The ontogeny of continuous quantity discrimination in zebrafish larvae (*Danio rerio*)

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ABSTRACT

Several studies have investigated the ontogeny of the capacity to discriminate between discrete numerical information in human and non-human animals. Contrarily, less attention has been devoted to the development of the capacity to discriminate continuous quantities. Recently, we set up a fast procedure for screening continuous quantity abilities in adult individuals of an animal model in neurodevelopmental research, the zebrafish. Two different sized holes are presented in a wall that divides the home tank in two halves and the spontaneous preference of fish for passing through the larger hole is exploited to measure their discrimination ability. We tested zebrafish larvae in the first, second and third week of life varying the relative size of the smaller circle (0.60, 0.75, 0.86, 0.91 area ratio). We found that the number of passages increased across the age. The capacity to discriminate the larger hole decreased as the ratio between the areas increased. No difference in accuracy was found as a function of age. The accuracy of larval zebrafish almost overlaps that found in adults in a previous study, suggesting a limited role of maturation and experience on the ability to estimate areas in this species.

KEYWORDS: Size discrimination; Zebrafish; Continuous extent; Spatial abilities.

INTRODUCTION

Cultural (Butterworth et al. 2011; Pica et al. 2004), cognitive (Price et al. 2012; Revkin et al. 2008) and developmental (Izard et al. 2009; Xu and Spelke 2000) psychology have provided multiple evidence for the existence of quantificational abilities that do not rely on language and culture. Such cognitive abilities, commonly referred to 'non-symbolic' quantificational skills, permit us to quickly solve many problems in everyday life, such as avoiding longer queues, selecting the shorter distance or the larger amount of food. Given the multiple advantages in terms of fitness and survival in the natural environment, quantification abilities are also shared by many vertebrate species (reviewed in Henik 2016).

There are two broad categories of quantificational skills, namely, estimation of discrete quantities (i.e. numerosity), and estimation of continuous quantities (e.g. distance, length, area, weight or duration). For what concerns the capacity to estimate numerosity, there is evidence that in our species it is innate and that it increases in precision across development even before the emergence of language, which occurs after 12-13 months of age (Fenson et al. 1994). Newborns can discriminate between two numbers up to a 0.33 numerical ratio (4 vs. 12 objects) (Izard et al. 2009); 6-months-old infants can discriminate a 0.50 ratio (8 vs. 16 objects) but not a 0.67 (8 vs. 12 objects) (Xu and Spelke 2000). Such ratio can be discriminated only later, at 10 months of age (Xu and Arriaga 2007). Our numerical acuity keeps improving throughout childhood, with 6-years olds discriminating a 0.83 ratio and adults discriminating a 0.90 ratio (Halberda et al. 2008; see Cordes and Brannon 2008 for a review).

Numerical abilities have been investigated in more than 50 species representing a wide range of vertebrate and invertebrate groups (e.g. Agrillo and Bisazza 2010; Beran and Parrish 2016). Developmental research is however limited to a few species. Inborn numerical abilities were found in domestic chicks tested with multiple experimental paradigms (e.g. Rugani et al. 2007, 2009, 2015). A small teleost fish, the guppy (*Poecilia reticulata*), demonstrated an innate ability to discriminate small numbers of social companions while their capacity to discriminate larger numerosities emerges later, as a result of both maturation and experience (Bisazza et al. 2010). Another study, using an operant conditioning procedure, showed that newborn guppies can discriminate between large numerosities in the first days of life (Piffer et al. 2013) but only if they could use multiple cues, such as number and cumulative surface area (Miletto Petrazzini et al. 2014).

For what concern continuous quantities, there is evidence that in humans this capacity improves across development too (reviewed in Feigenson 2007): 6-month old infants tested for their ability to discriminate between two areas could discriminate a 0.50 but not a 0.67 size ratio (Brannon et al. 2006). The latter ratio is discriminated at 10 months of age (Feigenson et al. 2002). Studies of continuous quantity estimation are rare in other species. Most evidences are indirect; for instance, it was found that different fish species discriminate their potential mates depending on their size (e.g. Bisazza et al. 2000; Dosen and Montgomerie 2004); also, size-assortative social grouping is widespread across animal species and seems the consequence of the ability to gauge conspecifics size (e.g. Barry et al. 2014; Pitcher 1986). Continuous quantity estimation abilities have been directly investigated in a half dozen species including sea lions (*Zalophus californianus*), carrion crows (*Corvus corone*), ruin lizards (*Podarcis sicula*) and Hermann's tortoises (*Testudo hermanni*) (Gazzola et al. 2018; Miletto Petrazzini et al 2017; Moll and Nieder 2014; Schusterman et al. 1965). As concerns fish, a recent work investigated in the guppy the ability to discriminate between different numbers of food items or between food items that differed in size. The study demonstrated that the guppies were able to discriminate both discrete and continuous quantities but were much more accurate in the latter task (Lucon-Xiccato et al. 2015). Although this ability is likely to have several functions even in young animals (e.g. social grouping, food choice, fighting assessment), there is a complete lack of studies regarding the ontogeny and the development of continuous quantity abilities in non-human species.

In humans, neuro-developmental and neuro-degenerative disorders are associated with a decline in numerical abilities and in the ability to estimate continuous quantities, for example the size, the weight and the distance of an object or the duration of an event (Barabassy et al. 2010; Brand et al. 2003; Girelli et al. 1999).

The zebrafish (*Danio rerio*) represents a well-established model in developmental, genetic and pharmacological research. This species is rapidly becoming a model for human neuro-developmental and neurodegenerative disorders (e.g. Brennan, 2011; Kalueff et al. 2014; Xi et al. 2011). The quasi-totality of these studies is conducted on embryos or in larvae in the first weeks of life. Larvae can be readily obtained in large numbers, allowing large-scale screening of neuroactive compounds and genotypes (e.g. Norton, 2013; Richendrfer et al. 2012). The rapid development of zebrafish favours the investigation of the developmental mechanisms whereas the transparency of the larvae allows the study of the neural circuits underlying behavioural and cognitive functions through in vivo calcium imaging (e.g. Sarvaiya et al. 2014). For these reasons, tools for modelling brain diseases in larval zebrafish are becoming increasingly available (e.g. Buckley et al. 2008; Paquet et al. 2009). Although cognitive deficits are important markers for many human neuropathologies (e.g. Levin et al 1989; Morris et al. 2001), there is currently a complete lack of protocols for measuring quantity estimation abilities in early developmental stages of this species.

