correct number of choices equal to or greater than 60% compared to the frequencies of each other individual corridor. The subjects, once they reached the learning criterion, were tested.

Test

In the test phase, the size of the apparatus used was different to the one used in the training phase. The total length of the apparatus has been reduced from 50 cm to 37 cm, creating a potential conflict between numerical and spatial information. In fact, the distance between the left side of the apparatus and the corridors (indicated by the arrows in Figure 26) had been calculated so that the 3rd corridor of the test sequence was at the same absolute distance, starting from the left side, where the 2nd corridor was in the training phase. In this way, if the fish, during the training, had used a spatial information, the same distance would bring it to the next one during test (the third). Differently, if the fish had referred to ordinal information, at test it would chose always the second corridor.
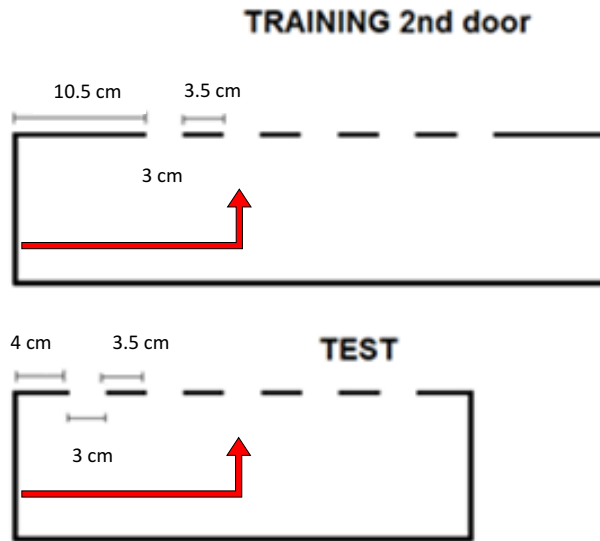
Figura 26. Schematic representation of the apparatus during the *training* and the *test*. At test, the total length of the apparatus changed creating a conflict between ordinal position and spatial distance. The red arrows show how the same spatial distance at training leads, at test, to the next corridor.

Each test consisted of a session of 8 test trials, divided into two days, four trials per day. In order to avoid any differential reinforcement, in the test all the doors were blocked (extinction procedure). At the beginning of each trial, the fish was placed into a transparent cylinder in the center of the apparatus, and once released, it was allowed to perform its exit attempts for 2 minutes, recording the number of choices for each corridor. If the animal did not make any choice in the two minutes, the time was extended as long as it did one, for a maximum of 10 minutes. The inter-test interval was 5 minutes, during which time the fish was free to swim into the comfortable environment. In the second day, the fish completed the test with the other four trials, preceded by a series of recall trials. If the fish, in the second test day, made many errors in the recall trials (due to the extinction procedure at test), it underwent a series of daily training sessions until it reached again the learning criterion. For each trial test, the first choice has been analyzed, as well as the total frequencies for each corridor in the first 30 seconds.

5.1.4. Results

Training

Results are shown in Figure 27. Fish were able to identify, given five identical corridors arranged sequentially, the only corridor that allowed the fish to go out of the apparatus. To reach the learning criterion, fish needed a mean of 176.29 ± 27.74 (mean $\pm$SEM) trials.

A repeated measure ANOVA with five corridors as within subject factor, revealed a statistically significant effect of the corridors [$F(4,24) = 28.483$, $p \leq 0.0001$]. A paired t test revealed a preferential choice for the second corridor compared to each other corridor (1st *vs.* 2nd: $t(6) = -6.683$, $p = 0.01$; 2nd *vs.* 3rd: $t(6) = 4.777$, $p = 0.003$; 2nd *vs.* 4th: $t(6) = 14.378$, $p \leq 0.0001$; 2nd *vs.* 5th: $t(6) = 10.783$, $p \leq 0.0001$). Other comparisons revealed that errors were primarily concentrated on the corridors close to the reinforced one, i.e. the first and the third corridor (1st *vs.* 4th: $t(6) = 2.913$, $p = 0.027$; 1st *vs.* 5th: $t(6) = 2.389$, $p = 0.054$; 3rd *vs.* 4th: $t(6) = 5.004$, $p = 0.002$; 3rd *vs.* 5th: $t(6) = 3.946$, $p = 0.008$). There were no significant differences between the first and the third corridors (1st *vs.* 3rd: $t(6) = -0.119$, $p = 0.909$) and between the fourth and the fifth (4th *vs.* 5th: $t(6) = 0.000$ $p = 1.000$).
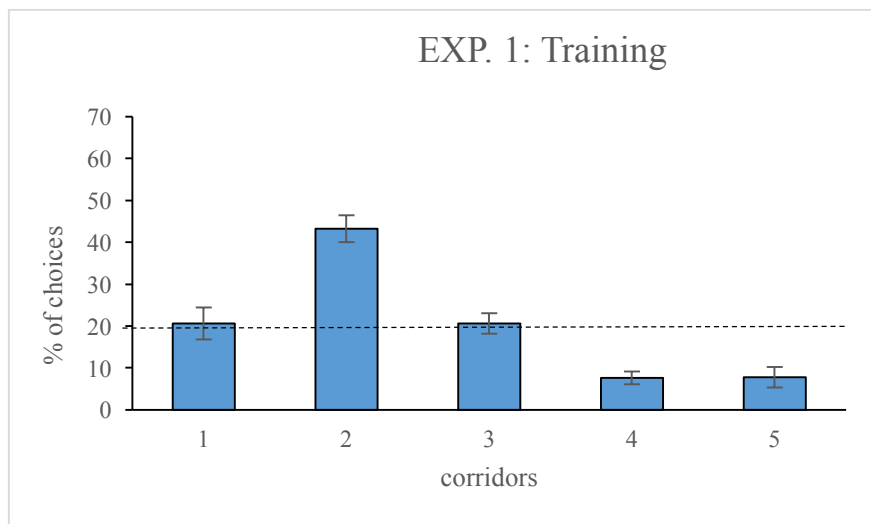

Figure 27. Percentages of total choices (mean $\pm$ SEM) for each corridor position in the last training session (learning criterion reached).

Test

The results at test are reported in Figure 28 and show the percentage of choices for each corridor taking into account only the first choices and the total choices in the first 30 seconds of trials.
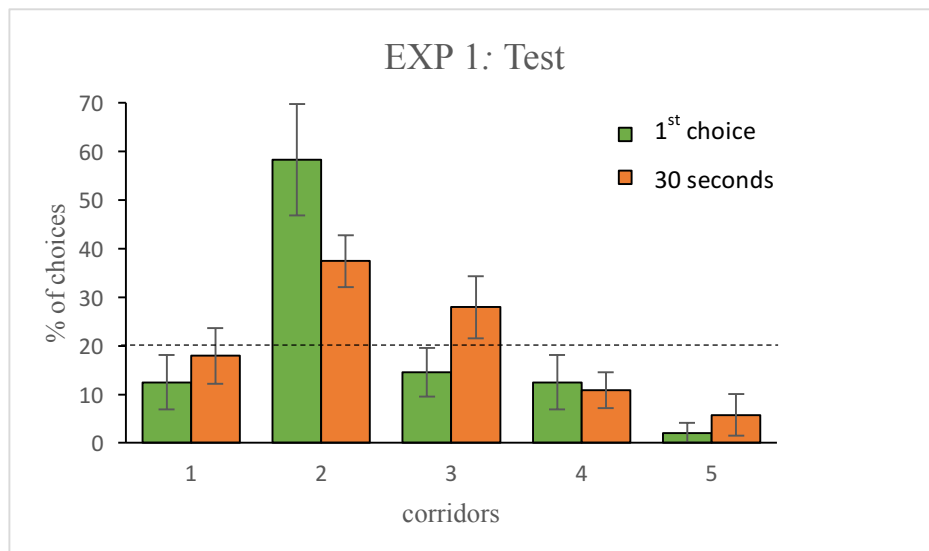

Figure 28. Percentages of choices (mean ± SEM) for each corridor position at test in the first trials and total frequencies in 30 seconds.

A repeated measure ANOVA with the five corridors as within subject factor, revealed a statistically significant effect of the corridors for both the first trials [$F_{(4,24)} = 10.694$, $p \leq 0.0001$] and the total of choices in 30seconds [$F_{(4,24)} = 4.638$, $p \leq 0.006$], showing heterogeneity associated with corridors. A paired t test on the first choices revealed a preferential choice for the second corridor compared to each other corridor (1st *vs.* 2nd: $t_{(6)} = -3.351$, $p = 0.015$; 2nd *vs.* 3rd: $t_{(6)} = 3.092$, $p = 0.021$; 2nd *vs.* 4th: $t_{(6)} = 3.653$, $p = 0.011$; 2nd *vs.* 5th: $t_{(6)} = 4.666$, $p = 0.003$). Other comparisons revealed significant differences between corridor 1 and 5 ($t_{(6)} = 2.500$, $p = 0.047$), and corridor three and five ($t_{(6)} = 3.240$, $p = 0.018$). No difference between the other corridors were observed (1st *vs.* 3rd: $t_{(6)} = -0.603$, $p = 0.569$; 1st *vs.* 4th: $t_{(6)} = 0.240$, $p = 0.818$; 3rd *vs.* 4th: $t_{(6)} = 0.812$, $p = 0.448$; 4th *vs.* 5th: $t_{(6)} = 1.188$ $p = 0.280$).

