1 Running Head: LOCAL VISUAL CUES IN TOADS

2

3

4 **Use of local visual cues for spatial orientation in terrestrial toads**

5 (*Rhinella arenarum)*: **The role of distance to a goal**

6

7 **M. Florencia Daneri**

8 **Instituto de Biología y Medicina Experimental (IBYME-CONICET),**

9 **Universidad de Buenos Aires (UBA) and Departamento de Biología, Bioquímica y**

10 **Farmacia, Universidad Nacional del Sur (UNS), Argentina**

11 **Emma B. Casanave**

12 **Instituto de Ciencias Biológicas y Biomédicas del Sur (INBIOSUR-CONICET-UNS)**

13 **Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur**

14 **(UNS), Bahía Blanca, Argentina**

15 **and**

16 **Rubén N. Muzio**

17 **Instituto de Biología y Medicina Experimental (IBYME-CONICET) and Universidad**

18 **de Buenos Aires (UBA), Argentina**

19

20 Corresponding autor:

21 Dr. Rubén N. Muzio
22 Grupo de Aprendizaje y Cognición Comparada
23 Laboratorio de Biología del Comportamiento
24 Instituto de Biología y Medicina Experimental (IBYME-CONICET)
25 Vuelta de Obligado 2490. CP 1428 - Buenos Aires
26 ARGENTINA
27 E-mail: rnmuzio@gmail.com
28

29 **Abstract**

30 The use of environmental visual cues for navigation is an ability present in many groups of

31 animals. The effect of spatial proximity between a visual cue and a goal on reorientation in

32 an environment has been studied in several vertebrate groups, but never previously in

33 amphibians. In this study we tested the use of local visual cues (beacons) to orient in an

34 open field in the terrestrial toad *Rhinella arenarum*. Experiment 1 showed that animals

35 could orient in space using two cues located near the rewarded container. Experiment 2

36 used only one cue placed at different distances to the goal and revealed that learning speed

37 was affected by the proximity to the goal (the closer the cue was to the goal, the faster toads

38 learned its location). Experiment 3 showed that the position of a cue results in a different

39 predictive value. Animals preferred cues located closer to the goal more than those located

40 farther away as a reference for orientation. Present results revealed, for the first time, that

41 (i) toads can learn to orient in an open space using visual cues, and that (ii) the effect of

42 spatial proximity between a cue and a goal, a learning phenomenon previously observed in

43 other groups of animals such as mammals, birds, fish and invertebrates, also affects

44 orientation in amphibians. Thus, our results suggest that toads are able to employ spatial

45 strategies that closely parallel to those described in other vertebrate groups, supporting an

46 early evolutionary origin for these spatial orientation skills.

47

48 *Keywords:* spatial learning, local visual cues, beacons, toads

49

50   Staying in one place or moving to another may mean accessing a resource or

51   avoiding a predator. In this sense, choosing where to settle and how to move to a new

52   location is clearly relevant for an animal. Much evidence suggests that natural selection has

53   shaped these decisions adaptively, and certain mechanisms are required to ensure that they

54   are effective. With the goal of reaching a desirable location, an animal needs to decide

55   where to go and how to get there (Barnard, 2004).

56   Animals use a range of strategies to navigate to a specific goal. These include the

57   use of a single salient cue (a beacon, usually a large local cue near the goal), path

58   integration, and learning a sequence of responses and creating a global, spatial

59   representation of environmental cues (a cognitive map). In the latter case, the goal is

60   defined by its spatial relationship to a number of different landmarks (usually distal cues

61   that surround an experimental space); such a map is highly adaptive, as the removal of any

62   single landmark does not necessarily disrupt navigation (Shettleworth, 2010). Another

63   possibility might be the use of a view-matching strategy of reorientation, where animals

64   would navigate so as to minimize the difference between the panoramic image of the

65   rewarded site and the panorama perceived from the current location. Recent comparative

66   research on insects and computer modelling suggest that this strategy could be used both by

67   insects and vertebrates (Pecchia & Vallortigara, 2010). According to Tommasi and

68   colleagues (2012), when encoding distance and direction from simple objects, the simplest

69   form of spatial orientation is that involving a goal directly associated with a visual object.

70   When the spatial location of a landmark coincides with the goal, the orientation behavior

71   simply requires that the navigating organism recognizes the landmark in its local space and

72   moves toward it.

73   There are studies conducted in vertebrates that focus on the use of proximal visual

74   cues to guide navigation. For example, Ingle & Sahagian (1973) revealed that goldfish can

75   learn to swim in a constant direction relative to proximal visual cues within a small

76   enclosure; this response was observed even if animals approached the choice point from

77   different directions (Salas et al., 1996). Fish also revealed sensitivity to environmental

78   shape and dimensions in spatial navigation (such as encoding of geometry and size of the

79   experimental space; see e.g., Lee et al., 2013, and Sovrano et al., 2005, 2007). López and

80   colleagues (2000, 2001) revealed that turtles (*Pseudemys scripta*) in a cue guidance

81   procedure solved a task by directly approaching to a single, individual intramaze cue

82   associated with a goal. If we analyze the phenomena, it seems that the behavioral control by

83   a visual cue increases as the absolute spatial proximity between the cue and a goal

84   decreases. There is evidence that animals rely preferentially on closer cues to locate a goal

85   (e.g., the work in digger wasps by Tinbergen & Kruyt, 1938). Spatial contiguity favors cues

86   at the goal or very near the goal as the best predictors of its location in rats (Chamizo &

87   Rodrigo, 2004). The fact that animals with the cue positioned closer to the goal learned the

88   task faster has also been observed in other mammals like dogs (Milgram et al., 1999). This

89   phenomenon seems to be quite general because is also present in invertebrates such as

90   honeybees (Cheng et al., 1987). Furthermore, Spetch (1995) showed in pigeons and

91   humans that the control over the response acquired by a cue at a given distance from the

92   target could be reduced or overshadowed by the presence of another cue closer to the target.

93   The same result was also observed in European jays, a food storing corvid (Bennett, 1993).

94   Chamizo and colleagues (2006) studied the competition between cues in spatial learning by

95   rats, analyzing the role of distance to the goal. The spatial proximity of a cue to the

96   platform (the goal) affected not only how well it could be used to locate the platform (better

97 when it was near), but also its ability to prevent learning about other cues (worse when

98 another cue was introduced to form a new configuration). Similar results were also

99 observed in invertebrates. In the study by Cheng et al. (1987), honeybees, trained to forage

100 at a small source of sucrose placed at a constant location within a particular array of cues,

101 weighed nearer cues more heavily than distant ones.

102 But knowledge about spatial learning in amphibians so far is scarce. This fact is

103 surprising because this is a particularly important group from an evolutionary standpoint,

104 since it lies at the transition between aquatic and terrestrial vertebrates (Macphail, 1982;

105 Muzio, 1999, 2012). Nevertheless, some work has been published demonstrating

106 amphibian spatial learning ability. For example, Adler (1980) showed that the green frog

107 (*Rana calamitans*) is capable of using static visual cues and memorized motor patterns as

108 orientation cues when trained and tested repeatedly in an arena. Lüddecke (2003) has also

109 reported spatial learning in the dendrobatid frog (*Colostethus palmatus*), using a situation

110 where animals had to orient within an arena with 24 different locations. In another study,

111 Sinsch (1987), using a displacement test in the common toad (*Bufo bufo*), established that

112 vision (use of environmental visual cues) was necessary to orient back to the start location.

