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Variation in contact chemical cues may mediate differential predator response in the colour polymorphic tortoise beetle, *Chelymorpha alternans*

Lynette R. Strickland[1] | **Donald Windsor**[2] | **Carla E. Cáceres**[3]

[1]Department of Biological Sciences, University of Memphis, Memphis, Tennessee, USA

[2]Smithsonian Tropical Research Institute, Panamá, Republic of Panama

[3]School of Integrative Biology, University of Illinois at Urbana-Champaign, Urbana, Illinois, USA

Correspondence
Lynette R. Strickland, Department of Biological Sciences, University of Memphis, Memphis, TN 38111, USA.
Email: slynn731@gmail.com

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Abstract

1. Differential predation on species with intraspecific colour variation has been explored in various systems and is often implicated as the driving force behind colour polymorphism maintenance.
2. Here, investigation done on whether predation contributes to the maintenance of extensive colour variation in the Neotropical tortoise beetle, *Chelymorpha alternans* (Chrysomelidae). Recorded predation rates on different colour pattern phenotypes by three common, generalist invertebrate predators, and identified potential chemical signals of unpalatability.
3. Predaceious mantids (Orthoptera, Mantidae) consumed no beetles, regardless of phenotype, whereas the giant orb-weaving spider (*Trichonephila clavipes*; Araneidae) consumed all three beetle phenotypes. The carton-nest ant, *Azteca chartifex* (Formicidae), displayed differential predation; the rufipennis phenotype of *C. alternans* was sometimes consumed, the metallic phenotype was never consumed, and the veraguensis phenotype was consumed in the first three encounters and subsequently discarded, suggesting a learned avoidance behaviour.
4. Using gas chromatography–mass spectrometry, it was determined that cuticular hydrocarbon profiles were similar between the metallic and militaris-a phenotypes. The rufipennis phenotype showed pronounced differences and displayed the greatest among-individual variation in elytral cuticular profiles.
5. Between-phenotype variation in chemical cues, and differences in how predators receive those cues, may mediate predator response, and play a role in maintaining colour variation in this species.

KEYWORDS
colour polymorphism, cuticular hydrocarbons, gas chromatography–mass spectrometry, invertebrate bioassays, predation

INTRODUCTION

Many of the world's extant insect species (estimated at 900,000 species, Janzen, 1976) are prey for one or more predator species (Cinel et al., 2020; Stork et al., 2015). This intense selection pressure has resulted in the evolution of various anti-predator defence mechanisms, from widespread tactics such as crypsis and anchoresis (seeking refuge) to taxon-specific responses such as thanatosis (death-feigning) and the ejection of toxic chemicals (Ruxton et al., 2004). Multiple predator avoidance strategies and communication cues are required

when a species is preyed upon by a suite of taxonomically distinct predators (Ratcliffe & Nydam, 2008). For example, communicating distastefulness to predators is beneficial, but can be difficult when predators differ in their prey detection strategies, visual perception, and levels/times of activity (Ratcliffe & Nydam, 2008; Stevens, 2007). Therefore, predator-deterrent signals often incorporate multiple modalities, including visual, auditory, olfactory, and chemical cues (Berenbaum & Miliczky, 1984; Bowdish & Bultman, 1993; Prudic et al., 2007).

This multimodal communication is well represented in the predator defence strategy of aposematism—the coupling of sequestered secondary toxins with an advertisement of unpalatability (Rojas et al., 2015). Most predator bioassays testing the efficacy of unpalatability in aposematic organisms have focused on avian predators as generally, they are visually oriented and have sophisticated visual systems (reviewed in Ruxton et al., 2004). However, a variety of predatory invertebrates can also distinguish unpalatable prey and avoid them in subsequent interactions (e.g., scorpions, spiders, coccinellids, mantids, carabids; Berenbaum & Miliczky, 1984). Moreover, the visual systems of terrestrial invertebrates can be quite sophisticated and are highly diverse compared to the relatively conserved visual systems of avian predators (Hart & Hunt, 2007; Land & Nilsson, 2012; Taylor et al., 2016). In addition, many of these predatory invertebrates rely on vision to detect prey (Gonzalez-Bellido et al., 2016; Hajek, 2004; Kelber, 2006; Taylor et al., 2016), as well as chemical cues on the surface of potential prey, to confirm identity (Ide et al., 2007; Rutledge et al., 2014). Cuticular hydrocarbons (CHCs) are contact chemical cues that provide important recognition information in mutualistic interactions including kin-recognition, sex discrimination, and breeding status (Drijfhout et al., 2010; Lihoreau & Rivault, 2009; Singer, 1998). However, the role of CHCs in predator–prey interactions is comparatively understudied. Investigations into how predatory insects use CHCs to identify prey from nonprey items can provide insights into predation shaped outside of the lens of visual processes (Koedam et al., 2011; Rutledge et al., 2014; Xue et al., 2018).

Tortoise beetles (Cassidinae) are a specialised subfamily within one of the largest animal families, Chrysomelidae (leaf beetles). Tortoise beetles are found worldwide but reach the greatest diversity in the Neotropics (Chaboo, 2007). Panamanian populations of the tortoise beetle, *Chelymorpha alternans*, have five genetically distinct colour pattern polymorphisms which co-occur across the Isthmus (Morrison & Windsor, 2018; Strickland et al., 2019). Previous work with this species has elucidated the pattern of inheritance for all five phenotypes, for full details, see Strickland et al. (2019). In brief, the 'metallic' phenotype has a red pronotum and elytra with metallic stripes, the 'militaris-a' and 'militaris-b' phenotypes display red colouration with black stripes (militaris-a with a variegated pronotum and militaris-b with a solid black pronotum), the 'veraguensis' phenotype has a red pronotum and elytra, and the 'rufipennis' phenotype has a black pronotum and red elytra. The striking colouration of *C. alternans*, their association with various plant species in the family Convolvulaceae, and their gregarious larval development (siblings remain clustered together from egg through pupation), are typical characteristics of aposematic organisms (Bowers, 1992; Morrison & Windsor, 2018; Ruxton et al., 2004). Although colouration has previously been termed 'aposematic' for this species (Strickland et al., 2019), the exact chemical components determining the degree of palatability/unpalatability have not been identified or functionally investigated. Moreover, the role of predation in maintaining adult colour polymorphisms has been understudied when compared with studies on predation at larval stages, which have several specialised defences, most notably, a 'faecal shield'—the accretion of exuviae and faecal material, carried by larvae as a chemical and mechanical deterrent (Eisner et al., 1967; Olmstead, 1991; Olmstead & Denno, 1993; Vencl et al., 2005; Windsor et al., 1992). Investigating whether differential predation works as a selective agent on adult colour phenotypes can provide further insights into the mechanisms maintaining phenotypic variation.