A paired t test on the total choices in the 30 seconds revealed significant differences between corridors 1 and 2 ($t_{(6)} = -4.066$, $p = 0.007$), corridors 2 and 5 ($t_{(6)} = 2.548$, $p = 0.044$), corridors 3

and 5 ($t(6)$ = 3.032, p = 0.023), corridors 4 and 5 ($t(6)$ = 3.567, p = 0.012), but not between corridors 1 and 3 ($t(6)$ = 0.383, p = 0.715), corridors 1 and 4 ($t(6)$ = 1.313, p =0.237), corridors 1 and 5 ($t(6)$ = 1.702, p = 0.140), corridors 2 and 3 ($t(6)$ = 1.912, p = 0.104), corridors 2 and 4 ($t(6)$ = 2.341, p = 0.058) and corridors 3 and 4 ($t(6)$ = 2.352, p = 0.057).

The results obtained in this first experiment revealed an ability of zebrafish to identify and chose an element over a series of identical elements. Moreover, fish demonstrated that the size of the environment did not affect the performance, nor even when it created a conflict with other information, such as the numerical one.

It is important to point out that, although the starting position of the fish was always in the center of the apparatus (confined into a cylinder), when released at the beginning of each trial, fish never showed a direct choice for one of the corridors, preferring to explore the environment swimming along the apparatus several times before attempting a choice.

Nonetheless, the result do not allow us to assert that the information used here by fish is a numerical one. This because another cue could have been used to identify the correct position, i.e. the inter-corridor distance, which in the test remained the same. We checked for this in the second experiment.

5.2. EXPERIMENT 2

In this second experiment, the training and test procedure were the same as in the experiment 1, except for the sizes of the apparatus. Fish were trained to identify the second corridor from the left and the tested into an apparatus in which the inter-corridor distance has been reduced, creating again a conflict between spatial and numerical information.

5.2.1. Subjects and apparatus

Seven naïve adult male of *Danio rerio* have been used in this experiment. Rearing condition were the same as experiment 1, as well as the procedure. Compared to the previous experiment, the apparatus used here was longer (76 cm), and the distance between each corridor changed (inter-corridor distance was 10 cm; Figure 29).
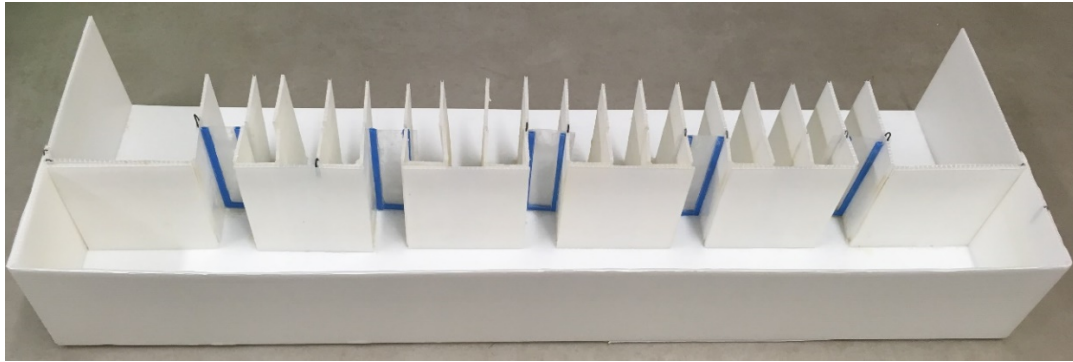
Figure 29. A Photograph of the apparatus used at training in the second experiment.

5.2.2. Procedure

Fish were trained to choose the second corridor counting from the left side, as in the first experiment. At test, the inter-corridor distance was manipulated, decreasing from 10 cm to 3 cm. As it is possible to see in Figure 30, this change created a strong contrast between ordinal rank and spatial distance. . Doing so, the distance between the 1st and the 2nd corridor at training, was the same as the distance between the 1st and the 3rd corridor at test. This contrast helped to further understand if the strategy used by the zebrafish at training was based on spatial distances or ordinal information.
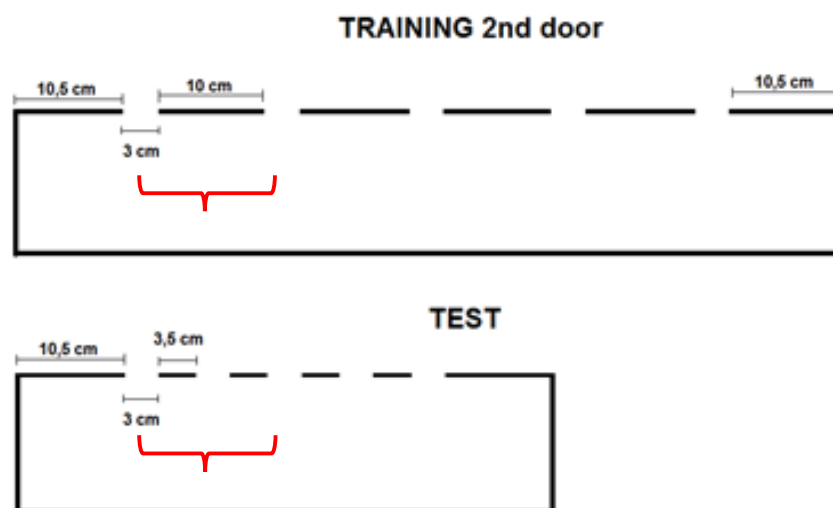

Figure 30. Schematic representation of the apparatus during the training and the test in the second experiment. At test, the distance between the corridors changed. The red curly brackets show how the same spatial distance at training and test leads to different corridors.

5.2.3. Results

Training

Results are shown in Figure 31. Fish were able to identify, given five identical corridors arranged sequentially, the only corridor that allowed the fish to go out of the apparatus. To reach the learning criterion, fish needed a mean of 108.43 ± 18.10 (mean $\pm$SEM) trials.

A repeated measure ANOVA with five corridors as within subject factor, revealed a statistically significant effect of the corridors [$F(4,24) = 40.279$, $p \leq 0.0001$]. A paired t test revealed a preferential choice for the second corridor compared to each other corridors (1st *vs.* 2nd: $t(6) = -7.126$, $p \leq 0.0001$; 2nd *vs.* 3rd: $t(6) = 7.940$, $p \leq 0.0001$; 2nd *vs.* 4th: $t(6) = 31.705$, $p = \leq 0.0001$; 2nd *vs.* 5th: $t(6) = 22.079$ $p \leq 0.0001$). Other comparisons revealed that errors were primarily concentrated on the corridors close to the reinforced one. In fact, the third corridor is chosen more than the fourth (3rd *vs.*4th: t(6) = 4.110, $p = 0.006$) and the fifth (3rd *vs.* 5th: $t(6) = 3.440$, $p = 0.014$), while the first one is chosen more than the fourth (1st *vs.* 4th: $t(6) = 2.596$, $p = 0.041$).
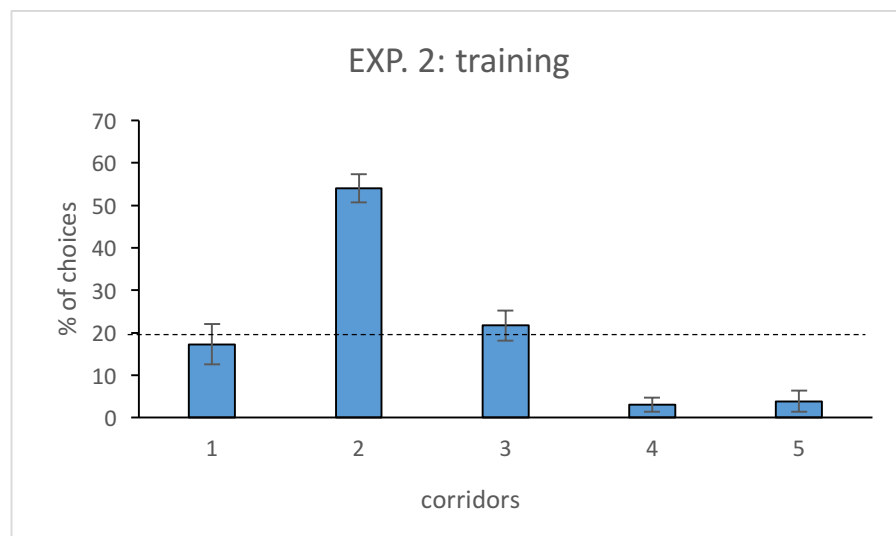

Figure 31. Percentages of total choices (mean values $\pm$ SEM) for each corridor position in the last training session (learning criterion reached).

There were no difference between the first and the third corridors ($t(6) = -0.645$, $p = 0.542$), between the first and the fifth ($t(6) = 2.282$, $p = 0.063$) and between the fourth and the fifth ($t(6) = -1.000$, $p = 0.356$).

