

26 **Summary statement**

27

28 Kissing-bugs use contact chemo-perception to avoid aversive substrates.
29 They can sensory distinguish between salty (sodium chloride) and bitter (caffeine)
30 tastes, but not between different salts (sodium and potassium chloride).

31

32 **Abstract**

33

34 Sensory aversion is an essential link for avoiding potential dangers. Here,
35 we studied the chemical perception of aversive compounds of different gustatory
36 modalities (salty and bitter) in the haematophagous kissing bug, *Rhodnius prolixus*.
37 Over a walking arena, insects preferred a substrate embedded with 0.3 M NaCl or
38 KCl rather than with distilled water. Same salts were avoided when prepared at 1 M.
39 When NaCl and KCl were confronted, no preferences were evinced by insects. A
40 pre-exposure to amiloride interfered with the repellency of NaCl and KCl equally,
41 suggesting that amiloride-sensitive receptors are involved in the detection of both
42 salts. Discriminative experiments were then performed to determine if *R. prolixus*
43 can distinguish between these salts. An aversive operant conditioning involving
44 either NaCl or KCl modulated the repellency of the conditioned salt, but also of the
45 novel salt. A chemical pre-exposure to the salts did not to modify their repellency
46 levels. When we crossed gustatory modalities by confronting NaCl to caffeine (*i.e.* a
47 bitter stimulus) no innate preferences were evinced. Aversive operant conditionings
48 with either NaCl or Caf rendered unspecific changes in the repellency of both
49 compounds. A chemical pre-exposure to Caf modulated the response to Caf but not
50 to NaCl, suggesting the existence of two independent neural pathways for the
51 detection of salts and bitter compounds. Overall results suggest that *R. prolixus*
52 cannot distinguish between NaCl and KCl but can distinguish between NaCl and Caf
53 and generalizes the response between these two aversive stimuli of different
54 gustatory modality.

55 1. Introduction

56

57 Innate aversion is a highly adaptive behaviour that plays a key role in the
58 survival of animals, as it can prevent them from suffering critical damages. Aversive
59 stimuli can promote avoidance or escape in an individual by directly provoking pain
60 (e.g. electric shock) or by alerting of the presence of a potential danger (e.g. alarm
61 pheromone). In both cases, the peripheral sensory system is in charge of
62 communicating the presence of a danger to the brain. Among the different sensory
63 modalities, gustatory aversiveness acquires a high relevance, as it can help in
64 preventing the ingestion of large quantities of toxic or harmful compounds that might
65 be present in food. Taste is a quite simple system that allows the detection and
66 differentiation of relative low number of stimuli as compared with the olfactory sense.
67 For example, animals can detect more than thousands of different olfactory
68 molecules, but they can only differentiate a dozen or less flavours (Liman *et al.*,
69 2014; McGann, 2017).

70

71 In insects, taste or contact chemoreception sensilla can be found assembled
72 in specific appendages such as proboscis, antennae or legs, but they can also be
73 present all along the insect's body (e.g. abdomen, ovipositor or wings). Even if they
74 are called "taste" sensilla, they can bear functions other than those involved in food
75 recognition, such as identification of suitable/unsuitable oviposition sites, detection
76 of toxic substrates, recognition of congeners and sexual partners, among other. In
77 any case, the functional unity of detection is the taste sensillum, which bears one
78 apical pore and a few neurons inside. Typically, one of the neurons is a mechano-
79 receptor that helps the animal to determine if the sensillum is contacting a surface
80 or not, while the other/s is/are chemo-receptors (Singh, 1997). This arrangement
81 can, however, change among species. Differently from the olfactory system in which
82 each neuron possesses one only type of receptor and hence it's specific for one
83 odour, many gustatory receptor (GR) types can be present in each gustatory
84 receptor neuron (GRN) (Freeman and Dahanukar, 2015). This is probably one of the

85 reasons why only few tastants can be discriminated by mammals and insects (Masek
86 and Scott, 2010; Spector and Kopka, 2002; Asparch *et al.*, 2016). Even if this could
87 be seen as a disadvantage, it might rather be an advantage for an animal, as it allows
88 to group chemical stimuli that communicate the same signal (for example “toxic”)
89 even if they have quite different molecular structures. For example, plants defence
90 against herbivory includes the production of numerous toxic compounds that have a
91 bitter taste (at least for humans) and generate aversive responses in animals. So
92 even without being able to discern if two bitter molecules are the same or not, it
93 would be useful for an animal simply to reject both of them. Contrarily, there are also
94 cases in which determining the chemical identity of a tastant can make the difference
95 between living or not. For example many alkaloids produced by plants to avoid being
96 eaten have different toxicity levels, for what if an animal’s taste system could
97 distinguish between them, in low-food availability conditions it might be very adaptive
98 to feed over plants producing the least toxic ones (Glendinning, 1994; Glendinning
99 *et al.*, 2001; Ayestaran *et al.*, 2010; Hurst *et al.*, 2014). In haematophagous insects
100 such as mosquitoes and kissing bugs, feeding responses were partially or even
101 completely inhibited by the addition of alkaloids to appetitive artificial diets (Kessler
102 *et al.*, 2014; Pontes *et al.*, 2014). Moreover, a walking substrate impregnated with
103 two different alkaloids (caffeine and quinine) was repellent for *R. prolixus* (Asparch
104 *et al.*, 2016). Although the avoidance response generated by caffeine and quinine
105 was quite similar, conditioning protocols allowed authors to determine that these
106 insects are able to discriminate among them.

107

108 Besides avoiding potentially toxic compounds, animals need to ingest the
109 correct amount of salts to maintain the ionic balance inside their bodies. Excessive
110 ingestion of salts can have as much harmful consequences as the absence of salts
111 in the diet. Therefore, the detection of salts and their concentrations in a potential
112 food source becomes relevant for most animals, reason why it normally guides the
113 decision making about feeding or not on a particular diet. In this search for
114 homeostasis, sodium chloride (NaCl) seems to be a main actor. In blood feeders

115 such as mosquitoes, kissing bugs, fleas and tse-tse flies, NaCl is crucial to elicit
116 gorging under artificial feeding conditions (Friend and Smith, 1977). However,
117 feeding rejection occurs if salt concentration is below or above vertebrate's blood
118 level (Pontes *et al.*, 2017; Galun *et al.*, 1963). In *R. prolixus*, NaCl detection seems
119 to be driven by amiloride-sensitive receptors present on the GRNs of the pharyngeal
120 sensilla (Pontes *et al.*, 2017). However, no information is available about the effects
121 that the detection of salts over the walking substrate might have on the behaviour of
122 such haematophagous insects.

123

124 This work aims to disentangle the capacity of a disease vector insect, the
125 haematophagous *R. prolixus*, to assess the gustatory quality of a walking substrate.
126 We firstly explored if they are capable of perceiving different concentrations of two
127 salts: NaCl and KCl. Then, we investigated if they are able to distinguish between
128 these two salts (*i.e.* two stimuli from the same gustatory modality) or between NaCl
129 and a bitter compound as caffeine (*i.e.* two stimuli from different gustatory modality).
130 Different experimental approaches were applied to analyse the discriminatory
131 capacities of *R. prolixus*. Along this work we assume that if a particular treatment
132 modulates the response to one stimulus but not to the other, insects are capable of
133 discriminating among them.

134

135 **2. Materials and methods**

136

137 **2.1. Insects**

138

139 *Rhodnius prolixus* (Heteroptera: Reduviidae: Triatominae) was reared in the
140 laboratory at constant 28 ± 1 °C, 60 ± 20 % relative humidity and 12 : 12 h. L / D
141 photoperiod cycle. Recently moulted 5th instar nymphs were kept unfed for 14 days
142 and randomly assigned to the different treatments along this work. Each nymph was

143 used only in one experiment and then returned to the rearing chamber. All animals
144 were handled according to the biosafety rules of the Hygiene and Safety Service of
145 the University of Buenos Aires.

146

147 Being nocturnal insects, all experiments were carried out during the first
148 hours of the insects' scotophase (*i.e.* 1 - 6 h. after lights were turned-off) in a dark
149 experimental room. The temperature of the room was set to 25 ± 1 °C for all assays.
150 This spatio-temporal arrangement allowed us to exclude external visual cues during
151 experiments and at the same time match the maximal activity period described for
152 triatomines (Lazzari, 1992).

153

154 **2.2. Stimuli**

155

156 Sodium chloride (NaCl), potassium chloride (KCl) and caffeine anhydrous
157 (Caf) were purchased from Biopack (Buenos Aires, Argentina). Amiloride
158 hydrochloride hydrate was purchased in Sigma-Aldrich® (St. Louis, U.S.A). The
159 different stimulus solutions (0.3 M and 1 M NaCl; 0.3 M and 1 M KCl; 0.1 M Caf; 1
160 mM amiloride) were prepared in distilled water, stored at 4°C and used in the same
161 week.