113 Another work by Dall'Antonia and Sinsch (2001), in the natterjack toad (*Bufo calamita*),

114 revealed that this species' orientation abilities relied on multisensory cues (visual,

115 magnetic, olfactory) in an octagonal arena. Finally, previous series of experiments on

116 spatial learning performed at our lab (Daneri et al., 2011) revealed that the terrestrial toad

117 *Rhinella arenaum* may use a fixed visual cue to solve a spatial challenge on a plus maze. In

118 this case, experimental animals learned to find a container full of water directly cued by a

119 proximal, intra-maze visual cue (beacon) located at the end of a reinforced maze arm. With

120   the exception of these earlier studies, there is no other information about maze learning

121   ability using visual cues in amphibians.

122   In order to determine the ability of amphibians to use local visual cues, and to reveal

123   if there is any effect of cue-proximity to the goal, we conducted three experiments in an

124   open field setting. Experiment 1 studied how toads orient in an arena using visual cues

125   located near a reward, Experiment 2 revealed the effect of different distances between a cue

126   and the goal, and Experiment 3 studied the competition between two cues in relation with

127   their distance to a goal.

128   **Experiment 1: Use of Local Cues**

129   The objective of our first experiment was to test the spatial abilities of the toad

130   *Rhinella arenarum* in a circular arena (an open field) and the use of proximal visual cues

131   (beacons) for spatial orientation.

132   ***Method***

133   ***Animals***. The subjects were eleven experimentally naive adult male toads, *Rhinella*

134   *arenarum* (formerly *Bufo arenarum*), captured in ponds around Bahia Blanca, Argentina.

135   This species is not listed as threatened (IUCN, 2010). Animals were maintained according

136   to the guidelines outlined by the NIH Guide for the Care and Use of Laboratory Animals.

137   Upon their arrival at the laboratory, toads were placed in cages (30 cm long, 21 cm wide

138   and 21 cm high), five to ten animals per cage, where they remained with continually

139   running tap water for at least two weeks. During the first week, subjects were treated with

140   antibiotics and antiparasitics mixed with commercial frog food (approximately 3 g per day

141   per animal). Then, animals were fed only with frog food (once a day during the second

142   week, and after that, once a week). The vivarium was kept at a temperature between 21 and

143   23°C, and under a 16:8 h light:dark cycle (light from 05:00 to 21:00 h). Before the start of

144   the experiment, animals were transferred to individual cages with ad libitum deionized

145   water. The day before pretraining, standard weights (weight of the hydrated animal with its

146   urinary bladder empty; Ruibal, 1962) of all animals were obtained. These weights varied

147   between 50 and 102 g. In order to induce water-search behavior, toads were dehydrated to

148   80% of their standard weights at the beginning of each pretraining and training session (a

149   standard method employed in this species; e.g., see Muzio et al., 1992, 2011).

150   *Apparatus*. The experimental environment was composed as an open field made of

151   opaque white Plexiglas; the floor was made of light brown rubber. The open field was 86

152   cm of diameter and its walls were 75 cm high. On the floor, four positions (goal points)

153   were established (North, West, East, and South). A plastic green water container (13 x 10 x

154   3 cm, L x W x H) was placed in each of the four goal points against the walls. The water

155   containers were filled with deionized water, but accessibility to the water was controlled by

156   adjusting the water level relative to a metallic grid placed into the container that served as

157   substrate (water level reached the surface of the grid or was underneath). This ensured

158   similar moisture level near both reinforced and nonreinforced containers.

159   The maze was surrounded by a white curtain that isolated it from any external visual

160   cues. The experimenter was hidden behind the curtain to observe and visually record the

161   animals' behavior (through a small window). Only one observer recorded the data of all the

162   experiments. The arena and the window positions were randomly rotated between

163   experimental sessions. The open field was cleaned between trials to avoid the use of any

164   uncontrolled intra-maze cues. Training was carried out in an experimental room kept at a

165   constant temperature and humidity (21-23 °C, 48-52% RH) and constant background white

166   noise (20-30,000 Hz).

167   ***Procedure.*** To accustom the animals to obtain water from the plastic green

168   containers in the experimental open field, all the toads received two pretraining sessions of

169   5 minutes (one per day). During these sessions, animals were free to move inside the open

170   field and had access to deionized water in all the plastic containers. Training started on the

171   following day. Each animal received 32 training sessions (one session per day of three trials

172   each).

173   Toads were randomly assigned to one of the following groups: Experimental ($n = 6$)

174   and Control ($n = 6$). Standard weights did not differ significantly between groups ($F(1, 9) =$

175   0.14, $p > 0.5$). One of the animals in the Experimental group died of unknown causes

176   during training (thereby, this group remained with $n = 5$). Toads of the Experimental group

177   were trained under a procedure in which the goal (the plastic container full of accessible

178   deionized water) was surrounded by two visual cues: a red rhombus (10 x 10 cm) on the

179   left (when facing the goal container) and a multicolored horizontally striped square (10 x 10

180   cm) on the right (Figure 1). Both cues were made of rubber sheet and placed on the wall 10

181   cm away and above the edge of the reinforced container. The position of the reinforced

182   container (with the visual cues) was randomly rotated between trials. Containers with

183   unreachable water were placed at the other three goal points. This procedure was designed

184   to prevent the animals from being guided to the correct goal box by a humidity gradient.

185   For animals in the Control group, the reinforced container was randomly located at any of

186   the four goal points with no visual cues associated with it. The use of the proximal visual

187   cues for spatial orientation was necessary to successfully solve the task for the

188   Experimental group.

189   At the beginning of each trial, every toad was covered by a cloth to prevent visual

190   access during the approach to the experimental arena and placed under a start container (12

191   x 12 x 8 cm, L x W x H) in the center of the open field. It remained under the start

192   container for 30 seconds until it was raised by hand, releasing the toad to move freely in the

193   open field and to choose among the four containers. As a consequence of their movements

194   during the 30 s waiting period, animals started each trial haphazardly facing different

195   directions. Furthermore, prior to each trial, toads were placed into the open field from

196   different positions and oriented in different directions in the start container, effectively

197   eliminating path integration or any other source of information to locate the goal other than

198   the two visual cues placed on the wall. Toads were then allowed to search in the open field

199   until one container was chosen (placing at least one limb on it) or for a maximum of 3 min.

200   The choice was recorded as Correct or Incorrect based on whether or not the toad arrived at

201   the container with reachable water. If the animal chose the correct container, 2 minutes of

202   water access was used as reinforcement. In the case of a incorrect choice (a container with

203   unreachable water) the animal was left for one minute on the metallic grid of the container,

204   then it was gently re-located back to the center of the open field and guided by hand to the

205   correct container in order to gain access to the water for 2 minutes (guided trial procedure).

206   Weight variation (g/100 g) was also recorded. Animals were weighed (in grams)

207   before and after each trial to assess the amount of water uptake that occurred during the

208   trial. The difference between these two weights was divided by the standard weight

209   computed before the first pretraining session and multiplied by 100 to provide a relative

210   measure of water uptake corrected for individual differences in body weight, as done in

211   previous studies (e.g., Muzio et al., 1992).