The results at test are reported in Figure 32 and show the percentage of choices for each corridor taking into account only the first choices and the total choices in the first 30 seconds of trials.
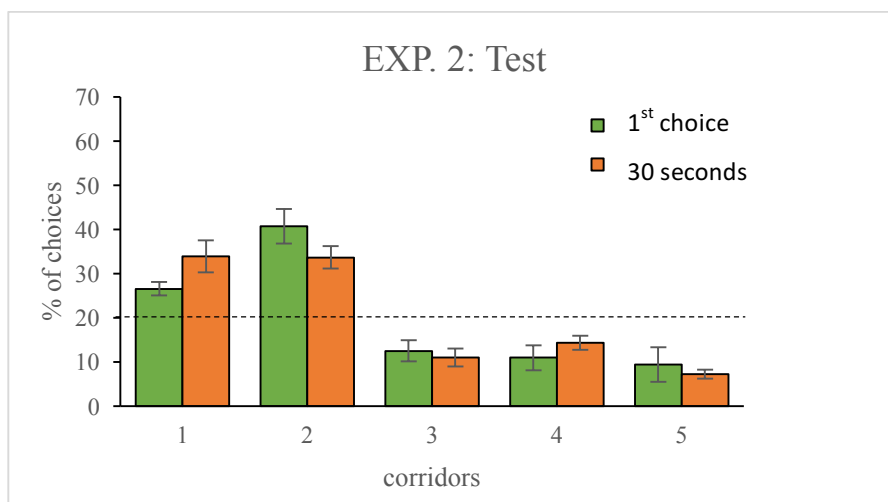

Figure 32. Percentages of choices (mean ± SEM) for each corridor position at test in the first trials and total frequencies in 30 seconds.

A repeated measure ANOVA with the five corridors as within subject factor, revealed a statistically significant effect of the corridors for both the first trials [$F_{(4,24)} = 12.385$, $p \leq 0.0001$] and the total of choices in 30 seconds [$F_{(4,24)} = 17.943$, $p \leq 0.0001$], showing heterogeneity associated with corridors. A paired t test on the first choices revealed a preferential choice for the second corridor compared to each other corridor (1st *vs.* 2nd: $t(6) = -3.376$, $p = 0.012$; 2nd *vs.* 3rd: $t(6) = 4.824$, $p = 0.003$; 2nd *vs.* 4th: $t(6) = 4.250$, $p = 0.005$; 2nd *vs.* 5th: $t(6) = 4.250$, $p = 0.005$) and for the first compared to each other corridor (1st *vs.* 3rd: $t(6) = 4.583$, $p = 0.004$; 1st *vs.* 4th: $t(6) = 4.382$, $p = 0.005$; 1st *vs.* 5th: $t(6) = 3.361$, $p = 0.015$). No significant difference between the other corridors were present (3rd *vs.* 4th: $t(6) = 0.548$, $p = 0.604$; 3rd *vs.* 5th: $t(6) = 0.281$, $p = 0.788$; 4th *vs.* 5th: $t(6) = 0.000$ $p = 1.000$.

A paired t test on the total choice in the 30 second revealed significant differences between corridors 2 and 3 ($t(6) = 4.454$, $p = 0.004$), corridors 2 and 4 ($t(6) = 8.579$, $p \leq 0.0001$), corridors 2

and 5 ($t(6) = 7.586$, $p \leq 0.0001$), between corridors 1 and 3 ($t(6) = 5.620$, $p = 0.001$), corridors 1 and 4 ($t(6) = 3.200$, $p = 0.019$), corridors 1 and 5 ($t(6) = 5.299$, $p = 0.002$ and between corridors 4 and 5 ($t(6) = 3.603$, $p = 0.011$). No significant difference were revealed between corridors 1 and 2 ($t(6) = -0.564$, $p = 0.593$), corridors 3 and 4 ($t(6) = -1.255$ $p = 0.256$) and corridors 3 and 5 ($t(6) = 1.146$, $p = 0.296$).

The results confirmed that zebrafish were able to identify a particular corridor at training. When then tested in a condition that created a conflict between numerical order and spatial distance among the corridors (2nd and 3rd), fish generalized the response in the new apparatus by correctly choosing the second corridor, even if that position was located at a different spatial distance. Beside the second corridor, it has been observed that the first corridor has been chosen more than the others. This result is not unexpected, in fact in an entire set of objects, those at the extremes are elements that are more salient than the others, reflecting a preference for the first corridor. The results obtained here suggest that fish did not use the spatial distance as a spontaneous strategy, preferring to follow ordinal numerical information.

5.3. EXPERIMENT 3

In a third experiment, we wanted to study how the fish's choice strategy changed when the number of elements between training and test increased. This also allowed us to control not only for the absolute spatial distances in terms of length, but also for the relative proportions that lies between the correct corridor and the entire length of the elements set. To do so a new test condition was used with the same group of subjects tested in the previous experiment.

5.3.1. Subjects and apparatus

In this experiment we tested the same group of *Danio rerio* that was used in the previous experiment. In this condition, the length of the apparatus remained the same, but the number of corridors changed from 5 to 9 (Figure 33).
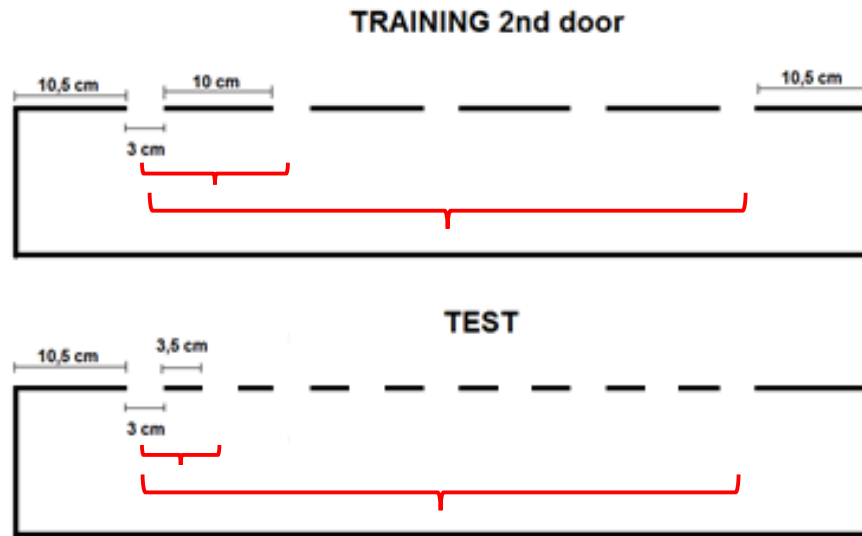
Figure 33. Schematic representation of the apparatus during the training and the test in the third experiment. At test the number of corridors changed, as well as the proportion between the corridors and the entire elements set.

5.3.2. Results

The results at test are reported in Figure 34 and show the percentage of choices for each corridor taking into account only the first choices and the total choices in the first 30 seconds of trials.
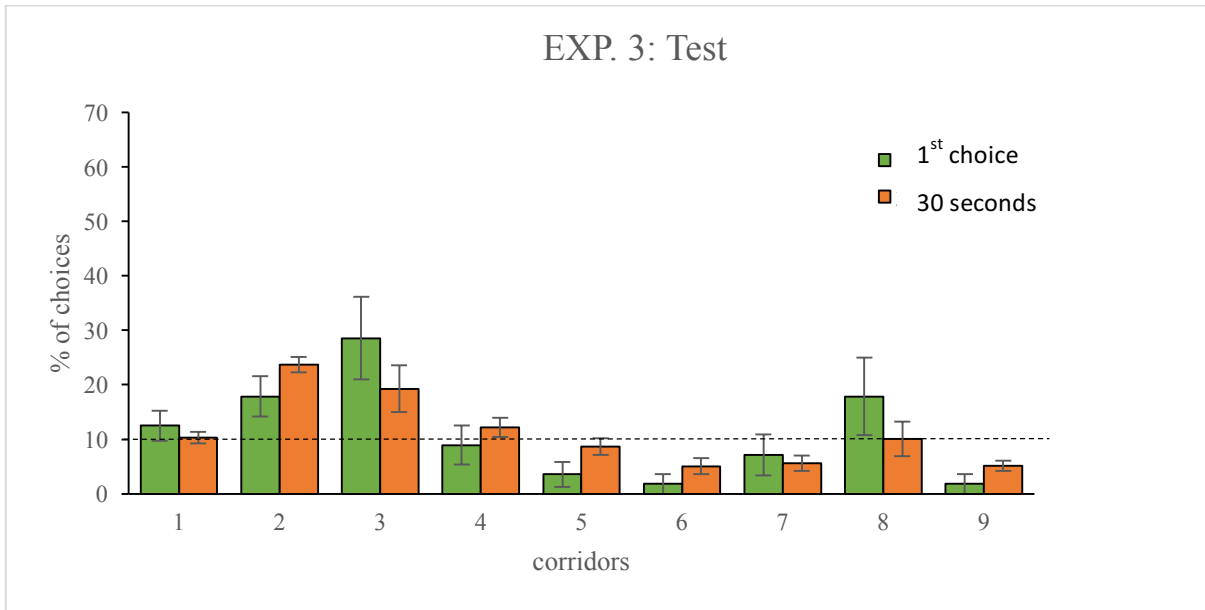
Figure 34. Percentages of choices (mean ± SEM) for each corridor position at test in the first trials and total frequencies in 30 seconds.

