

1 **Born to be asocial: newly-hatched tortoises spontaneously** 2 **avoid unfamiliar individuals**

3
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12 **ABSTRACT**

13 Individual recognition is important for modulating social interactions but it is not clear to what extent
14 this ability depends on experience gained through repeated interactions with different individuals. In
15 wild tortoises, evidence of social interactions is limited to behaviours performed years after hatching,
16 in the context of mating. To investigate the presence of abilities of individual recognition at the onset
17 of life in tortoises, we used hatchlings of two species (*Testudo marginata*, *Testudo graeca*) reared
18 with a single conspecific as unique social experience. When located in a novel environment together
19 with the familiar conspecific, tortoises reached the average distance expected by random trajectories.
20 On the contrary, tortoises tested with an unfamiliar conspecific first explored the mate, then actively
21 kept a distance significantly larger than expected by chance. These results show spontaneous abilities
22 of individual recognition in a non-social species at the onset of life, and active avoidance of unfamiliar
23 conspecifics. We suggest that this predisposed behaviour might be adaptive for young tortoises'
24 dispersal.

25

26 **Keywords:** individual recognition, tortoises, predispositions, social behaviour, hatchlings

27

28 **BACKGROUND**

29 Individual recognition requires to identify a specific organism according to its distinctive features.
30 This capacity is important to social responses in long-term social contexts [reviewed in ,1,2]:
31 individual recognition of mate and kin can induce a closer relationship to familiar individuals, in
32 neighbour-stranger discrimination it modifies responsiveness and aggression towards neighbours
33 compared to strangers, in dominance hierarchies triggers differential responses depending on the
34 relationship with the identified individual. Precocial avian species provide evidence of individual
35 recognition at the onset of life, since newly-hatched birds are adapted to recognize and follow social
36 partners after a brief exposure, through the mechanism of filial imprinting [3–6]. It is not clear
37 whether, beside filial imprinting, individual recognition is available at the onset of life, and whether
38 this ability is present in species with limited social habits.

39 We addressed these issues investigating newly-hatched tortoises, precocious animals that are known as
40 non-social. In fact, tortoises do not exhibit post-hatching parental care, mate promiscuously and do not
41 form pair bonds or cohesive social groups [7,8]. In wild tortoises, evidence of social interactions is
42 limited to behaviours that are performed when sexual maturity is reached, years after hatching, such
43 as courtship, mounting and nesting [see for instance ,9–12]. In captivity, adult tortoises housed
44 together with conspecifics show a capacity to follow the gaze of conspecifics [13] and to learn from
45 the actions of other individuals [14], suggesting that these animals possess capacities to deal with
46 social partners. It is not clear, though, whether these abilities emerge in captivity as a result of repeated
47 interactions or constitute the spontaneous behavioural repertoire of tortoises. Moreover, it is not clear
48 whether tortoises are capable of individual recognition and if this capacity is present at the onset of life.

49 To investigate the presence of spontaneous abilities of individual recognition, we used hatchlings of
50 two tortoise species (*Testudo marginata*, *Testudo graeca*). After hatching in individual
51 compartments, we raised young tortoises with a single conspecific as unique social experience, before
52 testing them in a novel environment together with a familiar tortoise (familiar condition) or an
53 unfamiliar tortoise (stranger condition). We expected to observe different responses to familiar vs.
54 unfamiliar individuals only if tortoises were capable of individual recognition. We observed a
55 different behaviour in familiar and stranger pairs, showing capabilities of spontaneous individual
56 recognition at the onset of life in these species. By using as experimental setup a circumscribed arena,
57 we were able to compare average pairwise distance with that expected with random trajectories and
58 to evaluate whether tortoises stayed closer or further than expected by chance. While familiar
59 tortoises progressively reached the chance distance, unfamiliar tortoises initially explored the
60 unfamiliar mate, then actively kept a distance larger than expected by chance.