162

163 **2.3. Taste preference assay: two-choice walking arena**

164

165 Individual assays were performed in a rectangular acrylic box (10 x 5 cm)
166 whose floor was completely covered with a filter paper (see upper panel of all
167 figures). A line was drawn on the paper at the centre of the arena to define two equal
168 square zones (5 x 5 cm). Over the paper of each zone 100 µl of a stimulus solution
169 (*i.e.* distilled water, 0.3 or 1M NaCl, 0.3 or 1M KCl or 0.1M Caf) were homogeneously

170 spread using a micropipette. In this way, according to the experimental series, the
171 following chemical two-choices were generated: H₂O/NaCl, H₂O/KCl, KCl/NaCl,
172 H₂O/Caf or Caf/NaCl. After one minute, an insect was gently located at the centre of
173 the arena and covered with an inverted flask for another 1 minute familiarization with
174 the experimental setup. Then, the flask was gently removed and the experiment
175 started. The position of the freely-walking insect in relation to the position of the
176 added stimuli was continuously recorded during 4 minutes using an infrared-
177 sensitive video-camera connected to a digital recorder. Note that the investigator
178 and the recording system (except the camera) were located outside the experimental
179 room to avoid the addition of potential cues that could be detected by the insects.

180

181 From the video recordings, the time spent in each side of the arena was
182 computed for each insect. A Preference Index (PI) was calculated as the time spent
183 at the stimulus “A” side (*St A*), minus the time spent at the stimulus “B” side (*St B*),
184 divided by the total experimental time:

185

$$186 \quad PI = \frac{T_{(St A)} - T_{(St B)}}{T_{(St A)} + T_{(St B)}}$$

187

188 PIs close to -1 or 1 show preference for “B” or “A”, respectively. PIs near 0
189 indicate lack of preference. Thirty replicates were performed for each experimental
190 series. Insects were used once and then discarded. In each experimental series, the
191 stimulus was assigned to the “left” or “right” side of the arena in a pseudorandom
192 manner.

193

194 **2.4. Discriminative experiments: pre-treatments**

195

196 Discriminative experiments were performed to discern if insects can
197 distinguish between different aversive stimuli. Different pre-treatments were applied
198 before the taste preference assays.

199

200 **2.4.1. Pre-exposure to amiloride**

201

202 Amiloride is a pyrazine known to block the detection of NaCl in animals by
203 interfering with the functioning of epithelial sodium channels (ENaC) (Kellengerger
204 and Schild, 2002). We pre-expose insects to amiloride to examine if there is a
205 modulation of the perception of NaCl, KCl or both salts.

206

207 Insects were individually exposed to amiloride inside a plastic vial (2 cm
208 diam., 3 cm height, see upper panel of Fig. 2) whose floor was covered with a filter
209 paper loaded with 100 μ l of H₂O (control series) or of 1 mM amiloride. One insect
210 was gently placed over the paper inside the vial and left to freely walk during 1
211 minute. During this time, legs, antennae, proboscis and/or other parts of the body
212 could contact the H₂O or the amiloride. The insect was then removed, maintained in
213 a different clean flask for 2 minutes and then transferred to the two-choice arena
214 where its chemical preference was tested as explain in section 2.3.

215

216 **2.4.2. Discriminative learning: operant aversive conditioning**

217

218 Associative learning protocols are widely applied in discriminatory studies,
219 as they can help in determining if an individual can detect as different two stimuli
220 generating the same behavioural output. It is assumed that if after a conditioning
221 protocol involving stimulus “A” there is a specific modulation of the response to “A”
222 and not to “B”, the individual can distinguish between “A” and “B”.

223

224 An operant aversive conditioning of 4 minutes was individually applied to the
225 insects over the experimental arena described in section 2.3. But, to deliver a
226 negative reinforcement to the insects, a vortex-type laboratory mixer was
227 mechanically attached to the arena (see upper panel of Figs. 3, 4, 7 and S1), allowing
228 us to voluntarily deliver a vibration disturbance via an external switch. For each
229 conditioning, 100 µl of the different compounds were loaded at each side of the arena
230 as explained before (see section 2.3), *i.e.* KCl/NaCl, H₂O/NaCl, H₂O/KCl or
231 Caf/NaCl. One of the compounds was predefined as the punished stimulus, the other
232 as the safe one (note that in the text and the figures a negative sign (-) is added
233 beside the stimulus punished during training). An insect was then located at the
234 centre of the arena, covered with an inverted flask for one minute for familiarization
235 with the experimental setup. Then the flask was gently removed, and the aversive
236 conditioning started. During the following 4 minutes of training, the insect could
237 control the occurrence of the mechanical disturbance by its position: every time it
238 entered the side of the arena loaded with the punished stimulus, the vortex mixer
239 was switched on, generating a mechanical disturbance for the insects. Conversely,
240 the vibration stopped whenever the insect entered the safe side of the arena.

241

242 Unpaired yoke control series were carried out to verify the associative origin
243 of the behavioural modulation. For this, during 4 minutes each individual received a
244 negative reinforcement independently from its position in the experimental arena
245 (note that in the text and the figures negative signs (-) are added beside both stimuli
246 during training). To match the intensity of the vibration received by insects of the
247 conditioning groups, the timing, frequency and duration of the vibration delivered to
248 yoke control insects were copied from the previously conditioned insect. In this way,
249 individuals of these series received the same amount of vibration than those from
250 the experimental series, but in this case dissociated from the position of the chemical
251 stimuli.

252

253 In all cases, the behaviour of each individual during the conditioning time
254 was registered in video as explained before, and the individual PIs were computed.
255 Once the conditioning ended, the insect was removed, put in an individual flask for
256 2 minutes and then transferred to the two-choice arena where its chemical
257 preference (*i.e.* without vibrations delivered) was tested as explain in section 2.3.
258 Note that for these series, separated PIs are registered for training (triangles in the
259 figures) and test phases (circles in the figures).

260

261 **2.4.3. Compound-specific chemical pre-exposure**

262

263 Long chemical exposures generally give rise to reduced responsiveness in
264 animals. Two possible processes might be responsible for this modulation: 1- a
265 peripheral sensory adaptation, in which receptors stop sending electrical information
266 to the brain and consequently the behavioural response decreases, 2- an
267 habituation, in which peripheral receptors send the electrical report to the brain but
268 this information is processed and the response also decreases. In any case, it is
269 assumed that if after an exposure to stimulus “A” there is a specific modulation of
270 the response to “A” and not to “B”, the individual can distinguish peripherally between
271 “A” and “B”.

272

273 A non-associative pre-exposure to NaCl, KCl or Caf was applied individually
274 to the insects before testing their chemical preferences. Pre-exposure procedure
275 was achieved in a plastic vial (2 cm diam., 3 cm height, see upper panel of Figs. 5,
276 8) whose floor was covered with a filter paper loaded with either 100 µl of 1 M NaCl,
277 of 1 M KCl or of 0.1 M Caf. In control series the paper was loaded with 100 µl of H₂O.
278 One insect was gently placed inside the vial over the paper and left to freely walk
279 during 60 minutes. During this time, legs, antennae, proboscis and/or other parts of

280 the body could contact the chemical stimuli. Once the pre-exposure ended, the
281 insect was removed, put in an individual flask for 2 minutes, and then its chemical
282 preference was tested as explained in section 2.3.

283

284 **2.5. Data and analyses**

285

286 Thirty insects were tested in each experimental series. One PI was
287 computed for each insect. The mean PI of each series was statistically compared
288 against the expected value if there were no chemical preferences, *i.e.* “0” by applying
289 One-Sample T-Tests ($\alpha = 0.05$). Normality and homoscedasticity were verified in all
290 data series. All figures represent the mean Preference Index (x-axis) and the
291 chemical compounds presented at each side of the arena (y-axis). Asterisks denote
292 statistical differences between the PI and the value 0 ($p < 0.05$).

293

294 **3. Results**

295

296 **3.1. Salt perception: concentration-dependent behaviour**

297

298 When the different salts were confronted to distilled water, *R. prolixus*'
299 responses were reliant on the tested concentrations. Insects preferred to walk over
300 the 0.3 M NaCl solution rather than over water (Fig. 1A, H₂O/NaCl, $T = 2.2$, $p < 0.05$).
301 A similar trend was observed for 0.3 M KCl (H₂O/KCl, $T = 1.8$, $p = 0.056$), although
302 the PI was borderline not significantly different from 0. Conversely, bugs avoided a
303 1 M solution of both salts (H₂O/NaCl, $T = -3.6$, $p < 0.01$; H₂O/KCl, $T = -3.3$, $p < 0.01$).

304

305 These results show that *R. prolixus* can detect the presence of both salts
306 over the walking substrate, and that the concentration perceived determines the type
307 of response evinced by these bugs. In fact, concentrations close to those found over
308 the skin of a potential host (*i.e.* around 0.1 M) were preferred, while higher ones,
309 which could probably alter negatively the internal homeostasis of the insect, were
310 repellent. However, regardless the concentration, and as the responses of insects
311 to both salts were quite similar, next experimental series were designed to determine
312 if these insects can detect both salts as different compounds or not. Experiments
313 presented here onwards were carried out using the aversive concentration of NaCl
314 and KCl, *i.e.* 1 M.

315

316 **3.2. Detection of NaCl and KCl: different or same receptors?**

317

318 **3.2.1. Innate response to the NaCl/KCl simultaneous presentation**

319

320 As a first attempt to examine if these bugs can detect NaCl and KCl as
321 different compounds, we offered them the possibility to choose between the aversive
322 concentration of both salts (*i.e.* 1 M). We assumed that the expression of any spatial
323 preference over the arena would indicate a differential perception of both stimuli.
324 However, in this situation insects exhibited no preference for one or the other salt
325 (Fig. 1B, $T = 1.5$, $p > 0.05$). This lack of preference exhibited could be the
326 consequence of two quite opposite situations: 1- insects' sensory system is unable
327 to distinguish between both salts, or 2- insects can detect both salts as different
328 compounds, but the triggered responses are similar in sign and intensity, *i.e.* NaCl
329 and KCl are equally repellent. Next experimental series were performed to tackle
330 this uncertainty.