A repeated measure ANOVA with the five corridors as within subject factor, revealed a statistically significant effect of the corridors for both the first trials [$F_{(8,48)} = 3.865$, $p = 0.001$] and the total of choices in 30seconds [$F_{(8,48)} = 9.544$, $p \leq 0.0001$], showing heterogeneity associated with corridors. A paired t test on the first choices revealed significant difference between corridors 1 and 6 ($t_{(6)} = 3.286$ $p = 0.017$), corridors 1 and 9 ($t_{(6)} = 3.286$ $p = 0.017$), corridors 2 and 5 ($t_{(6)} = 3.361$ $p = 0.015$), corridors 2 and 6 ($t_{(6)} = 3.576$ $p = 0.012$), corridors 2 and 7 ($t_{(6)} = 2.521$ $p = 0.045$), corridors 2 and 9 ($t_{(6)} = 4.500$ $p = 0.004$), corridors 3 and 5 ($t_{(6)} = 2.898$, $p = 0.027$), corridors 3 and 6 ($t_{(6)} = 3.041$, $p = 0.023$), corridors 3 and 9 ($t_{(6)} = 3.873$, $p = 0.008$) and corridors 4 and 6 ($t_{(6)} = 2.828$ $p = 0.030$). No other comparison were significant different.

A paired t test on the total choice in the 30 second revealed significant differences between corridors 1 and 2 ($t_{(6)} = -8.148$, $p \leq 0.0001$), corridors 1 and 3 ($t_{(6)} = -2.889$, $p = 0.028$), corridors 1 and 6 ($t_{(6)} = 2.680$, $p = 0.037$), corridors 1 and 7 ($t_{(6)} = 3.357$, $p = 0.015$), corridors 1 and 9 ($t_{(6)} = 3.742$, $p = 0.010$), corridors 2 and 4 ($t_{(6)} = 6.485$ $p = 0.001$), corridors 2 and 5 ($t_{(6)} = 6.931$, $p \leq 0.0001$), corridors 2 and 6 ($t_{(6)} = 8.800$, $p \leq 0.0001$), corridors 2 and 7 ($t_{(6)} = 9.721$, $p \leq 0.0001$), corridors 2 and 8 ($t_{(6)} = 4.103$ $p = 0.006$), corridors 2 and 9 ($t_{(6)} = 10.328$, $p \leq 0.0001$), corridors 3 and 5 ($t_{(6)} = 3.041$, $p = 0.023$), corridors 3 and 6 ($t_{(6)} = 3.269$, $p = 0.017$), corridors 3 and 7 ($t_{(6)} = 3.447$, $p = 0.014$), corridors 3 and 9 ($t_{(6)} = 3.333$, $p = 0.016$), corridors 4 and 6 ($t_{(6)} = 3.104$, $p = 0.021$), corridors 4 and 7 ($t_{(6)} = 2.680$, $p = 0.037$), corridors 4 and 9 ($t_{(6)} = 3.361$, $p = 0.015$). No other comparison were significant different.

By observing the first choices at test, the corridor mainly chosen was the third one, which corresponded during the test to the second one, according to a spatial information cue. The others two corridors mainly chosen were the 2nd and the 8th, which were the second elements counting from left and from right. However, this effect tended to decrease with time (test 30 seconds), showing a preference only for the 2nd and the 3rd corridor from left.

In this condition, the numerical information seemed to have a reduced priority in the generalization test, probably due to the strong variation of the elements set that almost doubled the number of corridors present (from 5 to 9 corridors).

The results suggest that both numerical and spatial cues were coded in the training phase, and when the environment underwent important variations, they could be used together to identify the correct corridor.

An interesting result was that, as first choices, the fish tend to choose both the second corridor from the left and from the right side (the 2nd and the 8th). This effect was totally absent in the two previous experiments, and a possible explanation is due to the elevate number of corridors introduced in this last condition. In fact, too many elements in a relative small apparatus could be not processed in the entire set, but only partially, and, since the most salient elements are the external ones, the fish could have based its choice strategy starting from both ends.

5.4. Discussion

The results reported in this study suggest that zebrafish can identify an element using its ordinal position in a series of identical elements, also showing that this strategy is spontaneously used when numerical and spatial cues are in contrast, providing that the set of elements does not undergo considerable changes, such as observed in experiment 3.

My results contribute to extend the limited evidence of the use of ordinal numerical information in fish (Miletto Petrazzini et al., 2015), adding to the already consolidated evidence in primates (Brannon & Terrace, 1998; Smith et al., 2003; Judge et al., 2005), non-primate mammals (Suzuki & Kobayashi, 2000; Jakkola et al., 2005) and birds (Emmerton et al., 1997; Pepperberg, 2006b; Rugani et al., 2007; Scarf et al., 2011).

In this study, zebrafish learned to identify the second corridor in a series of identical ones at the same distance, that allowed the fish to go out from the apparatus towards a comfortable environment. Subsequent tests have investigated whether fish had spontaneously encoded numerical or spatial cues to identify the correct corridor. Results showed that zebrafish did not rely on the total

size of the apparatus to identify the correct corridor, preferring to rely on the numerical cue, even when the spatial distance created a potential conflict with numerical position. The use of ordinal information was maintained even if the inter-distance among the corridors changed.

I also found that zebrafish, at test, tend to concentrate the attempt directed to the two positions adjacent to the correct one, in particular to the one closer to the beginning of the set (the first on the left). A possible explanation for this could be that fish identify the correct position on the basis of the closest end of the sequence, and this reflects a high number of attempt in the previous position rather than the subsequent one. This trend has been found also in rats (Suzuki & Kobayashi, 2000), chicks (Rugani et al., 2007) and another fish species (Miletto Petrazzini et al., 2015).

Finally, I found that when the number of elements of the set increased significantly (from 5 to 9, experiment 3), fish seemed to rely primarily on the spatial proportion cue, probably due to the difficulty to glimpse the entire series of elements, important for the use of numerical cue. However, fish showed also a preference for the ordinal positions of the elements, starting from the left side and, surprisingly, also from the right side of the set, a trend that disappeared with time, leaving only the second corridor from the right (ordinal cue) and the third corridor (spatial cue) mainly chosen. This last double preference for the 2nd and the 3rd element could be explained by supposing that numerical and spatial cues are used together to solve the task. However, another possible hypothesis is that the only spatial information is used at test (choice for the 3rd), while the attempts for the second corridor could be a bias to make errors more often on the previous position to the alleged correct one.

In summary, my experiments provide the first evidence in zebrafish of the use of ordinal numerical information, showing also a spontaneous preference over non-numerical cues. Despite this preference, spatial information could be also used by zebrafish when the numerical cue is less reliable, like when the fish cannot glimpse the entire series of elements.

In future studies, it would be of interest to investigate which are the limits of ordinal discrimination, by training fish to select ordinal positions higher than 2. In guppies it has been shown that precision decreases as the ordinal position increases (Miletto Petrazzini, 2015).

STUDY 4

Mapping Numerosities into space by Zebrafish

Humans seem to represent numbers along a mental number line (MNL) oriented from left to right (Dehaene, 2011). An experimental demonstration of the mental number line is the SNARC effect (Spatial-Numerical Association of Response Codes; Dehaene et al., 1993). In the SNARC effect, participants are faster in responding to small numbers when these are executed on the left side of space, and to large numbers when responses are executed on the right side of space.

It has been found that the orientation of the numbers may be influenced by cultural factors. Populations that have a different reading direction, such as Arabic people, show an inverted mental number line (Zebian et al., 2005). The direction of the MNL is influenced by cultural factor, but this does not exclude that a biological origin to orient numbers from left to right is present. Recently, similar phenomena have been described for non-symbolic numerosities in preverbal infants (Bulf, de Hevia & Macchi Cassia, 2015), primates (Adachi, 2014) and birds (Rugani et al., 2015).

Recently, the study of a MNL has also been investigated in fish (Triki & Bshary, 2017). Using the same protocol of Rugani and colleagues (2015) with chicks, the authors trained cleaner fish (*Labroides dimidiatus)* to find a food reward behind a panel depicting 5 elements. During the test, an identical pair of panels depicting either 2 elements or 8 elements were presented. Results did not show a preference to allocate small quantities on the left side and big quantities on the right side, providing no evidence that cleaner fish use a MNL.

The aim of this study was to investigate if zebrafish show a link between number and space in the mapping of numerosities. Zebrafish were first trained, using a discrimination task, to associate a target number with a reward. Differently than in the study of Triki & Bshary (2017), where no discrimination was required, here zebrafish were required to pay attention to the numerosity. Then zebrafish were tested in a preference choice test between two identical numerosities, but different to the training one, placed on the right and the left side of the experimental apparatus. We measured whether zebrafish spontaneously associated smaller numbers with the left space and larger numbers with the right space.

6.1. Method

6.1.1. Subjects

Adult males of zebrafish were used. Subjects were reared in an automated aquarium system (ZebTEC Benchtop, Tecniplast) in 3.5-L plastic tanks in isolated sex groups of 10 individuals. The water temperature was maintained at 26°C and the system were illuminated following a light cycle of 12 hours. Fish were fed three times a day with dry food (Sera GVG-Mix).

During the entire experiment, the subjects were kept separated one to each other into a 20-L plastic aquarium (23 x 38 x 25 cm) set with gravel and vegetation, mimicking a natural environment. Despite each animal was isolated, it could see the conspecifics from a net divider, in order to avoid potential negative effect due to the social deprivation. The range of fish total lengths was between 4 cm and 5 cm for both males and females.