Recently, we developed a simple procedure to test continuous quantity discrimination in adult zebrafish. The procedure is based on a spontaneous preference of fish to pass through the larger available breach (Santacà et al. 2020). We recorded small groups of zebrafish, composed of six adult females, that could move between the two compartments of their housing tank passing through a divider provided with two holes differing in size. In a first experiment, zebrafish significantly discriminated area ratios from 0.60 to 0.91 but their discrimination performance was found to decrease as the relative size of the smaller hole increased, in agreement with Weber’s law. In a second experiment, we measured the performance of individually tested fish. On average, performance of individually tested fish largely overlapped performance of fish tested in clusters. However, a large inter-individual variation in performance was observed in this experiment.

Very little is known about the quantificational abilities of zebrafish in the early stage of life. Two studies have found that zebrafish larvae were attracted by a small moving dot (a potential prey) but avoided a very large moving dot (a potential predator) (Bianco et al. 2011; Barker and Baier 2015) meaning that they are provided with at least some rudimental size discrimination ability. Neither of the two studies, however, investigated the developmental trajectory of such skill, nor they attempted to assess the threshold of discrimination.

Our study aimed to fill the gap in knowledge regarding the ontogeny and the developmental trajectory of continuous quantity discrimination, adapting the procedure developed for adults to study larval zebrafish. We observed the ability of larval fish to discriminate the larger hole to move from one compartment to another of their home tank. Zebrafish larvae were tested at three different ages, in the first, in the second and in the third week of life (7-, 14- and 21-days post fertilization respectively) varying the ratio of the smaller to the larger hole (0.60, 0.75, 0.86 and 0.91 area ratios). These information could also have practical applications as they may allow to study the cognitive deficits associated with neuropathologies in larval stages instead of adult zebrafish.

METHODS

Subjects

We tested one hundred and eighty zebrafish larvae (*Danio rerio*), in particular 30 groups of 6 larvae each for three different ages. Throughout this manuscript we used the standard age classification for zebrafish studies which start with the fertilization day and is expressed in days post fertilization or dpf (e.g. Bilotta and Saszik 2001). Since zebrafish hatch approximately 72 hours after fertilization, age from birth can be calculated subtracting three days from the number of dpf. We tested 10 groups larvae at 7 dpf, 10 groups of 14 dpf, and 21 dpf. The sex of the larvae was undetermined as sexual differentiation completes much later around week 11-12 post fertilization (Maac et al. 2003). The larvae used in our experiment originated from many different breedings from a wildtype strain bought by a local supplier in 2018 and maintained in our laboratory in a large population (>200 fish). The larvae were raised in several petri dishes in a solution of Fish Water 1x (0.5 mM $NaH_2PO_4*H_2O$, 0.5 mM $Na_2HPO_4*H_2O$, 1.5 gr Instant Ocean, 1 L de-ionized H_2O) and Methylene blue (0.0016gr/l). Until the beginning of the experiment, they were housed at a density of approximately 30 individuals each petri dish in the same room maintained at a temperature of 28.5 ± 1 °C. The room was illuminated according to a 14:10 h light:dark cycle. Larvae were fed twice a day with dry food (particle size: 0.75 mm) from the age of 6 dpf.

To avoid stressing the subjects, the standard length (from the snout to the caudal peduncle) and the width (from the left to the right side of the head) of each larvae was obtained from video recording of the experiment using the computer software Image J (https://imagej.nih.gov/ij/). For each larvae, we calculated the mean from three different video frames. The 7 dpf larvae were on average 3.962 ± 0.092 mm (mean ± SD) long and 0.612 ± 0.048 mm wide, the 14 dpf larvae were 4.397 ± 0.118 mm long and 0.732 ± 0.088 mm wide whereas the 21 dpf larvae were 4.565 ± 0.124 mm long and 0.918 ± 0.073 mm wide. The height at nape of the larvae could not be obtained from the video recordings; therefore, it was measured from a sample of larvae (n = 30) that did not participate in the study and consisted in the distance from ventral to dorsal, immediately posterior of head, perpendicular to the axis defined by the standard length. The 7 dpf larvae were on average 0.731 ± 0.061 mm (mean ± SD), the 14 dpf larvae were 0.914 ± 0.098 mm whereas the 21 dpf larvae were 1.022 ± 0.051 mm high.

Apparatus

The experiment was conducted in an hourglass-shaped apparatus (12 x 4.8 cm and 4 cm high) filled with 3.5 cm of Fish Water 1x (Figure 1). The apparatus was 3D printed with white PLA material. A central corridor (4.3 cm in length) connected the frontal and the posterior compartments. In the middle of the corridor, larvae could perform the task that consisted in the spontaneous passing through one of two holes of a plastic panel (3 x 3.2 cm; Figure 1). The panels were 3D printed with grey PLA material. Larvae could move from one compartment to the other one of the apparatus passing through the holes. One 0.72 W LED lamp was placed 1 cm above the frontal compartment, and one above the posterior one. We used four identical apparatuses at the same time. One video camera was placed above the central corridors of two adjacent apparatuses.

