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Larval-Ant Interactions in the Mojave Desert: Communication Brings Us Together

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LARVAL-ANT INTERACTIONS IN THE MOJAVE DESERT:

COMMUNICATION BRINGS US TOGETHER

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Abstract

Butterflies are a diverse and essential group of pollinators whose abundance is predominantly determined by growth and survival of their larvae. In the family Lycaenidae, many species participate in a larval-ant mutualism where ants feed on nutrient-rich nectar produced by larvae and, in turn, protect those larvae from predators. Emerging evidence indicates larval scent in the form of cuticular hydrocarbons (CHCs), not nectar production, drives this interaction. This study takes two approaches to investigate CHCs as the driver behind the larval-ant mutualism in three butterfly species native to southern Nevada: *Euphilotes bernardino martini* (Martin's blue), *Brephidium exilis* (western pygmy blue), and *Euphilotes ancilla* (Rocky Mountain dotted blue). First, behavioral assays are conducted to investigate larval-ant interactions in early and late larval instars both with live larvae and beads coated in larval extract. Second, the general composition of hydrocarbons found on the larval cuticles of these mutualist species is identified along with those from four species of attendant ants (*Forelius pruinosus, Camponotus spp, Crematogaster mormonum,* and *Linepithema humile*) to determine how varying CHC composition may be implicated in initiating and maintaining this important mutualism. In addition, this study addresses the novel hypothesis that early instar larvae produce a simple CHC suite to avoid ant aggression and then, in late instars, switch to producing a complex CHC suite that encourages ant interaction. This study finds partial support for CHC overlap in mutualist larvae and ants but does not find support for a developmental shift in CHC production.

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Section 1: Introduction

Mutualism Overview

The family Lycaenidae, the gossamer-winged butterflies, is the world's second largest butterfly family. More than one third of this family - over 2,700 species - participate in a well-documented mutualism with ants that is mediated by inter-specific communication including behavioral, auditory and chemical cues (Pierce et al. 2002). Since at least the 19th century, scientists and naturalists alike have documented this fascinating relationship (Newcomer 1912). Members of the Lycaenid family occur on six continents and larval-ant interactions occur wherever these butterflies are found. These interactions range from larval parasitism of ant colonies to loosely facultative larval-ant mutualism to obligate mutualism (Malicky 1970; Pech et al. 2004; Pierce et al. 2002; Thomas et al. 1998). In these unique mutualistic interactions, tending ants guard and protect myrmecophilous larvae from predators. In return, the ants are provided a nutritious liquid reward referred to as nectar (Ballmer and Pratt 1991; Devries 1998; Fiedler and Hagemann 1992; Hojo et al. 2014; Pierce et al. 2002).

The proposed benefits of this larval-ant mutualism are clear. Larvae participating in these interactions are expected to experience decreased predation, a possible decrease in attacks by parasitoids (though this is a highly debated effect – see Malicky 1970; Weeks 2003; and others), increased body size, and decreased development time (Cushman et al. 1994; Pierce et al. 1987 and 2002; Wagner 1993). Together, this implies that, with tending ants present, larvae develop a larger body size at a faster rate. A decrease in development time

means less time for a defenseless larva to be exposed to predators and parasitoids, further increasing survival rates. In addition, increased body size seen with attendant ants may have positive impacts on fecundity after these individuals reach adulthood (Elgar and Pierce 1988). Though it seems the proposed effects from this well-studied interaction would be strongly supported, studies show varying results both within and between larval species with respect to

Table 1. Summary of lycaenid larval ant-organs initially described by Malicky (1970) and their currently known functions.

Larval Organ/Adaptation	Function	Source
Dorsal nectar organ	Exocrine gland located on dorsum of 7th abdominal segment; produces nutrient-rich "nectar" considered to be the driver behind the larval-ant mutualism	Malicky 1970; Fiedler and Maschwitz 1987; Pierce et al 2002
Tentacular organs	Paired eversible structures located on dorsum of 8th abdominal segment; presumed to release/produce volatile compounds that can result in a variety of reactions including patrolling, aggression, and calming in attendant ants	Malicky 1970; Fiedler and Maschwitz 1987; Pierce et al 2002
Pore cupola organs	Single-celled epidermal glands ("lenticles") scattered over the lycaenid body surface in all species with few exceptions; potential appeasement organs that may secrete pacifying substances	Malicky 1970; Fiedler and Maschwitz 1987; Pierce et al 2002
Lack of "beat reflex"	In most lycaenid myrmecophiles, larvae lack typical reflex thrashing displayed when physically disturbed – likely because these movements typically evoke aggression from ants	Malicky 1970
Thickened cuticle	Cuticle can be 20-60 times thicker than in non-myrmecophilous larvae; composed mainly of tough endocuticle; structure described as a "goal adaptation" to mutualist ant mandibles wherein bites will be resisted and fold the cuticle inward to push vital organs toward center of larval body	Malicky 1970

each proposed benefit (Weeks 2003). Nevertheless, the larval-ant mutualism is ubiquitous and, though the effects on larvae are variable, the effect on ants is somewhat less variable.

For ants, the receipt of a nectar reward is a valuable trade for efforts spent guarding their larval mutualist. Though some ants that participate in this mutualism are omnivorous, and therefore may eat non-mutualist larvae, Fiedler and Hagemann (1992) found that it is in fact energetically more efficient for ants to consume larval nectar than to consume the larva itself. Larval behavior, including nectar secretion rates, can vary depending on the identity and aggression of tending ants (Axen 2000; Fiedler and Hagemann 1992). Variability in behavior is a valuable strategy as it is known that, especially for facultatively myrmecophilous larvae, the species, aggressiveness, and effectiveness of attendant ants can vary greatly both spatially and temporally in a single geographic range (Peterson 1995). In the case of variable ant partners for *Glaucopsyche lygdamus*, Axen (2000) showed that larger, more aggressive ants are given more nectar droplets than smaller, less aggressive ants except in the case of the smallest, least aggressive ant species. These ants, *Tapinoma sessile*, were given nearly twice the number of nectar droplets of the largest, most aggressive ant, *Formica obscuripes*. Thus, larvae apparently adjusted nectar secretion rates to the presence of different ant species, producing more nectar to entice and bind less protective ants (*T. sessile*) and producing less nectar to avoid attracting larger numbers of more aggressive partners (*F.* obscuripes) while still appeasing those partners present (Axen 2000).

In addition to variation in nectar droplet frequency, it has also been shown nectar composition varies by larval species and, in some cases, by attendant ant species. In a study on various plant nectar and honeydew sources performed in Australia, it was shown that ants will

preferentially consume and dominate these food sources if they contain amino acids, and that sucrose was the most valuable sugar present (Bluthgen et al. 2004). In a comparative study on larval nectar composition, Daniels et al. (2005) found that stronger larval mutualists may have significantly higher amounts and types of amino acids in their nectar than weak mutualists. The same study also found that weak mutualists may compensate for the lack of amino acids, a phylogenetically independent trait, by offering valuable sugars (in this case, melezitose). In either case, the amino acid patterns found in larval hemolymph were significantly different from those found in larval nectar (Daniels et al. 2005), suggesting a secondary reinforcement for nectar as a nutritive source for attendant ants over consumption of the larva itself. This supports the hypothesis of DeVries and Baker (1989), who proposed that larvae tended by insectivorous ants may produce nectar with similar nutrient content to arthropod prey (high amino acid and low sugar concentrations) and, conversely, larvae tended by nectarivorous ants may produce nectar that approximates the nectar of plants (typically low amino acid and high sugar concentrations).

In summary, research has shown that larval nectar provides valuable nutrients in exchange for protection from an ant guard and that, by increasing the value of the nectar, larvae may decrease the risk of ant aggression and increase ant attendance. However, recent work suggests some larvae may add something more than valuable sugars and amino acids to their nectar. One study has shown that *Narathura japonica* larval nectar may significantly decrease dopamine levels in their attendant ants, *Pristomyrmex punctatus* (Hojo et al. 2015). This decrease in dopamine appeared to decrease locomotion in attendant ants, assuring a standing guard, while increasing aggression in response to larval tentacular organ eversions

over time (also seen in some Riodinid larvae – see DeVries 1988). The authors suggest that this manipulation of the food-for-defense interaction between larvae and ants may indicate the relationship is parasitic, rather than mutualistic, in nature. While this discovery does shed new light on the role of larval nectar in larval-ant interactions, it does not necessarily mean that the relationship is parasitic in nature (Heil 2015), nor that the ants do not benefit from participating in the interaction more than they would otherwise (Fiedler and Hagemann 1992). One of the most important conclusions that can be made from this discovery is that larval nectar can play a variety of roles in the larval-ant mutualism, and that the variability in the production, composition, and presentation of nectar by larvae has thus far led researchers to conclude that larval nectar is the driver behind this interaction.

