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Exotic predators can sequester and use novel toxins from exotic non-coevolved prey

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Abstract

Defensive chemicals of prey can be sequestered by some coevolved predators, which take advantage of prey toxins for their own defence. The increase in the number of invasive species in the Anthropocene has resulted in new interactions among non-coevolved predator and prey species. While novelty in chemical defence may provide a benefit for invasive prey against non-coevolved predators, resident predators with the right evolutionary pre- adaptations might benefit from sequestering these novel defences. Here, we chose a well-known system of invasive species to test whether non-coevolved predators can sequester and use toxins from exotic prey. Together with the invasive prickly pear plants, cochineal bugs (*Dactylopius* spp.) are spreading worldwide from their native range in the Americas. These insects produce carminic acid, a defensive anthraquinone that some specialised predators sequester for their own defence. Using this system, we first determined whether coccinellids that prey on cochineal bugs in the Mediterranean region tolerated, sequestered, and released carminic acid in the reflex-bleeding. Then, we quantified the deterrent effect of carminic acid against antagonistic ants. Our results demonstrate that the Australian coccinellid *Cryptolaemus montrouzieri* sequestered carminic acid, a substance absent in its coevolved prey, from exotic cochineal bugs. When attacked, the predator released this substance through reflex-bleeding at concentrations that were deterrent against antagonistic ants. These findings reveal that non-coevolved predators can sequester and use novel toxins from exotic prey and highlight the surprising outcomes of novel interactions that arise from species invasions.

Keywords: Ants; Carminic acid; Chemical defence; Cochineal bugs; Coccinellid; Invasive species.

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Background

Chemical defence is used by a wide variety of organisms, including animals, plants, and microorganisms, to protect themselves against antagonists (Speed *et al.* 2012). Defensive chemicals can be synthesised *de novo* or sequestered from food (Blum 2012). The term "sequestration" refers to the ability of some organisms to take up and accumulate defensive chemicals from their diet (Opitz & Müller 2009; Duffey 1980). Sequestration requires specialised adaptations for uptake, storage, and tolerance of toxic substances from a food source (Beran & Petschenka 2022). In a strict sense, sequestration involves that i) the toxin is obtained from a suitable diet; ii) the toxin passes from the gut to other tissues where it accumulates; and iii) the toxin provides a benefit to the organism that sequesters it (Sloggett 2022). Sequestration of different classes of defensive chemicals requires unique physiological adaptations, thus sequestration is most often found in dietary specialists, where the predictability of toxins encountered in food has favored the emergence of such adaptations in the process of coevolution (Opitz & Müller 2009, Beran & Petschenka 2022). Interestingly, not all sequestering species have coevolved with their toxic diet, as adaptations to a toxin in one diet may facilitate host shifts to a distinct diet sharing the same toxin (Petschenka *et al.* 2022).

Dietary sequestration of defensive chemicals from plants by specialised herbivores is widespread and has been extensively studied (Duffey 1980; Nishida 2002; Opitz & Müller 2009; Zvereva & Kozlov 2016). In contrast, dietary sequestration of defensive chemicals from prey by predators is less frequent, perhaps due to the inherently lower degree of dietary specialisation of most predators. Among the exceptions are predators with a narrow dietary range and/or a close evolutionary relationship with their prey (Table 1). For example, in *Rhabdophis* snakes, *R. tigrinus* sequesters defensive steroids from toads, while *R. nuchalis* sequesters these substances from fireflies (Yoshida *et al.* 2020).

Several species belonging to four families of passerine birds are believed to sequester batrachotoxin alkaloids from *Choresine* beetles (Dumbacher *et al.* 2004; Bodawatta *et al.* et al. 2023). Several amphibian families acquire alkaloids from different sources of arthropods, including beetles, ants, or mites (Saporito *et al*. 2012). In arthropods, several insect species are considered canthariphilous and consume considerable amounts of tissue from Meloidae and Oedomeridae beetles to accumulate cantharidin (Dettner 1997). The coccinellid *Coccinella septempunctata* accumulates pyrrolizidine alkaloids from the aphid *Aphis jacobeae* (Witte 1990), while the coccinellid *Hyperaspis trifurcata* sequesters carminic acid from cochineal bugs, *Dactylopius* spp. Interestingly, at least two additional predator species from the native range of cochineal bugs sequester carminic acid from their prey, including the moth *Laetilia coccidivora* and the fly *Leucopis bellula* (Eisner *et al.* 1980, 1994).

The exponential increase of invasive arthropod species in the last century has brought together predator and prey species that have not coevolved, and toxic invasive prey species may expose predators to substances not previously encountered during their evolution (Meyerson & Mooney 2007; Keller *et al.* 2011; Seebens *et al.* 2017). The 'novel weapons hypothesis' proposes that novel biochemical weapons can have a stronger effect in their exotic range because new competitors or attackers have not evolved with them (Callaway & Ridenour 2004). While such chemical novelty in defence is expected to primarily benefit prey species, it may also offer unique opportunities for predators, especially if they possess pre-adaptations to facilitate sequestration of these chemicals. The prevalence of toxin acquisition from diet in coccinellids suggest that this group of predators may have physiological adaptations that allow the uptake and storage of exogen toxins, being likely predisposed to adopt novel sources of toxins for sequestration. Some coccinellid species are known to acquire defensive chemicals from non-coevolved prey.

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For instance, *Harmonia axyridis*, native to northeastern Asia, acquires alkaloids from other coccinellids native to Europe (Hautier *et al.* 2008). There is also anecdotal evidence that *Coccinella undecimpunctata*, native to central Asia, acquires cardenolides from the aphid *Aphis nerii*, native to the Mediterranean basin (Rothschild *et al.* 1973). However, it is doubtful that the two previous cases constitute sequestration, as the diet sources might be toxic for these coccinellids, and it is unknown whether they gain adaptive benefits from the novel toxins. Therefore, it has not been demonstrated that these substances confer a defensive benefit to coccinellids (Sloggett 2022).

Coccinellids have several defence strategies against antagonists, including behavioural, physical, and chemical defences (Majerus et al. 2007). The diversity of defensive compounds found in coccinellids suggest that chemical defence plays a prevalent role in coccinellid evolution (Schröder et al. 1998; Sloggett 2022). These defensive chemicals can be released by coccinellids in an antipredator defensive behaviour known as reflex bleeding (Daloze *et al.* 1994; Glisan King & Meinwald 1996; Pasteels 2007). When attacked, they contract their muscles to induce the expulsion of hemolymph through dedicated openings, bringing both endogenous and/or sequestered substances present in the hemolymph in contact with the attacking predator (Roux *et al.* 2017; Hillyer & Pass 2020). Any substance a coccinellid can accumulate and tolerate in its hemolymph thus has the potential to become an added layer of defence.