Procedure and stimuli

Since zebrafish is a highly social species, social isolation could alter the development of their normal behavioural repertoire, we studied fish in small social groups, the same condition in which this species is expected to solve this type of problem in nature (Forsatkar et al. 2017). The procedure consisted of two phases: a habituation phase and a test phase; both phases lasted two days. On day 1 of the habituation phase, six same-age larvae (7 dpf, 14 dpf or 21 dpf) were randomly selected from different maintenance petri dishes and were inserted in an experimental apparatus. On day 2, a grey panel with one central big hole (0.7 cm in diameter) was inserted in the middle of the apparatus to make the fish familiar with the procedure (i.e. passing through a hole to move from one side to the other). During these days, fish could habituate to the tank and swim through the hole. We video recorded the second day to ensure that the larvae had habituated to the experimental procedure passing through the hole. On the third and fourth day (test phase), four different panels with four types of size discrimination trials were randomly presented: ratio 0.60, ratio 0.75, ratio 0.86 and ratio 0.91 between the areas of the two holes (Figure 2). The value of the ratio represents the relative size of the smaller hole compared to the larger one that was maintained fixed in all four discriminations. A pilot experiment indicated that larvae easily pass through holes of 0.4 cm in diameter. However, since larvae have a poorer visual acuity than the adults (see discussion), to favour simultaneous visibility of the two holes even from a distance, we used holes above 0.6 cm of diameter (see Table 1 for details).

Each group was observed for eight consecutive hours during both days. Each ratio was presented for a total of 4 hours subdivided in two observations of 2 hours each; in one the bigger hole was presented on the frontal right side of the tank and in the other one on the frontal left side to check for any side bias. Each panel was presented once a day for each group and the sequence between groups was randomised. Before inserting a new panel, we waited until all larvae were near the two ends of the apparatus. From the video recordings, we scored the total number of passages through each hole for every panel presented for each cluster. Since both pre-test and test phases lasted two days, larvae of "7 dpf group" were 9-to-10-dpf at the moment of the cognitive measurement, those of "14 dpf group" were 16-to-17-dpf and those of "21 dpf group" were 23-to-24-dpf.

Statistical analyses

Analyses were performed in R version 3.5.2 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org). Each cluster of six larvae was considered as one datapoint with no distinction between the six larvae. All the data of each group are available in the supplementary file. Binomial tests were performed to compare the passages through the bigger hole in every ratio with chance level for all three ages separately and also pooled together. Post hoc power analyses were performed on the binomial tests using the 'binom.power' function of the 'binom' R package. A linear mixed-effects model (LMM, 'lmer' function of the 'lme4' R package) was performed to compare the total number of passages between the four ratios for the three larval ages. Such LMM was fitted with larvae group as random effect and with age and ratio as fixed effects; both fixed effects were categorical factors. To compare the performance (passages through the bigger hole) between the different ratios, between the three ages and the effect of the day, we used a LMM fitted with larvae group as random effect and with age, day and ratio as fixed effects; all three fixed factors were categorical factors. Subsequently, all pairwise comparisons were performed with Tukey post-hoc tests. We performed another LMM, equally fitted, to compare the results of the three ages with the results of the adults of the previous work (Santacà et al. 2020). Partial eta-squared (ηp^2) were used as an effect size statistic for LMMs (‘eta_sq’ function of the ‘sjstats’ package).

RESULTS

The mean number of passages was 136 ± 63 in 16 h of recordings for the 7dpf larvae, 337 ± 144 for the 14 dpf larvae and 720 ± 287 for the 21 dpf larvae. The LMM on the total number of passages revealed that the three larval ages significantly differed ($F_{(2, 27)} = 5.609$, $P < 0.01$, $\eta p^2 = 0.116$) while the effects of the ratio and the interaction age x ratio were not significant (ratio: $F_{(3, 81)} = 1.665$, $P = 0.181$, $\eta p^2 = 0.055$; age x ratio: $F_{(6,81)} = 2.144$, $P = 0.060$, $\eta p^2 = 0.131$).

Overall, 7 dpf larvae passed significantly more through the bigger hole in the ratios 0.60, 0.75, 0.86 but not in ratio 0.91; similarly, 14 dpf larvae passed significantly more through the bigger hole in the ratios 0.60, 0.75, 0.86 but not in ratio 0.91 whereas 21dpf larvae passed significantly more through the bigger hole in all the ratios presented (Figure 3). Table 2 shows the proportion of passages through the bigger hole, 95% Confidence Interval, binomial tests and post hoc power analyses for all ratios and for all ages. Pooling together the data of three ages, zebrafish larvae significantly discriminate the bigger hole in all the four ratios (all *P*-values < 0.001).

A LMM revealed significant difference in performances between the four ratios ($F_{(3, 189)} = 164.996$, $P < 0.001$, $\eta p^2 = 0.718$). A Tukey post hoc test revealed that all the pairwise comparisons between the ratios were statistically significant (all *P*-values < 0.05). The effects of the age and of the day were not significant (age: $F_{(2, 27)} = 0.412$, $P = 0.667$, $\eta p^2 = 0.004$; day: $F_{(1,189)} = 1.664$, $P = 0.199$, $\eta p^2 = 0.008$). All the interactions were not significant either (all *P* values > 0.333, all $\eta p^2 < 0.017$).

Comparing the results of the larvae with the adults' result of the previous study (Santacà et al. 2020), we found no significant effect of the age (LMM: $F_{(3, 32)} = 0.944$, $P = 0.431$, $\eta p^2 = 0.026$; Figure 3); also the interaction age x ratio was not significant ($F_{(2, 27)} = 1.443$, $P = 0.181$, $\eta p^2 = 0.108$).

DISCUSSION

Recently we showed that adult zebrafish can be very precise in continuous quantity discrimination, being able to detect also subtle size differences between two holes, such as 10% difference in area (Santacà et al. 2020). In this study we investigated the ontogeny and the developmental trajectory of this cognitive skill. To achieve our goal, we observed the spontaneous behaviour of groups of zebrafish larvae in the presence of holes differing in size.

