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METHODS PAPER

# Development and testing of a rapid method for measuring shoal size discrimination

**Tyrone Lucon-Xiccato**[1] · **Marco Dadda**[1] · **Elia Gatto**[1] · **Angelo Bisazza**[1]

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**Abstract** The shoal-choice test is a popular method to investigate quantity discrimination in social fish based on their spontaneous preference for the larger of two shoals. The shoal-choice test usually requires a long observation time (20–30 min), mainly because fish switch between the two shoals with low frequency, thus reducing the possibilities of comparison. This duration limits the use of the shoal-choice test for large-scale screenings. Here, we developed a new version of the shoal-choice test in which the subject was confined in a large transparent cylinder in the middle of the tank throughout the experiment to bound the minimum distance from the stimulus shoals and favour switching. We tested the new method by observing guppies (*Poecilia reticulata*) in a 4 versus 6 fish discrimination (experiment 1). The new method allowed for a faster assessment of the preference for the larger shoal (<5 min), resulting in potential application for large population screenings. Guppies switched five times more frequently between the two shoals and remained close to the first chosen shoal ten times less compared to experiments with the old method. In experiment 2, we found that with the new method guppies were able to discriminate up to 5 versus 6 fish, a discrimination that was not achieved with the classical method. This last result indicates that minor methodological modifications can lead to very different findings in the same species and suggests that caution should be exercised when interpreting inter-specific differences in quantitative abilities.

✉ Tyrone Lucon-Xiccato
tyrone.luconxiccato@unipd.it

[1] Dipartimento di Psicologia Generale, Università di Padova, Via Venezia 8, 35131 Padova, Italy

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## Introduction

A diversity of fish species live most of their lives in groups. Several social groups often coexist in the same area, and they may differ from each other in the size or phenotype of the members (e.g. age, sex, colour, body size). Therefore, an individual fish has the option of choosing which group to join, a decision that may have profound effects on fitness (e.g. Lindström and Ranta 1993; Rosenthal and Ryan 2005; Agrillo et al. 2008). Shoal size is one of the critical aspects that affect grouping decisions in fish, and it has been experimentally investigated in a large number of species. Because both dilution of individual predation risk and vigilance increase with increasing group size, larger groups are usually safer than smaller groups (Krause and Ruxton 2002). As a consequence, under perceived threat, prey fish often show a preference for the largest available group (Magurran and Pitcher 1987; Hager and Helfman 1991; Krause et al. 1998; Hoare et al. 2004).

Preference for the larger shoal has been recently used by cognitive ethologists as one of the main tools to study numerical abilities in fish (e.g. Agrillo and Dadda 2007; Gómez-Laplaza and Gerlai 2011, 2012; Bisazza et al. 2014a). Due to the practical difficulties of studying this behaviour in the wild, most experiments have measured social preferences in the laboratory using the three-chamber binary-choice test. Two groups of stimulus fish that differ in numerosity are placed in the two external chambers, and the subject is placed in the central chamber and is free to swim. Because the central chamber is unfamiliar and potentially unsafe, the subject is expected to show antipredator behaviour and to attempt to reach the larger shoal. Shoaling preference is assessed by measuring the relative time spent by the subject in close proximity to each shoal, usually by analysing the video recording of the experiment.

With this procedure, it has been found that *Girardinus falcatus, Pimephales promelas* and *Pterophyllum scalare* can discriminate between shoals formed by 1 versus 2 and 2 versus 3 fish (Hager and Helfman 1991; Agrillo and Dadda 2007; Gómez-Laplaza and Gerlai 2011). Guppies, *Poecilia reticulata*, and mosquitofish, *Gambusia holbrooki*, can distinguish more subtle shoal size differences, up to 3 versus 4 (Agrillo et al. 2008, 2012). Discrimination of shoal size occurs even when the access to non-numerical cues (such as density, cumulative surface, and amount of movement) is experimentally prevented (Dadda et al. 2009), although the presence of these cues has been proven to affect discrimination performance in diverse species of fish (e.g. Gómez-Laplaza and Gerlai 2012).

Compared with the other tests commonly used to study numerical abilities in fish, the shoal-choice test is relatively rapid to execute (Agrillo and Bisazza 2014). However, the time necessary to test one subject (usually 20–30 min; Gómez-Laplaza 2006; Agrillo et al. 2007) and to score its performance from the recordings makes it difficult to use the shoal-choice test for large-scale screenings (Patton and Zon 2001) such as those required to study the genetic bases of quantification mechanisms or population differences. The length of the test might be problematic for two further reasons. The prolonged exposure to a situation perceived as dangerous is likely to cause considerable stress to the fish (Chandroo et al. 2004). Then, in natural situations, discrimination of the larger shoal usually occurs very rapidly (Krause et al. 1998), and it is possible that, with long tests, other factors affect the choice of the subject.

