

Archerfish number discrimination

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20 **ABSTRACT**

21 Debates have arisen as to whether non-human animals actually can learn abstract non-
22 symbolic numerosness or whether they always rely on some continuous physical
23 aspect of the stimuli covarying with number. Here we investigated archerfish (*Toxotes*
24 *jaculatrix*) non-symbolic numerical discrimination with accurate control for co-varying
25 continuous physical stimulus attributes. Archerfish were trained to select one of two
26 groups of black dots (Exp. 1: 3 vs. 6 elements; Exp. 2: 2 vs. 3 elements); these were
27 controlled for several combinations of physical variables (elements' size, overall area,
28 overall perimeter, density and sparsity), ensuring that only numerical information was
29 available. Generalization tests with novel numerical comparisons (2 vs. 3, 5 vs. 8 and 6
30 vs. 9 in Exp. 1; 3 vs. 4, 3 vs. 6 in Exp. 2) revealed choice for the largest or smallest
31 numerical group according to the relative number that was rewarded at training. None of
32 the continuous physical variables, including spatial frequency, were affecting archerfish
33 performance. Results provide evidence of the spontaneous use of abstract relative
34 numerical information in archerfish for both small and large numbers.

35

36 **KEYWORDS:** number, numerical cognition, number discrimination, numerical rule,
37 number sense, archerfish.

38

39 INTRODUCTION

40 Non-symbolic numerical estimation is an important and well-studied cognitive ability that
41 allows humans and other animals to interact successfully with their surroundings. The
42 development of a “sense of number” is associated with fundamental biological needs
43 that in many ecological contexts allow animals to estimate how many companions or
44 enemies are around, or how much food is present in different patches - all important
45 information to maximize fitness and reproductive success in the wild [1].

46 Typically, in order to assess numerical abilities animals are requested to
47 discriminate between sets of visual stimuli differing in numerosity (review in [2]). This
48 can be done using spontaneous attractive natural stimuli such as food or social
49 companion, taking advantage of the animals’ natural and spontaneous tendency in
50 some ecological contexts to “go for more”. Alternatively, operant conditioning
51 procedures can be used that associate a particular set of stimuli with a reward.
52 Extensive evidence supports the use of numerical information in non-human primates
53 (e.g., [3–7]), as well as in other mammals (e.g., [8–13]), in birds (e.g., [14–19]), in
54 amphibians (e.g., [20,21]), in reptiles (e.g., [22,23]), in fish (e.g., [24–26]) and in
55 arthropods (e.g., [27–30]) (see for general reviews in vertebrates [1,31,32]).

56 Numerical discrimination seems to be supported by an “Approximate Number
57 System” (ANS, [33,34]), which discriminative accuracy is ratio-dependent in accordance
58 to Weber’s law (as the ratio between two numerosity increases, the discrimination gets
59 more difficult). Besides the ANS, an attentional working memory-based system has
60 been claimed for by some authors as providing precise representation of small numbers

61 (up to 3-4), the so-called “Object Tracking System” (OTS; [35]), though its generality for
62 non-human animals is debated (discussion in [31,36]).

63 Studies investigating the neural basis of number representation revealed
64 selectivity of response of neurons in some areas of the brain such as the parietal and
65 prefrontal cortex in humans [37,38] and in monkeys [39,40], the nidopallium
66 caudolaterale in crows [15,41] and the most caudal dorsal-central part of the pallium in
67 zebrafish [42,43], suggesting that common selective pressures led to convergent
68 evolution of numerical representation in different species [44,45].

69 However, one issue in all these experiments is that animals are dealing with sets
70 of physical elements, and thus numerical information is intrinsically melted with other
71 non-numerical properties of the stimulus, such as the area, the density or the spatial
72 frequency or the elements’ arrangement [46]. Recently, some debates have arisen
73 concerning whether bees use abstract numerical information or rather rely on sensory
74 properties of the stimulus for discrimination [47,48].

75 Taking advantage of the fact that we recently developed a sophisticated script for
76 the automatic generation of visual stimuli that can allow proper randomization and
77 control of continuous physical variables in number sense experiments [49], we decided
78 to perform some very precisely controlled experiments to check whether fish do use
79 number as abstract property.

80 We selected archerfish (*Toxotes jaculatrix*) for our study. These fish are well-
81 known for their particular hunting strategy, which consists of spitting at preys above the
82 water surface with a precise jet of water thrown with the mouth. This attacking repertoire
83 makes it very easy to train them to hit targets using operant conditioning (see e.g., [50]).

84 Still, to date, no studies in archerfish have explicitly investigated abstract numerical
85 abilities. Leibovich-Raveh et al. [51] and colleagues showed that when archerfish make
86 magnitude-related decisions, their choice is influenced by the non-numerical variables
87 that positively correlate with numerosity; for instance, when exposed to two groups of
88 dots differing in number and continuous physical information, archerfish spontaneously
89 selected the group containing the larger non-numerical magnitudes and smaller
90 numerosity, switching to the larger numerical set when positively correlated with all the
91 non-numerical magnitudes.