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63 METHODS

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65 Subjects and rearing conditions

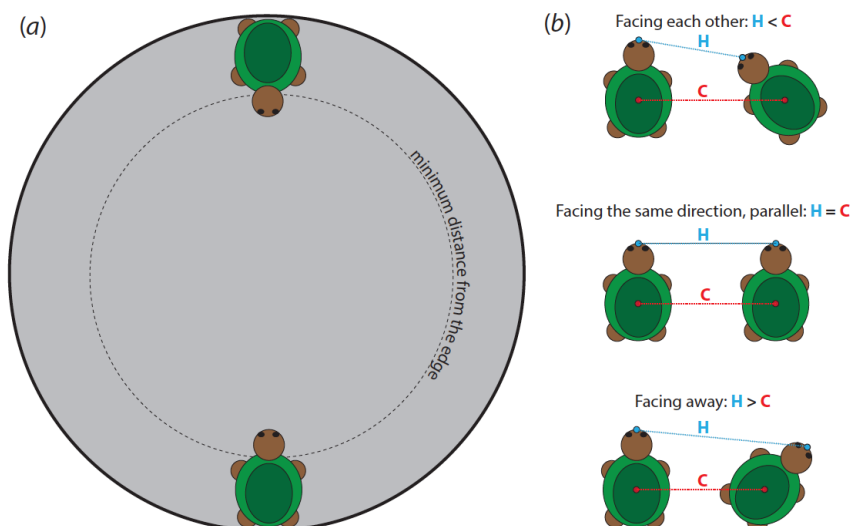
66 We observed 26 newly hatched tortoises of two species: 14 *Testudo graeca* and 12 *Testudo marginata*
67 individuals. Tortoises were about one-month old (24-43 days of age, average 27 days) at the moment
68 of the test. Eggs laid on the ground by tortoises were collected by the experimenters and incubated in
69 darkness at 31 °C +/-2 °C. Tortoises hatched in individual compartments and did not see any
70 conspecific for about 10 days (2-20 days, average 10 days) before being paired with a tortoise of the
71 same species. Tortoises were fed with green leaves and hydrated at least twice daily. Each pair was
72 housed in a square-shaped arena (20x20x12 cm) with the bottom covered with soil, leaves and straw.
73 Before the test, subjects did not see any other tortoise and did not interact with the experimenters.

74

75 Experimental apparatus

76 As experimental apparatus, we used a circular arena (∅ 25 cm, 10 cm high, Fig. 1a) with the bottom
77 covered with wet sand (0.5 cm). A Windows LifeCam© camera located on top of the centre of the
78 arena recorded tortoises' behaviour.

79



80

81 Figure 1.

82 (a) Schematic representation of the experimental apparatus with a pair located in the starting position. (b)
83 Pairwise distance between heads (H) and carapaces (C). When $H < C$ tortoises face each other, when $H = C$
84 tortoises are parallel, facing the same direction, when $H > C$ tortoises face away from each other.

85

86 **Procedure**

87 We first familiarized tortoises with a mate by keeping a pair in the same enclosure for about 22 days
88 (17-33 days, average 22.5 days). Tortoises were then tested in pairs of the same species. Each tortoise
89 was tested once or twice. The list of experimental pairs is shown in Table 1.

90 Before the beginning of the experimental session, we regulated the external temperature of the
91 subjects under a light, to make sure the temperature measured on the top of the carapace differed less
92 than 2 °C between tested individuals. We measured the carapace temperature with an infrared
93 thermometer. Immediately before the test, each individual was isolated for 5 minutes in an opaque
94 box. Subsequently, the experimental subjects were located in front of each other (a familiar or a
95 stranger tortoise, according to the experimental condition), facing the centre of the arena, at
96 diametrically opposed positions (the furthest possible distance within the arena). The behaviour was
97 recorded for 15 minutes from the moment in which one tortoise of the tested pair moved the first step.
98 We defined as the first step a movement of at least one leg that displaced the carapace. If both tortoises
99 did not move for more than 10 minutes, the session was aborted and repeated the subsequent day. To
100 score the behaviour of tortoises, we extracted one frame every 20 seconds (3 frames per minute) and
101 used ImageJ [15] to identify the location of the centre of the carapace and the tip of the head of each
102 tortoise in all frames.

103 For each pair we calculated, for 5 consecutive periods of three minutes (15 minutes overall), the
104 distance between the centroids of the carapaces (C), the distance between the tip of the heads (H) in
105 centimetres and the difference between these measures (H-C). The distance between centroids of the
106 carapaces provided and index of proximity irrespective of the relative orientation of the tortoises. The
107 difference between the distance of the centroids and the distance of the heads provided an index of
108 the relative orientation of the subjects: negative values indicate that tortoises are facing each other,
109 positive values that tortoises are facing away from each other (see Figure 1b).