To investigate the potential role of differential predation in maintaining colour polymorphisms in *C. alternans*, we performed a series of bioassays with three ecologically-relevant and widely distributed predators and three phenotypes of *C. alternans* (veraguensis, metallic, and rufipennis). These included at least three species of mantis, including *Stagmatoptera septentrionalis*, *Caridoptera brachyptera*, and *Pseudomusonia lineativentri* (all Mantodea: Mantidae), the carton-nest ant, *Azteca chartifex* (Hymenoptera: Formicidae), and the orb-weaving spider, *Trichonephila clavipes* (Araneae: Argiopidae). In addition, to test whether contact chemical cues differ between phenotypes which might influence predator response, we performed a CHC analysis using gas chromatography–mass spectrometry (GC–MS) with three phenotypes of *C. alternans* (militaris-a, rufipennis, and metallic). Specifically, we asked: (1) Do potential invertebrate predators avoid interacting with *C. alternans*? (2) Do phenotypes of *C. alternans* experience different levels of predation? (3) Do CHC profiles differ between phenotypes and potentially mediate invertebrate predator responses?

METHODS

Beetle stock and maintenance

Metallic, rufipennis, veraguensis, and militaris-a phenotypes of *C. alternans* (see Strickland et al., 2019 for full phenotypic details) were collected from host plants growing in the Gamboa, San Lorenzo, and David areas of Panama. Beetle stocks were housed in the Gamboa laboratories of the Smithsonian Tropical Research Institute (STRI), in plastic containers, with lids perforated to allow airflow and temperature regulation. All containers were exposed to natural light, temperature, and humidity, and provisioned daily with naturally-occurring fresh leaves of the preferred host plant *Merremia umbellata*. Beetles were moved weekly to recycled containers which were washed, and bleach sterilised. Coffee filters were placed in each new container to absorb excess moisture and collect feculae. Fungal pathogens were minimised by a light spraying of aqueous potassium sorbate.

Mantis

Praying mantises are fierce invertebrate predators who predominately feed on other insects but have also been shown to feed on small reptiles, mammals, and birds (Nyffeler et al., 2017). Mantises often co-occurred in many of the same habitats and collection sites as *C. alternans* (personal observation). Adult mantises of species large enough to attack the beetles were collected in the vicinity of Gamboa and housed in the laboratory in black mesh enclosures (30 × 30 × 30 cm). Eighteen full-grown individual mantises, were assigned to one of three phenotypes of *C. alternans* (metallic, rufipennis, or veraguensis; six replicates per phenotype) and were offered a single beetle after a 24-h starvation period. Each mantis underwent a single trial because they did not do well when kept in laboratory conditions for longer periods.

Mantises were moved into plastic containers (14 1/8″ × 7 3/4″ × 4 7/8″) and allowed a 1-hour acclimation period (to adjust to the new container) before the beginning of trials. After this, a single beetle was placed into the trial container, and response time (measured as time to first head swivel towards the beetle), and consumption or rejection of the beetle, were recorded. The time between a consumption/rejection behaviour and the first head swivel was calculated as the handling time. A prior study by Berenbaum and Miliczky (1984), using mantises as predators to assess unpalatability in milkweed bugs, found that when mantises reject prey items, they strike and grasp the prey in their forelimbs and then hurl the item away, vigorously shaking both forelimbs. As this predator consumed no beetles, this behaviour was also used as a rejection response for the trials. Mantises were observed until at least one rejection response had occurred, or for 90 min. Those individuals who did not respond within 90 min were placed back into their containers and given another 24-h starvation period (only two individuals did not respond on the first day). Individuals who showed a rejection response were tested for satiation with a moth (of several species collected from light traps in the same geographic area) and released at their original collection sites. All trials were recorded with a Canon Powershot G12 for later observation.

Orb-weaving spider (*T. clavipes*)

Orb-weaving spiders are opportunistic predators highly attentive to web conspicuousness and construction, removing unwanted items (prey or debris) from their webs and reconstructing up to two-thirds of their web every night (Nentwig & Spiegel, 1986; Zschokke et al., 2006). Females of the orb-weaving spider, *T. clavipes* were collected from Gamboa and San Lorenzo, Panama, from June through August 2016, and kept in the laboratory in black mesh enclosures (30 × 30 × 30 cm) for at least 24 h. This holding period allowed females to build a web and to acclimate to surroundings. In total, 18 females were used, with six females assigned to each of the three beetle phenotypes. To assess the potential for learned avoidance behaviour, each spider was presented with a single beetle of her assigned phenotype in each of five trials (30 beetles/phenotype over all trials). Each trial was separated by at least 24 h (and no more than 48 h), and all spiders were released where they were originally collected after being used in our study for 1.5 weeks.

A pair of forceps was used to transfer beetles directly to spider webs. This transfer as well as the natural movement of the beetle attempting to escape the web, was enough to alert the spider to a potential prey item. The amount of time it took for the spider to move towards the beetle after placement (response time), handling time (amount of time spent with the beetle, which ended with returning to the centre of the web), and the decision to consume the beetle (or not) was recorded for each trial. All trials were recorded with a Canon Powershot G12 for later observation.

Carton-nest ants (*A. chartifex*)

Carton-nest ants are generalist tropical forest predators in Panama and throughout Mesoamerica, and have been used as predators in previous bioassays (along with the closely related species, *Azteca lacrymosa*), including other tortoise beetles and larvae of *C. alternans* (Hammer & Van Bael, 2015; Vencl et al., 2005, 2011). Carton-nest ants were identified around Gamboa, Panama, by their characteristic tear-drop shape, well-traversed foraging paths leading from the nest to the ground, and location near the tops of trees (Dejean et al., 2008). Nine colonies were identified and fitted with a wooden platform on the tree trunk near the foraging path of the ants. Platforms were left in place for a week to allow the ant colony members to acclimate to the new structure. Each ant colony was assigned one of three beetle phenotypes (metallic, rufipennis, and veraguensis) and went through 10 successive trials with different individuals. Two hours before the start of all trials, chunks of canned tuna were placed on the platforms to attract ants, and was then removed 90 min later. A recently frozen beetle (30 min prior to trial) was placed in the centre of the platform and the response time (time until the first antennation), handling time (time from first antennation until removal from the platform), and consumption were recorded for each trial. Any colony members carrying the beetle up the tree and into the nest were used as a proxy for consumption. Rejection of the beetle as a prey item was recorded when the ants hurled the beetle from the platform in the opposite direction from the base of the tree. All beetles received either an acceptance or rejection response. A frozen cricket was placed on the platform after an acceptance or rejection of the beetle to test for satiation.