In our experiment we found that larvae of all ages prefer to pass through the larger hole. It is unclear whether they used a linear measure (diameter or circumference) or the area of the hole to estimate its size. In humans, the Weber function is similar for linear measures and for areas but, since the surface area of a figure increases with the square of its linear size, the difference between the areas of two figures is more discernible than the difference between their linear dimensions (e.g. Eriksen and Hake 1955; Miller 1956; Rule 1969). Accordingly, it seems wise to consider that our subjects were estimating up to a 0.91 ratio between areas rather than up to a 0.95 ratio between diameters or between circumferences (see table 1).

There appears to be only minor differences between the three ages. Binomial tests showed that 7 and 14 dpf larvae discriminated up to 0.86 ratio; only the 21 dpf larvae demonstrated to discriminate the most difficult ratio (0.91) as adult zebrafish do (Santacà et al. 2020). One may be tempted to conclude that the precision to make continuous quantity discrimination increases across development. However, descriptive data showed quite a similar performance among the three ages and LMM on the number of passages through the holes indicated that this measure increased as a function of age, with 21 dpf larve doing more than 5-fold number of passages than 7 dpf larvae. This result is in accordance with previous studies showing that locomotor activity of larval zebrafish steadily increases during development (e.g. Colwill and Creton 2011; Fuiman and Webb 1988). Therefore, the differences here reported in the binomial tests are likely to be due to the different statistical power of the binomials tests rather than reflecting true differences in the precision of cognitive systems underlying quantity discrimination. This was confirmed by the post hoc power analyses and by the LMM on the proportion of choices for the larger hole that showed no difference as a function of age even when the data of adults are included in the analyses. Our experimental approach proved to be highly effective since both larvae and adults demonstrated a high discrimination ability since they both discriminated between two areas that differ for less than 10%. To our knowledge, beside humans (Agrillo et al. 2013), such precise size quantitative discrimination has been found only in sea lions trained to a size-discrimination task (Schusterman et al. 1965). One subject discriminated between two geometrical shapes up to a 0.88 area ratio and the other up to 0.94 ratio.

Interestingly, we found that the performance of all three age groups significantly decreased when the ratio between the smaller and the larger hole increased, ranging from 74% (0.60 ratio) to 54% (0.91 ratio). This aligns with a large body of evidence showing that the capacity to make relative quantity judgments in human and non-human animals (both discrete and continuous) obeys to Weber’s law, which states that the ability to discriminate two stimuli could depends mainly on their relative difference, rather than their absolute difference (e.g. Feigenson 2007; Ditz and Nieder 2016). However, our study was not designed to verify the adherence of larval zebrafish to Weber's law. In fact, the size ratio and the absolute size of the smaller hole covaried so that it is difficult to assess the relative influence of these two dimensions on the performance and prevent a definite conclusion on a Weber’s law effect. In addition, when a spontaneous preference paradigm is used, it is not easy to separate the contribution of motivation and cognition (see discussion below) as they are both predicted to have similar effects on performance as the ratio changed.

One may argue that our experiment does not allow distinguishing between the discrimination and the motivation to prefer the bigger hole. Indeed, this is an important limit of all cognitive studies that use spontaneous preference paradigms. For example, if an animal is observed to choose the larger of two food quantities, this also implies that it can discriminate between them. On the contrary, the lack of a preference could be due to either a limit in discriminability or to the fact that both quantities are enough to satisfy its requirements. This factor could be a limit in our study too. While it is clear that larval zebrafish are able to discriminate the bigger hole even when the difference is small, our experiment cannot precisely indicate the causes of the decrease in performance with increasing similarity between holes. This could be due to limits in the capacity to appreciate small size differences or could be partly or entirely due to a reduced benefit of choosing the larger of two holes as difference between them decreased.

The limitations of the spontaneous preference paradigms can be circumvented by adopting an alternative procedure based on discrimination learning. In this paradigm, as motivation to obtain a reward is considered independent from the stimulus to discriminate, a lack of choice should only reflect the upper limit of discriminative ability. Yet, serious limitations characterize this approach too, as it is time-consuming and potentially very stressful for the animal; in addition, extensive training can lead to the recruitment of neurocognitive systems that are not normally involved in such task (Agrillo and Bisazza 2014). In our case, a further limitation is that procedures to train larval zebrafish to this task are presently unavailable.

Our study does not provide information about the strategy used by the larvae to solve this task. There are essentially two ways that they could have used: the first one could consist in the relative comparison made by visually estimating the two holes from a distance, the second one could consist instead in taking an independent decision for each hole, by estimating its size using vision or other sense organs (e.g., the lateral line). The former implies they possess a system for relative quantity judgements, a mechanism that have been shown in the new-borns of another teleost species but only for numerical judgements (Piffer et al. 2013). The latter would require that they use an egocentric reference system (e.g. measuring distance between their body and the edge of the hole) or in alternative comparing the size of each hole with a pre-set threshold (either innate or learned). Based on what is currently known about the physiology of their visual system, it is unclear whether they could have used a visual relative judgement. Adult zebrafish are known to possess an excellent visual acuity (i.e. 0.56-0.58 cycles per degree; Mueller and Neuhauss 2010, Tappeiner et al. 2012) but the only measurement available for larval zebrafish regards fish tested two days after hatching, at 5dpf (Haug et al. 2010). At this stage they have poor vision, approximately one third of adults, being able to distinguish two objects only if at least 3 degrees apart. It is doubtful if this acuity would allow to perceive the small size difference proposed in our experiment (i.e. 400 µm; see Table 1), unless they get very close to the holes, making a simultaneous estimate of the two holes impractical. However, the youngest larvae we used in our experiments were twice as old (9-10 dpf) compared to the larvae used in the cited study. It is unknown how rapidly the visual system develops but an important phase in the development of nervous system occurs around 6 dpf when larvae start to show a richer behavioural repertoire and begin to catch live preys (Fero et al. 2011). We have only indirect information about visual acuity at this stage of development. McElligott and O'Malley (2005) have studied the kinematic of prey capture in 6-8 dpf larvae feeding on paramecia. Prey capture is visually guided, and many prey tracking movements initiate when a prey is between 2.5 to 3 mm from the fish. Considering that a paramecium is 200-250 µm long, larvae of this age are certainly capable of perceiving even the smaller hole used in this study (7600 µm) from any position within the apparatus. It is unclear whether they would be able to appreciate the smallest difference between hole's diameters that was only twice the average length of a paramecium. However, if we consider that larvae likely estimated the area rather than the diameter of the holes (see discussion above) the difference in areas is well above the minimum discernible area of their prey (4.9 mm^2 and 0.02 mm^2 respectively).