331

332 **3.2.2. Inhibition of NaCl and KCl detection by amiloride**

333

334 We tested here if amiloride can block the detection of salts in *R. prolixus* and
335 if its action is specific for the detection of NaCl or if it interferes with the detection of
336 KCl too. We assume that if the effect is specific for one salt, insects can distinguish
337 between them.

338

339 Insects pre-exposed to H₂O avoided both salts when faced to H₂O (Fig. 2A,
340 H₂O/KCl, $T = -5.7$, $p < 0.001$; H₂O/NaCl, $T = -4.4$, $p < 0.001$) and exhibited no
341 preference when confronted to each other (KCl/NaCl, $T = 1$, $p > 0.05$). These control
342 series show that there is no effect of pre-exposing insects to H₂O. However, after a
343 pre-exposure to amiloride, insects presented random distributions over the arena in
344 all cases (Fig. 2B, H₂O/KCl, $T = -0.4$, $p > 0.05$; H₂O/NaCl, $T = -1.0$, $p > 0.05$;
345 KCl/NaCl, $T = 0.4$, $p > 0.05$), evincing an unspecific lost in their capacity to perceive
346 both salts.

347

348 Amiloride showed to efficiently inhibit salt detection in *R. prolixus*. The lack
349 of specificity for NaCl or KCl suggests that the detection of both salts might be
350 achieved by the same or at least by similar receptors, sensitive to amiloride
351 blockage.

352

353 **3.2.3. Aversive conditioning with salts**

354

355 Taking in consideration the results of sections 3.2.1 and 3.2.2, which show
356 that NaCl and KCl generate the same avoidance response (see Figs. 1, 2), we
357 applied operant aversive protocols to find out if *R. prolixus* can distinguish between

358 these salts. We assume that the occurrence of a salt-specific conditioning indicates
359 the existence of a differential detection pathway.

360

361 We firstly confronted KCl with NaCl during trainings, pairing the mechanical
362 punishment to one of the salts, aiming to generate a specific aversive association
363 during subsequent tests. Our results show that the mechanical vibration was indeed
364 perceived as a negative reinforcement by bugs, as during conditionings insects
365 avoided the punished side of the arena regardless if it contained KCl or NaCl (Fig.
366 3A, triangle KCl/NaCl(-), $T = -8.0$, $p < 0.001$; Fig. 3B, triangle KCl(-)/NaCl, $T = -4.6$,
367 $p < 0.001$). However, in subsequent KCl/NaCl tests, insects exhibited no preference
368 for one or the other salt, evincing that the conditioning did not affect their innate
369 behaviour, (Fig. 3A, circle KCl/NaCl, $T = 0.3$, $p > 0.05$; Fig. 3B, circle KCl/NaCl, $T =$
370 0.4 , $p > 0.05$).

371

372 Three different situations could explain this lack of preference during tests:
373 1- insects could be detecting both salts as different but the conditioning protocol
374 could not be efficient (*e.g.* too short in duration, salience of the conditioned stimulus
375 too low, negative reinforcement not enough, etc.), failing in modifying the innate lack
376 of preference, 2- insects could be incapable of distinguishing between salts and then
377 perceive an homogeneous substrate during both, training and test, or 3- insects
378 could distinguish between salts and the conditioning protocol could be efficient, but
379 they are generalizing what they have learnt for one salt to the other salt. Next series
380 intend to separate and discard some of these options.

381

382 To determine if they are indeed capable of learning under our experimental
383 conditions, we applied a similar conditioning protocol but involving only one salt and
384 H₂O during training. As we know from previous series that 1 M NaCl and KCl are
385 aversive for these insects (see Fig. 1A), the negative reinforcement was applied at

386 the H₂O side of the arena. Once again, the mechanical vibration was efficient as
387 negative reinforcement, even if insects had to remain in the salty side of the arena
388 (Fig. 4A, triangle H₂O(-)/NaCl, T = 5.2, p < 0.001; Fig. 4B, triangle H₂O(-)/KCl, T =
389 5.4, p < 0.001). During tests insects continued to prefer the salty side of the arena
390 rather than the H₂O side (Fig. 4A, circle H₂O/NaCl, T = 2.5, p < 0.05; Fig. 4B, circle
391 H₂O/KCl, T = 3.1, p < 0.01). Surprisingly, they also preferred the side of the arena
392 containing a salt that was not presented during training (Fig. 4A, circle H₂O/KCl, T =
393 3.2, p < 0.01; Fig. 4B, circle H₂O/NaCl, T = 3.1, p < 0.01). Regardless the salt used
394 during the aversive conditioning, no preferences were expressed in tests in which
395 both salts were confronted (Fig. 4A, circle KCl/NaCl, T = 0.5, p > 0.05; Fig. 4B, circle
396 KCl/NaCl, T = 0.5, p > 0.05).

397

398 These results clearly show that *R. prolixus* can modify their chemical
399 preferences after an aversive operant conditioning, *i.e.* they can learn to avoid
400 punished stimuli. However, results evince that the observed experience-dependent
401 plasticity is not compound specific, for what we still cannot discern if *R. prolixus* is
402 not capable of distinguishing between these two salts, or if they can distinguish, they
403 can learn, but they generalize between salts (*i.e.* distinguishing peripherally between
404 them but transferring what they have learnt for one stimulus to the other).

405

406 **3.2.4. Chemical pre-exposure to salts**

407

408 We applied a chemical pre-exposure to NaCl or KCl and analysed then if
409 this treatment had or not an effect over the behaviour of *R. prolixus* confronted to
410 the pre-exposed salt, to the new salt or to both. We assume that the occurrence of
411 a salt-specific effect indicates the existence of a differential detection pathway.

412

413 Insects were exposed during 60 minutes to H₂O, NaCl or KCl. Immediately
414 after their preferences were tested by confronting the same salt or the new one to
415 H₂O or to each other. Results show that the three chemical pre-exposures (*i.e.* to
416 H₂O, NaCl and KCl) failed in modulating the innate responses of insects to the salts.
417 Bugs continued to avoid NaCl and KCl presented against H₂O and evinced no
418 preference when the contingency NaCl/KCl was presented (Fig. 5A, H₂O/KCl, $T = -$
419 2.9 , $p < 0.01$; H₂O/NaCl, $T = -3.8$, $p < 0.001$; KCl/NaCl, $T = -0.6$, $p > 0.05$; Fig. 5B,
420 H₂O/KCl, $T = -2.2$, $p < 0.05$; H₂O/NaCl, $T = -2.1$, $p < 0.05$; KCl/NaCl, $T = -0.02$, $p >$
421 0.05 ; Fig. 5C, H₂O/KCl, $T = -2.5$, $p < 0.05$; H₂O/NaCl, $T = -2.3$, $p < 0.05$; KCl/NaCl,
422 $T = -0.7$, $p > 0.05$). These results support the hypothesis that *R. prolixus* cannot
423 distinguish between NaCl and KCl.

424

425 **3.3. Discrimination between aversive compounds of different gustatory** 426 **modality, *i.e.* salty and bitter**

427

428 **3.3.1. Innate responses to Caffeine**

429

430 Results presented in previous sections show that *R. prolixus* avoids walking
431 over an aqueous solution of 1 M NaCl (see Fig. 1). Here we show that an aqueous
432 solution 0.1 M of the alkaloid Caf is also avoided by *R. prolixus* (Fig. 6, H₂O/Caf, T
433 $= -5.5$, $p < 0.001$). However, when this alkaloid is confronted to 1 M NaCl, the insects
434 evinced no preference, remaining the same time at each side of the arena (Fig. 6,
435 Caf/NaCl, $T = -0.6$, $p > 0.05$).

436

437 These results evince that *R. prolixus* can sense the presence of caffeine on
438 the walking substrate, and that this perception generates an avoidance, which is
439 similar in intensity to that exerted by NaCl. Consequently, the question arises again
440 about the capacity of *R. prolixus* to distinguish between these two aversive

441 compounds of radically different chemical identity, *i.e.* a salt and an alkaloid. Next
442 experimental series involving associative and non-associative discriminatory
443 learning protocols were designed to answer this question.

444

445 **3.3.2. Aversive associative conditioning with salts and alkaloids**

446

447 For these experimental series we applied a similar operant aversive protocol
448 to that described in section 3.2.3, but pairing in this case the negative reinforcement
449 with the salt, *i.e.* NaCl(-), or with the bitter compound, *i.e.* Caf(-). In both training
450 periods the mechanical disturbance was effective causing insects to avoid the
451 punished side (Fig. 7A, triangle Caf/NaCl(-), $T = 8.4$, $p < 0.001$; Fig. 7B, triangle Caf(-
452)/NaCl, $T = -6.6$, $p < 0.001$). However, during subsequent tests insects did not evince
453 any change in their innate behaviour, *i.e.* they showed no preferences (Fig. 7A, circle
454 Caf/NaCl, $T = 1.0$, $p > 0.05$; Fig. 7B, circle Caf/NaCl, $T = -1.0$, $p > 0.05$).