Bioassay statistical analysis

To assess differences in predator behavioural response to phenotypes of *C. alternans*, two-way analysis of variances (ANOVAs), followed by a Tukey's post hoc comparison, were performed using the *car* (Fox & Weisberg, 2019), *multcompview* (Hothorn et al., 2008), and *emmeans* (Lenth, 2022) packages in *R* v. 3.5.1. An overall model assessed the

effects of predator type (spider, ant, and mantis) and beetle phenotype (metallic, rufipennis, and veraguensis), on average response time (in minutes), and average handling time (in minutes). As the consumption data were binomial, a logistic regression was used to assess the effects of predator type and beetle phenotype on overall consumption. However, since mantises consumed none of the beetles, this results in complete separation within the model (this category contains entirely zero counts). To account for this a bias-reduced logistic regression was performed using the R package *brglm2* (Kosmidis, 2021). Separate two-way, repeated-measures ANOVAs were performed using the *tidyverse* (Wickham et al., 2019), and *lmerTest* (Kuznetsova et al., 2017) packages to assess the effects of phenotype, trial, and an interaction, on response time and handling time for the orb-weaving spiders and carton-nest ants. As these two predators underwent multiple trials, changes in either behavioural trait over multiple encounters could indicate a learned behaviour and signal unpalatability or distastefulness in *C. alternans*. Moreover, consumption rates of the three beetle phenotypes from each trial were also examined. Because the consumption data were both binomial (consumed or not consumed) and repeated, a generalised estimating equation (GEE) was performed to assess the effects of trial and phenotype on consumption for spiders and ants individually. This analysis was performed using the package *geepack* in *R* (Halekoh et al., 2006).

Gas chromatography–mass spectrometry

To assess whether phenotypes differ in their CHC profiles, elytral CHCs were extracted from three phenotypes using a hexane extraction protocol modified from Beran et al. (2014). All beetles were taken from a large breeding population of *C. alternans* maintained at STRI facilities in Gamboa, Panama. This population was reared in facilities that are partially exposed to ambient external conditions and fed freshly-collected leaves from their host plant, *M. umbellata*. Extractions were performed in May and July of 2018 on virgin adults (males and females) between 55 and 60 days after oviposition. Before extraction, beetles were starved for 12 h and sacrificed by freezing at −20°C for 45 min, then thawed for 15 min. Elytra from each beetle were removed using sterile, sharp-ended forceps, and soaked in 1 ml *n*-hexane (>96% purity) for 15 min at room temperature in 3 ml V-vials. Extracts were transferred to a separate 3 ml vial, and soaked a second time in 0.5 ml *n*-hexane for 10 min and transferred to the same vial. The solvent was condensed to 1 ml using a gentle, purified nitrogen stream.

Extracts were analysed with GC–MS according to Darragh et al. (2017) using a Hewlett-Packard model 5977 mass-selective detector connected to a Hewlett-Packard GC model 7890B, with a Hewlett-Packard ALS 7693 autosampler at the STRI Tupper facility. Aliquots of 1ul for each sample were performed in splitless mode at 250°C. Fused silica capillary columns (30 m × 0.25 mm, 0.25 μm; Agilent, Santa Clara, CA) were used with helium as the carrier gas at a constant flow of 1.2 ml/min. The oven temperature program started at 50°C, held for 3 min, and then increased at a rate of 5°C/min until 320°C was reached and then held again for 5 min. From each sample, individual peak area relative to total peak area was computed for each reported peak and those with less than 0.1% of the total peak abundance were discarded from further analysis. Information on peak retention time and area were obtained using the open-source software, Automated Mass Spectral Deconvolution, and Identification System. A non-metric multidimensional scaling ordination was performed on peak relative abundance based on the Bray–Curtis similarity matrix to visualise differences between phenotypes and sexes. This was performed using the 'metaMDS' function in the *vegan* package in *R* v. 4.0.5 (Oksanen et al., 2017). To assess which compounds (peaks) contributed to chemical variation between phenotypes, the function 'envfit' was used with 1000 permutations at a *p*-value cutoff of 0.001. To assess differences in chemical variation between phenotypes, an Analysis of Similarity was performed using a Bray–Curtis similarity matrix with 10,000 permutations. The function 'envfit' was also run to corroborate results, again using a Bray–Curtis similarity matrix and 10,000 permutations. This was followed with post hoc pairwise comparisons using the function 'pairwise.perm.manova' in the package *RVAideMemoire*, with a Bonferroni correction to identify which groups differed (Darragh et al., 2020; Hervé, 2018).

RESULTS

Predators differ in their overall response to and interaction with *C. alternans*

The three invertebrate predators differed in their behavioural responses to phenotypes of *C. alternans* (measured as response time and handling time, Figure 1 and Table S1), as well as in their overall consumption of beetles. The carton-nest ants were the quickest predator to respond, typically within a matter of seconds, while mantises had the longest response time ($F_{2,36} = 5.75$, $p < 0.01$; Figure 1a). However, there was no effect of beetle phenotype ($F_{2,36} = 1.24$, $p > 0.05$) or an interaction between beetle phenotype and predator ($F_{4,36} = 0.39$, $p > 0.05$) when comparing response times. Post hoc analyses show differences in response times between mantis and spider predators ($p < 0.05$) as well as mantis and ant predator ($p < 0.05$), but not between ants and spiders ($p > 0.05$). There were also differences in handling time between predators with mantises having the shortest handling time, carton-nest ants having the longest, and orb-weaving spiders spending an intermediate amount of time of the three predators ($F_{2,36} = 312.28$, $p < 0.001$; Figure 1b). Handling time also differed as a function of beetle phenotype ($F_{2,36} = 13.87$, $p < 0.001$), as well as an interaction between predator type and beetle phenotype ($F_{4,36} = 17.06$, $p < 0.001$). Post hoc analyses reveal differences between mantis and spider predators ($p < 0.0001$), mantis and ant predators ($p < 0.0001$), as well as spider and ant predators ($p < 0.0001$). Handling time showed differences between metallic and rufipennis beetle phenotyeps ($p < 0.0001$), as well as rufipennis and veraguensis phenotypes ($p < 0.0001$), indicating that the rufipennis phenotype experienced increased handling times for some predators (Figure 1b).