Here, we chose a well-known system of invasive species to test whether non-coevolved coccinellids can sequester novel toxins from exotic prey and use these substances in reflex bleeding for a potential adaptive benefit. The cochineal bugs *Dactylopius* spp. are invasive in many countries around the world and exclusively feed on likewise invasive prickly pear plants (*Opuntia* spp.). The two genera are native to the Americas but were translocated around the globe as part of intercultural and economic exchanges (Chávez-

119　Moreno *et al.* 2009) . Nowadays, prickly pears and cochineal bugs are present in extensive

120　continental areas of Africa, Asia, Europe, and Oceania, as well as various islands (Mazzeo

121　*et al.* 2019). Cochineal bugs produce carminic acid, a substance exploited by humans as

122　red dye (Lloyd 1980). This anthraquinone plays a defensive role for cochineal bugs and

123　is highly deterrent against ants, which are among the most abundant potential predators

124　of cochineal bugs in their area of origin (Eisner *et al.* 1980, 1994). It is unclear if carminic

125　acid is deterrent for other predator species, although few non-specialist predators are

126　observed feeding on cochineal bugs in their native range (Gilreath & Smith Jr 1988;

127　Vanegas-Rico *et al.* 2010a; Kelly *et al*., 2022). Among the main specialist predators, the

128　coccinellid *H. trifurcata* feeds on cochineal bugs and by sequestering carminic acid is

129　itself defended from predatory ants (Eisner *et al.* 1994). In the cochineal invaded areas,

130　several native and naturalised coccinellids have been reported to feed on cochineal bugs

131　(Pettey 1950; Greathead 1972; El-Aalaoui *et al.* 2019b; Mazzeo *et al.* 2019; Mendel *et*

132　*al.* 2020). These observations suggest that coccinellids may generally be able to tolerate

133　carminic acid, which in turn may facilitate sequestration even by these non-coevolved

134　predators.

135　Based on these observations, we designed a series of experiments to test whether non-

136　coevolved predators that feed on exotic cochineal bugs can sequester carminic acid and

137　release it on demand to repel their potential enemies. We first identified and quantified

138　the resident predators that exploit cochineal bugs in the Mediterranean basin, an area

139　invaded by cochineal bugs. Then, we determined whether the most abundant predatory

140　coccinellid tolerated, sequestered, and released carminic acid in the reflex-bleeding.

141　Finally, we quantified the deterrent effect of carminic acid against antagonistic ants

142　present in the invaded area.

143　**Methods**

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Exploitation of cochineal bugs by resident predators

Ten prickly pear clusters infested by cochineal bugs were located and selected in semi-natural areas or field margins in eastern Spain (Supplementary materials, Figure S1). We sampled the clusters during two consecutive years (2020 and 2021) in late spring (10/06 – 20/06), mid-summer (27/07 – 5/08) and mid-autumn (15/10 – 26/10). In each prickly pear cluster, sixty cladodes were observed and those infested by more than ~50 cochineal bugs were sampled to identify potential predators. In each infested cladode, cochineal bugs were identified down to species level, and the predators preying on them were counted and identified down to species level. In addition, arthropods feeding on prickly pear extrafloral nectaries and arthropods observed on other parts of the cladode (not on cochineal colonies or extrafloral nectaries) were also identified and counted.

Initial observations identified *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) as the most abundant predator feeding on cochineal colonies. Previous laboratory studies found that this diet is suitable for *C. montrouzieri* (Baskaran *et al.* 1999; El-Aalaoui *et al.* 2019a; Zim *et al.* 2023). To confirm that cochineal bugs are a suitable and a persistently exploited diet for *C. montrouzieri* in Mediterranean basin, we determined whether *C. montrouzieri* consistently feeds on cochineal bugs, complete its development and persist for several generations feeding on this prey under field conditions. To do so, two clusters with high abundance of this species were selected and sampled every two weeks from April 2020 to January 2022. These clusters were in the localities of Godella [39º33'12.1''N 0º25'43.9''W 74 m above sea level (a.s.l.)] and Náquera (39º39'58.1''N 0º25'32.2''W 242 m a.s.l.). In each cluster, 60 cladodes infested by cochineal bugs were sampled at each sampling date and location, and the number *C. montrouzieri* was recorded per developmental stage.

Sequestration of carminic acid by *C. montrouzieri*

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169 To determine whether *C. montrouzieri* sequesters carminic acid when feeding on

170 cochineal bugs, we first quantified the concentration of carminic acid in the reflex-blood

171 of *C. montrouzieri* larvae and adults fed on cochineal bugs *versus* fed on mealybugs under

172 laboratory conditions. The presence of carminic acid in the reflex-blood (haemolymph)

173 would imply that the substance passes from the gut to other tissues and can be potentially

174 used as an added layer of defence. Adult *C. montrouzieri* were obtained from Koppert

175 Biological System and placed in plastic rearing boxes (20 x 20 x 20 cm, 50 adults per

176 box) for oviposition. Adults were fed on *Ephestia kuehniella* eggs that were renewed

177 every three days, polyester wadding (Rolta Soft®) was used as oviposition substrate

178 (Maes *et al.* 2014a) and moistened cotton wool was provided as water source. Polyester

179 wadding was inspected every three days and eggs were collected and placed individually

180 in 8 cm plastic petri dishes (with muslin on top) and maintained in a climatic chamber at

181 $25 \pm 1°C$, $75 \pm 5\%$ HR, photoperiod 14:10. Newly emerged *C. montrouzieri* larvae were

182 fed either on nymphs of the mealybug *Planococcus citri* (Risso) (Hemiptera:

183 Pseudococcidae) as control, or on nymphs of the cochineal bug *Dactylopius opuntiae*

184 (Cockerell) (Hemiptera: Dactylopiidae). Both prey were offered *ad libitum* and renewed

185 every three days. A piece of wet cotton wool was used as a water source. To have

186 sufficient prey stock, both preys were reared in the laboratory. *Planococcus citri* was

187 collected from citrus trees and reared on lemon fruits, while *D. opuntiae* was collected

188 from prickly pear plants and reared on prickly pear cladodes. Conditions in both cases

189 were $25 \pm 1°C$, $75 \pm 5\%$ RH, photoperiod 14:10.