455

456 Even knowing from previous series that our conditioning protocol is effective
457 to generate a spatial memory in these insects, these results do not allow us to
458 determine if *R. prolixus* is not able to distinguish between NaCl and Caf, or if they
459 can do it but they generalize what they have learnt for one compound to the other.
460 In the next series we deal with this question by applying a chemical pre-exposure to
461 each compound and analysing its specificity.

462

463 **3.3.3. Chemical pre-exposure to salts and alkaloids**

464

465 Following the 60 minutes chemical pre-exposure to H₂O, NaCl or Caf, the
466 chemical preferences of insects were tested. No effects were evinced after pre-
467 exposing to H₂O (Fig. 8A, H₂O/NaCl, $T = -3.8$, $p < 0.001$; H₂O/Caf, $T = -5.6$, $p <$

468 0.001; Caf/NaCl, $T = 0.1$, $p > 0.05$) or NaCl (Fig. 8B, H₂O/NaCl, $T = -2.2$, $p < 0.05$;
469 H₂O/Caf, $T = -3.4$, $p < 0.01$; Caf/NaCl, $T = 0.7$, $p > 0.05$). In both cases the avoidance
470 of NaCl and Caf when confronted to water, and the lack of preference when the salt
471 and the alkaloid were presented simultaneously were similar to those exhibited by
472 naïve insects (see Figs. 1, 6). However, when insects were pre-exposed to Caf, the
473 aversive response to Caf vanished (Fig. 8C, H₂O/Caf, $T = 1.0$, $p > 0.05$) but the
474 avoidance of NaCl remained intact, effect evinced when confronted to H₂O or to Caf
475 (H₂O/NaCl, $T = -4.3$, $p < 0.001$; Caf/NaCl, $T = 3.6$, $p < 0.01$).

476

477 These results, in which the effect of a pre-exposure to Caf is compound-
478 specific, demonstrate that *R. prolixus* can distinguish between the salt NaCl and the
479 alkaloid Caf, and suggest the existence of two different sensory pathways involved
480 in the detection of aversive compounds of different gustatory modality. Additionally,
481 we can validate our method to analyse the taste discrimination in *R. prolixus*,
482 enforcing the evidences presented before in which we suggest that NaCl and KCl
483 are not distinguished by these insects.

484

485 **3.4. Yoke control series**

486

487 When applying operant conditioning protocols, unpaired yoke controls
488 assure that the experience dependent modulation observed during conditioning is
489 an associative process and not a merely effect of one of the stimuli presented alone.
490 In our experiments, yoke insects were submitted to a pseudoconditioning in which
491 the occurrence of the mechanical punishment was delivered independently from the
492 insect's chemical choice. As expected, these insects showed a random distribution
493 during trainings regardless the stimulus added at each side of the arena (Fig. S1A,
494 triangles KCl(-)/NaCl(-), $T = -0.01$, $p > 0.05$; KCl(-)/NaCl(-), $T = -1.0$, $p > 0.05$; Fig.
495 S1B, triangles H₂O(-)/NaCl(-), $T = -0.01$, $p > 0.05$; H₂O(-)/KCl(-), $T = 0.8$, $p > 0.05$;
496 Fig. S1C, triangles Caf(-)/NaCl(-), $T = 1.0$, $p > 0.05$; Caf(-)/NaCl(-), $T = 1.0$, $p > 0.05$).

497 The lack of preference when the aversive compounds were confronted to water
498 could be generated by the un-associated delivery of the mechanical disturbance,
499 which could be interfering with the innate chemical preferences of these bugs. As in
500 previous sections, it seems that the negative reinforcement is somehow stronger
501 than the chemical preferences.

502

503 During subsequent tests, the animals evinced a behaviour similar to that
504 expressed by naïve ones, distributing randomly over the arena when two aversive
505 compounds were presented in simultaneous (Fig. S1A, B, circles KCl/NaCl, $p > 0.05$
506 in 4 cases; Fig. S1C, circles Caf/NaCl, $p > 0.05$ in 2 cases), and avoiding the aversive
507 stimulus (*i.e.* NaCl and KCl) when confronted to H₂O (Fig. S1B, circles H₂O/NaCl, p
508 < 0.05 in 2 cases; H₂O/KCl, $p < 0.05$ in 2 cases).

509

510 The results of yoke controls confirm that in conditioning groups showed
511 before (see sections 3.2.3 and 3.3.2) the modulation of the behaviour expressed in
512 the tests can be explained by an association between the chemical stimulus and the
513 negative reinforcement and not by a non-associative modulation caused by the
514 chemical exposure or the mechanical disturbance alone.

515

516 **4. Discussion**

517

518 In this work we examined the capacity of *R. prolixus* to perceive aversive
519 chemical compounds and to distinguish between stimuli of the same or of different
520 gustatory modalities, *i.e.* salty (NaCl and KCl) and bitter (Caf). Results here
521 presented strongly suggest that *R. prolixus* is not able to distinguish between the two
522 salts: NaCl and KCl. Conversely, we demonstrate that these insects can distinguish
523 between salts and alkaloids, probably by detecting these compounds of different

524 gustatory modality via different gustatory receptors. Still, electrophysiological
525 recordings would be needed to confirm our findings.

526

527 Differently from olfactory sensory neurons, gustatory neurons of insects can
528 bear different types of receptors in the surface of the same dendrite. As a result,
529 different molecules (e.g. “A” and “B”) reaching different receptors of the same neuron
530 can generate the same electric transduction to higher order brain structures, causing
531 a deficit in the capacity to distinguish between A and B. This apparent disadvantage
532 may in fact be an adaptive value, as animals might only need to detect diverse
533 compounds as a unique stimulus signaling a potential harm and categorize them
534 as “aversive” independently from their chemical identity. In an extreme case, all
535 aversive chemical stimuli could be perceived equally as negative inputs, and evoke
536 the same escape/reject response.

537

538 There are many reports presenting evidence that mammals can distinguish
539 between certain salts (Spector *et al.*, 1996). In our work we show that *R. prolixus* is
540 not able to differentiate between NaCl and KCl, but it could discriminate between
541 NaCl and a bitter alkaloid such as the caffeine. These results confirm that, as it
542 happens in most animals, it is quite difficult for *R. prolixus* to distinguish between
543 different compounds of the same gustatory modality (e.g. salty) and more feasible
544 to distinguish between tastants of different modalities (e.g. salty and bitter).

545

546 Commercially available repellents for haematophagous insects typically
547 make use of the olfactory sense of these insects to keep them away from our skin.
548 However, the gustatory sense is by far less taken into consideration for the chemical
549 formulation of repellents. We show here the existence of non-toxic contact repellents
550 (as salts and bitter compounds) that could be used simultaneously with olfactory
551 stimuli as to obtain a multimodal aversive product. Odours acting at a longer distance

552 and tastants at a shorter range could significantly increase the repellency and
553 eventually the biting deterrence efficiency of a final product. Moreover, being barely
554 volatile compounds, they could probably present a higher effective action along time
555 than odours. Still, experiments combining stimuli from both sensory modalities would
556 be needed to confirm this suggestion.

557

558 Learning and memory has been shown to occur in most animals. The
559 importance of learning and memory in haematophagous insects has been suggested
560 already more than 60 years ago, when Nielsen and Nielsen (1953) described that
561 mosquitoes return to places where they had previously fed, a behaviour known as
562 homing behaviour. Other authors also suggested the existence of a spatial memory
563 in mosquitoes, as they reported that in natural environments they are capable of
564 associating particular odours of the environment with favourable oviposition sites or
565 hosts (Alonso *et al.*, 2003; Chilaka *et al.*, 2012; Kaur *et al.*, 2003; McCall and Eaton,
566 2001; McCall *et al.*, 2001; McCall and Kelly, 2002; Menda *et al.*, 2012; Mwandawiro
567 *et al.*, 2000; Sanford and Tomberlin, 2011; Tomberlin *et al.*, 2006; Vinauger *et al.*,
568 2014). Alonso and Schuck-Paim (2006) discuss about particular cases found in the
569 literature in which authors erroneously ascertain the existence of learning and
570 memory processes in haematophagous insects. Many advances have been made
571 in understanding the cognitive abilities of haematophagous insects other than
572 mosquitoes in the last decade. For example, in a controlled experimental setup, the
573 kissing bug *R. prolixus* started to walk towards or against an originally neutral odour
574 after an appetitive or aversive conditioning, respectively (Vinauger *et al.*, 2011a, b).
575 Same authors found that even if *R. prolixus* did not present a preference when
576 odours from a live rat or quail were presented simultaneously, an aversive
577 conditioning generated an aversion to odours from the punished host (Vinauger *et*
578 *al.*, 2012). Taking advantage of the proboscis extension response elicited by
579 triatomine insects confronted to a warm surface, Vinauger and collaborators (2013)
580 demonstrated that the PER of *R. prolixus* can be modulated by non-associative and
581 associative learning forms. In a completely different context, it was shown that the

582 escape response of *R. prolixus* to the alarm pheromone can be widely modulated by
583 associative and non-associative conditioning protocols (Minoli *et al.*, 2013). In a
584 feeding context, it was reported that the addition of bitter compounds to an appetitive
585 diet inhibits the feeding behaviour of *R. prolixus* (Pontes *et al.*, 2014). Same authors
586 also found that a brief pre-exposure to bitter compounds negatively modulates the
587 motivation of bugs to feed on an appetitive solution. Additionally, it was shown that
588 triatomines' cognitive abilities follow a circadian rhythm, performing well during the
589 night, but not during the day (Vinauger and Lazzari, 2015). As well, studying the
590 repellent effect of new non-toxic molecules for *R. prolixus*, Asparch and collaborators
591 (2016) found that bugs are innately repelled by different bitter molecules, and that
592 this repellence can be modulated by associative and non-associative forms of
593 learning. Indeed, after an aversive operant conditioning, the behaviour of *R. prolixus*
594 changed from avoidance to indifference or even to preference, according with the
595 protocol applied (Asparch *et al.*, 2016). In a completely different behavioural context,
596 Mengoni and collaborators (2017) showed that the innate attractive response to
597 faeces of the kissing bug *Triatoma infestans* can be switched to avoidance if an
598 aversive conditioning protocol is applied. Finally, Minoli and collaborators (2018)
599 were able to demonstrate that the learning efficiency of *R. prolixus* under an aversive
600 operant conditioning tested over a spatial preference walking arena, is highly
601 dependent on the sensory modality of the conditioned stimulus.

