BEHAVIORAL ECOLOGY OF PARASITOID DIET BREADTH AND INSECT DEFENSES

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Dedication

To the most rarely-observed, under-appreciated, and misunderstood creatures inhabiting this pale blue dot. I hope that revealing your stories will bring out the best in us.

Abstract

One of the primary challenges animals face is consuming enough nutrients of sufficient quality that they might realize their reproductive potential. In response to this challenge, many strategies have evolved, varying in the types and specificity of the foods consumed. Why do some animals consume a variety of foods (generalists), while others have extremely narrow diets (specialists)? What causes these divergent strategies to evolve? These questions form a central, yet unresolved theme of biology. To address them, I've used comparative studies rooted in behavioral ecology, chemical ecology, and natural history to better understand how physiological and behavioral trade-offs might limit a species' diet breadth.

My work utilizes two closely related parasitoid wasps that attack butterfly pupae: *Pteromalus puparum*, reported to attack over forty hosts in the field, and *P. cassotis*, an apparent specialist on monarch butterflies. Parasitoid wasps are an ideal group for studying the evolution of diet breadth strategies because they are hyper-diverse, ecologically ubiquitous, and have enormously variable host ranges, even amongst closely related species. Individual endoparasitoids spend their entire larval development inside of a single insect host, almost always killing this host before emerging as free living adults. Therefore, a common measure of a parasitoid species' diet breadth is its range of suitable host species.

In Chapter 1, I provide the first detailed reports of parasitism of monarch butterfly pupae by *P. cassotis*. Using field experiments in the northern U.S. and observational data from wild-collected pupae in the southern U.S., we report occurrences and brood characteristics of this host-parasitoid interaction across a broad geographic area. I also discuss several lines of evidence which suggest that *P. cassotis* is a specialist on monarchs. This chapter serves as a starting point for conservation-related investigations of the potential impacts of *P. cassotis* on monarch populations, which have declined severely in recent decades.

In Chapter 2, I test multiple hypotheses regarding the potential merits and trade-offs associated with the evolution of generalist *versus* specialist dietary strategies. Using no-choice trials, female *P. cassotis* and *P. puparum* were presented ten butterfly species as potential hosts. I measured each species preference for, and performance on each host. Our results supported the preference-performance hypothesis, but did not support the trade-off hypothesis. As predicted by the novel diet breadth mistakes hypothesis, the generalist was more likely than the specialist to accept unsuitable hosts, while the specialist was more likely than the generalist to reject suitable hosts. The frequency of these mistakes in nature is unknown. These findings suggest that foraging

mistakes may not only be consequences of diet breadth strategies, could also reciprocally influence the evolution of such strategies. This research has implications for organisms in rapidly changing environments and parasitoid-based biological control because it demonstrates that specialist foragers may retain the physiological ability and evolutionary potential for host switches, while generalist foragers may cause difficult-to-measure non-target mortality.

Not all hosts were found to be suitable for the development of a either parasitoid, and those hosts known to sequester plant defense compounds into their own bodies were especially likely to be unsuitable. In Chapter 3, I investigate the potential role of milkweed butterflies' sequestered cardenolides as a defense mechanism against parasitoids by testing the preference and performance of *P. cassotis* and *P. puparum* on milkweed butterfly pupae containing high, low, or zero sequestered cardenolides. More toxic monarchs were more likely to survive encounters with the specialist, but only because this wasp was less likely to attack more toxic hosts. After attack, neither host survival nor the emergence of parasitoids was affected by host toxicity, although the specialist produced smaller broods and experienced lower survival on more toxic hosts. *P. puparum* was unable to develop in monarchs of high or low toxicity, and was also unsuccessful when attacking a cardenolide-free danaid butterfly, *Euploea core*, though it frequently killed both species. These findings do not rule out the possibility that sequestered plant toxins may be one mechanism preventing successful parasitism of monarchs by the generalist, but suggest that other mechanisms are also involved.

The challenge of consuming an optimal diet inspired the first three chapters of this dissertation. In Chapter 4, I investigate the opposing side of this challenge: avoiding mortality caused by natural enemies. In conducting the work that makes up the first three chapters of this dissertation, I observed butterfly larvae and pupae performing behaviors that I hypothesized to be defensive in nature, such as choosing protected pupation sites, color matching, and vigorous wiggling when contacted by a parasitoid. Insect pupae are relatively immobile and are often presumed to be highly vulnerable to natural enemies. In fact, previous reviews of insect defenses had only briefly considered morphological and physiological aspects of pupal defense, but the role of behavior in pupal defense had largely been ignored. In Chapter 4, I attempt to fill this gap in the literature. By bringing together dozens of studies showing that insect pupae likely benefit from a variety of behaviors performed before pupation (by the larva or pre-pupa), behaviors of the pupa itself, and behaviors of conspecific and heterospecific individuals. Some of these behaviors include the construction of protective enclosures and devices, behavioral enhancement of crypsis and mimesis, evasive movements, the use of biting mandibles and "gin-traps," and

intraspecific interactions including mutualisms and host manipulation by parasitoids. All the behaviors described at least plausibly function in defense against would-be natural enemies of insect pupae, yet the adaptive potential of many of these behaviors remain untested. I discuss the hypothetical costs and benefits of the evolution of a pupal stage and the evolutionary success of the holometabolous insects and suggest that more complete investigations of these behaviors will improve our understanding of insect population dynamics in natural and agricultural systems and insects' evolutionary histories.

Taken together, this work provides a broad and novel perspective on the role of behavior in predator-prey interactions. It supports many other recent studies demonstrating the importance of behavior as an important determinant of animal diet breadth and as an overlooked aspect of the defense of insect pupae. This research provides the first details on the natural history and chemical ecology of interactions between *P. cassotis* and the monarch butterfly, provides a novel perspective on insect defenses, and suggests that behavioral decisions and mistakes may be as, or even more important than physiological trade-offs in shaping animal diet breadth.

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Chapter 1:
New Reports that Monarch Butterflies (Lepidoptera: Nymphalidae, *Danaus plexippus* Linnaeus) are Hosts for a Pupal Parasitoid (Hymenoptera: Chalcidoidae, *Pteromalus cassotis* Walker)

Summary

Monarch butterflies are one of the best studied non-pest lepidopterans, serving as a model for migration, chemical ecology, and insect conservation. Despite the intensity with which the larvae and adults have been studied, the cryptic pupal stage is often difficult to study in the wild. It is perhaps due to this difficulty that researchers have largely overlooked monarchs' interactions with a pupal parasitoid, *Pteromalus cassotis*. Using field experiments in the northern U.S. and observational data from wild collected pupae in the southern U.S., we report occurrences of this host-parasitoid interaction at sites Minnesota, Georgia, Oklahoma, Texas, Virginia, and Wisconsin. At sites in Minnesota, rates of parasitism of experimentally placed monarch were highly variable, ranging from 60% in 2010 to 0% in 2013 and 2014 (median = 7.3%). Observations of wild-collected pupae suggest that rates of parasitism may near 100% at some sites in the southern U.S. The number of wasps emerging from a single host ranged from 1-425 (mean = 71). Later dissections of hosts revealed that, in some cases, dead parasitoids remained inside the host as larvae, pupae, and/or adults. Within a host, wasp sex ratios were typically female-biased (median = 91% female), as is common in gregarious parasitic hymenopterans. Infected monarch pupae at a site in Oklahoma produced more wasps per host, more male-biased sex ratios, and had higher survival than hosts from other sites. We discuss the possibility that *P. cassotis* is a specialist on monarchs and perhaps closely related species, based on monarchs' sequestered cardenolides, published host records, and evidence for correlated population dynamics of this host and parasitoid.

Introduction

The monarch butterfly (*Danaus plexippus*, Linnaeus 1758, Lepidoptera: Nymphalidae) is perhaps the best recognized lepidopteran in the world. It serves as a model organism for insect migration (Guerra *et al.* 2014), insect conservation (Guiney and Oberhauser 2008), and is the focus of many citizen science projects (Oberhauser *et al.* 2015a). Because monarch larvae sequester toxic cardiac glycosides from their milkweed hosts (*Asclepias* spp.) and retain these chemicals as adults (Parsons 1965, Reichstein *et al.* 1968), their interactions with natural enemies have been of particular interest to chemical ecologists (Brower *et al.* 1967, Brower *et al.* 1968, Brower 1984, Malcolm 1994). Immature monarchs are attacked by a wide variety of invertebrate predators including mantids, polistes wasps, ants, lacewings, soldier bugs, and spiders (reviewed by Oberhauser *et al.* 2015b), but the frequency of these interactions and potentially latent effects of feeding on sequestered cardenolides are largely unknown (but see Rafter *et al.* 2013). Monarchs also suffer mortality from parasitoids, the best studied of which are tachinid flies, most commonly *Lespesia archippivora* (Riley 1871, Arnaud 1978), although several other Tachinidae also attack monarchs (Oberhauser *et al.* 2017). *L. archippivora* females oviposit onto 2nd to 5th instar monarch larvae. The fly larvae emerge from 5th instar caterpillars and pupate in the soil. These flies parasitize ~13% of monarchs across the US, and 1-3 larvae typically emerge from a single host (Oberhauser *et al.* 2007). While predation and parasitism of monarch eggs, larvae, and adults has been well-documented, natural enemies of monarch pupae, which are cryptic and difficult to locate in the field, have received much less attention.

Here, for the first time in over a century, we report parasitism of monarch pupae by a gregarious parasitoid wasp, *Pteromalus cassotis*, Walker 1847 (Hymenoptera: Chalcidoidae, *syn. P. archippi* Howard), at several locations over multiple years in the eastern United States. The genus *Pteromalus* (473 spp.) is nested within the tribe Pteromalinae (314 genera, 2073 spp.), within the family Pteromalidae (588 genera, 3506 spp., likely polyphyletic) (Noyes *et al.* 2017). Most species within the genus *Pteromalus* are tiny parasitoids that attack larvae and pupae of coleopteran, lepidopteran, and dipteran hosts (Noyes *et al.* 2017). *P. cassotis* is a gregarious endoparasitoid of lepidopteran pupae whose biology and host-associations are relatively unknown. To our knowledge, the only publication describing parasitism of monarchs by *P. cassotis* is a 126-year old anecdotal report of a single chrysalis that produced over 50 adult wasps (Gillette 1888).

Using field experiments in the northern U.S. and observational data from wild collected pupae in the southern U.S., we report occurrences of this host-parasitoid interaction at sites Minnesota, Georgia, Oklahoma, Texas, and Wisconsin. We report the proportion of parasitized hosts and (where available) summaries of brood characteristics, including the number of wasps emerged, the proportion surviving to emerge from the host, and sex ratios within wasp broods.

Overview of P. *cassotis* parasitism of monarchs

In ongoing studies, we have learned that a single *P. cassotis* female can produce >100 offspring from a single monarch host (Figure 1). Superparasitism (an event where multiple conspecific females parasitize the same host) is possible, but the relative frequencies of single parasitism and superparasitism in the field are unknown. For simplicity, we refer to any group of offspring that developed in the same host as a 'brood', while acknowledging that these offspring may be descendants of multiple females. Approximately 14-20 days after oviposition, the adult wasps emerge from the host via one or two small exit holes. Under laboratory conditions, adults emerge over the course of several hours and mating occurs immediately. There is considerable variation in adult size, both within and between broods, and females are typically much larger than males. Sex ratios are typically strongly female biased, as is common in gregarious parasitoid species that practice sib-mating (Godfray 1994). There are several potential outcomes of *P. cassotis* oviposition in a monarch host: 1) the host ecloses, with or without deformity, 2) some or all of the parasitoids emerge as adults, always killing the host, 3) both host and parasitoids fail to reach adulthood, though parasitoid development is evident from a dissection, or 4) the host dies and dissection reveals no signs of wasp development, so that the cause of death is ambiguous. Pupal diapause and overwintering of the wasps can also occur within monarch hosts, though less than 10% of parasitized hosts produced adult wasps in the spring after being overwintered outdoors in clear plastic containers in Minnesota.

Experimental exposure of monarch pupae in the field

We first documented parasitism of monarchs by *P. cassotis* during the summer of 2008, when we placed lab-reared monarch pre-pupae and pupae at several sites in St. Paul and Roseville, Minnesota and at one site in St. Croix County, Wisconsin. To expose these pupae, we waited until the caterpillars had spun their silken pads and hung from the lids of their rearing

containers, and then we hung the lids ~1 meter above and parallel to the ground. These pupae were placed in natural or semi-natural habitats (e.g. native prairie, pollinator gardens, and buffer strips near horticultural plots) for 7-10 days, after which pupae were brought into the laboratory and individually monitored for butterfly or wasp emergence. Of the 340 monarch pupae that were recovered from the field, zero pupae from the Minnesota sites were parasitized, but one pupa from the Wisconsin site produced *P. cassotis* wasps. Since 2008, these field experiments have continued in Minnesota only (approximately 70km from the WI site). We modified the methods such that wandering larvae, pre-pupae, and pupae were placed in groups of 10-50 in closed screened cages. These cages allow parasitoid access, but prevent caterpillars from wandering away and larger predators from removing the pupae (Oberhauser *et al.* 2015b). Cages were placed at the same or similar sites to those used in 2008, and the same exposure and monitoring methods were followed. In all years, if neither host nor parasites had emerged after 30 days, we dissected the hosts to determine the cause of death. The fate of hosts was recorded as either eclosed, successfully parasitized, unsuccessfully parasitized, or dead due to ambiguous cause. Hosts from which any wasps emerged were also dissected to determine the proportion of parasitoids that died as visible larvae, pupae, or adults inside of the host. Therefore, when reporting the mean proportion of emerged per brood, the proportions of emerged wasps from all hosts known to be parasitized (whether or not wasps emerged successfully) were included. One limitation of this procedure is that we could not account for wasp eggs or larvae that did not develop to a stage visible with a dissecting microscope. Eggs and small larvae are not visible at low microscope power and may have disintegrated by the time of dissection, but later instar larvae, pupae, and adult wasps inside of hosts are easily distinguished. Thus, we cannot know the total number of eggs oviposited into a host. Similarly, hosts that died for unknown reasons may have contained imperceptibly small eggs or larvae inside, but our calculations of proportion emerged could not account for these ambiguities.

Parasitism was highly variable between sites and years in Minnesota and Wisconsin. Yearly parasitism rates ranged from 0 to ~60%, with a median of 7.3% (Table 2). Of the 1,128 monarch pupae placed and recovered from field experiments in 2009-2012, 198 pupae produced adult *P. cassotis* and an additional 89 pupae, when dissected, revealed parasitoids inside. No monarchs were parasitized in 2013 or 2014 (of the 702 exposed in the field). During the summers of 2015 and 2016 we placed a total of 451 pupae, of which, 5.5% were successfully parasitized

and an additional 1.8% were unsuccessfully parasitized. A fraction (>10%) of parasitoid broods found via dissection contained live larvae and/or possibly living pupae, but most contained desiccated larvae, pupae, and/or adults that had died within the host.

Observational reports of *P. cassotis* parasitism of monarchs

Wild monarch pupae harboring *P. cassotis* parasitoids have also been found by researchers and citizen scientists in Georgia, Oklahoma, Texas, Virginia, and Pennsylvania. These observational data provide interesting comparisons to the experimental data from the northern U.S. Although finding pupae in the field is typically uncommon, immature monarchs occasionally reach high densities, allowing pupae to be more easily located by researchers. At one such site in Stillwater, Oklahoma, all 18 pupae found during September of 2013 were parasitized. Four hosts were monitored for parasitoid emergence *in situ*, and the remaining 14 were collected and monitored indoors. In 2014, lab-reared monarch pupae were placed in the same area (N=10 spring, N=10 fall). Approximately half of these experimentally placed pupae were depredated by ants, and the rest eclosed, suggesting that few or none were parasitized.

High monarch densities and high rates of parasitism by *P. cassotis* can also occur during winter months at year-round monarch breeding sites found along the Gulf and Atlantic Coasts of the U.S. (D. Satterfield, unpublished). Researchers with Project Monarch Health (Altizer 2015 <*www.monarchparasites.org*>) have monitored one site near Savannah, Georgia annually from 2012-2014. At this site, which contains non-native *Asclepias curassavica*, these researchers collected monarch pupae and monitored them indoors for emergence of butterflies or parasitoids. Of pupae collected during January 2-5, 2012, 22 of 39 were parasitized by *P. cassotis*. Of those collected January 6-7, 2013, 37 of 44 were parasitized. Of those pupae that were apparently not parasitized by *P. cassotis*, only one survived to eclosion. The other six were either parasitized by tachinid flies (larval parasitoids), or died for unknown reasons. On January 5-6, 2014, 64 monarch pupae were collected at this site and sent to the University of Minnesota, where they were reared for host or parasitoid emergence. If neither butterfly nor wasps had emerged within 30 days of field collection, pupae were dissected to determine the cause of death. Of the 64 pupae, 41 were successfully parasitized, 4 were unsuccessfully parasitized (desiccated wasps discernible via dissection), 19 died for unknown reasons at various stages of development, and no

butterflies successfully eclosed. Citizen scientists at this site also provided photos of up to five *P. cassotis adults* on (or near) a monarch pre-pupa and of a single wasp parasitizing a fully formed pupa.

P. cassotis parasitism of monarchs has also been observed at sites with *A. curassavica* in San Antonio, Texas (D. Satterfield, pers. comm.). On March 31, 2012 a Monarch Larva Monitoring Project volunteer collected and reared eight monarch pupae, five of which were successfully parasitized. These wasp offspring and host carcasses were discarded, which prevented any further study of wasp performance within these hosts.

Since 2008, several citizen scientists from Wisconsin, Minnesota, Texas, Pennsylvania, and Virgina provided anecdotal reports of parasitism of monarchs by pupal parasitoids to the Monarch Larva Monitoring Project (MLMP). In every case, the volunteers delivered dead wasps to the Monarch Lab at the University of Minnesota, where they identified as *P. cassotis*.

P. cassotis brood characteristics

Brood size, sex ratios, and the proportion of broods that successfully emerged varied greatly within and between years, and from site to site. Wasp broods from 2008-2014 were fully quantified, while only the fate (not quantity and sex ratio) of those broods from field experiments in 2016 and 2016 were recorded. The site in Oklahoma produced the greatest numbers of wasps per host (ANOVA, $F[2,212]=50.8$, $p<0.0001$, TukeyHSD: MN=GA<OK, Figure 2A), as well as the highest proportions of males (ANOVA, $F[2,212]=12.73$, $p<0.0001$, TukeyHSD: MN=GA, OK=GA, GA>MN, Figure 2B). This pattern follows the prediction that sex ratios will produce increasingly more males as superparasitism increases because of increased local mate competition and declining host quality per capita (Hamilton 1967, King 1987). Interestingly, these large broods from the Oklahoma site also had the highest proportion of wasp emergence per host (ANOVA, $F[2,212]=3.085$, $p<0.0478$, TukeyHSD: MN=GA=OK, Figure 2C), suggesting that resource limitation is not a significant source of larval mortality, at least at the naturally occurring densities we observed. We did not measure wasp size, but our observations suggest that individuals from broods with many individuals were smaller per capita than individuals from broods with fewer individuals. The Oklahoma site had, on average, three times more wasps per host than those found in experimentally placed hosts at Minnesota sites, yet mortality inside of Minnesota hosts was higher than either of the southern sites. It is important to note that larval host

plants vary by region. Milkweed characteristics that vary by region may influence host traits such as size or toxicity, which could indirectly affect parasitoid success.

Discussion

P. cassotis has been reportedly reared from at least nine butterfly (nymphalids, papilionids, and pierids) and one moth species (Table 1). To our knowledge, the most recent publication that mentions a natural host associate of this parasitoid was published over 40 years ago; Drummond *et al.* (1970) reported that *P. cassotis* uses *Chlosyne lacinia* as a host in Texas, but a rate of parasitism was not provided. To date, *P. cassotis* has been reported in 18 U.S. states east of the Rocky Mountains, as well as California, but many of these listings lack host-association data (GBIF 2014, Noyes *et al.* 2017) (Figure 3). It is likely that some of the published host records were only observed under laboratory conditions (e.g. Burks 1975). While these host records are potentially valuable for understanding the behavioral and physiological determinants of parasitoids' host ranges, they may also represent factitious or ecologically irrelevant interactions (Harvey *et al.* 2012). This paucity of host association data brings into question the host specificity of *P. cassotis.*

Because *P. cassotis* has been reported from a broad geographic range that overlaps many butterfly host species, it is not clear if the somewhat limited host range is a result of low sampling effort by experimenters, high host specificity of the parasitoid, or both. *P. cassotis* may well utilize numerous butterfly species in the wild, but observations of parasitism may be limited by the difficulty of studying (often cryptic) non-pest lepidopteran pupae, leading to a perceived limited host range. Alternatively, given the hypothesis that toxic, specialist herbivores are exploited by specialist natural enemies (Bernays & Graham 1988, Gauld *et al.* 1992, Gauld *et al.* 1994, Stireman & Singer 2003, Sznajder & Harvey 2003), *P. cassotis* may be a specialist on cardenolide-sequestering monarchs and, perhaps, closely related species. In support of this idea, the development of a specialist parasitoid wasp, *Cotesia melitaearum*, was unaffected by host plant iridoid glycoside concentrations when reared on its specialist caterpillar host, *Melitaea cinxia*, while more toxic host plants negatively affected the development of two generalist caterpillars and two subsequent generalist parasitoids (Reudler *et al.* 2011). This study, and others (Campbell & Duffey 1979, 1981, Barbosa *et al.* 1991, El-Heneidy *et al.* 1988, Lampert *et al.*

2011), suggest that generalist parasitoids are often more susceptible to host plant chemistry variation than specialists.

