

1 **On the adaptive meaning of chemically insignificant cues in multitrophic**
2 **caterpillar-ant-plant symbioses**

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4 Luan Dias Lima^{1,2}, Diego Santana Assis², Fábio Santos do Nascimento² and Lucas
5 Augusto Kaminski^{1,3*}

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7 ¹Programa de Pós-graduação em Biologia Animal, Departamento de Zoologia,
8 Universidade Federal do Rio Grande do Sul — UFRGS, 90650-001, Porto Alegre, RS,
9 Brazil.

10 ²Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Departamento de Biologia,
11 Universidade de São Paulo — USP, 14040-901, Ribeirão Preto, SP, Brazil.

12 ³Núcleo de Ecologia e Biodiversidade, Instituto de Ciências Básicas e da Saúde,
13 Universidade Federal de Alagoas — UFAL, 57072-900, Maceió, AL, Brazil.

14 ORCiDs: LDL, 000-0001-5414-3427; DSA, 0000-0002-8131-2617; FSN, 0000-0002-
15 3731-7111; LAK, 0000-0002-6468-0960

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20 *Author for correspondence: Lucas A. Kaminski

21 Email: lucaskaminski@yahoo.com.br

22 Ants use cuticular hydrocarbons (CHCs) as chemical cues for recognition, which
23 are exploited by myrmecophiles to usufruct the benefits of the social habit. We aimed to
24 identify the functional role of CHCs of two riodinid caterpillar species that obligately
25 associate with different multitrophic ant-plant symbioses. Experiments of ant acceptance
26 of caterpillars showed the concerted action of larval-ant-organs that produce liquid
27 rewards (tentacle nectary organs, TNOs) and luring signals (anterior tentacle organs,
28 ATOs) was key to ant appeasement and larval survival. Experiments changing the
29 symbiosis were often lethal for the caterpillars, mainly after emptying the secretions of
30 their TNOs and their ATOs were never activated. Chemical profiles of caterpillars were
31 insignificant. Field bioassays with chemically insignificant palatable insect prey attracted
32 fewer ants, indicating that insignificant cues may reduce the probability of ant attacks.
33 Thus, caterpillars control the emission of signals of ant-organs during symbiosis, whereas
34 the cues of CHCs are silenced. It is possible that a trade-off exists between signals under
35 control and non-controlled cues. Chemical insignificance may allow for a crucial period
36 for caterpillars to make tactical decisions to appease aggressive ants and could be
37 extended to other communication channels according to the sensorial universe of the
38 receiver.

39 **Keywords:** adaptive resemblance, biosemiotics, chemical transparency, defensive
40 strategy, mutualism, myrmecophily

41 1. Background

42 Ants have existed since the Upper Cretaceous, approximately 100 million years ago, and
43 constitute a dominant insect group in terms of abundance and biomass and are the main
44 invertebrate predators in terrestrial ecosystems [1]. These insects, as with other eusocial
45 insects, use intraspecific recognition systems and can distinguish between nestmates and
46 non-nestmates using mainly chemical cues, namely compounds present in their cuticle
47 called cuticular hydrocarbons (CHCs) (e.g. [2–4]). However, some organisms that
48 interact with ants, i.e., myrmecophiles, can conceal information of chemical cues,
49 allowing them to live with ants without being attacked and exploiting the benefits of the
50 social habit, either protection, food, or shelter [1–2]. This intimacy and/or integration into
51 ant nests and foraging territories on plants can be obtained through chemical strategies
52 that include adaptive resemblance (such as mimicry, crypsis or masquerade) or not, such
53 as chemical insignificance or conspicuousness [5–6].

54 Among adaptive resemblance strategies, chemical mimicry *sensu* [5] occurs when
55 an organism is detected by a receiver (ant, in this case) as something interesting, resulting
56 in action by the receiver. There are several examples of chemical mimicry strategy in the
57 literature, mainly of social parasites that mimic the profiles of their host ants (e.g. [3,7–
58 9]). These cases can be classified as aggressive mimicry (*sensu* [10]), since the emitter
59 mimics the receiver to obtain benefits that directly harm the fitness of the receiver colony.
60 Other strategies of adaptive resemblance include chemical crypsis, which occurs when
61 the emitter organism is not detected due to the blending of its chemical cues with the
62 background or chemical masquerade, when the organism is detected, but misidentified as
63 something uninteresting, and thus not causing a reaction by the receiver [5]. These two
64 kinds of strategies are broadly classified as chemical camouflage and have been reported
65 for herbivorous insects as a way to reduce ant attacks (e.g. [6,11–14]).

66 Chemical insignificance occurs when an organism has few chemical cues due to
67 the suppression or low concentration of cuticular compounds, which hampers its detection
68 [5,15–16]. This strategy has been reported for some social parasitic ants and one
69 caterpillar species (see [3,17]). Besides these strategies, ants can learn to recognise the
70 CHC profiles of mutualistic partners, even when they are neither congruent with those of
71 the ants nor the host plants, and thus avoid attacking them [18–20]. These non-congruent
72 profiles may constitute conspicuousness strategy (*sensu* [6]).