One of the reasons for extending observation in laboratory experiments is that the shoal-choice setting only approximately reproduces the natural situation. This may cause a large imprecision in the measure, especially at the beginning of the test, that the experimenters try to circumvent by extending the length of the observation. One of the main issues observed at the beginning of the test is the low frequency of switching between the two shoals, which arguably reduces the possibility to compare the two options and assigns a large weight to the first choice made by the subject. For example, two recent shoal-choice experiments found an average of 5.94 switches between the two shoals in 15 min and 4.23 switches in 8 min, respectively (Agrillo et al. 2012; Lucon-Xiccato et al. 2016). Another study found an average of 5.29 min spent near the first shoal chosen before the first switch (Lucon-Xiccato and Dadda, unpublished results). One of the causes for the reduced switching frequency at the beginning of the test appears to be that, after it is released in the middle of the tank and took the first shoaling decision, the subject is very close to one of the two shoals and is in a relatively safe location. Even if the subject now perceives that the farther shoal is larger, it needs to cross a long distance alone to reach the larger shoal. Both guppies and three-spined sticklebacks, *Gasterosteus aculeatus*, have been experimentally shown to prefer the near shoal, even if the far shoal is larger (Tegeder and Krause 1995; Mühlhoff et al. 2011).

Here, we aimed to develop a new version of the shoal-choice test, which allows a rapid assessment of shoal size discrimination ability. In particular, we hypothesised that time of permanence near one shoal decreases and selectivity increases by preventing the subject from approaching the stimulus shoal too closely, and we tested whether, using this expedient, the time required to assess the performance would be reduced. To bound the minimum distance from stimulus shoals, we confined the subject in a large transparent cylinder in the middle of the tank, and we took the time spent attempting to join each shoal as a measure of preference. This solution has already been used in mate choice studies to prevent the subject from approaching the stimulus fish too closely (Plath et al. 2008). We also used two novel features adopted in two recent shoal-choice experiments (Dadda et al. 2015; Lucon-Xiccato et al. 2016). To deal with the problem of pheromone released by previously tested individuals, the subject compartment received a continuous water supply from a large undisturbed population of guppies kept in seminatural conditions. To obtain well-acclimated stimulus fish, we permanently housed the stimulus shoals in their compartments.

In the first experiment, we measured the ability of guppies to discriminate 4 versus 6 fish with our new method, focussing especially on the initial phase of the test. We chose the 4 versus 6 contrast because it is challenging for guppies. Two studies found guppies were able to discriminate 4 versus 6 fish (Dadda et al. 2015; Lucon-Xiccato et al. 2016), but two other studies reported that guppies failed to discriminate this contrast (Agrillo et al. 2012; Bisazza et al. 2014a).

We also compared the results of experiment 1 with those obtained from three previous studies on the same guppy population that adopted two different shoal-choice methods (Agrillo et al. 2012; Bisazza et al. 2014a; Lucon-Xiccato et al. 2016). Two early studies (Agrillo et al. 2012; Bisazza et al. 2014a) adopted a classical shoal-choice procedure. The focal fish was released in the middle of the tank, and two shoals were confined in the two lateral sectors. The third work (Lucon-Xiccato et al. 2016) aimed at studying sex differences in shoal size discrimination and used two of the innovations described in the present work: the water flow from a tank with a large guppy population and the stimuli permanently housed in the stimulus tank. However, in the work by Lucon-Xiccato et al. (2016) the subject was not confined in a large cylinder throughout all the test; the subject was kept for 2 min in a small cylinder in the middle of the tank to observe the two shoals and then released in the tank to choose the preferred option.

In a second experiment, we looked for the upper limit of shoal size discrimination with the new method by observing guppies in more subtle discriminations (4 vs. 5 and 5 vs. 6 fish).

## Materials and methods

### Experiment 1

#### *Subjects*

The subjects were 32 adult females bred in 400-L holding tanks made of grey plastic in our laboratory at Dipartimento di Psicologia Generale (Università di Padova, Italy). We used 20 additional females of the same population as stimulus. The guppies were descendants of wild guppies caught in a high predation-risk environment in the lower Tacarigua River in Trinidad. At the time of the experiment, the subjects were 4–6 months old (standard length: approximately 22 mm). In the holding tanks, fish were kept in large mixed-sex groups (approximately 50 individuals per tank). We provided a gravel bottom and abundant natural plants to resemble natural conditions. We also provided water filters and 36-W fluorescent lamps (12-h:12-h light/dark photoperiod). Water temperature was kept constant at 26 ± 1 °C. Three times per day the guppies were fed commercial food flakes (Fioccomix, Super Hi Group, Ovada, Italy) and *Artemia salina* nauplii, which was the only interaction these guppies had with humans before the experiments. Each subject was used only once and, after the test, it was released in a tank and kept for breeding purposes.