Mechanisms for Mutualism

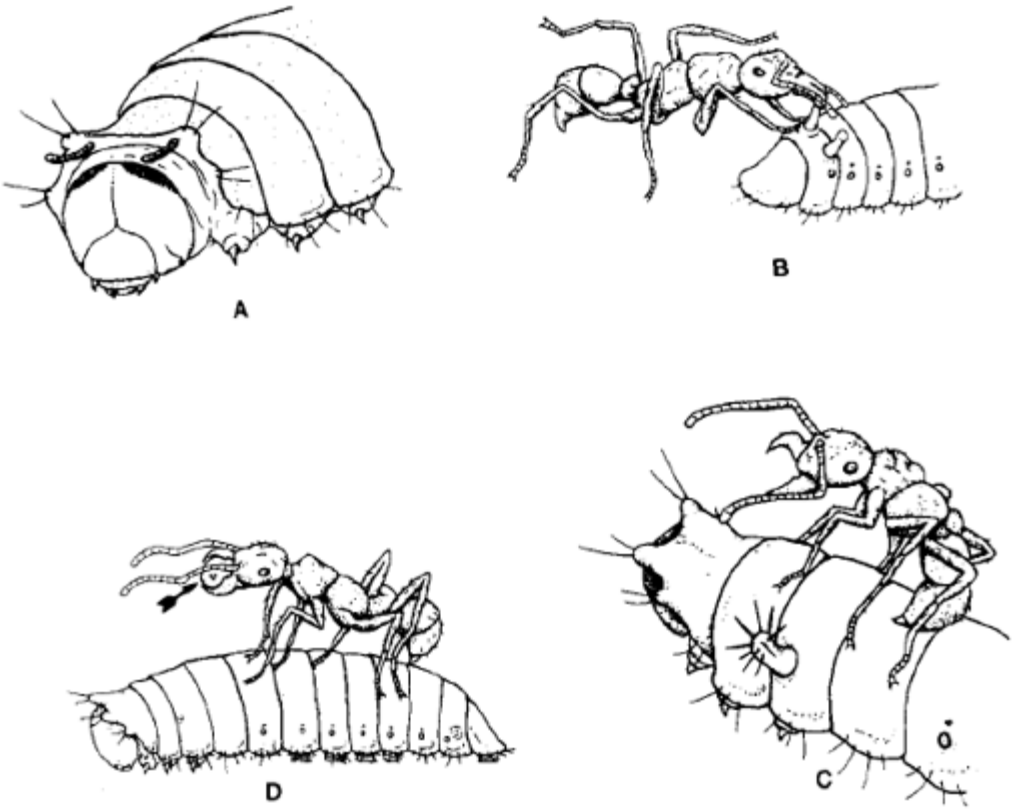
Many studies have explored the details of the larval-ant mutualism. This interaction occurs on a gradient where the strength of the interaction can range from weakly facultative to strongly facultative to obligate. The ability of larvae to reciprocate and appease tending ants may play a role in this variation.

Tending by larval ants is easily distinguished from exploration by antennal activity. When ants are merely exploring their environment, slow antennal movement is observed. This is called “groping” (Malicky 1970). However, when an ant encounters an object of interest, quick and repeated antennation is observed. This is called “palpation” and is distinct from groping by more than just speed (Malicky 1970). During palpation, ants hold their antennae at

an acute angle (Malicky 1970) and this angle generally places the tips of the antennae closer to the mandibles than during groping. Other activity displayed by ants, such as incidental contact lasting less than 1 second, resting atop a larva, preening atop a larva, or aggression are not considered to be part of tending behavior (Ballmer and Pratt 1991).

Palpation of larvae is also easily noticeable due to the fact that ants will palpate certain areas of interest on a larva more than others (Malicky 1970). Because larval nectar production can easily be observed on the dorsum of the larva with low-level magnification, and palpation commonly occurs here, it has long been assumed that the dorsal nectar organ (DNO) is responsible for driving this interaction (Newcomer 1912 and others). This is an especially compelling hypothesis when larvae with multiple species of attendant ants are observed. For example, in the Wenatchee Mountains of Washington state, larvae of the blue butterfly *Euphilotes ancilla* have been observed to be tended by no less than fifteen different species of ants from three different subfamilies throughout larval development (Peterson 1995). Nevertheless, not all larvae that are tended by ants have a DNO, so something else must play a role in this interaction (Malicky 1970). In 1970, Malicky described in detail the larval organs present in late larval instars that induced palpation of larvae. These organs are: the dorsal nectar organ (DNO), tentacular organs (TOs), and pore cupola organs (PCOs). Malicky also summarized the various adaptations myrmecophilous larvae have developed as a result of their associations with ants (summarized in Table 1).

Figure 1. The process of "enticement and binding". A) larva calls ant via sound production B) larva rewards ant with nectar secretion from DNO after initial contact C) larva utilizes intermittent TO eversions to maintain alert state in ant guard D) larva maintains ant guard through continued use of primary ant organs (DNO and TOs). *Image adapted from DeVries 1988.*



In 1988, DeVries described in larvae of the myrmecophilous riodinid *Thisbe irenea* a process named "enticement and binding" wherein larvae use larval ant organs to attract attendant ants and then "bribe" them to continue to tend rather than leave and forage elsewhere (Figure 1). The steps of the enticement and binding process are reported as follows:

1. Larvae call and attract ants via sound production
2. Larvae reward ants with a nectar secretion shortly after initial contact

3. Larvae utilize intermittent tentacular organ eversions to put ants into an alert state. In *T. irenea*, these organs produce semiochemicals that induce an alarm state in attendant ants
4. Continued use of the primary ant organs binds ants to larvae, maintaining their ant guard for extended periods of time

Lycaenid larvae are known to produce calls as well, although not through the use of vibratory papillae or specialized epicranial structures as in the riodinids, and these calls can be highly variable and produced by mutualist and non-mutualist species alike (DeVries 1991; Schonrogge et al. 2017; Travassos and Pierce 2000). In light of this inconsistency, there is still the question of whether a universal mechanism by which larvae can attract and bind a standing ant guard exists.

Mutualism Breakdown

Interestingly, most studies on larval-ant interactions focus on larvae in the third instar and later (hereafter referred to as "late instar larvae"). Rarely are larvae in instars one and two (hereafter referred to as "early instar larvae") mentioned. This is presumably because, as in larvae of the family Riodinidae, early instar lycaenid larvae do not have functional "ant organs" that secrete nectar (DeVries 1989; DeVries 1991). This revelation raises several important questions. Namely, how do potential attendant ants interact with early instar larvae? If nectar is the driver in this relationship, there must be some way in which early instar larvae can appease ants that encounter them to escape attack or predation. More importantly, when ants

encounter early instar, non-reciprocating, myrmecophilous larvae, what is the implication for the larval-ant mutualism if early instar larvae do not provide a reward? What prevents the lack of a food reward from precipitating mutualism breakdown?

One of the three main hypotheses regarding the breakdown of a mutualism focuses on reciprocity, where a cooperative benefit is given from partner A (here, larvae) to partner B (here, ants) in exchange for a net fitness benefit (Sachs and Simms 2006). Reciprocity relies on partner fidelity, or repeated interactions between partners, and thus breakdown can occur when one partner sanctions the other (Edwards et al. 2006; Kiers et al. 2003; Pellmyr and Huth 1994; Sachs and Simms 2006). In the case of early instar myrmecophilous larvae that are unable to provide a benefit to their partner, the lack of a nectar reward may act as a sanction in the mutualism. Thus, it would seem the variability in benefit exchange where ants sometimes encounter non-cooperative partners and sometimes encounter cooperative partners would foster a highly facultative, or optional, relationship that could often cease altogether. However, many lycaenid larval-ant mutualisms are obligate or stable despite the fact that early instars are incapable of providing a nectar reward. Therefore, there must be a secondary driver behind the larval-ant mutualism aside from nectar that allows partner ants to both recognize potential reciprocating partner larvae and maintain partner fidelity in the face of variable partner reward responses.

This study seeks to determine what factors in addition to nectar influence larval-ant mutualisms. In an attempt to begin addressing the gap in information on the relationship between early instar larvae and mutualist ants, this study will examine the drivers behind the larval-ant mutualism in both early and late instar larval stages. First, behavioral assays will be

used to assess mutualist ant behavior toward both naturally rewardless early instar larvae and typical late instar larvae that provide a reward. In addition, the question of a second reinforcer for the larval-ant mutualism will be addressed using chemical analysis of larval and ant semiochemical suites present on the cuticle.

Inter-Species Communication as a Driver

With such variation in presence, and even function, of larval ant organs, it seems there must be some other underlying mechanism that aids all mutualist larvae in communicating with a variety of potential attendant ants. Recently, a series of laboratory experiments yielded interesting new information about what may drive this interaction (Hojo et al. 2014).

Using larvae of the genus *Narathura japonica*, which are tended in the field by *Pristomyrmex punctatus* ants, behavioral assays were conducted in which *P. puncatatus* behaviors toward intact larvae capable of providing a nectar reward, larvae rendered rewardless, and beads coated in larval cuticular extract were observed. Two populations of *P. punctatus* ants were used – one "experienced" group that had previous exposure to mutualist larvae and one "inexperienced" group that had no previous exposure to mutualist larvae. The authors made several significant observations with these assays:

1. Prior exposure to larvae and the number and frequency of nectar droplets released from the DNO are important for inducing tending by ants
2. Experienced ants will tend mutualist larvae that have been rendered rewardless, indicating that nectar is not the driver behind this interaction

3. Experienced ants will tend glass beads coated in hydrocarbons extracted from the cuticle of mutualist larvae
4. Ants use simple associative learning to determine when to tend larvae where a recognizable cuticular hydrocarbon (CHC) profile, paired with a nectar reward, induces tending

These findings point to CHCs as a driver for ant interaction with mutualist larvae. This is compelling as CHCs, unlike ant organs, are produced on all larval cuticles. Thus, CHCs may be considered a universal trait of larvae and one that ants could have learned to recognize over time.