73 Myrmecophilous butterflies (Lepidoptera: Lycaenidae and Riodinidae) live in
74 association with ants during their immature stages (reviewed in [21]). In general, the ant-
75 caterpillar interaction is trophobiotic, a kind of mutualistic interaction mediated by sugar-
76 rich liquids that attract and serve as food resources for the ants, which in return offer
77 protection against natural enemies or clean their partner caterpillars [1,20,22–23]. Such
78 caterpillars use multimodal channels of communication to deal with ants, including
79 tactile, chemical, and acoustic signalling, through morphological adaptations called ant-
80 organs [22, 24–27]. Cues of CHCs can also mediate chemical strategies. Studies with the
81 chemical profiles of myrmecophilous caterpillars with different kinds of interactions with
82 ants (commensalism, mutualism, or parasitism), different degrees of specificity
83 (facultative or obligate), and different feeding habits (herbivorous, omnivorous or
84 carnivorous) have identified several kinds of chemical strategies (e.g. [6,17,28–29]).
85 Thus, myrmecophilous caterpillars are excellent models for studying the ecology and
86 evolution of different chemical strategies mediated by cuticular compounds [30].

87 Here, we assessed for the first time the chemical strategies mediated by CHCs of
88 two caterpillar species of the family Riodinidae. Several species of this family engage in
89 obligate trophobiotic interactions with ants (e.g. [31–32]), but the composition and
90 function of the CHCs of these caterpillars are unknown. The studied species use similar

91 host plants but obligately interact with different aggressive ant species that are
92 phylogenetically distant (figure 1). Thus, the aim of this study was to perform chemical
93 analyses and experiments in the field to identify the functional role of the CHC profiles
94 of these caterpillars. Studies with species in the sister family Lycaenidae showed that
95 trophobiotic caterpillars (that produce caloric rewards) generally have conspicuous CHCs
96 that may appease ant aggressiveness or be associated with resource availability [6,20,33].
97 Based on this information, we hypothesised that the CHC profiles in the two caterpillar
98 species would be informative to their respective tending ants and that, for this reason,
99 they would be recognised as mutualistic partners and would not be attacked.

100

101 **2. Methods**

102 **(a) Study site and organisms**

103 The study was performed at the Serra das Araras Ecological Station (EESA) located in
104 Porto Estrela (15°40'18" S; 57°13'47" W), state of Mato Grosso in the Center-West
105 region of Brazil. The vegetation of EESA constitutes a transition between the Cerrado
106 savanna and the Amazon forest [34–35]. The Amazon influence is stronger in the wetter
107 areas of semideciduous forest and gallery forest and many caterpillar-ant-plant systems
108 are typically Amazonian (e.g. [36]). Collections were carried out in the gallery forest of
109 the Salobro river and Boca do José. Caterpillars were sampled in these sites by visually
110 scanning vegetation. Sampling focused on two species of myrmecophilous butterflies
111 abundant at EESA: *Juditha molpe* (Hübner, [1808]) and *Nymphidium chione* Bates, 1867
112 (Lepidoptera: Riodinidae). The caterpillars of these butterflies are classified as obligate
113 myrmecophilous; *J. molpe* lives in association with *Dolichoderus bispinosus* (Olivier,
114 1792) (Dolichoderinae) and *N. chione* with *Pheidole biconstricta* Mayr, 1870

115 (Myrmicinae) (LAK, pers. observation) (figure 1). As observed for other obligate
116 myrmecophilous riordinids, the caterpillars of *J. molpe* and *N. chione* are polyphagous
117 throughout their geographic distribution, using mainly host plants bearing extrafloral
118 nectaries [37–41]. The main host plants used by *J. molpe* and *N. chione* in areas of gallery
119 forest at EESA are two species of *Inga* (Fabaceae). Plants of this genus have conspicuous
120 extrafloral nectaries and attract many ants [42]. The ants, *D. bispinosus* and *P.*
121 *biconstricta* (Myrmicinae), are top dominant ants in Neotropical forests and monopolise
122 liquid food sources on vegetation [43–45]. In the study site, both species visit the
123 extrafloral nectaries of *Inga* spp. but never occur together on the same plant (LAK,
124 unpublished data).

125 Samples of the studied organisms were also collected for subsequent chemical
126 analyses: *J. molpe* (first instar $n = 6$; second instar $n = 8$; third instar $n = 3$; fourth instar
127 $n = 2$; last instar $n = 1$), *N. chione* (third instar $n = 9$; fourth instar $n = 4$; last instar $n = 7$),
128 *D. bispinosus* ($n = 100$), *P. biconstricta* ($n = 100$), *Inga* sp. 1 ($n = 10$), and *Inga* sp. 2 (n
129 $= 10$). These samples were frozen and kept at about -20°C until extraction of their
130 cuticular compounds and subsequent chemical analyses.