The extreme diversity of parasitic organisms is often attributed to the strong, disruptive selection pressures and presumed tradeoffs associated with specialization on different hosts (Price 1980), which can lead to host-associated differentiation and speciation (Stireman *et al.* 2006). Recently, the combined use of DNA barcoding and host-association data has revealed high levels of host-specific, cryptic species diversity among previously presumed generalist parasitoids (e.g. Smith *et al.* 2008). This growing awareness of cryptic diversity among morphologically similar species only exacerbates the problem that insect parasitoids and hosts are frequently misidentified, which can lead to the publication of false relationships which are difficult or impossible to ascertain and correct later (Shaw 1994). In the case of the hyperdiverse family *Pteromalidae*, misidentifications are probably relatively common, even among classical biological control agents (e.g. Gibson *et al.* 2006). Forty-seven species within the genus *Pteromalus* are known to occur in the U.S. (Noyes *et al.* 2017). Of these, *P. puparum* is well known because it has been used a biological control agent of *Pieris* crop pests and is reported to parasitize a wide range of non-pest lepidopterans (e.g. Lei *et al.* 1997, Barron *et al.* 2003). Furthermore, it is morphologically very similar to *P. cassotis*; the distinguishing character of *P. cassotis* is the female's yellow femora, compared to the darker femora of *P. puparum* (Howard 1889). It is plausible that, for example, *P. cassotis* specimens could have been misidentified as *P. puparum*, which would have led to an overestimation of *P. puparum's* host range and an underestimation of *P. cassotis'.*

Finally, because the population dynamics of specialist parasites are tightly linked to those of their hosts, correlated population dynamics may provide indirect evidence for specialization (Hassell & May 1986, Bjørnstad *et al.* 2001). Monarch population measures include the area occupied by adult monarchs overwintering in Mexico as well as observations of immature monarchs in the breeding range. Both the area occupied by monarchs in Mexico (Vidal & Rendón-Salinas 2014) and the mean density of eggs per milkweed stem (Stenoien *et al.*, 2015) indicate that the eastern migratory population of monarchs has been at or near historic lows since December of 2012. The fact that no parasitism of monarchs by *P. cassotis* was recorded in Minnesota during 2013 and 2014 suggests that their population dynamics may be linked and that *P. cassotis* may indeed be a specialist on monarchs, at least in the northern U.S. Field studies

including on a greater variety of hosts and careful identifications are needed to differentiate the true host specificity of this parasitoid.

While predation and parasitism of monarch eggs, larvae, and adults has been well-documented, few have documented natural enemies of monarch most cryptic life stage, the chrysalis. We report, for the first time in over a century, the parasitism of monarch butterflies by the pupal parasitoid *P. cassotis.* While many invertebrates are known to cause immature monarch mortality, we have little data on the fitness related effects of consuming cardenolide rich prey. However, we have found that *P. cassotis* not only cause mortality, but thrive on monarch hosts. We have documented this host parasitoid interaction in two northern and three southern U.S. states east of the Rocky Mountains, and these are the first records of *P. cassotis* in GA, OK, VA, and WI. Data from our longest term sites demonstrate highly variable rates of parasitism from year to year. We observed that monarch hosts in Oklahoma produced the largest numbers of offspring per host, the least female biased sex ratios, and relatively high survival to adulthood compared to hosts parasitized in Minnesota and Georgia. While the host specificity of *P. cassotis* remains largely unknown, the fact that it parasitizes a chemically defended host, has a limited host range(at least as reported in the literature), and that its population dynamics may be linked to monarch population dynamics suggest it could be a specialist on monarchs. Finally, given the petition to the USFWS to list the species as threatened (Center for Biodiversity *et al.* 2014), it will be especially important to assess the impact of all sources of monarch mortality, including that of *P. cassotis.*

Tables

Table 1. Nine butterfly and one moth species have been documented as hosts of *P. cassotis*. Taxonomic family and relevant citations are provided for each host species.

Lepidopteran Host	Family	Reference
Danaus plexippus	Nymphalidae	Gillette 1888
Chlosyne lacinia	Nymphalidae	Drummond 1970
Limenitis archippus	Nymphalidae	Schaffner and Griswold 1934
Limenitis arthemis astyanax	Nymphalidae	Schaffner and Griswold 1934
Euphydryas chalcedona sperryi	Nymphalidae	CAS Ent. Collection Database 2015
Euphydryas editha	Nymphalidae	CAS Ent. Collection Database 2015
Papilio polyxenes	Papilionidae	Schaffner and Griswold 1934
Papilio cresphontes	Papilionidae	Burks 1979
Pieris spp.	Pieridae	Burks 1975
Thyridopteryx ephemeraeformis	Psychidae	Berisford and Tsao 1975

Table 2. Summary of results of parasitism experiments in Minnesota (all years) and Wisconsin (2008 only). Pupae and pre-pupae were placed in cages in habitats suitable for monarchs, recovered 7-10 days later, and monitored for the emergence of butterflies or wasps. Exposure methods differed during 2008 (see text).

Year	Earliest placement	Latest Placement	Earliest Parasitism	Latest Parasitism	Total exposed	Total parasitized	Percent Parasitized
2008	7/21	7/23	7/22	7/22	340	1	0.3%
2009	7/10	8/22	8/6	8/20	313	13	4.2%
2010	8/24	9/29	8/24	9/29	309	188	60.8%
2011	8/29	9/1	8/29	9/1	467	67	14.3%
2012	7/7	7/12	7/7	7/12	73	18	24.7%
2013	7/15	9/6	-	-	235	0	0%
2014	6/10	9/22	-	-	367	0	0%
2015	6/19	10/4	7/14	9/5	228	17	7.5%
2016	7/25	9/15	8/24	9/15	233	16	6.9%

Figures

Figure 1. Photo of *P. cassotis* female ovipositing into monarch pupa under lab conditions (left) and her offspring emerging from the host, approximately fifteen days later (right).

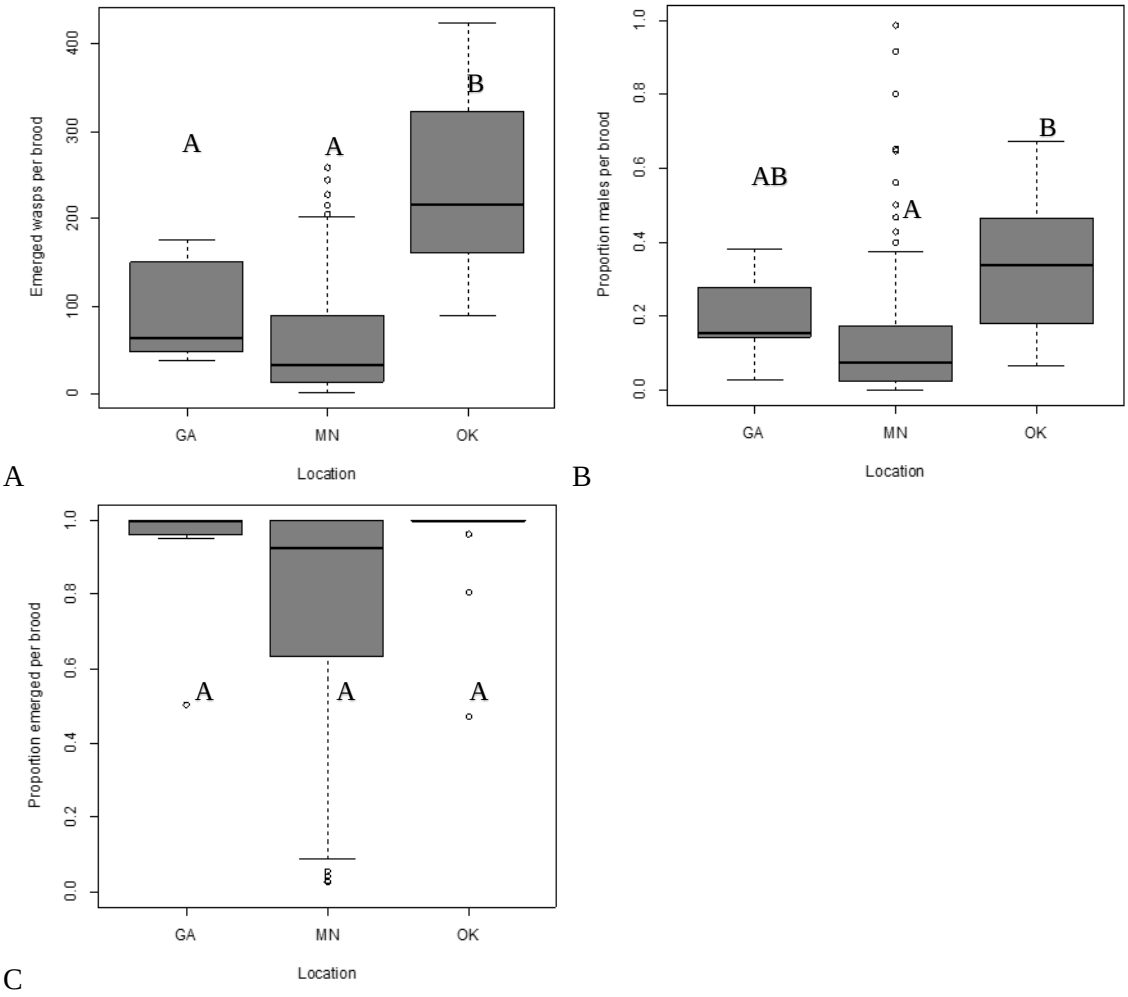

Figure 2. A) Brood size, B) the sex ratio as a proportion of males per brood, and C) the proportion of individuals within a brood that successfully emerged as adults are shown for each site for which brood data of successfully parasitized hosts were available (2008-2012). Boxplot whiskers extend 1.5xIQR from the first and third quartiles. Sample sizes: MN=194, GA=7, OK=14. Letters indicate statistical groupings of measurement variables as detected by Tukey's HSD; locations that do not share a letter are significantly different.

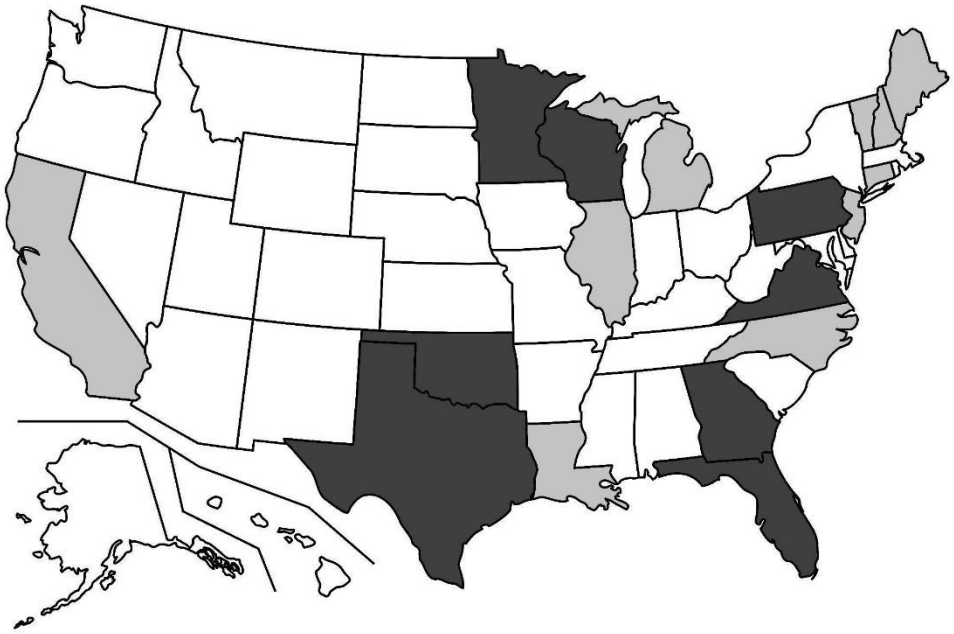

Figure 3. *P. cassotis* has now been recorded in eighteen US states. Parasitism of monarchs has been confirmed in eight states (black), and observed to be present, but not confirmed as a parasitoid of monarchs in ten additional states (grey) (assembled from GBIF 2014, Noyes *et al.* 2017, and references therein). Prior to this study, the range of *P. cassotis* was not known to include Georgia, Oklahoma, Wisconsin, or Virginia.

Supplementary Text

While this dissertation is written by the dissertation author, a similar version of this manuscript lacking data from 2015 and 2016 has been published with the following authorships and affiliations:

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Chapter 2:
Comparative studies of a generalist and specialist parasitoid reveal asymmetrical constraints on potential and realized host range

Summary

Why do some animal species consume a wide variety of foods, while others are dietary specialists? Furthermore, what are the consequences of these divergent life history strategies? We used two parasitoid wasps (generalist: *Pteromalus puparum*, specialist: *Pt. cassotis*) and several butterfly species as potential hosts in no-choice trials to test three hypotheses: the trade-off hypothesis, the preference-performance hypothesis, and the diet breadth mistakes hypothesis, which predicts that generalists and specialists tend to make opposite foraging mistakes. We predicted that generalists would be more likely to accept unsuitable hosts (errors of commission), and specialists more likely to reject suitable hosts (errors of omission). When comparing the brood size and survival of both wasps in hosts known to be suitable to the generalist, we found little support for the trade-off hypothesis. The specialist performed as well on atypical hosts as typical hosts and performed as well or better than the generalist on the generalist's hosts. Additionally, both parasitoids exhibited positively correlated preference and performance across hosts, though the generalist more readily accepted unsuitable hosts. Finally, the generalist was more likely to accept unsuitable hosts, while the specialist was more likely to reject suitable hosts. The frequency of these mistakes in nature is unknown, but these findings suggest that foraging mistakes may be both causes and consequences of diet breadth, with implications for conservation and biological control.

Introduction

Animals exhibit a wide range in the types and variety of foods consumed, and the context-dependent terms "specialist" and "generalist" mark the ends of this continuum. The questions of why organisms vary so widely in diet breadth and the resulting consequences of these differences pervade ecological and evolutionary biology (Hutchinson 1957, Roughgarden 1972, Tilmon 2008). Existing hypotheses for evolutionary specialization as opposed to generalization invoke resource availability, physiological constraints, defense, and foraging behaviors, yet the challenge of understanding the evolution of diet breadth remains.

Dietary specialization is common among parasitic organisms such as bacteria, lice, intestinal worms, and herbivorous insects (e.g. butterfly larvae and aphids). Most herbivorous insects can predictably be found feeding on a small number of closely-related or physiologically-similar plant species. Furthermore, most herbivorous insects are hosts of parasitoids, insects which spend their entire development feeding on a single host before developing into free living adults (Godfray 1994, Quicke 1997). Most parasitoids also utilize relatively few host species, but differ from true parasites in that they always kill their hosts (Hawkins 2005). Because larvae feed on only one host during development, a simplified measure of a parasitoids' diet breadth is its range of suitable host species.

Three broad factors determine a parasitoid's niche breadth: 1) the physiological suitability of hosts, 2) parasitoids' host recognition and acceptance behaviors, and 3) ecological opportunities (i.e., spatial and temporal coincidence of hosts and parasitoids) (Figure 1). The *potential host range*, illustrated by the entire blue circle in Figure 1, includes all hosts suitable for complete offspring development and depends on interactions between host defenses and the parasitoid's ability to overcome these defenses (Vinson & Iwantsch 1980). However, when determining the potential host range, experimenters might unknowingly exclude suitable hosts, or oviposition might not occur (Shaw 1994, Strand & Obrycki 1996). Experimentally transferring parasitoid eggs between hosts is rarely feasible, especially for endoparasitioids, which often inject immuno-suppressant venom or polyDNA viruses with their eggs (Pennacchio & Strand 2006, Asgari 2006). In contrast to the *potential host range*, the *realized host range* only includes hosts used successfully in the field (Harvey *et al.* 2012). Potential hosts may be excluded from the realized host range if they do not co-occur with the parasitoid due to geographic or phenological differences (Stireman & Singer 2003); in these cases, there is no ecological opportunity for

parasitoid attack (areas outside of the yellow circle in Figure 1). Parasitoid host searching and acceptance behaviors may also cause potential hosts to be ignored or rejected in the field (areas outside of red circle in Figure 1). Of particular interest are spaces A and B in Figure 1, which represent cases of parasitoids missing opportunities to use suitable hosts (A) because they do not recognize or accept them, or wasting resources by ovipositing in physiologically unsuitable hosts (B).

The best known, and perhaps most intuitive, explanation for differences in resource breadth is the *trade-off hypothesis*, summarized by the proverb: "A jack of all trades is master of none" (MacArthur & Connell 1966, Levins 1968, Wilson & Yoshimura 1994, Asplen *et al.* 2012). Foundational to this hypothesis is the presumed evolutionary difficulty of simultaneously adapting to multiple resources because any increase in performance associated with one resource is expected to cause reduced performance on others due to negative genetic correlations (Dethier 1954, Falconer 1960, Rausher 1983). Therefore, from an intraspecific perspective, specialists are expected to be highly efficient on a narrow portion of a resource gradient, but perform poorly outside of that range, while generalists are expected to be moderately efficient across a broad portion of a resource gradient (MacArthur 1972). Furthermore, from an interspecific perspective, a specialist is expected to outperform any generalist on the specialist's typical resource (Bush 1975, Futuyma & Moreno 1988). Finally, a generalist is expected to outperform a specialist on any resource utilized only by the generalist, because even intermittent selection experienced by the generalist is expected to outweigh the lack of selection experienced by the specialist on these resources. These hypothesized trade-offs have been used to explain the high host specificity characteristic of most phytophagous and parasitoid insects (Price *et al.* 1980, Jaenike 1990, Godfray 1994, Quicke 1997, Strand & Obrycki 2006).

The *preference-performance hypothesis* aims to explain the evolution of foraging strategies and behavioral specialization. Following optimality theory (Stephens & Krebs 1986), this hypothesis originated with phytophagous insects, but is equally relevant to parasitoid oviposition behaviors (Charnov & Stephens 1988). Female parasitoids need to assess host suitability factors such as species, life stage, and nutritional quality (Vinson 1976), and natural selection should shape oviposition preferences toward those hosts that yield the greatest fitness and away from hosts that yield poor fitness.

When empirically tested, both the trade-off hypothesis and the preference-performance hypothesis reveal that physiological performance and host choice behaviors are not always correlated as predicted. Tests of the trade-off hypothesis have shown that many specialists perform well on novel or atypical hosts, suggesting that a narrow diet breadth does not necessarily trade off with physiological performance across hosts (Futuyma & Moreno 1988, Fry 1996, Palaima 2007, Gompert *et al.* 2015). Furthermore, tests of the preference-performance hypothesis have shown that foragers generally prefer the best resources, but sometimes have high preferences for poor or unsuitable hosts (errors of commission) or low preferences for high quality hosts errors of omission) (Gripenberg *et al.* 2010, Hufnagel *et al.* 2016, Konig *et al.* 2016, Fei *et al.* 2017). Desneaux *et al.* (2009) tested this hypothesis by measuring the performance and foraging behaviors of an aphid parasitoid (*Binodoxys communis*) across 20 host species. They found that, in general, the sting rate of *B. communis* positively correlated with host suitability. However, mistakes were evident in frequent attacks of *Aphis nerii*, a chemically-defended host in which offspring fail to develop. The acceptance of poor quality hosts has also been demonstrated in other systems (e.g. Janssen 1989, Heimpel *et al.* 2003).

Here, we propose and test the novel *diet breadth mistakes hypothesis*, which states that different foraging strategies employed by generalists and specialists lead to predictable types of errors. If generalists have a broad host range at least in part because they have broad acceptance criteria for hosts they encounter, then we expect they will be more likely than specialists with narrower host acceptance criteria to over-accept poor quality hosts (area B in Figure 1, errors of commission). Equivalently, if specialists have narrow realized host ranges at least in part because they have narrower foraging strategies or acceptance criteria, we expect that they will be more likely than generalists to reject hosts that could support the development of their offspring (area A in Figure 1, errors of omission). A meta-analysis of preference-performance relationships in phytophagous insects found that female preference for high quality plants was stronger in oligophagous than polyphagous species, providing some support for that the diet breadth mistakes hypothesis, though without knowing the suitability of plants tested, we can't know which behaviors are optimal or sub-optimal (Gripenberg *et al.* 2010).

We tested these three hypotheses using two gregarious endoparasitoids of the genus *Pteromalus* (Hymenoptera: Chalcidoidae) that attack lepidopteran pupae. Based on a broad literature search and personal observations, these parasitoids differ greatly in their realized host

range (Table 1). *Pteromalus puparum* has been released around the world as a biocontrol agent of the crop pests *Pieris rapae* and *P. brassicae* (Moss 1933; Barron *et al.* 2003; Benson *et al.* 2003). This species was probably intentionally introduced to North America from Europe during the mid-to-late 1800s, though it is possible its native range included North America (Scudder 1889, Muesenbeck *et al.* 1951, Clausen 1956, Benson *et al.* 2003, Gibson & Gillespie 2006). It is currently distributed throughout the US (Lasota & Kok 1986). In addition to pierids, it is reported to use 48 lepidopteran hosts, many of which are pierid and nymphalid butterflies, as well as some papilionids and members of various moth families (Noyes 2017).