But are cuticular hydrocarbons a viable explanation? Can they be used for inter-species communication? Insect hydrocarbons are used for a variety of purposes and are ubiquitous throughout insects. In particular, CHCs play valuable roles in prevention of water loss, nest-mate recognition, mating cues, chemical mimicry, and even recognition of aphids by ants (Barbero 2016; Gibbs 1998; Gibbs et al. 2003; Howard and Blomquist 2005; Takahashi and Gassa 1995; Torres et al. 2007; Simmons et al. 2014). This last example best supports the hypothesis of inter-species communication playing a role in the larval-ant mutualism as aphid-ant interactions are well-studied (Stadler and Dixon 2005; Lang and Menzel 2011; Endo and Itino 2013). As in parasitic myrmecophiles, it could be that larvae produce a CHC profile that mimics attendant ant CHCs (Elmes et al. 2002). However, as mutualist larvae are not recognized as ant brood or carried to the nest, they would need to mimic the general colony odor, or *gestalt*, of their attendant ants rather than the CHC profile of a particular caste within the nest (van Zweden et al. 2010). This new, universal answer creates a new question: if CHCs are

universal, and not all larvae reciprocate, how do non-mutualist larvae avoid aggression from, and predation by, ants they encounter?

Hojo and colleagues (2014) conducted a second set of experiments in their study utilizing the non-myrmecophilous larval species *Lycaena phlaeas*. Using the same assays with *L. phlaeas* that had been performed with *N. japonica*, another set of significant findings was produced. Notably,

1. Experienced ants do not spend more time tending non-mutualist larvae than inexperienced ants (where experienced ants have previously been exposed to *L. phlaeas*)
2. Ants do not learn to associate the CHCs of non-mutualist larvae with a nectar reward and do not show increased tending when a nectar reward is paired with non-mutualist CHCs

These findings indicate that non-mutualist larvae may be missing a critical communication component found in mutualist larval CHCs. Indeed, Hojo and colleagues found that non-mutualist larvae have more simple (straight-chained, non-branching, non-modified) CHCs than mutualist larvae. Apparently, simple CHCs can reduce ant aggression during encounters with these potential predators. The authors proposed that the more complex suites of hydrocarbons (double-bonded, branching, modified) found on mutualist larval cuticles are more similar to hydrocarbons found on ant exoskeletons and, thus, make mutualist larvae better able to communicate with ants. The authors even hypothesize that larvae exhibiting a higher degree of mutualism with ants (i.e. highly facultative or obligate) will be more competitive attractors of

ant attendants than larvae with a lesser degree of mutualism (i.e. moderately to weakly facultative).

A New Answer to an Old Question

Inter-species communication as a driver for the larval-ant mutualism is an intriguing concept. Not only does this account for variation in larval ant organs, but the interspecific communication hypothesis may explain why some larvae are more highly tended than others. As with all new ideas, this hypothesis also raises new questions.

As has been noted, no matter the degree of mutualism, early instar mutualist larvae (instars one and two) are typically not tended by ants. Previously, evidence would suggest this is because these larvae are incapable of producing a nectar reward. But, if some species which lack a DNO altogether are found tended, why would early instars with apparently undeveloped DNOs not utilize other ant organs to elicit a tending response? As noted by Ballmer and Pratt (1991), there is high variation in the presence of ant organs across larval species. Thus, not all early instar mutualists may be capable of using alternative ant organs to communicate with tending ants. If this is the case, it would follow that early instar larvae rely on some other method to decrease ant aggression.

Following the new inter-specific communication hypothesis (Hojo et al. 2014), it would not be unreasonable to expect that mutualist larvae undergo a developmental shift wherein early instar mutualists produce simple CHCs that decrease ant aggression or mimic some entity commonly encountered by, and innocuous to, ants whereas late instar mutualists, now with

functional DNOs, produce complex CHCs that encourage ant interaction. A developmental shift may also help prevent mutualism breakdown by providing a clear distinction between larvae of the same species that can or cannot reciprocate in the larval-ant interaction.

This study will directly test two hypotheses by use of behavioral assays and chemical analysis of cuticular hydrocarbons. First, the claims of Hojo et al. (2014) will be tested to determine whether mutualist larvae evolve a matching subset of complex CHCs that overlap with attendant ant species depending on their degree of mutualism. Second, the developmental shift hypothesis will be tested to determine whether early larval instars produce a different subset of CHCs than late instars of the same species to prevent aggression from potential attendant ants as well as mutualism breakdown. Finally, this study will explore possible mechanisms for mimicry or avoidance utilized by early instar larvae. This is a novel approach to a long-studied interaction that will bring a new perspective to well-developed field.

Section 2: Methods

Selection of Study Species

This study aims to determine the driving factors behind the larval-ant mutualism from early to late instars and on a scale from weakly facultative to strongly facultative mutualism. Local, southern Nevada butterfly and ant species were chosen for ease of collection. Butterflies of the subfamily Polyommatinae (family Lycaenidae) were ideal due to relatively high abundance and the varying degrees of mutualism seen throughout local species.

The butterfly species chosen to represent the weakly facultative mutualist was *Euphilotes bernardino martini* (Mattoni, 1954). This species can be found in abundance in southern Nevada and frequents the host plant *Eriogonum fasiculatum,* the California buckwheat (Austin and Leary 2008). *E. bernardino martini* was rated by Ballmer and Pratt (1991) as a weakly facultative mutualist, being tended by ants in their experiment only 2% of the time. To represent the strongly facultative mutualist, *Brephidium exilis* (Boisduval, 1852) was chosen. Ballmer and Pratt (1991) rated *B. exilis* as a strongly facultative mutualist after observing its tending 98% of the time in laboratory experiments. *B. exilis* frequents host plant species from the family Amaranthaceae (Thacker 2004; Pittaway et al. 2006; Austin and Leary 2008) but was collected for this study from flowers of *Calliandra eriophylla*, a member of the family Fabaceae.

Ants were either collected with larvae (for hydrocarbon extraction) or from a single ant nest at a pre-determined location (for behavioral assays). Ants collected for hydrocarbon

extraction are identified in Table 2. Ants collected for behavioral assays were identified as the Argentine ant, *Linepithema humile,* Mayr 1868 (Wheeler and Wheeler 1986). Ants from a colony found at the base of a *C. eriophylla* plant near the laboratory were used as they consistently exhibited tending behavior toward *B. exilis* larvae in the field. Ants collected tending larvae at distant field sites displayed unreliable behavior transported to the lab and could not be used for behavioral assays. Utilizing *L. humile* individuals from a single, nearby nest known to display tending behavior allowed collection of data that more closely represented what is observed in the field (personal observation). Except in its native South American range,

L. humile is an invasive species world-wide, especially in Mediterranean climates (Gordon and Human 1996; Torres et al. 2007). It was introduced to North America in the early 1800s and was first recorded in Nevada in 1953, where its presence was noted in Boulder City, roughly 25 miles east of Las Vegas (Tsutsui and Case 2001; Wheeler and Wheeler 1986). *L. humile* is an omnivore known to be highly competitive in its introduced range and workers are known to be carnivorous, granivorous, consumers of plant floral and extrafloral secretions, and tenders of honeydew-producing insects (Bernays and Cornelius 1989; Bond and Slingsby 1984; Holway 1998; Wheeler and Wheeler 1986).

Table 2. Ant species collected for hydrocarbon extraction. Ant identification was made using the key published by Wheeler and Wheeler 1986. Larval species tended by each ant species at time of collection is noted.

Ant Species Collected	Larval Species Tended
Forelius pruinosus	*Euphilotes bernardino martini* *Euphilotes ancilla*
Camponotus spp	*Euphilotes bernardino martini*
Crematogaster mormonum	*Euphilotes ancilla*
Linepithema humile	*Brephidium exilis*

Field Sites

Euphilotes Bernardino martini and associated tending ants were collected south of Boulder City, Nevada in washes along Nelson Road, located east of Nevada Highway 95. *Euphilotes ancilla* and associated tending ants were collected near Willow Creek, Nevada on the host plant *Eriogonum umbellatum*, the sulphur-flower buckwheat. *Brephidium exilis* and *Linepithema humile* individuals were collected from two *C. eriophylla* plants located on the main campus of the University of Nevada, Las Vegas.

Larval and Ant Collection

Larvae were located by searching host plant inflorescences. Once found, all larvae were collected by trimming the inflorescence from the host plant, placing it in a clean plastic cup, and sealing the cup with a lid. Each larva was housed in its own cup. Ants actively tending larvae were collected with this method and kept in the same container as their larval partner.

Ants collected for behavioral assays were located near the entrance of a single *L. humile* nest. Ants were collected by suction as they entered or left the nest and transferred to a single plastic container with *C. eriophylla* leaves and flowers to provide cover and moisture.

Cuticular Hydrocarbon Extraction

Hydrocarbon extraction was performed using hexane. Prior to extraction, all instruments were rinsed with hexane to prevent cross contamination. For each sample, a larva

or ant was placed in a glass test tube with about 1mL of hexane and allowed to sit for two minutes. At the end of this time, a glass pipette was used to remove the hexane solution from the test tube. The solution was then placed in a 2mL glass vial and evaporated either fully or partially using nitrogen gas. Samples were then either stored in a freezer (vials with hexane solution) or stored at room temperature (vials with hexane fully evaporated) until analysis. A "Control" sample was performed and sent for analysis. The Control was created by following the same procedures for hydrocarbon extraction in the absence of a sample organism. This control was performed to identify possible contaminant hydrocarbons introduced to samples in the course of collection.