131

132 (b) Experiments of ant acceptance of caterpillars

133 Experiments were performed on ant acceptance of caterpillars to assess the role of ant-
134 organs in ant-caterpillar interactions and the obligatoriness of this symbiosis. The
135 experiments were performed on host plants in the field and consisted of two parts. For the
136 control (without changing the associated ant), final instar (fourth or fifth) caterpillars of
137 *J. molpe* and *N. chione* were removed from their host plants and transferred to another
138 host plant of the same species occupied by their associated ant, i.e. *J. molpe* with *D.*

139 *bispinosus* ($n = 17$) and *N. chione* with *P. biconstricta* ($n = 15$). For the treatment (change
140 to the non-associated ant), the caterpillars were transferred to another host plant of the
141 same species occupied by the non-associated ant species, i.e., *N. chione* with *D.*
142 *bispinosus* ($n = 15$) and *J. molpe* with *P. biconstricta* ($n = 16$). All caterpillars were placed
143 in small plastic pots with food, using careful manipulation with sterilized entomological
144 tweezers, and being kept for 24 h without ants prior to carrying out the experiments. The
145 caterpillars were then placed on plants in a manner that allowed the ants enough time to
146 spontaneously find them. This practice was fundamental because quick movements
147 and/or disturbances on the vegetation near the ants can immediately trigger aggressive
148 responses from them, which would invalidate the acceptance trail.

149 The experimental plants (*Inga* spp.) were selected along a transect of
150 approximately 1000 m based on plant species and presence of associated ants.
151 Observations were made for 5 min, starting from the first contact between a worker ant
152 and the placed caterpillar, which could occur after a variable amount of time. Behavioural
153 responses were observed after these initial contacts and were classified at the end of the
154 5 min into two categories: 1) acceptance: ants tending the caterpillars without biting while
155 antennating the ant-organs, i.e., tentacle nectary organs (TNOs), and anterior tentacle
156 organs (ATOs) are constantly activated as described in the ‘enticement and binding’
157 process (*sensu* [25]); or 2) rejection: ants attacking the caterpillars with biting followed
158 by recruitment of other ants. Each caterpillar and each plant were used only once in the
159 experiments. Moreover, the action of ant-organs was observed *ad libitum* [46] during the
160 acceptance experiments as well as their effects on the tending ants.

161

162 (c) **Chemical analyses**

163 The cuticular chemical composition of the studied ants, caterpillars, and host plants was
164 assessed by extracting the compounds following [6] by immersing the samples for 5 min
165 in 5 ml of hexane (99%, SupraSolv, Merck, Germany), followed by removal from hexane
166 with entomological tweezers that were previously cleaned with hexane. Pools of about 10
167 individuals (workers) per sample were used for tending ants, while for the caterpillars,
168 one individual was used when it was in the last instar or a pool of up to five individuals
169 when they were from first to the fourth instar. The cuticular compounds of the host plants
170 were extracted following the same steps as for the insects, but with 20 ml of hexane. Each
171 obtained extract was later evaporated under a fume hood and stored at -20°C until the
172 moment of analyses when they were redissolved in hexane (95%, *n*-hexane, Macron,
173 USA) and subsequently submitted to sonic baths.

174 All samples were analysed in a combined gas chromatography-mass spectrometer
175 GC-MS (GC-QP2010 Plus, Shimadzu, Japan) equipped with a silica capillary column
176 and using helium as a carrier gas following [47]. The conditions of the analyses were:
177 injection performed manually with 2 μL using a glass syringe; splitless injection; oven
178 temperature initially set at 150°C increasing $3^{\circ}\text{C}/\text{min}$ until reaching 250°C , which was
179 maintained for 10 min; injector temperature of 220°C ; temperatures of detectors and
180 transfer lines were 250°C and 200°C , respectively; ionization energy of 70 eV and a range
181 of 40–600 amu, with a scanning interval of 0.3 s.

182 Data were analysed with GC-MS software for Windows (Shimadzu Corporation),
183 and the compounds were identified based on their mass spectra and with the aid of a
184 standard solution (Sigma-Aldrich Chemical Co., USA) with synthetic linear
185 hydrocarbons from C_{21} to C_{40} , as well as by consulting the Wiley and NIST Libraries
186 database. Compounds that were present in less than half of the individuals belonging to a

187 group, and compounds contributing less than 0.5% to the total compounds, were excluded
188 from the analyses.

189

190 **(d) Bioassays with chemically significant and insignificant model prey**

191 Our initial hypothesis was that the cuticular compounds of the caterpillars would be
192 informative (i.e. their own chemical signature) and, for this reason, they would be
193 recognised and would not be attacked by the ant species they interact. However, the
194 chemical analyses suggested that these caterpillars are chemically insignificant (see
195 Results). Thus, we aimed to test the adaptive value of the chemical insignificance in the
196 myrmecophily by performing field bioassays to examine the behavioural responses of the
197 two ant species to the cuticular compounds of larvae of *Tenebrio molitor* Linnaeus, 1758
198 (Coleoptera: Tenebrionidae) a model of a palatable insect prey to be offered to the ants.
199 We hypothesised that the chemically insignificant larvae would be less attractive and
200 recognised as potential prey, reducing attack time and probability. The larvae used were
201 previously killed by freezing at -20°C and had a drop of hexane dripped on the cuticle
202 (control) (chemically significant, with their normal CHCs) or that had their cuticular
203 compounds extracted by hexane during 10 min at room temperature (treatment)
204 (chemically insignificant, with reduction of CHCs). According to [48], the time of 10 min
205 was sufficient to remove virtually all CHCs of *Blattella germanica* (Linnaeus, 1767)
206 (Blattellidae) nymphs also working for Lepidoptera caterpillars [12].