190 To quantify the concentration of carminic acid in the reflex-blood of *C. montrouzieri*

191 larvae, five larvae fed on *P. citri* and eight fed on *D. opuntiae* were used two days after

192 reaching the third larval instar (5-6 mm length). The dorsal side of the abdomen of the

193 larvae was gently touched with a fine brush to obtain the reflex-blood (Grill & Moore

194   1998; Sato *et al.* 2009). To quantify the concentration of carminic acid in the reflex-blood

195   of *C. montrouzieri* adults, five adults fed on *P. citri* and five fed on *D. opuntiae* were used

196   four days after adult emergence (3.7-4.2 mm length). Since adults have a hardened

197   integument, fine brush touching was often insufficient to trigger reflex-bleeding, thus one

198   leg of the adult was touched with a hot needle (Happ & Eisner 1961; Peck 2000). In all

199   the cases, a glass microcapillary (1µL Drummond Microcaps®, Sigma-Aldrich) was used

200   to collect and measure the volume of reflex-blood (Knapp *et al.* 2018, 2020). Once

201   collected, each reflex-blood sample (between 0.03 - 1.34 µL) was immediately diluted in

202   350 µL of 100% MeOH. All the samples were stored at -20ºC until analysis.

203   The analysis and quantification of carminic acid was performed by high performance

204   liquid chromatography (HPLC) using Alliance equipment (Waters, Barcelona, Spain)

205   equipped with a 2695 separation module, a 2996 photodiode detector (DAD), a ZQ2000

206   single quadrupole mass detector, a thermostatic column oven and an automatic injector.

207   Extracts were separated using a C18 Tracer Excel 5 µm 120 OSDB reverse phase column

208   (250 mm x 4.6 mm) (Teknokroma, Barcelona, Spain). Elution was carried out using a

209   phase gradient composed of acetonitrile (Phase A) and 0.6% acetic acid (Phase B), using

210   LC-MS grade solvents. Conditions were as follows: start with 10% A for 2 min; a linear

211   increase to 75% A in the following 28 min; and returned to the initial conditions

212   maintaining 10% A for 5 minutes, with a total experiment time of 35 min at a flow rate

213   of 1 mL/min and an injection volume of 10 µL. For MS, negative electrospray conditions

214   were used and data was processed using Empower 2 software (Waters, Spain). Carminic

215   acid was identified by comparison of its retention time, UV-Vis and mass spectral

216   characteristics with an analytical standard (Sigma Aldrich®, Purity ≥ 90% HPLC, CAS

217   Number: 1260-17-9). Concentrations were determined using an external calibration curve

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218   with carminic acid (RT = 11.4 min; [M-H]$^-$ = 491 m/z). The concentrations obtained were

219   expressed in weight percentage (mg/100 mL of sample).

220   Once the concentration of carminic acid was determined for laboratory-reared *C.*

221   *montrouzieri*, we collected third-instar larvae feeding on *D. opuntiae* in the field to

222   confirm and quantify sequestration of carminic acid under natural conditions. We

223   followed the same methodology as above. One-way ANOVA was used to compare the

224   carminic acid volume collected from larvae and adults fed on the different diets and the

225   carminic acid concentration between laboratory-reared and field-collected larvae fed on

226   *D. opuntiae*.

227   **Deterrence of carminic acid against *C. montrouzieri* potential enemies**

228   To determine whether carminic acid can be used by *C. montrouzieri* as a chemical weapon

229   and potentially benefit the predator against its antagonists in the invaded area, we

230   performed predation and feeding assays using the ant *Lasius grandis* Forel

231   (Hymenoptera: Formicidae), which is native from the Mediterranean basin and we

232   frequently observed visiting prickly pears during our observations. In eastern Spain, *L.*

233   *grandis* is among the most abundant ants and its presence has been shown to negatively

234   affect coccinellid abundance (Mestre *et al.* 2016). Ants in the genus *Lasius* are known to

235   attack coccinellids in other regions (Marples 1993; Takizawa & Yasuda 2006) and the

236   presence of aggressive ants has been suggested to avoid the establishment of *C.*

237   *montrouzieri* in some areas (Greathead 1972).

238   *Release of carminic acid against ants under field conditions*

239   First, we tested whether *C. montrouzieri* larvae exhibit reflex-bleeding, and therefore

240   release carminic acid, when attacked by *L. grandis* under field conditions. We used third-

241   instar larvae that had been found feeding on cochineal bugs in the field, and individually

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242 placed them in *L. grandis* trails close to an ant nest in a local organic citrus orchard. We

243 tested a total of 20 larvae, and for each larva we quantified the number of ant-attacks and

244 defensive behaviours of *C. montrouzieri* larvae. Three defensive behaviours were

245 recorded: fled before ant attack, fled during ant attack, and reflex-bleeding. The

246 observations lasted from the release of the larvae until they managed to escape and moved

247 away from the ant trail. These observations never exceeded 3 minutes. For each repetition,

248 a different larva was used (*i.e*., the same larva was not tested multiple times).

249 *Deterrence of carminic acid against ants*

250 Second, we determined whether the ant deterrence toward *C. montrouzieri* reflex-blood

251 is likely due to carminic acid sequestration. Following the methodology of Eisner et al.

252 (Eisner *et al.* 1980), we offered ants a pairwise choice between 10^{-1} M aqueous sucrose

253 solution (control) and 10^{-1} M aqueous sucrose solution with carminic acid at four

254 concentrations: 10^{-4}, 10^{-3}, 10^{-2} and 10^{-1} M. Each concentration was tested separately and

255 was replicated five times. For each replicate, we used a rectangular plastic board with

256 eight feeding depressions, four depressions in two rows, separated by 5 cm. Control and

257 carminic acid solutions were alternated in each row (Figure S2). After placing the board

258 on the ground, we waited 15 minutes to allow ants to locate the food sources. Then, the

259 total number of ants at each solution was counted at 2-minute intervals for 20 minutes.

260 We averaged the numbers of the 11 sequential snapshot observations, and calculated the

261 deterrence rate of the experimental solution as [1-(mean number of ants feeding on the

262 carminic acid solution / mean number of ants feeding on control solution)]. To rule out

263 the possibility that the ants discriminated against the experimental solutions because of

264 their color, tests were conducted in darkness by placing a cardboard box with open bottom

265 over the boards. This was because the ant *L. grandis* is also active at night (Pekas et al.

266 2011). Observations were done through a translucid red-coated paper on top of the box.

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267   A Generalized Linear Model (GLM) with binomial error structure was used to evaluate

268   the effect of carminic acid concentration on the deterrence against ants. Deterrence rate

269   was used as response continuous variable and carminic acid concentration in the sucrose

270   solution was used as predictor continuous variable. All statistical analyses were carried

271   out in R, version 4.2.2 (R Core Team, 2022). We used the package "ggplot2" (Wickham,

272   2016) to build the graphs.