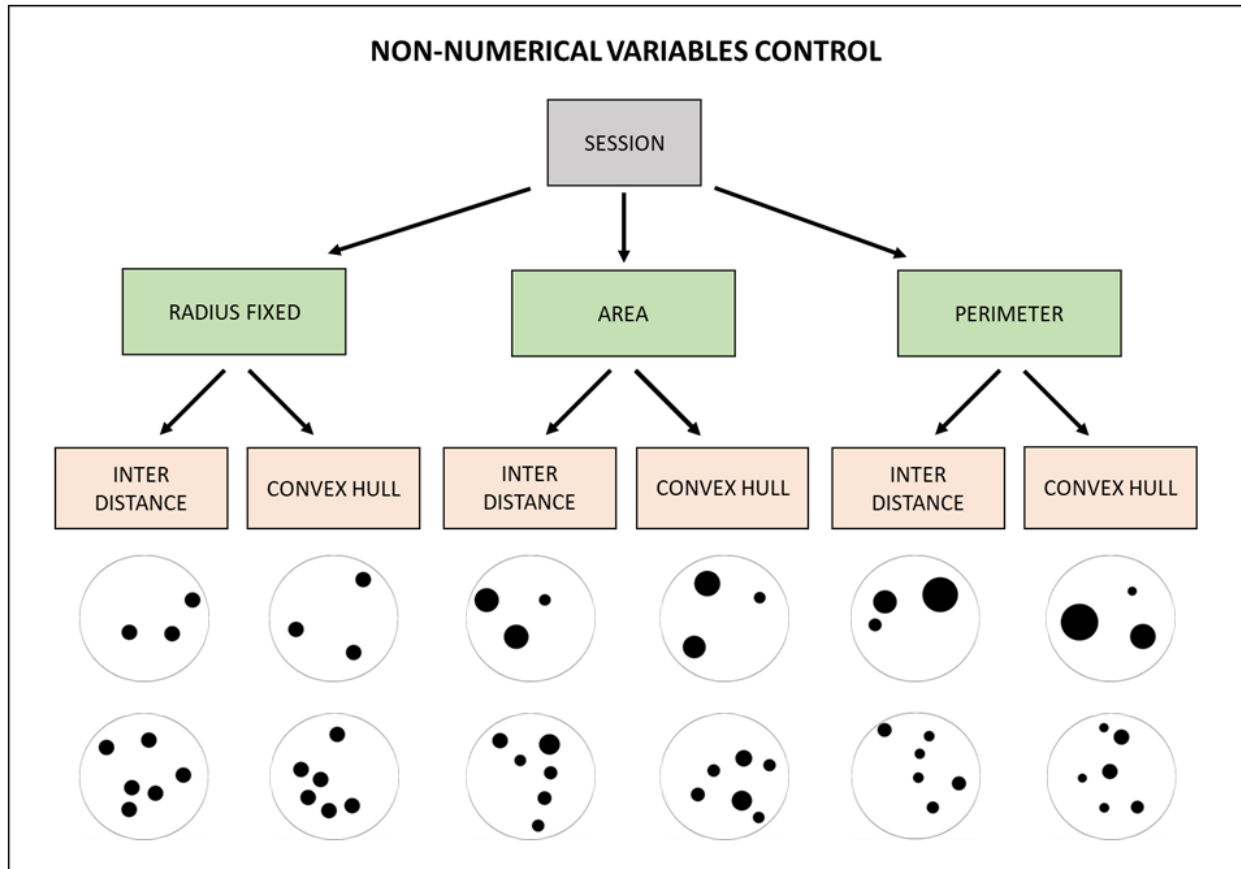
92 Related to magnitude discrimination, archerfish also proved to be able to associate
93 different geometric shapes with different food quantities [52]; this would support the
94 existence of a system dealing with magnitudes, although a specific role of numerical
95 information remains unclear.

96 In our study archerfish were trained to select one of two arrays, involving either a
97 small and a large numerosity (Exp. 1: 3 vs. 6 elements) or small numerosities only (Exp.
98 2: 2 vs. 3 elements). After reaching a learning criterion, archerfish were tested with
99 novel numerical comparison (2 vs. 3, 5 vs. 8 and 6 vs. 9 in Exp 1; 3 vs. 4, 3 vs. 6 in Exp
100 2) to check whether the rule they used in the training phase was based on a relative
101 judgement (select the “largest” or “smallest” group) or on an absolute judgment (select a
102 specific number of item). All of the different continuous physical variables such as
103 radius, total area, total perimeter, convex hull and inter-distance were carefully
104 controlled for and alternately balanced across trials, ensuring that the animals could not
105 rely on them to perform their judgment (Figure 1). Furthermore, a statistical analysis
106 was run for *a posteriori* evaluation of whether any of these variables influence the

107 archerfish responses, confirming that they were not used as a cue for numerical
108 evaluation.

109

110



111 **Figure 1.** Schematic representation of the non-numerical physical controls applied to
112 the stimuli in each session.
113

114

115

116 RESULTS

117 *EXPERIMENT 1*

118 Eight archerfish were trained to discriminate between two groups of black dots in a 3 vs.
119 6 numerical comparison; four fish were trained to select the number 3, while the other
120 four were rewarded with the number 6. No difference has been found in the number of
121 trials needed to reach the learning criterion between the group trained with 3 elements
122 (mean \pm SEM= 451.25 \pm 106.77) and the group trained with 6 elements (mean \pm SEM=
123 413.25 \pm 73.14) (Independent Samples t-Test: $t(6)=0.294$, $p=0.779$).