207 The larvae were glued with white glue on leaves of removed branches of the *Inga*
208 host plants of the studied caterpillars. Entomological tweezers, cleaned with hexane, were
209 used to manipulate the larvae. The larvae were placed inside a small polystyrene foam
210 cooler with rigid refrigerant bottles and kept cold until the moment of the bioassays. The

211 removed branches with the experimental glued larvae were offered on the ground, at the
212 base of the plants where ants were foraging, such that the ants could spontaneously find
213 them. Pairs of control and treatment larvae were offered simultaneously, at a distance of
214 at least 30 cm to avoid the recruitment effect of one larva on the other. A video of 5 min
215 was recorded for each bioassay using a camera (Handycam Hdr-CX405 HD, Sony,
216 Japan), starting from the first contact between a worker ant and the glued larva. The
217 videos were later analysed and the total number of ants that touched a larva until the end
218 of the bioassay was registered. Thirty replicates of this bioassay were performed (15 for
219 each ant species).

220

221 (e) **Statistical analyses**

222 The behavioural responses of the ants in the caterpillar acceptance experiments
223 were assessed by performing the chi-square test (χ^2) for comparison (or heterogeneity)
224 between the proportions of the total number of caterpillars being accepted or rejected
225 (control and treatment). The tested hypothesis was that the proportion of caterpillars
226 accepted and rejected did not differ significantly between the control and treatment
227 according to ant species.

228 The videos of the bioassays with chemically significant and chemically
229 insignificant larvae were analysed by a Recruitment Index (RI) of the ants modified from
230 [12]. The RI (mean \pm SE) was calculated as the percentage of ants that touched larvae of
231 the total number of ants recruited for the larvae (control and treatment) during 5 min.
232 With the total number of ants recruited the RI was calculated as follows:

233

$$\text{RI} = (\text{TL}/\text{TL}+\text{CL}).100;$$

234 where RI is the Recruitment Index; TL is the number of ants that touched the treatment
235 larva and; CL is the number of ants that touched the control larva. Thus, RIs values of 50
236 or near 50 indicate no difference in the recruitment between control and treatment, those
237 higher than 50 indicate higher recruitment for treatment larva, and those lower than 50
238 indicate higher recruitment for control larva.

239 Moreover, the chi-square test (χ^2) was also performed, as previously mentioned,
240 for the bioassay videos of chemically significant larvae (with CHCs) and chemically
241 insignificant larvae (with reduction of CHCs), but without subtracting 0.5 and comparing
242 the total number of recruited ants for larvae (control and treatment). The hypothesis tested
243 was that the proportion of ants recruited did not differ significantly between the control
244 and treatment according to ant species.

245 We followed the methodology of [6] the percentages of absolute abundance of the
246 cuticular compound extracts were calculated considering them as 100%. These data were
247 then used to calculate the quantity of each separate compound (relative abundance)
248 expressed as a percentage of the total occurrence of a class of substance. A principal
249 component analysis (PCA) was performed using these data to verify similarities or
250 differences among them. All analyses were performed using R environment [49].

251

252 **3. Results**

253 **(a) Experiments of ant acceptance of caterpillars**

254 The caterpillar acceptance experiments (control) showed that *J. molpe* and *N. chione* are
255 almost always accepted by their associated ants when transferred to another host plant
256 (figures 1 and 2a), i.e., the caterpillars successfully appeased the ants. There was no

257 heterogeneity regarding ant species, which means they had similar behavioural responses
258 considering the caterpillars.

259 The caterpillars used the concerted action of their ant-organs to appease the
260 tending ants. The ants did not recognise the caterpillars immediately, only when they
261 antennated the region near the ant-organs. The TNOs were promptly everted after rapid
262 antennation (palpation), liberating a drop of secretion that was imbibed by the ants. After
263 this first contact with the TNOs, ants started being recruited and the ATOs were everted.
264 The action of this organ promoted excitement in the ants, which started “protecting” the
265 caterpillar and reestablishing the symbiosis (figures 1*c,f*).

266 On the other hand, there was a significant difference between the aforementioned
267 controls and treatments ($\chi^2 = 35.41$; $df = 1$; $p < 0.001$) for the experiments transferring
268 caterpillars (treatment) between plants occupied with different ant species, which was
269 often lethal for the two caterpillar species (figure 2). As in the control group, the
270 caterpillars were firstly ignored until the ants found the TNOs. The, the antennating
271 resulted in a drop of nectar being secreted that was imbibed by the ant. This appeasement
272 lasted for a short amount of time, until the emptying of the secretions from the TNOs or
273 when the ants antennated the region near the ATOs, which were never everted during the
274 treatments. The emptying of the secretions of the TNOs seemed to result in more
275 inspection of the caterpillar by the ants followed by biting. From this moment on, the ants
276 started attacking the caterpillars and recruiting other ants (figures 2*b,d*). During the time
277 that these experiments lasted, nearly all the caterpillars were killed. For *J. molpe*
278 caterpillars with *P. biconstricta* (treatment), even soldiers with their robust mandibles,
279 and who do not usually climb the plants, were recruited to help tear the caterpillars apart.