273   **Results**

274   **Exploitation of cochineal bugs by resident predators**

275   All identified cochineal bugs belonged to the species *D. opuntiae*. The most abundant

276   predator observed in cochineal colonies was the coccinellid *C. montrouzieri*, and it was

277   observed feeding in seven out of the ten sampled sites (Table 2). Density of *C.*

278   *montrouzieri* and geographical distribution increased from spring to autumn. Two other

279   coccinellids, *Chilocorus bipustulatus* (Linnaeus) and *Scymnus interruptus* Goeze, were

280   found on cochineal colonies in one site each, but their abundance and distribution were

281   extremely low. In addition, four ant species were identified in cochineal infested

282   cladodes: *L. grandis, Tapinoma nigerrimum* (Nylander)*, Crematogaster scutellaris*

283   (Olivier) and *Camponotus lateralis* (Olivier). These ants were observed feeding on

284   prickly pear extrafloral nectaries and walking on the cladode, but they were not observed

285   walking on or attacking cochineal colonies (Table S1). Other predator species were also

286   identified feeding on the extrafloral nectar or searching and resting on the cladode, but

287   their abundance was low (Table S1; Figure 1G).

288   All larval instars and adults of *C. montrouzieri* were found feeding on cochineal bugs

289   (Figure 1 and 2), and individuals pupated in prickly pear clusters infested by cochineal

290   bugs (Figure 1). The first individuals of *C. montrouzieri* on cochineal colonies were

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291   observed between May and June. The density of this coccinellid increased throughout the

292   summer and peaked in September-October (Figure 2). Multiple developmental stages of

293   the predator *C. montrouzieri* coexisted in cochineal colonies from May to December in

294   2020 and 2021 (Figure 1 and 2). The proportion of the different developmental stages

295   was very heterogeneous across the year.

296   **Sequestration of carminic acid by *C. montrouzieri***

297   Touching of the dorsal side of *C. montrouzieri* larvae with a fine brush caused the release

298   of reflex-blood droplets at multiple reflex-bleeding sites, and not necessarily from the

299   touched site. The volume of reflex-blood collected from laboratory-reared larvae was

300   0.34 ± 0.08 µL when fed on *P. citri,* and 0.66 ± 0.15 µL when fed on *D. opuntiae*. This

301   volume did not significantly differ between both diets ($F_{1, 11} = 2.5$, $P = 0.14$). The volume

302   of reflex-blood collected from field-collected larvae fed on *D. opuntiae* was 1.01 ± 0.12

303   µL. This volume did not differ significantly from laboratory-reared larvae fed on *D.*

304   *opuntiae* ($F_{1, 17} = 3.37$, $P = 0.08$). The color of the larvae reflex-blood ranged from yellow

305   to orange in larvae fed on *P. citri*, and from red to garnet in larvae fed on *D. opuntiae*

306   (Figure 3). Most adults did not perform reflex-bleeding when touched with a fine brush,

307   but all of them did when one of their legs was touched with a hot needle. Reflex-blood

308   droplets were released by adults at multiple reflex-bleeding sites, such as leg joints and

309   elytral margins. The volume of reflex-blood collected from laboratory-reared adults was

310   0.074 ± 0.02 µL when fed on control *P. citri*, and 0.098 ± 0.02 µL when fed on *D.*

311   *opuntiae*. This volume did not significantly differ between diets ($F_{1, 8} = 0.79$, $P = 0.4$).

312   The color of the reflex-blood collected from adults ranged from achromatic to pale-ivory

313   in *C. montrouzieri* fed on both *P. citri* or *D. opuntiae*.

314   Carminic acid was not detected in the reflex-blood of *C. montrouzieri* larvae fed on *P.*

315   *citri* nymphs, whereas it was detected in all *C. montrouzieri* larvae fed on *D. opuntiae*

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nymphs, both in laboratory-reared and field-collected larvae (Figure 3). The concentration of carminic acid in the reflex-blood was 0.35 ± 0.09 % for larvae fed on *D. opuntiae* under laboratory conditions, and 0.6 ± 0.19 % for larvae found in *D. opuntiae* colonies in the field. Carminic acid concentration was not significantly different between laboratory-reared and field-collected larvae ($F_{1,\ 17} = 1.19$, $P = 0.29$). Carminic acid was not detected in the reflex-blood of *C. montrouzieri* adults fed either on *P. citri* or on *D. opuntiae* in the laboratory.

Deterrence of carminic acid against *C. montrouzieri* potential enemies

Release of carminic acid against ants under field conditions

Out of the twenty *C. montrouzieri* cochineal-fed larvae placed on *L. grandis* trails, ten fled before being attacked by ants, while the remaining ten were attacked by ants. Of these, seven larvae fled after ants removed some of the waxy filaments covering the larva. The final three larvae were persistently attacked by ants, and these larvae responded by reflex-bleeding (Figure 1B). Invariably, the attacking ants aborted the attack as soon as they become in contact with reflex-blood and dedicated themselves to grooming, resulting in no successful predation event by ants in this assay.

Deterrence of carminic acid against ants

Deterrence of carminic acid in sucrose solution against *L. grandis* increased with concentration ($F_{1,\ 22} = 374.25$; $P < 0.001$; Figure 4; *Deterrence = 1/(1+Exp(-(-5.5+3.2(5+log(Carminic acid concentration [M in 0.1M Sucrose]))))))*. At a concentration of 10^{-2} M (0.5% of weight, the average concentration previously found in *C. montrouzieri* reflex-blood), deterrence of carminic acid against *L. grandis* was absolute.

Discussion

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340 Our study demonstrated that predators could tolerate and sequester defensive chemicals

341 from non-coevolved prey and use them as deterrents against antagonists. The coccinellid

342 predator *C. montrouzieri* was the most abundant species feeding on cochineal colonies in

343 our two-year field study in the Mediterranean basin, one of the many areas invaded by

344 cochineal bugs in the world. The larvae of *C. montrouzieri* tolerated and sequestered

345 carminic acid from the cochineal bugs and used it as deterrent against Mediterranean ants.

346 *Cryptolaemus montrouzieri* is native to Australia and was introduced to more than 64

347 countries, including the Mediterranean basinHaga clic o pulse aquí para escribir texto., to

348 suppress important mealybug pests (Kairo *et al.* 2013). Although mealybugs are likely

349 the preferred and optimal prey of *C. montrouzieri* (Maes *et al*., 2014b), it has a wider host

350 range that includes other native and exotic prey, including cochineal bugs in the

351 introduced range (Kairo *et al.* 2013). *Cryptolaemus montrouzieri* has been observed

352 feeding on cochineal bugs in colonized areas such as South Africa (Greathead 1972) and

353 Israel (Mendel *et al.* 2020), and it can complete its development feeding exclusively on

354 cochineal bugs (Baskaran *et al.* 1999; El-Aalaoui *et al.* 2019a; Zim *et al.* 2023). Our

355 results further confirmed that cochineal bugs, specifically *D. opuntiae*, is an adequate diet

356 to *C. montrouzieri*, and all larval instars and adults of the coccinellid were observed

357 feeding on this exotic prey. Moreover, *C. montrouzieri* completed multiple non-discrete

358 generations on *D. opuntiae*, and increased its density under field conditions from May to

359 October. While *C. montrouzieri* is present in the study area since 1927, when it was

360 imported from Australia (Gómez-Clemente 1928), *D. opuntiae* was firstly detected in

361 2007 (MAPA 2007), expanding and increasing in abundance in the following years

362 (Rodrigo et al. 2010; Ben-Dov and Sánchez-García 2015). Our study demonstrates that

363 *C. montrouzieri* has rapidly adapted to exploit this exotic prey in western Mediterranean

364 basin.