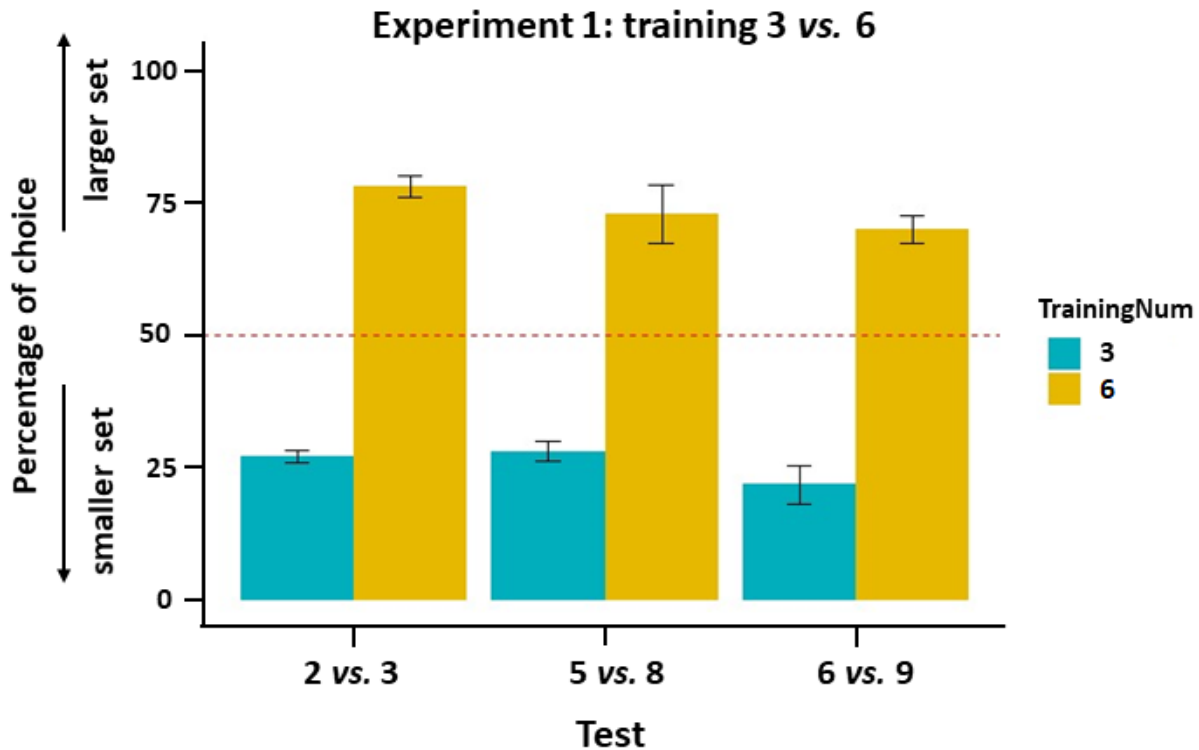
124 Once the learning criterion was reached, all the fish performed three different tests.

125 TEST 1: This test was the main discriminator to understand whether at training fish
126 represented numerosity as relative or absolute. Fish trained to select the smallest
127 number 3 at training (i.e. the smallest set in the 3 vs. 6) were presented at test with a
128 novel discrimination 2 vs.3, while fish trained to select the number 6 at training (i.e.,
129 largest set in the 3 vs. 6) were tested with a 6 vs. 9 condition. The use of “relative”
130 information (go for the smallest or largest) should lead the fish to choose the novel
131 numerosity at test, while the use of “absolute” information would reflect in the choice of
132 the stimulus with the same number of elements as at training.

133 TEST 2: The second test aimed to clarify the role of the incorrect (i.e.,
134 unrewarded) training stimulus and its relevance for the fish. When fish are trained to
135 select the numerosity 3, thus avoiding number 6, once presented with the new
136 comparison 6 vs. 9 (or *vice versa* 2 vs. 3, if trained to select 6), do they choose the
137 group according to the relative information even if it coincides with the absolute
138 numerosity to avoid at training?

139 TEST 3: In the last test, fish behaviour was observed in a comparison involving
140 novel numerosities never experienced during the training, i.e., 5 vs. 8. This allowed
141 observing whether zebrafish applied a relative representation (go for the “smallest” or
142 “largest”), or if the choice was at the chance level, since no absolute numerical
143 information experienced at training was present here.

144 Results at tests for Experiment 1 are reported in Figure 2. Choices for the relative
145 numerosity were analyzed using a generalized linear mixed model fit by maximum
146 likelihood (Laplace Approximation), binomial GLMM with a logit link in R. Four fixed
147 effects (type of Training -3 or 6 dots-; type of Test -2 vs. 3, 5 vs. 8 and 6 vs. 9-; type of
148 geometrical control -radius fixed, overall area controlled, overall perimeter controlled-;
149 type of spatial disposition control -inter-distance controlled; convex-hull controlled-) and
150 one random intercept effect (fish) were considered. Analysis on the random effect
151 showed not to affect the model and no significant differences were found between
152 effects of groups, nor group interactions (comparisons between different models
153 considering various effects and interactions reported always $p > 0.05$, suggesting to
154 adopt the simplest model described by the only choice with no contribution from any
155 effects). Only a trend for the contribution of the type of geometrical control was
156 observed, driven by a non-significant difference between the “radius fixed” and “overall
157 area controlled” conditions (post-hoc non-parametric tests adjusted with Tukey method:
158 $p = 0.063$); within this trend, every single condition was statistically significant by chance
159 level in the direction of the relative choice (“radius fixed”: probability of success =
160 71.18%, $p < 0.001$; “overall area controlled”: probability of success = 81.25%, $p < 0.001$;
161 “overall perimeter controlled”: probability of success = 73.61%, $p < 0.001$).