Pteromalus cassotis has received much less study than *P. puparum*, with nine lepidopteran species recorded as hosts, though one or more species may have only been observed under lab conditions (Noyes 2017, Burks 1975). It most commonly attacks nymphalids, but has also been reared from papilionids and a bagworm (Psychidae). Over 100 years ago, Gillette (1888) submitted an anecdotal report of a monarch chrysalis that produced over fifty *P. cassotis* offspring. Since this report, Drummond *et al.* (1970) reported that *P. cassotis* is probably the primary source of pupal mortality of *Chlosyne lacinia* (Nymphalidae) in Texas, but few other publications have mentioned *P. cassotis* in association with a host. In 2008, our lab 'rediscovered' *P. cassotis* as a parasitoid of monarch butterflies in Minnesota and Wisconsin (Oberhauser *et al.* 2015), and this host-parasitoid interaction has since been confirmed in several other states across the Eastern US (Stenoien *et al.* 2015, McCoshum *et al.* 2016, Stenoien unpublished).

These lists of host associations gathered from the published literature may be flawed by a sampling bias towards *P. puparum*, as well as incorrect species identifications of hosts or parasites, but serve as a useful starting point in assessing the host specificity of these parasiotids. Based on the large difference in reported hosts, and because toxic, specialist herbivores are typically exploited primarily by specialist natural enemies (Bernays and Graham 1988; Gauld *et al.* 1992, 1994; Stireman & Singer 2003), we are confident that, between this pair of wasps, *P. puparum* is the relative generalist, and *P. cassotis* is a relative specialist on monarchs and perhaps other milkweed butterflies (Danainae).

Using no-choice trials, we offered the parasitoids several butterfly host species, which represented three families and four subfamilies (Nymphalidae: Nymphalinae, Nymphalidae: Danainae, Pieridae: Pierinae, and Papilionidae: Papilioninae). The geographic ranges of most

hosts tested overlap with the parasitoids' known geographic ranges. Some host species had been previously reported as hosts for one or both parasitoids, while others had not (Table 1). We measured rates of host acceptance and the physiological performance (brood size and offspring survival) of each parasitoid with each host.

We leveraged these host-parasitoid interactions to test three predictions of the trade-off hypothesis. When a host is attacked, 1) *P. cassotis* will perform best in its typical host, *Danaus plexippus*, and more poorly in all other hosts; 2) *P. cassotis* will outperform *P. puparum* on its typical hosts; and 3) *P. puparum* will outperform *P. cassotis* on all hosts reported to be realized hosts of *P. puparum* but not *P. cassotis.*

We also tested the preference-performance hypothesis, which predicts that each parasitoid should exhibit a positive correlation between the average performance attained on a given host, and its relative preference for that host.

Based on previous tests of the physiological trade-off hypothesis and the preference-performance hypothesis, we expected some deviation from a perfect correlation between preference and performance by each parasitoid across all tested hosts. Specifically, the diet breadth mistakes hypothesis predicts that a specialist (*P. cassotis*) will more likely to reject or ignore suitable hosts, while a generalist (*P. puparum*) will be more likely to accept unsuitable hosts. We tested these predictions by measuring the deviations in preference based on an expected "optimal" preference-performance relationship across hosts.

Methods

Insect collection and rearing

In these experiments, the preference and performance of *P. puparum* and *P. cassotis* were compared across ten species of butterfly host pupae (Table 1). Laboratory populations of both parasitoids were established field-infected hosts. The *P. puparum* colony was started from *Pieris rapae* pupae which were placed in the field in Roseville, MN during September of 2013. The *P. cassotis* colony consisted of wasps from *D. plexippus* hosts and contained a mix of sources: Oklahoma (October 2013), Georgia (January 2014), Minnesota (August 2015 and September 2016), and Florida (July 2016). All parasitoid lineages were maintained with sib-sib matings. All experiments were conducted between September 2013 and December 2016.

All butterfly larvae were reared at low to moderate densities on live host plants or fresh clippings (host plants listed in Table 1). Locally-collected butterfly species were reared in a greenhouse, while those not locally collected were reared in growth chambers (*Euploea core* and *Battus philenor*, per specifications of USDA APHIS permit # P526-160112-040). Most pupae tested were first generation offspring from wild-collected butterflies, although for *D. plexippus* and *P. rapae*, we sometimes used 2nd and 3rd generation offspring, taking care to avoid inbreeding. Day length was approximately 16 hours and temperatures ranged from 18-32^{o}C in the greenhouse and 18-25^{o}C in the growth chambers. In general, host plants were grown from seed in a greenhouse, and fertilized every two weeks, although some host plants were grown outdoors.

When close to pupation, larvae were transferred to 16 ounce clear plastic deli containers with perforated lids, moved into the lab, and fed leaves of the host plant daily until pupation (~2.5% were transferred to 32 ounce containers, though container size did not seem to affect outcomes). All nymphalid species typically pupated on the underside of the lid of the deli container. Pierids typically pupated on the underside of the lid or side of the container. Papilionids were provided a stick propped at approximately 45 degrees on which to pupate, though a few pupated on the side or underside of the lid of the container.

General protocols

After removing frass and unconsumed plant matter, trials were conducted in the same containers in which the larvae were fed during their final stadium. Pupae were exposed to one or two naïve wasps within 24 hours of pupal ecdysis (mean days since ecdysis=0.32, sd=0.29). Trials with two wasps always contained sisters from the same brood. Wasps were 1-25 days old (mean=8.27, sd=5.55) and provided with a ~1cm^3 sponge soaked in 20% honey water. All trials lasted 1.8-4.2 days (mean=2.79, sd=0.34). In most cases, the natal host of experimental *P. puparum* was *P. rapae* (~97.5%, ~2.5% other species) and the natal host of *P. cassotis* was *D. plexippus* (~80%, ~10% from *P. rapae* and ~10% other host species).

During all trials, at least three observations (typically twice daily for a total of 4-8 observations) were made to determine whether the wasp(s) was standing on the host (observations of a wasp crawling onto and then off of the host within <~30 seconds were considered 'off' during that observation). When observed more closely, standing on a host was typically associated with an inserted ovipositor, though this was not always possible to confirm

due to the wasp's position and depth of insertion. Video recordings of host-parasitoid interactions demonstrate that parasitoid handling time often lasts 12-24 hours, and observations of a wasp on a host correlates well with confirmed oviposition events, so detection of attempted parasitism with these methods is likely (CS, unpublished), so we considered any host viewed in contact with a host to have attempted oviposition.

Upon removal of the wasp(s), most (~92%) pupae were massed, then reattached to the container to allow successful eclosion. Nymphalids were reattached by tying string around the cremaster and taping the string to the inside of the lid. Pierids were reattached with double-sided tape to the bottom of the container. Papilionids were either massed on the stick and then the stick massed and subtracted, or massed and reattached to the container using tape at the posterior end of the organism and to reattach the silken girdle. We recorded the date of emergence, as well as the number and sex of all emerged wasps. If neither host nor parasites had emerged after 30 days, hosts were dissected to determine the cause of death. Host fate was recorded as eclosed, successfully parasitized, unsuccessfully parasitized, or died due to ambiguous cause. Hosts from which any wasps emerged were dissected to determine the proportion of parasitoids that died as visible larvae, pupae, or adults (including sex) inside of the host. Therefore, when reporting the mean proportion of emerged wasps per brood, all hosts known to be parasitized are included, whether or not any wasps emerged successfully. A limitation of this procedure is that we could not account for wasp eggs or very small larvae that did not develop to a stage visible with a dissecting microscope. Eggs and small larvae may have disintegrated by the time of dissection, but larger larvae, pupae, and adult wasps inside hosts are easily distinguished. Thus, we cannot know the total number of eggs oviposited into a host when parasitoids emerged or had died in the host. Similarly, hosts that died for unknown reasons may have contained imperceptible eggs or larvae. We did not count offspring or dissect the host for some trials which resulted in successful parasitism if they represented highly replicated host-parasitoid combinations. Specifically, we did not count wasps and dissect hosts for 100 *P. cassotis-D. plexippus*, 202 *P. cassotis-Pieris rapae*, 317 *P. puparum-P. rapae*, and 5 *P. puparum-Colias philodice* trials.

By combining observations of attempted oviposition with the outcome of the trial, the attempted parasitism of the wasp(s) in each trial was scored as either successful, unsuccessful, or no attempt. Trials in which wasps successfully emerged were scored as 'successful' whether or not the wasp was observed on the host. 'Unsuccessful' outcomes included trials in which the

maternal wasp(s) was seen ovipositing, but the host did not produce viable wasps, as well as trials in which all wasps were found dead inside of the host. Trials for which we never saw the wasp in contact with the host that resulted in an emerged butterfly or pupa that died due to an unknown cause were scored as 'no attempt.' To give some context for these categorizations, 21.5% of the 205 trials in which *P. cassotis* was not seen on a *D. plexippus* host resulted in either successful or unsuccessful parasitism. Of the 677 trials in which *P. cassotis* was seen a *D. plexippus* host, 7.2% survived to become butterflies. For *P. puparum*, of 142 trials in which the wasp was not seen on a *P. rapae* host, 57% of these hosts were either successfully or unsuccessfully parasitized. Of the 939 trials in which *P. puparum* was seen a *P. rapae* host, 2.2% survived to become butterflies. When *P. cassotis* was paired with alternative suitable hosts, 22.8% of trials in which the wasp was not seen on the host resulted in parasitism, while 13.6% of trials in which the wasp was seen on the host resulted in butterfly survival. When *P. rapae* was paired with alternative suitable hosts, 29.6% of trials in which the wasp was not seen on the host resulted in parasitism, while 4.6% of trials in which the wasp was seen on the host resulted in butterfly survival. Therefore, it seems that our observations on alternative hosts are approximately equally likely to detect attempted parasitism events. The differences in detection of parasitism on typical hosts may be explained by a shorter handling time required of *P. puparum* on the small *P. rapae* hosts compared to the longer handling time induced upon *P. cassotis* when attacking much larger *D. plexippus* hosts.

Determination of potential suitability of each host for each parasitoid

No-choice trials with one or two female wasps were used to determine if hosts were suitable for parasitoid development (Table 3). In no-choice trials, a parasitoid was presented with a single host, and attempts to parasitize that host were recorded. A host was considered suitable for a parasitoid species if $\geq$10% of trials in which the wasp was seen in contact with the host resulted in successful parasitism. Hosts that were seen with wasp(s) in contact, but that were successful in <10% of these trials, were deemed "poor/unsuitable" hosts. If a host-parasitoid combination never resulted in observed oviposition attempts or parasitoid offspring, the suitability of such a pair could not be determined and was removed from further analyses.

Sample sizes between host species and between one- and two-wasp trials varied widely for several reasons. First, trials of each wasp with its typical host are especially highly replicated

because we were interested in the interactions between these wasps and their typical hosts and because we maintained our wasp colonies on these hosts. Second, obtaining large numbers of some hosts was logistically difficult. Finally, we used two wasps in some trials because each parasitoid species was reluctant to attempt parasitism on certain hosts. By using two wasps instead of one, we hoped to increase the likelihood of attempted parasitism to better determine the suitability of those hosts.

Test of the Trade-Off Hypothesis

Comparisons of physiological performance between the two parasitoid species and across all hosts were made using the same trials as were used for the determination of physiological suitability of hosts. However, only trials in which parasitism was attempted were used to determine the performance of a given parasitoid-host species pair, so as not to conflate a lack of preference for low performance, which would occur if averaging across all trials without regard to oviposition behavior.

There are many ways to measure the performance of parasitoids; each is informative, but none perfectly measures fitness. The simplest performance metric we employed was the relative frequency of success (wasp emergence) and failure (no wasp emergence) when we observed the wasp on the host. We did not differentiate between failed attempts that resulted in wasp death, host death without evidence of wasps inside, or butterfly emergence.

We also considered four brood-level performance metrics: 1) the number of emerged wasps per brood, 2) an adjusted measure of the quantity of wasps per brood, which partially corrects for differences in sex ratio through a reduction in the value of males in accordance with species-specific differences in the relative average mass of individuals of each sex, 3) the number of emerged wasps per gram of host, and 4) the proportion of wasps surviving to emergence. The adjusted measure of wasp quantity was calculated by weighing groups of dried male and female wasps representing 30+ broods of both species from their typical hosts, and calculating the ratio of the mean mass of males to females (*P. cassotis*: 310 males weighed 0.04178 g, 2584 females weighed 0.79203 g, therefore mean male mass/female mass = 0.431; *P. puparum*: 318 males weighed 0.03716 g, 712 females weighed 0.16338 g, therefore mean male mass/female mass = 0.509). Larger brood size, larger adjusted brood size, more wasps per gram of host, more female biased sex ratios, and higher survival were interpreted as higher performance.

Except for the proportion of wasps surviving to emergence, each of these metrics was compared using two sets of trials: 1) all trials in which we had observed oviposition, and 2) only trials in which we knew parasitism had occurred, either because adult wasps emerged or dead wasps were found in the dissected host pupa. We did not compare the proportion surviving to emergence in the first set of trials because the number of eggs and small larvae that failed to develop to a point discernable via dissection was unmeasurable. All hosts suitable for the generalist parasitoid were included in these analyses except *V. atalanta*, for which the performance of the specialist parasitoid was unknown due to lack of attempted parasitism. *Papilio polyxenes* and *B. philenor* were also excluded because they were poor or unsuitable hosts for both parasitoids.

To test the prediction that *P. cassotis* would perform best in its typical host (*D. plexippus*) and more poorly in all other hosts, we compared the frequencies of success and failure when *P. cassotis* attempted parasitism on its typical host *vs.* all other hosts combined. We also tested the frequencies of success and failure on its typical host versus every other host species separately. Lastly, we tested for differences in the four brood-level performance metrics between *D. plexippus* and all other hosts.

To test the prediction that *P. cassotis* would outperform *P. puparum* on the specialist's typical host (*D. plexippus*), we compared the relative frequency of successful parasitism when observed in contact with the host. Because *P. puparum* never successfully parasitized *D. plexippus*, we did not compare brood-level performance metrics.

To test the prediction that *P. puparum* would outperform *P. cassotis* on hosts reported to be realized hosts of *P. puparum* but not *P. cassotis*, we compared the relative frequency of success when observed in contact with the host, pooled across all host species and within each host species individually. Finally, we tested for differences in the four performance metrics between the two parasitoids within each host when observed in contact with the host.

The relative frequencies of wasp success and failure when a wasp was observed in contact with the host were compared using Fisher Exact Tests. Follow-up tests performed on differences within or between individual host species were Bonferroni corrected to account for multiple comparisons. We did not account for the number of wasps in a trial because frequencies of successful attempted parasitism within any host-parasitoid combination were similar between trials with one and two wasps (Table 3).

Brood size, adjusted brood size, and wasps per gram of host (rounded to the nearest integer of wasp count) were analyzed using a hurdle model implemented via the pscl package (Jackman 2015) in R (R Core Team, version 3.3.3). These models used a binomial distribution for the zero *vs.* positive portion of the model and a negative binomial distribution for the count portion of the model. Survival was modelled using a binomial distribution with a logit link function. All models included the interaction between parasitoid and host species, and the age and number of wasps in the trial as covariates. We then used Tukey-adjusted pairwise comparisons of least square means via the lsmeans package (Lenth 2016) to determine whether differences within or between hosts were significant, depending on the specific predictions made. Because hurdle models are invalid when a level of a predictor variable includes only zeros, and *P. puparum* never successfully produced a brood from *D. plexippus*, we used Wilcoxon Rank Sum Tests to compare brood level metrics of the parasitoids on this host.

We considered incorporating hosts' phylogenetic relationships as a covariate, but the lack of an existing tree with all ten butterflies, the limitation imposed by small sample sizes of several clades in our dataset, and the apparent incompatibility of phylogenies with hurdle distributions prevented us from doing so.

Test of the Preference-Performance Hypothesis

To test the preference-performance hypothesis, we used data from the same trials to calculate the mean number of wasps that emerged from trials in which parasitism was attempted (wasp seen on host) from every combination of host and parasitoid. We used these mean performance values as predictors of parasitoid attack in logistic regressions for each parasitoid species, with wasp age as a covariate.

Tests of the Diet Breadth Mistakes Hypothesis

The diet breadth mistakes hypothesis predicts that a specialist (*P. cassotis*) will be more likely than a generalist to reject suitable hosts, while a generalist (*P. puparum*) will be more likely than a specialist to accept poor or unsuitable hosts. We used data from trials with one wasp to test these predictions using three different analyses.

Our first approach was to simplify the variation in performance across hosts into two categories, suitable and poor/unsuitable, as determined previously. We considered cases in which

a wasp did not oviposit into a suitable host and cases in which a wasp did oviposit into a poor or unsuitable host as mistakes. We compared the frequency of mistakes committed by both parasitoids on suitable and poor/unsuitable hosts using Bonferroni corrected Fisher Exact Tests.

Our second approach was to model parasitoid acceptance behavior as influenced by the potential suitability of a given host species for a given parasitoid. We used logistic regressions with potential suitability, parasitoid species identity, and their interaction, as well as wasp age as predictors of attack. Performance attained by each wasp on each host was represented by normalized brood size score bounded between 0 and 1, relative to the mean brood size attained when attempting parasitism on the best host in the set. A significant effect of parasitoid species identity would indicate that, given hosts of equivalent suitability, one parasitoid species is more likely to accept it, while the other is more likely to reject it. if the specialist was more likely to reject suitable hosts and the generalist more likely to accept unsuitable hosts, these results would support the diet breadth mistakes hypothesis. An interaction between suitability and parasitoid species identity would indicate that these differences in foraging behaviors are more pronounced on one end of the host quality spectrum than the other.

Our third approach was to use the maximum mean performance (brood size) attained by a parasitoid across all hosts tested to generate an equation where, given the mean performance on any host, it would generate an expected preference for that host, expressed as the proportion of trials expected to result in attempted parasitism. Furthermore, for both wasp species, we calculated the deviation between the expected and actual proportions that attempted parasitism on each host species and tested whether these deviations were different from zero. This approach assumes that preference should be 100% for the host species that yields the highest performance, and that the preference for any given host should be proportional to its performance on that host (Equation 1). It also assumes that optimal behavior will be expressed by the collective (average) decision of many individuals, rather than a uniform response to a specific set of stimuli. All host-parasitoid combinations were included in this analysis except for *P. cassotis* with *V. atalanta* and *N. antiopa* because this parasitoid never attacked these hosts in one-wasp trials, so we could not estimate performance. *P. cassotis* also never attacked *B. philenor* in one wasp trials, but because it was unsuitable in two wasp trials, we assigned it a mean brood size of zero for this analysis.

Equation 1: $\text{Expected preference}_{i,j} = (1/\text{performance}_{i,b}) * \text{performance}_{i,j}$

Expected preference = expected proportion of no-choice trials in which parasitism should have been attempted; performance = mean number of emerged wasps when parasitism was attempted. i = wasp species, j= host species, and b=the host species which yields the greatest performance for wasp species 'i'.

Results

Determination of the physiological suitability of each host for each parasitoid

In general, the Nymphalinae were suitable for both parasitoids, the Papilionidae were (contrary to previous reports) poor or unsuitable for both parasitoids, and the Danainae were suitable only for *P. cassotis* (Table 2). All four Nymphalinae (*Junonia coenia, Nymphalis antiopa, Vanessa atalanta, Vanessa cardui*) and both Pieridae (*Pieris rapae, Colias philodice*) were suitable hosts for *P. puparum*, though *D. plexippus*, *Euploea. core*, and *Battus philenor* were never successfully parasitized. *Papilio polyxenes* was very infrequently successfully parasitized by *P. puparum*, despite many attempts. Seven species were suitable hosts for *P. cassotis*, all of which are Nymphalinae, Danainae and Pieridae. Both papilionids, *B. philenor* and *Papilio. polyxenes*, seem to be poor or unsuitable hosts, though the infrequency of attempted parasitism on these hosts prevents us from declaring them as wholly unsuitable for *P. cassotis*. Finally, the status of *V. atalanta* as a potential host of *P. cassotis* remains unknown because no wasp successfully parasitized, nor was observed attempting to parasitize this potential host species.

Trade-Off Hypothesis

Prediction 1: Specialist performs best on typical host

Support for the prediction that the specialist would perform best on its typical host varied based on the performance metric considered. Some metrics indicate that *P. cassotis* performs as well or better on *D. plexippus* than on alternative hosts, while other metrics indicate no difference or even better performance on alternative hosts.

The relative frequencies of success when attempting parasitism on the typical host *vs.* all other hosts combined were not significantly different. *P. cassotis* was successful in 76.4% of attempts on *D. plexippus*, and 80.1% of all attempts on other hosts (Fisher Exact Test, p=0.0984,

Table 3). When the frequency of success on *D. plexippus* was compared to each other host species individually, five were statistically indistinguishable from *D. plexippus.* The only difference was that successful parasitism was more likely on *J. coenia* than *D. plexippus* (Fisher Exact Test, p=0.0056, Bonferroni corrected alpha=0.05/6=0.0083).