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365 *Cryptolaemus montrouzieri* larvae acquired carminic acid, a substance absent in its native

366 range, when feeding on the exotic cochineal bugs and released this substance through

367 reflex-bleeding when attacked. The sequestration of defensive chemicals by predatory

368 insects from prey has been rarely documented (Table 1). Among the few known cases,

369 three specialist, likely coevolved, predators of cochineal bugs sequester carminic acid:

370 the moth *Laetilia coccidivora (Comstock)* (Lepidoptera: Pyralidae), the fly *Leucopis*

371 *bellula* Williston (Diptera: Chamaemyiidae), and the coccinellid *H. trifurcata* (Eisner *et*

372 *al.* 1980, 1994). The repeated adoption of carminic acid for sequestration by these

373 predators suggests that there is a large adaptive benefit to be gained from this trait.

374 Sequestration by predators from non-coevolved prey such as in our present study is even

375 more rare, with the clearest example besides our own being the coccinellid *Harmonia*

376 *axyridis,* which sequesters alkaloids from other coccinellids (Hautier *et al.* 2008). It thus

377 seems likely that coccinellids in general have pre-adaptations for sequestering of

378 defensive chemicals, and that carminic acid specifically is a worthwhile target for

379 sequestration.

380 Carminic acid has only been reported to be produced by two genera of scale insects native

381 to America (*Dactylopius* spp.) and Eurasia (*Porphyrophora* spp.) (Wouters & Verhecken

382 1989; Cooksey 2019), while it has not been found in the native range of *C. montrouzieri*.

383 However, some Australian scale insects produce pigments which are structurally similar

384 anthraquinones (Brown 1975; Banks *et al.* 1976a;1976b; Cameron *et al.* 1981a;1981b;

385 Rasmussen *et al*., 2018). It is therefore possible that *C. montrouzieri* is adapted to

386 sequester other anthraquinones from native prey. The ability to sequester defensive

387 compounds from native prey remains to be investigated in this system. Such pre-

388 adaptations might have facilitated the ability to sequester carminic acid from novel prey

389 without requiring additional adaptations.

16

Our results showed that *C. montrouzieri* larvae released carminic acid when attacked by ants and, at concentrations found in the reflex-blood, this compound caused absolute deterrence against the Mediterranean ant *L. grandis*. Reflex-bleeding of chemical defence plays a prevalent role for coccinellid defence against antagonists such as ants (Daloze *et al.* 1994; Glisan King & Meinwald 1996; Majerus *et al.* 2007; Pasteels 2007). *Lasius* ants are aggressive and dominant predators that usually attack coccinellids that feed on hemipterans (Marples 1993; Takizawa & Yasuda 2006; Majerus *et al.* 2007). In eastern Spain, *L. grandis* is among the most abundant ant species (Beltrà *et al.* 2017; Plata *et al.* 2023a), and its presence has a strong negative effect on coccinellids, especially on immature stages (Mestre *et al.* 2016). Ants can cause significant mortality of *C. montrouzieri* larvae (Mansour *et al.* 2012), and the presence of aggressive ants has been suggested to prevent the establishment of *C. montrouzieri* in some areas (Greathead 1972). Thus, the sequestration of carminic acid might facilitate the establishment of *C. montrouzieri* in ecosystems where ants play a dominant role. In our study-system, cochineal bugs were not tended by ants, likely because of carminic acid deterrence. This makes it unlikely for the coccinellid to be attacked by ants while feeding on cochineal colonies. However, ants were abundant feeding on extrafloral nectar of prickly pear plants, and as confirmed by our field observations they can attack coccinellids when moving away from cochineal colonies. Further research should study whether the ability to sequester carminic acid can significantly reduce the mortality caused by ants and other antagonists of *C. montrouzieri*.

Sequestration of carminic acid by *C. montrouzieri* larvae must involve uptake of the compound via the gut membrane. Interestingly, carminic acid was not detected in the reflex-blood of *C. montrouzieri* adults, which could be due to a more rapid detoxification or excretion system, or a less transmissive gut in adults. We observed that the feces of *C.*

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montrouzieri adults fed on cochineals were garnet, indicating that carminic acid is excreted. This pattern of sequestration in the larvae and excretion in the adult is also found in the cochineal-specific predator *H. trifurcata* (Eisner *et al.* 1994), and may reflect general physiological adaptations of coccinellids. The adults of *C. montrouzieri* and *H. trifurcata* produce deterrent defensive chemicals such as the alkaloid euphococcinine, (Brown & Moore 1982; Eisner *et al.* 1986), but it is unknown if their larvae are similarly defended. If larvae lack alkaloid-producing glands, they might instead rely on sequestering chemicals from their prey. Similar to *H. trifurcata* (Eisner *et al.* 1994), larvae of *C. montrouzieri* sequestered carminic acid at concentrations that results in absolute deterrence to antagonistic ants. However, further research is needed to understand if these larvae can actively control carminic acid concentrations in their hemolymph, or if concentrations are determined by prey content alone.

An alternative explanation for the absence of carminic acid in adults would be that sequestration of carminic acid might have a physiological cost for *C. montrouzieri*, as occurs in other insects that sequester toxins (Sloggett *et al.* 2009; Sloggett & Davis 2010; Reudler *et al.* 2015; Züst *et al.* 2018; Sloggett 2022). Unlike larvae, the cost of storing exogenous toxins could outweigh the potential benefit for coccinellid adults, that are equipped with defence mechanisms not found in larvae, including a hardened cuticle, wings for escape, or glands that secrete endogenous toxins (Majerus *et al.* 2007). Cochineal bugs are a suitable diet for *C. montrouzieri*, that is able to complete its development feeding exclusively on this diet, however, the development of *C. montrouzieri* is slower when fed on cochineal bugs than on mealybugs, suggesting a potential cost (Baskaran *et al.* 1999). Nonetheless, the high and constant abundance of *C. montrouzieri* in cochineal colonies in our field study, compared to previous field studies upon mealybugs in crops from the same region (Pérez-Rodríguez *et al.* 2019; Plata *et al.*

18

440   2022, 2023a), suggests that the ecological benefits of this diet are higher than the cost.