#### *Apparatus*

The experimental apparatus was similar to the one adopted in previous shoal-choice experiments with guppies (Agrillo et al. 2012; Bisazza et al. 2014a) and other species (Agrillo and Dadda 2007; Gómez-Laplaza and Gerlai 2011). It consisted of three adjacent glass tanks (60 × 40 × 35 cm) filled with 25 cm of water (Fig. 1). The central tank ('subject tank') housed the subject during the experiment, while the other two tanks ('stimulus tanks') housed the two stimulus shoals. The long walls and the bottom of the subject tank were covered with green plastic panels. The apparatus was placed in a completely dark room, and the subject tank received light indirectly from the two stimulus compartments. Luminance measured with Gossen Mavo-Monitor USB photometer was 0.85 cd/m$^2$ in the cylinder and 105 cd/m$^2$ in the front compartments of the stimulus tanks.

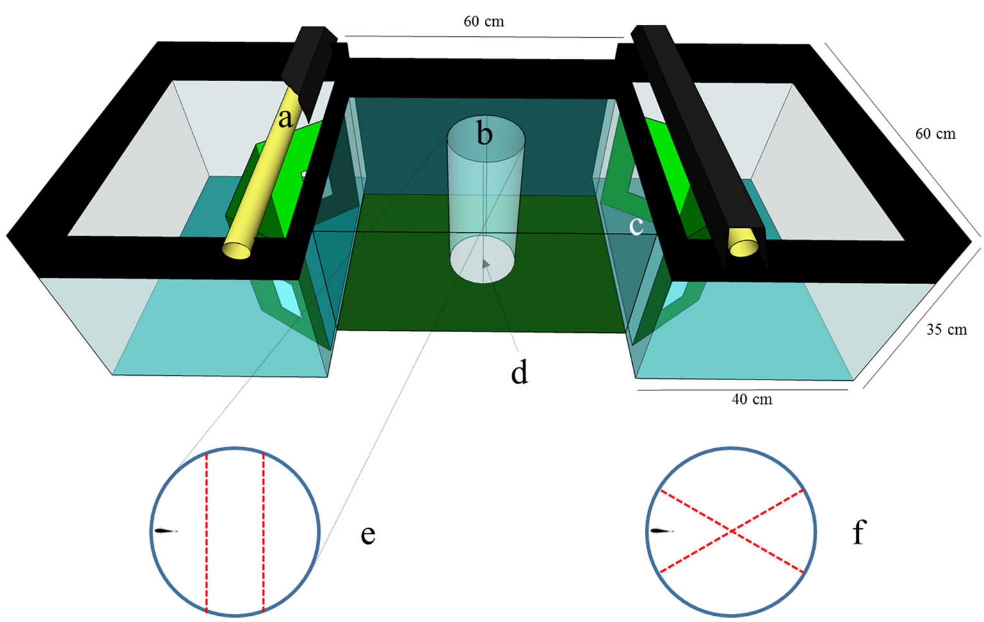


**Fig. 1** Apparatus adopted in our study. The two lateral tanks housed the stimulus shoals. **a** Fluorescent lamp; **b** transparent cylinder that housed the subject; **c** front compartment to present the stimulus shoals to the subjects; **d** fissures to allow water flow into the cylinder; **e** aerial view of the cylinder with the three sectors used to measure the preference of the subject in the main analysis; **f** aerial view of the cylinder with the four sectors used to measure the preference of the subject based on its orientation

Unlike previous studies, a transparent cylinder (height: 30 cm; diameter: 15 cm) limited the movements of the subject to the central part of the subject tank for the whole experiment and allowed the subject to reach a minimum distance of 22.5 cm from the stimulus tanks. The cylinder was made with two acetate sheets connected with paper clips. The connections were always kept on the lateral side so that they did not hamper the sight of the stimulus shoals. Under the cylinder, the bottom of the tank was made of white plastic to facilitate the tracking of the subject. To allow water flow into the cylinder, we left a 1-mm fissure in correspondence of each connection of the two acetate sheets by preventing the sheets to completely overlap (Fig. 1), and we also left a similar fissure between the cylinder and the bottom of the tank. Moreover, before each trial we lifted the cylinder to completely change the internal water.

Two additional features of our apparatus were based on two studies on shoal choice recently performed in our laboratory (Dadda et al. 2015; Lucon-Xiccato et al. 2016). The first of these features regards the water used for the subject compartment, which was supplied from a 400-L tank with the same characteristics as the holding tanks and with a large population of guppies (approximately 50 individuals, both sexes, all ages). Two pumps in this tank provided a constant 1.5-L/min flow of water to the subject tank, and water in excess was drained from a hole (diameter 2 cm) on the bottom of the subject tank and pumped back to the population tank.