In the last decade, zebrafish is rapidly gaining popularity as a model of human neuropathologies, due to a greater ease in dissecting the genetic and physiological basis of these diseases. Zebrafish models have been established for studying neurodegenerative diseases such as Alzheimer's disease, neurodevelopmental disorders such as schizophrenia, genetic or autoimmune diseases that affects nervous system such as multiple sclerosis (e.g. Kulkarni, Yellanki, Medishetti, Sriram, Saxena, Yogeeswari, 2017; Newman, Ebrahimie, Lardelli, 2014). Some of these types of neurodegenerative diseases are frequently associated with a decline in the ability to estimate quantities. The deficits involve both numeracy (e.g. Gandini et al. 2009) and the estimation of continuous quantities, for example size, weight or distance of an object (e.g. Barabassy et al. 2010; Brand et al. 2003). One of the advantages of studying zebrafish is the possibility to investigate pathological phenomena very early, soon after birth or in the first weeks of life. A remarkable example is the development of zebrafish lines with alterations of TAU protein functioning that induce early neuronal disturbances and cell death, and recapitulated, in the first days of life, the key pathological features of human TAU-related pathologies such as Alzheimer's disease (Paquet et al. 2009). Our experimental procedure has been shown in previous study on adults to have a good retest reliability and to be unaffected by experience (Santacà et al. 2020). Moreover, the procedure is relatively fast and records the spontaneous behaviour of fish in their home tank, avoiding stressing subjects with frequent transfers from home cage to the experimental tank. Here we showed that it can be administered since the first week of life. In the light of these characteristics, this test appears suitable to measure longitudinal changes on cognitive abilities for example to examine the effects of pharmacological treatments on neurodegenerative processes using larval zebrafish as model, instead of adult zebrafish.

REFERENCES

Agrillo C, Bisazza A (2014) Spontaneous versus trained numerical abilities A comparison between the two main tools to study numerical competence in non-human animals. J Neurosci Meth 234:82-91

Agrillo C, Bisazza A (2018) Understanding the origin of number sense: a review of fish studies. Philos T R Soc B, 373:20160511

Agrillo C, Piffer L, Adriano A (2013) Individual differences in non-symbolic numerical abilities predict mathematical achievements but contradict ATOM. Behav Brain Funct 9:26

Barabassy A, Beinhoff U, Riepe MW (2010) Cognitive estimation in aged patients with major depressive disorder. Psychiat Res 176:26-29

Barker AJ, Baier H (2015) Sensorimotor decision making in the zebrafish tectum. Curr Biol 25:2804-2814

Barry M, Shanas U, Brunton DH (2014) Year-round mixed-age shelter aggregations in Duvaucel's geckos (*Hoplodactylus duvaucelii*). Herpetologica 70:395-406

Beran MJ, Parrish AE (2016) Going for more: Discrete and continuous quantity judgments by nonhuman animals. In Continuous Issues in Numerical Cognition (pp. 175-192). Academic Press.

Bianco IH, Kampff AR, Engert F (2011) Prey capture behavior evoked by simple visual stimuli in larval zebrafish. Frontiers in Systems Neuroscience 5:101

Bilotta J, Saszik S (2001) The zebrafish as a model visual system. Int J Dev Neurosci 19:621-629

Bisazza A, Manfredi S, Pilastro A (2000) Sexual competition, coercive mating and mate assessment in the one-sided livebearer, *Jenynsia multidentata*: are they predictive of sexual dimorphism?. Ethology 106:961-978

Bisazza A, Piffer L, Serena G, Agrillo C (2010) Ontogeny of numerical abilities in fish. PLoS One, 5:e15516

Brand M, Kalbe E, Fujiwara E, Huber M, Markowitsch HJ (2003) Cognitive estimation in patients with probable Alzheimer’s disease and alcoholic Korsakoff patients. Neuropsychologia 41:575-584

Brannon EM, Lutz D, Cordes S (2006) The development of area discrimination and its implications for number representation in infancy. Developmental Sci 9:F59-F64

Buckley CE, Goldsmith P, Franklin RJ (2008) Zebrafish myelination: a transparent model for remyelination?. Dis Model Mech 1:221-228

Butterworth B, Reeve R, Reynolds F (2011) Using mental representations of space when words are unavailable: studies of enumeration and arithmetic in indigenous Australia. J Cross Cult Psychol 42:630-638

Colwill RM, Creton R (2011) Locomotor behaviors in zebrafish (*Danio rerio*) larvae. Behav Process, 86:222-229

Cordes S, Brannon EM (2008) Quantitative competencies in infancy. Developmental Sci, 11:803-808

Ditz HM, Nieder A (2016) Numerosity representations in crows obey the Weber–Fechner law. P Roy Soc B-Biol Sci, 283(1827), 20160083

Dosen LD, Montgomerie R (2004) Female size influences mate preferences of male guppies. Ethology 110:245-255

Eriksen CW, Hake HW (1955) Multidimensional stimulus differences and accuracy of discrimination. J Exp Psychol 50:153

Feigenson L (2007) The equality of quantity. Trends Cogn Sci, 11(5), 185-187

Feigenson L, Carey S, Spelke E (2002) Infants' discrimination of number vs continuous extent. Cognitive Psychol, 44:33-66

Fenson L, Dale PS, Reznick JS, Bates E, Thal DJ, Pethick SJ, Stiles J (1994) Variability in early communicative development. Monogr Soc Res Child i-185