Behavioral Assays

All behavioral assays focused on ant response and were performed using individuals from the species *Linepithema humile*. Three types of assays were conducted: 1) larval assays, 2) bead assays, and 3) choice assays. For all three assay types, observations took place in 35mm petri dishes and lasted 5 minutes. Lids were used to decrease the likelihood of ant escape and Teflon (brand: DuPont Teflon Non-Stick Dry-Film Lubricant) was used to coat the sides of the dish to prevent ants from spending time on the petri dish lid. Observations were made to ensure the Teflon did not impact ant behavior. A new dish was used for each assay.

All behavioral assays followed a standard format modelled after Ballmer and Pratt (1991) and Hojo et al. (2014). First, five *L. humile* individuals were placed in the petri dish and given 5 minutes to acclimate. The dish was placed on white paper as this decreased the amount

of time ants spent attempting to reach the lid (personal observation). Next, a stimulus (larva or larval-scented bead) and a blank (host plant flower or hexane-rinsed bead) were randomly placed in the petri dish at locations roughly equidistant from each other and the edges of the dish. Directly following addition of the stimulus and blank, a 5-minute video recording of the entire dish was taken.

For larval assays, both *Euphilotes bernardino martini* and *Brephidium exilis* individuals were used. For both species, early and late instar larvae were observed (n=8 early and 5 late instars for *E. bernardino;* n=3 early and 3 late instars for *B. exilis*). In larval assays, the blank was a single flower taken from the same inflorescence on which the larva was collected.

In bead and choice assays, cuticular extract from *Euphilotes bernardino* was used to coat stimulus beads. Both assays used 3mm diameter, clear glass beads as stimuli or blanks. Beads used for stimuli were coated in larval extract by reconstituting evaporated samples with 100µL hexane, applying 50µL to each bead, and allowing the hexane to evaporate. Beads used as blanks were rinsed thoroughly with hexane and allowed to dry. Bead assays tested ant response to early or late cuticular extract (n=3 early instars, n=5 late instars) compared to a blank.

Choice assays measured ant response to beads coated in early or late larval extract. Ant preference was inferred by a comparison of time spent on each bead when both beads were presented in the same dish. Four choice assays were performed. In all choice assays, beads coated in larval cuticular extract were re-used to ensure enough sample was left for chemical analysis.

Chemical Analysis

Cuticular hydrocarbons from raw cuticular extracts were analyzed using gas chromatography with mass spectrometry (GC-MS). Samples were submitted to Millis Scientific, Inc. (Report No. SE-40749-1) where a Waters/Micromass Quatro GC mass spectrometer interfaced to a ThermoElectron Trace GC gas chromatograph was used following silica gel purification that purified the cuticular extract by separating out CHCs. Samples were run through a 30 m 0.25mm ID DB-5 column with the exception of a non-insect control sample that was run through a DB-1 column. For all samples, helium gas was used as a carrier at 1ml/min splitless with a 2µL injection volume. See Appendix for GC ramping conditions and MS parameters (Table A1 and Table A2). AMDIS software was used to identify target components, retention indices (RI), and integral ion counts (peak areas). A known alkane mixture was used to obtain a reference point. Compound levels were obtained by fitting to a one point calibration curve.

Data Analysis

Five variables were quantified during analysis of behavioral assay videos: dish time, flower time, larval time, blank time, and tending time. Total time (in seconds) spent engaging in each behavior by each ant in the assay dish of was recorded at least 3 times per assay and then averaged for each assay to give total ant seconds (Ballmer and Pratt 1991). Dish time was measured as the total time ants spent on the dish rather than interacting with stimuli or blanks.

Flower time reflected the total amount of time ants spent interacting with anything in the dish. Larval time denoted the total amount of time ants spent on any larva or bead coated in larval extract, depending on the assay type. Blank time indicated the amount of time ants spent on any blank in the dish (i.e. flower without larva, bead rinsed in hexane). Tending time represented the amount of time ants spent actively tending any larva or bead coated in larval extract. Active antennation of the larva, walking back and forth across the larva, and alarm response (not observed in this study) are considered ant attendance (Ballmer and Pratt 1991). Total ant seconds for each assay, represented as an average of at least 3 timings per variable, can be found in Table 3. These data were analyzed using Mann-Whitney U and Wilcoxon Signed-rank tests in SPSS (IBM SPSS Statistics Version 25). P-values generated for behavioral assay analyses (Larval Assays, Bead Assays, and Choice assays) were tested for false positives using the Bonferroni sequential method where p-values were tested by category of comparison (i.e. in Larval Assays, all Blank Flower vs Larval Flower p-values were corrected together, all Larval Flower vs Larva p-values were corrected together, etc.) (Rice 1989).

Results from GC-MS analysis of cuticular extracts were organized using Microsoft Excel. All data were put into an Excel sheet organized by Kovat's Retention Index (RI) values with sample ID attached. Any RI values within two integers of each other were considered the same compound for sorting purposes. RI values were averaged across these groupings and a new average value was calculated to create presence/absence tables for all compounds present for all sampled individuals.

Analyses of these tables were performed using Principal Components Analysis (PCA) in SPSS. While these data are considered non-linear, this analysis has been shown to be robust

(Kolenikov and Angeles 2004; Zuur et al. 2010). RI's with loading values greater than 0.55 and less than -0.55 in the PCA were considered to be important contributors to the overall pattern of each component. Comparisons of groups from the PCA (ants, all larvae, early larvae, and late larvae) were made using Mann-Whitney U tests in SPSS.

Table 3. Mean tending time with standard error for Larval, Bead, and Choice assays. Tending time is measured in total ant seconds. Means are presented with standard error. Rows indicate mean time ants spent tending each item during a given assay. Columns represent means calculated per category (All = pooled between both larval species; EBM = means from assays performed with *Euphilotes bernardino martini*; BE = means from assays performed with *Brephidium exilis*).

		All Larvae	All Early Instars	All Late Instars	All EBM Larvae	Early Instar EBM Larvae	Late Instar EBM Larvae	All BE Larvae	Early Instar BE Larvae	Late Instar BE Larvae
Larval Assays	Blank Flower	31.2 ± 8.6	32.0 ± 14.4	30.1 ± 6.3	39.0 ± 11.9	41.1 ± 19.0	35.7 ± 9.3	14.3 ± 4.1	7.9 ± 4.9	20.8 ± 4.4
	Larval Flower	38.8 ± 6.5	35.5 ± 7.1	43.3 ± 12.6	45.3 ± 7.1	44.3 ± 7.6	46.9 ± 15.3	24.8 ± 13.0	12.2 ± 3.2	37.4 ± 25.9
	Larval Tending	98.7 ± 38.3	9.5 ± 9.0	221.2 ± 71.3	52.7 ± 26.7	0.63 ± 0.63	136.2 ± 52.1	198.1 ±100.6	33.2 ± 33.2	363 ± 149.4
Bead Assays	Blank Bead				9.0 ± 3.5	10 ± 5.1	8.3 ± 5.2			
	Larval Bead				26.1 ± 6.1	17.6 ± 6.5	31.3 ± 8.5			
Choice Assays	Early Instar Bead				31.5 ± 10.1					
	Late Instar Bead				24.5 ± 4.0					

Section 3: Results

Typical larval-ant interactions in late instar *E. bernardino martini* and *B. exilis* are similar to *T.* irenea- ant interactions described by DeVries (1988) when he coined the term enticement and binding. Roughly half of all field encounters with late instar *E. bernardino martini* larvae, and all encounters with late instar *B. exilis* larvae, involved a single larva being tended by one to five ants. During tending in the field, ants patrolled larval inflorescences, patrolled around and on top of larvae, and imbibed nectar. Some ants focused solely on the dorsal nectar organ of the larva and positioned themselves so that their mandibles were directly above the DNO at all times (Figure 2). These same behaviors were observed with late instar larvae in the lab. In either setting, few tentacular organ eversions were seen and these eversions never caused "excited runs" or elicited aggression in ants.

Figure 2. Ants tend a late instar *Euphilotes enoptes* larva. The larva's head is oriented into the center of the inflorescence and an ant positions itself so that its mandibles hover above the dorsal nectary organ. Photo Credit: Daniel Thompson.

In contrast, no early instar larvae were found tended by ants in the field. This was true even in the case of *B. exilis,* where early instar larvae were sometimes found on the same plant as late instar larvae that were actively being tended. In the lab, nine out of eleven early instar larvae were never tended in behavioral assays (of the two tended, one *E. bernardino* was tended for 5 seconds and one *B. exilis* was tended for 99.7 seconds out of a 300 second trial time). When observed under a microscope, no dorsal nectar organ or tentacular organs could be located on any early instar larvae in this study. These observations confirm the assertion that early instar larvae lack the organs that mediate the larval-ant interaction (DeVries 1989, 1991). While early instar larvae were not tended by mutualist ants, they also avoided being treated as prey in larval assays or in the field. Instead, they were typically ignored.

The distinct difference between larval-ant interactions in early instar larvae and late instar larvae is interesting. This is especially the case in light of the inter-specific communication hypothesis recently presented by Hojo and colleagues (2014). A lack of ant interaction with early instar larvae, coupled with the absence of the nectar-producing DNO, would apparently implicate larval nectar as the driver behind the larval-ant interaction as a lack of nectar in early instars appears to preclude tending.