6.1.2. Apparatus

The experimental apparatus consisted of a "diamond shaped" arena (28 x 25 x 19 cm) made of white plastic material (Poliplak, see Figure 35a). On the longest side of the apparatus, two specular rectangular exit doors were carved (1.5 x 4.5 cm, located 4.5 cm from the floor), connecting the intern of the apparatus with an outer region. The apparatus was indeed inserted into a larger opaque tank (35 x 49 x 27 cm) set with gravel, plants, and two female conspecifics. In this way, animals were strongly motivated to go out of the apparatus and reach the comfortable environment. Under each exit door there was a removable white plastic card, depicting a precise number of orange square stimuli (Figure 35b). Between the two doors, a protruding plastic vertical partition was placed as a divider, creating a left and a right area.
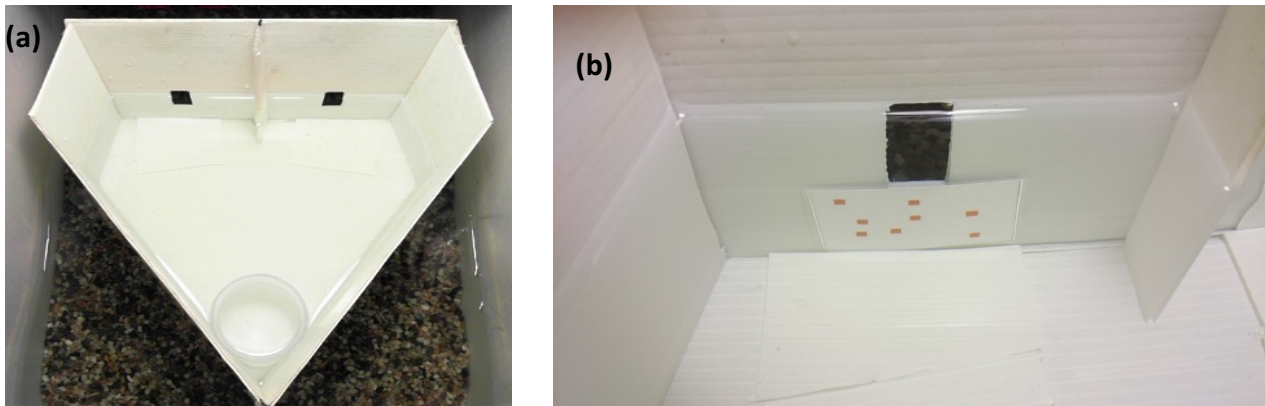
Figure 35. (a) A photograph of the apparatus used. **(b)** Particular of the door with a card depicting 8 elements.

The water was maintained at a constant temperature of 25°C and filtered by a pump and filter system (Micro Jet Filter MCF 40). The apparatus was lit by a fluorescent 60-W lamp, and a webcam (Microsoft LifeCam Studio) recorded the fish behavior from above (50 cm) the setup.

6.1.3. Procedure

The experiment comprised two parts: *training* and *test*.

Training

In the training phase, just one of the two exit-doors was open, while the other was blocked from the outside by a thin transparent plastic sheet. The opened door was identifiable by a plastic card (depicting a defined number of elements) located under it (Figure 35b) while under the blocked one there was a white card without elements.

Each animal was trained with individual daily sessions of 10 trials. Before starting each session, the fish was taken from the housing aquarium and left for 5 minutes outside of the apparatus into the comfortable environment. At the beginning of each test, the fish was confined into a transparent plastic cylinder in front of the stimulus (Figure 36). After a short wait of 15 seconds, the cylinder was lifted up, allowing the fish to move freely within the apparatus. A correction method has been used: if the fish made a wrong choice, it was allowed to change it until it was able to exit, or until the overall time allowed for the trial elapsed (maximum 15 minutes). An attempt choice was considered done when the fish approached with its snout a door, touching the transparent panel in the case of the wrong door, or passing through the door in correspondence of

the correct one. The reward received after each trial depended from the animal performance: when the correct corridor was chosen as first attempt, it received a 6 minute interval, food and social companions; when the animal made one or more choices for the wrong exit, it received 3 minutes interval, no food and no conspecifics; in case of no response during the maximum time of the trial (15 minutes), the fish was given a pause-time of 5 minutes.

The position of the reinforced exit (left or right) as well as the spatial disposition of the elements changed randomly among trials. It was really important to focus the attention of the fish only on the numerosity of the stimuli, making it the only reliable strategy to identify the correct exit.

At the end of each session, the number of total frequencies in each corridor was calculated in the 10 trials. Learning was considered complete when the subject presented, for two consecutive daily sessions, a frequency of the correct number of choices equal to or greater than 70% on the total frequencies. Once the learning criterion was reached, the fish was ready for the testing phase.

Test

The testing phase consisted of four trials with no reinforce (both doors were closed). In each trial, two identical card stimulus, but with a different numerosity than in the training phase, were located on the right and left exits. In front of each door, a white plastic board located on the floor delimitated a restricted area close to them (highlighted in Figure 37). This area has been used in the test to codify the first approach by the fish towards one of the two stimuli (right or left). The shape area has been delineated with the aim to create a precise and restricted left and right area close to the stimulus, avoiding a central neutral area.

Before each trial, the fish was placed into the cylinder, and after 20 seconds of observation, the fish was released and the first approach was registered. To be considered as a choice, the fish had to enter in one of the two areas with the entire body. The interval between test trials was 5 minutes, during which the fish was free to swim in the comfortable environment. For each subject, the approach in the first trial, as well as the total approaches in the four trials, were analyzed.

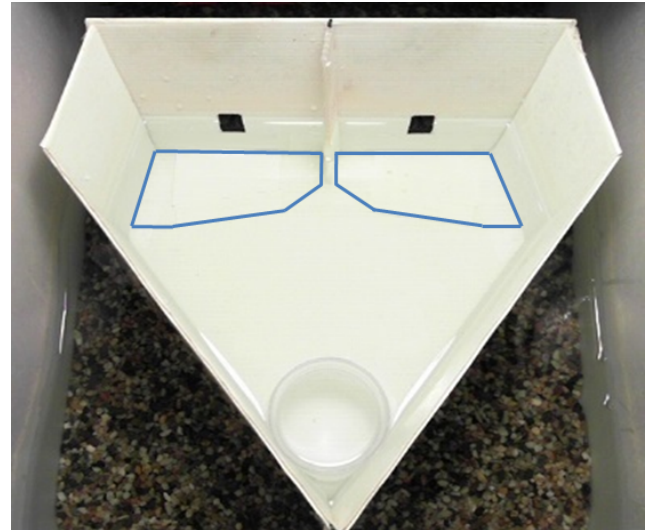**Figure 36**. Example of trial (left door reinforced).

Figure 37. Choice areas used in the test.

6.2. EXPERIMENT 1

Thirteen male fish were trained to obtain a reward by choosing a panel depicting 5 orange squares. When fish learned the task, two test (divided in two days) were proposed: in one test two panels, with a lower numerosity (2 squares) than in the training, were placed under the left and under the right door; in the other test, two panels with a larger numerosity (8 squares) than in the training were used (see figure 38). In this experiment, each square side was long 3 mm.

Half of the fish performed first the lower numerosity test and then the larger numerosity test, the other half vice versa. The aim of this first experiment was to see if zebrafish would spontaneously associate a smaller number (2) with the left space and a larger number (8) with the right space.
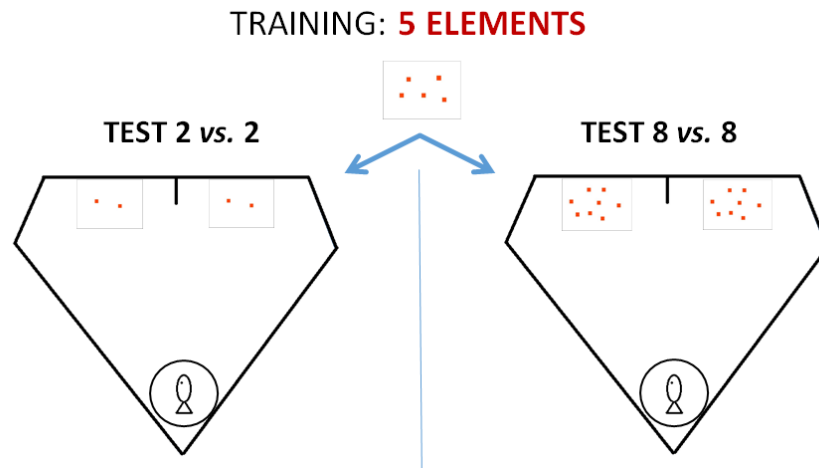
TRAINING: **5 ELEMENTS**

TEST 2 *vs.* 2   TEST 8 *vs.* 8

Figure 38. Schematic representation of the first experiment. Fish were trained to discriminate 5 elements. After, fish were tested with two identical numerosities, but smaller (2) or bigger (8) than training.