212   When the trial was finished, the toad was removed from the maze and returned to its

213   home cage for a 2-min inter-trial interval. This movement also served to disorient the

214   animals between trials. An acquisition criterion of a minimum of 5/9 correct choices during

215   three consecutive sessions was used (i.e., 55.56 % correct trials in the last three sessions,

216   thus exceeding by more than twice what would be expected by chance). After 24 training

217   sessions the criterion was reached, and an overtraining and testing phase then began (8

218   additional sessions). During this period, animals received four trials per session: three

219   training trials plus one probe trial. Presentation of the probe trials were distributed pseudo-

220   randomly among the training trials (probe trials never occurred on the first or last trial).

221   Probe trials were designed to rule out that the toads were locating the goal using only visual

222   cues emanating from the correct water container; therefore, during these trials, all the

223   containers were in the unreachable water condition (i.e., during probe trials toads were not

224   reinforced). For each probe trial, the container chosen was recorded and the response

225   classified as "Correct" or "Incorrect" with respect to the original position of the reachable

226   container during training trials.

227   Analysis of variance (ANOVA) with repeated measures, followed by pairwise

228   comparisons of groups based on the Least Significant Difference (LSD) Test, was applied

229   for statistical analysis of the data. In all cases, significance was evaluated by setting the

230   alpha value at less than 0.05.

231   ***Results and Discussion***

232   Figure 2 presents the percentage of correct responses per training session for each

233   group. Each data point is the average of the data collected from the animals of a particular

234   group. We conducted an ANOVA with a between-subjects factor (condition) and a within-

235   subjects factor (session number). An overall analysis of the acquisition period showed a

236   significant effect for groups ($F(1, 9) = 66.94$, $p < 0.001$, partial $\eta^2 = 0.88$). In the first

237   session both groups displayed a similar performance ($F(1, 9) = 0.04$, $p > 0.5$), and did not

238 choose any of the four containers more than expected by chance (groups vs. chance F(2,

239 14) = 0.17, p > 0.5). But during the last three acquisition sessions (22, 23 and 24) the

240 performance of Experimental group was significantly better than the one of the Control

241 group ($F(1, 9) = 14.27$, $p < 0.01$, partial $\eta^2 = 0.61$). Animals in the Experimental group

242 usually began the trial wandering around the start area and then facing towards the

243 container of choice. Performance of this group suggests that they learned how to locate the

244 goal, in contrast with the poor learning of the Control group. In addition, during the last

245 three acquisition sessions, toads from the Experimental group also showed a clear

246 preference for the correct quadrant associated with the rewarded container directly cued by

247 the two visual cues (58% of choices; Figure 3A). In contrast, animals of the Control group

248 chose equally across all quadrants.

249   As typically observed (e.g., Muzio et al., 1992), weight variation (measured on a

250 trial-by-trial basis) demonstrated that reward resulted in a significant amount of body

251 weight change that paralleled the toads' better performance across training trials (data not

252 shown). Weight variation means (g/100g) were 1.98 +/- 0.09 for the Experimental group,

253 and 1.47 +/- 0.05 for the Control group.

254   The general performance of both Experimental and Control groups during the

255 overtraining and testing period was not affected by the introduction of the probe trials (i.e.,

256 significant differences between groups were maintained, $F(1, 9) = 32.16$, $p < 0.001$, partial

257 $\eta^2 = 0.78$; and no changes across sessions nor interaction were observed, $ps > 0.5$). These

258 probe trials were applied to determine whether toads solved the task on the basis of a visual

259 cue-guided response. An ANOVA reveals significant differences in the mean correct

260 choices of both groups across test trials ($F(1, 14) = 39.41$, $p < 0.001$, partial $\eta^2 = 0.73$; see

261   Figure 3B). This fact indicates that the visual cues had controlled the toads' behavior in the

262   Experimental group, guiding them toward the goal container.

263   **Experiment 2: Effect of Distance from a Visual Cue to the Goal**

264   In the previous experiment we showed that toads are capable of using visual cues

265   for spatial orientation. Experiment 2 explored the effect of the distance from a visual cue to

266   the goal on learning performance.

267   *Method*

268   ***Animals and apparatus****. Eighteen experimentally naive adult male toads were

269   obtained and maintained as described in the previous experiment. Standard weights varied

270   between 98 and 125 g and were not statistically different across groups (ANOVA, $F(2, 15)$

271   $= 0.09, p > 0.5$). Other conditions of maintenance and apparatus used were the same as in

272   Experiment 1.

273   ***Procedure****. Pretraining was the same as described in Experiment 1. Training started

274   the following day. All animals received a total of 24 training sessions (one session per day,

275   also of three trials each). Toads were randomly assigned to one of three groups: Above ($n =$

276   6), Near 10 ($n = 6$) and Near 30 ($n = 6$). Animals of the Above group had the accessible

277   water container directly cued by a visual cue (beacon) located 10 cm above the container on

278   the wall of the open field. Animals of the Near 10 and Near 30 groups had the cue

279   displaced 10 and 30 cm respectively to the right (when facing the water container; Figure

280   4). The cue used in all cases was a multicolored horizontally striped square (10 x 10 cm)

281   made of rubber (one of the cues used in Experiment 1). For all trials, the cue and associated

282   container was placed at randomly changing goal points relative to the starting location.

283   Similar containers with unreachable water were always placed at the other three goal

284   points. Thus, animals could only solve the task using the visual cue as a reference. Toads

285   could not use the presence of reward as cue because, as in Experiment 1, it was also

286   ensured the constancy of sensory cues from water in the four containers, both reinforced

287   and nonreinforced (see Apparatus and Procedure sections of Experiment 1,

288   reachable/unreachable water). Other procedural details were the same as described in

289   Experiment 1.

290   ***Results and Discussion***

291   Figure 5A shows the percentage of correct responses for each group. An overall

292   analysis of the training period revealed significant effects for group ($F(2, 15) = 22.19$, $p <$

293   0.001, partial $\eta^2 = 0.74$), across sessions ($F(23, 345) = 6.35$, $p < 0.001$, partial $\eta^2 = 0.30$),

294   but no interaction between factors ($F(46, 345) = 0.85$, $p > 0.5$).

295   Weight variation means (g/100g) were also recorded and were 1.38 +/- 0.08 for the

296   Above group, 1.20 +/- 0.07 for the Near 10 group, and 1.32 +/- 0.08 for the Near 30 group.

297   In order to identify the source of the difference in correct choices observed among

298   the groups, the number of sessions necessary to reach the learning criterion was analyzed

299   (Figure 5B). An ANOVA revealed significant differences among the groups ($F(2, 15) =$

300   45.90, $p < 0.001$, partial $\eta^2 = 0.86$); all the three groups required a different number of

301   sessions to reach criterion (LSD test, all comparisons $p < 0.005$). These results reveal that

302   learning rate changes with the distance between the goal and the visual cue: the closer the

303   visual cue to the goal the faster an animal learns to locate the goal (i.e., fewer sessions to

304   reach criterion).

305   **Experiment 3: Predictive Value and Competition between Visual Cues**

306   The fact that animals weighed nearer cues more heavily than distant ones was

307   previously observed in a wide variety of groups of animals (see introduction) but never

308   before in amphibians. Thus, the Experiment 3 was designed in order to test in toads the

309   competition between two visual cues in relation to their distance to the goal, analyzing how

310   the distance to the goal affects the predictive value of each cue.