162
163 **Figure 2.** Percentage of choice for the larger/smaller set (mean \pm SEM) displayed by
164 fish in the novel comparison tests for the groups trained to select the smaller (3) or
165 larger (6) set.
166

167 Overall, considering the previous discussion, a binomial test shrinking all the data
168 together was performed to investigate the final findings: fish showed a strong significant
169 preference for the relative numerosity (probability of success: 74.3%, $p < 0.001$).

170 The result obtained in Experiment 1 showed that archerfish, when trained to select
171 one of two simultaneously displayed groups of dots with different numerosities (i.e., 3
172 vs. 6 dots), use a relative numerical rule to perform novel numerical comparisons.
173 These results confirm findings in other fish species such as angelfish [55] and guppy
174 [56] but they are different from those obtained in bees which showed instead a
175 preference for the absolute number [57]. An important difference between fish and bees
176 studies is related to the numerical comparison used: respectively large numbers (> 4

177 elements) for fish and small numbers (≤ 4 elements) with bees. This might engage
178 different systems (see Introduction) thus explaining the discrepancy. The training
179 discrimination used here in Experiment 1 involved two numbers (3 vs. 6) that belong
180 one to the hypothesized “small” and the other to “large” systems, respectively. This is
181 different than in previous fish studies which involved only large numerosities; thus, it
182 remains to be tested how fish would deal when trained with small numerosities only. In
183 principle, the presence of a large number in the comparison in Exp. 1 may be enough to
184 lead the archerfish to follow a relative rule. If trained with a numerical discrimination
185 involving only small numbers, would the animals still use a relative numerosity
186 judgement or would they turn to absolute judgement? This was tested in Experiment 2.

187

188 *EXPERIMENT 2*

189 Four subjects were trained to select the largest number in a 2 vs. 3 comparison (i.e., the
190 number 3). Fish judgment was then observed in two tests (i.e. 3 vs. 4 and 3 vs. 6)
191 involving a comparison between the previously trained numerosity (3) and a novel
192 numerosity (4 or 6).

193 All fish reached the learning criterion, showing an ability to discriminate between
194 the two numbers (trials to criterion \pm SEM = 506.5 ± 97.8). Results at test are reported in
195 Figure 3. A GLMM model with three fixed effects (type of test -3 vs. 4 and 3 vs. 6-; type
196 of geometrical control -radius fixed, overall area controlled, overall perimeter controlled-;
197 type of spatial disposition control -inter-distance controlled; convex-hull controlled-) and
198 one random intercept effect (fish) showed no random effect of fish, neither significant
199 differences between groups or groups' interactions (Chi-Square tests between all

200 different models with different effects and interactions report always $p > 0.05$, suggesting
201 to adopt the simplest model based on the only fish choice -for the relative or absolute
202 number- and no effects of controls).

203 An Exact binomial test considering a merge of the data showed a highly
204 significant preference for the relative number (probability of success: 69.79%, $p <$
205 0.001).

206 In Experiment 2, archerfish showed to be able to discriminate between two
207 different numerical groups of dots within the small numerical range. At test, fish
208 preferred the novel numerosity to the familiar 3 items, in both 3 vs. 4 and 3 vs. 6
209 comparisons, confirming the use of a relative rather than absolute numerical rule. This
210 evidence does not match with findings in bees, tested in the same numerical conditions,
211 suggesting that the spontaneous engagement of relative/absolute rule to extract
212 numerical information may be guided by different ecological pressures experienced by
213 different species in their phylogenetic history. The spontaneous use of relative rules
214 suggests that among fish, it is more important to learn a general rule that is applicable
215 to novel comparisons. It cannot be excluded that this strategy is adopted because it
216 could be less demanding as to memory load than an absolute judgement strategy.

217



218
219 **Figure 3.** Percentage of choice for the larger/smaller set (mean \pm SEM) displayed by
220 fish in the novel comparison tests for the group trained to select the larger (3) set.
221

222 Considering the results of Experiment 2 and Experiment 1, it is apparent that
223 archerfish can easily discriminate between small and large numerosity using the same
224 rules, providing evidence in favour of a unique system underlying numerical
225 discrimination as found in other fish species [26,58].