Regarding the four brood-level performance metrics, *P. cassotis* performed well on *D. plexippus* by some metrics, but poorly by others. Comparisons of least square means derived from the various models revealed that, after accounting for the age and number of wasps in a trial, *P. cassotis* achieved relatively large raw and adjusted brood sizes (Figure 2A,B,D,E) on *D. plexippus*, though not significantly larger than E. *core* or *J. coenia*, or hosts with small sample sizes, such as *V. cardui* and *N. antiopa.* Contrary to prediction, *P. cassotis* achieved relatively small numbers of offspring per gram of host on *D. plexippus* relative to other hosts (Figure 2C,F). For these three metrics, patterns were generally similar regardless of whether trials in which parasitism was assumed or guaranteed were considered. Finally, *P. cassotis* survival in *D. plexippus* was moderate: lower than in two other species, indistinguishable from two species, and greater than two species (Figure 2G).

Prediction 2: Specialist outperforms generalist on specialist's typical host

P. puparum attempted parasitism of *D. plexippus* hosts 71 times, but never succeeded in producing offspring, nor even developed to a point discernable in a dissection. Therefore, *P. cassotis* was more likely than *P. puparum* to succeed when attacking *D. plexippus* (76.4% *vs.* 0%, Fisher Exact Test, p<0.001, Table 2).

Prediction 3: Generalist outperforms specialist on generalist's hosts

When attempting parasitism on suitable hosts, *P. puparum* was marginally more successful than *P. cassotis* (83.76% *vs.* 80.31%, Fisher Exact Test, p=0.0635, Table 2). When the frequency of success of each parasitoid was compared on each host individually, five hosts yielded statistically indistinguishable rates of success. However, *P. puparum* was more likely than *P. cassotis* to successfully parasitize *P. rapae* (Fisher Exact Test, p=0.0074, Bonferroni corrected alpha=0.05/6=0.0083, Table 2).

Despite this slight advantage in success frequency to *P. puparum*, significant differences among brood-level performance metrics typically favored *P. cassotis. P. cassotis* achieved larger

raw and adjusted mean brood sizes in all five hosts in which successful parasitism occurred for both parasitoid species, and these differences were significant for two or three hosts, depending on the dataset analyzed (Figure 3A,B,D,E). Due to missing host mass data, fewer comparisons of host use efficiency were possible, although *P. cassotis* produced more wasps per gram of host than *P. puparum* in two hosts, while the other hosts were equivalent or not comparable (Figure 3C,D). Regarding brood survival to adulthood, *P. cassotis* outperformed *P. puparum* in *N. antiopa* and *J. coenia*. The only significant difference in favor of *P. puparum* was brood survival when attacking *P. rapae* (Figure 3G).

Preference-Performance Hypothesis

As predicted by the Preference-Performance Hypothesis, both parasitoid species were more likely to attempt parasitism of hosts which yielded larger broods, on average (*P. puparum:* $p<0.001$, $n=1453$, *P. cassotis:* $p<0.001$, $n=1982$. Figure 4, Table 3.). Wasp age was a significant negative predictor of attack for *P. cassotis*, but not *P. puparum.*

Diet Breadth Mistakes Hypothesis

For our first test of the diet breadth mistakes hypothesis, we classified hosts into suitable and poor/unsuitable categories, and tested the relative frequency of mistakes under the assumption that suitable hosts should always be attacked and poor/unsuitable hosts should never be attacked. As predicted, *P. cassotis* was more likely to reject suitable hosts (Fisher Exact Test, $p<0.001$, odd ratio = 7.57, $n_{Pc}=1946$, $n_{Pp}=1246$, Figure 5A) and *P. puparum* more likely to attack poor or unsuitable hosts (Fisher Exact Test, $p<0.001$, odds ratio = 4.35, $n_{PC}=49$, $n_{PP}=199$, Figure 5A). It is worth noting that within each parasitoid species, attack behaviors varied considerably with host species identity, even between species in the same suitability category (Figure 5B). Host suitability was determined using one- and two-wasp trials, but this analysis considered only one-wasp trials, which is why *N. antiopa* and *B. philenor* were able to be categorized, despite no attempts of parasitism by *P. cassotis* in one-wasp trials. Full results are given in Table 2.

For our second test of this hypothesis, we fit a logistic regression to determine if parasitoid species identity, the mean relative performance of each host-parasitoid species interaction, and the interaction between these variables were significant predictors of wasp attack.

We also included wasp age and the interaction between wasp age and parasitoid species identity as covariates, based on the results of the preference-performance hypothesis.

As in the preference-performance regressions, relative potential performance on a host was a strong positive predictor of wasp attack (Table 4). As predicted by the diet breadth mistakes hypothesis, if all else was equal, *P. puparum* was significantly more likely than *P. cassotis* to attack a host of any relative suitability. Furthermore, the positive interaction between *P. puparum* and mean relative performance, coupled with the positive main effect of *P. puparum*, indicates that a high proportion of *P. puparum* wasps attack hosts on which they attain moderate or higher performance, while a high proportion of *P. cassotis* attack only occurs for hosts on which they can attain high performance. Because *P. cassotis* attained larger maximum brood sizes, this effect would be even stronger if we modeled absolute, rather than normalized, mean brood sizes. Finally, the negative effect of wasp age was strongest for *P. cassotis*, as was shown in the tests of the preference-performance hypothesis.

For our third test of this hypothesis, we assumed that within a parasitoid species, the proportion of individuals that attack a host should be proportional to their mean performance on that host, relative to the mean performance on their best host. Under the diet breadth mistakes hypothesis, we expected that the proportion of generalists attacking any host would be higher than expected and that the proportion of specialists attacking any hosts would be lower than expected. We obtained usable preference and performance data for ten and seven hosts of *P. puparum* and *P. cassotis*, respectively (Figure 6). The deviations in the proportion of *P. puparum* individuals attacking hosts were significantly greater than zero (pseudomedian = 0.304, Wilcoxon Signed Rank Test, two tailed, p=0.0488), but the deviations in the proportion of *P. cassotis* individuals attacking hosts was not significantly different from zero (pseudomedian = -0.084, Wilcoxon Signed Rank Test, two tailed, p=0.4469). Therefore, the generalist behaved as predicted by the diet breadth mistakes hypothesis, but the specialist's behavior across these seven hosts did not deviate statistically from the expected relationship between preference and performance.

Discussion

Differences in physiological performance and ovipositional preferences between host species contribute to variation in host specificity and host ranges of insect parasitoids. In this

study, we compared the performance and decisions of a specialist (*P. cassotis*) and a generalist (*P. puparum*) when paired with several butterfly host species to determine the importance of physiological trade-offs and foraging behaviors as causes and consequences of their realized host range (Figure 1). The generalist readily accepted most hosts, even those that were poor or unsuitable, while the specialist ignored or rejected many hosts, even those in which it could perform well. Therefore, the realized host range of the generalist was mostly constrained by the physiological ability to develop in all hosts attacked, while the realized host range of the specialist was constrained by its inability or unwillingness to oviposit into alternative hosts.

Our results provide little support for the intuitively appealing trade-off hypothesis (see also Via 1990, Fox & Caldwell 1994, Gompert *et al.* 2015). As predicted by this hypothesis, the specialist produced larger broods from its typical host than most alternatives. However, per other performance measures including survival and host-use efficiency, the specialist performed as well or better on alternative hosts as its typical host. Additionally, while the specialist vastly outperformed the generalist on *D. plexippus,* the specialist's preferred host, the specialist also performed as well or better than the generalist on most of the hosts previously reported to be used only by the generalist. The finding that *P. cassotis* did not suffer physiological trade-offs in atypical hosts suggests that negative genetic correlations due to antagonistic pleiotropy do not affect the ability to develop in different hosts. If mutation accumulation, genetic drift, or the evolution of novel defenses by alternative hosts are mechanisms that cause specialists to perform poorly on atypical hosts, it is possible that there simply has not been enough evolutionary time for *P. cassotis* to lose the ability to develop in alternative hosts since behaviorally specializing on milkweed butterflies. An alternative explanation is that *P. cassotis* is more polyphagous than is indicated by host records, and performed well on alternative hosts because it frequently experiences selection on a broad suite of hosts, possibly including those tested here.

Our results support the preference-performance hypothesis. Both parasitoids were more likely to parasitize hosts from which they could produce larger broods, though it is worth noting that, regardless of suitability, the generalist was more likely to attack hosts of any suitability than the specialist. This hypothesis has been extensively tested in plant-insect systems, and a recent meta-analysis demonstrated that in most cases, offspring perform better on preferred plant types and females lay more eggs on plant types conducive to offspring performance (Gripenberg *et al.* 2010). While host-parasitoid interactions have received much attention, tests of the hypothesis in

these sysmtes are generally supportive (e.g. Brodeur *et al.* 2003, Desneaux *et al.* 2009, Harvey *et al.* 2014 but see Henry *et al.* 2005). Host choice behaviors are thought to be evolutionarily labile and ample evidence suggests that behavior is a common mechanism by which specialization proceeds (Futuyma & Moreno 1988, Ravigné *et al.* 2009). It is also possible that behavior could drive the evolution of performance; Pre-existing preferences for certain host types could result in selection for increased performance on those hosts.

Finally, we found strong support for the newly-proposed diet breadth mistakes hypothesis. Generalists have been shown to use more broadly conserved cues in assessing hosts, while specialists require more specific cues to recognize and accept hosts (Meiners *et al.* 2000, Steidle *et al.* 2001), which may explain why the generalist was more likely to make errors of commission, while the specialist was more likely to make errors of omission.

Somewhat unexpectedly, the specialist *P. cassotis* was so choosy that, even under conditions designed to encourage oviposition, this species never attempted to parasitize two of the species presented to it. This disallowed us from estimating performance on these hosts, and forced us to remove them from the analysis. *Nymphalis antiopa* was suitable for *P. cassotis* in trials with two wasps, but was never attacked in trials with one wasp, so we could not derive a performance estimate for this pair. *Vanessa atalanta* was also never attacked by *P. cassotis*, though the closely related *V. cardui* was suitable. If either of these hosts is suitable for *P. cassotis*, repeated rejections of them represent errors of omission, and excluding them from the analysis would have falsely skewed the results toward the null hypothesis. Further experiments between *P. cassotis* and these hosts, perhaps including manipulations designed to encourage oviposition, such as the provision of cues from *P. cassotis'* typical host, might reveal that they are potential hosts.

Another limitation of this study involves our metrics for preference and performance. To measure preference, we used a binomial response variable (attack or not), but a continuous response variable (such as the latency to oviposit) could provide a more precise and relevant measure of host preferences. To measure performance, we used brood size, but ignored offspring size, which is likely to influence fitness (Visser 1994). Additionally, it is possible that brood size is not a truly independent measure of performance if, for example, females are willing to attack a less preferred host but place fewer eggs because it is less-preferred. Despite these potential issues,

brood size is the most direct measure of fitness available and was largely corroborated by our other performance metrics.

We found that the generalist's realized host range was primarily constrained by the physiological inability to attack some hosts, rather than by the behavioral rejection of suitable hosts. Herbivore-sequestered plant allelochemicals are known to play important roles in host parasitoid interactions and might explain why some hosts were unsuitable (Turlings & Benrey 1998, Ode 2006, Price *et al.* 1980). Of the hosts tested, *D. plexippus, E. core*, and *B. philenor* are known to employ chemical defenses, and none of these hosts were susceptible to parasitism by *P. puparum* (Parsons 1965, Rothschild *et al.* 1978, Malcolm & Rothschild 1983, Sime *et al.* 2000, Agrawal *et al.* 2012). Chemical defenses may also limit the physiological suitability of hosts for the specialist because, while *P. cassotis* frequently attacks danaids (here, *D. plexippus* and *E. core*), which contain sequestered cardenolides and endogenously produced cardioactive compounds, this parasitoid was apparently unable to develop in *B. philenor*, which sequesters aristolochic acids (Sime *et al.* 2000).

The physiological suitability of hosts seems to be a secondary constraint on the realized host range of the specialist; the primary constraint is the infrequent acceptance of most alternative hosts, regardless of their potential suitability. If behavior is driving the evolution of *P. cassotis*' specialization on monarch hosts, over time, this species might lose the ability to parasitize alternative hosts due to the evolution of host defenses, mutation accumulation, or drift, but this has not yet happened.

While a parasitoid's potential host range is defined by the physiological suitability of hosts, this study demonstrates that behavior can act as a filter that limits parasitoids' realized host ranges (Figure 1). Several authors have recognized that although genetic trade-offs in larval performance generally do not explain high degrees of host specificity, other types of performance-related trade-offs and ecological factors might allow higher fitness on one host than on others (Thompson 1996, Scheirs *et al.* 2005). Janz and Nylin (1997) provided evidence that host choice behavior can limit herbivores' realized host ranges by showing that although a generalist and specialist herbivore performed equally on a poor-quality host plant, only the specialist discriminated against it. Similarly, Brodeur *et al.* (2003) found that the potential host range of two *Cotesia* parasitoids includes more host species than constitute the realized host range in the field, suggesting that female behavior, rather than host suitability, limits the realized host

range. As a mechanism for observations such as these, Bernays (2001) argued that because choosing a host requires the ability to process multiple sensory inputs, generalists are less efficient at host selection than specialists, and are therefore at a selective disadvantage, partially explaining the commonality of host-specific parasitic insects. This selective advantage erodes, however, if the specialist's preferred host becomes scares, suggesting that behavioral trade-offs related to perception, cognition, and learning might drive the evolution of generalist *vs.* specialist strategies, rather than physiological trade-offs.

An important aspect of the discussion of optimal and sub-optimal behavior is the difficult problem of quantifying the fitness-related costs to a forager of making errors of either omission or commission (van Baalen & Hemerik 2008). In the context of parasitoid foraging, the costs of an error of omission are opportunity costs, because the forager has forgone an opportunity to reproduce. The cost of an error of commission includes the energy and nutrients invested in the eggs, potential risk of injury or death associated with an oviposition bout, and the opportunity costs associated with investing time and eggs into an unprofitable host when those resources could have otherwise been used to forage for a more profitable host. The costs of both types of errors also depend upon the quality of the host in question and the likelihood of future oviposition, which is influenced by ecological factors (e.g. the availability of suitable hosts in the environment), life history traits (e.g. whether the forager can produce additional eggs during her lifetime), and status of the forager (e.g. whether egg- *vs* time-limited).

Another important question raised by this study is the frequency of foraging mistakes in natural systems. Our experimental set-up represented an extremely simplified environment. By placing the parasitoid within ~10 cm of the host, we removed the normal process of host searching. Due to differences in micro-habitat use in a natural setting, it is possible that each parasitoid might never or only rarely encounter some of the hosts in this study. If so, errors of commission might be rare. Errors of commission are known to occur in a few taxa. Although inferring some aspects of parasitoid foraging behavior in the field is possible (see Heimpel & Casas 2008), detecting errors of commission committed by parasitoids in the field is often difficult because parasitoid offspring might die as eggs or small larvae and the host may or may not survive such an attack. If the host is killed, the cause of mortality may be difficult or impossible to differentiate from other possible causes of death. It is therefore surprising and particularly relevant that *P. puparum*, after introduction as a biological control agent in New

Zealand, was reported to attack monarch butterfly pupae, "although it apparently fails to develop within," (Ramsay 1964, p15). Recently, it has been discovered that indigenous scelionine egg parasitoids attack, fail to develop, and sometimes cause abortion of brown marmorated stink bug eggs, *Halyomorpha halys*. This pentomatid is a polyphagous pest in North America and experiences very low rates of successful parasitism by native egg parasitoids, though lab experiments show that native parasitoids readily accept these hosts, and recent field experiments suggest that attack rates in the field have probably been considerably underestimated (reviewed in Abram *et al.* 2016).

Errors of commission have also been observed to be committed by monarch butterflies, which oviposit onto two invasive swallow-worts, *Vincetoxicum nigrum* and *V. rossicum*. Both species are unsuitable, and field, lab, and cage studies have demonstrated that, while these plants are not preferred over suitable native host plants, females may place up to 25% of their eggs on them (DiTomasso & Losey 2003, Casagrande & Dacey 2007). Finally, many water-loving insects have found themselves in evolutionary traps (Schlaepfer *et al.* 2002) that cause errors of commission in oviposition or habitat choice. Certain reflectance patterns of polarized light, a formerly reliable cue of open water, are also produced by a variety of manmade surfaces (e.g. black asphalt, crude oil lakes, glass). Due to the misinterpretation of these cues, mayflies, caddisflies, water beetles, damselflies, and dragonflies sometimes attempt to utilize these unsuitable object as oviposition sites or habitats (reviewed by Horváth *et al.* 2009). It is worth noting each of these examples involves recently altered ecological conditions, such as change caused by the introduction of a new species or anthropogenic element.

If specialists are efficient at finding and attacking their preferred hosts such that they realize their potential fecundity, the frequency of omission of alternative hosts might be high, though the costs might be low. However, many specialists have been found to perform well on alternative hosts in laboratory studies, but rarely use alternative hosts in the field (Futuyma & Moreno 1988). If a specialist's preferred resource becomes scarce or extinct, the specialist will be forced to find and accept alternative resources or become extinct as well. However, when ecological changes are rapid, it is possible that the evolution of foraging behaviors will not keep pace (Levins 1968). Indeed, specialist species are at higher risk of extinction than generalists during periods of rapid global change, and co-extinctions of hosts and parasites have the potential to greatly alter ecosystem processes during the current mass extinction event defining the

Anthropocene (Clavel *et al.* 2011, Strona 2015). North American monarch butterflies have experienced significant population declines over the past 20 years, measurable by adult overwintering counts and immature abundance during the breeding season (Rendón-Salinas *et al.* 2017, Stenoien *et al.* 2015b). There is evidence that monarch and *P. cassotis* population dynamics are linked (Stenoien *et al.* 2015a, Chapter 1), suggesting that this parasitoid strongly prefers monarchs as hosts and that it may be under selection to expand its realized host range.

Thus far, we have focused on foraging mistakes as *consequences* of a given diet breadth strategy, but they could also serve as *causes* of differences in diet breadth. If, over evolutionary time, a specialist loses its ability to parasitize alternative hosts (perhaps due to the previously mentioned potential mechanisms), then trade-offs would arise and the omission of these hosts would become optimal behaviors, reinforcing their exclusion. Therefore, if hosts become less suitable due to their lack of use by a specialist, the rejection of suitable hosts could eventually reinforce a specialist's narrow host range over evolutionary time.

A generalist's mistake of ovipositing into poor or unsuitable hosts will remain a poor decision and, if the genetic variation exists, will be continually selected against unless the parasitoid acquires a beneficial mutation that increases performance on these hosts or a host evolves to become susceptible. Despite this typically negative feedback associated with errors of commission, there could be selective advantages reinforcing the use of broadly conserved oviposition cues. For example, if a generalist is likely to encounter more host species than it has the cognitive capacity to discriminate between and most hosts in the environment are at least moderately suitable, a generalist strategy may be appropriate (Dukas 1998). Broad host acceptance criteria could also be advantageous if the availability of different types of hosts is unpredictable or if the forager is time- rather than egg-limited.

We found *Papilio polyxenes*, a species previously reported as a host for both parasitoids, to be a poor or unsuitable host for both parasitoids. This result could be an artefact of our experimental design. Alternatively, some published host records may be incorrect, possibly due to misidentification of parasitoids. Incorrect host records would not be terribly surprising, as cryptic diversity related to high levels of host specificity and revealed by DNA barcoding have been found in other groups of parasitoid wasps (Smith *et al.* 2008). Pteromalidae is an extremely diverse and notoriously difficult clade to reconstruct and could contain cryptic diversity related to high levels of host specificity (Desjardins *et al.* 2007, Heraty *et al.* 2013).

We also discovered that several hosts of previously unknown suitability for one or both parasitoids are, in fact, suitable. Six new hosts were found to be physiologically suitable for *P. cassotis* and one new host for *P. puparum*. We do not know the frequency with which these interactions occur in the field, but these findings demonstrate the evolutionary potential for host shifts or host range expansion. Understanding the relationship between potential and realized host ranges and predicting host range shifts or expansions is important for safe and successful applications of biological control agents, as well as predicting impacts in natural systems that are are rapidly changing due to land use change, climate change, and invasive species.

Given that parasitoids are extremely diverse, ubiquitous across ecosystems, and vary greatly in their relative host range, they are a useful system for studying the causes and consequences of the evolution of diet breadth strategies (Askew 1968, Shaw 1994). Our findings and the newly proposed diet breadth mistakes hypothesis, however, are potentially applicable and relevant to others' comparative studies of resource use strategies including as diet breadth, host range, or habitat use. The diet breadth mistakes hypothesis could be tested using existing datasets, especially from studies of preference-performance relationships of foraging predators, and oviposition decisions of herbivores and parasitoids.

Tables

Table 1. Information regarding the ten potential hosts tested against two *Pteromalus* parasitoids.