The second additional feature regarded stimulus tanks. We modified the stimulus tanks, dividing them into a front compartment (40 × 22 cm) and a back compartment (60 × 18 cm) by means of green plastic (Fig. 1). The back compartment was provided with gravel bottom, abundant plants, water filter and heater. The front compartment was the only part of the stimulus tank visible from the subject tank. Each front compartment was illuminated by a 15-W fluorescent lamp. An opaque lid covered each lamp on three sides to prevent it from lightning the room or the subject tank. Each stimulus tank housed permanently a group of 10 female guppies. These guppies were matched for body size with the subjects. Outside the experiments, stimulus guppies could freely access both front and back compartments by two guillotine doors.

### *Procedure*

Thirty minutes before the experiment, the required number of stimulus guppies was confined in the front compartment by closing the guillotine doors. The position of the larger shoal was randomized between the two stimulus tanks across subjects to counterbalance potential differences between the stimulus fish. The subject was netted from the holding tank, transported in a plastic jar and gently inserted in the cylinder. We recorded the behaviour of subjects for 15 min with a camera placed 70 cm above the experimental tank. The recording started after the subject was inserted in the cylinder; this was necessary to avoid familiarisation with the subject tank and to study the antipredator response (i.e. preference for the larger shoal) that guppies express in novel environments. Two subjects showed freezing behaviour and did not move for the entire experiment. They were discarded and substituted.

### *Analysis of the recordings*

The choice of the subjects was analysed from the digital recordings played back on a computer screen. In a pilot experiment with a cylinder of this size (approximately 6 guppy's body length), we observed that guppies spent more than 80% of their time in the two zones closest to the stimuli, swimming against the transparent cylinder in the attempt to reach one of the two shoals. This behaviour did not include the up-down movements usually reported for escaping attempts and thrashing, but rather resembled the behaviour of guppies with the old shoal-choice method when they swim against the transparent partition to reach the stimuli. However, we cannot exclude that the swimming behaviour towards the stimuli was at least partially due to the willingness to escape from the cylinder. Guppies crossed the central sector of the cylinder only to reach the opposite side and swim towards the opposite shoal. Based on the pilot experiment, we virtually divided the section of the cylinder in three zones with equal longitudinal extension (5 cm, corresponding to more than 2 guppy's body lengths), two choice sectors facing the stimulus shoals and one central, neutral sector (Fig. 1). We obtained this division by superimposing three lines on the computer screen by means of a computer software originally developed in our laboratory ('Overlap', written in Delphi 5 Borland). The fish was considered to stay in one sector when the snout was within that area, but usually the subject was completely within the choice area when trying to reach one of the two shoals. As in previous studies, video recordings were analysed using a computer program originally developed in our laboratory ('Ciclic Timer', written in Delphi 5 Borland) by an experimenter that operated on the computer keyboard. The experimenter was blind with respect to the position of the larger shoal. The software calculated the time spent in each sector of the cylinder. As a measure of preference for the larger shoal, we computed the proportion of time spent in the sector near the larger shoal over the total time spent in the two sectors near the two shoals. We also analysed the proportion of time spent in the central sector because this measure is used as a proxy of motivation to shoal: when highly motivated to shoal, fish are expected to spend little time in the central sector

(Lucon-Xiccato and Dadda 2016a; Miletto Petrazzini and Agrillo 2016). To investigate the temporal trend of preference, we set the computer software to obtain an output divided in 3 blocks of 5 min each (Lucon-Xiccato and Dadda 2016b; Lucon-Xiccato et al. 2016). Subsequently, we repeated the analysis of the recordings focussing on the first minute and the initial 3 min of test.

To validate our approach, we conducted three further analyses on a subsample of 16 randomly chosen recordings. For the first analysis, a new blind experimenter scored the recordings in order to calculate the inter-rater reliability of our measure of preference. The second analysis consisted of a more detailed behavioural examination on the initial 5 min. In this latter analysis, we measured the time spent by the subject swimming against the transparency in the direction of one of the two stimulus compartment, i.e. in the area subtended by a central angle of 60° (Fig. 1). The preference index was calculated as before. Finally, we examined the number of switches between the two choice areas and the permanence of the subjects in the choice area chosen at first.

### Comparison with previous methods

The three previous studies used for the comparison tested guppies from the same population tested in the present work. The recent study by Lucon-Xiccato et al. (2016) was performed in the same apparatus used for the present work, but without the cylinder during the test; the two early studies used an apparatus with a 60 × 36 × 35 cm subject tank (Agrillo et al. 2012; Bisazza et al. 2014a). For the two early studies, we used the pooled data ($N = 110$) after checking the absence of a significant difference between the performance of guppies in these two experiments (independent samples $t$ test: $t_{108} = 0.943$, $P = 0.348$). Regarding the recent study by Lucon-Xiccato et al. (2016), data were originally scored grouped in blocks of 4 min each; we therefore re-analysed the initial 15 min of the trial of female guppies tested with female shoals ($N = 24$) from the video recordings using the procedure described for experiment 1 (main analysis in 3 blocks of 5 min each; subsequent detailed analysis of the first minute and the initial 3 min). Preference for the larger shoal was always arcsine-square-root-transformed before the analysis (Sokal and Rohlf 1995). For the work by Lucon-Xiccato and colleagues and the work by Agrillo and colleagues, we also analysed the frequency of switching between the two shoals; we could not analyse this variable in the experiment by Bisazza and colleagues, because it was not reported in the original study and we do not possess the video recordings.