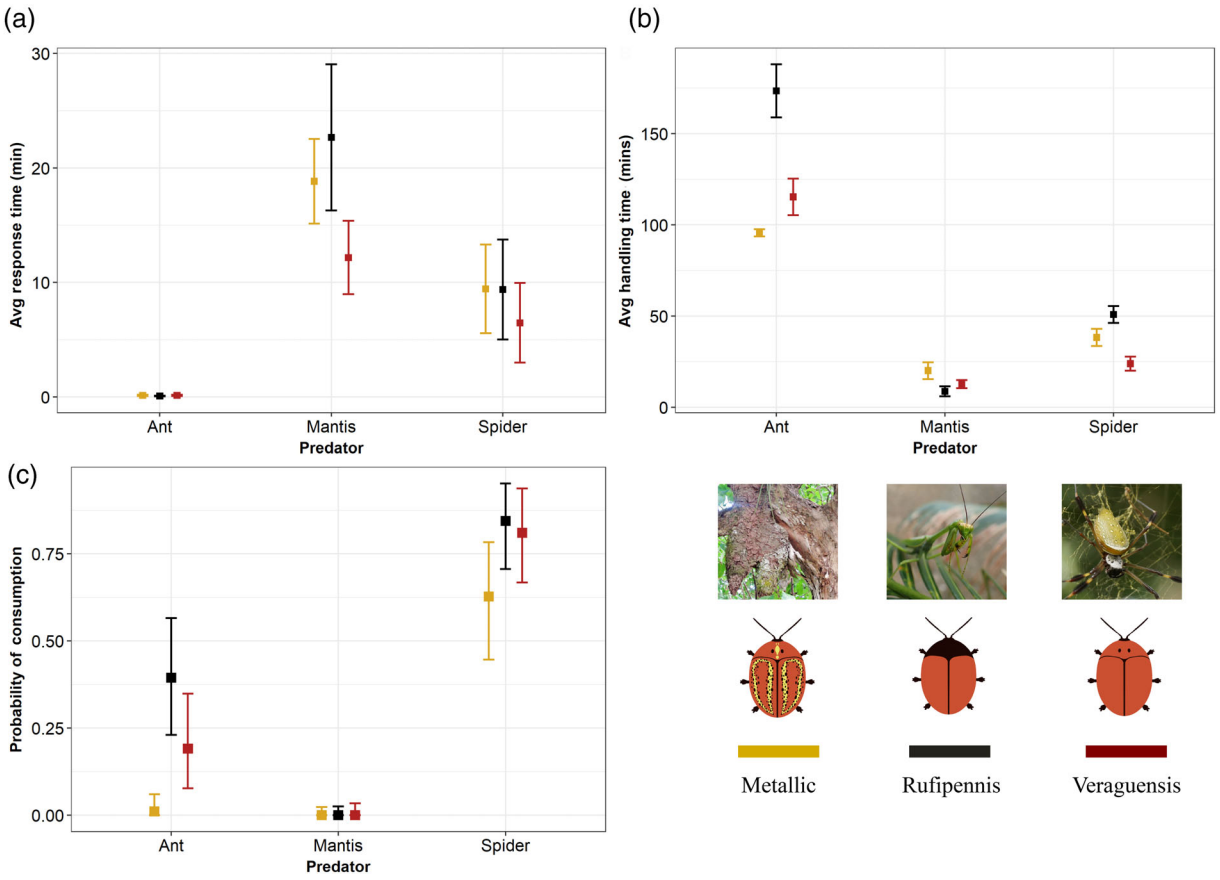

FIGURE 1 Response time, handling time, and consumption of predators when offered different beetle phenotype as prey. (a) Average response time for carton-nest ants (in seconds), mantises (in minutes), and orb-weaving spiders (in minutes) for each phenotype. (b) Average handling time (in minutes) for each predator. (c) Probability of consumption for each predator

Differences in foraging behaviours also translated to among-predator differences in total consumption as shown by the bias-reduced logistic regression ($\chi^2 = 87.188$, d.f. $= 2$, $p < 0.0001$; Figure 1c), with an effect of beetle phenotype ($\chi^2 = 16.663$, d.f. $= 2$, $p < 0.001$; Figure 1c), but no significant interaction between predator and phenotype ($\chi^2 = 2.918$, d.f. $= 4$, $p > 0.05$; Figure 1c) indicating that phenotype is as protective regardless of predator. Post hoc analyses show that the metallic phenotype is consumed less often than the rufipennis phenotype ($p < 0.01$), but that metallic and veraguensis ($p > 0.05$), and rufipennis and veraguensis ($p > 0.05$) individuals are consumed at similar rates. Overall, orb-weaving spiders, consumed the largest number of beetles (68 of the 90 presented, 76%), followed by carton-nest ants, (18 of the 90 presented, 20%). In contrast, mantises consumed none of the beetles presented. Between orb-weaving spiders and carton-nest ants, approximately 45% of the rufipennis phenotype was consumed, followed by 35% of the veraguensis phenotype, and only 21% of the metallic phenotype. Although mantis predators consumed no beetles, all control moths were captured within 6 min and readily consumed (Table S2). Controls were consumed by spiders and ant predators as well (Table S3 for spiders and Table S4 for ants).

As mantises could only be tested for one trial, the first trial for every predator bioassay was evaluated separately to assess whether this influenced consumption differences between predators. When considering only the first trial of every predator (45 total), 11 of the 18 spiders consumed the first beetle presented (61%), 3 of the 9 ant colonies consumed the first beetle presented (33%), while none of the mantises consumed the beetle. A bias-reduced logistic regression (again used due to the binomial nature of the data) using only predator responses from trial 1, indicates that there are persistent differences in consumption between the predators tested here ($\chi^2 = 20.7701$, d.f. $= 2$, $p < 0.0001$, Figure S1). However, there was no effect of beetle phenotype ($\chi^2 = 1.2991$, d.f. $= 2$, $p > 0.05$) or an interaction between the effects of predators and phenotype ($\chi^2 = -0.5613$, d.f. $= 4$, $p > 0.05$).

Predators differ in response to *C. alternans* over multiple encounters

Of the 90 beetles placed into webs, spiders consumed 22 metallic individuals, 25 rufipennis individuals, and 24 veraguensis individuals (Figure 1c). In each trial, the spider flipped the beetle onto the soft dorsal side, inserted her chelicera, and discarded the beetle's elytra and pronotum. Beetles that were not consumed were removed from