Fero K, Yokogawa T, Burgess HA (2011) The behavioral repertoire of larval zebrafish. In: Zebrafish Models in Neurobehavioral Research, Humana Press, Totowa, New York, pp 249-291

Forsatkar MN, Safari O, Boiti C (2017). Effects of social isolation on growth, stress response, and immunity of zebrafish. Acta Etholog 20:255-261

Fuiman LA, Webb PW (1988) Ontogeny of routine swimming activity and performance in zebra danios (Teleostei: Cyprinidae). Anim Behav 36:250-261

Gandini D, Lemaire P, Michel BF (2009) Approximate quantification in young, healthy older adults', and Alzheimer patients. Brain Cognition 70:53-61

Gazzola A, Vallortigara G, Pellitteri-Rosa D (2018) Continuous and discrete quantity discrimination in tortoises. Biol Letters 14:20180649

Girelli L, Luzzatti C, Annoni G, Vecchi T (1999) Progressive decline of numerical skills in Alzheimer-type dementia: A case study. Brain Cognition 40:132-136

Halberda J, Mazzocco MM, Feigenson L (2008) Individual differences in non-verbal number acuity correlate with maths achievement. Nature 455:665

Haug MF, Biehlmaier O, Mueller KP, Neuhauss SC (2010) Visual acuity in larval zebrafish: behavior and histology. Frontiers Zool 7:8

Henik A (Ed) (2016) Continuous issues in numerical cognition: How many or how much. Academic Press, Cambridge

Izard V, Sann C, Spelke ES, Streri A (2009) Newborn infants perceive abstract numbers. Proceedings of the National Academy of Sciences 106:10382-10385

Kalueff AV, Echevarria DJ, Stewart AM (2014) Gaining translational momentum: more zebrafish models for neuroscience research. Prog Neuro-psychoph 55:1-6

Kulkarni P, Yellanki S, Medishetti R, Sriram D, Saxena U, Yogeeswari P (2017) Novel Zebrafish EAE model: A quick in vivo screen for multiple sclerosis. Mult Scler Relat Dis 11:32-39

Levin BE, Llabre MM, Weiner WJ (1989) Cognitive impairments associated with early Parkinson's disease. Neurology 39:557-557

McElligott MB, O'Malley DM (2005) Prey tracking by larval zebrafish: axial kinematics and visual control. Brain Behav Evolut 66:177-196

Miletto Petrazzini ME, Agrillo C, Piffer L, Bisazza A (2014) Ontogeny of the capacity to compare discrete quantities in fish. Dev Psychobiol 56:529-536

Miletto Petrazzini ME, Fraccaroli I, Gariboldi F, Agrillo C, Bisazza A, Bertolucci C, Foà A (2017) Quantitative abilities in a reptile (*Podarcis sicula*). Biol Letters 1320160899

Miller GA (1956) The magical number seven, plus or minus two: Some limits on our capacity for processing information. Psychol Rev 63:81-97

Moll FW, Nieder A (2014) The long and the short of it: Rule-based relative length discrimination in carrion crows, *Corvus corone*. Behav Process, 107:142-149

Morris JC, Storandt M, Miller JP, McKeel DW, Price JL, Rubin EH, Berg L (2001) Mild cognitive impairment represents early-stage Alzheimer disease. Arch Neurol-Chicago 58:397-405

Mueller KP, Neuhauss SC (2010) Quantitative measurements of the optokinetic response in adult fish. J Neurosci Meth 186:29-34

Newman M, Ebrahimie E, Lardelli M (2014) Using the zebrafish model for Alzheimer's disease research. Frontiers in Genetics 5:189

Norton WHJ (2013) Toward developmental models of psychiatric disorders in zebrafish. Front Neural Circuit 7:79

Paquet D, Bhat R, Sydow A, Mandelkow EM, Berg S, Hellberg S, ... Haass C (2009) A zebrafish model of tauopathy allows in vivo imaging of neuronal cell death and drug evaluation. J Clin Invest 119:1382-1395

Pica P, Lemer C, Izard V, Dehaene S (2004) Exact and approximate arithmetic in an Amazonian indigene group. Science 306:499-503

Piffer L, Miletto Petrazzini ME, Agrillo C (2013) Large number discrimination in newborn fish. PLoS One, 8:e62466

Pitcher TJ (1986) Functions of shoaling behaviour in teleosts. In:The behaviour of teleost fishes, Springer, Boston, pp 294-337

Price GR, Palmer D, Battista C, Ansari D (2012) Nonsymbolic numerical magnitude comparison: Reliability and validity of different task variants and outcome measures, and their relationship to arithmetic achievement in adults. Acta Psychol 140:50-57

Revkin SK, Piazza M, Izard V, Zamarian L, Karner E, Delazer M (2008) Verbal numerosity estimation deficit in the context of spared semantic representation of numbers: A neuropsychological study of a patient with frontal lesions. Neuropsychologia 46:2463-2475

Richendrfer H, Pelkowski SD, Colwill RM, Creton R (2012) On the edge: pharmacological evidence for anxiety-related behavior in zebrafish larvae. Behav Brain Res 228:99-106

Rugani R, Fontanari L, Simoni E, Regolin L, Vallortigara G (2009) Arithmetic in newborn chicks. P Roy Soc B-Biol Sci 276:2451-2460

Rugani R, Regolin L, Vallortigara G (2007) Rudimental numerical competence in 5-day-old domestic chicks (*Gallus gallus*): identification of ordinal position. J Exp Psychol Anim B 33:21

Rugani R, Vallortigara G, Regolin L (2015) At the root of the left–right asymmetries in spatial–numerical processing: from domestic chicks to human subjects. J Cogn Psychol 27:388-399

Rule SJ (1969) Subject difference in exponents from circle size, numerousness, and line length. Psychon Sci 15:284-285

Santacà M, Caja T, Miletto Petrazzini ME, Agrillo C, Bisazza A (2020) Size discrimination in adult zebrafish (*Danio rerio*): Normative data and individual variation. Sci Rep-UK 10:1-12