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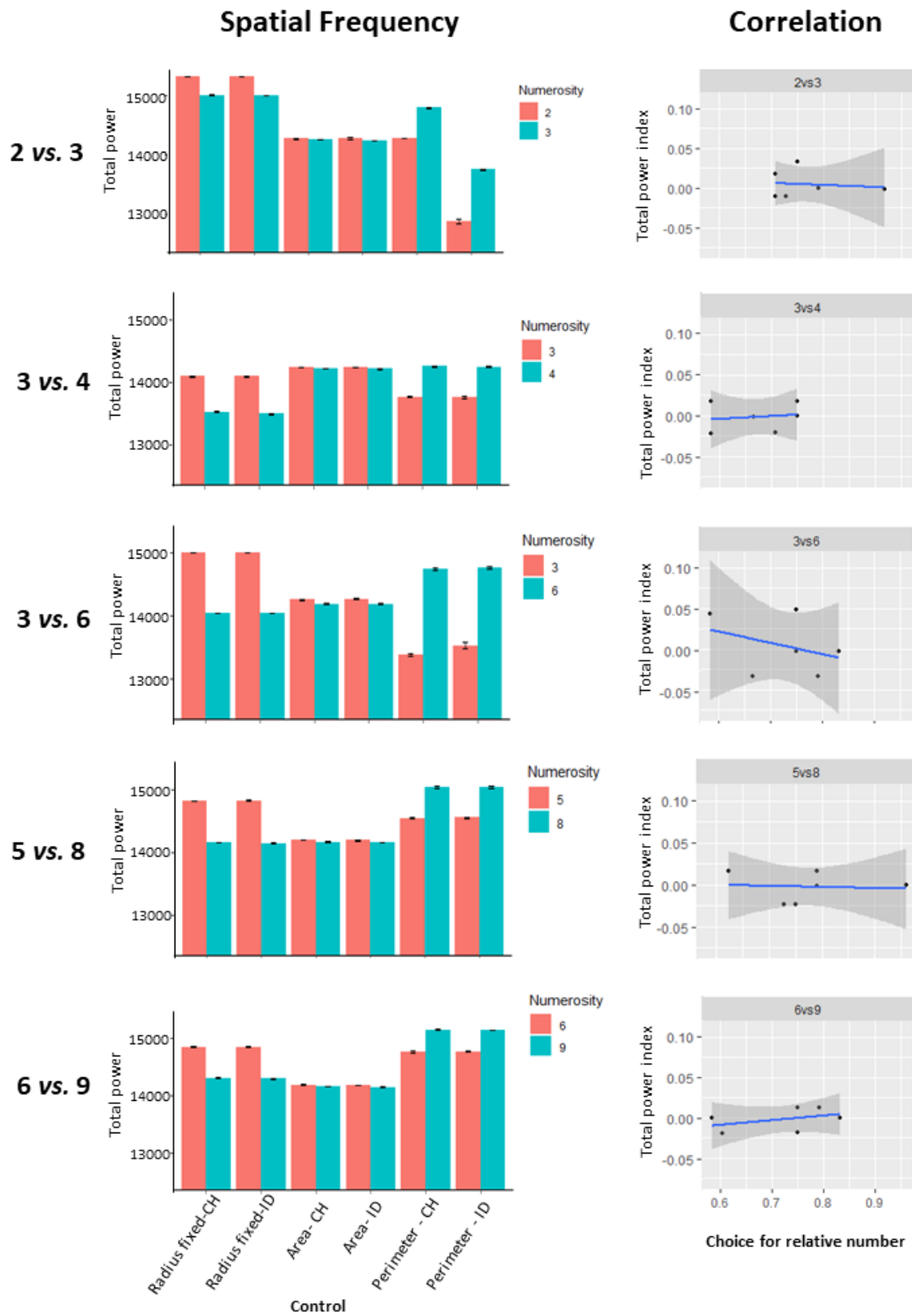
227 *NUMEROSITY AND SPATIAL FREQUENCY*

228 The stimuli used in our Experiments were visual collections of black dots differing in
229 numerosity. As described in the method section, for each numerical comparison, the
230 physical properties of each array were equalized for the geometry (radius, area and
231 perimeter) and spatial disposition (inter-distance (density) and convex-hull; see Figure

232 1). Since we are dealing with images, each figure could also be described in terms of
233 spatial frequency. Spatial frequency can be thought of as the number of repeating
234 elements in a pattern per unit distance, and it is mathematically described by the Fourier
235 transform theory. No control was applied to the spatial frequency of our stimuli. Thus, in
236 order to check whether spatial frequency could influence archerfish choice, we
237 calculated spatial frequency variation across all different numerosities and control
238 conditions (see Methods section). Within each numerical test comparison, different
239 spatial frequencies were found (see Figure 4). The different constraints applied to the
240 stimuli (control of the area, perimeter or elements radius) showed to influence differently
241 the spatial frequency between the two numerosities. In detail, when the elements' radius
242 was fixed between the two numerical arrays, the total power of the spatial frequency
243 was higher in the smaller group than in the larger one, while the opposite was found in
244 the groups in which the overall perimeter was balanced (total power higher in the more
245 numerous group). Interestingly, this trend was maintained in all the numerical
246 comparisons used, irrespective of the number of elements to be compared.

247 To investigate the influence of spatial frequency in the numerical task, we
248 analyzed whether a correlation between the performance accuracy and the spatial
249 frequency was apparent, for all possible control configurations (see Methods section).
250 Results are reported in Figure 4, showing no correlations between any comparison (test
251 2 vs. 3: $r(4) = -0.17$, $p = 0.83$; test 3 vs. 4: $r(4) = 0.15$, $p = 0.77$; test 3 vs. 6: $r(4) = -0.35$,
252 $p = 0.50$; test 5 vs. 8: $r(4) = -0.08$, $p = 0.88$; test 6 vs. 9: $r(4) = -0.42$, $p = 0.41$).

253 These data strongly suggest that the spatial frequency was not influencing archerfish
254 performance in the numerical task.