602

603 This work enriches the knowledge about gustatory perception in an
604 haematophagous vector insect, and highlights the utilization of the learning process
605 as an indispensable tool for the determination of their discrimination capacities.
606 Moreover, taking in consideration that *R. prolixus* is an insect-vector of a human
607 disease and that its genome has been recently sequenced (Mezquita *et al.*, 2015),
608 it can become a promising model in the learning and memory field. Knowledge about
609 the gustatory system of vector insects and its inherent plasticity provides
610 opportunities to develop new sustainable methods to reduce or even prevent vector-
611 host contacts and disease transmission.

612 **5. Symbols and abbreviations**

613

614 GR: Gustatory receptor

615 GRN: Gustatory receptor neuron

616 H₂O: Distilled water

617 NaCl: Sodium chloride

618 KCl: Potassium chloride

619 Caf: Caffeine anhydrous

620 12 : 12 h. L / D: 12 hours of light and 12 hours of darkness

621 PI: Preference index

622 ENaC: Epithelial sodium channels

623 (-): sign added besides the stimulus punished during training

624

625 **6. Acknowledgements**

626

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628 maintaining the insect colony.

629

630 **7. Author contributions**

631

632 S.Ma. performed experiments, analysed data and wrote manuscript.

633 A.C performed experiments.

634 Y.A. performed experiments.

635 R.B. designed experiments, wrote manuscript.

636 S.Mi. designed experiments, analysed data and wrote manuscript.

637

638 **8. Competing interests**

639

640 No competing interests declared.

641

642 **9. Funding**

643

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646

647

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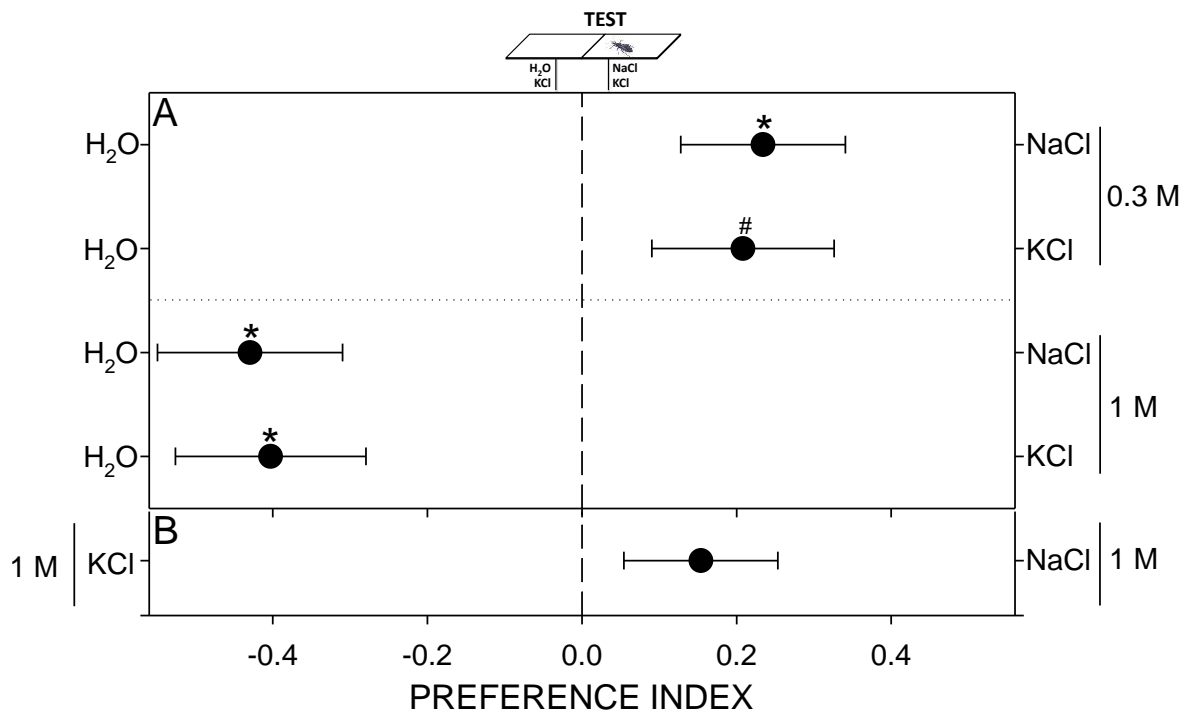
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- 757

758 11. Figures and legends

759

760 **Figure 1. Concentration-dependent responses to NaCl and KCl.** Insects preferred low (0.3 M)
761 and avoided high (1 M) concentrations of both salts confronted to H₂O (A). Insects exhibited no
762 preference when both salts were confronted (B). The Preference Index expresses the relative time
763 spent at each side of the arena: 0 = equal time at each side, -1 and 1 = full time spent at the left or
764 right side of the arena, respectively. Each point represents the mean (\pm s.e.m.) of 30 replicates.
765 Asterisks denote statistical differences ($p < 0.05$) after a One-Sample T-Test with expected value =
766 0. Numeral shows a p-value of 0.056.

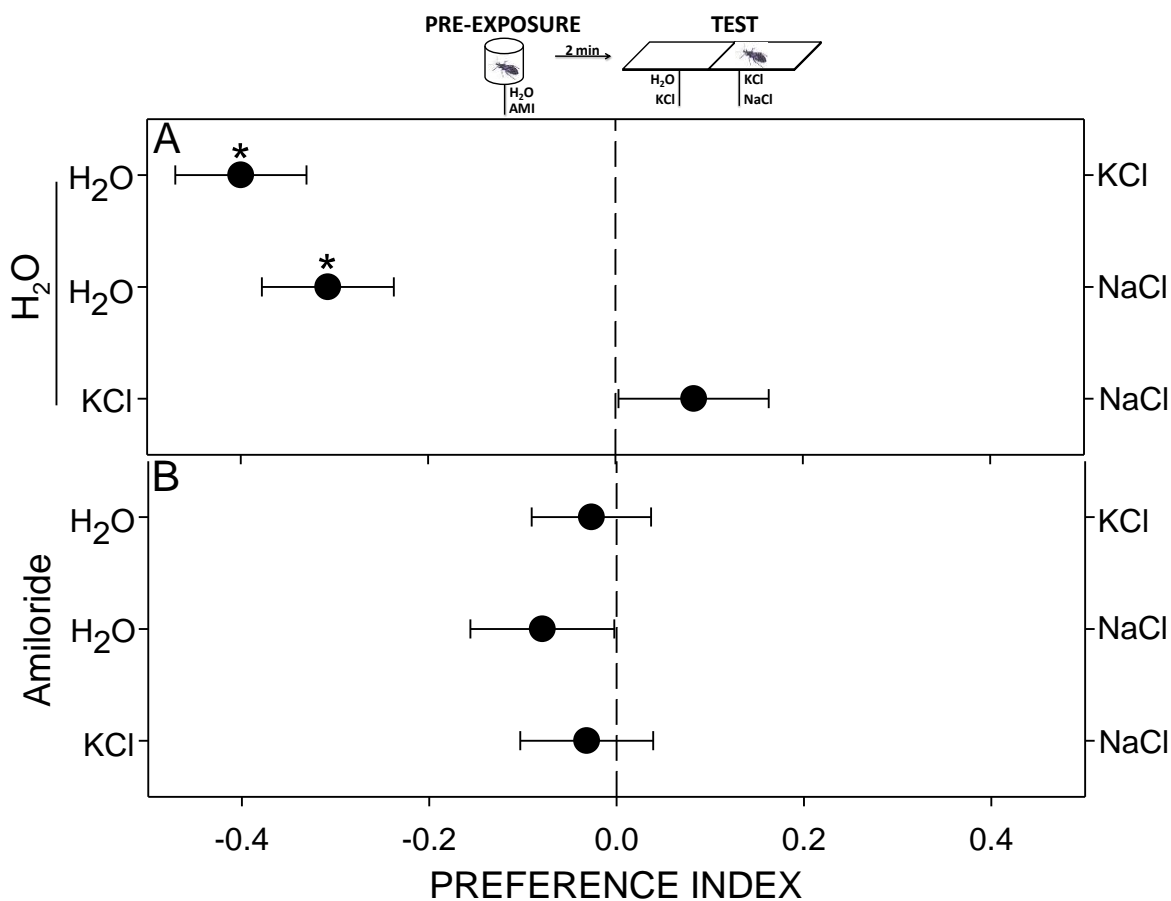


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769

770 **Figure 2. Amiloride blocks salt perception.** A pre-exposure to H₂O did not modify the innate
771 repellence of insects to both salts (A). A pre-exposure to amiloride interfered with the perception of
772 both salts (B). The Preference Index expresses the relative time spent at each side of the arena: 0 =
773 equal time at each side, -1 and 1 = full time spent at the left or right side of the arena, respectively.
774 Each point represents the mean (\pm s.e.m.) of 30 replicates. Asterisks denote statistical differences (p
775 < 0.05) after a One-Sample T-Test with expected value = 0.