6.2.1. Results

The results at test are reported in Figure 39 and show the percentage of choices for the left area in the first trial and in the four trials for both the smaller and the larger tests. In the first trial, fish preferred to approach the left panel when the numerosity at test decreased (test 2 vs. 2). When the numerosity at test was larger (8 elements) the performance was at the chance level. A Fisher's exact test, was performed in order to compare the two conditions, revealing no significant difference ($p = 0.4110$).
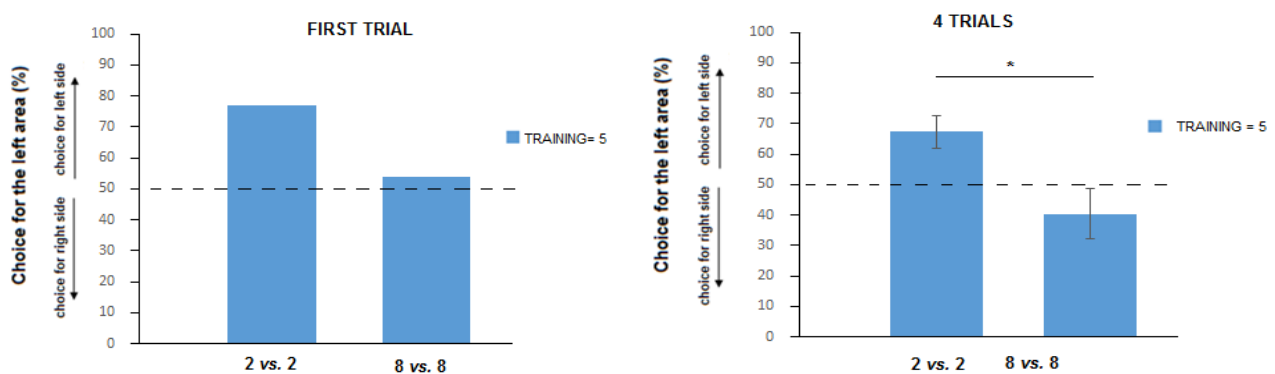
Figure 39. Results of the experiment 1: percentage of choice for the left area in the first trial and percentage of choice (mean ± SEM) in the total trials, in the 2 *vs.* 2 and 8 *vs.* 8 test.

Analyzing the total choices in the four trials, for each fish, an index of choice for the left area was calculated. One-sample two-tailed *t* tests revealed that zebrafish choices were significantly greater for the left area when, at test, the numerosity was smaller (2 *vs.* 2 condition: $t(12) = 3.323$, *p*

= 0.006). On the other hand, there was no significant difference in fish tested in 8 *vs.* 8 condition ($t(12)$ = -1.162 p = 0.268), although there was a good trend towards the right area. However, a paired sample *t*-test revealed a significant difference between the two groups ($t(12)$ = 2.214, p = 0.047).

This first experiment revealed that zebrafish, when tested with a smaller numerosity (2) than in the training (5), preferred to approach the left area, while when tested with a larger one (8) there was only a trend for the right side. A possible explanation of this result is that, since the animals performed two tests, they could have interfered which each other, reducing the number-space mapping. As a result, the comparison between 5 and 8 elements may have been more affected because of the ratio, which is less advantageous than the comparison between 5 and 2.

6.3. EXPERIMENT 2

A fundamental characteristic of the MNL, that was investigated in this second experiment, is its relativity. This property means that the same number could assume a smaller value or a bigger value related to a target number. For instance, the number 5 is bigger than the number 2, which is placed on the left side of the MNL, while the same number is smaller if referred to the number 8, assuming in this case a left space mapping (Figure 40).

In this experiment, one group of seven male zebrafish was trained with 2 elements and one group of eight males was trained with 8 elements. Then, both groups performed the same test 5 *vs.*5.

Figure 40. Schematic representation of the Experiment 2: two groups of fish were trained with 2 or 8 elements ant then tested with 5 elements.
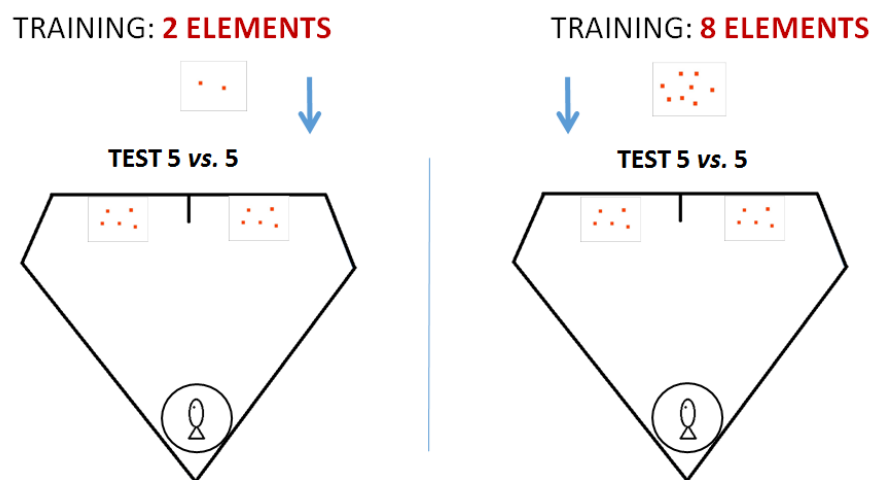
6.3.1. Results

The results at test are reported in Figure 41 and show the percentage of choices for the left area in the first trial and in the four trials, for the two different trainings (8 or 2 elements). Fish trained with 8 elements, in the 5 *vs.* 5 test, preferred as first trial to approach the left panel, while those who were instead trained with a smaller numerosity (2), at test approached the right area. A Fisher's exact test was performed in order to compare the two conditions, revealing a significant difference ($p = 0.0406$).
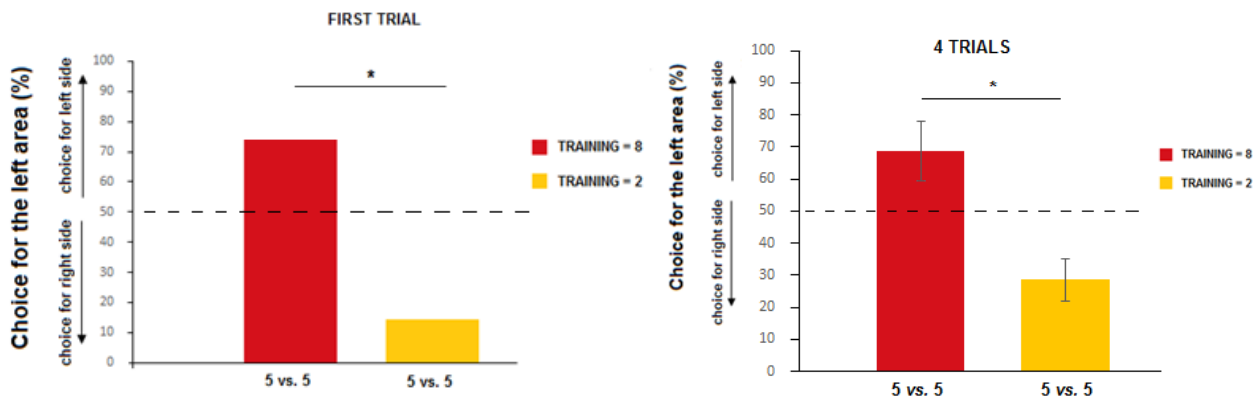

Figure 41. Results of the experiment 2: percentage of choice for the left area in the first trial and percentage of choice (mean values ± SEM) in the total trials test, for the training with 8 and 2 elements.

Analyzing the total four trials, one-sample *t*-test (two-tailed) revealed that zebrafish choices were significantly greater for the right area passing from smaller to larger numerosity (from 2 to 5 elements; $t(6) = -3.286$, $p = 0.017$), but not the opposite (from 8 to 5 elements; $t(8) = 2.049$, $p = 0.080$). An independent *t*-test with type of training as between subjects factor has revealed a strong significant difference between the two groups ($t(13) = -3.479$, $p = 0.004$).

In this experiment, zebrafish showed that the same numerosity at test (5) is processed as smaller or larger, compared to the numerosity seen at training. Fish showed again more difficulties in the discrimination between 5 and 8 element than 2 and 5 elements, probably due to the different ratio among the two comparisons.

6.4. EXPERIMENT 3

In this third experiment we controlled the effect of non-numerical cues on number-space mapping. As known, when numerosities increase, there are physical variables that co-vary with them. In this condition we controlled for the overall length of the stimulus.

6.4.1. Training 2-8 elements, test 5 *versus* 5

We used the same numerical comparison as in the previous experiment, training two groups with 2 and 8 elements, and testing them in a 5 *vs.* 5 test, with the substantial difference that the contour length was equalized between training and test (Figure 42). One group has been trained with two squares (each side long 7.5 mm), and tested with 5 squares (each side long 3 mm), so that the overall perimeter for both group of stimuli was 60 mm. The second group was trained with 8 squares (each side long 3 mm), and tested with 5 squares (each side long 4.8 mm). The overall perimeter for both groups was 96 mm.