441   The enhanced defense against ants and other antagonists may be one of these benefits.

442   Besides *L. grandis,* the low abundance of other predators suggests that carminic acid

443   might be deterrent against other species that are potential antagonists of *C. montrouzieri*.

444   While our results suggest that *C. montrouzieri* gained a defensive benefit from carminic

445   acid sequestration, there could be additional benefits for feeding on cochineal colonies.

446   First, the availability of cochineals throughout the year (Rodrigo *et al.* 2010) provides *C.*

447   *montrouzieri* with a stable food source, while the presence of mealybugs is more seasonal

448   (Plata *et al.* 2023b). Second, some insecticides applied in crops harm *C. montrouzieri*

449   (Planes *et al.* 2013), while cochineal bugs occur in semi-natural areas or field margins

450   where pesticides are not applied. Third, the large cottony mass produced by the cochineal

451   bug *D. opuntiae* might induce oviposition in *C. montrouzieri*, as do empty mealybug

452   ovisacs (Kairo *et al.* 2013). Further work is needed to identify the most likely drivers of

453   the close association between *C. montrouzieri* and cochineal bugs in the invasive range.

454   **Conclusions**

455   Our work showed that *C. montrouzieri* takes advantage of the exotic cochineal bugs as a

456   new abundant prey, and apparently benefits from an enemy-free space gained from a

457   novel defensive weapon, carminic acid. Moreover, *C. montrouzieri* can release this toxin

458   for its own defence against antagonists in its invasive range. As far as we know, this

459   represents the first documented case of a defensive use of a sequestered toxin from exotic

460   prey by non-coevolved predators. The ability of sequester novel toxins from exotic diets

461   may occur in other arthropods, including other trophic levels. In herbivorous insects,

462   toxin sequestration is typically highly selective and depends on specific plant sources (eg.

463   Boppré 1990; Pasteels *et al*. 1990; Züst & Agrawal 2016; Birnbaum & Abbot 2018;

464   Petschenka *et al.* 2022). However, it is known that some herbivore species can sequester

toxins from exotic plants (Muchoney *et al.* 2022). Likewise, the sequestration of novel toxins from exotic prey might also occur in other animals pre-adapted to sequester exogenous toxins, such as some tetrapod vertebrates (Savitzky *et al.* 2012). These novel weapons might provide a benefit for some animals and affect the trophic structure of native food webs. We suggest that these more 'hidden' consequences of species invasions be considered in ecological risk assessment and biological control.

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Conflict of interest statement

There are no competing interests to declare.

Ethics statement

This work followed the Association for the Study of Animal Behaviour / Animal Behavior Society Guidelines for the Use of Animals in Research. The procedures used are clearly described and conform to the legal requirements of the country in which the work was carried out and to all institutional guidelines.

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488 **Data archiving statement**

489 Data and code available from Dryad repository: https://doi.org/10.5061/dryad.rjdfn2zj1

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770   **Table 1.** Cases of defensive chemicals acquisition from prey by predators.

| Phylum	Predator	Acquired toxin	Prey	Reference
Vertebrata	Poison frogs (Dendrobatidae Mantellidae, Bufonidae Myobatrachidae, Eleutherodactylidae)	Alkaloids	Melyrid beetles, ants, millipedes, mites, moths.	Dumbacher 2004; Saporito, 2012; Santos 2016; Rodríguez 2011
	Passerine birds (Ifritidae, Pachycephalidae, Oriolidae, Oreoicidae)	Batrachotoxin alkaloids	Melyrid beetles	Dumbacher 2004; Jønsson 2008; Bodawatta 2023
	Snakes (*Rhabdophis*)	Bufadienolides steroids	Frogs	Kojima 2015; Hutchinson 2012; Yoshida 2020
	Snakes (*Thamnophis*)	Tetrodotoxin	Salamandridae (*Taricha*)	Williams 2012
Ctenophora, Acoelomorpha, Platyhelminthes, Mollusca	Cnidarian predators	Nematocysts	Cnidarians	Goodheart 2016
Insects	Fireflies (*Photuris* sp.),	Bufedienolides steroids	Fireflies (*Photinus* sp.)	Eisner 1997
	Canthariphylous predators (Anthicidae, Staphylinidae, Pyrochroidae, Ceratopogonidae, Anthomyiidae*)*	Cantharidine	Meloidae and Oedemeridae beetles	Dettner 1997
	Lacewings (Chrysopidae)	Cardiac glycosides cardenolides	*Aphis nerii*	Rothschild 1973
	Coccinella undecimpunctata	Cardiac glycosides cardenolides	*Aphis nerii*	Rothschild 1973
	Coccinella septempunctata	Pyrrolizidine alkaloids	*Aphis jacobaeae*	Witte 1990
	Harmonia axyridis	Alkaloids (Adaline and precocinelline)	*Adalia bipunctata* and *Coccinella septempunctata*	Haultier 2008
	Laetillia coccidivora	Carminic acid (anthraquinone)	Cochineal bugs	Eisner 1980
	Leucopis bellula	Carminic acid (anthraquinone)	Cochineal bugs	Eisner 1994
	Hyperaspis trifurcata	Carminic acid (anthraquinone)	Cochineal bugs	Eisner 1994
	Cryptolaemus monstrouzieri	Carminic acid (anthraquinone)	Cochineal bugs	This study

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Table 2. Abundance of arthropods preying upon cochineal bugs in the ten prickly pear sites from eastern Spain sampled in spring, summer, and autumn of 2020 and 2021. Represented by mean (± SE) number of arthropods per cochineal colony. The proportion of prickly pear sampling sites where each predator species was present is represented in brackets.

| Year | Season | Predators on cochineal colonies | | |
		Cryptolaemus montrouzieri	*Chilocorus bipustulatus*	*Scymnus interruptus*
2020	Spring	0.027 ± 0.016 (3/10)		
	Summer	0.133 ± 0.060 (5/10)		
	Autumn	0.600 ± 0.254 (7/10)		
2021	Spring	0.007 ± 0.004 (4/10)		
	Summer	0.160 ± 0.079 (5/10)	0.003 ± 0.003 (1/10)	
	Autumn	0.363 ± 0.180 (6/10)		0.002 ± 0.002 (1/10)