### Experiment 2

In experiment 2, we observed 24 female guppies in the discrimination of 4 versus 5 fish and 24 female guppies in the discrimination of 5 versus 6 fish. Apparatus and procedure were the same as experiment 1. Based on the results of experiment 1, in experiment 2 we recorded the subjects for 5 min. We also measured the number of switches between the two choice areas in a random subsample of 12 subjects for each discrimination. Two subjects were discarded and substituted because they showed freezing behaviour.

## Results

### Experiment 1

Guppies spent 83.50 ± 5.88% ($M$ ± SD percentage) of time in the two choice sectors facing the stimulus shoals. We compared preference for the larger shoal with chance level (50%) with one-sample $t$ tests. When considering the entire observation period (15 min), subjects showed a significant preference for the larger shoal (58.43 ± 18.65%, one-sample $t$ test: $t_{31} = 2.549$, $P = 0.016$; Fig. 2). During the first block of time (min 1–5), subjects spent significantly more time near the larger

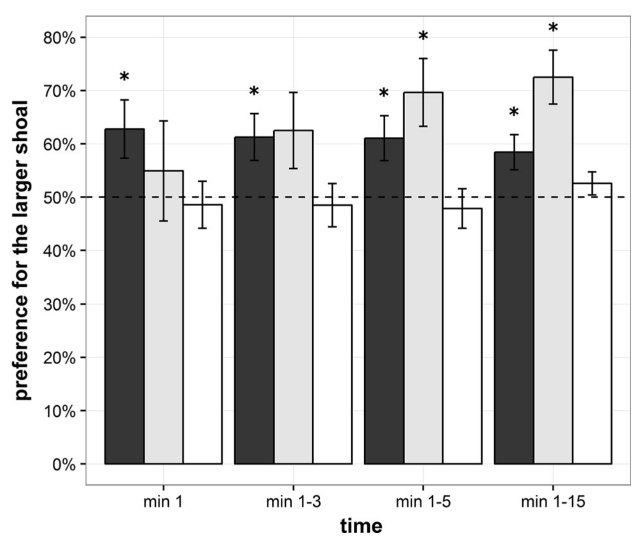


**Fig. 2** Comparison of the performance ($M$ ± SEM preference for the larger shoal in the 4 versus 6 fish discrimination) with three different methods. *Dark bars* new method, experiment 1 of the present study. *Grey bars* method used by Lucon-Xiccato et al. (2016) that shared two features with the present work but not the use of the large cylinder. *White bars* classical method, pooled data from Agrillo et al. (2012) and Bisazza et al. (2014a). *Asterisks* indicate that the preference is significantly above chance (50%, *dashed line*)

shoal (61.05 ± 23.87%, $t_{31} = 2.621$, $P = 0.013$; Fig. 2). Conversely, the preference for the larger shoal did not significantly differ from 50% during the remaining blocks of minutes (block 2, min 6–10: 56.74 ± 22.35%, $t_{31} = 1.671$, $P = 0.105$; block 3, min 11–15: 57.51 ± 27.81%, $t_{31} = 1.583$, $P = 0.124$; Fig. 2). The absence of a significant preference for the larger shoal in blocks 2 and 3 could be due to the fact that guppies tended to increase the time spent in the central sector as time passed (block 1, min 1–5: 12.63 ± 7.50%, block 2, min 6–10: 18.8 ± 7.84%, block 3, min 11–15: 18.1 ± 8.05%; repeated measures ANOVA: $F_{2,62} = 9.584$, $P < 0.001$), suggesting a decrease in motivation to shoal.

To test whether with our new method shoal size preference can be assessed also with shorter time intervals, we performed further analysis on the initials 3 min and the first minute of the test. Preference for the larger shoal was significant in the initial 3 min (61.27 ± 24.89%; $t_{31} = 2.601$, $P = 0.014$), and even considering only the first minute (62.77 ± 30.95%; $t_{31} = 2.410$, $P = 0.022$; Fig. 2).

The reliability test showed that the primary measure of preference based on the position of the fish in the cylinder was robust and repeatable. The scores of the preference for the larger shoal assessed across the 15 min of test by the two experimenters were almost identical (2.63 ± 2.85% difference between the two scores, calculated as | [score of experimenter A − score of experimenter B]|/[mean between the scores of experimenter A and experimenter B] × 100). Furthermore, there was a strong correlation between the two scorers (Pearson's $r = 0.991$, $P < 0.001$). The secondary, more refined, measure of preference based on the orientation of the subject was highly correlated with the primary measure of preference based on the position of the fish in the cylinder (Pearson's $r = 0.965$, $P < 0.001$). The number of switches between the two shoals was 38.25 ± 12.17 across the 15 min of test. The duration of guppies' permanence in the choice area of the first chosen shoal was 31.56 ± 41.92 s.