280

281 **(b) Chemical analyses**

282 Samples of the caterpillars showed very low concentrations of CHCs, and so an extract
283 was performed with all previously used samples totalling 20 caterpillars of each species
284 to identify the compounds that remained in very low concentrations (electronic
285 supplementary material, figure S1, table S1). The compounds identified in these extracts
286 were mainly *n*-alkanes (C₂₅–C₂₉) for *J. molpe* and (C₂₁–C₃₁) for *N. chione* (electronic
287 supplementary material, figure S1, table S1). The compounds identified in the ants were
288 in a much greater quantity and concentration, with branched alkanes being the main
289 compounds (electronic supplementary material, figure S1, table S1). There were
290 significant differences between the cuticular compounds of the two species of caterpillars
291 ($p < 0.001$) and also between those of caterpillars and ants ($p < 0.001$) (electronic
292 supplementary material, figure S2). The compounds of the plants were not identified as
293 they were visually much very and in a much greater quantity and concentration than those
294 of the caterpillars, which shows that the caterpillars are not chemically camouflaged
295 (electronic supplementary material, figure S1).

296

297 **(c) Bioassays with chemically significant and insignificant model prey**

298 There were differences in the recruitment of *D. bispinosus* and *P. biconstricta* to *T.*
299 *molitor* larvae between control and treatment with a tendency for more ant to recruitment
300 to larvae with CHCs (control): 19.93 ± 3.92 for *D. bispinosus* on control and 10.6 ± 2.8
301 on treatment; 64.13 ± 15.87 for *P. biconstricta* on control and 50.73 ± 14.26 on treatment
302 ($\chi^2 = 53.31$; $df = 13$; $p < 0.001$) (figure 3).

303 However, there was heterogeneity regarding ant species ($\chi^2 = 406.76$; $df = 13$; p
304 < 0.001). There was higher recruitment of *D. bispinosus* for larvae of *T. molitor* with

305 CHCs than to larvae with reduced CHCs ($RI = 35.21 \pm 6.82$) and there was a clear
306 tendency for higher recruitment to larvae with CHCs ($\chi^2 = 42.79$; $df = 14$; $p < 0.001$)
307 (figure 3a).

308 The recruitment of *P. biconstricta* was also higher for larvae with CHCs than those
309 with reduced CHCs ($RI = 43.45 \pm 5.95$), however, there was not a clear tendency for
310 higher recruitment to larvae with CHCs (figure 3b).

311

312 **4. Discussion**

313 The studied caterpillar species were generally accepted by their associated tending ants
314 and killed when they were transferred to host plants occupied by non-associated ants.
315 Experiments changing myrmecophilous riodinid caterpillars from their associated ants
316 have demonstrated that they may be accepted by some ant species, but sometimes
317 attacked by others [31,50–51]. In these studies, failure in signalling has been proposed as
318 a factor that could trigger the observed negative reactions, a hypothesis that is
319 corroborated by the experiments of the present study as the caterpillars controlled the
320 emission of specific signals from their ant-organs. There is evidence that ants use CHCs
321 to recognise nestmates and non-nestmates and attack species with which they do not have
322 mutualistic interactions (e.g. [1,15,52]). It has been shown that some CHCs of caterpillars
323 associated with many ant species (facultative myrmecophily) may trigger aggressive
324 responses or appeasement in different ant species [8].

325 The caterpillars studied here are presumed to have mutualistic interactions with
326 ants, so our initial hypothesis that caterpillars would have informative CHC profiles, as
327 reported for other trophobiotic species (e.g. [6,18–20]), was not confirmed. The strategies
328 of chemical mimicry and chemical camouflage were discarded due to the low

329 concentrations and number of compounds and incongruence with the CHC profiles of the
330 ants or host plants. Moreover, the present study demonstrates, through chemical analyses,
331 that the caterpillars use the chemical insignificance strategy, thus silencing their cuticular
332 chemical cues. Chemical insignificance has been reported as an effective strategy to
333 decrease recognition and attacks by ants [3,5,15]. This strategy has been commonly
334 reported for several ant parasite taxa, i.e., ants, arachnids, isopods, collembolans,
335 coleopterans, dipterans, gastropods, and zygentomans [3 and references therein,8,53–55].
336 Within Lepidoptera, this strategy has already been reported for the pupa of *Feniseca*
337 *tarquinius* (Fabricius, 1793) [28] and the caterpillar of *Arhopala zylda* Corbet, 1941 [17].
338 However, the adaptive basis of this chemical insignificance has never been tested and
339 continues to be a little-understood phenomenon among myrmecophilous organisms.