Butterfly host	**Family , subfamily**	**Host plants**	**Pupal mass (grams, mean±SD)**	**Known host of *P. puparum*?**	**Known host of *P. cassotis?***
Danaus plexippus	Nymphalidae, Danainae	*Asclepias* spp.	1.13 ± 0.22	No, unsuccessful (Ramsay 1964)	Yes (Gillette 1888)
Euploea core	Nymphalidae, Danainae	*Nerium oleander*, *A. incarnata*	0.80 ± 0.11	No	No
Nymphalis antiopa	Nymphalidae, Nymphalinae	*Salix nigra*	0.88 ± 0.17	Yes (Shaw *et al.* 2009)	No
Vanessa cardui	Nymphalidae, Nymphalinae	*Plantago lanceolata*	0.49 ± 0.11	Yes (Stefanescu *et al.* 2011)	No
Vanessa atalanta	Nymphalidae, Nymphalinae	*Urtica dioica*	0.59 ± 0.15	Yes (Muesenbeck *et al.* 1951)	No
Junonia coenia	Nymphalidae, Nymphalinae	*Plantago lanceolata*	0.43 ± 0.07	No	No
Papilio polyxenes	Papilionidae, Papilioninae	Various Apiaceae	0.82 ± 0.26	Yes (Peck 1963)	Yes (Peck 1963)
Battus philenor	Papilionidae, Papilioninae	*Aristolochia macrophyla*	1.16 ± 0.20	No	No
Colias philodice	Pieridae, Pierinae	*Trifolium* spp.	0.24 ± 0.04	Yes (Muesenbeck *et al.* 1951)	No
Pieris rapae	Pieridae, Pierinae	*Brassica oleracea*	0.13 ± 0.02	Yes (Scudder 1889)	No

Table 2. Frequencies of outcomes for each host-parasitoid species combination, separated by trials with one or two wasps.

	Pteromalus cassotis								*Pteromalus puparum*							
	Attempts: 1 wasp trials			Attempts: 2 wasp trials					Attempts: 1 wasp trials			Attempts: 2 wasp trials				
	None	Fail	Success	None	Fail	Success	Total trials	**Host class**	None	Fail	Success	None	Fail	Success	Total trials	**Host class**
Danaus plexippus	19.6%	18.7%	61.7%	7.4%	23.5%	69.1%	1 w: 840 2 w: 136	**Suitable**	55.9%	44.1%	0%	20%	80%	0%	1 w: 152 2 w: 5	**Not Suitable**
Euploea core	8.3%	25%	66.7%	0%	100%	0%	1 w: 12 2 w: 1	**Suitable**	16.7%	83.3%	0%	0%	100%	0%	1 w: 12 2 w: 1	**Not Suitable**
Nymphalis antiopa	100%	0%	0%	61.1%	16.7%	22.2%	1 w: 52 w: 18	**Suitable**	22.2%	33.3%	44.4%	15%	40%	45%	1 w: 9 2 w: 20	**Suitable**
Vanessa cardui	82.4%	5.9%	11.8%	50%	0%	50%	1 w: 17 2 w: 2	**Suitable**	0%	21.4%	78.6%	-	-	-	1 w: 14 2 w: 0	**Suitable**
Vanessa atalanta	100%	0%	0%	-	-	-	1 w: 67 2 w: 0	**N/A**	91.7%	0%	8.3%	-	-	-	1 w: 12 2 w: 0	**Suitable**
Junonia coenia	11.9%	7.1%	81%	0%	0%	100%	1 w: 42 2 w: 8	**Suitable**	6.25%	15.6%	78.1%	0%	25%	75%	1 w: 32 2 w: 8	**Suitable**
Papilio polyxenes	76.2%	23.8%	0%	59.3%	40.7%	0%	1 w: 42 2 w: 27	**Not Suitable**	17.1%	80%	2.9%	0%	96.3%	3.7%	1 w: 35 2 w: 27	**Not Suitable**
Battus philenor	100%	0%	0%	87.5%	12.5%	0%	1 w: 7 2 w: 8	**Not Suitable**	55.5%	44.4%	0%	50%	50%	0%	1 w: 9 2 w: 6	**Not Suitable**
Colias philodice	25.3%	10.1%	64.6%	0%	50%	50%	1 w: 79 2 w: 6	**Suitable**	7.7%	19.2%	73.1%	0%	0%	100%	1 w: 52 2 w: 5	**Suitable**
Pieris rapae	50.9%	10.4%	38.7%	26%	13%	61%	1 w: 951 2 w: 77	**Suitable**	5.9%	14.6%	79.6%	2.1%	12.5%	85.4%	1 w: 1127 2 w: 48	**Suitable**

Table 3. Logistic regression model outputs of the test of the preference-performance hypothesis for *P. cassotis* (A) and *P. puparum* (B).

A. *P. cassotis*

	Estimate	Std. Error	z value	p value
Intercept	-0.77756	0.144534	-5.38	<0.001
Relative performance (0-1)	3.598921	0.246753	14.585	<0.001
Wasp age (days)	-0.06102	0.009548	-6.391	<0.001

B. *P. puparum*

	Estimate	Std. Error	z value	p value
Intercept	0.121507	0.195892	0.62	0.535
Relative performance (0-1)	4.891393	0.344146	14.213	<0.001
Wasp age (days)	-0.00586	0.014537	-0.403	0.687

Table 4. Results of logistic regression of the likelihood of attack based on characteristics of the wasp, performance on a particular host, and relevant interactions between these characteristics. *Pteromalus puparum* is more likely than *P. cassotis* to attack hosts of any quality. *Pteromalus puparum* also demonstrates a more positive relationship between host quality and preference, such that nearly all hosts of moderate or greater quality are accepted by *P. puparum*, while only those hosts of highest quality are very readily accepted by *P. cassotis* (shown by the interaction term).

	Estimate	Std. Error	z value	p value
Intercept	-0.77756	0.144536	-5.38	<0.001
Relative performance (0-1)	3.598921	0.246763	14.585	<0.001
Wasp: P. puparum	0.899063	0.243443	3.693	<0.001
Wasp age (days)	-0.06102	0.009548	-6.391	<0.001
Relative performance * P. puparum	1.292472	0.423472	3.052	0.002273
Wasp age * P. puparum	0.05516	0.017393	3.171	0.001517

Figures

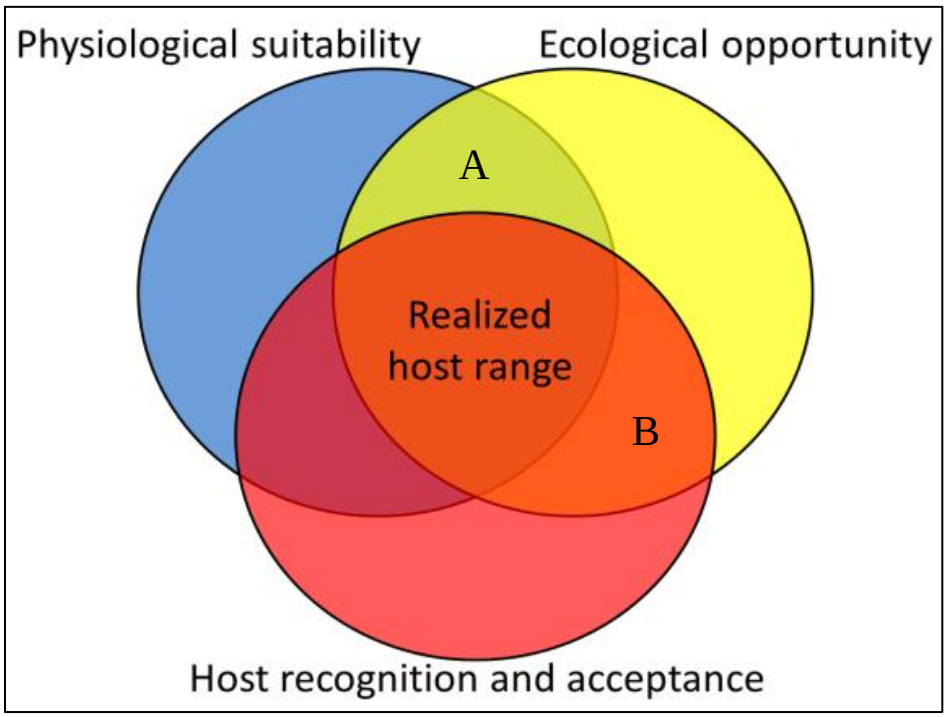


Figure 1. A parasitoid's realized host range includes hosts that are physiologically suitable, recognized and accepted, and overlapping in space and time with the parasitoid. A represents missed opportunities. B represents unsuccessful parasitism.

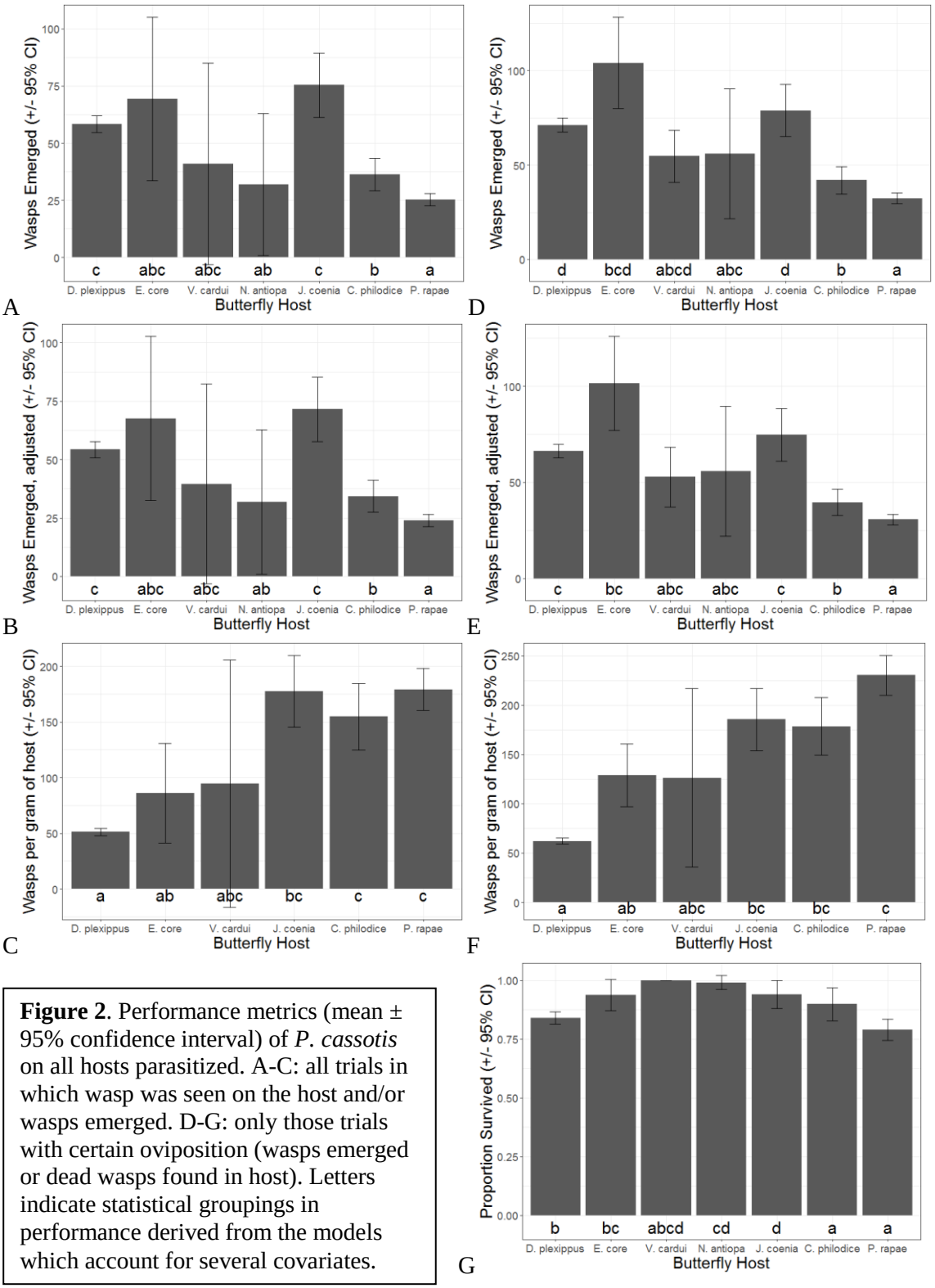


Figure 2. Performance metrics (mean ± 95% confidence interval) of *P. cassotis* on all hosts parasitized. A-C: all trials in which wasp was seen on the host and/or wasps emerged. D-G: only those trials with certain oviposition (wasps emerged or dead wasps found in host). Letters indicate statistical groupings in performance derived from the models which account for several covariates.

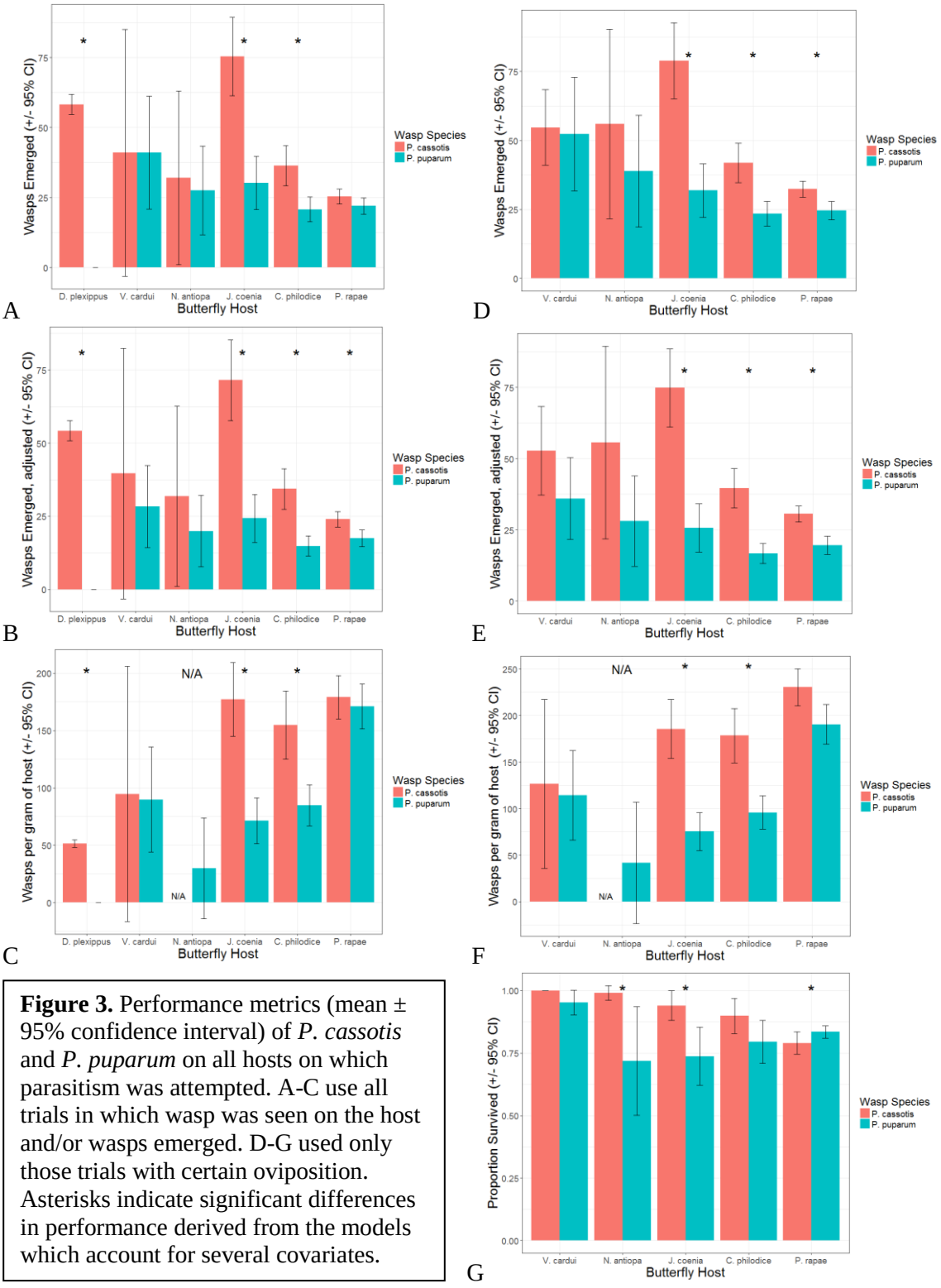


Figure 3. Performance metrics (mean ± 95% confidence interval) of *P. cassotis* and *P. puparum* on all hosts on which parasitism was attempted. A-C use all trials in which wasp was seen on the host and/or wasps emerged. D-G used only those trials with certain oviposition. Asterisks indicate significant differences in performance derived from the models which account for several covariates.

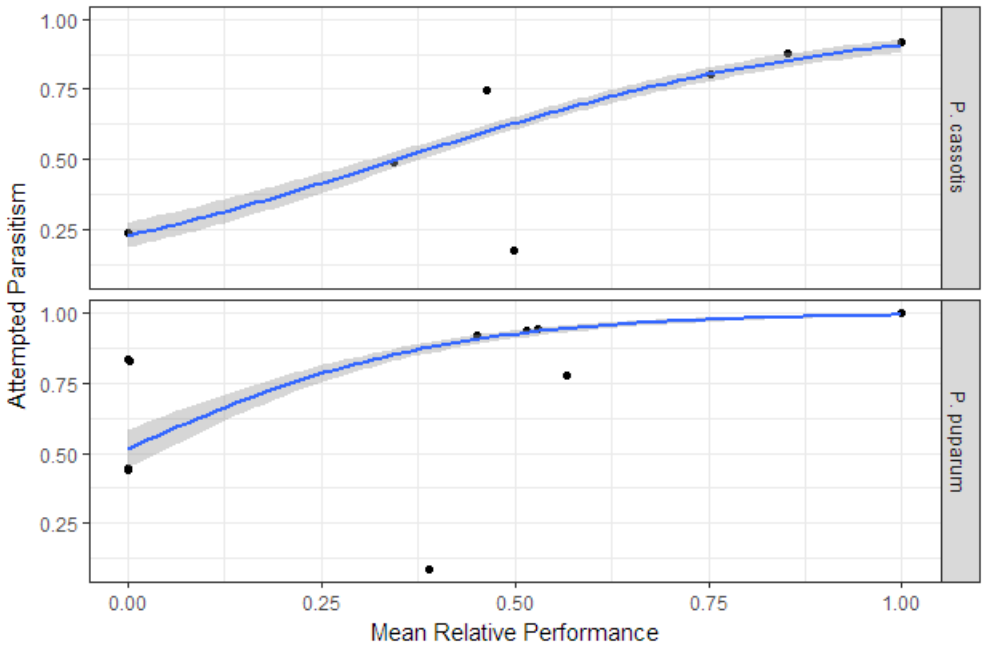
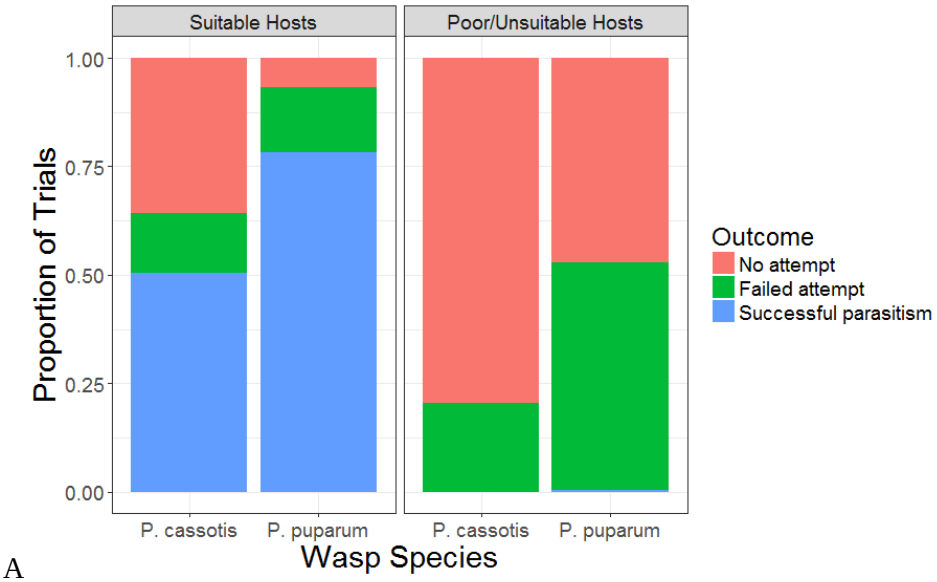
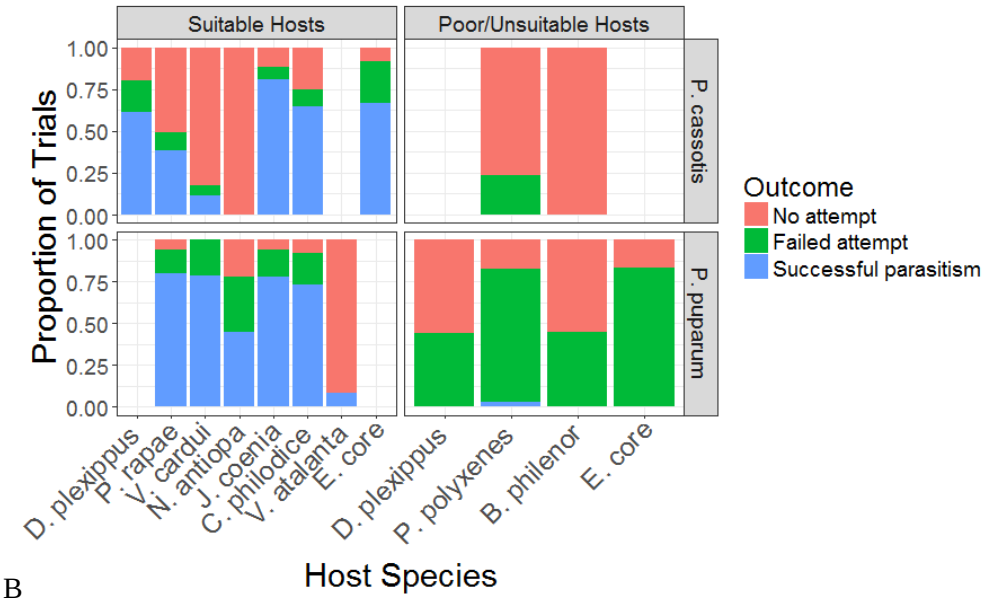


Figure 4. Logistic regression of the likelihood of parasitoid attack based on the relative brood size of a given host species. This single variable representation of the model does not show the negative effect of wasp age for *P. cassotis*. Mean maximum brood sizes (where relative performance =1): *P. cassotis*= 75.64 on *E. core*. *P. puparum* = 41.07 on *V. cardui*.