256 **Figure 4.** The histograms (on the left) show the spatial frequency (Total power) for each
257 numerical comparison among the different control groups (non-numerical variables
258 control). The different constraints applied to the stimuli (control of the area, perimeter or
259 elements radius) showed to influence the spatial frequency between the two compared
260 numerosities. The regression lines (on the right) show the correlation between fish'
261 performance accuracy (choice for the relative numerosity) and the spatial frequency
262 (total power index between the two total power values), for all numerical comparisons.
263

264 DISCUSSION

265

266 Overall, our results showed that when trained to select a specific group of elements
267 between two numerical arrays, archerfish spontaneously generalize at test to novel
268 numerical comparison according to a relative numerical rule (select the largest/smallest)
269 rather than an absolute numerical rule (select the specific number of items). These
270 findings are in agreement with previous results from other fish species and humans
271 [55,56], while differing with respect to bees [57].

272 Interestingly, archerfish uses a general relative judgement even when trained to
273 discriminate between numerosities that belong to different systems, namely small and
274 large numerosities (for a review see [61]). In Experiment 1, archerfish were trained with
275 a 3 vs. 6 contrast and then observed in test conditions with a 2 vs. 3, 6 vs. 9 and 5 vs. 8
276 comparison. In all the tests, archerfish showed to spontaneously use a general relative
277 rule. In Experiment 2, subjects' performance was observed in a numerical discrimination
278 involving at training only small numerosities (i.e., 2 vs. 3). Once again, at test, fish
279 followed the relative rule, selecting the largest group in the test comparisons 3 vs. 4 and
280 3 vs. 6, thus ignoring the absolute number of elements (3).

281 Taken together, our results support the hypothesis of a unique system for
282 representing numerosities in archerfish, working both for small and large numbers,
283 obeying the ANS. Evidence from other fish species supports this claim [26,58].

284 The reason for which archerfish primarily rely on the relative information of
285 numerical groups may have ecological reasons, being more adaptive in a natural
286 environment that constantly require numerical/quantity judgement. Selecting the largest
287 social group of companions or the largest food patch are easy rules that can be more
288 efficient than using an absolute rule. Moreover, the use of relative information may be
289 less cognitively expensive (in terms of memory load) than the absolute one, since it
290 does not require storing the information about the precise number of elements: the
291 discrimination could work on a simple relative comparison between numerosities,
292 guided by the numerical ratio between the two. Nevertheless, the engagement of
293 relative rules requires a good level of abstraction and the creation of a general rule to be
294 applied to [62].

295 In fish, the use of an absolute rule may not be as convenient as the relative one,
296 given that in most ecological contests there is not a specific optimal amount of food,
297 partners or companions. However, this seems not to be the case for species such as
298 bees, which showed instead a spontaneous use of absolute numerical information,
299 suggesting that this rule may be more informative and useful in their ecological
300 environment. Similar evidence has been found in spiders, that, in a natural predatory
301 strategy context, settle their attack based on the specific number of conspecifics at the
302 nest [29].

303 Note, however, that the spontaneous use of a relative or absolute rule does not
304 imply that animals are unable to use both. Vertebrates can be trained to learn a specific
305 number of items in a set if forced to do it [6,56,63,64]. Similarly, bees can be trained to
306 the numerical concepts of “greater than” or “smaller than” [47]. The spontaneous
307 engagement of one of the two criteria is therefore justified probably by a combination of
308 natural constraints and/or less cognitive demand motivations that better fit for the
309 individuals' fitness in their particular niches of adaptation.

310 Lastly, with respect to the main question of our paper, the results showed that
311 archerfish are capable of abstract numerical discrimination, not influenced by other
312 continuous physical variables. We tested archerfish with numerical arrays well
313 controlled for all the possible non-numerical variables (e.g., total area, perimeter, inter-
314 distance (density), convex hull), thus ensuring that the discrimination made by the
315 animals was based on purely numerical information. The results of the statistical
316 analyses showed no influence whatsoever of the different control conditions on the fish
317 choices. Moreover, we showed that even the different spatial frequencies of the stimuli
318 were not influential on archerfish performance. The total power of the spatial frequency
319 has been described in the literature to positively increase with numerosity [48]; however,
320 in our stimuli, the different geometrical constraints showed that it can be reversed as
321 well. Moreover, elements area and perimeter seem to play a crucial role in the
322 distribution of the spatial frequencies' energy with respect to the elements disposition
323 (inter-distance and sparsity). All our analyses suggested that the amplitude component
324 of the spatial frequency was not influencing archerfish numerical evaluation during our
325 experiments.

326 Note, however, that in all studies carried out so far (including our own analysis),
327 the focus was on the amplitude of the spatial frequency as the main component, which
328 provides information on the alternation rate of different elements in the image. It is likely
329 that a more specific role on computation of numerosity is played by the spatial
330 frequency phase component (related to elements' spatial coherence and
331 distribution) which directly relates to figure-ground aggregation and unity formation.

332 In conclusion, our results provide clear evidence that under conditions of strict
333 control of continuous physical variables archerfish can encode an abstract concept of
334 number to support relative numerical judgement for both small and large numerosities.