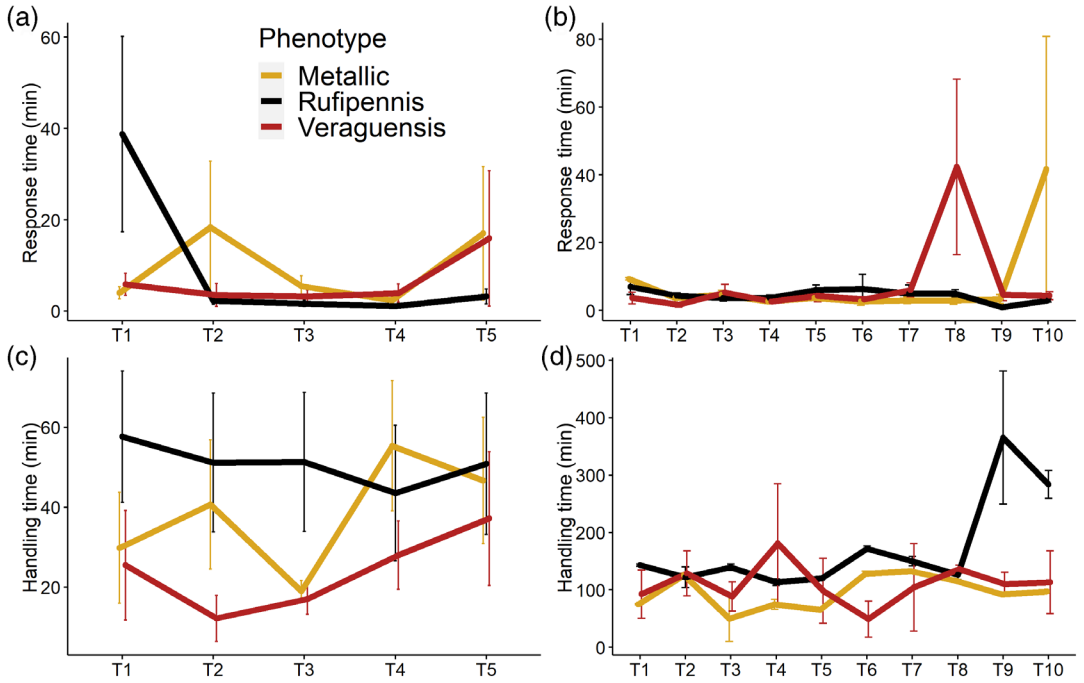

FIGURE 2 Response time and handling time of predators when offered different beetle phenotypes as prey over multiple trials. (a) Response time across all trials for orb-weaving spiders (in minutes) and (b) carton-nest ants (in seconds). (c) Handling time for orb-weaving spiders (in minutes) and (d) carton-nest ants (in minutes) across all trials. T, trial

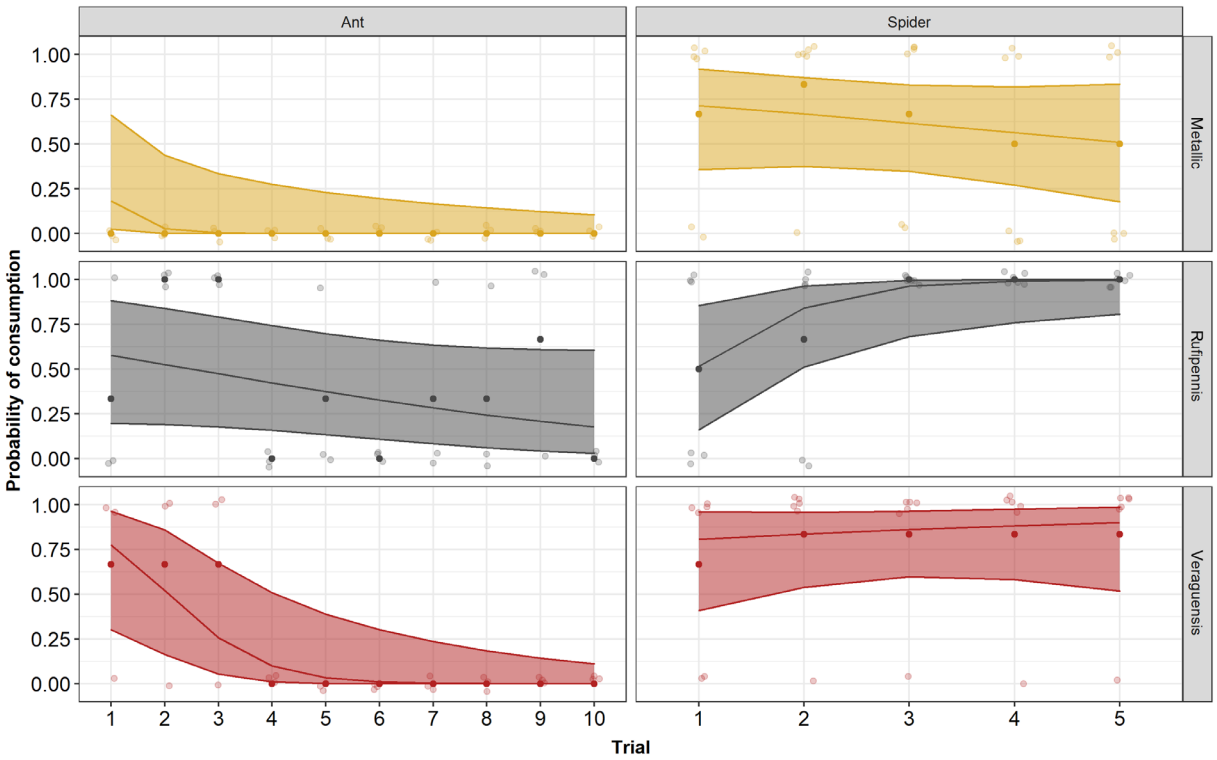

FIGURE 3 Probability of consumption of different beetle phenotypes by ants and spiders over multiple trials. Solid lines represent best fit of logistic regression model and ribbons represent 95% confident intervals for the model. Bold points in each panel show the average, faded points are the raw values for that trial.

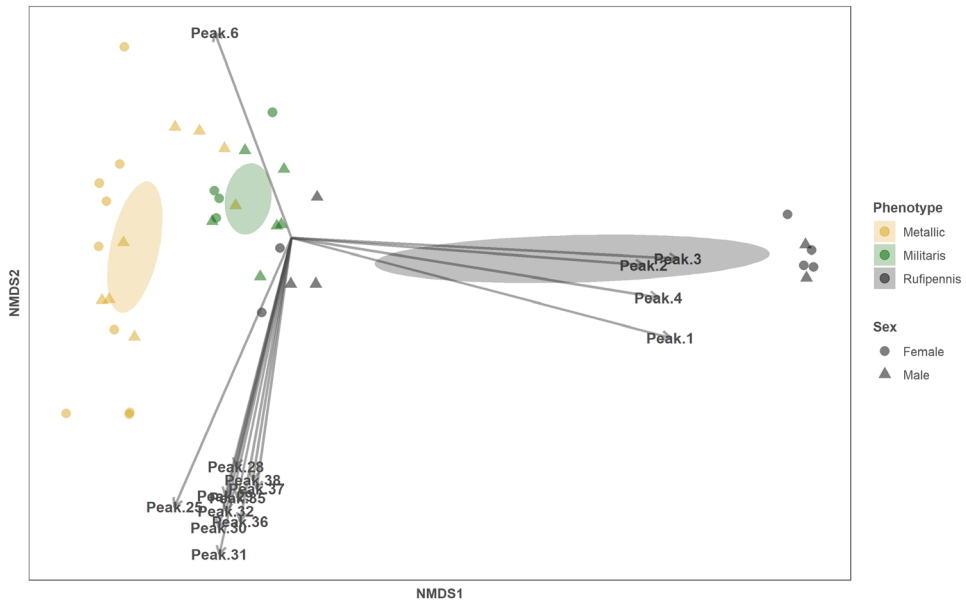

FIGURE 4 Non-metric multidimensional scaling (NMDS) biplot illustrating variation in cuticular hydrocarbon profiles for beetle phenotypes, using the relative abundance of elytral compounds. There are 16 compounds (peaks), shown as black arrows, that contribute to overall chemical variation ($p = 0.001$). The ellipses around each cluster represent 95% CIs. Stress = 0.096

the web by the spider through a cutting behaviour (involves the spider cutting the web around the beetle, causing the beetle to fall from the web immediately or loosens the webbing enough that the beetle can manoeuvre and escape on its own) and then was removed from the mesh enclosure by the observer.