A



B

Figure 5. Proportional outcomes of trials for both wasp species with A) data from all hosts pooled into suitable and unsuitable categories and B) each host species within those categories. The proportion of trials resulting in attack is significantly greater for *P. puparum* with both suitable and unsuitable hosts.

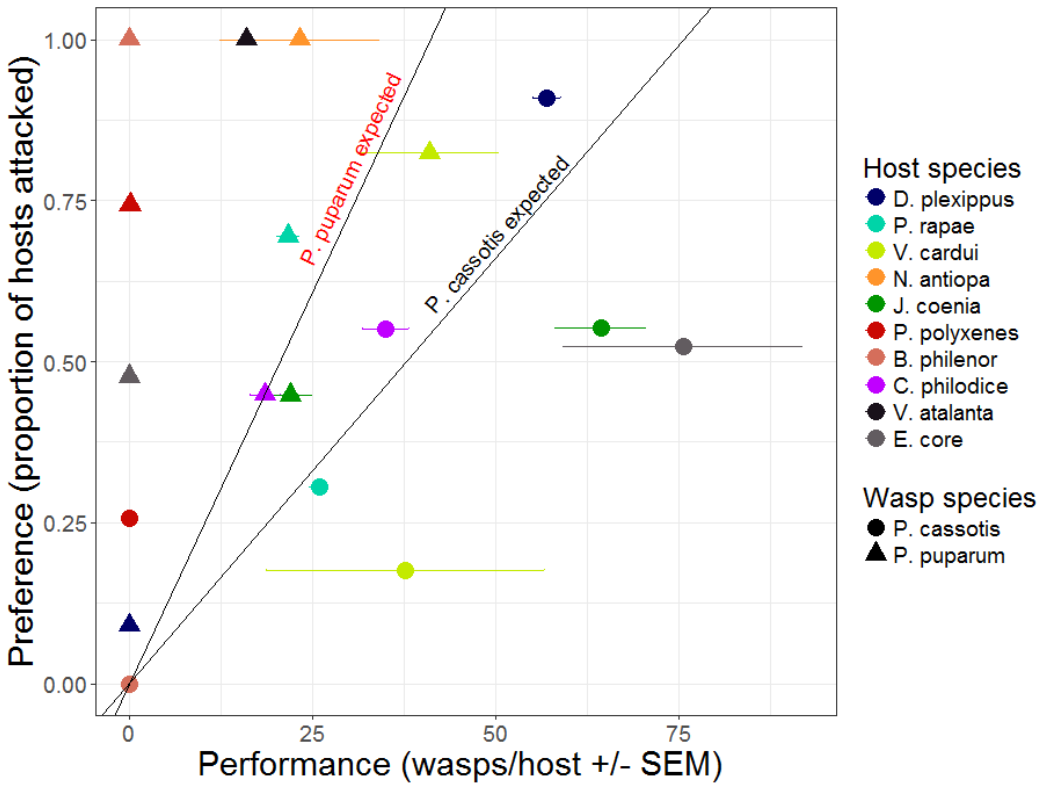


Figure 6. Expected and actual relationships between preference and performance for both parasitoids. Expected relationships are given by Equation 1, which assumes that a parasitoid's best host in the set should be accepted by all females, that unsuitable hosts should be accepted by no females, and that hosts that yield intermediate brood sizes should be accepted proportionally to the brood size attained on the best host. Error bars indicate SEM (incalculable for *P. puparum* with *V. atalanta* due to n=1 parasitized host).

Supplementary Tables

Table S1. Model outputs that correspond to equivalently lettered graphs in Figure 2, intra-specific comparisons of *P. cassotis* performance across hosts. Significant effects are denoted with the following codes: '*'<0.05, '**' <0.01, '***' <0.001.

A.				
Count portion				
(Intercept)	4.39547	0.0778	56.496	<0.0001***
Host: P. rapae	-0.7429	0.04667	-15.918	<0.0001***
Host: V. cardui	-0.2898	0.32483	-0.892	0.372285
Host: N. antiopa	-0.7372	0.28682	-2.57	0.010162*
Host: J. coenia	-0.0694	0.08962	-0.775	0.438418
Host: C. philodice	-0.4767	0.08498	-5.61	<0.0001***
Host: E. core	0.25815	0.19717	1.309	0.190455
Wasp age (days)	-0.0371	0.00409	-9.079	<0.0001***
Number wasps	0.20496	0.05976	3.43	0.000604***
Log(theta)	1.21873	0.05191	23.478	<0.0001***
Zero portion				
(Intercept)	1.75586	0.26323	6.670 2	<0.0001***
Host: P. rapae	-0.3946	0.14976	-2.635	0.00841**
Host: V. cardui	0.23834	1.15881	0.206	0.83704
Host: N. antiopa	-1.0982	0.79446	-1.382	0.16688
Host: J. coenia	1.44214	0.60575	2.381	0.01728*
Host: C. philodice	0.65555	0.34871	1.88	0.06012.
Host: E. core	-0.4851	0.62102	-0.781	0.43476
Wasp age (days)	-0.073	0.01266	-5.765 8	<0.0001***
Number wasps	-0.1353	0.20247	-0.668	0.50403
B.				
Count portion				
(Intercept)	4.37227	0.08157	53.605	<0.0001***
Host: P. rapae	-0.7352	0.04899	-15.008	<0.0001***
Host: V. cardui	-0.2456	0.34047	-0.721	0.47073
Host: N. antiopa	-0.6514	0.30047	-2.168	0.03017*

Host: J. coenia	-0.0533	0.09409	-0.567	0.57075
Host: C. philodice	-0.4619	0.08908	-5.186	<0.0001***
Host: E. core	0.29572	0.20681	1.43	0.15275
Wasp age (days)	-0.0387	0.00426	-9.073	<0.0001***
Number wasps	0.17228	0.06283	2.742	0.00611**
Log(theta)	1.12119	0.05183	21.634	<0.0001***
Zero portion				
(Intercept)	1.75586	0.26323	6.67	<0.0001***
Host: P. rapae	-0.3946	0.14976	-2.635	0.00841**
Host: V. cardui	0.23834	1.15881	0.206	0.83704
Host: N. antiopa	-1.0982	0.79446	-1.382	0.16688
Host: J. coenia	1.44214	0.60575	2.381	0.01728*
Host: C. philodice	0.65555	0.34871	1.88	0.06012.
Host: E. core	-0.4851	0.62102	-0.781	0.43476
Wasp age (days)	-0.073	0.01266	-5.765	<0.0001***
Number wasps	-0.1353	0.20247	-0.668	0.50403
C.				
Count portion				
(Intercept)	4.25341	0.08178	52.008	<0.0001***
Host: P. rapae	1.36043	0.04808	28.293	<0.0001***
Host: V. cardui	0.65195	0.32924	1.98	0.047689*
Host: N. antiopa	0.93335	0.09126	10.227	<0.0001***
Host: J. coenia	1.12026	0.08554	13.097	<0.0001***
Host: C. philodice	0.60782	0.20259	3	0.002697**
Host: E. core	-0.0384	0.00426	-9.013	<0.0001***
Wasp age (days)	0.21239	0.06223	3.413	0.000642***
Number wasps	1.15757	0.05082	22.776	<0.0001***
Zero portion				
(Intercept)	1.82511	0.26826	6.804	<0.0001***
Host: P. rapae	-0.4588	0.15249	-3.008	0.00263**
Host: V. cardui	0.22408	1.15891	0.193	0.84668
Host: N. antiopa	1.42685	0.60597	2.355	0.01854*
Host: J. coenia	0.63343	0.34898	1.815	0.06951.
Host: C. philodice	-0.5054	0.62121	-0.814	0.41592
Host: E. core	-0.0725	0.01296	-5.598 2	<0.0001***

Wasp age (days)	-0.183	0.20446	-0.895	0.37084
Number wasps				
D.				
Count portion				
(Intercept)	4.39547	0.0778	56.496	<0.0001***
Host: P. rapae	-0.7429	0.04667	-15.918	<0.0001***
Host: V. cardui	-0.2898	0.32484	-0.892	0.372356
Host: N. antiopa	-0.7372	0.28681	-2.57	0.010162*
Host: J. coenia	-0.0695	0.08962	-0.775	0.438316
Host: C. philodice	-0.4767	0.08498	-5.61	<0.0001***
Host: E. core	0.25815	0.19717	1.309	0.190456
Wasp age (days)	-0.0371	0.00409	-9.079	<0.0001***
Number wasps	0.20495	0.05976	3.43	0.000604***
Log(theta)	1.21873	0.05191	23.478	<0.0001***
Zero portion				
(Intercept)	2.96672	0.39497	7.511	<0.0001***
Host: P. rapae	-0.4881	0.22483	-2.171	0.029922*
Host: V. cardui	14.614	2281.72	0.006	0.99489
Host: N. antiopa	14.2832	1978.09	0.007	0.994239
Host: J. coenia	1.4781	1.02289	1.445	0.14845
Host: C. philodice	0.78564	0.61278	1.282	0.199812
Host: E. core	14.273	1395.61	0.01	0.99184
Wasp age (days)	-0.0611	0.01849	-3.303	0.000958***
Number wasps	-0.3067	0.2972	-1.032	0.302175
E.				
Count portion				
(Intercept)	4.37227	0.08156	53.605	<0.0001***
Host: P. rapae	-0.7352	0.04899	-15.008	<0.0001***
Host: V. cardui	-0.2456	0.34047	-0.721	0.47073
Host: N. antiopa	-0.6513	0.30047	-2.168	0.03018*
Host: J. coenia	-0.0533	0.09409	-0.567	0.57075
Host: C. philodice	-0.4619	0.08908	-5.186	<0.0001***
Host: E. core	0.29572	0.20681	1.43	0.15275
Wasp age (days)	-0.0387	0.00426	-9.073	<0.0001***

Number wasps	0.17228	0.06283	2.742	0.00611**
Log(theta)	1.1212	0.05183	21.634	<0.0001***
Zero portion				
(Intercept)	2.96672	0.39497	7.511	<0.0001***
Host: P. rapae	-0.4881	0.22483	-2.171	0.029922*
Host: V. cardui	14.614	2281.72	0.006	0.99489
Host: N. antiopa	14.2832	1978.09	0.007	0.994239
Host: J. coenia	1.4781	1.02289	1.445	0.14845
Host: C. philodice	0.78564	0.61278	1.282	0.199812
Host: E. core	14.273	1395.61	0.01	0.99184
Wasp age (days)	-0.0611	0.01849	-3.303	0.000958***
Number wasps	-0.3067	0.2972	-1.032	0.302175
F.				
Count portion				
(Intercept)	4.25341	0.08178	52.008	<0.0001***
Host: P. rapae	1.36042	0.04808	28.293	<0.0001***
Host: V. cardui	0.65196	0.32925	1.98	0.047686*
Host: J. coenia	0.93335	0.09126	10.227	<0.0001***
Host: C. philodice	1.12026	0.08554	13.097	<0.0001***
Host: E. core	0.60782	0.20259	3	0.002697**
Wasp age (days)	-0.0384	0.00426	-9.013	<0.0001***
Number wasps	0.2124	0.06223	3.413	0.000642***
Log(theta)	1.15757	0.05082	22.776	<0.0001***
Zero portion				
(Intercept)	3.0048	0.39783	7.553	<0.0001***
Host: P. rapae	-0.5147	0.22723	-2.265	0.02351*
Host: V. cardui	14.6371	2281.12	0.006	0.99488
Host: J. coenia	1.49077	1.02295	1.457	0.145026
Host: C. philodice	0.80069	0.61285	1.307	0.19138
Host: E. core	14.2784	1395.45	0.01	0.991836
Wasp age (days)	-0.0625	0.0186	-3.357	0.000788***
Number wasps	-0.3416	0.29771	-1.147	0.251189
G.				
(Intercept)	3.57075	0.05392	66.224	<0.0001***

Host: P. rapae	-0.5313	0.03883	-13.684	<0.0001***
Host: V. cardui	13.4551	121.985	0.11	0.9122
Host: N. antiopa	3.13069	1.00294	3.122	0.0018**
Host: J. coenia	1.74894	0.14533	12.034	<0.0001***
Host: C. philodice	-0.4629	0.06273	-7.379	<0.0001***
Host: E. core	0.16312	0.14855	1.098	0.2722
Wasp age (days)	-0.0487	0.00295	-16.476	<0.0001***
Number wasps	-0.6168	0.0366	-16.853	<0.0001***

Table S2. Model outputs that correspond to equivalently lettered graphs in Figure 3, inter-specific comparisons between *P. cassotis* and *P. puparum* performance across hosts. Significant effects are denoted with the following codes: '*'<0.05, '**' <0.01, '***' <0.001.

A.				
Count portion				
(Intercept)	3.310878	0.095326	34.732	<0.0001***
Wasp: P. puparum	-0.25666	0.049355	-5.2	<0.0001***
Host: V. cardui	0.343892	0.34944	0.984	0.32506
Host: N. antiopa	-0.14646	0.314101	-0.466	0.64101
Host: J. coenia	0.639942	0.101901	6.28	<0.0001***
Host: C. philodice	0.19834	0.09659	2.053	0.04003*
Wasp age (days)	-0.0216	0.003657	-5.908	<0.0001***
Number of wasps	0.442878	0.083491	5.305	<0.0001***
P. puparum*V. cardui	0.184482	0.395522	0.466	0.64091
P. puparum*N. antiopa	0.422744	0.347535	1.216	0.22383
P. puparum*J. coenia	-0.48842	0.150954	-3.236	0.00121**
P. puparum*C. philodice	-0.33302	0.137166	-2.428	0.01519*
Log(theta)	1.076678	0.04803	22.417	<0.0001***
Zero portion				
(Intercept)	1.06736	0.30809	3.464	0.000531***
Wasp: P. puparum	0.68183	0.14875	4.584	<0.0001***
Host: V. cardui	0.54061	1.16224	0.465	0.641828
Host: N. antiopa	-0.43938	0.81633	-0.538	0.590407
Host: J. coenia	1.9211	0.61082	3.145	0.00166**
Host: C. philodice	0.92593	0.356	2.601	0.009298**
Wasp age (days)	-0.03261	0.01122	-2.905	0.003675**
Number of wasps	-0.14795	0.26378	-0.561	0.57488
P. puparum*V. cardui	-0.65682	1.33768	-0.491	0.623417
P. puparum*N. antiopa	-0.53204	0.8876	-0.599	0.548897
P. puparum*J. coenia	-1.70036	0.7461	-2.279	0.022667*
P. puparum*C. philodice	-0.79529	0.5096	-1.561	0.118617
B.				
Count portion				
(Intercept)	3.22627	0.10337	31.211	<0.0001***
Wasp: P. puparum	-0.44131	0.0537	-8.219	<0.0001***
Host: V. cardui	0.33749	0.37731	0.894	0.371069
Host: N. antiopa	-0.03668	0.33919	-0.108	0.913886

Host: J. coenia	0.6504	0.11027	5.898	<0.0001***
Host: C. philodice	0.16869	0.10438	1.616	0.106082
Wasp age (days)	-0.0136	0.00401	-3.392	0.000695***
Number of wasps	0.42333	0.09094	4.655	<0.0001***
P. puparum*V. cardui	0.08669	0.42797	0.203	0.839484
P. puparum*N. antiopa	0.24245	0.37595	0.645	0.519003
P. puparum*J. coenia	-0.50058	0.16362	-3.059	0.002218**
P. puparum*C. philodice	-0.42571	0.14932	-2.851	0.004358**
Log(theta)	0.91744	0.04879	18.802	<0.0001***
Zero portion				
(Intercept)	1.06736	0.30809	3.464	0.000531***
Wasp: P. puparum	0.68183	0.14875	4.584	<0.0001***
Host: V. cardui	0.54061	1.16224	0.465	0.641828
Host: N. antiopa	-0.43938	0.81633	-0.538	0.590407
Host: J. coenia	1.9211	0.61082	3.145	0.00166**
Host: C. philodice	0.92593	0.356	2.601	0.009298**
Wasp age (days)	-0.03261	0.01122	-2.905	0.003675**
Number of wasps	-0.14795	0.26378	-0.561	0.57488
P. puparum*V. cardui	-0.65682	1.33768	-0.491	0.623417
P. puparum*N. antiopa	-0.53204	0.8876	-0.599	0.548897
P. puparum*J. coenia	-1.70036	0.7461	-2.279	0.022667*
P. puparum*C. philodice	-0.79529	0.5096	-1.561	0.118617
C.				
Count portion				
(Intercept)	5.3228	0.09751	54.586	<0.0001***
Wasp: P. puparum	-0.17147	0.05024	-3.413	0.000643***
Host: V. cardui	-0.827	0.35162	-2.352	0.018673*
Host: J. coenia	-0.45179	0.10284	-4.393	<0.0001***
Host: C. philodice	-0.30745	0.09664	-3.181	0.001466**
Wasp age (days)	-0.02294	0.00364	-6.303	<0.0001***
Number of wasps	0.40384	0.08575	4.709	<0.0001***
P. puparum*V. cardui	0.08466	0.39781	0.213	0.831466
P. puparum*J. coenia	-0.56096	0.15131	-3.707	0.000209***
P. puparum*C. philodice	-0.46425	0.13585	-3.417	0.000632***
Log(theta)	1.03624	0.04417	23.462	<0.0001***
Zero portion				
(Intercept)	0.97296	0.32516	2.992	0.00277**
Wasp: P. puparum	0.74801	0.15299	4.889	<0.0001***
Host: V. cardui	0.56349	1.16255	0.485	0.62789

Host: J. coenia	1.96359	0.61133	3.212	0.00132**
Host: C. philodice	0.95069	0.35688	2.664	0.00772**
Wasp age (days)	-0.02864	0.01168	-2.452	0.0142*
Number of wasps	-0.1224	0.28237	-0.433	0.66468
P. puparum*V. cardui	-0.70046	1.33817	-0.523	0.60067
P. puparum*J. coenia	-1.78447	0.74644	-2.391	0.01682*
P. puparum*C. philodice	-0.85283	0.51092	-1.669	0.09508.
D.				
Count portion				
(Intercept)	3.31088	0.095326	34.732	<0.0001***
Wasp: P. puparum	-0.25666	0.049355	-5.2	<0.0001***
Host: V. cardui	0.343877	0.349438	0.984	0.32507
Host: N. antiopa	-0.14647	0.3141	-0.466	0.64098
Host: J. coenia	0.639942	0.101901	6.28	<0.0001***
Host: C. philodice	0.198339	0.096591	2.053	0.04003*
Wasp age (days)	-0.0216	0.003657	-5.908	<0.0001***
Number of wasps	0.442878	0.083491	5.305	<0.0001***
P. puparum*V. cardui	0.184503	0.395521	0.466	0.64087
P. puparum*N. antiopa	0.422759	0.347534	1.216	0.22381
P. puparum*J. coenia	-0.48842	0.150955	-3.236	0.00121**
P. puparum*C. philodice	-0.33302	0.137166	-2.428	0.01519*
Log(theta)	1.076677	0.048031	22.417	<0.0001***
Zero portion				
(Intercept)	2.28591	0.4119	5.55	<0.0001***
Wasp: P. puparum	0.34006	0.213	1.596	0.1104
Host: V. cardui	15.04865	2279.027	0.007	0.9947
Host: N. antiopa	15.06165	1978.09	0.008	0.9939
Host: J. coenia	2.0498	1.02917	1.992	0.0464*
Host: C. philodice	1.18517	0.62153	1.907	0.0565.
Wasp age (days)	-0.02641	0.0155	-1.704	0.0884.
Number of wasps	-0.37548	0.34505	-1.088	0.2765
P. puparum*V. cardui	-0.62054	2572.035	0	0.9998
P. puparum*N. antiopa	-15.6015	1978.09	-0.008	0.9937
P. puparum*J. coenia	-2.16958	1.14166	-1.9	0.0574.
P. puparum*C. philodice	-0.79285	0.82244	-0.964	0.335
E.				
Count portion				