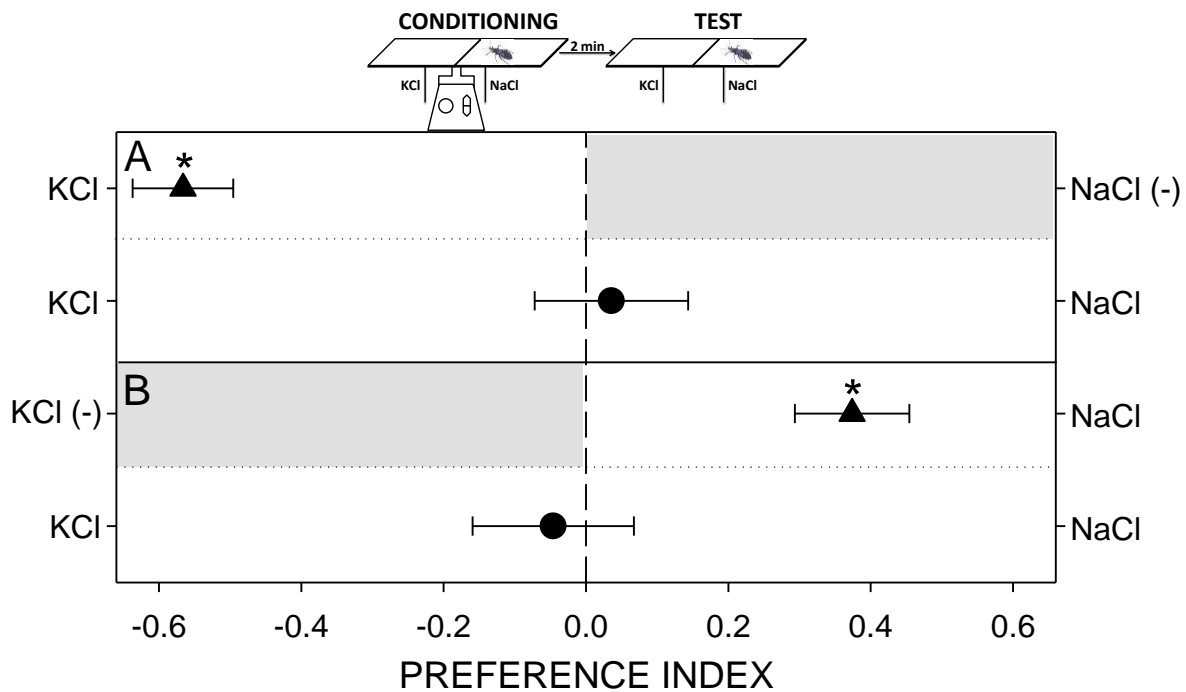


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779 **Figure 3. Salts discriminative assays through an operant conditioning protocol with NaCl(-) or**
780 **KCl(-).** During trainings (triangles in A and B) insects avoided the punished side of the arena (grey
781 shadowed) regardless if it was loaded with NaCl or KCl. During tests (circles in A and B), no
782 modifications of the innate behaviour were evinced, *i.e.* no preference when NaCl and KCl were
783 confronted. The Preference Index expresses the relative time spent at each side of the arena: 0 =
784 equal time at each side, -1 and 1 = full time spent at the left or right side of the arena, respectively.
785 Each point represents the mean (\pm s.e.m.) of 30 replicates. Asterisks denote statistical differences (p
786 < 0.05) after a One-Sample T-Test with expected value = 0.

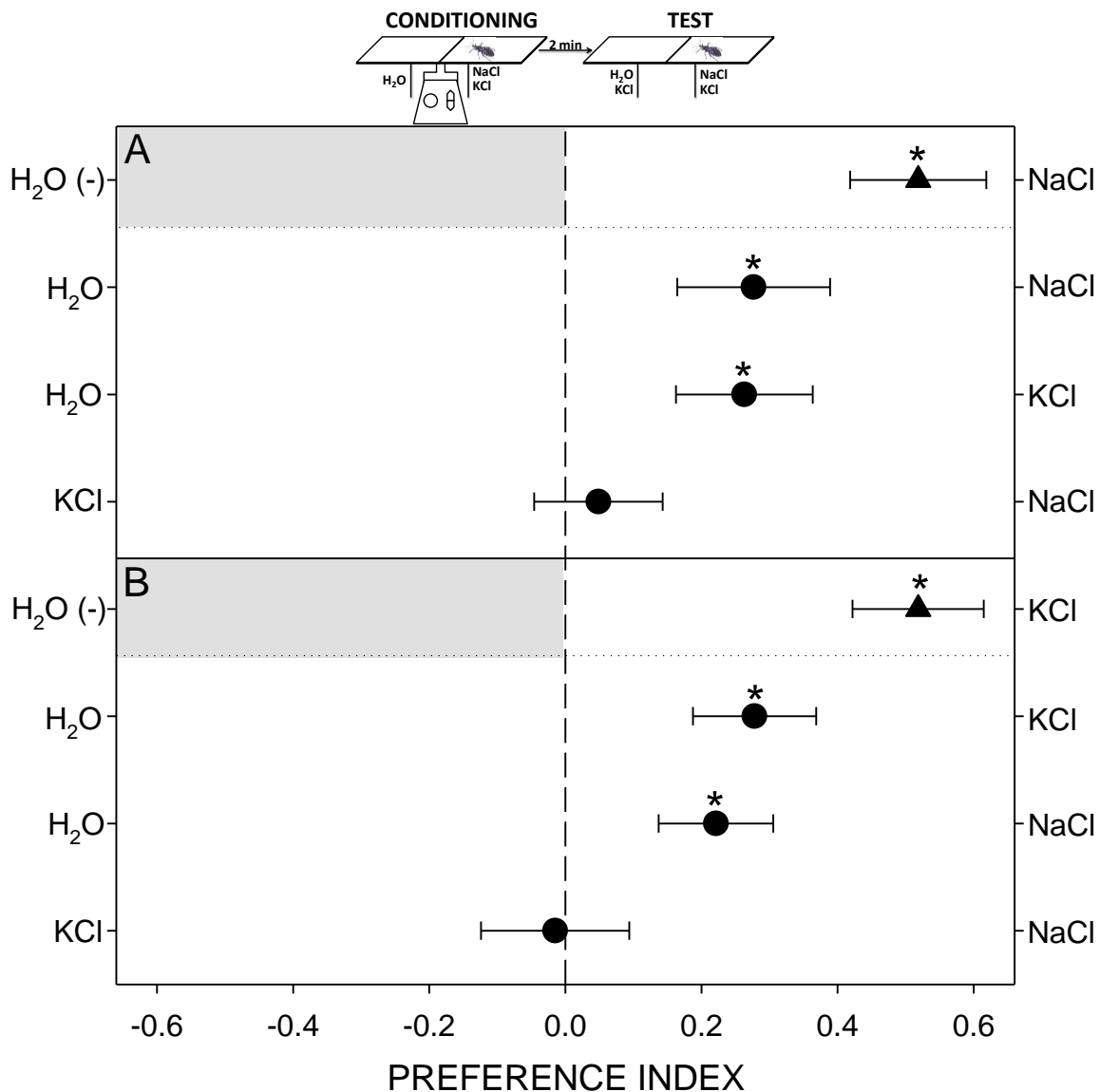


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790 **Figure 4. Salts discriminative assays through an operant conditioning protocol with H₂O(-).**
 791 During trainings (triangles in A and B) insects avoided the punished side of the arena (grey
 792 shadowed). During tests (circles in A and B), they preferred the salty side of the arena regardless if
 793 it contained the same salt used during training or the novel one. When confronted, no preference for
 794 one or the other salt was evinced. The Preference Index expresses the relative time spent at each
 795 side of the arena: 0 = equal time at each side, -1 and 1 = full time spent at the left or right side of the
 796 arena, respectively. Each point represents the mean (\pm s.e.m.) of 30 replicates. Asterisks denote
 797 statistical differences ($p < 0.05$) after a One-Sample T-Test with expected value = 0.