311   *Method*

312   *Animals and apparatus*. Fourteen experimentally naive adult male toads were

313   obtained and maintained as described in the previous experiment. Standard weights varied

314   between 80 and 130 g and were not statistically different across groups ($F(1, 8) = 0.30$, $p >$

315   0.5). Other conditions of maintenance and the apparatus used were the same as in the

316   previous experiments.

317   *Procedure*. Pretraining was the same as described in Experiment 1. Training started

318   the following day. This training protocol consisted of the same training session procedure

319   as in the two previous experiments (one session per day of three trials each). Training was

320   divided into two periods: acquisition (number of sessions to reach the learning criterion of

321   5/9 correct choices during three consecutive sessions) and overtraining and testing (a phase

322   of 6 additional sessions). Toads were randomly assigned to one of two groups: Near-Near

323   ($n = 7$) and Near-Far ($n = 7$). Animals of the Near–Near group were trained under a

324   procedure in which the container with accessible water was surrounded by two visual cues:

325   a red rhombus (10x 10 cm) on the left and a multicolored horizontally striped square (10 x

326   10 cm) on the right when facing the cues (Figure 6, Left panel). The cues were placed on

327   the wall 10 cm away and up from the edge of the reinforced container (same condition as

328   the Experimental group in Experiment 1). The position of the cues was inverted (i.e.,

329   rhombus on the right and square on the left) for three animals of this group for control

330   purposes. Animals of the Near-Far group had one of the cues also positioned at 10 cm

331   distant from the reinforced container (near cue) on the left, but the other one (far cue) was

332   located at the same height but between the opposite container and the container to the right

333   (Figure 6, Right panel). In this case, the near cue was the rhombus for four animals and the

334   square for three of them for control purposes.

335   As the goal container was placed at a randomly changing goal point relative to the

336   starting point, for each trial the only way to reach the reinforced container was by using the

337   visual cues as a reference. Other procedural details were the same as described in

338   Experiment 1. Once the acquisition criterion was reached, the overtraining and testing

339   phase began. The probe trials were intended to determine the predictive value of each

340   visual cue according to its position relative to the goal. Tree types of probe trials were

341   presented, two times each: 1) with both cues present, 2) with the cue on the right present

342   and 3) with the cue on the left present. Water reward was removed during the probe trials

343   (toads were not reinforced). Depending on the container chosen, the response was classified

344   as either "Correct" or "Incorrect".

345   The percentage of correct responses per training session and body weight variation

346   were recorded during acquisition. Unfortunately, for technical reasons, the weight variation

347   data from four animals were lost. As such, the weight analysis only includes five animals in

348   each group. During the test trials weight variation was not recorded as the animals did not

349   have access to water.

350   ***Results and Discussion***

351   Figure 7A shows the percentage of correct responses during training sessions for

352   each group. A global analysis with a repeated-measures ANOVA for acquisition revealed

353   significant differences between groups ($F(1,12) = 9.19$, $p < 0.05$, partial $\eta^2 = 0.43$) and

354   across sessions ($F(15, 180) = 2.64$, $p < 0.005$, partial $\eta^2 = 0.18$), but no interaction effect (p

355   > 0.5). This result suggests that animals in both groups learned the location of the goal, but

356   with some differences in their performance. The two groups reached the acquisition

357   criterion at different times: the Near-Near group took an average of 16 sessions to reach

358   criterion, while Near-Far group took 18 sessions. In order to determine the source of this

359   between group difference during the acquisition period, learning rate (number of sessions to

360   reach criterion) was analyzed (Figure 7B). An ANOVA revealed significant differences

361   ($F(1, 12) = 17.45$, $p < 0.005$, partial $\eta^2 = 0.59$), suggesting that the Near-Near group needed

362   statistically fewer training sessions to locate the goal than the Near-Far group (16 sessions

363   versus 18 sessions, respectively). According to these results, the cues presented to the

364   animals in the Near-Near group seem to be more effective in learning to locate the goal,

365   allowing animals to learn the task and orient faster (i.e., these toads took fewer sessions to

366   reach criterion).

367   Means of weight variation (g/100g) were also recorded and were 1.16 +/- 0.06 for

368   Near-Near group, and 1.15 +/- 0.06 for Near-Far group.

369   During the overtraining period the presentation of the probe trials did not affect

370   performance in either group (group, session and interaction factors, all *ps>0.5*). By

371   analyzing data from the different testing conditions, we established the predictive value of

372   each cue (Figure 8). An ANOVA revealed nonsignificant differences between the groups'

373   performance in each testing condition with two cues ($F(1, 12) = 0.16$, $p > 0.5$) or the cue

374   available on the left -the near one for both groups- ($F(1, 12) = 1.09$, $p > 0.1$). When the test

375   involved the cue on the right –in this case, near for one group and far for the other-

376   significant differences appeared ($F(1, 12) = 8.33$, $p < 0.05$, partial $\eta^2 = 0.41$). The results

377   suggest that both cues seemed to be equally salient for animals of the Near-Near group;

378   they easily oriented using either of them alone. On the other hand, the cue on the right (far

379   from the container) seemed to provide very little predictive value for animals of the Near-

380   Far group. Hence, when the right cue was only available, animals found it difficult to locate

381   the goal (in the latter case, their choice distribution did not differ from chance; groups vs.

382   chance $F(1, 12) = 6.25, p > 0.1$).

383   **General Discussion**

384   The goal of this study was to investigate cue-based navigation in an amphibian, the

385   terrestrial toad *Rhinella arenarum*, under laboratory conditions and provide novel

386   information for comparisons with findings in other groups of animals. In our series of three

387   experiments we proved that an open field with intramaze visual cues can be used to test the

388   importance of different visual cues for orientation by the toad *Rhinella arenarum*.

389   Results of Experiment 1 reveal that toads can use proximal intramaze visual cues as

390   beacons to orient in space in an open field situation. The association between the visual

391   cues and the position of the reinforced container could be easily established by toads in the

392   Experimental group after 24 training sessions. This learning strategy enabled toads to

393   maximize water gain during exposure to the reinforcement.

394   From the results of Experiment 2 it may be concluded that one visual cue

395   (independent of its distance to the goal -above, 10 or 30 cm away-) was sufficient to orient

396   toward the goal. However, a graded effect of distance was observed during acquisition.

397   Increasing distance with respect to the goal negatively affected the rate of acquisition; thus,

398   the number of sessions necessary to reach the learning criterion was larger. As previously

399   observed in mammals (Chamizo & Rodrigo, 2004), in the absence of any other relevant or

400   causally related event, learning was possible even at relatively long distances between the

401   cue and the goal. In addition, this experiment reveals for the first time that this phenomenon

402   is also present in amphibians.

403   Finally, the results of Experiment 3 accord with those expected on the basis of

404   previous observations in mammals, birds, fish and invertebrates (see introduction): in

405   amphibians increasing the distance from visual cues to the goal also diminishes their

406   predictive value. This fact suggests that cues closer to the goal are considered more reliable

407   for spatial orientation and "overshadow" more distant cues.