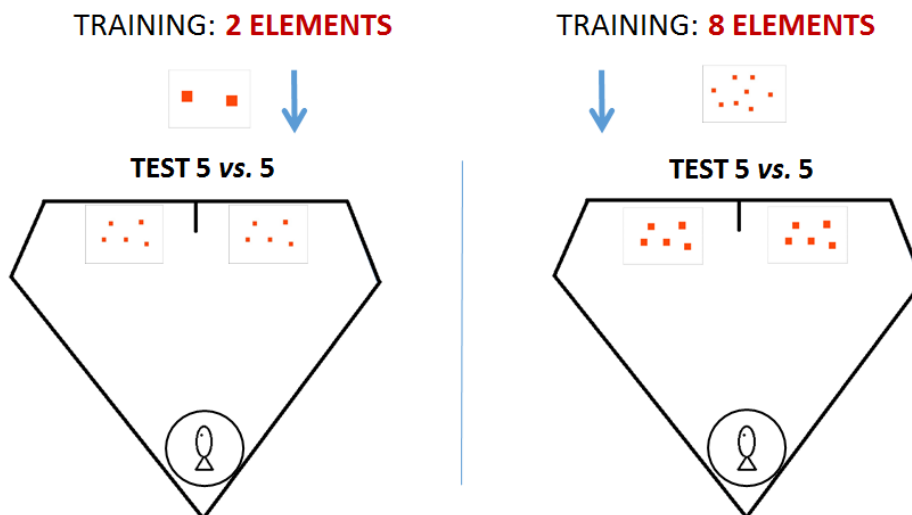**Figure 42.** Schematic representation of the Experiment 3: two groups of fish have been trained with 2 or 8 elements and then tested with 5 elements. The overall perimeter at training and test has been equalized.

Another important aspect about this condition is that when the perimeter is equalized, this create at the same time a negative correlation between overall area and number. This means that

when the numerosity decreases, the area increases and vice versa. For example, in the first condition, with the same perimeters, the overall area of the 2 elements was larger (112.5 mm^2) than the one of the 5 elements (45 mm^2). In the other condition, 8 elements had an overall area of 72 mm^2, while 3 elements had an area of 192 mm^2. We could take advantage of this opposite correlation to see if fish spontaneously base their choice on the numerosity or the total area.

One group of eight males fish was trained with 2 elements and one group of ten males fish was trained with 2 elements. Both groups were then tested in a 5 *vs.* 5 comparison.

6.4.2. Results

The results at test are reported in Figure 43 and show the percentage of choices for the left area in the first trial and in the four trials, for the two different trainings (8 or 2 elements). The results obtained show that, fish trained with 8 elements and tested with a smaller numerosity (5 elements) show a slightly approach toward the left panel, while the group trained with a smaller numerosity (2) than in the test (5) strongly approach the right area. A comparison between the two conditions revealed a significant difference (Fisher's exact test, $p = 0.043$).

Figure 43. Percentage of choice for the left area in the first trial and percentage of choice (mean values ± SEM) in the total trials test, for the training with 8 and 2 elements.

Results for the total choices show that zebrafish choices were significantly greater for the right area passing from a smaller to a larger numerosity (from 2 to 5 elements; $t(9) = -3.000$, $p = 0.015$), but not the opposite (from 8 to 5 elements; $t(7) = 0.552$, $p = 0.598$). An independent t-test with the type of training as a between subjects factor has revealed a strong significant difference between the two groups ($t(16) = -2.273$, $p = 0.037$).

When passing from 2 elements to 5, fish preferred to approach the right side, suggesting the use of numerical magnitudes rather than non-numerical cues such as area or perimeter. On the other hand, fish trained with 8 stimuli and tested with 5 showed just a little bias (as in the experiment 2) for the left side.

6.4.3. Training 8 elements, test 3 *versus* 3

This control condition has been developed with the aim to clarify whether the lack of number-space mapping when fish were trained with 8 elements and then tested in a 5 *vs.* 5 choice was due to a ratio between the two numbers not bigger enough. To do so, in this experiment it has been used a ratio similar to the one present in the comparison between 2 and 5 elements (ratio=0.4).

A group of nine male zebrafish were trained with 8 elements (each square side long 3 mm), and tested with 3 *vs.* 3 squares (each side long 8 mm). The overall perimeter for both groups was 96 mm, while the overall area of the 8 elements was 72 mm^2, and the one for the 3 elements was 192 mm^2 (Figure 44). The ratio between the two numerosities was 0.375.

Figure 44. Fish were trained with 8 elements and tested with 3 elements. The contour length between training and test is balanced.

6.4.4. Results

Results are reported in Figure 45. In the first trial, fish showed a preferential approach to the left panel. However, this was not confirmed by analyzing the total trials, where zebrafish's choices were not significant ($t(8) = -0.316$, $p = 0.760$).
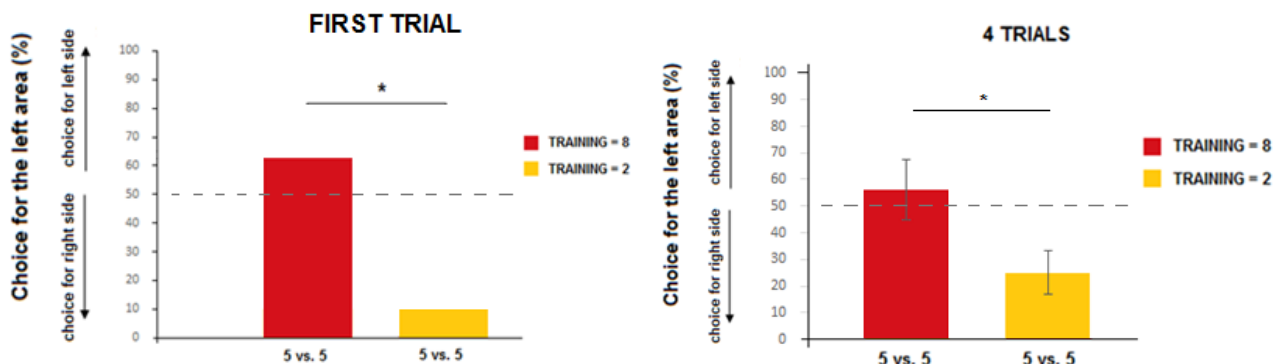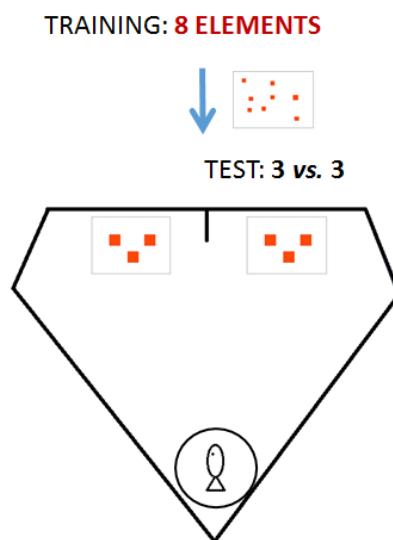

Figure 45. Percentage of choice for the left area in the first trial and percentage of choice (mean ± SEM) in the total trials test.

When tested with a smaller numerosity (3) than in the training (8), in this condition fish showed a preferential approach in the first trial test, but not in the total of trials. Despite the ratio used here (ratio=0.375), that was even more advantageous than the one used in the discrimination between 2 and 5 elements (ratio=0.4), results did not show a number-space mapping.

It should be noted, however, that in this last condition, the difference between the area of the 3 elements and the area of the 8 elements was strongly different (Δ areas =120 mm^2), almost the double than the one in the other condition proposed between 2 and 5 elements (Δ areas = 67.5 mm^2). A possible explanation is that although the ratio may be enough to map numerosites into space, the strong difference of area could create a tendency to use surface area rather than number as reference magnitude.

6.5. Discussion

The results obtained in this study suggest that zebrafish might associate numerosities with space. When trained to identify a particular target number of elements, fish spontaneously associate novel numerosities onto space, with small numerosities to the left area, and large numerosities to the right area. However, the number-space mapping seems to be affected also by other non-numerical magnitudes.

After training with 5 elements, zebrafish spontaneously associate a smaller numerosity (2 elements) with the left area, but showed only a trend for the same preference for the right side when the numerosity increased (8 elements). In this experiment, however, the same fish were tested with both types of changes and this may have interfered with the mapping.

When tested in a between-subjects design, zebrafish trained with 2 elements and tested with 5, spontaneously associated this numerosity with the right area. The opposite trend was found when fish were trained with 5 and then tested with 8 elements, although the preference was not as strong as the one between 2 and 5. The ratio could play an important role in the spatial numerical mapping. In fact, the ratio between 5 and 8 (ratio=0.625) is less advantageous than the one between 2 and 5 (ratio=0.4), making the discrimination more difficult.

When contour length was equalized, zebrafish preferred to approach the right side when the numerosity at test (5) was bigger than at training (2). This result exclude that fish focused their choices using the total area, due to its negative correlation with the numerosities. However, if the difference of the total area between the two numerical elements change considerably, it may influence the number-space association, as hypothesized in the contour length control discrimination between 8 and 3 elements.

The results provide further evidence that the number space mapping is not exclusive to mammals (Adachi, 2014) and birds (Rugani et al., 2015) but is observed also in fish. Recently it has been argued that fish do not possess a mental number line, providing evidence that the cleaner fish *Labroides dimidiatus* fail in a similar test (Trili & Bshary, 2017). However, the paradigm used by the authors, replicating the one used with chicks (Rugani et al., 2015), presents substantial differences compared to the one used here in zebrafish. In fact, cleaner fish were trained to find food behind a panel depicting a target number of elements, procedure that did not ensure that fish payed enough attention to the elements, because it was not necessary in order to reach the reward. In my experiment, I have used a training discrimination where the fish had to select the correct panel (the one with the elements depicted on it) to receive a reward. It should not be excluded that the different performance between the two fishes may be related to the difference between the

species. The Cleaner fish, differently to zebrafish, is a salt water fish, and lives in a strongly different environment. It would be interesting to further investigate the presence of a rudimental number line in that species, using the same paradigm of zebrafish. Moreover, future studies in zebrafish are necessary in order to understand better which is the role of numerical ratio and physical variables in the number-space mapping.