335 MATERIALS AND METHODS

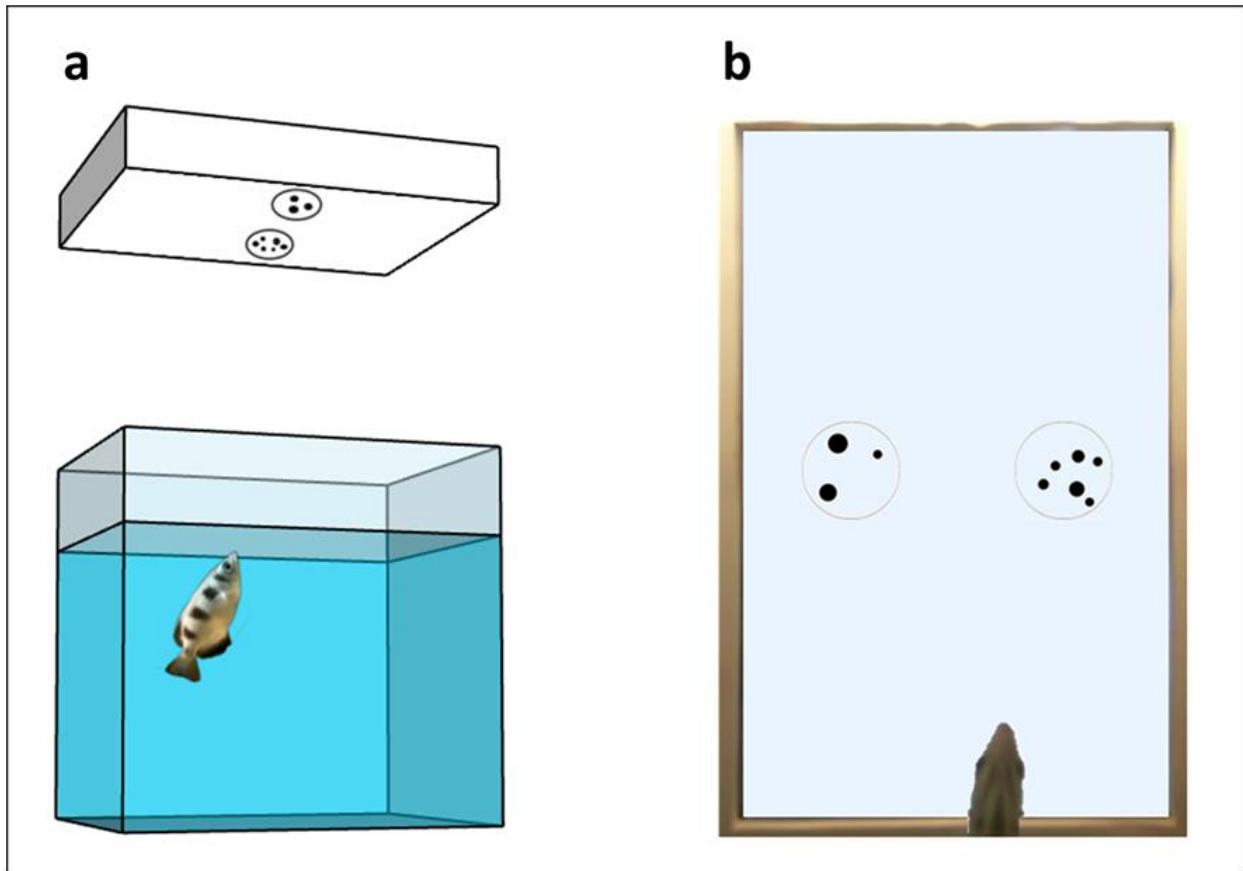
336 *SUBJECTS AND REARING CONDITIONS*

337 Sixteen adult archerfish, *Toxotes jaculatrix* (fish size ranged between 8 and 10 cm in
338 length) were provided by a local commercial supplier ("Acquario G di Segatta Stefano").
339 Four animals were excluded because they did not show any consistent motivation in
340 hitting the screen. A group of fish (N=8) took part in Experiment 1, while a second group
341 (N=4) took part in Experiment 2. All fish were housed in large aquariums (100 x 40 x 40
342 cm) in groups of 10 individuals. Prior to the experiment, each archerfish was moved into
343 individual aquaria (40 x 30 x 50 cm) filled with freshwater maintained at 25°C and
344 enriched with gravel and a shelter. Water quality was kept by suitable filters (Sera fil
345 60). The system was illuminated under a 10:14 light /dark cycle (Sylvania luxline plus
346 F36W/840 cool white). Fish were fed with food pellets (Hikari cichlid gold baby pellet).

347 **APPARATUS**

348 Both the apparatus and the training method were set up based on previous studies
349 conducted with archerfish on visual discrimination tasks (i.e., [52,53]). Each
350 experimental tank consisted of a rectangular aquarium with a monitor screen located
351 above it (20", DELL 2009Wt), held at 30 cm from the water level (Figure 5a). Each tank
352 was surrounded by white opaque panels to ensure that the fish was not distracted by
353 external cues. Each tank was raised 8 cm off the table thanks to lateral supports,
354 allowing the positioning of a video camera under the centre of the pavement's tank to
355 record a bottom view of the fish and the screen (see video example in the
356 supplementary materials).

357



358

359 **Figure 5.** a) Schematic representation of the experimental setup; b) Bottom view of
360 the tank from the camera placed below the tank's pavement.
361

362 *STIMULI*

363 The stimuli presented in the training phase consisted of groups of black dots confined
364 into a black outline circle (6 cm diameter). The dots size was ranging between 3 and 12
365 mm, and the visual angle was in the range 0.43° and 1.72° , which has been proven to
366 be well perceived by archerfish [54]. In every trial, a couple of stimuli was
367 simultaneously presented in the centre of the screen (horizontally aligned to the shortest
368 monitor's side, see Figure 5b). All the stimuli were created using the software GeNEsIS
369 [49], a Matlab program that allows to create numerical collections of stimuli controlled
370 for several non-numerical magnitudes. Given that it is mathematically impossible to
371 balance all the non-numerical magnitudes simultaneously in two different numerical
372 groups, different sets of stimuli were created for each numerosity, controlling for some
373 visual physical property; all the possible properties were covered across the different
374 sets during a session (see Figure 1 for a view of all the combinations applied in a
375 session). Pictures from each set were randomly presented, making the numerical
376 information the only reliable cue to differentiate the two stimuli across all the various
377 trials.