340 In the present study, caterpillars were observed using their ant-organs when
341 discovered by ants. Glands that produce caloric rewards (TNOs) were active regardless
342 of the tending ant species. However, other factors, such as luring signals from caterpillar
343 ATOs, may have influenced associated ants not to attack during the trials but were not
344 used with non-associated ants. The concerted use of ant-organs was also observed for the
345 riodinid caterpillar *Thisbe irenea* (Stoll, 1780), which could appease associated ants yet
346 never evert ATOs when attacked [25].

347 The myrmecophilous riodinid species of the subtribes Lemoniadina and
348 Nymphidiina (*sensu* [56]) have TNOs that secrete nutritional substances and ATOs that
349 are everted during symbiotic interactions with ants [25,57–59]. According to [17], the
350 caterpillars of *Arhopala* (Lycaenidae) generally have dorsal nectary organs (DNOs) that
351 produce nectar rewards analogous to the TNOs in riodinid caterpillars, posterior tentacle
352 organs (TOs) analogous to ATOs in riodinid caterpillars [51], and pore cupola organs
353 (PCOs) that supposedly produce substances that decrease attacks by the ant

354 *Crematogaster decamera* Forel, 1910. Indeed, other authors have shown that DNO
355 secretions from lycaenids can appease ants [60].

356 The chemical insignificance strategy has arisen independently in the two butterfly
357 families of species with trophobiotic caterpillars on plants, as well as in other
358 myrmecophiles with social parasitic lifestyles [3,17,28]. In addition to chemical analysis,
359 the results of the field bioassays show that the removal of CHCs decreases the recruitment
360 of ants to a palatable model insect, which suggests a possible adaptive value of the
361 chemical insignificance in this case by allowing a crucial period of time for the caterpillar
362 to decide on tactics to use to appease aggressive ants. This can be added to the fact that
363 ants will even prey on mutualistic aphids when they do not secrete sugar-rich liquids (e.g.
364 [61–62]). In the present study, caterpillars that were transferred to another host plant were
365 attacked and killed by non-associated tending ants. This suggests that other factors must
366 be acting in the establishment of stable caterpillar-ant symbioses, but reinforces the
367 importance of a chemical strategy for caterpillars to keep close to aggressive ants and
368 avoid recognition as prey, thereby reducing the possibility of attacks. Caterpillars of the
369 present study were also observed using secretions as a way to keep close and appease
370 ants, as well as avoid attacks, which worked only with their associated ant partner.
371 Something similar was observed by [17], when *Arhopala* caterpillars were transferred to
372 other plants, although different for *A. zylida* which also used the chemical insignificance
373 strategy and was ignored by ants. The riodinids studied here may use the same tactics as
374 *Arhopala* caterpillars as they also have ant-organs and secrete nectar rewards for
375 aggressive associated ants to avoid attacks.

376 According to [25] the ATO chemistry is important for caterpillars to be accepted
377 by certain species of ants and it does not function with others that kill them. This could
378 be related to chemical signals that are specific to their associated ants and do not work

379 with ants from different subfamilies as they may have coevolved. This is because there
380 are chemical cues that can provide information unintentionally and can act directly as
381 precursors for the evolution of chemical signals that evolved due to a specific effect,
382 providing information intentionally, and work because the receiver's response also
383 evolved [63–64]. Thus, understanding the difference between cues and signals is essential
384 to understanding the role of CHCs in myrmecophilous systems as cues and signals can
385 act simultaneously [64] and can interfere with the fitness of myrmecophiles.

386 The present study found an expression of chemical signals from ant-organs that
387 are intentional, however, the cuticular chemical cues do not allow this expression. The
388 results reveal a possible trade-off between controlled signals and non-controlled cues in
389 obligate multitrophic caterpillar-ant-plant symbioses. In this context, the insignificance
390 of chemical cues may be adaptive in multitrophic interactions that involve complex
391 multimodal signals by avoiding deaths in unpleasant encounters with non-associated ants.

392 The insignificance of chemical cues is a strategy that could be extended to other
393 channels of communication according to the sensorial universe of the receiver and the
394 degree of intimacy of the interaction. Caterpillars use a multimodal system to deal with
395 ants [27], but this was not evaluated in the present study. For example, the apparent loss
396 of the ability to produce sounds by caterpillars of some species of *Nymphidium* (see [26])
397 may be related to selection for cue insignificance. Another remaining question is whether
398 chemical insignificance can also decrease attacks of other natural enemies such as
399 chemically oriented predators and parasitoids.

400

401 **Ethics.** The collection and transport of the specimens were authorised by Sistema de
402 Autorização e Informação em Biodiversidade (SISBIO) (licence no. 62345-1).

403 **Data Accessibility.** The data that support the findings of this study are openly available
404 on Figshare [65]. Supplementary information is provided in the electronic supplementary
405 material [66].