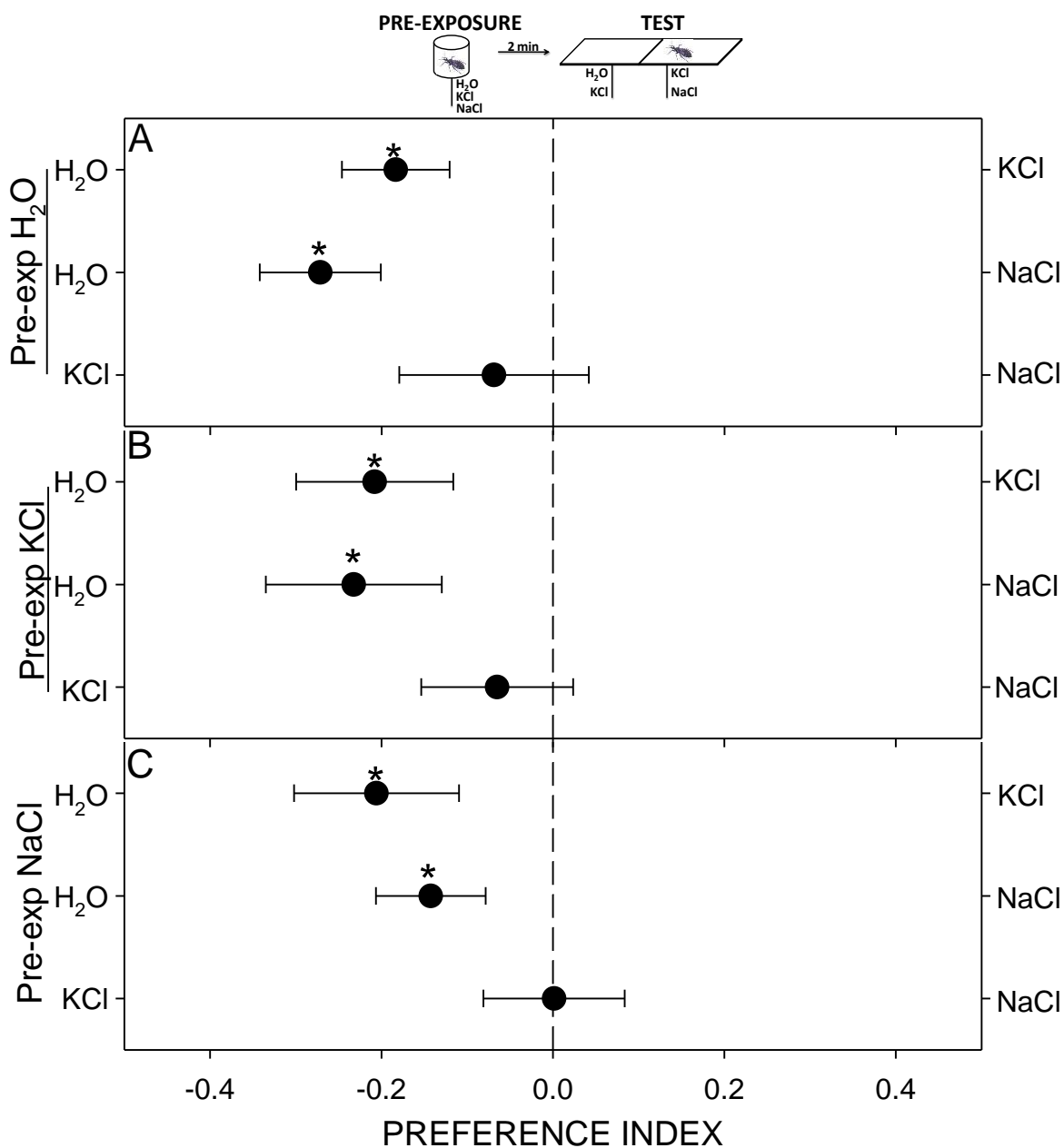


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800

801 **Figure 5. Pre-exposure to salts: effect on the perception of NaCl and KCl.** The innate repellence
 802 of insects to both salts was not modified by a pre-exposure to H₂O (A), KCl (B) or NaCl (C). The
 803 Preference Index expresses the relative time spent at each side of the arena: 0 = equal time at each
 804 side, -1 and 1 = full time spent at the left or right side of the arena, respectively. Each point represents
 805 the mean (\pm s.e.m.) of 30 replicates. Asterisks denote statistical differences ($p < 0.05$) after a One-
 806 Sample T-Test with expected value = 0.

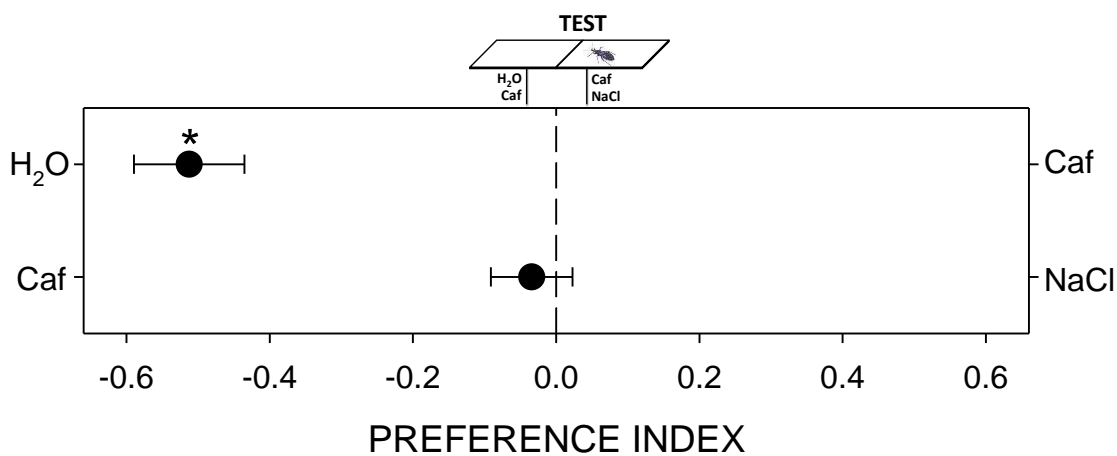


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809

810 **Figure 6. Innate responses to Caf.** Insects avoided the caffeine when confronted to H₂O but
811 exhibited no preference when it was simultaneously presented with NaCl. The Preference Index
812 expresses the relative time spent at each side of the arena: 0 = equal time at each side, -1 =
813 full time spent at the left or right side of the arena, respectively. Each point represents the mean (\pm
814 s.e.m.) of 30 replicates. Asterisks denote statistical differences ($p < 0.05$) after a One-Sample T-Test
815 with expected value = 0.

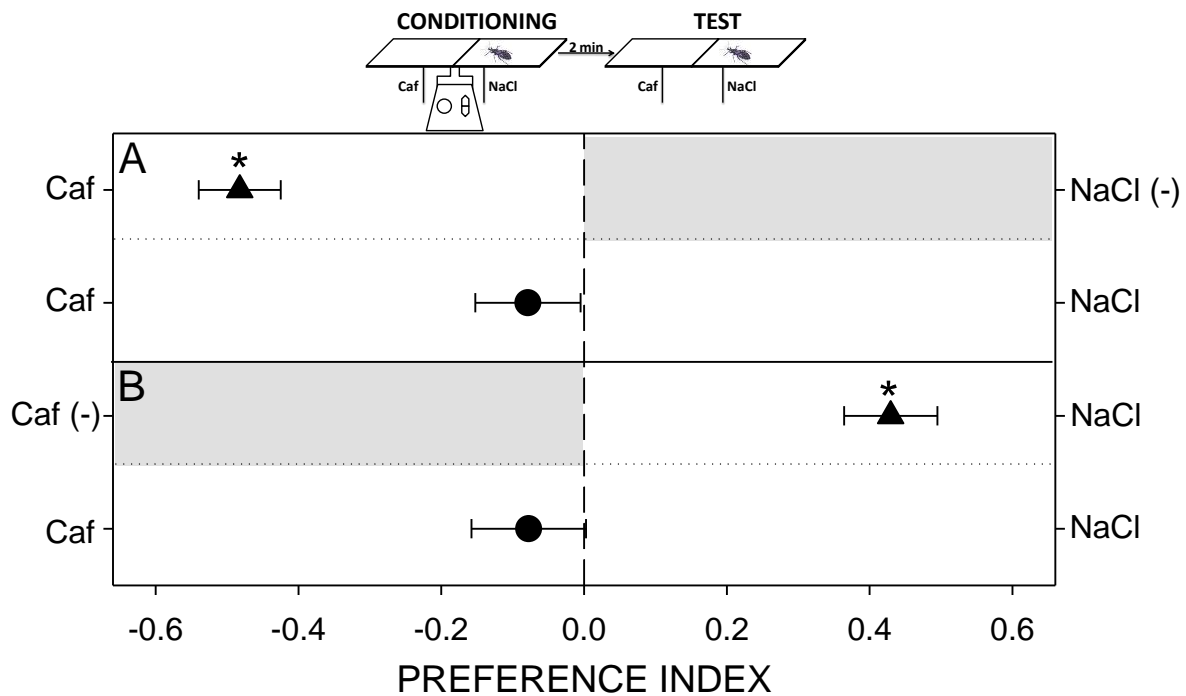


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819 **Figure 7. Discriminative assays between aversive stimuli of different gustatory modality**
820 **through an operant conditioning protocol with NaCl(-) or Caf(-).** During trainings (triangles in A
821 and B) insects avoided the punished side of the arena (grey shadowed) regardless if it was loaded
822 with NaCl or Caf. During tests (circles in A and B), no modifications of the innate behaviour were
823 evinced. The Preference Index expresses the relative time spent at each side of the arena: 0 = equal
824 time at each side, -1 and 1 = full time spent at the left or right side of the arena, respectively. Each
825 point represents the mean (\pm s.e.m.) of 30 replicates. Asterisks denote statistical differences ($p <$
826 0.05) after a One-Sample T-Test with expected value = 0.