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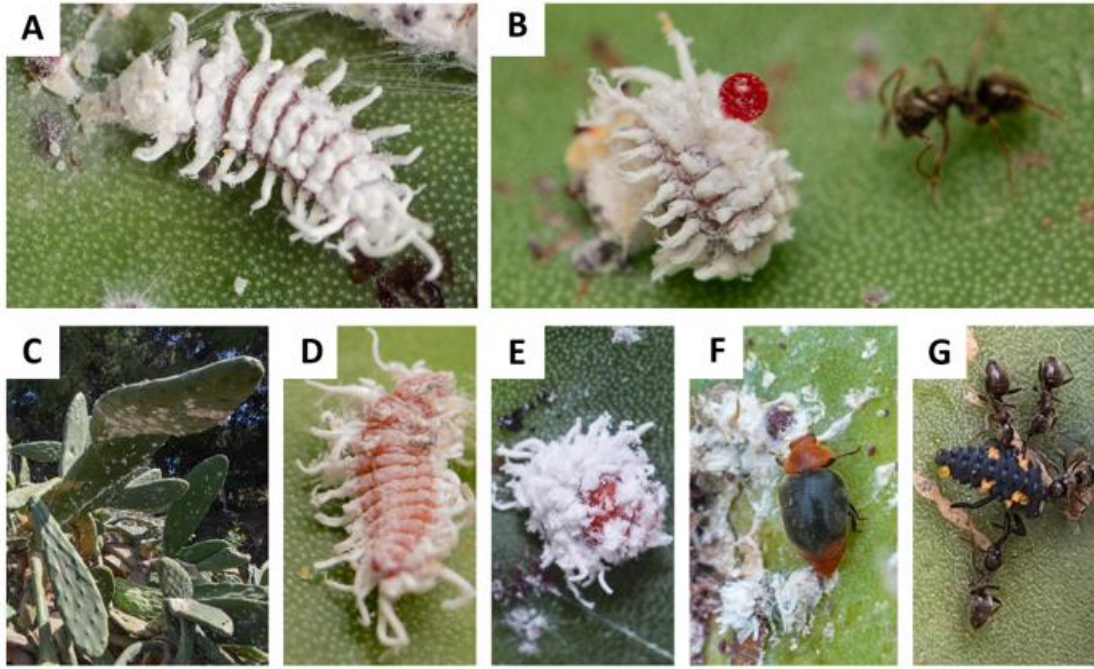
Figure 1. Field observations on prickly pear clusters. A) Larva of *C. montrouzieri* feeding on cochineal bugs. B) Larva of *C. montrouzieri* performing reflex-bleeding after an attack of *L. grandis*, which was deterred. C) Prickly pear plant infested by cochineal bugs. D) Red color exhibited by larvae ventral side. E) *Cryptolaemus montrouzieri* newly formed pupa, exhibiting red color after losing the wax. F) Adult of *C. montrouzieri* feeding on cochineal bugs. G) *Lasius grandis* ants attacking a larva of the coccinellid *Coccinella septempunctata* while feeding on prickly pear extrafloral nectar.
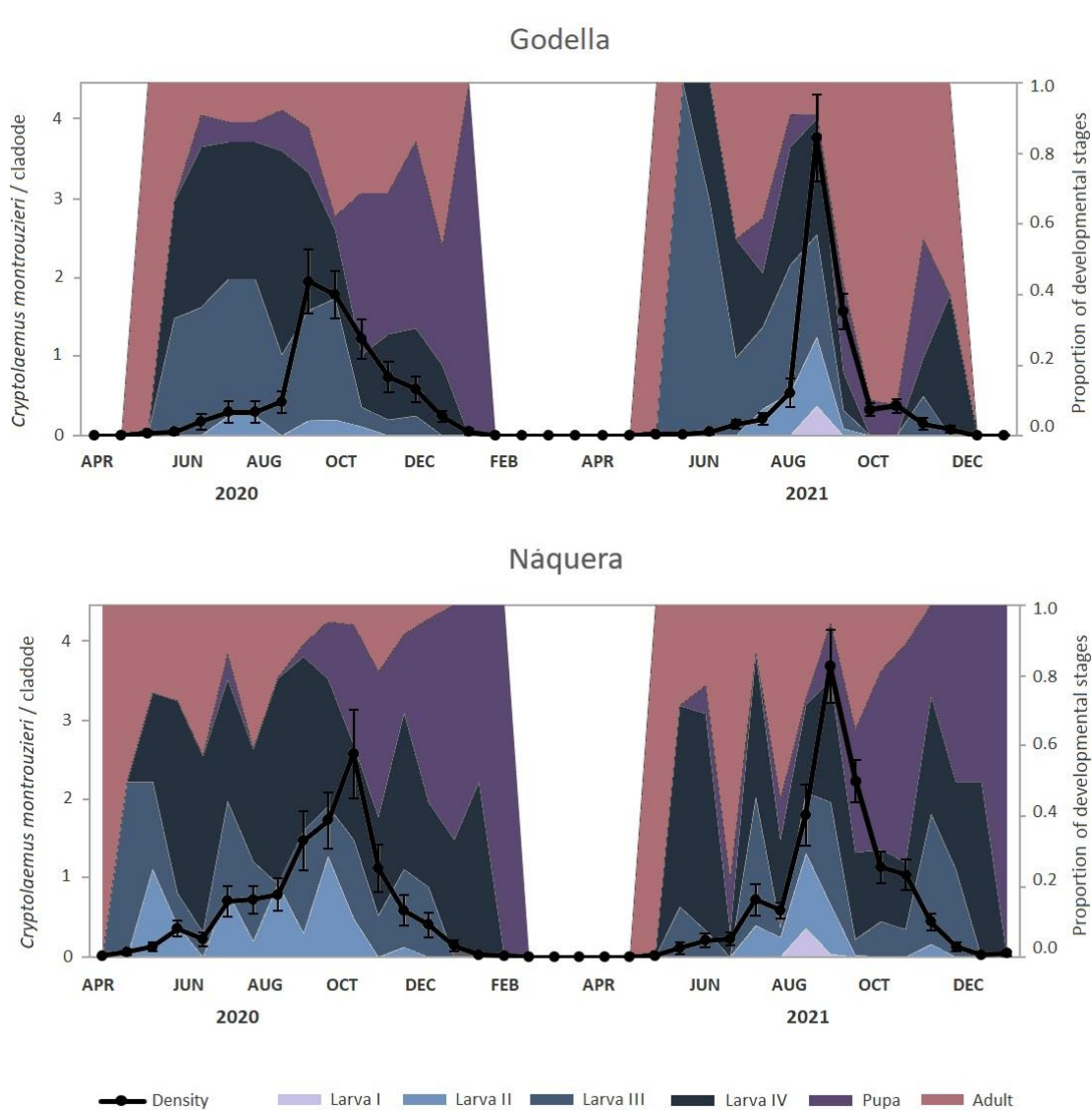
Figure 2. Density and phenology of *Cryptolaemus montrouzieri* feeding on cochineal bugs across two consecutive years (2020 and 2021). The vertical bars above and below the density line indicate the standard error. Two prickly pear clusters were selected for this study because a high number of *C. montrouzieri* had been previously observed.