408   In this sense, our present results in toads confirm that proximity to the goal is

409   determinant to establish cue relevance during spatial orientation, as predicted by the

410   Rescorla and Wagner model (1972). Moreover, the terrestrial toad *Rhinella arenarum* relies

411   mainly on proximal visual cues when orienting toward a water source in an open field

412   situation. Thus, our data in toads contribute not only to understanding how amphibians

413   process spatial information to locate a goal, but also shed light on this understudied

414   vertebrate class, the first to adapt to terrestrial life. Overall, our current results in

415   amphibians are in agreement with other data obtained from several non-mammal

416   vertebrates, e.g., fish and reptiles, suggesting that the ability to learn and rely on visual cues

417   to orient toward and reach a goal has an ancient evolutionary origin.

418   **References**

419   Adler, K. (1980). Individuality in the use of orientation cues by green frogs. *Animal*

420   *Behaviour, 28*, 413-425. http://dx.doi.org/10.1016/s0003-3472(80)80050-9

421   Barnard, C. (2004). *Animal Behaviour. Mechanism, Development, Function and Evolution*.

422   Pearson Prentice Hall.

423   Bennett, A. T. D. (1993). Spatial memory in a food storing corvid. *Journal of Comparative*

424   *Physiology A*, *173*, 193-207. http://dx.doi.org/10.1007/bf00192978

425   Chamizo, V. D. & Rodrigo, T. (2004). Effect of absolute spatial proximity between a

426   landmark and a goal. *Learning and Motivation*, *35,* 102-114.

427   http://dx.doi.org/10.1016/s0023-9690(03)00059-6

428   Chamizo, V. D., Manteiga, R. D. Rodrigo, T., & Mackintosh, N. J. (2006). Competition

429   between landmarks in spatial learning: The role of proximity to the goal. *Behavioural*

430   *Processes*, *71*, 59-65. http://dx.doi.org/10.1016/j.beproc.2005.11.003

431   Cheng, K., Collett, T. S., Pickhard, A., & Wehner, R. (1987). The use of visual landmark

432   by honeybees: Bees weight landmarks according to their distance from the goal.

433   *Journal of Comparative Physiology A*, *161*, 469-475.

434   http://dx.doi.org/10.1007/bf00603972

435   Dall'Antonia, P. & Sinsch, U. (2001). In search of water: orientation behaviour of

436   dehydrated natterjack toads, *Bufo calamita. Animal Behaviour*, *61*, 617-629.

437   http://dx.doi.org/10.1006/anbe.2000.1627

438   Daneri, M. F., Casanave, E. B. & Muzio, R. N. (2011). Control of spatial orientation in

439   terrestrial toads (*Rhinella arenarum*). *Journal of Comparative Psychology*.

440   http://dx.doi.org/10.1037/a0024242.

441   Ingle, D. & Sahagian, D. (1973). Solution of a spatial constancy problem by goldfish.

442   *Physiological Psychology*, *1*, 83-84. http://dx.doi.org/10.3758/bf03326873

443   IUCN (2010) IUCN Red List of Threatened Species. Version 2010.4.

444   http://dx.doi.org/10.1080/14888386.2007.9712825

445   Lee, S. A., Vallortigara, G., Flore, M., Spelke, E. S. & Sovrano, V. A.. (2013). Navigation

446   by environmental geometry: the use of zebrafish as a model. *The Journal of*

447   *Experimental Biology, 216,* 3693-3699. http://dx.doi.org/10.1242/jeb.088625

448   López, J. C., Rodríguez, F., Gómez,Y., Vargas, J.P., Broglio, C. & Salas, C. (2000). Place

449   and cue learning in turtles. *Animal Learning and Behavior, 28*, 360-372.

450   http://dx.doi.org/10.3758/bf03200270

451   López, J. C., Gómez, Y., Rodríguez, F., Broglio, C., Vargas, J.P. & Salas,C. (2001). Spatial

452   learning in turtles. *Animal Cognition, 4*, 49-59.

453   http://dx.doi.org/10.1007/s100710100091

454   Lüddecke, H. (2003). Space use, cave choice and spatial learning in the dendrobatid frog

455   *Colostethus palmatus*. *Amphibia-Reptilia, 24*, 37-46.

456   http://dx.doi.org/10.1163/156853803763806920

457   Macphail, E. M. (1982). *Brain and intelligence in vertebrates*. Oxford University Press.

458   http://dx.doi.org/10.2307/1422556

459   Milgram, N., Adams, B., Callahan, H., Head, E., Mackay, B., Thirlwell, C. & Cotman, C.

460   (1999). Landmark Discrimination Learning in the Dog. *Learning & Memory*, *6*, 54-

461   61.

462   Muzio, R. N. (1999). Aprendizaje instrumental en anfibios. *Revista Latinoamericana de*

463   *Psicología*, *31*, 35-47.

464 Muzio, R. N. (2012). Aprendizaje en anfibios, el eslabón perdido: Un modelo simple

465   cerebral en el estudio de conductas complejas. *Cuadernos de Herpetología, 27(2),*

466   Online.

467 Muzio, R. N., Pistone Creydt, V., Iurman, M., Rinaldi, M. A., Sirani, B. & Papini, M. R.

468   (2011). Incentive or habit learning in amphibians? *PLoS ONE, 6(11),* e25798.

469   doi:10.1371/journal.pone.0025798

470 Muzio, R. N., Segura, E. T., Papini, M. R. (1992). Effect of schedule and magnitude of

471   reinforcement on instrumental acquisition and extinction in the toad, *Bufo arenarum.*

472   *Learning and Motivation*, *23,* 406-429. http://dx.doi.org/10.1016/0023-

473   9690(92)90004-6

474 NIH Guide for the Care and Use of Laboratory Animals. Eighth Edition. 2011. National

475   Academies Press Ed.

476 Pecchia, T. & Vallortigara, G. (2010). View-based strategy for reorientation by geometry.

477   *The Journal of Experimental Biology, 213*, 2987-2996.

478   http://dx.doi.org/10.1242/jeb.043315

479 Rescorla, R. A. & Wagner, A.R. (1972). A theory of Pavlovian conditioning: variations in

480   the effectiveness of reinforcement and nonreinforcement. In A. H. Black and W. F.

481   Prokasy (Eds.), *Classical Conditioning II: Current Research and Theory* (pp. 64-99).

482   New York: Appleton Century Crofts.

483 Ruibal, R. (1962). The adaptive value of bladder water in the toad, *Bufo cognatus*.

484   *Physiological Zoology*, *35*, 218-223.

485 Salas, C., Rodríguez, F., Vargas, J. P., Durán, E., & Torres, B. (1996). Spatial learning and

486   memory deficits alter telencephalic ablation in goldfish trained in place and turn maze

487   procedures. *Behavioral Neuroscience, 110*, 965-980. http://dx.doi.org/10.1037//0735-

488   7044.110.5.965

489   Shettleworth, S. J. (2010). *Cognition*, *Evolution, and Behavior*. Second Edition. New York:

490   Oxford University Press.

491   Sinsch, U. (1987). Orientation behavior of toads (*Bufo bufo*) displaced from the breeding

492   site. *Journal of Comparative Physiology A*, *161*, 715-727.

493   http://dx.doi.org/10.1007/bf00605013

494   Sovrano, V. A., Bisazza, A. & Vallortigara, G. (2005). Animals' use of landmarks and

495   metric information to reorient: effects of the size of the experimental space.

496   *Cognition, 97*, 121-133.

497   Sovrano, V. A., Bisazza, A. & Vallortigara, G. (2007). How fish do geometry in large and

498   in small spaces. *Animal Cognition, 10*, 159-168.