Larval Assays

Behavioral assays using early instar and late instar larvae (hereafter referred to as "Larval Assays") were conducted to confirm that larval and ant behaviors in the lab were similar

to those in the field and to test the hypothesis that early instar mutualist larvae are tended significantly less than late instar mutualist larvae. These assays provide a direct comparison of weakly facultative and strongly facultative interactions. As for the first objective, larval behavior was not observed to be significantly different in the lab than in the field. Ant behavior, however, was somewhat affected. Ants placed in petri dishes tended to focus on escape or cease movement even in the presence of late instar larvae that were producing nectar. In particular, ants spent a significant amount of time on the lid of the dish. Teflon was used to prevent ants from reaching the lid. However, this did not prevent them from focusing on reaching the lid. The addition of white paper beneath the petri dish minimized attempts to reach the lid of the dish (presumably by eliminating the positively phototactic response of ants attempting to escape a dark lab bench through a bright dish lid) and encouraged exploration. In addition, the use of multiple ants in each dish encouraged movement and natural behaviors (Ballmer and Pratt 1991; Hojo et al. 2014). As a result, there were no assays in which all ants were inactive and typical foraging ant behaviors (exploration through groping, interaction with nestmates, and larval tending) were observed.

Three comparisons of tending by ants were made in the larval assays: 1) blank flower vs larval flower, 2) larval flower vs larva, and 3) blank flower vs larva. Overall, both by species and pooled, there was no difference in tending of blank flowers as opposed to larval flowers (Table 4). This lack of preference indicates that ants are not attracted to these larval host plants due to mutualist larvae, but rather to forage for nectar.

Table 4. Larval Assay analysis. Mean tending time (in total ant seconds) is shown below p-values generated by the Mann-Whitney U test. P-values with (*) denote significance with the Mann-Whitney U test, p-values with (**) denote significance after correction with the sequential Bonferroni method (corrected p-values can be found in Table A3). Abbreviations are as follows: EBM = *Euphilotes bernardino martini*, BE = *Brephidium exilis*, BF = blank flower, LF = larval flower, L = larva.

	BF vs LF	**LF vs L**	**BF vs L**
All Larvae	p=0.234 31.2 vs 38.8	p=0.506 38.8 vs 98.7	p=0.525 31.2 vs 98.7
All Early Instars	p=0.243 32.0 vs 35.5	p=0.002** 35.5 vs 9.5	p=0.001** 32.0 vs 9.5
All Late Instars	p=0.721 30.1 vs 43.3	p=0.015* 43.3 vs 221.2	p=0.005** 30.1 vs 221.2
All EBM Larvae	p=0.336 38.98 vs 45.3	p=0.125 45.3 vs 52.8	p=0.139 38.98 vs 52.8
Early Instar EBM	p=0.382 41.1 vs 44.3	p=0.002** 44.3 vs 0.6	p=0.001** 41.1 vs 0.6
Late Instar EBM	p=0.841 35.7 vs 46.9	p=0.222 46.9 vs 136.2	p=0.095 35.7 vs 136.2
All BE Larvae	p=0.937 14.3 vs 24.8	p=0.394 24.8 vs 198.1	p=0.394 14.3 vs 198.1
Early Instar BE	p=0.4 7.9 vs 12.2	p=0.7 12.2 vs 33.2	p=0.7 7.9 vs 33.2
Late Instar BE	p=1.0 20.8 vs 37.4	p=0.1 37.4 vs 363	p=0.1 20.8 vs 363

Ant attendance to flowers in comparison to larvae did yield interesting results. By species, no significant results were found except for in early instar *Euphilotes bernardino martini* larvae. Here, ants preferred to tend blank flowers or flowers with an early instar larva on them compared to tending the larva itself (Blank Flower vs All Early Instar Larvae, n=8; Mann-Whitney U test, U=3, p=0.001, d.f.=7; Larval Flower vs All Early Instar Larvae, n=8; Mann-Whitney U test, U=4.5, p=0.002, d.f.=7, respectively) with mean larval tending in either scenario equaling less than one second (see Table 3). Results supported the hypothesis that early instar larvae are tended significantly less than late instar larvae (see Table 4). Ants spent roughly the same amount of time on flowers in the pooled tests but spent significantly more time tending

late instar larvae and less time tending early instar larvae. Although this study produced significant results only in pooled tests, the means for each test, combined with observations from Larval Assays indicate a larger sample size may yield significance without pooling data.

Bead Assays

Bead assays were conducted to determine whether ants would interact with and tend glass beads coated in larval extract (see Table 5). A pooled comparison of early and late larval instar beads to blank beads produced a significant result wherein ants tended beads coated in larval scent significantly more than beads rinsed with hexane (Blank Bead vs All EBM Beads, n=8; Mann-Whitney U test, U=11.5, p=0.028, d.f.=7).

Table 5. Bead Assay analysis. Mean tending time (in total ant seconds) is shown below p-values generated by the Mann-Whitney U test. P-values with (*) denote significance with the Mann-Whitney U test, p-value correction with the sequential Bonferroni method did not find significance for any bead assay comparison. Value noted with (***) considered marginally significant. Abbreviations are as follows: EBM = *Euphilotes bernardino martini*, BB = blank bead, LB = larval bead.

	Blank Bead vs Larval Bead
All EBM Beads	p=0.028* 8.95 vs 26.1
Early Instar EBM Beads	p=0.4 10 vs 17.6
Late Instar EBM Beads	p=0.056*** 8.3 vs 31.3

By instar, results from bead assays supported results from larval assays: ants prefer to tend beads coated in late larval extract over blank beads (Blank Bead vs Late Instar EBM Bead, n=5; Mann-Whitney U test, U=3.5, p=0.056, d.f.=4) and show no tending preference for early

instar larval beads over blank beads (Blank Bead vs Early Instar EBM Bead, n=3; Mann-Whitney U test, U=2, p=0.4, d.f.=2).

Choice Assays

Choice assays were conducted to determine if there is a true preference for late instar larval cuticular extract over early instar larval cuticular extract. This test may be useful for understanding the roles and interaction of larval behavior and larval "scent". Despite an apparent disregard for early larval scent in bead assays, choice assays showed that tending ants have no preference for early larval cuticular extract over late larval cuticular extract (Early Instar EBM Bead vs Late Instar EBM Bead, n=4; Mann-Whitney U test, U=7, p=0.886, d.f.=3, mean early instar bead tending time=31.5s ± 10.1s [SE], mean late instar bead tending time=24.5s ± 4.0s [SE]). It is important to note that late instar beads were also used in Bead Assays before the Choice Assays were performed the same day and thus signal interference from exploration by other ants was a possible factor in this analysis, though trail pheromones of ants are known to dissipate in as little as two minutes if not reinforced (Cardé and Millar 2009; Traniello 2009).

Table 6. Choice Assay analysis. Mean tending time (in total ant seconds) is shown below p-values generated by the Mann-Whitney U test. P-values with (*) denote significance with the Mann-Whitney U test, p-values with (**) denote significance after correction with the sequential Bonferroni method. Abbreviations are as follows: EBM = *Euphilotes bernardino martini*, BF = blank flower, LF = larval flower, L = larva, LB = larval bead.

	BF vs LB	**LF vs LB**	**L vs LB**
All EBM Larvae	p=0.575 26.9 vs 26.1	p=0.123 48.7 vs 26.1	p=0.401 85.1 vs 26.1
Early Instar EBM	p=0.593 12.3 vs 17.6	p=0.109 51.7 vs 17.6	p=0.109 0 vs 17.6
Late Instar EBM	p=0.5 35.7 vs 31.3	p=0.5 46.9 vs 31.3	p=0.138 136.2 vs 31.3

Assay Comparisons

Comparisons of larval assay results to bead assay results were used to test whether ant response to a larva differed from ant response to the cuticular extract of that same larva. This would again add to the understanding of the role of larval behavior in ant attendance and its potential interaction with larval "scent". Three of these comparisons produced interesting results: 1) blank flower to larval bead, 2) larval flower to larval bead, and 3) larva to larval bead. None of these comparisons, either pooled or by instar, showed a significant difference in tending times (Table 6). Ants appear to recognize flowers, larvae, and larval extract equally as indicators of a food reward.

GC-MS Analysis

The GC-MS analysis of 22 cuticular extract samples from 6 early instar larvae, 6 late instar larvae, and 10 ants resulted in a total of 90 retention times. The compounds present

were C10 to C40 hydrocarbons, the majority of which were *n*-alkanes, as identified by Kovat's Retention Indices (RI). Unless otherwise stated, compounds have been identified as simple or complex by use of RI values alone. Any RI falling on an even 100's value plus or minus 6 (i.e. RI 2200±6, RI 2300±, etc.) is presumed to be an *n*-alkane based on results from alkane standards analyzed (Gapeev and Yeh 2016). However, it should be noted that RI values for complex compounds could overlap with these values, depending on which modifications have been made to the molecule (Carlson et al. 1998). Each sample had a minimum of 14 and a maximum of 91 compounds. There were 30 compounds specific to larvae and 6 compounds specific to ants (see Table 7). Of the compounds specific to larvae, Kovat's Retention Indices indicate 3 are *n*-alkanes (simple CHCs) and the remaining 27 are modified alkanes (complex CHCs). All six of the compounds specific to ants are complex CHCs as determined by RI. A review of ant CHCs has revealed that ants most commonly produce CHCs with odd chain lengths and with modifications at odd carbon numbers as opposed to producing CHCs with even chain lengths or modifications at even numbered carbons (Martin and Drijfhout 2009). Overall, larvae and ants share similar ratios of simple:complex hydrocarbons on their cuticles (Simple compounds, n=22; chi-square goodness of fit test, χ^2=7.25, d.f.=1; Complex compounds, n=22; chi-square goodness of fit test, χ^2=2.13, d.f.=2) (see Table 8). There is no difference across any group between the average ng of CHCs present per sample, the average number of CHCs present per individual, or the average chain length of CHCs (determined by the weighted average of compounds present, calculated as the sum of RI value x % in sample) as verified by Mann-Whitney U tests (see Table A4 in Appendix).