406 **Authors' contributions.** L.D.L.: conceptualization, data curation, formal analysis,
407 funding acquisition; investigation; methodology; software; validation; visualization;
408 writing – original draft; writing – review & editing; D.S.A.: data curation; formal
409 analysis; software; validation; visualization; writing – review & editing; F.S.N.: funding
410 acquisition; investigation; project administration; resources; supervision; validation;
411 visualization; writing – review & editing; L.A.K.: conceptualization; data curation;
412 funding acquisition; investigation; methodology; project administration; resources;
413 supervision; validation; visualization; writing – original draft; writing – review & editing.
414 All authors gave final approval for publication and agree to be held accountable for the
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435

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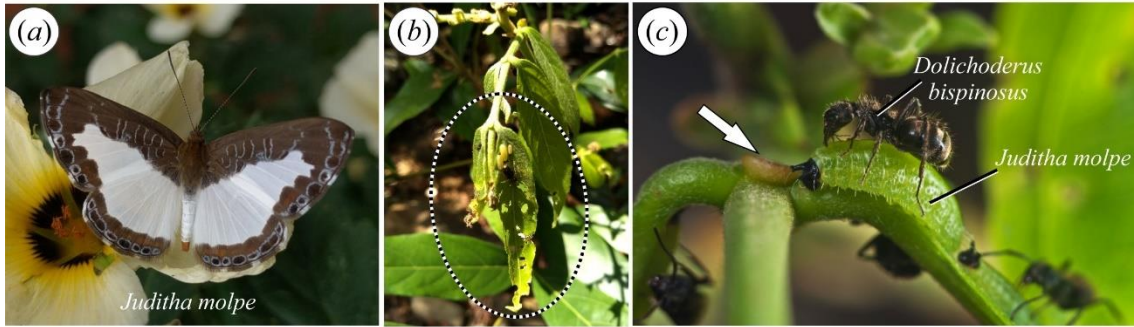
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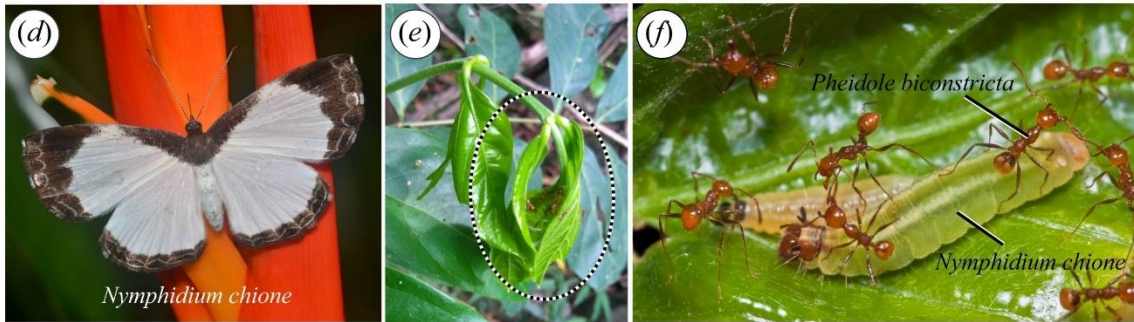
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Multitrophic “*Juditha molpe*–*Dolichoderus bispinosus*–*Inga*” symbiosis



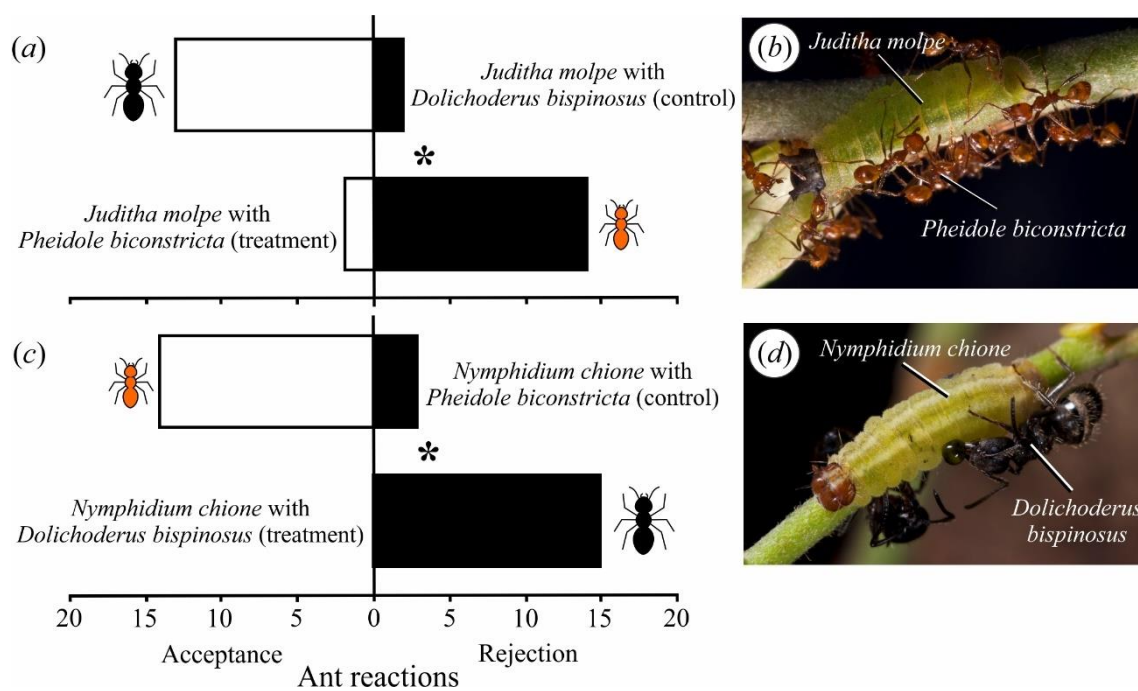
Multitrophic “*Nymphidium chione*–*Pheidole biconstricta*–*Inga*” symbiosis