There were no changes in behaviour by spiders towards any *C. alternans* phenotype over repeated trials. Response times remained constant over multiple encounters ($F_{4,60} = 1.344$, $p > 0.05$; Figure 2a), did not differ between phenotypes ($F_{2,60} = 0.187$, $p > 0.05$) and did not show a significant interaction between trial and phenotype ($F_{8,60} = 1.714$, $p > 0.05$) as shown by a repeated measures ANOVA. Although handling time did differ among phenotypes ($F_{2,60} = 4.4807$, $p < 0.05$; Figure 2c), there was no difference after multiple encounters ($F_{4,60} = 0.5759$, $p > 0.05$) nor an interaction between the two ($F_{8,60} = 0.5086$, $p > 0.05$). Results of the GEE show no effect of trial (d.f. = 4, $\chi^2 = 2.66$, $p > 0.05$) or of phenotype (d.f. = 2, $\chi^2 = 3.74$, $p > 0.05$; Figure 3) on consumption.

When considering the ant predator, there were likewise no changes in response time through multiple encounters with *C. alternans* ($F_{9,54} = 1.754$, $p > 0.05$; Figure 2b) and no differences in response time as a function of beetle phenotype ($F_{2,6} = 0.199$, $p > 0.05$), nor an interaction between the two ($F_{18,54} = 1.201$, $p > 0.05$). There were three extreme outliers that were removed; however, these did not change the results (analyses with outliers: no effect of phenotype, $F_{2,6} = 0.479$, $p > 0.05$; of trial, $F_{9,54} = 1.114$, $p > 0.05$, or an interaction, $F_{18,54} = 1.405$, $p > 0.05$). Handling time for ants, did not change over multiple trials ($F_{9,54} = 1.79$, $p > 0.05$; Figure 2d), but differed between phenotypes ($F_{2,6} = 16.25$, $p < 0.01$) and also showed a phenotype by trial interaction ($F_{18,54} = 2.055$, $p < 0.05$). Moreover, ants did not consume all phenotypes indiscriminately as the metallic phenotype was completely avoided (d.f. = 3, $p < 0.0001$; Figure 3). Consumption differed between predators, and changed over multiple trials for carton-nest ants. The probability of consumption differed between ant and spider predators as shown by the logistic regression and decreased after multiple encounters for ant predators (d.f. = 9, $p < 0.001$; Figure 3). However, there was no interaction between trial and beetle phenotype (d.f. = 18, $p > 0.05$) as shown by the GEE (Figure 3).

Differences in CHC profiles among beetle colour phenotypes

As chemically oriented predators often rely on contact chemical cues to recognise potential prey items, CHC profiles from phenotypes of *C. alternans* were evaluated to determine whether this may mediate behavioural differences in predator response. Phenotypes do show differentiation based on CHC profile ($F_{2,35} = 7.91$, $p < 0.001$) with phenotypes clustering distinctly (Figure 4; pairwise comparisons: metallic-militaris, $p = 0.0015$; metallic-rufipennis, $p = 0.0003$; militaris-rufipennis, $p = 0.0012$). No distinct chemical differences between males and females were observed ($p > 0.05$, Figure 4). There were 16 chemical compounds (peaks) which differed in their abundance among phenotypes ($p = 0.001$), and differentiated phenotypic clusters. Peaks 1–4 differentiate the cluster of six rufipennis individuals (Figure 4) and 3 of these were identified as, (1) *n*-tricosane, (2) *n*-tetracosane, and (3) *n*-hexacosane, with the highest degree of variation in the relative abundance of these peaks occurring in the rufipennis phenotype (Figure 5 and Table S5). The differences in chromatograms are shown in Figure S2.

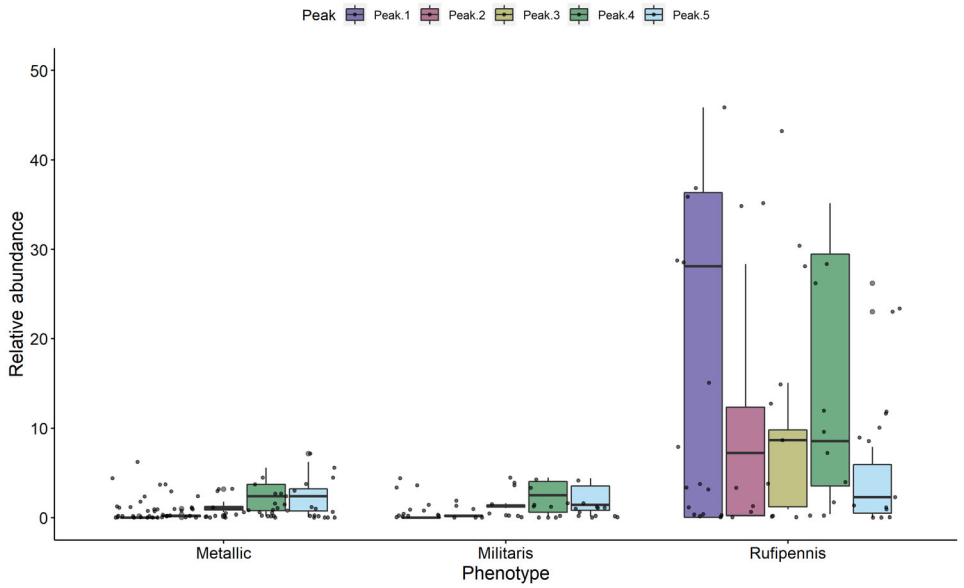

FIGURE 5 Five elytral compounds (peaks) differ in abundance among beetle phenotypes, and contribute to overall variation in elytral cuticular hydrocarbon profile.

DISCUSSION

The three colour pattern phenotypes of *C. alternans* tested here elicited distinct responses from the three invertebrate predators used in this study. There are behavioural differences in response time and handling time between predators. In addition, each beetle phenotype elicited different levels of predation from the different predators. Moreover, there were distinct differences in contact chemical cues, measured as CHC profiles, between phenotypes, and within phenotypes. Interestingly, the phenotype which elicited the most variable response from predators, also displayed the greatest amount of variation in CHC profile.

The differences between response time and handling time between the three predators may be due to differences in prey detection strategies and life-history characteristics (Abrams, 2000). Mantises are visually oriented predators, sit-and-wait predators, which may result in the longer response time (Rossoni & Niven, 2020; Yamawaki, 2017). The much longer handling time displayed by the ants when compared to the mantises and spiders may be a direct result of solitary versus cooperative living and decision-making in these predators (Sudd, 1965).