### Comparison with previous methods

With the classical method (Agrillo et al. 2012; Bisazza et al. 2014a), guppies did not discriminate between shoals made of 4 versus 6 fish considering the overall 15 min ($t_{109} = 1.227$, $P = 0.223$), the initial 5 min ($t_{109} = 0.614$, $P = 0.541$), the initial 3 min ($t_{109} = 0.461$, $P = 0.646$), or the first minute of test ($t_{109} = 0.362$, $P = 0.718$; Fig. 2). With the method adopted by Lucon-Xiccato et al. (2016) which shares two features with our new method, guppies showed a significant preference for the larger shoal in the overall 15 min and the initial 5 min of the test ($t_{23} = 4.409$, $P < 0.001$ and $t_{23} = 2.758$, $P = 0.012$, respectively), but not in the initial 3 min or in the first minute of test ($t_{23} = 1.597$, $P = 0.124$ and $t_{23} = 0.547$, $P = 0.589$, respectively; Fig. 2). When we compared the preference for the larger shoal across the three experiments, we found a significant difference in the overall 15 min (one-way ANOVA: $F_{2,163} = 8.786$, $P < 0.001$) and in the initial 5 min ($F_{2,163} = 3.705$, $P = 0.027$), but not in the 3-min and 1-min intervals ($F_{2,163} = 1.840$, $P = 0.162$; $F_{2,163} = 1.167$, $P = 0.314$, respectively). The lack of statistical difference across the three methods in the first minute and the initial 3 min of test may be partly related to the fact that the variance in the preference for the larger shoal appeared to increase as the time interval considered for the analysis decreased (Bartlett test: Lucon-Xiccato and colleagues: $P = 0.030$; classical method: $P < 0.001$; present work: $P = 0.052$; Fig. 2).

The comparison with the previous studies revealed also indirect evidence of increased switching. The frequency distribution plot of the preference for the larger shoal in the initial 5 min of the test resembles a normal distribution in our study (Fig. 3a); conversely, it is skewed in the study by Lucon-Xiccato et al. 2016; Fig. 3b), and clearly bimodal in experiments with the classical method, suggesting that here fish tend to remain close to the first chosen shoal (Agrillo et al. 2012; Bisazza et al. 2014a; Fig. 3c).

To confirm this increased switching, we calculated the frequency of switching per minute in our work and we compared it with that observed in previous works (Agrillo et al. 2012; Lucon-Xiccato et al. 2016) using a one-way ANOVA. We found a significant difference between the frequency of switching across the three studies ($F_{2,53} = 96.590$; $P < 0.001$). A Tukey post hoc test revealed that the frequency of switching observed with our method (2.55 ± 0.81 switches per minute) was significantly greater than the one observed with previous methods (Agrillo and colleagues: 0.40 ± 0.20 switches per minute, $P < 0.001$; Lucon-Xiccato and colleagues: 0.36 ± 0.44 switches per minute, $P < 0.001$), but there was no difference between the two previous methods ($P = 0.975$).

### Experiment 2

In the 4 versus 5 discrimination, guppies significantly preferred the larger shoal (62.82 ± 26.23%; $t_{23} = 2.120$, $P = 0.045$; Fig. 4). Subjects appeared to choose the larger shoal also in the 5 versus 6 discrimination (54.88 ± 19.01%; Fig. 4), but here the preference was not significantly greater than chance ($t_{23} = 1.306$, $P = 0.204$). We compared the preference for the larger shoal in the initial 5 min of the experiment 1 (4 vs. 6) and the two discriminations of the experiment 2 with a one-way ANOVA. This analysis found no significant difference between the three discriminations ($F_{2,77} = 0.679$, $P = 0.510$; Fig. 4), suggesting that, although not significantly achieved, the 5 versus 6