499   Spetch, M. L. (1995). Overshadowing in landmark learning: Touch-screen studies with

500   pigeons and humans. *Journal of Experimental Psychology: Animal Behavior*

501   *Processes*, *21*, 166–181. http://dx.doi.org/10.1037//0097-7403.21.2.166

502   Tinbergen, N., & Kruyt, W. (1938). Über die Orientierung des Bienenwolfes (philanthus

503   triangulum Fabr.). III. Die Bevorzugung bestimmter Wegmarken. *Zeitschrift für*

504   *vergleichende Physiologie*, *25*, 292-334.

505   Tommasi, L., Chiandetti, C., Pecchia, T., Sovrano, V. A. & Vallortigara, G. (2012). From

506   natural geometry to spatial cognition. *Neuroscience and Biobehavioral Reviews, 36*,

507   799–824. http://dx.doi.org/10.1016/j.neubiorev.2011.12.007

508

509 **Footnote to title page**

516   Correspondence concerning this article should be addressed to Dr. Rubén N. Muzio,

517 Grupo de Aprendizaje y Cognición Comparada. Laboratorio de Biología del

518 Comportamiento, Instituto de Biología y Medicina Experimental (IBYME-CONICET).

519 Vuelta de Obligado 2490, CP 1428 - Ciudad de Buenos Aires, ARGENTINA. E-mail:

520 rnmuzio@gmail.com

521

Figure Legends

522

523   *Figure 1*. Diagram of the open field environment used, showing the spatial relationship

524   among the four plastic green water containers (placed North, West, East, and South), only

525   one of which (light blue) would be accessible to deionized water as reward. Toad drawing

526   indicates the central start position used at the beginning of each trial. Schematic is shown

527   for the Experimental group condition of Experiment 1, with the two visual cues on the

528   walls (a red rhombus on the left and a multicolored horizontally striped square on the right

529   when facing the cues and the reinforced water container). The actual reinforced water

530   container position (with the visual cues) was randomly rotated across trials. For the Control

531   group there were no visual cues on the walls.

532

533   *Figure 2*. Percentage of correct responses during acquisition (24 sessions), and overtraining

534   and testing (8 sessions) in the Experimental (n = 6) and Control (n = 6) groups of

535   Experiment 1. Means and confidence intervals (alpha = 0.05) are plotted.

536

537   *Figure 3.* **Panel A**: Distribution of percentage of correct quadrant choices across the last

538   three acquisition trials (i.e., when animals had reached criterion) for the Experimental and

539   Control groups of Experiment 1. Quadrants were differentiated as Correct (containing the

540   rewarded container), Right, Left and Opposite (according to relative position with respect

541   to the correct container quadrant). **Panel B**: Mean percentage of correct choices across test

542   trials (8 tests) for both groups. Means and confidence intervals (alpha = 0.05) are plotted.

543

544   *Figure 4.* Spatial relationship between the goal (rewarded container) and the cue (a

545   multicolored horizontally striped square) for the three groups of Experiment 2. Above

546 group had the goal directly cued 10 cm above it. Near 10 and Near 30 groups had their cues

547 displaced 10 and 30 cm, respectively, to the right.

548

549 *Figure 5*. **Panel A**: Percentage of correct responses during acquisition (24 sessions) for the

550 Above (n = 6), Near 10 (n = 6) and Near 30 (n = 6) groups of Experiment 2. **Panel B**:

551 Number of sessions to reach the learning criterion for each of the three groups. Means and

552 confidence intervals (alpha = 0.05) are plotted.

553

554 *Figure 6*. Schematic of the open field environment used for the toads of Experiment 3,

555 showing the spatial relationship among the four plastic green water containers (placed

556 North, West, East, and South), with one (light blue) containing accessible deionized water

557 as reward, and the two possible positions of the visual cues on the walls. Group Near-Near

558 had the two cues (a multicolored horizontally striped square on the left and a red rhombus

559 on the right) relatively near to the reinforced water container (Left panel). Group Near-Far

560 had one of the two cues (the multicolored horizontally striped square back on the left)

561 relatively farther away from the goal, and the other cue (the red rhombus on the right)

562 relatively near to the goal (Right panel). Toad drawing indicates the central start position

563 used at the beginning of each trial.

564

565 *Figure 7*. **Panel A**: Percentage of correct responses during acquisition (16 and 18 sessions),

566 and overtraining and testing (6 sessions) for the Near-Near (n = 7) and Near-Far (n = 7)

567 groups of Experiment 3. **Panel B**: Number of sessions to reach the learning criterion for

568 both groups of Experiment 3. Means and confidence intervals (alpha = 0.05) are plotted.

569

570   *Figure 8.* Percentage of correct choices during test trials (6 tests, 2 each for the 3 test

571   conditions for all animals) for the Near-Near and Near-Far groups of Experiment 3. Means

572   and confidence intervals (alpha = 0.05) are plotted.
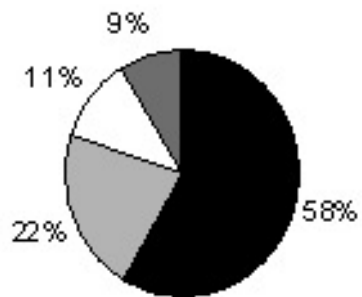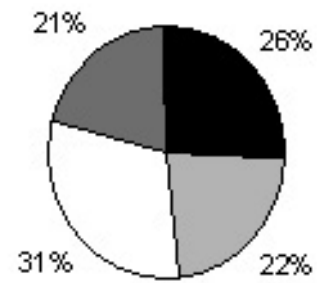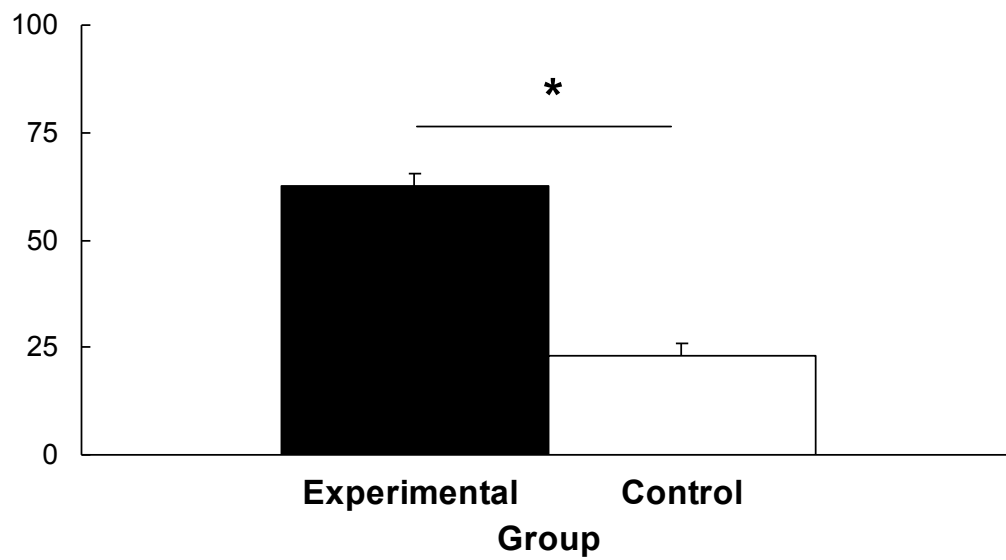
573