To begin the analysis of cuticular hydrocarbons, all clusters of sets of Kovat's Retention Index (RI) values that differed by less than 2 units were identified and assigned the same average RI value within samples and across individual larvae and ants. This led to a dataset of 90 unique RI values. It should be noted that it is possible for some of the compounds combined into one average RI value to be different than its cohorts as modifications (methyl branches, double bonds, etc) made to *n*-alkanes can impact their retention time and, therefore, RI value leading to the possibility for a simple and complex hydrocarbon to share an RI value (Carlson et al. 1998). Construction of presence/absence tables across all specimens revealed the following patterns: 1) compounds present in all individuals; 2) compounds present in all individuals of a given species, class, or instar (i.e. all ants, all larvae, all early instar larvae, all late instar larvae); and 3) variable compounds present in some individuals but not consistently related to any category or species.

Table 7. Compounds unique to either larvae or ants. Compounds presumed to be simple (non-modified n-alkanes) as determined by RI value are denoted with (*).

	Kovat's RI Value		
Present in early and late instar larvae only	1257	2967	3530
	1493	2978	3538
	1641	3033	3543
	1794*	3157	3547
	1994*	3223	3670
	2130	3244	3694*
	2667	3252	3710
	2733	3409	3756
	2864	3430	3882
	2959	3471	3953
Present in ants only	2584	3347	3588
	2676	3510	3763

For the first pattern, two compounds were found in the cuticular extracts of all larvae and all ants sampled. These compounds, average RI ~2904 and ~3103, are likely n-alkanes based on RI and mass spectral ionization patterns. These compounds were also found in the extract control based on presumed carbon chain length (*n*-C29 and *n*-C31). In addition to these compounds, compound identified in the non-insect control sample were: squalene, *n*-C27, *n*-C28, *n*-C30, and *n*-C32 (Gapeev and Yeh 2016). Squalene is expected to be a contaminant (Gapeev, personal communication). The remaining four compounds were found in some but not all samples analyzed. There are multiple ways this can be interpreted since the non-insect control was analyzed at a separate time from the insect samples and was analyzed using a different GC column type (Gapeev and Yeh 2016). First, it is possible these compounds contaminated the samples during collection, though their intermittent presence does not make this necessarily likely. Alternatively, contamination could have occurred at the injector port during GC analysis. In the case of this sample-to-sample cross-talk, these compounds may not have contaminated the samples at all since the non-insect control was run at a different time. Because the sample contamination appears to be intermittent and the compounds involved could be produced by insects, compounds with RI 2700, RI 2800, RI 3000, and RI 3200 were not excluded from analysis.

For the second pattern, multiple compounds were found that were shared across all early instar larvae, all late instar larvae, or all late instar larvae and all ants. Three compounds were shared by all early larvae (RI ~3256, ~3346, and ~3728). These compounds were present in some ants and late larvae but with no consistent pattern. RI 3256 was found in 7 late larvae or ants, RI 3346 was found in 4 late larvae or ants, and RI 3728 was found in 11 late or ants

(n=16). The RI values and mass spectral ionization patterns of these compounds indicate they are modified alkanes ("complex" compounds with possible double bonds or methyl branches) and thus are good candidates to be used for communication.

Table 8. Compounds present in each group. Number of compounds present in all individuals of a group are noted. Type of compounds (simple or complex) present each group are also noted.
*Compounds presumed simple or complex based on Kovat's RI value

	Total	Present in All	Simple*	Complex*	Simple/Complex	Simple: Complex
All Larvae	86	2	27	59	0.45	1 : 2.1
Early Instar Larvae	74	3	25	49	0.51	1 : 1.96
Late Instar Larvae	75	14	24	51	0.47	1 : 2.13
Ants	62	6	24	38	0.63	1 : 1.58

All late larvae shared fourteen compounds. All ants shared eight compounds. Two of the compounds shared by all ants were also shared by all larvae (RI 2904 and 3103). The remaining six compounds shared by all ants, five of which are presumed n-alkanes, were also shared by all late larvae. These are RI ~2605, ~2703, ~2799, ~3005, ~3198, and ~3882 (complex compound). The remaining eight compounds shared by all late larvae were not shared by every member of any other group (Table 9). No compounds were shared among all early larvae and all ants nor between all early larvae and all late larvae.

Further analysis of the clustering or association of compounds within individuals was explored in Principal Components Analysis (PCA). The first four principal components (PCs) generated with PCA provided groupings of compounds found with one another (strong positive loadings on PC) or consistently absent (strong negative loadings on PC) in the presence of other compounds (Table 10). The two compounds, RI 2904 and 3103, shared by all larvae and all ants did not covary in a predictable manner with respect to any of the four PCs. Two of the

compounds shared by all early larvae appear in the PCA results. RI 3256 and RI 3346 load positively on PC1 (loadings are 0.74 for both) and occur in the absence of three other compounds: RI 3198, RI 3382, and RI 3291 (loadings are -0.614, -0.614, and -0.738, respectively). RI 3198 (an n-alkane) and RI 3382 (a modified alkane) are shared by all late larvae and all ants while RI 3291 (a modified alkane) is shared by all late larvae.

Table 9. Compounds shared by all individuals of each group. Note that all compounds shared by all ants were also shared by all late larvae.

	Kovat's RI Value
Shared by All Larvae	2904.7 3103.75
Shared by All Early Instar Larvae	3256.45 3346.45 3728.41
Shared by All Late Instar Larvae	2300.7 2399.81 2503.96 2819.8 2864.66 3291.36 3483.33 3597.4
Shared by All Late Instar Larvae and All Ants	2605.15 2703.55 2798.96 3004.88 3198.56 3382.83
Shared by Early Instar Larvae and Ants	N/A

Although Principal Components Analysis generated several patterns of co-occurrence of compounds, most of these patterns do not appear to be related to functional groupings that can explain ant responses to larvae. Mann-Whitney U tests revealed significant differences between mean scores of only two groups across all four Principal Components. Significant differences were seen between early instar larvae and ants on PC1 (Early Instars 10.4 ± 3.1 [SE], n=6 and Ants 3.1 ± 2.4 [SE], n= 10; Mann-Whitney U test, U=11, p=0.04, d.f.=15). Examination of PC1 loadings indicate that the high mean for early larvae is associated with the presence of the two compounds shared by all early instar larvae (RI 3256 and RI 3346) and the absence of one compound (RI 3291) shared by all ants. Although the low PC1 mean for ants indicates ants do not typically have these compounds, it is difficult to interpret this pattern with respect to potential inter-species communication except to note that if either of these compounds found in all early instar larvae can be detected by ants, the compounds could suppress ant aggression or be an indicator of a rewardless partner larva.

In addition, late instar larvae and ants had significantly different mean scores for PC3 (Late Instars 5.16 ± 0.8 [SE], n=6 and Ants 2.5 ± 0.6 [SE], n=10; Mann-Whitney U test, U=9.5, p=0.02, d.f.=15). As there were few significant loadings in this Principal Component, it is difficult to interpret this pattern beyond the acknowledgement that these compounds were more often present in late instar larvae than in ants. This difference does not appear to indicate a significant potential for use of these compounds in inter-specific communication.

Table 10. Results from Principal Component Analysis with Kovat's RI values for each compound listed. All RI values in bold are shared among all of some group (early instars, late instars, late instars and ants).

Principle Component Loadings Results			
< -0.55	**PC 1**		**> 0.55**
3198.46		3066.43	3442.25
3291.36		3077.85	3456.94
3382.87		3162.81	3499.28
		3174.36	3521.35
		3256.45	3552.62
		3267.76	3618.6
		3300.1	3644.11
		3317.3	3678.88
		3346.45	3788.05
		3358.93	4060.5
		3395.35	
		3413.26	
	PC 2		
		1593.12	**2704.04**
		1600.16	**2799.2**
		1896.71	**2819.8**
		1999.75	
		2099.51	
		2200.22	
		2300.7	
		2503.96	
		2605.17	
	PC 3		
			2934.47
			2953.5
			3130.55
	PC 4		
1593.12			3153.3
			3336.31

Section 4: Discussion

Larval-ant mutualisms are a well-studied interaction in which ants tend larvae in exchange for a nutritious nectar reward. Although this interaction has been studied for over 100 years (Newcomer 1912), there is still much that remains a mystery. The mechanisms by which ants find and recognize their larval mutualists are not yet broadly understood. While it is widely accepted that larval nectar is critical for ant attendance, recent research has called into question the notion that nectar is the driver behind this interaction (Hojo et al. 2014). Rather than larval nectar driving the interaction, it is recognized as a reinforcement to ants learning to discriminate larval scents (Hojo et al. 2014). Hojo and colleagues suggest that ants learn to recognize larval scents in the form of hydrocarbons on the larval cuticle. In their study, results were consistent with the inference that mutualist larvae had more complex cuticular hydrocarbons (CHCs) than non-mutualist larvae and that ants recognize these mostly complex suites of CHCs that are more similar to their own.