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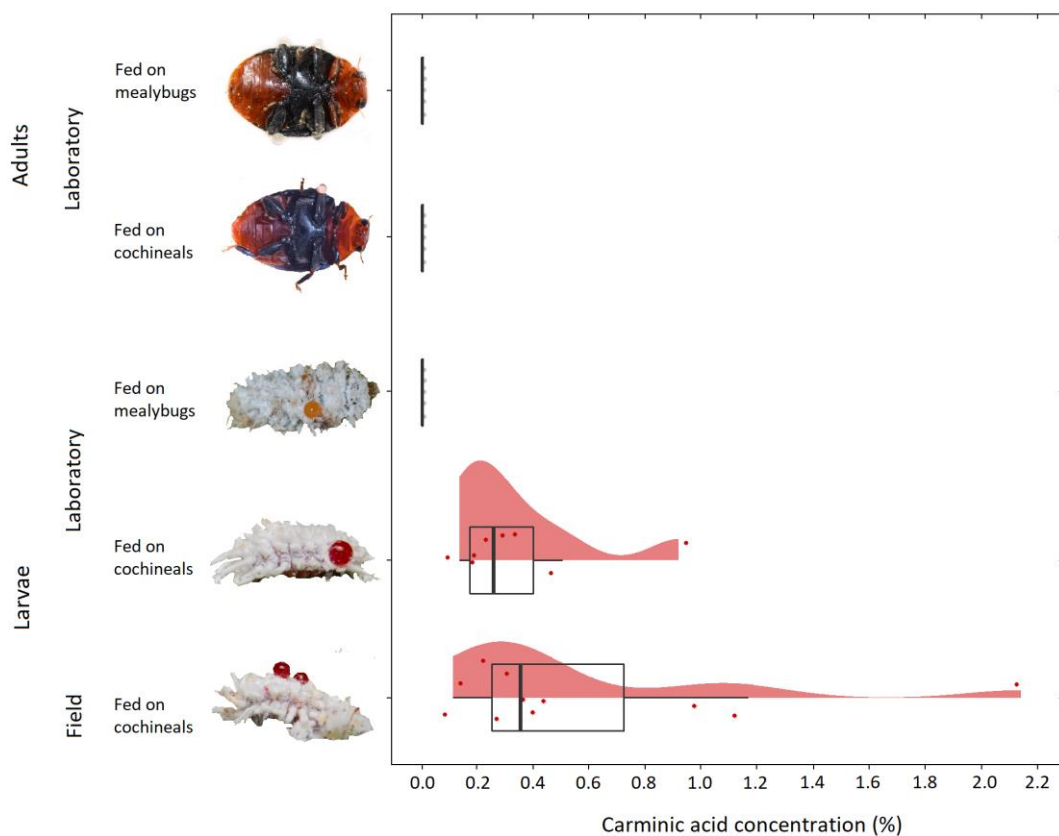
Figure 3. Concentration of carminic acid in the liquid expelled through reflex-bleeding (reflex-blood) by adults and third instar larvae of *Cryptolaemus montrouzieri* fed on the mealybug control diet (*Planococcus citri*) and fed on cochineal bugs (*Dactylopius opuntiae*). Points represent the concentration of carminic acid in the reflex-blood of each individual. Rectangles represent the second and third quartiles and the inner vertical line represent the median value. The lower and upper quartiles are shown as horizontal lines on either side of the rectangle. Half-violin plots represent the probability density of each data set.

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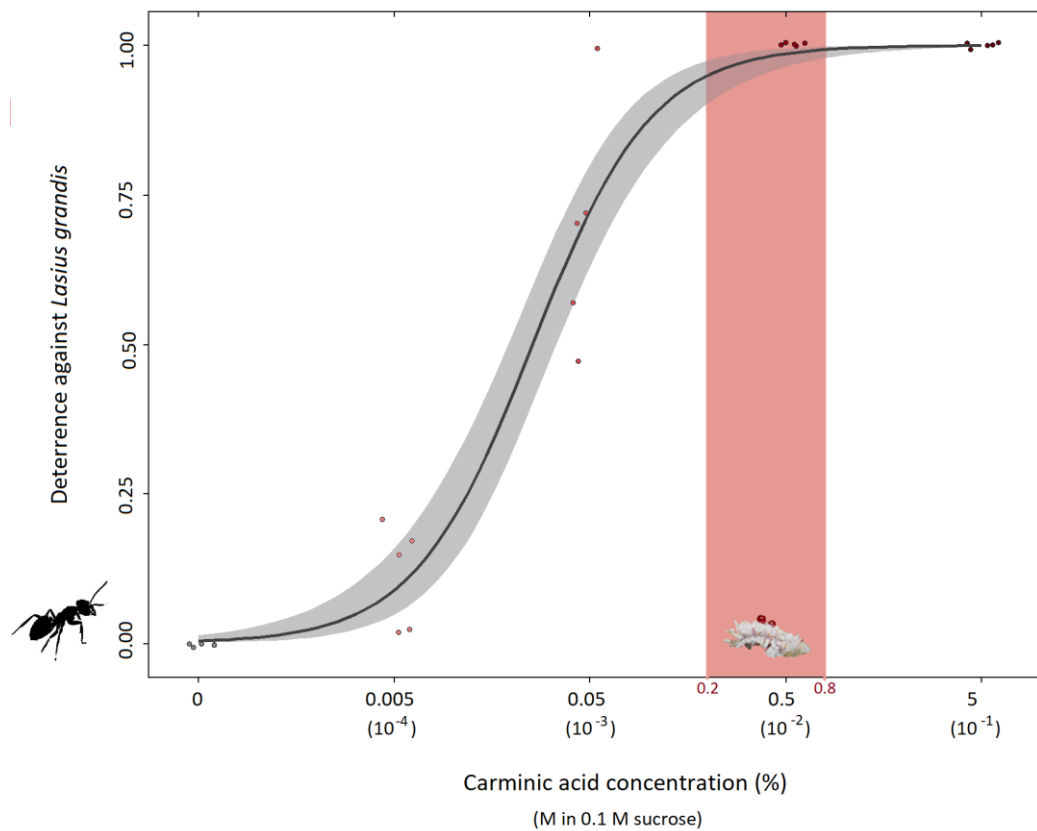
Figure 4. Effect of carminic acid concentration on the deterrence against the Mediterranean ant
Lasius grandis when it has access to sucrose food sources with different carminic acid
concentrations. Each point represents the deterrence calculated as [1-(mean number of ants
feeding on the carminic acid solution / mean number of ants feeding on control solution)] during
a 20-minute test. Line represents the generalized linear model (GLM) binomial distributed. Grey
area represents 0.95 confidence interval. Red shaded area represents the concentration of carminic
acid found in the reflex blood of *C. montrouzieri* larvae fed on cochineal bug colonies in the field.

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