574   **Figure 1**
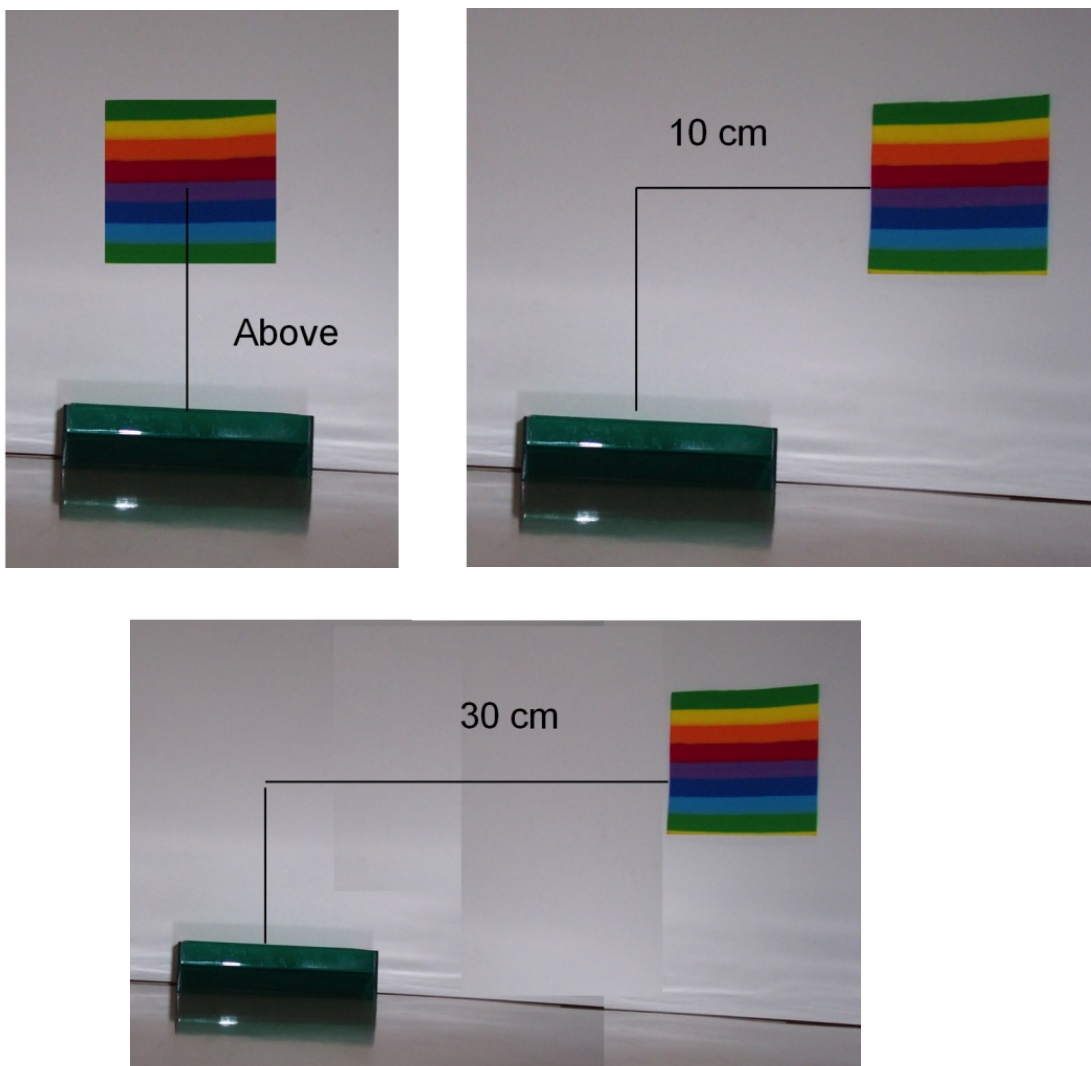
575
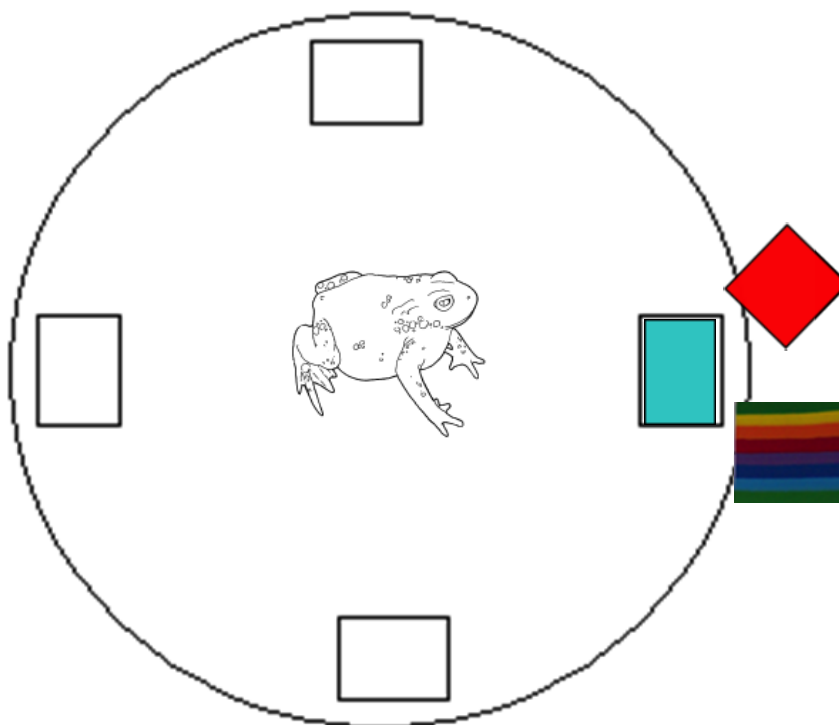
Experimental Group