378 *GENERAL PROCEDURE*

379 Pre-training phase

380 Before starting the experiment, fish underwent a pre-training phase in which they were
381 gradually habituated to spit (hit with a jet of water) at the training stimulus on the screen.
382 This was accomplished throughout a shaping procedure to facilitate the task. The

383 silhouette of an insect was initially presented, inducing the fish reaction to spit at the
384 prey; once hit, fish were rewarded with a food pellet. The insect was gradually replaced
385 by a black dot and finally with the effective training stimulus. Once the fish
386 accomplished all these stages, the training phase was initiated.

387 Training phase

388 Fish were trained to spit at the correct target presented on the monitor above the tank.
389 The stimuli to discriminate consisted of two groups of dots with different numerosity.
390 Every trial started with the appearance of a blinking black square (1.6 cm, three blinks
391 of 100 milliseconds) at the centre of the screen to catch the fish's attention towards the
392 screen. Then, the two training stimuli were displayed one next to the other (distance 7
393 cm) on the two sides of the monitor. Only one of the two numerosities was rewarded
394 with a food pellet when hit, while the choice for the incorrect stimulus caused the stop of
395 the trial, which in every case, in absence of choice, was stopped after 5 minutes. At the
396 end of each trial, the screen was cleaned from the water drops and a new trial started.
397 In the first training session only, a corrective method was applied: the stimuli remained
398 on the screen until the subject selected the correct target, even if the incorrect stimulus
399 was hit, allowing the fish to correct its choice.

400 Fish were trained with daily sessions of 48 trials, in which continuous physical
401 variables were controlled and changed according to the scheme reported in Figure 1,
402 and the position of the target stimulus on the screen (right-left) was randomized. Fish
403 generally responded 70% to 100% of the trials. The learning phase was considered
404 completed when the fish reached a learning criterion of at least 75% of correct choices

405 for two consecutive days (binomial test: $p < 0.01$), allowing the fish to take part in the
406 test phase.

407

408 Test phase

409 Generally, each test condition consisted of the presentation of a couple of stimuli with a
410 novel numerical comparison, aiming to see if the numerosity target learned in the
411 training phase was represented as a relative or an absolute numerical information. Each
412 test was composed of 24 probe trials not rewarded, divided into three testing days of 8
413 trials. In each test session, the 8 test trials were shuffled and interspersed with
414 rewarded recall training trials (32 recall in total), to maintain the fish motivation high
415 during the whole test duration. The order of the tests was randomized among the fish to
416 exclude that the performance could be influenced by their order. At the end of each test,
417 the fish underwent a complete daily session of retraining to further exclude potential
418 interference among the tests.

419 Statistical analyses and data analysis

420 Data were analyzed using R software (R-4.1.0). In Experiment 1, an independent t-test
421 was used to compare the number of trials to reach the criterion between the two groups
422 at training. At test (Exps. 1 and 2), choices for the relative numerosity were analyzed
423 using a generalized linear mixed model fit by maximum likelihood (Laplace
424 Approximation), binomial GLMM with a logit link. A binomial test was used to compare
425 the distribution of the choices for the relative and absolute numerosities.

426 To obtain an estimate of the spatial frequency we adopted an approach already
427 performed in other studies [48,59,60]: the fast Fourier transform of our images was

428 calculated, a radial average of the signal amplitude in the frequency domain was
429 performed, and lastly, all the frequency contributions of its power spectrum were
430 summed up. In this way, a value related to the total energy of each frequency
431 component inside a given image is obtained.

432 To investigate the influence of spatial frequency in the numerical task, we analyzed
433 whether a correlation between the performance accuracy (choice for the relative
434 numerosity) and the spatial frequency (normalized total power difference between the
435 two compared numerosities) was apparent, for all possible control configurations. To
436 compare two numerosities we reported a normalized difference (total power index)
437 between the two total power values (difference between the total power of the biggest
438 numerosity and the smallest, divided by their sum). All the frequency calculations were
439 performed with a custom script in Matlab, while the statistical comparisons were
440 calculated in R. For each of them a Pearson's correlation coefficient was calculated
441 comparing the choice for the relative numerosity and the normalized difference between
442 numerosities (as explained above).

443

444 **Ethical regulation**

445 The present research was carried out at the Animal Cognition and Neuroscience
446 Laboratory (ACN Lab) of the CIMEC (Center for Mind/Brain Sciences), at the University
447 of Trento (Italy). All husbandry and experimental procedures complied with European
448 Legislation for the Protection of Animals used for Scientific Purposes (Directive
449 2010/63/EU) and were approved by the Scientific Committee on Animal Health and
450 Animal Welfare (Organismo Preposto al Benessere Animale, OPBA) of the University of
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452 **Competing interests**

453 We declare we have no competing interests.

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459 **Authors' contribution**

460 D.P. and G.V conceived the study. D.P. G.V and M.Z. designed the experiment. D.P.
461 performed the experiments. All authors interpreted the data and contributed to the
462 manuscript writing.

463

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