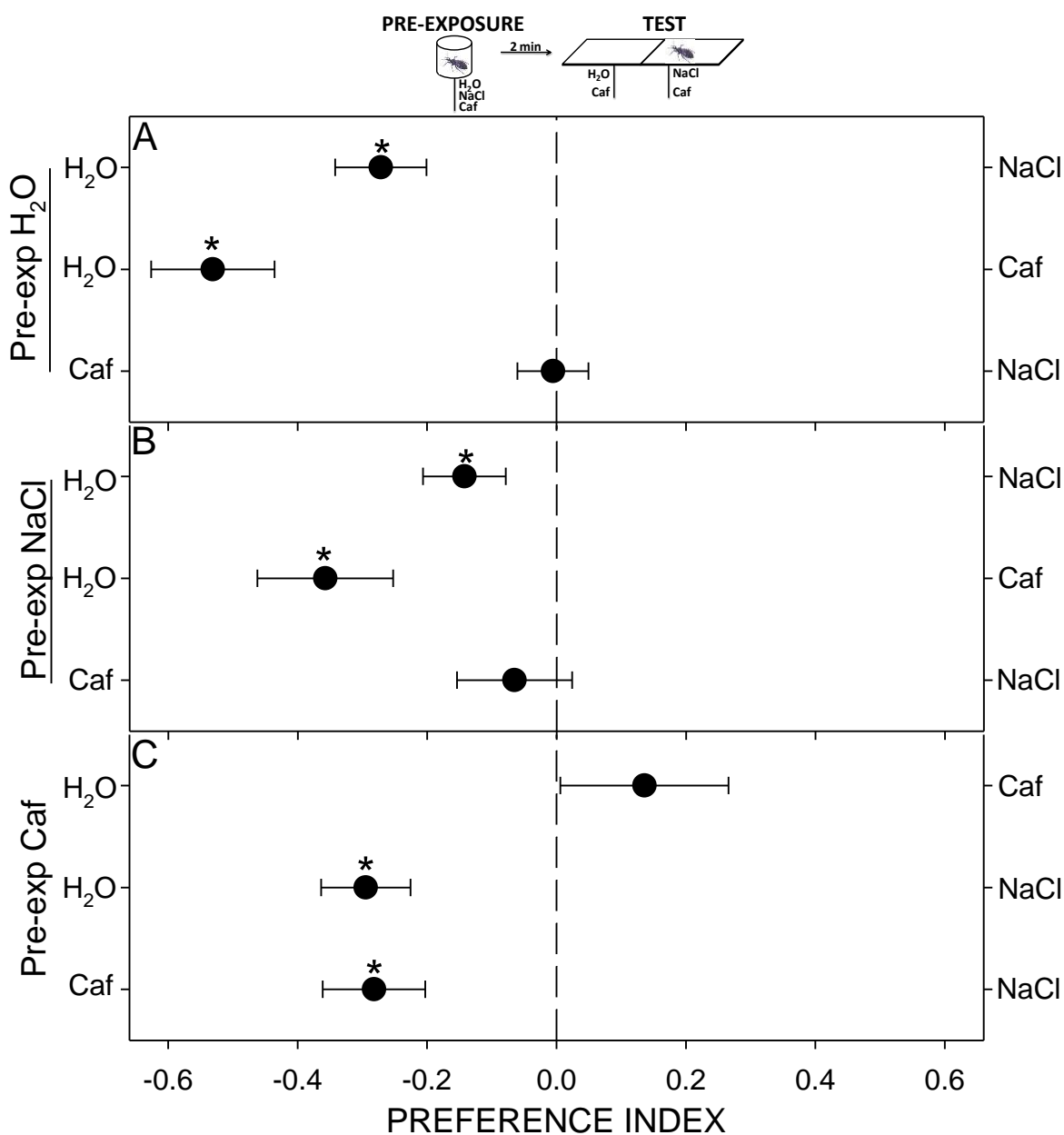


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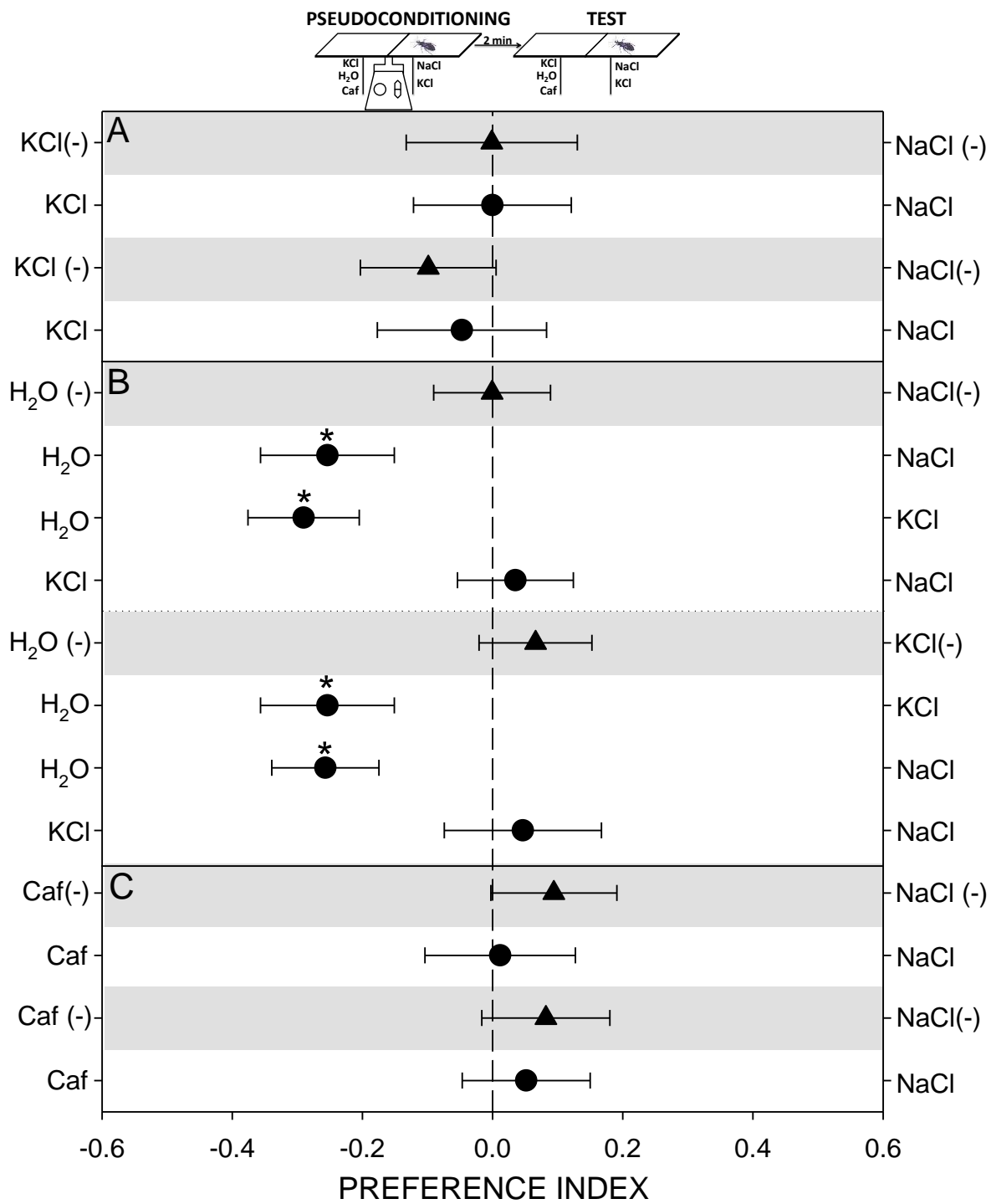
830 **Figure 8. Discriminative assays between aversive stimuli of different gustatory modality**
 831 **through a pre-exposure to NaCl or Caf.** A pre-exposure to H₂O (A) or NaCl (B) did not modify the
 832 innate repellence of insects to the salt nor to the alkaloid. However, a pre-exposure to Caf (C)
 833 interfered specifically in the perception of the alkaloid but not of the salt. The Preference Index
 834 expresses the relative time spent at each side of the arena: 0 = equal time at each side, -1 and 1 =
 835 full time spent at the left or right side of the arena, respectively. Each point represents the mean (\pm
 836 s.e.m.) of 30 replicates. Asterisks denote statistical differences ($p < 0.05$) after a One-Sample T-Test
 837 with expected value = 0.



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840 **Figure S1. Operant yoke control experiments: unpaired presentation of the chemical stimulus**
841 **and the mechanical disturbance.** During trainings (triangles in A, B and C), no chemical preferences
842 were observed (grey shadows evince that mechanical punishment could be delivered at both sides
843 of the arena). During tests (circles in A, B and C), no modifications of the innate behaviours were
844 displayed. (A) Yoke control of Fig. 3. (B) Yoke control of Fig. 4. (C) Yoke control of Fig. 7. The
845 Preference Index expresses the relative time spent at each side of the arena: 0 = equal time at each
846 side, -1 and 1 = full time spent at the left or right side of the arena, respectively. Each point represents
847 the mean (\pm s.e.m.) of 30 replicates. Asterisks denote statistical differences ($p < 0.05$) after a One-
848 Sample T-Test with expected value = 0.



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