110
111

Species	Condition	Pair
<i>T. graeca</i>	Familiar	15-16
<i>T. graeca</i>	Familiar	17-21
<i>T. graeca</i>	Stranger	11-13
<i>T. graeca</i>	Stranger	12-14
<i>T. graeca</i>	Stranger	23-31
<i>T. graeca</i>	Stranger	25-42
<i>T. graeca</i>	Stranger	37-24
<i>T. marginata</i>	Familiar	22-28
<i>T. marginata</i>	Familiar	3-4
<i>T. marginata</i>	Familiar	5-6
<i>T. marginata</i>	Familiar	7-8
<i>T. marginata</i>	Stranger	1-10
<i>T. marginata</i>	Stranger	9-2
<i>T. graeca</i>	Familiar	11-12
<i>T. graeca</i>	Familiar	13-14
<i>T. graeca</i>	Familiar	23-24
<i>T. graeca</i>	Familiar	25-31
<i>T. graeca</i>	Familiar	37-42
<i>T. graeca</i>	Stranger	16-17
<i>T. marginata</i>	Familiar	1-2
<i>T. marginata</i>	Familiar	9-10
<i>T. marginata</i>	Stranger	3-6
<i>T. marginata</i>	Stranger	5-8
<i>T. marginata</i>	Stranger	7-4

112

113 Table 1. List of experimental pairs by Species and Condition.

114

115 **Identification of the distance expected with random trajectories**

116 To calculate the chance pairwise distance between tortoise centroids (carapace centroid) and between
117 tortoise heads (tip of the head) we implemented a Fermi-like estimation method. Tortoises were
118 simulated as circles with radius 1.82 cm with a circular head of radius 0.5 cm. To obtain the chance
119 carapace distance, we simulated the random positions of 2.5×10^7 pairs of tortoises uniformly
120 distributed within an arena of radius 12.5 cm (the same size used in the experiments), and calculated
121 their Euclidean pairwise distances. For the chance head distance, we first simulated the position of
122 the carapace centroid, then simulated a random orientation of the head across 360° around the
123 centroid, and finally moved 1 cm away from the edge of the carapace. To avoid overlaps between
124 individuals, we dropped pairs closer than twice the radius of an average tortoise (carapace). To
125 exclude overlaps with the edge of the arena, we dropped positions of individuals closer to the edge
126 than the radius of the tortoise.

127 We repeated the simulation 20 times and obtained a chance carapace distance of 8.84 cm and a chance
128 head distance that converged to 8.91 cm. All simulations were run with a custom MATLAB code.
129

130

131 **Data analysis**

132 We investigated the relationship between the dependent variables (Overall distance run, Distance
133 between centroids, Distance between heads) and the independent variables Condition (familiar vs.
134 stranger), Species (*T. marginata*, *T. graeca*) and Time (1-3, 4-6, 7-9, 10-12, 13-15 minute) as fixed
135 effects and Pair as random effect. Initially, we included the full set of explanatory variables and
136 interactions and progressively found the minimum adequate model using F-tests for models fitted
137 using maximum likelihood. For the variables Distance between centroids and Distance between
138 heads, that showed a significant interaction between Condition and Time, we run post-hoc analyses
139 of variance for the first and second half of the test separately. We used one sample t-tests against the
140 chance level obtained in the simulation to check for significant departures of the distance between
141 centroids and of the distance between heads, independent t-test to compare distance between
142 conditions at specific time points and paired t-test to compare distance within condition at two time
143 points. Significance was set at $p < 0.05$. Analysis were conducted with R (version 3.2.1), for model-
144 fitting we used the nlme package.
145

146

146 **Ethics**

147 All experiments comply with the current Italian and European Community laws for the ethical
148 treatment of animals. The experimental procedures were approved by the Ethical Committee of the
149 Fondazione Museo Civico Rovereto (Italy).
150