The orb-weaving spider, is the only predator used that consistently consumed individuals of *C. alternans*, regardless of trial or phenotype. This may be due, in part, to the injection-style of predation exhibited by orb-weaving spiders (Robinson & Mirick, 1971). The elytra (the colour bearing portion) of the beetle can convey prey status through the presence of CHCs. However, spiders punctured beetles on their soft dorsal side, injecting enzymatic fluids to liquefy the tissue, and then discard the elytra and pronotum. As a result, orb-weaving spiders may never come in contact with noxious compounds emitted by *C. alternans*.

Mantises exhibit an adverse behavioural response to *C. alternans* with no individual in our bioassays apparently considering *C. alternans* as potential prey. Mantises are opportunistic predators, known to consume other arthropods, and have even been reported to consume small vertebrates, including certain birds, lizards, and mammals (Nyffeler et al., 2017). As mantises visually detect prey and are highly tactile predators, they can likely detect visual cues as well as contact chemical cues from *C. alternans*, but the ability to discern the importance of each mechanism was outside of the scope of this study. High mortality in preliminary trials precluded using individual mantises in more than one trial. However, it would have been interesting to note if mantises would consume beetles after several days of starvation as observed in the Berenbaum and Miliczky (1984) study, which found that mantises vomited during or soon after consuming the bugs but would nonetheless eat them after several days.

Interestingly, the three phenotypes used elicit very different responses from the carton-nest ants. In every colony tested and over every trial, there was never an individual of the metallic phenotype consumed. Only two colonies in the first three trials consumed the veraguensis phenotype, and in subsequent trials, all individuals were discarded. This particular pattern is indicative of learned avoidance behaviour that is typically seen when predators sample potential prey items, learn of their unpalatability, and then avoid them in subsequent encounters (Skelhorn & Rowe, 2006; Svennungsen & Holen, 2007). In contrast, the rufipennis phenotype received the most variable treatment of all three phenotypes used for carton-nest ant bioassays. In some trials, every colony presented with this phenotype consumed the individual, but in subsequent trials, none of the colonies consumed the individual presented. This variation in response to the rufipennis phenotype may be partly due to the variation in the genotype of individuals used for these studies. The metallic phenotype is only expressed when two recessive 'r' alleles affecting colour expression are inherited (rr) and thus all individuals have the same genotype at this locus. Moreover, all of the veraguensis individuals used for this study were heterozygotes (Vr). However, the rufipennis individuals used for these studies were either homozygous or heterozygous (RR or Rr),

but the genotype itself was not known at the time of the predation trials. Although we did not perform a chemical analysis on the same individuals used in the predator bioassays, results of the CHC study show that individuals of the rufipennis phenotype display the most pronounced variation in elytral chemical profiles, and perhaps these cues were detected by the ants and used to deduce levels of palatability.

Because all predators used in these bioassays were wild-caught individuals, it is possible that previous encounters influenced the responses of the individuals here. In the case of the orb-weaving spiders (*T. clavipes*), individuals may have learned of their ability to bypass beetle defences from prior experience. Because this predator builds its web along microhabitats similar to where the host plant of *C. alternans* grows, it is likely that beetles can fall into orb-weaver webs or have been encountered in their environments.

Aposematic signalling relies on unpalatability, which may be acquired through de novo production of toxins or the sequestration of host plant secondary metabolites (Ruxton et al., 2004). One of the underlying theoretical expectations of aposematic signalling is that individuals within a population will converge on an 'optimal' phenotype as predators learn to avoid specific signals and patterns, thus eroding variation over time (Mallet & Singer, 1987; Sherratt, 2008). However, persistent variation in aposematic signalling has been observed in a variety of taxa including; *Heliconius* butterflies (Langham, 2004), dendrobatid frogs (Rojas & Endler, 2013), and coccinellid beetles (Wheeler et al., 2015); to the extent that variation in aposematic signalling may be the norm rather than the exception (Briolat et al., 2019). The presence of persistent polymorphisms under a scenario where the consequences of divergence are expected to be quite high (decreased signal recognition resulting in increased predation) provides an ideal setting for investigating the interactions between the ecological and genomic factors maintaining intraspecific phenotypic variation (Briolat et al., 2019; Summers et al., 1997). However, documenting aposematic organisms can be quite difficult. Studies must show that the organism is (1) chemically and/or structurally defended (2) advertises this defence with a warning signal (colouration, odours, sounds) (3) and that the signal is used by predators to detect unpalatability and deters predation (Briolat et al., 2019; Rojas et al., 2015; Ruxton et al., 2004).

A potential explanation for polymorphic variation in aposematic signalling may be a direct result of variation in unpalatability, such that more conspicuous individuals or phenotypes are more toxic (Ruxton et al., 2004). Studies with two species of ladybird beetles, *Coccinella septempunctata* and *Harmonia axyridis*, have shown a positive correlation between elytral colouration and sequestered alkaloid content, such that individuals with higher levels of elytral carotenoid content (more red colouration) also had higher levels of defensive alkaloids (Bezzerides et al., 2007; Blount et al., 2012). In paper wasps, *Polistes dominula*, individuals that were more brightly coloured were also more toxic, indicating honest signal variation (Vidal-Cordero et al., 2012). It is possible that phenotypic variation in *C. alternans* is correlated with variable levels of palatability.

The five genetically distinct phenotypes of *C. alternans* do show some geographic partitioning, with the metallic and rufipennis phenotypes being the most widely distributed, occurring on both the western and eastern ends of the Isthmus of Panama. The veraguensis phenotype is found throughout populations in western Panama, including the Azuero peninsula, and the militaris-a and militaris-b (the militaris-b phenotype was not used in these studies) phenotypes are found from Central Panama through eastern Panama and into Colombia (see Strickland et al., 2019 for full details on phenotype distribution and colour pattern inheritance).

Research into other mechanisms contributing to the maintenance of colour variation in this species has examined the effects of nonrandom mating in adult phenotypes and differences in larval survival between assortative and disassortative parental pairings (Strickland et al., 2021). Adult females have also been shown to eject spermatozoa droplets of undesirable males after mating, suggesting that cryptic female choice may also be a significant selective mechanism for this species (Rodriguez, 1995). Although individuals mate randomly for colour pattern phenotype, it is possible that individuals use CHC profile as a mating cue. A reciprocal study using the transference of CHC mixtures between phenotypes, or using glass beads as models, could help differentiate the effects of colour pattern and CHC profile on mate choice. The adult stages of a closely related species, *Chelymorpha cribraria*, has also been described as having extensive colour pattern polymorphisms with several phenotypes displaying the same elytral and pronotal colouration (metallic, militaris-a, and militaris-b). It is hypothesized that *C. cribraria* is a part of a mimicry complex with other beetles in the families Coccinelidae and Chrysomelidae throughout Central and South America (Vasconcellos, 1988). It is possible that both species are involved in a beetle colour pattern mimicry complex, however, no further work has been done on this. At the larval stages, other selective pressures act on individuals, most notably the effects of parasitoids, as some populations experience parasitization rates as high as 50% (Cuignet et al., 2008). Moreover, the endocellular bacterium *Wolbachia* has been shown to have some pronounced effects in *C. alternans*, with some populations experiencing single and double infections. As *Wolbachia* can affect reproduction through cytoplasmic incompatibility when two individuals infected with different strains mate, this could be a factor affecting population distributions across Panama (Keller et al., 2004).