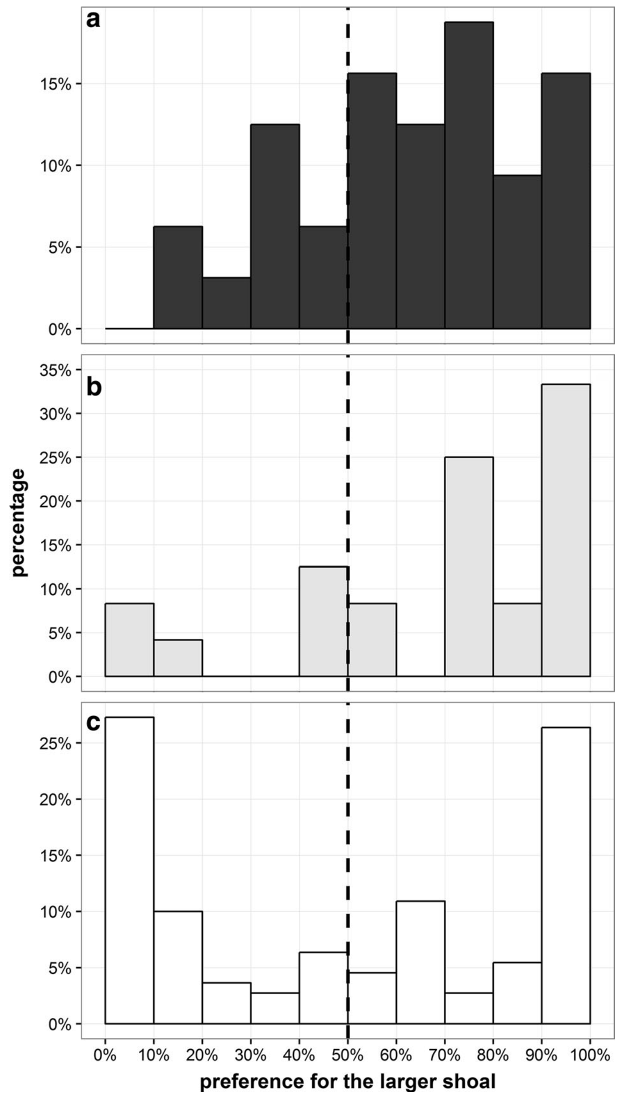


**Fig. 3** Frequency distribution plot of the preference for the larger shoal (4 vs. 6 fish) in the initial 5 min assessed with **a** the new method (experiment 1), **b** the method used by Lucon-Xiccato et al. (2016) and **c** the classical method used by Agrillo et al. (2012) and Bisazza et al. (2014a)

discrimination might represent the threshold of guppies' shoal size acuity.

Finally, we ran a generalised linear model (with Poisson error distribution) to compare the number of switches during the initial 5 min of test of the three numerical discriminations tested in this work. We found that numerical discrimination significantly affected the number of switches ($\chi^2_2 = 20.319$, $P < 0.001$). A Tuckey post hoc test revealed that guppies switched between the two shoals with similar frequency in the 4 versus 6 and the 4 versus 5 discrimination (4 vs. 6: 14.13 ± 7.04 switches; 4 vs. 5: 11.92 ± 6.36 switches; $P = 0.249$); however, guppies switched more often in the 5 versus 6 discrimination

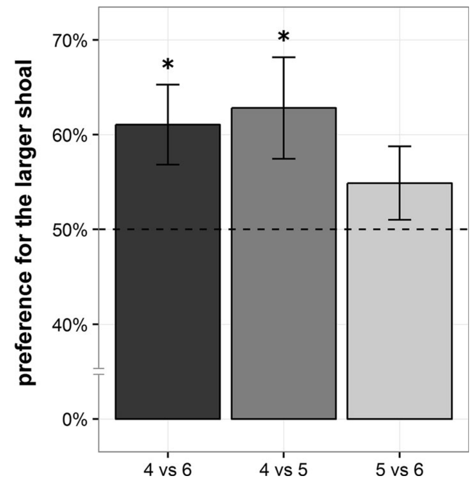


**Fig. 4** Preference for the larger shoal ($M \pm$ SEM) in the three discriminations investigated with our new method (experiment 1: 4 vs. 6; experiment 2: 4 vs. 5 and 5 vs. 6). *Asterisks* indicate that the preference is significantly above chance (50%, *dashed line*)

(18.92 ± 10.61 switches) than in the two easier discriminations (4 vs. 6: $P = 0.005$; 4 vs. 5: $P < 0.001$).

## Discussion

In the classical shoal-choice test, shoaling preference is usually assessed with extended periods of time (e.g. up to 30 min; Agrillo et al. 2007), mainly to counter the measurement error due to the initial low mobility of the subjects. In this study, we developed and tested a new version of the shoal-choice test, which was aimed at obtaining a rapid assessment of shoal size discrimination ability. Our new method was expected to favour more frequent switching between stimulus shoals and hence more selectivity since the beginning of the trial.

The results of experiment 1 showed that shoaling preference can be assessed very quickly with our method. Female guppies were able to discriminate between shoals of different size after only 5 min of testing. Indeed, our data indicated that even shorter intervals can be used to assess quantity discrimination. The preference for the larger shoal was significant considering the initial 3 min of the test and even in the first minute, although variance appears to increase with decreasing time interval. Guppies tested with the old method did not significantly select the larger shoal in such reduced intervals.