151

152 **RESULTS**

153

154 **Distance between carapace centroids**

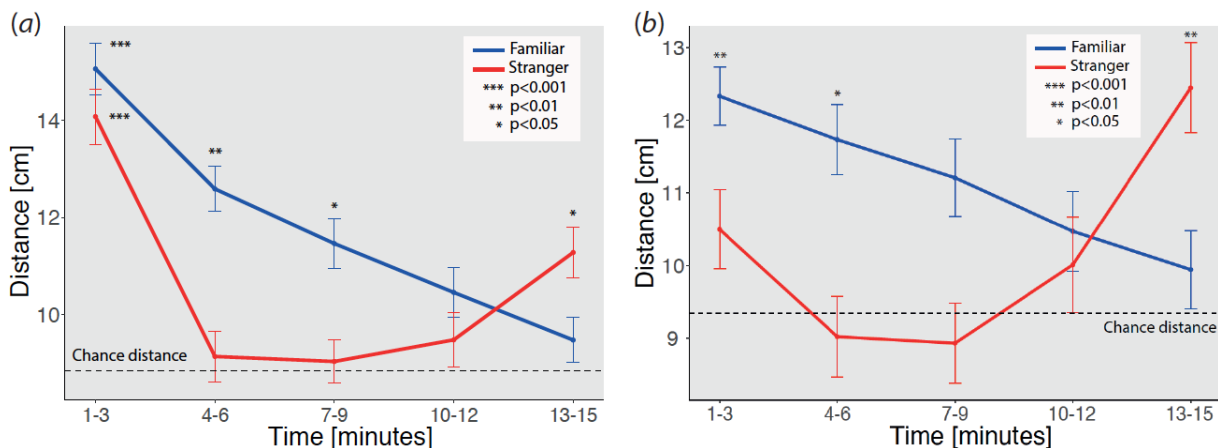
155 Distance between carapace centroids of the tortoises was analysed with Condition, Species and Time
156 as fixed effects and Pair as random effect, using a linear mixed effect model fitted using maximum
157 likelihood. The minimum adequate model included Condition, Time and their interaction: Condition
158 ($F_{1,22}=0.444$, $p=0.512$), Time ($F_{4,88}=7.694$, $p<0.0001$), Condition x Time ($F_{4,88}=3.003$, $p=0.023$), see
159 Figure 2A.

160 As post-hoc tests, we run one analysis of variance for the first and one analysis of variance for the
161 last half of the experiment, using Condition as between factor and Time as within factor. In the first
162 part of the experiment there was no significant main effect of Condition ($F_{1,22}=0.411$, $p=0.528$) a
163 significant main effect of Time ($F_{2,44}=7.50$, $p=0.002$) and no significant interaction ($F_{2,44}=1.559$,
164 $p=0.222$). In the second half of the experiment there was no significant main effect (Condition
165 $F_{1,22}=2.967$, $p=0.100$; Time $F_{2,44}=1.730$, $p=0.189$) but a significant interaction Condition x Time

166 ($F_{2,44}=3.613$, $p=0.035$). As reported in Table 2, while in the familiar condition tortoises progressively
 167 approached the chance level distance, in the stranger condition tortoises initially approached the other
 168 subject and then moved significantly further than the chance distance. In all time points and
 169 conditions, carapace pairs were significantly closer than the average starting distance (20.74 cm).
 170

Condition	Time [minutes]	Carapace distance [cm]	t	p
Familiar	1-3	15.07	5.068	<0.001
Familiar	4-6	12.59	3.252	<0.01
Familiar	7-9	11.46	2.179	0.050
Familiar	10-12	10.46	1.335	0.207
Familiar	13-15	9.41	0.650	0.528
Stranger	1-3	14.08	5.592	<0.001
Stranger	4-6	9.13	0.244	0.812
Stranger	7-9	9.03	0.228	0.824
Stranger	10-12	9.48	0.605	0.559
Stranger	13-15	11.28	3.006	0.013

171
 172 Table 2
 173 Average carapace distance in centimetres, t and p level for one-sample t-tests against the chance carapace
 174 distance (8.84 cm) obtained by simulated data.
 175
 176



177
 178 Figure 2.
 179 Average distance in centimetres by Condition in Time. (a) Average carapace distance. (b) Average head
 180 distance. The dashed line indicates the chance distance calculated with Fermi-like simulations. Significance
 181 against the chance level is indicated by asterisks: *** $p<0.001$, ** < 0.01 , * $p<0.05$.
 182