Sarvaiya VN, Sadariya KA, Rana MP, Thaker AM (2014) Zebrafish as model organism for drug discovery and toxicity testing: A review. Veterinary Clinical Science 2:31-38

Schusterman RJ, Kellogg WN, Rice CE (1965) Underwater visual discrimination by the California sea lion. Science 147:1594-1596

Tappeiner C, Gerber S, Enzmann V, Balmer J, Jazwinska A, Tschopp M (2012) Visual acuity and contrast sensitivity of adult zebrafish. Front Zool 9:10

Xi Y, Noble S, Ekker M (2011) Modeling neurodegeneration in zebrafish. Curr Neurol Neurosci 11:274-282

Xu F, Arriaga RI (2007) Number discrimination in 10-month-old infants. Brit J Dev Psychol 25:103-108

Xu F, Spelke ES (2000) Large number discrimination in 6-month-old infants. Cognition 74:B1-B11

ETHICAL NOTES: The experiments were approved by the Ethical Committee of the Università di Padova (protocol n. 61/2018) and adhered to the current legislation of the country in which they were performed (Italy, Decreto Legislativo 4 Marzo 2014, n. 26).

CONFLICT OF INTEREST STATEMENT: We declare no conflict of interests.

DATA AVAILABILITY STATEMENT: The data that support the findings of this study are available from the corresponding author upon request.

TABLE

Table 1. Hole diameters and size ratios used in this study.

Diameter of larger hole	Diameter of smaller hole	Ratio between the diameters	Ratio between the areas
0.8 cm	0.62 cm	**0.78**	**0.60**
0.8 cm	0.69 cm	**0.86**	**0.75**
0.8 cm	0.74 cm	**0.93**	**0.86**
0.8 cm	0.76 cm	**0.95**	**0.91**

Table 2. Performance of the three ages. Mean proportion of choices for the bigger hole, 95% Confidence Interval and Binomial Tests for all ratios and for the three ages.

Larval age	Ratio	Proportion of choices for the bigger hole (mean ± SD)	95% Confidence Interval	Binomial Tests	Post hoc power analyses
7 dpf	0.60	0.766 ± 0.034	0.742, 0.790	$P < 0.05$	$1 - \beta = 1.000$
	0.75	0.636 ± 0.072	0.585, 0.687	$P < 0.05$	$1 - \beta = 1.000$
	0.86	0.594 ± 0.071	0.543, 0.644	$P < 0.05$	$1 - \beta = 0.870$
	0.91	0.549 ± 0.071	0.507, 0.582	$P = 0.073$	$1 - \beta = 0.280$
14 dpf	0.60	0.736 ± 0.066	0.689, 0.783	$P < 0.001$	$1 - \beta = 1.000$
	0.75	0.649 ± 0.069	0.600, 0.699	$P < 0.001$	$1 - \beta = 1.000$
	0.86	0.568 ± 0.064	0.522, 0.614	$P < 0.001$	$1 - \beta = 0.901$
	0.91	0.519 ± 0.074	0.466, 0.572	$P = 0.124$	$1 - \beta = 0.152$
21dpf	0.60	0.716 ± 0.062	0.671, 0.760	$P < 0.001$	$1 - \beta = 1.000$
	0.75	0.642 ± 0.075	0.589, 0.696	$P < 0.001$	$1 - \beta = 1.000$
	0.86	0.594 ± 0.026	0.575, 0.612	$P < 0.001$	$1 - \beta = 1.000$
	0.91	0.546 ± 0.046	0.513, 0.578	$P < 0.001$	$1 - \beta = 0.980$

FIGURES

Figure 1. Experimental apparatus. a) Lateral and b) aerial view of the experimental context. The apparatus was composed of a movable test panel in the middle of the corridor that divided the apparatus in a frontal and a posterior compartment.

Figure 2. Experimental panels. Subjects were presented with panels showing pairs of holes with four different ratios between the areas within each pair: ratio 0.60 (a), ratio 0.75 (b), ratio 0.86 (c) and ratio 0.91 (d).

Figure 3. Comparison of the results of the three larval ages and the adults of the previous work (Santacà et al. 2020). The Y-axis refers to the proportion of choices for the bigger hole in the four ratios tested (ratio 0.60, ratio 0.75, ratio 0.86, ratio 0.91). Bars represent the standard error.

Figure 1.