In conclusion, the evidence reported in this study contributes to prove that the disposition to map numerosities from left to right exists independently of cultural factors and could be biologically determined. Rugani and colleagues (2015) suggest that the spatial mapping of numbers may be a universal cognitive strategy available among animals, and such direction from left-to-right may be due to brain asymmetry and a right hemisphere dominance in visuospatial tasks.

7. GENERAL CONCLUSION

The results reported in this thesis contribute to provide evidence of a wide range of numerical abilities in the fish zebra (*Danio rerio*). Besides supplying support to the hypothesis of an ancient phylogenetic origin of numerical abilities among vertebrates, these results have particular relevance because obtained in an animal model with a great potential for future implications in the study of the genetic bases of numerical cognition.

In the first study, discrimination of quantity (magnitude) was investigated using a spontaneous shoaling behavior preference. The results obtained showed that zebrafish were able to discriminate between small and large numerical ranges and the main factor affecting the precision of the discrimination was the ratio between the sets to be discriminated. Zebrafish abilities to discriminate between two numerousness would increase as the ratio between them increases, in accordance with the Weber's law (Gallistel & Gelman, 1992). In particular, zebrafish discriminate up to 2:3 ratio (0.67), failing with a 3:4 ratio (0.75), both for small and large numerical comparisons. The same limit has been found in other fish species such as redtail splitfin (*Xenotoca eiseni*; Stancher et al., 2013) and angelfish (*Pterophillum scalare*; Gómez-Laplaza & Gerlai, 2011b), while other species are even more accurate, until a 0.75 ratio (*Poecilia reticulata*; Agrillo et al., 2012a; *Gambusia holbrooki*; Agrillo et al., 2008a). However, these results, differently from those obtained in zebrafish, were documented only for small numerical discriminations (e.g. 1 *vs.* 2, 2 *vs.* 3 and 3 *vs.* 4). When large numerical discriminations were presented (over 4 elements), fish showed a decline of the performance, with discrimination up to 0.5 ratio (*Poecilia reticulata*; Agrillo et al., 2012a; *Gambusia holbrooki*; Agrillo et al., 2008a; *Xiphophorus elleri*; Buckingham et al., 2007) or up to 0.56 ratio (*Pterophillum scalare*; Gómez-Laplaza & Gerlai, 2011b). The results supported the possible existence of two numerical systems: an object file system (OFS), for representing small values ($\leq$ 3–4) and an approximate number system (AMS), for representing large magnitudes (> 4). This two-systems evidence has been observed in other animal species (e.g., chimpanzees, see Tomonaga & Matsuzawa, 2002; birds, see Rugani et al., 2008; honeybees, see Dacke & Srinivasan, 2008). The main finding that favors the existence of two systems was found in the failure to discriminate sets across a large-small boundary such as in 1 *vs.* 4 or 2 *vs.* 4 discrimination, the so called "boundary effect" (infants: Feigensons et al., 2002; fish: Piffer et al., 2012). What I found in my study is different: zebrafish showed not only good performance discrimination over small and large discrimination, maintaining the same 0.67 ratio limit, but they were also able to discriminate a comparison that crossed the boundary of the two hypothesized systems (2 *vs.* 4). This suggest that the

results obtained in zebrafish appear to be in agreement with the idea of a single mechanism for dealing with approximate estimation of the magnitude of numerosity, that corresponds to the ANS or to some equivalent. Similarly to zebrafish, other animals studies suggest a continuity in the processing of small and large numerosities (e.g., non-human primates, see Brannon & Terrace,1998, Cantlon & Brannon, 2007, Judge et al., 2005, Smith et al., 2003; birds, see Rugani et al. 2009; 2011;2013).

A limit of this experiment, as well as in all other studies that make use of social conspecifics as stimuli, is the lack of control of the continuous physical variables (e.g. area, boundaries length, or amount of movement) that could play a crucial role in the discrimination process. Further experiments have to be carried out with the aim to control for non-numerical variables, using for example artificial stimuli. This would help us to understand also if the accuracy in discrimination changes between social and non-social stimuli, due to possible different motivations.

The second part of the thesis showed that zebrafish could track one-by-one additions of sequential single elements. This ability is particularly interesting because it provides evidence that fish can do mental transformations over numerical values. To do so, I have proposed, for the first time in fish, a discrimination between two sets of conspecific shoals, differing in numerosity, involving arithmetic addition. The paradigm proposed was inspired to the ones used in infants (Wynn,1992), mammals (primates; Hauser et al., 1996; Uller & Hauser, 2001; Santos et al., 2005; West et al., 2002) and birds (Rugani et al., 2009). The results obtained showed that zebrafish are able to solve such a complex task, tracking one by one conspecifics that hide behind two opaque screens and preferring the group with the bigger numerosity. Compared to the previous study, discrimination is less accurate, showing a limit that does not exceed 1:2 ratio. This lower discrimination was expected, given the difficulty of the task.

An unexpected result occurred when the total time presentation between the two groups of elements was equalized. Fish preferred always the group in which the elements moved faster, even in condition previously not discriminated (e.g. 2 *vs.* 3 elements). A possible explanation for these findings is that seeing conspecifics with reduced motility may be interpreted as less attractive, because joining a slower shoal creates a potential oddity effect (Landeau & Terborgh, 1986; Theodorakis, 1989), while active fish may be more confusing to predators.

One of the future developments of this study will aim to clarify if and how the proto-arithmetic addition ability could be helped by the different amount of time necessary to present the sets of elements.

Besides the use of paradigms that make use of spontaneous ability, to overcome the potential limitation of these tests, I also made use of training procedures to study numerical abilities in zebrafish. In general, the use of operant conditioning revealed that zebrafish can learn numerical discriminations, though with more difficulties in comparison with other fish species, such as angelfish, guppies or redtail splitfin (see Agrillo et al., 2012). One general aim for future work with zebrafish would be to develop better training paradigms for zebrafish.

The third part of this thesis aimed to study ordinal numerical ability, i.e. zebrafish were required to identify an element on the basis of its ordinal position in a series of identical ones. I found that ordinal information was spontaneously used over the spatial one, when at test the distance among the elements varied in comparison to a learned position, creating a conflict between ordinal position and spatial distance. Fish showed however difficulty when the set of elements changed, such as when the number of elements almost doubled (from 5 to 9).

Evidence of ordinal abilities in fish has been found only in another fish species, the guppies (*Poecilia reticulata*: Miletto Petrazzini et al., 2015). A direct comparison between the two species revealed important differences. Zebrafish learned to identify correctly the second of a series of five elements, while guppies learned to discriminate very well the third element over twelve objects in a shorter number of trials (less than one half). This difference could be related to the different paradigm used or could be related to the species.

Future studies should also investigate whether zebrafish show differences in learning an element position that has to be identified on a left or right oriented series, investigating if fish would be facilitated when the element to be identified starts from the left end of the series rather than from the right end, as it has been observed in rhesus monkeys (Drucker & Brannon, 2014) and chicks (Rugani et al., 2009, 2016), evoking an ancestral left-right number space mapping.

The role of space in mapping numerosities was investigated in the last part of the thesis. Using an adaptation of the experiment of Rugani and colleagues (2015) with chicks, I investigated if zebrafish, once trained to identify a target number, spontaneously associated a smaller number with the left space and a larger number with the right space. The results suggest that numerosities are mapped onto space in zebrafish, showing the same trend as reported in chicks (Rugani et al., 2015) and primates (Adachi, 2014). A potential limitation of the use of mapping strategies in zebrafish is probably related to the ratio between the training and test numerosities. In fact, zebrafish showed no significant preference in discrimination with a 0.625 ratio (5-8 elements) rather than a 0.4 ratio (2-5 elements) which showed a left-to right numerosities mapping. Control conditions of this experiment confirmed that the overall perimeter and the overall area did not strongly influence the orientation of

the supposed mental line. However, zebrafish are not completely unaffected by changes in surface areas of the stimuli. Indeed, when the area considerably increases in absolute terms, correlating negatively with numerosity, the numerical elaboration could be influenced with a consequent loss of mapping onto space.

Very recently, a study in the cleaner fish (*Labroides dimidiatus*) showed no number-space mapping (Triki & Bshary, 2017). The authors accurately duplicated Rugani's study with chicks (2015), showing that cleaner fish failed in a mental number line test. This result is totally in contrast with my findings in zebrafish, that showed instead a connection between numbers and space. A potential and crucial difference between my study and the ones by Triki and Bshary could be related to the familiarization phase, that in the cleaner fish is only a familiarization with a target number, while in zebrafish I required an active discrimination. This could have created differences in attending to numerosities in the stimulus. To confirm this it will be important to replicate my paradigm with cleaner fish, in order to see whether the different performance is due to the experimental paradigm or if there is a difference among fish species.

In conclusion, my results contribute to give further information and evidence about numerical cognition abilities in a fish species that could be potentially useful in understanding the neural and genetic basis of numerosities. *Danio rerio* is the best animal model available to study the ontogenetic development of numerical cognition. By conducting behavioral studies in wild-type and mutants animals, and by using neurobiological techniques, it would be possible to perform further work aiming to identify the neural mechanisms underlying these abilities.

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