636

637 **Figure 1.**

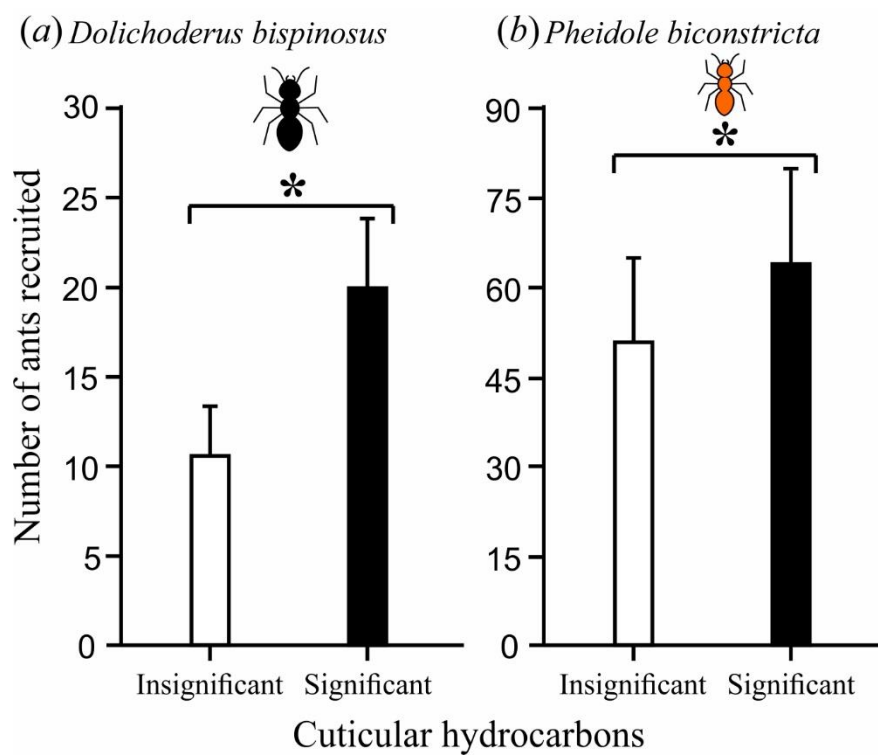
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639

640 **Figure 2.**

641



642

643 **Figure 3.**

644

645 **Figure legends**

646

647 **Figure 1.** The studied multitrophic caterpillar-ant-plant symbioses: (a) *Juditha molpe*
648 adult; (b) *Inga* branch with *J. molpe* caterpillars and *Dolichoderus bispinosus* ants
649 (dashed ellipse); (c) detail of *J. molpe* caterpillars and *D. bispinosus* ants, note the *Inga*
650 extrafloral nectary (white arrow); (d) *Nymphidium chione* adult; (e) *Inga* branch with *N.*
651 *chione* caterpillars and *Pheidole biconstricta* ants (dashed ellipse); (f) detail of *N. chione*
652 caterpillars and *P. biconstricta* ants. Photos by J. Movia[©] (a), H. Soares Júnior[©] (c,f), and
653 by L.L. Mota[©] (d).

654

655 **Figure 2.** Ant reactions in ant acceptance of caterpillar experiments on *Inga* spp. (a)
656 *Juditha molpe* caterpillars with associated tending ant *Dolichoderus bispinosus* (control)
657 and with non-associated *Pheidole biconstricta* (treatment); (b) detail of *J. molpe*
658 caterpillar attacked by *P. biconstricta* workers; (c) *Nymphidium chione* caterpillars with
659 associated tending ant *P. biconstricta* (control) and with non-associated *D. bispinosus*
660 (treatment); (d) detail of caterpillar attacked by *D. bispinosus* workers, note the
661 hemolymph droplet leaking through the caterpillar tegument. (*) = significant values (χ^2
662 = 35.41; $df = 1$; $p < 0.001$). Photos by H. Soares Júnior[©] (b,d).

663

664 **Figure 3.** Number of ants recruited in bioassays with palatable model prey (larvae of
665 *Tenebrio molitor*): chemically insignificant (with reduction of cuticular hydrocarbons,
666 white bars) and chemically significant (with cuticular hydrocarbons, black bars). (a)

667 *Dolichoderus bispinosus*; (b) *Pheidole biconstricta*. Values are means \pm SE; (*) =
668 significant values ($\chi^2 = 53.31$; $df = 13$; $p < 0.001$).