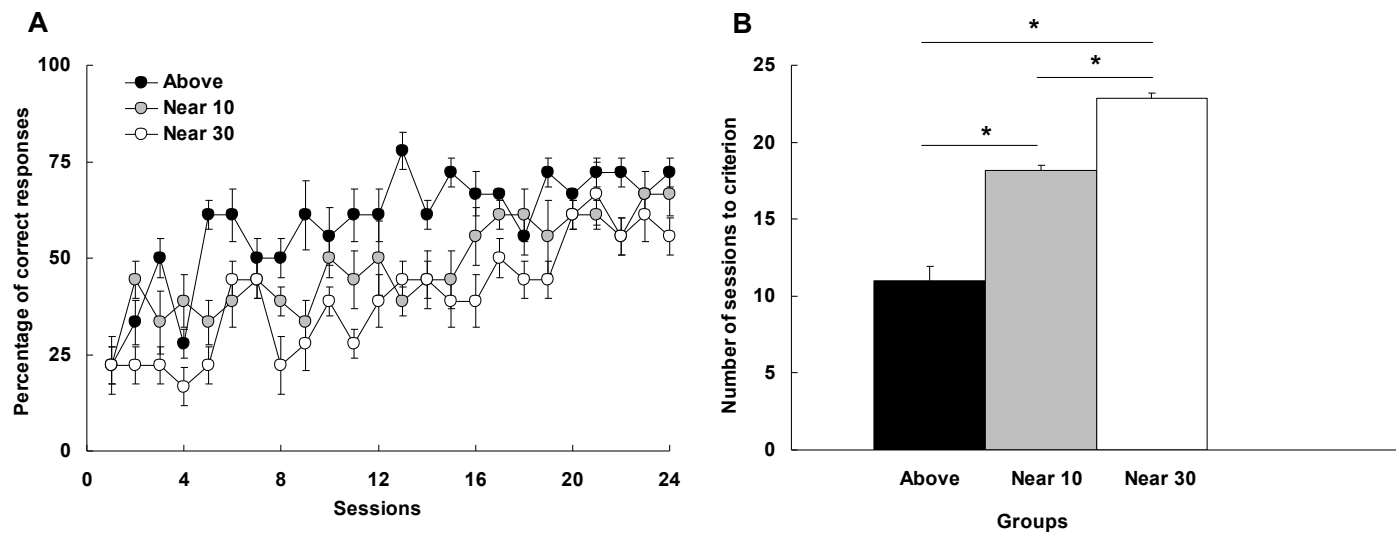576   **Figure 2**

577
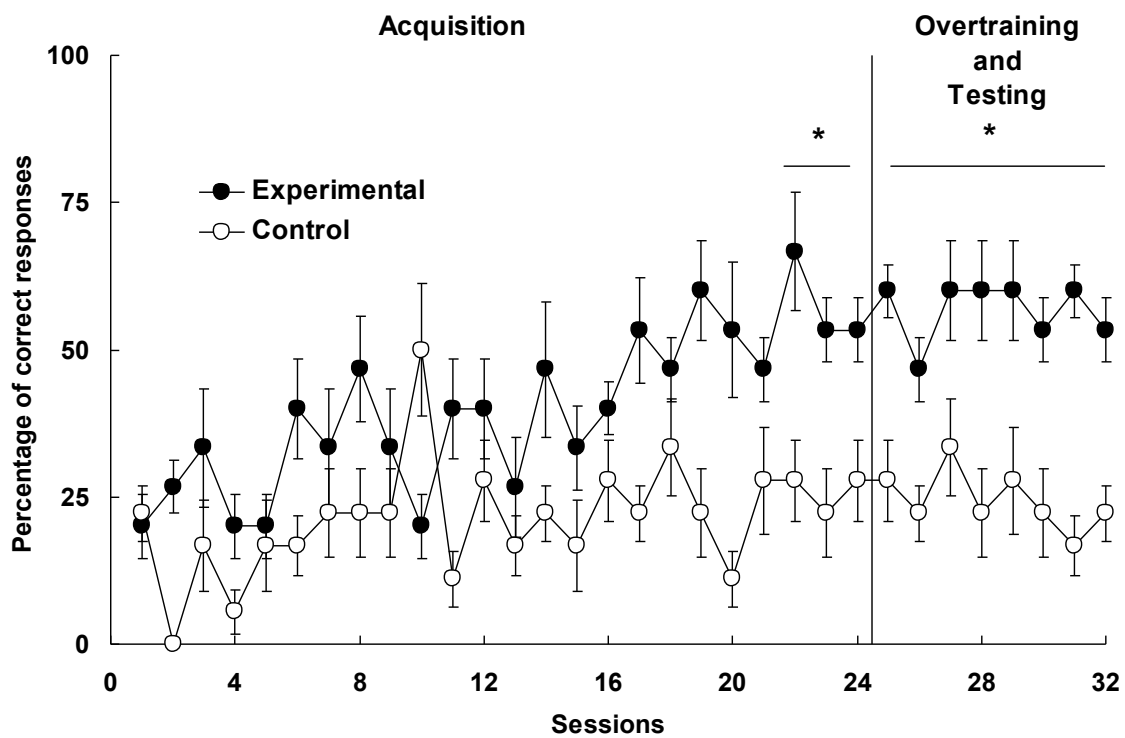
A

Experimental Group

Control Group

9%
11%
22%
58%

■Corect
■Right
□Opposite
■Let

21%
26%
31%
22%

B

Percentage of correct choices

100

75

*

50

25

0

Experimental   Control
Group

580   **Figure 4**
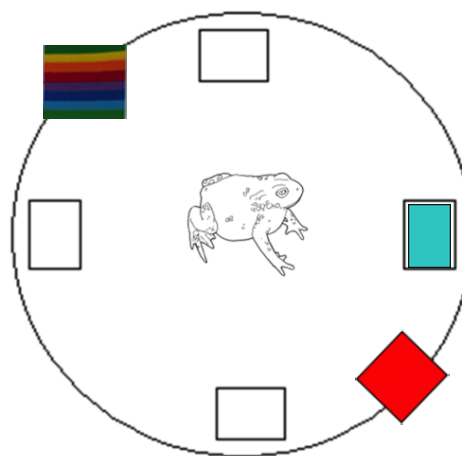
581

Above

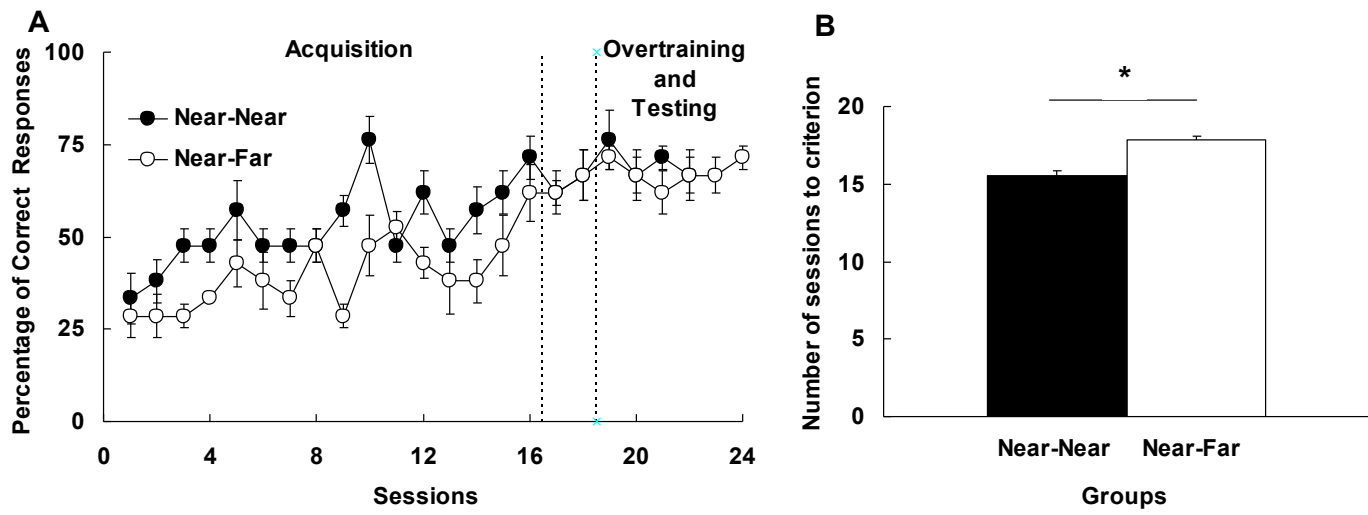10 cm

30 cm

582   **Figure 5**

583

584   **Figure 6**

585

Near - Near   **Near - Far**

586 **Figure 7**
587

588   **Figure 8**

589