CHCs have been shown to act as recognition signals in mate choice for identifying both hetero- and conspecifics. However, their role in predator–prey interactions is relatively understudied. One study assessing CHC profiles of the leaf beetle, *Altica viridicyanea*, show that CHCs serve as the pivotal cue to the predatory stink bug, *Zicrona caerulea* (Xue et al., 2018). In addition, the nest-provisioning wasp, *Cerceris fumipennis*, uses CHCs as a kairomone to specifically identify buprestid beetles (Rutledge et al., 2014); and the digger wasp, *Trachypus boharti*, likely uses CHC cues to identify its prey (specifically males of the stingless bee, *Scaptotrigona postica*) (Koedam et al., 2011). Thus, additional attention needs to be given to the role of CHCs in predator–prey interactions.

In studies assessing relationships between CHC profiles and levels of parasitization, octocosane, docosane, and tricosane elicit high levels of egg-laying in the parasitoid wasp, *Trichogramma chilonis* (Padmavathi & Paul, 1998). In a separate study assessing two species of *Trichogramma*, *Trichogramma brasiliensis*, and *Trichogramma exiguum*, pentacosane and hexacosane elicited a very high parasitoid

activity index, followed by docosane, tricosane, heneicosane, hexatriacontane, and tetracosane (Paul et al., 2002). This would indicate that these particular hydrocarbons have high kairmonal activity, for these parasitoids, and perhaps for others as well. Tricosane has also been shown to increase the rate of parasitization by *Trichogramma pretiosum* and *Trichogramma achaeae* (Gross et al., 1975). In whole, this would indicate that specific hydrocarbons and their mixtures can provide cues to predators, parasites, and conspecifics as to the suitability of an individual as a potential prey item, host, or mate.

Two caveats should be addressed here. Freshly frozen beetles were used in the ant bioassays to prevent beetles from running or flying off the platform before the experiment could begin. Because mantis and spider bioassays were performed in small, enclosed containers, this was not a constraint in these experiments. The colouration of *C. alternans* takes several days to fade after death, so flash-freezing beetles over a short time scale (30 min prior to the start of trials) does not affect colouration (L.R. Strickland, personal observation, July 15, 2016). In addition, because CHCs are highly stable and non-volatile compounds, their degradation is expected to be slow. A study assessing CHC profiles of Sacrophagidae flies (Diptera) showed that wild-caught specimens and museum specimens collected up to 117 years prior to analysis could be identified by their CHC profiles (Moore et al., 2021). This suggests that CHC profiles are highly reliable for identification, and flash-freezing beetles should not affect the CHC profiles and corresponding ant responses in this study. Previous predator bioassays with *A. chartifex* and larvae of a variety of tortoise beetle species, including *C. alternans*, showed that live and freshly-killed prey were both readily consumed (Vencl et al., 2005). Therefore, changes in behaviour (lack of movement) should not affect the results presented here.

Second, predator bioassays were conducted over the course of two field seasons, with spider bioassays conducted in July and August of 2016, and ant and mantis bioassays conducted in July and August of 2017. It is possible that temporal differences may have affected results, however as both studies happened during the rainy season, in the same locale (Gamboa, Panama), it is unlikely that this would produce the specific trends seen in this study.

Taken together, these results support that *C. alternans* is indeed an aposematic organism, advertising unpalatability to potential predators. Phenotypes do receive different treatment from predators, indicating different fitness levels between phenotypes. For instance, it would seem that the veraguensis and metallic phenotypes have similar levels of overall palatability. However, the metallic phenotype elicited avoidance by ants and mantises before consumption, whereas ants sampled multiple individuals of the veraguensis phenotype before learning of their unpalatability. An important note, only three of the five phenotypes of *C. alternans* were used for predator studies. Future studies should assess differences in all phenotypes and take into consideration predatory bugs (Hemiptera), as well as potential avian predators. As these beetles likely face a diverse array of invertebrate and vertebrate predators, there remains much to be done in understanding the ecological factors involved in polymorphic maintenance within this system.

AUTHOR CONTRIBUTIONS

Lynette R. Strickland conducted all experiments and data collection, participated in data analyses and preparation of the manuscript. Donald Windsor contributed to the experimental design and preparation of the manuscript. Carla E. Cáceres contributed to data analyses and preparation of the manuscript.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

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ORCID

Lynette R. Strickland https://orcid.org/0000-0003-2940-4685
Donald Windsor https://orcid.org/0000-0003-2769-2601
Carla E. Cáceres https://orcid.org/0000-0003-0532-2941

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1. The total number of beetles consumed over all trials/phenotype. *Azteca chartifex* had three separate colonies/phenotype (9 colonies total). Mantises had six individuals/phenotype. *Trichonephila clavipes* had six individuals/phenotype. The average amount of consumption was taken by dividing the total eaten, by the number of individuals tested for that predator. The mean, SEs and 95% CIs are also shown.

Table S2. The amount of time (rounded to nearest minute) until the first strike for each mantis, whether or not the individual consumed the beetle and moth (response) and the phenotype of the beetle.

Table S3. Each individual of *Trichonephila clavipes* and the amount of time (rounded to nearest minute) for initial movement toward the beetle, the handling time (minutes) and whether the beetle was consumed for each trial (response). The overall mean and SD are also shown as well as the mean and SD/phenotype.

Table S4. Each colony of *A. chartifex* and the amount of time (rounded to nearest second) until the first antennation (contact); the phenotype of the beetle presented and whether the beetle was consumed for each trial (response). The total mean and SD are shown as well as the mean and SD/phenotype. The last column shows the colonies response to the presented control cricket (eaten = Y, rejected = N).

Table S5. The relative abundances (%) of the three most commonly identified peaks from each sample.

Figure S1. Differences in the probability of consumption of beetle phenotypes among predators for the first trial only, as shown by a bias reduced logistic regression ($\chi^2 = 20.7701$, d.f. = 2, $p < 0.0001$).

Figure S2. The GC–MS chromatogram of two representative individuals. The *x*-axis shows retention time (minutes), and the *y*-axis shows peak abundance. (A) The chromatogram of a metallic female, representative of the individuals that formed the large group cluster. (B) The chromatogram of a rufipennis female, representative of the six individuals who clustered separately.

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