(Intercept)	3.22626	0.10337	31.211	<0.0001***
Wasp: P. puparum	-0.44131	0.0537	-8.219	<0.0001***
Host: V. cardui	0.33753	0.37732	0.895	0.371022
Host: N. antiopa	-0.03669	0.33919	-0.108	0.91385
Host: J. coenia	0.65041	0.11027	5.899	<0.0001***
Host: C. philodice	0.16867	0.10438	1.616	0.106114
Wasp age (days)	-0.0136	0.00401	-3.392	0.000695***
Number of wasps	0.42334	0.09094	4.655	<0.0001***
P. puparum*V. cardui	0.08665	0.42798	0.202	0.839552
P. puparum*N. antiopa	0.24246	0.37595	0.645	0.518973
P. puparum*J. coenia	-0.50058	0.16362	-3.059	0.002218**
P. puparum*C. philodice	-0.42569	0.14932	-2.851	0.00436**
Log(theta)	0.91744	0.04879	18.802	<0.0001***
Zero portion				
(Intercept)	2.28591	0.4119	5.55	<0.0001***
Wasp: P. puparum	0.34006	0.213	1.596	0.1104
Host: V. cardui	15.04865	2279.027	0.007	0.9947
Host: N. antiopa	15.06165	1978.09	0.008	0.9939
Host: J. coenia	2.0498	1.02917	1.992	0.0464*
Host: C. philodice	1.18517	0.62153	1.907	0.0565.
Wasp age (days)	-0.02641	0.0155	-1.704	0.0884.
Number of wasps	-0.37548	0.34505	-1.088	0.2765
P. puparum*V. cardui	-0.62054	2572.035	0	0.9998
P. puparum*N. antiopa	-15.6015	1978.09	-0.008	0.9937
P. puparum*J. coenia	-2.16958	1.14166	-1.9	0.0574.
P. puparum*C. philodice	-0.79285	0.82244	-0.964	0.335
F.				
Count portion				
(Intercept)	5.32281	0.09751	54.586	<0.0001***
Wasp: P. puparum	-0.17148	0.05024	-3.413	0.000642***
Host: V. cardui	-0.82699	0.35162	-2.352	0.018674*
Host: J. coenia	-0.45178	0.10284	-4.393	<0.0001***
Host: C. philodice	-0.30744	0.09664	-3.181	0.001467**
Wasp age (days)	-0.02294	0.00364	-6.303	<0.0001***
Number of wasps	0.40384	0.08575	4.709	<0.0001***
P. puparum*V. cardui	0.08464	0.39781	0.213	0.831506
P. puparum*J. coenia	-0.56097	0.15131	-3.707	0.000209***
P. puparum*C. philodice	-0.46426	0.13585	-3.418	0.000632***

Log(theta)	1.03625	0.04417	23.462	<0.0001***
Zero portion				
(Intercept)	2.25595	0.4244	5.316	<0.0001***
Wasp: P. puparum	0.36853	0.21723	1.696	0.0898.
Host: V. cardui	15.0858	2277.363	0.007	0.9947
Host: J. coenia	2.10326	1.03	2.042	0.0412*
Host: C. philodice	1.21022	0.62245	1.944	0.0519.
Wasp age (days)	-0.0213	0.01598	-1.333	0.1825
Number of wasps	-0.41669	0.35801	-1.164	0.2445
P. puparum*V. cardui	-0.63701	2570.659	0	0.9998
P. puparum*J. coenia	-2.21787	1.14231	-1.942	0.0522.
P. puparum*C. philodice	-0.81324	0.82359	-0.987	0.3234
G.				
(Intercept)	2.752857	0.070842	38.859	<0.0001***
Wasp: P. puparum	0.334985	0.043767	7.654	<0.0001***
Host: V. cardui	13.75261	117.8087	0.117	0.9071
Host: N. antiopa	3.927517	1.004489	3.91	<0.0001***
Host: J. coenia	2.343422	0.149433	15.682	<0.0001***
Host: C. philodice	-0.06195	0.070379	-0.88	0.3787
Wasp age (days)	0.001177	0.003612	0.326	0.7445
Number of wasps	-0.63504	0.057904	-10.967	<0.0001***
P. puparum*V. cardui	-13.0327	117.8089	-0.111	0.9119
P. puparum*N. antiopa	-3.09719	1.016166	-3.048	0.0023**
P. puparum*J. coenia	-2.57837	0.172436	-14.953	<0.0001***
P. puparum*C. philodice	-0.5184	0.108146	-4.794	<0.0001***

Chapter 3:
Influence of herbivore-sequestered cardenolides on interactions between milkweed butterflies and parasitoid wasps

Summary

Plant allelochemicals have long been recognized for their roles in plant defense, as a limiting factor in the host range of herbivorous insects, and as a facilitator of ecological and co-evolutionary dynamics within tri-trophic systems of plants, herbivores, and natural enemies. We used no-choice trials to test the preference and performance of two species of parasitoid, an apparent specialist (*Pteromalus cassotis*) and a known generalist (*Ptermalus puparum*), on milkweed butterfly (*Danaus plexippus* and *Euploea core*) pupae containing high, low, or no sequestered cardenolides. These methods allowed us to determine whether variation in the concentration of herbivore-sequestered plant toxins affects host survival and physiological suitability for either parasitoid. We hypothesized that more toxic hosts would experience higher survival and cause poorer performance of both parasitoids, and that these patterns would be stronger for the generalist. We found that monarch survival was higher when attacked by the generalist and that this species was unable to attack monarchs of high or low toxicity. More toxic monarchs were more likely to survive encounters with the specialist, but only because the specialist was less likely to attack them. After attack, neither host survival nor the emergence of parasitoids was affected by host toxicity, but the specialist produced smaller broods and experienced lower survival on more toxic hosts. When attacking a related milkweed butterfly that does not sequester cardenolides into the pupal stage, the specialist was successful, while the generalist was unsuccessful. These findings do not rule out the possibility that sequestered plant toxins may be one mechanism preventing successful parasitism of monarchs by the generalist, but suggest that other mechanisms are also at play. Results are discussed in the context of tri-trophic plant defense, preference-performance relationship of foraging insects, and natural enemies of the monarch butterfly.

Introduction

Novel plant allelochemicals have long been recognized for their roles in plant defense and as a limiting factor in the host range of herbivorous insects (Dethier 1954). However, the complete efficacy of such a novel defensive strategy is often temporary, overcome by the subsequent co-evolution of detoxification or tolerance by one or more lineages of insect herbivores (Ehrlich & Raven 1964). Eventually, these toxins may serve as feeding stimulants for specialist insect herbivores and, in a co-evolutionary twist, many insects have evolved the ability to cope with, sequester, and redeploy plant allelochemicals in their own defense (Blum 1981, Nishida 1995, Nishida 2002; Glendinning 2007, Opitz & Müller 2009). Once insect herbivores evolve the ability to co-opt their hosts' defensive chemistry, the allelochemicals have the potential to facilitate co-evolutionary dynamics between the second and third trophic levels (herbivores and their natural enemies) that are similar to those facilitated between the first and second trophic levels (plants and herbivores) (Price *et al.* 1980).

Herbivore-sequestered plant toxins are often effective in defense against vertebrate predators, but studies of their effects against invertebrate natural enemies have only recently become more common (Duffey 1980, Ode 2006). Plant allelochemicals can directly protect the herbivore from natural enemies if they make it toxic (Greenblatt & Barbosa 1981, Duffey *et al.* 1986), or have indirect effects if they make it less nutritionally valuable (Hare & Luck 1991, Ode *et al.* 2004). Endoparasitoids are expected to be especially vulnerable to sequestered allelochemicals because they spend their entire larval development surrounded by and feeding on host tissues, leading to two related predictions: 1) Because specialists are more likely to have evolved the ability to tolerate the allelochemicals, hosts' chemical defenses more strongly limit generalist than specialist parasitoids. 2) Parasitoids which target specialist, chemically-defended herbivores are often specialists themselves. These predictions are primary components of the *nasty host hypothesis* (Gauld *et al.* 1992, Gauld & Gaston 1994). In support of this idea, the development of a specialist parasitoid wasp, *Cotesia melitaearum*, was unaffected by host plant iridoid glycoside concentrations when reared on its specialist caterpillar host, *Melitaea cinxia*, while more toxic host plants negatively affected the development of two generalist caterpillars and two subsequent generalist parasitoids (Reudler *et al.* 2011). This study, and others (Campbell & Duffey 1979, 1981, Barbosa *et al.* 1991, El-Heneidy *et al.* 1988, Lampert *et al.* 2011), suggest

that generalist parasitoids are often more susceptible to variation in host plant allelochemistry than specialists.

Monarch larvae sequester cardenolides from milkweed host plants (*Asclepias* spp.) and retain these chemicals as adults (Parsons 1965, Rothschild *et al.* 1966, Brower 1984, Malcolm 1995, Agrawal *et al.* 2012). Cardenolides are bitter tasting steroids that inhibit neural and cardiac ion channels in most animals, though monarchs and several other specialist insect herbivores of milkweeds demonstrate insensitivity to these chemicals due to convergent evolution resulting from point mutations in the alpha subunit of the sodium-potassium ATPase (Holzinger & Wink 1996, Dobler *et al.* 2011, 2015). Cardenolide concentrations within monarchs increase monotonically with host plant concentrations, but have relatively high sequestration even from low cardenolide plants (Malcolm 1995). Toxicity of monarchs to bird predators was first documented using captive blue jays (Brower *et al.* 1967). Subsequent studies showed that intra-specific variation in sequestered plant toxins leads to a "palatability spectrum"; adult monarchs containing fewer cardenolides are more readily consumed by blue jays and pigeons, and greater quantities of the butterflies must be consumed in order to induce vomiting (Brower *et al.* 1968, Roeske *et al.* 1976, Dixon *et al.* 1978). The effects of variation in monarch toxicity has since been examined for mice and other species of birds (Brower & Fink 1985, Glendinning 1993).

Many generalist insect predators including ants, soldier bugs, and spiders consume monarch eggs, larvae, and pupae (Zalucki & Kitching 1982, Prysby 2004, Oberhauser *et al.* 2015), but the frequency of these interactions and potentially latent toxic effects of cardenolides are unknown. In fact, the effectiveness of sequestered cardenolides has been studied for only a few invertebrate enemies of monarchs. Rayor (2004) found that foraging *Polistes dominulus* wasps generally preferred monarch larvae raised on lower toxicity milkweeds, although the relationship was not as consistent as predicted. Chinese mantids, *Tenedora sinensis*, remove the gut contents of monarch larvae before consuming the body, a behavior that is not performed with non-toxic Lepidoptera (Rafter *et al.* 2013). While gut contents differed in the type of cardenolides present, the overall concentration of cardenolides in the discarded gut contents was similar to that of the monarch body (Rafter *et al.* 2013). A follow-up study found that mantids suffered no apparent acute or long-term consequences of consuming monarch larvae (Rafter *et al.* 2017). In fact, mantids that fed on the greatest biomass of monarch larvae showed an increase in

reproductive condition, which the authors hypothesized was due to nutritional advantages of a relatively mixed diet (Rafter *et al.* 2017).

Parasitoids are probably the most important source of mortality for herbivorous insects (Godfray 1994, Hawkins *et al.* 1997). At least seven species of tachinid flies have been reared from wild-collected monarch larvae, though *Lespesia archippivora* is the most common and thoroughly studied monarch parasitoid (Oberhauser *et al.* 2017). Still, little is known about the influence of cardenolides in tachinid-monarch interactions. In general, it seems that milkweed species and relative cardenolide concentrations influence host and parasitoid success, though not always as expected (Prysby 2004). Hunter *et al.* (1996) showed that the cardenolide content of monarch host plants did not influence the likelihood of parasitism, but the number of tachinid adults per host decreased with cardenolide concentration, suggesting that cardenolides may influence the survivorship of parasitoid larvae. Oberhauser *et al.* (2015) demonstrated that penetration of a host does not always result in successful parasitism or host death and that larvae reared on the most toxic host plant species were marginally more likely to survive infection by *L. archippivora* than larvae reared on less toxic host plants.

Pteromalus cassotis (Hymenoptera, Walker 1847) has long been known recognized as a parasitoid of monarchs, and eight other butterfly species have also been recorded as hosts, mostly Nymphalids (Gillette 1888, Muesenbeck *et al.* 1951, Peck 1963, Burks 1975, Burks 1979, CAS Entomology Collection Database 2015, Noyes 2017). To date, only a few studies on interactions between *P. cassotis* and monarchs have been published. Stenoien *et al.* (2015) described rates of monarch parasitism and brood characteristics (number of offspring, sex ratio, and apparent survival) for multiple locations in the U.S. Field experiments using monarchs reared in a greenhouse on either *A. syriaca* (low cardenolides) or *A. curassavica* (high cardenolides) showed that monarchs reared on lower toxicity host plants were more likely to be parasitized (87% vs 60%), but this difference could have resulted from greater preference for, survival on, or both for lower cardenolide hosts (Oberhauser *et al.* 2015).

Here, we present research exploring host-parasitoid interactions between two closely related parasitoids of butterfly pupae, *Pteromalus cassotis* and *P. puparum* (Hymenoptera: Chalcidoidae), and two danaid butterflies, *D. plexippus* and *Euploea core. Pteromalus puparum* was once released around the world as a biocontrol agent of the crop pests *Pieris rapae* and *P. brassicae* (Moss 1933, Lasota & Kok 1986, Barron *et al.* 2003, Benson *et al.* 2003). In addition to

pierids, *P. puparum* is reported to use 48 lepidopteran hosts, many of which are nymphalid butterflies, and is thus no longer employed for biological control (Muesenbeck *et al.* 1951, Peck 1963, Noyes 2017). Ramsay (1964) observed unsuccessful attacks of monarchs by *P. puparum* in the field in New Zealand, resulting in host death and failure of the wasps to develop, though the host plant and number of observed attempted parasitism attempts is not clear. Based on field data suggestive of linked population dynamics (Stenoien *et al.* 2015), and because toxic, specialist herbivores are hypothesized to primarily be exploited by specialist natural enemies (Bernays & Graham 1988, Gauld *et al.* 1992, Gauld & Gaston 1994, Stireman & Singer 2003), it is likely that *P. cassotis* is a specialist on monarchs, and, perhaps, related species.

Because *D. plexippus* sequesters cardenolides in concentrations that reflect the concentrations in host plants, we reared them on two species of milkweeds (genus *Asclepias*) to be relatively high or low in overall cardenolide concentration. *Euploea core* is a danaid found in South Asia and Australia. Like monarchs, *E. core* larvae sequester cardenolides from host plants (Apocynaceae), but unlike monarchs, do not retain the cardenolides into the pupal or adult stages due to an impermeable midgut epithelium (Malcolm & Rothschild 1983, Petschenka & Agrawal 2015). Before this study, it was unknown whether either *P. cassotis* or *P. puparum* can develop in *E. core* hosts.

We compared the influence of monarch cardenolide sequestration levels on host survival, parasitoid foraging behaviors, and parasitoid performance using both wasp species. We hypothesized that 1) greater concentrations of herbivore-sequestered plant toxins would increase butterfly survival against both species of parasitic wasp, and 2) parasitism of more toxic hosts would result in decreased parasitoid performance, measured by the frequency of successful parasitism, total brood size, female brood size, survival to emergence, adult lifespan, and fecundity in the next generation. After finding that *P. puparum* was incapable of parasitizing monarchs regardless of host plant, we also tested whether both parasitoids are capable of parasitizing *E. core*, a related danaid that does not sequester cardenolides into the pupal stage. We expected the performance of *P. cassotis* to be similar between monarch and *E. core* hosts and hypothesized that if cardenolides are the sole mechanism preventing *P. puparum* from succeeding in monarch butterflies, then *P. puparum* would be able to reproduce in *E. core* hosts.

Methods

Butterflies

Monarch colonies were established each summer from wild-caught butterflies in Minnesota and maintained in mesh cages in a greenhouse. Matings were controlled to avoid inbreeding. *Euploea core* were shipped as pupae from Australia and maintained for one generation in growth chambers (per specifications of USDA APHIS permit # P526-160112-040). We allowed monarch butterflies to oviposit on host plants, then randomly assigned eggs to feed on one of two species of milkweed. All butterfly larvae were reared at densities of 1-15 larvae per potted host plant, with reduced density as the larvae matured. Midway through the fifth stadium, we transferred larvae to clear 16-ounce plastic deli containers with perforated lids, moved them into the lab, and fed them fresh clippings of the same host plant species. Most pupae tested were first generation offspring from wild-collected butterflies, although for *D. plexippus*, we sometimes used 2nd and 3rd generation offspring. Day length was approximately 16 hours and temperatures ranged from 18-32^{o}C in the greenhouse, 20-24^{o}C in the lab, and 18-25^{o}C in the growth chambers.

Plants

Host plants were grown from seed in a greenhouse, and fertilized biweekly. Monarch larvae were fed either *Asclepias incarnata* or *A. curassavica*, low and high cardenolide milkweeds (mean cardenolide value for *A. incarnata* =14, *A. curassavica* = 1055 μg/0.1 g dry weight of leaf tissue [Malcolm 1990].) *Asclepias incarnata* seeds were collected from naturally occurring plants in Minnesota, and *A. curassavica* seeds purchased from OutsidePride.com, LLC. These host plants are morphologically similar, yet occur on opposite ends of the milkweed cardenolide concentration spectrum. Petschenka and Agrawal (2015) found that, of eight milkweeds tested, monarchs reared on *A. incarnata* had in the lowest concentration of hemolymph cardenolides and those reared on *A. curassavica* had the highest concentration (<0.01 and >0.21 μg μl^{-1}, respectively). In this same study, *E. core* did not have detectable levels of hemolymph cardenolides when reared on any host plants. We chose to rear *E. core* on *Nerium oleander* because it is known to develop on this host in the wild; *N. oleander* seeds were purchased from a grower in Florida via Ebay.com.

Parasitoids

Both parasitoid species were collected from field-infected hosts and used to establish laboratory populations. The *P. puparum* colony was started from *Pieris rapae* pupae placed in the field in Roseville, MN during September 2013. The *P. cassotis* colony consisted of wasps from *D. plexippus* hosts that were collected in four U.S. states: Oklahoma (October 2013), Georgia (January 2014), Minnesota (August 2015 and September 2016), and Florida (July 2016). All experiments were conducted between September 2013 and December 2016.

Pupal cardenolide measurements

To verify whether the cardenolide content of monarch pupae varied as expected based on their larval host plant, we randomly chose four monarch pupae reared on each host plant and sacrificed them for measurement of total cardenolide content. These pupae were freeze dried (FreeZone Cascade Benchtop Freeze Dry System; Labconco Corp.), and the cardenolides extracted. Cardenolides from each sample were run through high-performance liquid chromatography (HPLC) at Western Michigan University, using digitoxin and *Calotropis procera* extracts as internal standards. Cardenolide concentration in the butterfly tissue from both treatments was determined by summing all peaks from HPLC output (Rasmann *et al.* 2009). Peaks were considered cardenolides if they had a symmetrical absorbance maximum detected between approximately 207 and 222nm (Malcolm and Zalucki 1996). We did not measure the cardenolide content of *E. core* hosts because several previous studies have detected no sequestered cardenolides in this species (e.g. Malcolm and Rothschild 1983, Petschenka & Agrawal 2015).

Experimental protocols

After removing frass and unconsumed plant matter, trials were conducted in the same containers in which the larvae were fed during their final stadium. Pupae were exposed to one naïve wasp within 24 hours of pupal ecdysis (mean days since ecdysis=0.27, sd=0.41). Wasps were 1-25 days old (mean=7.6, sd=5.43) and provided with a ~1cm^3 sponge soaked in 20% honey water. All trials lasted between 1.8 and 4.2 days (mean=2.67, sd=0.41). In all cases, the natal host of *P. puparum* wasps was *P. rapae*, and in most cases, the natal host of *P. cassotis* wasps was *D. plexippus* (92%, plus 6.2% from *P. rapae* and 1.8% from *Colias philodice*). In total, 497

monarchs were exposed to *P. cassotis* (249 fed *A. incarnata*, 248 fed *A. curassavica*), 161 monarchs were exposed to *P. puparum* (88 fed *A. incarnata*, 73 fed *A. curassavica*), and 188 monarchs were controls to determine background rates of pupal mortality (96 fed each host plant). *Euploea core* pupae were exposed in 21 trials, 11 with *P. cassotis* and 10 with *P. puparum* wasps.

During all trials, at least three observations (but typically twice daily, mid-morning and again in the late afternoon, for a total of 5-6 observations per trial) were made to determine whether the wasp was standing on the host (observations of a wasp crawling onto and then off the host within <~30 seconds were considered 'off' during that observation). Standing on a host was typically associated with an inserted ovipositor, though this was not always possible to confirm due to the wasp's position and depth of insertion. Parasitoid handling time was usually 12-24 hours and observations of wasps on hosts correlate well with oviposition in typical hosts (CS, unpublished), so we considered any wasp viewed in contact with a host to have attempted oviposition.

Upon removal of the wasp, most pupae (91%) were weighed, then reattached to the container to allow successful eclosion. Chrysalides were reattached by tying string around the cremaster and taping the string to the inside of the lid. Those that were not massed were not removed or reattached. We recorded the date of emergence, as well as the number and sex of all emerged wasps. If neither host nor parasites had emerged after 30 days, hosts were dissected to determine the cause of death. Host fate was recorded as eclosed, successfully parasitized, unsuccessfully parasitized, or died due to ambiguous cause. Hosts from which any wasps emerged were also dissected to determine the proportion of parasitoids that died as visible larvae, pupae, or adults (including sex) inside of the host. Therefore, when reporting the mean proportion of emerged wasps per brood, all hosts known to be parasitized are included, regardless of whether any wasps emerged successfully. A limitation of this procedure is that we could not account for wasp eggs or larvae that did not develop to a stage visible under a dissecting microscope. Eggs and small larvae may have disintegrated by the time of dissection, but later instar larvae, pupae, and adult wasps inside hosts are easily distinguished. Thus, we cannot know with certainty the total number of eggs oviposited into a host. Similarly, hosts that died for unknown reasons may have contained imperceptible eggs or larvae. We are missing brood size, sex, and survival data

for 41 of the 497 *P. cassotis-D. plexippus* trials which resulted in successful parasitism (26 fed *A. incarnata*, 15 fed *A. curassavica*).