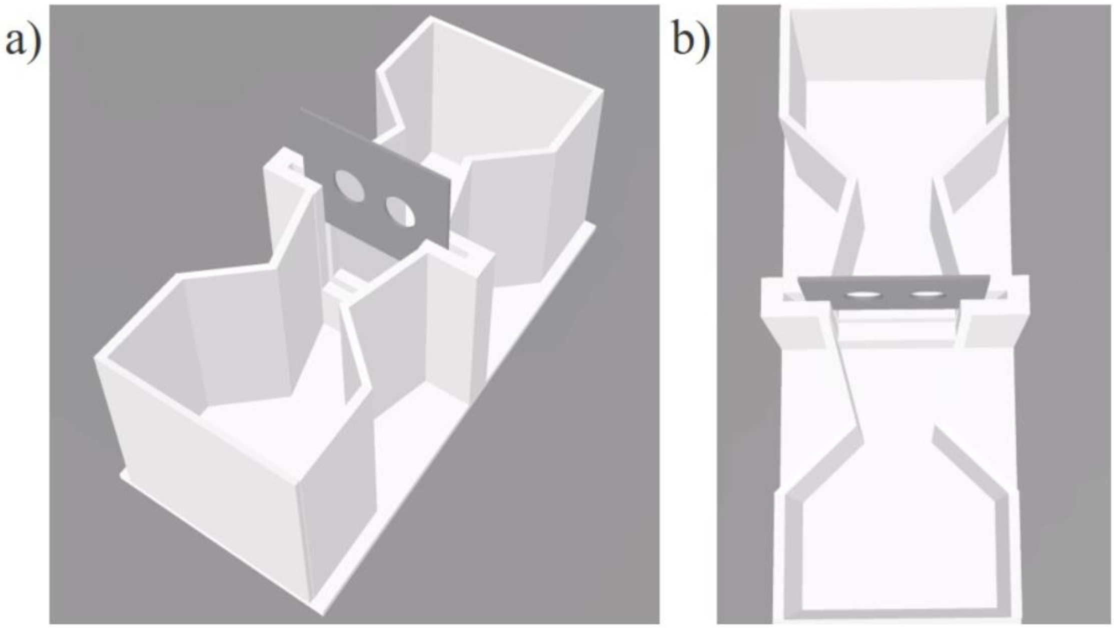
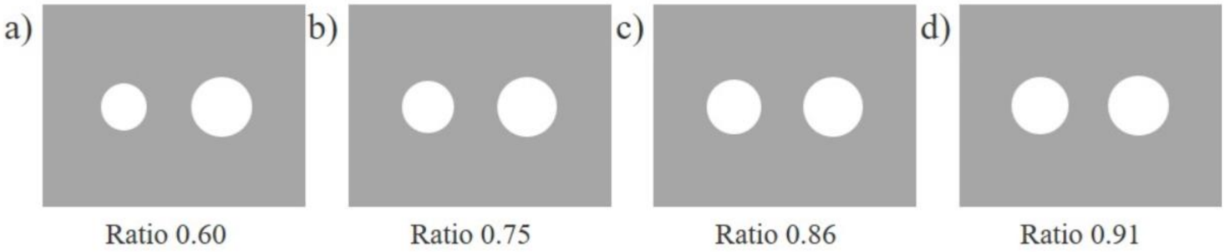
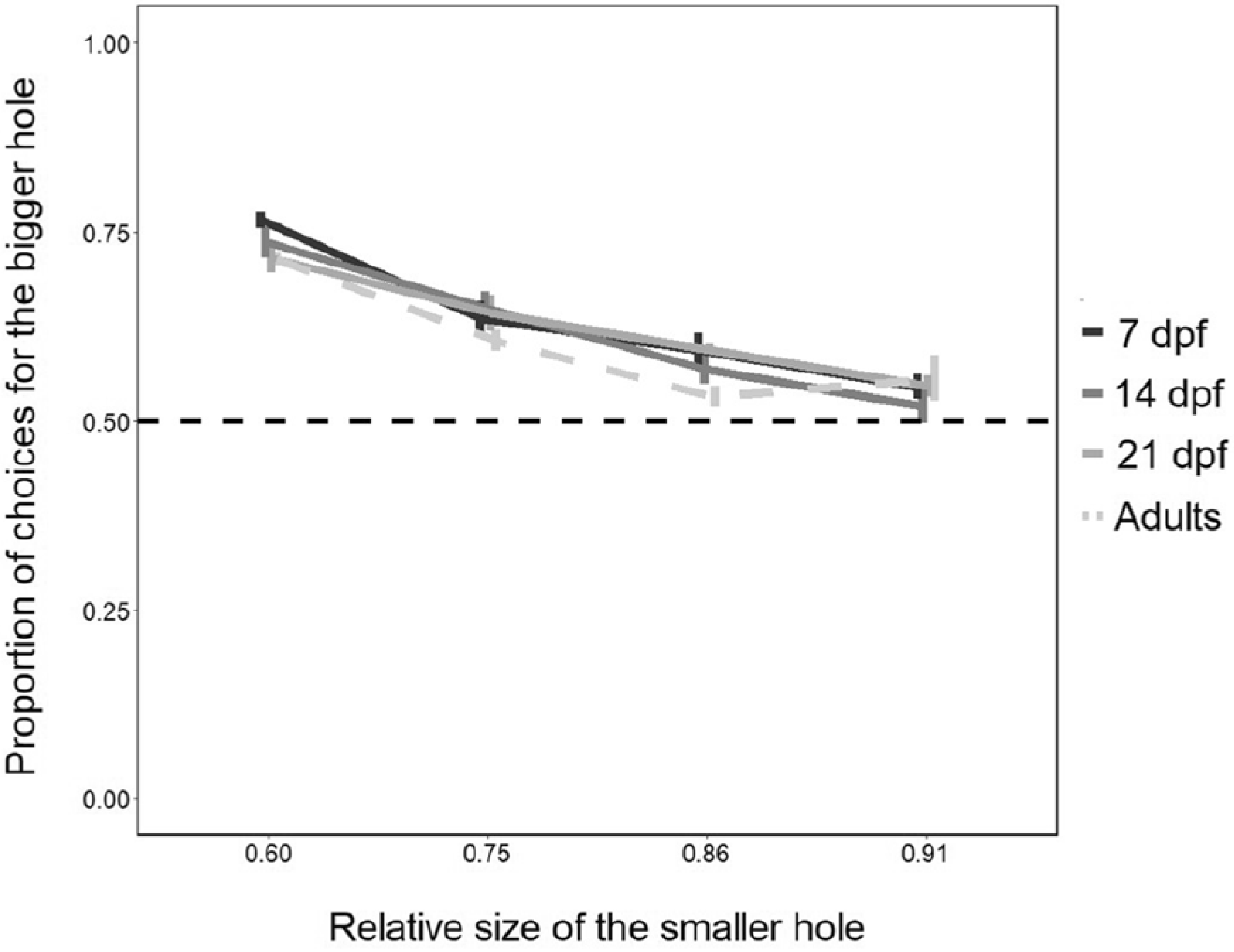


Figure 2.



Ratio 0.60 Ratio 0.75 Ratio 0.86 Ratio 0.91

Figure 3.


Proportion of choices for the bigger hole
1.00
0.75
0.50
0.25
0.00
0.60
0.75
0.86
0.91
Relative size of the smaller hole
7 dpf
14 dpf
21 dpf
Adults