A rich exploration of the larval-ant mutualism can be found in the literature, but these studies only address larvae in late and final instars. Few studies that measure ant attendance in the field mention larvae in early instars (instars one and two) but do not measure tending rates of these early instars (Peterson 1995). Even in-depth analyses of larval “ant organs” and their functions address late instars only (Malicky 1970; DeVries 1991; Ballmer and Pratt 1991). While the small size and resulting collection difficulties associated with early larval instars may play a role in some of these information gaps, the need for studies that provide this information is clear. This study focused on early and late instar larvae by testing two hypotheses. First, it

describes the general CHC composition of mutualist larvae in early and late instars to address the claims that mutualist larvae mimic the CHC composition of attendant ants (Hojo et al. 2014). Second, it explores a novel hypothesis that mutualist larvae undergo a developmental shift wherein they produce a mostly simple suite of CHCs in early instars to avoid ant aggression and mutualism breakdown and, in late instars, produce a mostly complex suite of CHCs that overlap more closely with attendant ants. Ultimately, this study verified that early instar larvae are not tended by ants, that early instar larvae lack important ant organs such as dorsal nectar organs, and examined CHC profiles to determine whether early larval instar CHCs differed from late larval instar CHCs and addressed whether rewardless, early instar mutualist larvae are able to avoid aggression and predation by mutualist ants by mimicking the reported simple CHC profile of non-mutualist larvae.

For the most part, early instar larvae do not receive any attention from mutualist ants in the field or the laboratory. In larval assays, any flower in the assay dish was tended significantly more than any early instar larva – even a flower with an early instar larva on it. On closer examination, it was confirmed that early instar larvae of *Euphilotes bernardino martini* and *Brephidium exilis* lack at least two organs critical in the enticement and binding of attendant ants – the dorsal nectar organ and tentacular organs (DeVries 1988). These organs are not present in first and second instar larvae but are found in third and fourth instar larvae. Tending times of third and fourth larval instars (late instars) were significantly than tending times of first and second larval instars (early instars) in behavioral assays. In assays with late instar larvae, larvae were consistently tended more than any flower in the assay dish. Clearly, ants recognize late instar larvae as individuals that can provide a nutritious nectar reward but do not recognize

early larvae of the same species in the same way. This difference in tending of larval instars by ants is consistent with ants learning to associate some cue present in late larvae with the nectar reward these late larvae provide. It would follow that either this cue is present in late larvae only or that early larvae may have a unique cue indicating they cannot provide a reward. Alternatively, both of these scenarios could be true and contribute to ant differentiation of early instar and late instar larvae.

Previous studies have shown that glass objects coated in larval cuticular extract, specifically CHCs, can elicit recognition responses in ants (Torres 2007 and references therein). In addition, the *L. humile* ants used for this experiment are known to consume homopteran honeydew and floral and extrafloral secretions (Bernays and Cornelius 1989; Wheeler and Wheeler 1986), indicating *L. humile* ants are able to recognize mutualists and utilize nectar from a variety of sources for energy. While Larval Assays indicate attendant ants will use floral nectar in the presence of early instar larvae but prefer nectar from late instar larvae, Bead Assays suggest ants may differentiate between early and late instar larvae by some cue aside from their nectar reward. In Bead Assays, no tending difference between early instar beads and blank beads compared to marginally significant tending results of late instar beads over blank beads indicate attendant ants can differentiate early instar and late instar mutualist larvae by cuticular hydrocarbons alone. Because the influences of larval behavior and nectar rewards on ants are removed from the interaction in Bead Assays, these results indicate the recognition cue allowing ants to recognize reciprocating, late instar mutualist larvae may be found in cuticular hydrocarbon composition.

Ants learn to recognize nestmates through a three-step recognition process (Bos and d'Ettorre 2012). First, a colony "label" is produced that consists of a set of cuticular hydrocarbons present on each colony member that is unique to the colony and creates what is known as a colony odor, or *gestalt* (Bos and d'Ettorre 2012; van Zweden et al. 2010; Barbero 2016). Perception of this label occurs when one individual encounters another and compares their scent to a memory of the colony CHC composition, also known as the "template" (Bos and d'Ettorre 2012). The difference between the CHC template and the encountered individual's CHCs is compared and used to determine what action the ant will take. When an encountered individual's CHC composition is sufficiently different from the CHC template the ant will attack, but if there is no difference or an insufficient difference, the encountered individual will typically be either accepted or ignored (Bos and d'Ettorre 2012). Nestmate recognition has a heritable component, but CHCs can also be passed from colony member to colony member during the positive interaction events of trophallaxis or grooming (van Zweden et al. 2010; Barbero 2016). While colony *gestalt* allows for general nestmate recognition, additional variation is found in CHC patterns that align with differences in sex, caste, developmental stage, and status (Barbero 2016).

Colony odor is seldom consistent within colonies and ants must be able to use continuous positive associative learning to recognize nestmates (Barbero 2016; Bos and d'Ettorre 2012). This commonly results in recognition errors that may allow easy exploitation of the template recognition system when positive events are associated with sufficiently similar CHC profiles to that of the colony (Barbero 2012). If this is the case, late instar larvae should have a CHC profile that overlaps with ants, allowing them to exploit the template recognition

system and bind ants through positive associative learning experiences (i.e. by providing a nutritious nectar reward). In this case, late instar larval CHC profiles should be similar enough to attendant ant CHCs to avoid aggression but have some key differences that create a cue ants can learn to recognize. Early instar larvae could either share the majority of this similar late instar CHC profile with some exceptions that would comprise their non-reciprocating cue, have a template difference insufficient to induce aggression, or have a mostly simple suite of CHCs that decreases aggression in potential attendant ants (Hojo et al. 2014).

Analysis of larval CHCs showed both early and late instar larvae have roughly a 2:1 ratio of complex to simple cuticular CHCs. Thus, early instar larvae are not using suites of mostly simple CHCs to decrease aggression in mutualist ants as non-mutualist *Lycaena phlaeas* larvae are thought to do (Hojo et al. 2014). Greene and Gordon (2007) showed that both *L. humile* and *Aphaenogaster cockerelli* ants required a combination of at least two hydrocarbon structural classes to elicit a species recognition response and concluded that recognition does not depend on a single compound or set of compounds in a profile, but rather on the mixture of structural classes present. It may be that high ratios of complex to simple hydrocarbons are required for ant recognition of mutualist larvae so that sufficiently varied CHC profiles are present for mutualist recognition by ants. In a 2009 review, Martin and Drijfhout report that the only hydrocarbon classes present in *L. humile* ants are alkanes, monomethyl alkanes, and alkenes (see also Greene and Gordon 2007). They note that *n*-alkanes and monomethyl alkanes are ubiquitous across the 78 ant species surveyed and suggest their presence as a flexible waterproof layer over the cuticle makes them necessary for water loss prevention rather than for use in communication. They note, however, that some monomethyl alkanes may be used as

communication signals but that their ubiquitous nature would make them general indicators only. Interestingly, Martin and Drijfhout also reported that most of the ants in their review produced either alkenes or dimethyl hydrocarbons, but rarely both. This may indicate that mutualist larvae, which can interact with a wide variety of attendant ants based on location, could be exploiting the most ubiquitous compounds present across ant species to communicate: *n*-alkanes and monomethyl alkanes. In *Lasius niger* ants, recognition of various species of mutualist aphids is dependent on *n*-alkane composition in particular (Lang and Menzel 2011). Larval use of ubiquitous ant CHC compounds for recognition would seem to be the only way for larvae to be universally recognized as a partner (late instars) or avoid aggression due to a high template-label differential (early instars). This is because colony *gestalt*, and not just individual ant odor, differs across colonies and is unstable over time within each colony (Bos and d'Ettorre 2012; Peterson 1995; Axen 2000; and others). However, more research is needed as mutualist lycaenid larval cuticular hydrocarbon profiles are not well-studied to date.

If variations in larval cuticular hydrocarbon profiles are the cues used by ants to differentiate between non-reciprocating early instar larvae and reciprocating late instar larvae, variations in early instar and late instar CHC profiles should be examined. This study found that all late instar larvae shared five simple (*n*-alkane) and one complex (modified alkane) CHCs with all ants. This set of shared compounds, composed of two structural classes of CHCs, could be a cue that allows ants to recognize late instar larvae as mutualist partners that are capable of providing a nectar reward. Conversely, early instar larvae shared no compounds with all ants but did have three complex compounds shared only among all early larvae. Results of Principal

Components Analysis show two of these compounds should typically be found in the absence of one simple CHC shared by all ants, and two complex compounds shared by all ants and all late instar larvae. These two compounds shared by early instar larvae in the absence of at least one compound found in late instar larvae only could be a cue that allows ants to recognize early instar larvae as non-reciprocating partners incapable of providing a nectar reward. This combination of compounds unique to early larvae could also be a cue that suppresses ant aggression. Some mechanism that allows ants to differentiate or learn the difference between reciprocating late instar larvae and non-reciprocating early instar larvae is vital to the prevention of mutualism breakdown due to a developmental delay in reciprocity. Whether compounds shared by late instar larvae and ants comprise a cue ants can attach to a positive stimulus (receipt of nectar) or compounds found in early instar larvae only allow these non-reciprocating partners to be ignored and avoid aggression, some consistent difference should be found across species that maintains this mutualism. Identification of compounds unique to each group (early instar larvae, late instar larvae, ants), especially those found in early instar larvae only, should be undertaken. Once these compounds are identified, analysis of their potential effects through behavioral assays should be performed to determine what role these variations in larval CHC suites may play.