183 Distance between heads

184 Distance between head tips was analysed with Condition, Species and Time as fixed effects and Pair
 185 as random effect, using a linear mixed effect model fitted using maximum likelihood. The minimum
 186 adequate model included Time as only significant main effect Time ($F_{4,92}=3.428$, $p=0.015$), see
 187 Figure 2B. As post-hoc tests we run one analysis of variance for the first and one analysis of variance
 188 for the last half of the experiment, using Condition as between factor and Time as within factor. In
 189 the first part of the experiment there was no significant main effect of Condition ($F_{1,22}=1.730$,
 190 $p=0.202$) of Time ($F_{2,44}=0.712$, $p=0.49$) and no significant interaction ($F_{2,44}=0.201$ $p=0.819$). In the
 191 second half of the experiment there was no significant main effect (Condition $F_{1,22}=2.485$, $p<0.001$;
 192 Time $F_{2,44}=0.702$, $p=0.501$) but a significant interaction Condition x Time ($F_{2,44}=4.267$, $p=0.020$). As
 193 reported in Table 3, while in the familiar condition tortoises progressively approached the chance

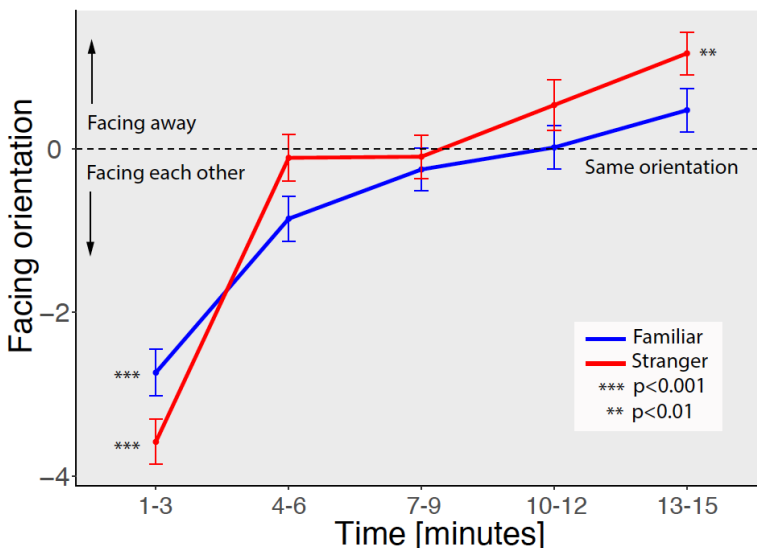
194 level distance, in the stranger condition tortoises initially approached the other subject and then
 195 moved significantly further than the chance distance. In all time points and conditions, head pairs
 196 were significantly closer than the average starting distance (14.87 cm).
 197

Condition	Time [minutes]	Head distance [cm]	t	p
Familiar	1-3	12.33	3.890	0.002
Familiar	4-6	11.73	2.36	0.036
Familiar	7-9	11.21	1.670	0.115
Familiar	10-12	10.47	1.047	0.316
Familiar	13-15	9.849	0.498	0.627
Stranger	1-3	10.50	1.134	0.283
Stranger	4-6	9.023	-0.268	0.794
Stranger	7-9	8.93	-0.418	0.684
Stranger	10-12	10.01	0.553	0.592
Stranger	13-15	12.44	3.261	0.009

198
 199 Table 3
 200 Average head distance in centimetres, t and p level for one-sample t-tests against the chance carapace distance
 201 (9.35 cm) obtained by simulated data, grouped by Condition and Time point (in minutes).
 202

203 Facing orientation

204 The facing orientation, measured as difference between carapace distance and head tips was analysed
 205 with Condition, Species and Time as fixed effects and Pair as random effect, using a linear mixed
 206 effect model fitted using maximum likelihood. We did not observe significant effects or interactions.
 207 However, limiting the analysis to the first two time-points we observed a significant effect of Time
 208 ($F_{1,22}=16.22$, $p<0.001$) no significant effect of Condition ($F_{1,22}=1.436$, $p=0.24$) and a significant
 209 interaction Condition x Time ($F_{1,22}=5.521$, $p=0.028$), see Figure 3. As showed by one-sample t-tests
 210 against the chance level (0), tortoises showed a different propensity to face each other during the test:
 211 at time point 1-3 minutes, tortoises of both conditions exhibited a significant preference to face each
 212 other (familiar: $t_{12}=-5.146$, $p<0.001$; stranger: $t_{10}=-8.858$, $p<0.001$), while at time point 12-15 minutes
 213 tortoises in the stranger condition had a significant preference to face in different directions
 214 ($t_{12}=3.363$, $p=0.007$) while tortoises in the familiar condition did not ($t_{10}=1.077$, $p=0.303$). These
 215 effects suggest in the stranger condition tortoises initially oriented more towards the experimental
 216 mate than familiar tortoises, while in the second part of the test tortoises in the stranger condition
 217 oriented less towards the other experimental subject than familiar tortoises.