Our analysis suggests that the greater efficiency of our method at the beginning of the test is related to the fact that guppies switched often between the two shoals, approximately five times more frequently than with the previous methods (Agrillo et al. 2012; Lucon-Xiccato et al. 2016). Further evidence of the same effect is that in our experiment the average time spent by subjects close to the first chosen shoal was around half a minute, which is ten times shorter than that observed in a previous experiment (Lucon-Xiccato and Dadda 2016a). The increased frequency of switching is likely to favour the comparison between the two shoals and therefore a more accurate assessment. The same increased switching rate could in theory be obtained by greatly reducing the length of the subject tank. However, as a side effect, this would considerably reduce the distance between the two stimulus shoals. As a consequence, the subjects might perceive themselves as part of a single large shoal and thus reduce their selectivity. Interestingly, in experiment 2, we also found that frequency of switching increased as the ratio between the two shoals increased. It has often been assumed that animals should increase sampling to obtain high-quality information (Stephens 2008; Chittka et al. 2009). Accordingly, guppies might need to switch more between two shoals with similar size (5 vs. 6 fish) in order to compare the two options and eventually identify the larger shoal.

A rapid assessment of shoal size discrimination ability can have multiple advantages. For instance, it is possible to use the new method in studies that involve a large number of subjects, such as those aimed at disclosing subtle individual differences (Cote et al. 2012) or screening a large population for genetic studies (Patton and Zon 2001). Equally important, in nature, shoaling decisions are likely to occur very rapidly (Krause et al. 1998). A fast shoal-choice test is therefore expected to measure the cognitive processes normally activated to solve shoal size discrimination under natural conditions. Beyond the obvious time saving for researchers, a fast test also minimises exposure of the subject to the unfamiliar environment, reducing potential stress (Cachat et al. 2010). This is very important considering that, for the subject, being enclosed in the cylinder might be *per se* more stressful than freely swimming in the apparatus.

It should be said that, despite the initial advantage of our new method, when considering intervals longer than 3 min, guppies were more accurate with the recent method adopted by Lucon-Xiccato et al. (2016). In this version of shoal choice, fish were free to swim in the subject tank after a 2-min acclimatisation period in a small cylinder; however, similarly to our method, water was supplied from a tank with a large guppy population, and stimuli lived in the stimulus tanks to favour habituation. These two latter features thus appear to be enough to allow a greater accuracy of guppies but do not solve the problem of initial low performance as the use of the cylinder does. Our method is perhaps more suitable for fast assessment of discrimination abilities, but the method used by Lucon-Xiccato et al. (2016) could be considered a valid alternative when time is not constrained or a greater accuracy level is required. Further, the method by Lucon-Xiccato and colleagues might be preferred in experiments studying ecological aspects of shoal choice or the behavioural response of the subject fish (e.g. aggression) because subjects are free to swim and might express more spontaneous behaviours.

The new method that we developed not only allows a fast assessment of shoal size discrimination abilities, but also appears to disclose significant discrimination for numerical ratio that normally are not achieved by fish using classical shoal-choice methods (Agrillo and Dadda 2007; Agrillo et al. 2008, 2012; Gómez-Laplaza and Gerlai 2011). In experiment 2, guppies achieved a 4 versus 5 fish discrimination, a numerical comparison that to date guppies have been shown to solve only with extensive training procedures (Bisazza et al. 2014b). This result does not necessarily indicate that with our new method the numerical accuracy of guppies is equivalent to that observed with extended training. Indeed, Bisazza et al. (2014b) prevented guppies from solving the discrimination using the non-numerical cues that covary with number (e.g. the area of the stimuli), but we did not. Our guppies might have exploited both numbers and other cues to identify the larger shoal (Gómez-Laplaza and Gerlai 2012), and the use of multiple cues is known to affect numerical discrimination performance (Agrillo et al. 2009).

The findings of experiment 2 on the maximum numerical acuity of guppies raise the issues of the comparability of results obtained from studies that adopted different methodologies. The importance of methodological differences is a well-known issue in experiments that employ discrimination-learning procedures. Frequently, the result varies depending on the type of learning mechanism involved (e.g. classical vs. operant conditioning: Brembs and Heisenberg 2000), the type of stimuli used (e.g. real objects vs. pictures: O'Hara et al. 2015) or other features such as length of training and type of contingency (Wickens et al. 1970; Giurfa et al. 2003). Guppies have been recently found to achieve enhanced discrimination-learning performances by adopting an 'ecological' training method that resembles the natural foraging behaviour of this species (Bisazza et al. 2014b; Gatto et al. 2016). Our present study suggests that cognitive tests which are not based on discrimination-learning procedures and exploit spontaneous behaviours are not immune to the issue of methodological differences. Indeed, in experiment 2 we showed that subtle methodological differences can affect the measurement of discrimination accuracy in guppies. Since many differences between different studies on the same species or between different species may be due to this issue, greater attention to methodological details is required in future research.

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**Compliance with ethical standards**

**Conflict of interest** The authors declared no conflict of interest.

**Ethical standard** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The experiments complied with the law of the country (Italy) in which they were performed (Decreto legislativo 4 marzo 2014, n. 26). The experimental procedure has been approved by Università di Padova ethical committee (protocol n. 32/2015).

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