Even with a cue that distinguishes early larval instars from late larval instars, and a CHC template close enough to the colony *gestalt* to avoid aggression (Bos and d'Ettorre 2012), early instar larvae should have some mechanism for defense against attack from would-be attendant ants that may choose to carry early instar larvae back to the nest as a prey item. This is especially true in the case of the larvae in this study, as *L. humile* ants are known to be

generalist predators and prey on a variety of caterpillar species (Bernays and Cornelius 1989). Larval species in this study are highly camouflaged and exhibit little movement. They are typically found feeding on the buds and flowers of host plants and position their bodies so that their head is pointed toward the center of a flower or inflorescence and their posterior end, and associated ant organs, are oriented toward the outer edge of the flower or inflorescence. When disturbed, members of the butterfly subfamily Polyommatinae do not display the common lepidopteran defensive strategies of rearing up, curling, walking away, or falling off the host plant to escape (personal observation; Bernays and Cornelius 1989; Malicky 1970). Rather, these larvae remain relatively motionless in the face of disturbance both before and after contact (personal observation; Bernays and Cornelius 1989; Malicky 1970). *L. humile* ants found generalist larvae most palatable followed by cryptic specialists with a relatively narrow host range (such as the Polyommatinae) and aposematic specialist species. They noted that one likely reason cryptic specialist species were able to avoid predation by *L. humile* was that their behavioral response to a threat, holding still, apparently resulted in a lack of recognition of the larvae as prey (Bernays and Cornelius 1989). Thus, it is possible that the highly cryptic early instar larvae in this study were able to avoid aggression from mutualist ants using a unique CHC composition that identified them as a partner or decreased ant aggression coupled with a behavior mechanism that excluded them from being considered a prey item. This same combination of factors could have played a role in the lack of ant tending observed by Hojo et al. (2014) with non-mutualist *L. phlaeas* larvae that share the same cryptic coloration and slug-like body form of the larvae in the present study.

This study verifies a lack of crucial ant organs in early instar lycaenid larvae and documents a lack of tending in early instar larvae compared to late instar larvae. This study does not reject the claims of Hojo et al. (2014) that mutualist larvae evolve a matching subset of complex CHCs that overlap with attendant ant species, but qualifies that in facultative species CHC overlap is seen in late instar larvae only and is only seen to some extent (6 of 62 compounds present in ants were also present in late larvae). This study does not support the hypothesis that a developmental shift occurs in mutualist larvae wherein early instar larvae produce mostly simple CHCs later shift to producing a more complex suite of CHCs when they are able to provide attendant ants with a nectar reward. Rather, this study provides evidence that the larval-ant mutualism does not break down in the face of variable partner reciprocation and suggests several mechanism for maintenance of the mutualism. Early instar larvae may produce a unique set of CHCs that may identify them as non-reciprocating partners and prevent the perception of partner sanctions against ants in the absence of a nectar reward, decrease ant aggression, or be sufficiently similar to general ant colony *gestalt* to escape aggression. Further study is needed to investigate cuticular hydrocarbon profiles and patterns across developmental stages of lycaenid mutualist larvae. In addition, identification of individual compounds rather than compound class alone should be undertaken as this may provide an opportunity to directly test the impact of each cuticular hydrocarbon or set of hydrocarbons rather than inferring their roles. Finally, the three compounds found to be unique to early instar larvae should be identified and their potential presence within ant nests on members not of the worker caste (sampled here) or on innocuous objects workers commonly encounter should be

examined as these could play a vital role in the survival of early instar larvae and the prevention of mutualism breakdown.

Appendix

Table A1. GC ramping conditions for DB-5 column (all samples but Control) and DB-1 column (for sample Control). Helium gas was used for a carrier at 1 mL/min splitless with injection volume 2µL. All data taken from Gapeev and Yeh 2016.

DB-5 Column		**DB-1 Column**	
Injector temperature	250 C	Injector temperature	250 C
Initial oven temperature	40 C	Initial oven temperature	0 C
Initial hold	1 min	Initial hold	5 min
Ramp I	40 C/min	Ramp I	40 C/min
Final temperature I	180 C	Final temperature I	100 C
Ramp II	10 C/min	Ramp II	5 C/min
Final temperature II	320 C	Final temperature II	200 C
Final temperature II hold	5 min	Ramp III	10 C/min
		Final temperature II	300 C
		Final temperature II hold	5 min

Table A2. MS parameters as listed by Gapeev and Yeh 2016.

Ionization and ion polarity	EI+
Scan rate	2 scans/sec
Mass range	50-500 Da
Ion source temperature	180 C
Transfer line temperature	320 C

Table A3. Corrected p-values for Larval Assay analysis. As in Table 4, mean tending time (in total ant seconds) is shown below p-values generated by the Mann-Whitney U test. P-values in these columns with (*) denote significance with the Mann-Whitney U test, p-values with (**) denote significance after correction with the sequential Bonferroni method. Columns labeled as Corrected show adjusted p-values after application of sequential Bonferroni method and p-values with (**) denote significance. Abbreviations are as follows: EBM = *Euphilotes bernardino martini*, BE = *Brephidium exilis*, BF = blank flower, LF = larval flower, L = larva.

	BF vs LF	**BF vs LF Corrected**	**LF vs L**	**LF vs L Corrected**	**BF vs L**	**BF vs L Corrected**
All Larvae	p=0.234 31.2 vs 38.8	p= 2.1	p=0.506 38.8 vs 98.7	p=1.0	p=0.525 31.2 vs 98.7	p=1.0
All Early Instars	p=0.243 32.0 vs 35.5	p=1.0	p=0.002** 35.5 vs 9.5	p=0.02**	p=0.001** 32.0 vs 9.5	p=0.01**
All Late Instars	p=0.721 30.1 vs 43.3	p=1.0	p=0.015* 43.3 vs 221.2	p=0.10	p=0.005** 30.1 vs 221.2	p=0.04**
All EBM Larvae	p=0.336 38.98 vs 45.3	p=1.0	p=0.125 45.3 vs 52.8	p=0.63	p=0.139 38.98 vs 52.8	p=0.57
Early Instar EBM	p=0.382 41.1 vs 44.3	p=1.0	p=0.002** 44.3 vs 0.6	p=0.02**	p=0.001** 41.1 vs 0.6	p=0.01**
Late Instar EBM	p=0.841 35.7 vs 46.9	p=1.0	p=0.222 46.9 vs 136.2	p=0.89	p=0.095 35.7 vs 136.2	p=0.57
All BE Larvae	p=0.937 14.3 vs 24.8	p=1.0	p=0.394 24.8 vs 198.1	p=1.0	p=0.394 14.3 vs 198.1	p=1.0
Early Instar BE	p=0.4 7.9 vs 12.2	p=1.0	p=0.7 12.2 vs 33.2	p=1.0	p=0.7 7.9 vs 33.2	p=1.0
Late Instar BE	p=1.0 20.8 vs 37.4	p=1.0	p=0.1 37.4 vs 363	p=0.60	p=0.1 20.8 vs 363	p=0.57

Table A4. Quantitative comparison of CHC properties. For each comparison, p values and means (measured in total ant seconds) for each group are shown. Means are listed respectively for each category. SE is listed in parenthesis after each mean.

	ng CHCs	Weighted Avg	Number Compounds
Early Instar vs Late Instar	p=0.699 760.4 (292) vs 391.1 (99.0)	p=0.699 681.8 (295.6) vs 320.4 (96)	p=0.394 55 (9.8) vs 44.5 (8.8)
Early Instar vs Ants	p=0.147 760.4 (292) vs 228.6 (101.4)	p=0.093 681.8 (295.6) vs 274.9 (123.6)	p=0.118 55 (9.8) vs 33.9 (5.3)
Late Instar vs Ants	p=0.118 391.1 (99.0) vs 228.6 (101.4)	p=0.22 320.4 (96) vs 274.9 (123.6)	p=0.368 44.5 (8.8) vs 33.9 (5.3)

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Colorado Mesa University

Academic Experience

- NSF Research Experience for Undergraduates, Ecuador May 2012 to August 2012
  - Collected larvae for experiments on plasticity of larval development along an elevation gradient in Napo Province, Ecuador
  - Assisted in leading an Earthwatch team in larval collection and identification

Teaching Experience

- Graduate Teaching Assistant, Principles of Modern Biology II Fall 2014 to Spring 2017
  - Prepared lectures and led laboratory activities for classes of 15-25 students
  - Created and graded weekly course assessments to ensure students understood material and stayed on track
  - Assisted laboratory coordinator in editing laboratory manual for content and clarity Spring 2017
- Undergraduate Mentor Summer 2015 to Spring 2017
  - Mentored six undergraduate students in data collection in the field and laboratory to study a larval-ant mutualism
- Alternative Break Trip Program Student Leader, Catalina Island March 2015
  - Co-led a group of 10 students on a conservation-based volunteer trip during Spring Break
  - Prepared and presented relevant conservation information in an informal setting intended to generate discussion

Outreach

- UNLV Graduate Rebel Ambassador Fall 2015 to Spring 2017
- Y.O.U. Science Fair Outreach December 2014 to February 2015

Awards

- Invitation to speak at Inspiration, Innovation, Impact reception hosted by UNLV Graduate College, Spring 2017
- Finalist in Three Minute Thesis competition at UNLV, Fall 2016

- Honorable mention in Science Track A for presentation at UNLV GPSA Research Forum, Spring 2016
- Second place in Science Track A for presentation at UNLV GPSA Research Forum, Spring 2015
- First place in Sciences Track 2B for presentation at the Colorado Mesa University Student Showcase, Spring 2013

Grants

- Awarded research grants by the UNLV Graduate and Professional Student Association for Spring and Fall 2015