218
 219 Figure 3.

220 Facing orientation by Condition in Time. Negative values indicate tortoises facing each other, positive values
221 opposite orientation. Significance against the chance level (same orientation, facing in the same direction) is
222 indicated by asterisks: *** $p < 0.001$, ** < 0.01 .

223

224 **DISCUSSION**

225 Individual recognition, the ability to recognize particular individuals, has been mainly investigated in
226 contexts of repeated interactions, such as kin recognition, neighbour-stranger recognition, dominance
227 hierarchies [reviewed in ,1,2]. Recognition of specific individuals is documented in different taxa,
228 such as fish [16,17], mammals [18,19], reptiles [20,21], birds [22–24], invertebrates [25,26]. Since in
229 most cases affiliative or competitive interactions recurred several times before recognition was tested,
230 it is not clear to what extent individual recognition requires an individual to have experience with
231 multiple subjects. The case of filial imprinting in precocial avian species is a notable exception:
232 through this learning mechanism, chicks of the domestic fowl promptly recognize familiar individuals
233 after a single exposure [3–6] and exhibit affiliative responses towards the imprinting stimulus. Chicks
234 and other social species exhibit several predispositions for social behavioural soon after hatching
235 [27], and these behaviours have a genetic component [28]. It is not known, though, whether exposure
236 to a single individual is sufficient to elicit individual recognition at the onset of life in other species,
237 including non-social animals. Showing abilities of individual recognition in tortoise hatchlings would
238 suggest a certain independence of individual recognition from complex social experience and the
239 possibility that this trait has evolved in contexts other than repeated social interactions.

240 Here, we documented the first evidence of spontaneous individual recognition in hatchlings of two
241 species of tortoises (*Testudo graeca* and *Testudo marginata*) previously exposed to a single
242 conspecific. At test, tortoises were placed in a novel environment, so that no territoriality was present
243 for the test arena. Tortoises of both species showed strikingly different behavioural responses in the
244 two conditions. While pairs of familiar tortoises appeared to ignore each other in the chosen
245 trajectories, and progressively reached the chance level distance, pairs of stranger tortoises initially
246 approached each other much faster than familiar pairs, then walked away from each other and reached
247 a distance significantly larger than expected by chance. Interestingly, in the first part of the test
248 unfamiliar individuals approached each other faster than familiar individuals, suggesting an initial
249 interest in the unfamiliar conspecific. This observation is supported by the fact that tortoises in the
250 unfamiliar condition faced more the other individual than tortoises in the familiar condition.

251 We hypothesize that the spontaneous avoidance of unfamiliar tortoises in hatchlings might be an
252 adaptation to help dispersal of the clutch. In the wild, it has been documented that *T. marginata* and
253 *T. graeca* lay multiple clutches per year (1-4) and each clutch contains 3-7 eggs [29,30] (in captivity,
254 we observed larger clutch size, up to 12 eggs per individual in *T. marginata*). Hence, without active
255 dispersal hatchlings would quickly saturate the carrying capacity of the environment, moreover,
256 without dispersal there would be a greater exposure to predation of the entire clutch. In line with this
257 idea, the range of hatchlings has little overlap [31] and females of *T. graeca* increase mobility before
258 or after nesting, producing a wide dispersal of nests laid by each individual [32]. Overall, our results
259 show that non-social species, such as land tortoises, possess the capacity for individual recognition
260 at the onset of life, with very limited experience with other individuals. This suggests that individual
261 recognition abilities might have evolved in contexts different than repeated social interactions.

262

263

264 **Competing interests**

265 We have no competing interests.

266

267 **Authors' contributions**

268 E.V. and G.S conceived the project; G.S., E.V. and S.D. designed the experiment; S.D. carried out
269 the experiment; E.V. M.C. and S.D. analysed the data; M.C. developed the simulations; E.V. drafted
270 the paper; all authors revises the manuscript and gave final approval for publication.

271

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