The experimental wasps' outcome in each trial was scored as either successful, unsuccessful, or no attempt. Trials in which wasps successfully emerged were scored as 'successful', regardless of whether the wasp was observed on the host. 'Unsuccessful' outcomes were made up of trials in which the maternal wasp(s) was seen ovipositing, but the host did not produce viable wasps, plus trials in which all wasps were found dead inside of the host. Trials for which we never saw the wasp in contact with the host and that resulted in an emerged butterfly or pupa that died due to an unknown cause were scored as 'no attempt.'

To measure the effect of larval diet on the fate of monarch hosts, we compared the relative frequency of successful eclosion versus death in all trials, separately for each parasitoid. To test whether differences in butterfly survival across host plants were a result of parasitoid foraging behaviors, we compared the relative frequency of attempted parasitism for each parasitoid. To avoid confounding preference with performance, we then looked only at the subset of trials in which parasitism was attempted. These trials allowed us to test whether larval diet affected survival to adulthood via physiological processes alone.

For all remaining analyses described below, we used only those trials in which parasitism was attempted. To address whether hosts are more likely to survive parasitism attempted by the generalist than specialist, we compared the relative frequency of butterfly success. To test whether the physiological performance of either parasitoid depends on monarchs' larval diet, we compared the relative frequency of successful parasitism on hosts fed each host plant species. We did not differentiate between failed attempts that resulted in wasp death, host death without evidence of wasps inside, or butterfly emergence.

Pteromalus puparum never successfully parasitized *D. plexippus*. For *P. cassotis*, however, we further compared total brood size, female brood size, survival to adulthood, and development time based on host's diet. We also measured whether the host's diet had indirect effects on emerging parasitoids' lifespan and fecundity. The indirect effect of host type was compared by including only those trials in which the parent was the offspring from an earlier trial (this subset included 148 of the 367 *P. cassotis*-monarch trials in which parasitism was attempted). This allowed us to test for the latent effect of the diet of the host from which the parent emerged while controlling for the effect of the diet of the host which it would later attack.

Finally, we compared the lifespan of *P. cassotis* based on host diet. We randomly chose 20 females from two *A. incarnata*-fed hosts and two *A. curassavica*-fed hosts that had all emerged at known times on the same day. Each wasp was maintained in a 40-dram polystyrene tube with no food or water in a growth chamber (LD 12:12, 18^{o}C), and location within the chamber was randomized daily. We checked the survival of each wasp in the morning and evening (at approximately 7 AM and 7 PM). All those that had died overnight were ascribed a time of death of 2 AM and those that died during the day were ascribed a time of death of 2 PM.

Monarchs are highly efficient at sequestering cardenolides from low-toxicity plants, making it nearly impossible to rear monarchs that are devoid of cardenolides on any species of milkweed (Brower *et al.* 1967, Malcolm 1995). To determine if the mere presence, rather than relative concentration, of cardenolides prevents *P. puparum* from successfully parasitizing monarch hosts, we exposed *E. core* to *P. puparum*. *Euploea core hosts* were also exposed to *P. cassotis* as a control, with the expectation that *P. cassotis* would perform similarly on *E. core* and monarch hosts. To maximize the difference in treatments, we compared the performance of *P. cassotis* on *E. core* to its performance on *A. curassavica*-fed monarch hosts.

Statistical analyses

The concentrations of monarch's sequestered cardenolides between diet treatments were compared using a Wilcoxon Rank Sum Test. The relative frequencies of butterfly success, wasp success, and attempted parasitism were compared using Fisher Exact Tests, with Bonferonni adjusted alpha levels whenever multiple tests were performed on a given contingency table. Brood size measures were modelled using hurdle models implemented via the pscl package (Jackson 2015) in R (R Core Team, version 3.3.3). These models used a binomial distribution for the zero *vs.* positive portion of the model and a negative binomial distribution for the count portion of the model. Survival to adulthood was modelled using binomial distributions with a logit link function. Finally, a linear model was applied to the lifespan data with host plant as a fixed effect and brood identity as a random effect. All models included the pupa's host plant, mass, time since pupal ecdysis, and the wasp's age as predictors. The model of survival to adulthood included the total number of emerged and detectable unemerged wasps as a covariate, in case maternal preferences resulted in differences in the number of offspring invested in a host, which could, in turn, affect the success of the brood due to Allee effects or competition. The

model of developmental time also included the number of wasps in the brood, as high densities within hosts increase rates of development in these species (Stenoien, unpublished). Finally, the model of latent effects of host type on fecundity included the parental wasp's host's diet as an interaction term with the focal host's diet. We used Tukey-adjusted pairwise comparisons of least square means via the lsmeans package (Lenth 2016) to determine whether differences between host plant treatments were significant.

Results

Pupal cardenolides

Cardenolide concentrations were significantly greater in pupae reared on *A. curassavica* (range = 3.73-11.33 µg per 0.1g of host mass) than those reared on *A. incarnata* (range = 0.22-1.35 µg per 0.1g of host mass) (W=16, p = 0.029). The concentration in pupae reared on *A. curassavica* was nearly an order of magnitude greater than those reared on *A. incarnata*, and indicates that the host plant treatment resulted in two distinct levels of cardenolides sequestered into the pupal stage.

Are monarchs that contain more sequestered cardenolides more likely to survive encounters with parasitoids?

When considering all trials, regardless of wasp behavior, monarchs fed *A. curassavica* were more likely than those fed *A. incarnata* to survive encounters with *P. cassotis* (Figure 1, Fisher Exact Test p = 0.0047, odds ratio = 0.533). Monarchs' sequestered cardenolides had no effect on survival when exposed to *P. puparum* (Figure 1, Fisher Exact Test p = 0.86).

Is the likelihood of parasitoid oviposition affected by sequestered cardenolide concentration?

Pteromalus cassotis females were more likely to attempt parasitism of monarchs that had fed on *A. incarnata* than monarchs that had fed on *A. curassavica* (Figure 2, Fisher Exact Test, p = 0.0093, odds ratio = 1.83). Monarch host plant had no effect on the likelihood of attack by *P. puparum* (Figure 2, Fisher Exact Test, p = 0.63).

When parasitism is attempted, are monarchs that contain more sequestered cardenolides more likely to survive attacks by either parasitoid?

When considering only those trials in which parasitism was attempted, regardless of host plant, monarchs were more likely to survive attacks by *P. puparum* than *P. cassotis* (Figure 3, for both host plants $p < 0.001$, odds ratio > 15). Contrary to expectations, monarch host plant had no effect on the likelihood of host survival when facing either parasitoid (Figure 3, Fisher Exact Tests, *P. cassotis* $p = 0.53$, *P. puparum* $p = 0.34$). Attempted parasitism by the generalist, although it never resulted in successful parasitism, still resulted in monarch mortality; monarchs attacked by *P. puparum* survived only ~ 60% of trials (*vs*. ~ 97% survival for control monarchs).

Does the performance of either wasp depend on monarchs' larval diet?

P. puparum never successfully parasitized monarch hosts reared on either host plant, nor developed to a larval stage discernable via dissection (Figure 3).

Host diet had no effect on the overall success rate of attempted parasitism by *P. cassotis* wasps (Figure 3, Fisher Exact Test $p = 0.468$). However, *P. cassotis* performed better in hosts reared on *A. incarnata* than on *A. curassavica* as measured by several brood-level and offspring performance metrics. In trials where parasitoids attacked the host, broods reared from *A. incarnata*-fed hosts were comprised of significantly more total offspring (Figure 4A, Table 1A). Interestingly, sex ratios were slightly more female-biased in *A. curassavica*-fed hosts (mean±SE = 0.85±0.02 for *A. curassavica vs* 0.83±0.02 for *A. incarnata*), but *A. incarnata*-fed hosts still produced significantly more female offspring, on average (Figure 4B, Table 1B).

Brood size metrics derive from both preference- and performance-related processes (maternal investment decisions and survival, respectively). Therefore, to control for maternal preferences in our survival model, we included the total number of emerged and dead wasps as an estimate of total maternal investment. We found that *P. cassotis* invests more offspring, on average, in *A. incarnata*-fed hosts (mean±SE = 60.9±3.47 for *A. curassavica vs* 72.1±3.55 for *A. incarnata*). We also found an apparent Allee effect; larger broods of *P. cassotis* experienced higher survival, on average, than smaller broods (Courchamp *et al.* 2008). Even after controlling for these effects, broods reared from *A. incarnata*-fed hosts had significantly higher survival than broods reared from *A. curassavica*-fed hosts (Figure 4C, Table 1C).

Host type did not appear to influence development time (Figure 4D, Table 1D). Regarding potential latent effects of host plant on wasp performance, there was no detectable effect of host diet on adult lifespan (Figure 4E, Table 1E). There was, however, an effect of the mother's developmental environment; females reared from *A. incarnata*-fed hosts produced significantly larger broods than females reared from *A. curassavica*-fed hosts (Figure 4F, Table 1F).

There were strong and consistent effects of the covariates between models. In general, younger wasps, more recently eclosed pupae, and larger pupae were predictive of increased wasp performance (Table 1). Also, most of the significant effects in the hurdle models were detected in the count portion, rather than the binomial portion, of the model, suggesting further that wasp success as a binary variable is less affected by host diet than the continuous measures of wasp success (Table 1A,B,C,F).

Does cardenolide presence prevent P. puparum from successfully parasitizing monarchs?

In all ten trials in which *P. puparum* attempted parasitism of *E. core* hosts, they were unsuccessful in producing offspring or developing to a larval stage discernable upon dissection (Figure 5). Therefore, the performance of *P. puparum* on *E. core* was indistinguishable from its performance on monarch hosts.

To test the most contrasting concentrations of cardenolides, we compared *P. cassotis* performance on *E. core* with that on monarchs reared on *A. curassavica* only. *A.* curassavica-reared monarchs and *E. core* are approximately equivalent hosts, although our small sample of *E. core* hosts limits inferences. The likelihood of successfully attempted parasitism was similar between hosts (8/11 on *E. core*, compared to 394/518 on *A. curassavica*-reared monarchs, Fisher Exact Test, $p = 0.73$). *Euploea core* pupae are smaller than monarchs (mean±SD in grams = 0.81±0.09 and 1.13±0.21, respectively). *Pteromalus cassotis*' mean brood size from *E. core* hosts was smaller overall (mean±SE = 39.6±11.9; 48.7±2.1), and the number of offspring per gram of host was slightly larger for *E. core* than monarch hosts (mean±SE = 49.1±14.8; 43.4±1.9), though neither of these differences were statistically significant. A binomial logistic regression indicated that survival of *P. cassotis* to adulthood also did not differ between *E. core* and monarch hosts or depend on host size ($p=0.886$).

Discussion

Host ranges of herbivorous insects and parasitoids are limited by physiological and morphological defenses of hosts, including defensive chemistry (Ehrlich & Raven 1964, Godfray 1994, Ode 2006, Gauld & Gaston 1994). Monarchs provide a model system for studying the role of plant allelochemicals in tri-trophic interactions, yet little is known about the role of sequestered cardenolides in interactions with invertebrate natural enemies. Here, we provide the first investigation of the defensive function of milkweed butterflies' sequestered cardenolides when experimentally exposed to two species of parasitic wasps.

We first verified that monarchs reared on a more cardenolide-rich milkweed, *A. curassavica*, sequestered greater concentrations of cardenolides into the pupal stage than monarchs reared on a less cardenolide-rich milkweed, *A. incarnata*. Our key findings were: 1) Monarchs were unsuitable hosts for the generalist, *P. puparum*, and were more likely to survive parasitism attempted by the generalist than the specialist parasitoid. 2) Overall, monarchs reared on *A. curassavica* were more likely to survive encounters with the specialist, *P. cassotis*, but only because *P. cassotis* wasps were less likely to attempt parasitism on the *A. curassavica*-fed hosts. 3) When parasitism was attempted, neither butterfly survival nor parasitoid success was influenced by host diet. 4) Although parasitoid success (a binary metric) did not depend on host diet, *P. cassotis* brood size, survival, and brood size in the next generation were all negatively affected when attacking *A. curassavica*-fed hosts. 5) Lastly, *P. puparum* was unable to parasitize *E. core*, even though *E. core* lacks sequestered cardenolides, while *P. cassotis* performed similarly in this host as in *A. curassavica*-fed monarchs.

A growing body of evidence shows that herbivore-sequestered plant toxins can negatively affect the performance of both specialist and generalist parasitoids (Barbosa *et al.* 1986, Ode 2006, Harvey *et al.* 2007, Gols & Harvey 2009) When *P. cassotis* attempted parasitism, the likelihood of successful parasitism did not depend on host diet, but attacks of hosts reared on a less cardenolide-rich host plant resulted in approximately 15% more offspring, 5% higher survival to adulthood, and 15% larger broods in the next generation. Because these hosts were reared on live plants, they represent ecologically relevant ranges of cardenolide concentrations, although further investigation of the effects of higher concentrations of foliar cardenolides could utilize less-fertilized *A. curassavica* plants (Couture *et al.*, 2010, Agrawal *et al.* 2012), or milkweeds containing even higher levels of cardenolides, such as *A. masonii*, *A.*

albicans, or A. labriformis (Malcolm 1990). Finally, milkweeds and danaid butterflies vary not just in their concentrations, but also in the types of cardenolides present. Cardenolides vary in their sidechains and polarity, and these characteristics could also influence the outcome of host-parasitoid interactions (Agrawal *et al.* 2012).

The performance of *P. puparum* is affected by glucosinolates sequestered by *Pieris brassicae* reared on various *Brassica oleracae* cultivars, although no *B. oleracae* diet rendered hosts completely unsuitable (Harvey *et al.* 2011). While we cannot know for certain whether cardenolides are the sole mechanism that prevents *P. puparum* from developing in monarch hosts, several manipulations, including simultaneous exposure to up to five female wasps and simultaneous multiparasitism with *P. cassotis*, all resulted in failed parasitism by *P. puparum*, indicating that monarchs truly are unsuitable hosts for this parasitoid (Stenoien, unpublished). If the presence of sequestered cardenolides was the sole mechanism that prevented this generalist from developing in monarch hosts, then *P. puparum* should have been able to develop in *E. core* hosts, but this was not the case. Potential explanations for the generalist's inability to develop in *E. core* hosts include: 1) Monarchs and *E. core*, which are in the same subfamily, might have similar immunological defenses which *P. puparum* cannot overcome. 2) Although *E. core* pupae do not contain cardenolides, they do contain unidentified, endogenously produced cardioactive compounds, which may provide a defensive function against *P. puparum* (Rothschild *et al.* 1978, Malcolm & Rothschild 1983). Monarch pupae also contain these endogenously produced cardioactive compounds (Malcolm & Rothschild 1983), which could be the sole mechanism, or a barrier in addition to cardenolides, preventing parasitism of *D. plexippus* and *E. core* by *P. puparum*.

Herbivores could benefit either directly or indirectly from sequestering plant secondary compounds. We did not find evidence of a direct benefit of herbivore-sequestered plant toxins; monarch survival did not depend on host diet when attacked by either parasitoid. However, indirect benefits of sequestered plant toxins could include influences on natural enemy foraging behavior, if, for example, the toxins affect host detectability or perceived quality. Thus, parasitoids may discriminate against hosts based on the host plant upon which the host fed, leading to differences in host survival that are independent of differences in the physiological suitability of a host due to host plant. We found evidence of such an indirect benefit of increased host toxicity in interactions between monarchs and *P. cassotis*, and this benefit was mediated by host plant-dependent parasitoid foraging behavior. Because sequestered toxins influence the mean

survival and brood size, *P. cassotis* may have evolved to discriminate against more toxic hosts. An alternative explanation is that these parasitoids might rarely encounter hosts reared on *A. curassavica*, which is not native to the U.S., or *A. curassavica* grown under our experimental conditions, and that the parasitoids were less likely to recognize and accept these hosts. Both explanations suggest that the parasitoids are capable of discriminating between hosts, with a preference for the host type that yields the highest performance. An association between parasitoid foraging preference and offspring performance has also been detected in these and other parasitoids (Stenoien Chapter 2, Desneaux *et al.* 2009), although for some parasitoids, there seems to be no relationship (Gols *et al.* 2009).

Few studies have examined the roles of monarch diet on their palatability or nutritional quality for invertebrate natural enemies. Our results indicate that the consumption of more toxic host plants diminished the quality of monarch hosts for the specialist endoparasitoid *P. cassotis.* Because *P. puparum* was unable to develop in monarchs and *E. core* hosts, we cannot know the role of cardenolides in monarch defense against *P. puparum*, as there could be at least one additional defense mechanism shared by both danaid hosts. *Pteromalus cassotis* performance results mirror those found in one study of tachinid fly parasitism, where dietary cardenolides did not affect the likelihood of parasitism and host death, but did seem to decrease brood size and parasitoid survival (Hunter *et al.* 1996). However, Oberhauser *et al.* (2015) found that monarchs that consumed more toxic milkweed species were more likely to survive tachinid fly attacks. A few other parasitoids have occasionally been reported to reproduce in monarchs, including *Brachymeria lasus* in Australia (Zalucki & Freebairn 1982), as well as *Brachymeria ovata* (Halstead 1988) and *Trichogramma minutum* (Peck 1963) in North America. While it is not clear if parasitism by these species is rare or simply rarely observed and reported, it would be interesting to investigate whether host suitability for these parasitoids also varies with host plant.

Mortality rates for monarch eggs and larvae have been investigated in the lab and field by various research groups (Nail *et al.* 2015 and citations therein). The causes and frequency of monarch pupal mortality, however, are poorly understood, perhaps because monarch pupae are cryptically colored and difficult to locate in the field. Monarch pupal mortality due to *P. cassotis* has been measured by placing groups of pupae in the field in Minnesota and Wisconsin, as well as via opportunistic collection of naturally occurring pupae in parts of the southern U.S. (Oberhauser *et al.* 2015, Stenoien *et al.* 2015). Rates of parasitism between 0-100% have been

recorded, with a great deal of temporal and spatial variation (Stenoien *et al.* 2015). Clearly, more detailed and geographically expansive studies are needed to understand the population-level effects of *P. cassotis* and other sources of monarch pupal mortality, including from *P. puparum*. While *P. puparum* cannot reproduce successfully in monarchs, it does cause monarch mortality, at least under lab conditions. The frequency of host mortality due to attack by incapable parasitoids is an interesting avenue for study, although difficult to study in the field. In our study, monarch mortality caused by *P. puparum* resembles that caused by bacterial or viral infections. Other examples of attempted parasitism of unsuitable hosts that result in host death have been documented, and have spurred a debate about their potential use as biological control agents (Abram *et al.* 2016).

Many questions remain regarding the role of milkweed butterflies' cardioactive compounds and their interactions with Pteromalid parasitoids. We showed that *P. cassotis* can survive and develop on hosts containing variable levels of cardenolides, and that, unlike their monarch hosts (Petschenka & Agrawal 2015), *P. cassotis* performance is affected by host plant. Whether *P. cassotis* larvae avoid, excrete, sequester, degrade, or in some other way minimize the negative effects of cardenolides remains to be seen. It would be particularly interesting if this entomophagous insect has a modifed midgut or sodium potassium pumps, as do many insect herbivores of Asclepiads (Després *et al.* 2007, Dobler *et al.* 2012); such modifications would represent convergent evolution in response to plant toxins at multiple trophic levels. Finally, it would be interesting to investigate interactions between *P. cassotis* and other parasites and parasitoids of monarchs. For example, tachinid-infected pupae die upon parasitoid emergence, and the ability to discriminate against such pupae would increase *P. cassotis* fitness. Additionally, monarchs infected with the protozoan parasite *Ophryocystis elektroscirrha*, which requires development of the host to the adult stage to complete its lifecycle, experience higher survival when infected with *L. archippivora* (Sternberg *et al.* 2011). It would be interesting to determine the same is true for *P. cassotis.*

Conclusions

Parasitic wasps are the most important source of mortality for herbivorous insects and are commonly used as biological control against a variety of insect pests (Quicke 1997, Hawkins *et al.* 1997). Despite the ecological and economic importance of plant-herbivore-parasitoid

interactions in natural and agricultural systems, defining and predicting host ranges of parasitoids remains a challenge, although the role of host plant chemistry is increasingly recognized as an important determinant of host susceptibility and parasitoid performance (Price *et al.* 1980). Our tri-trophic approach to this issue is one of few studies that has attempted to clarify the ecological significance of milkweed butterflies' diet and chemistry when encountering invertebrate natural enemies. Our results suggest that sequestered cardenolides may provide protection from a generalist parasitoid by deterring oviposition and halting the development of offspring once attacked. Furthermore, when facing an apparent specialist parasitoid, our results suggest that host plants with varied cardenolide content and concentrations directly influence parasitoid performance and may indirectly influence host survival through altered foraging behavior.

Tables

Table 1. Model outputs for brood-level and latent effects of monarch host plant on *P. cassotis* performance. Significant effects are denoted with the following codes: '*'<0.05, '**' <0.01, '***' <0.001.

A. Total wasps emerged

	Estimate	Std. Error	z-value	p-value
Count model				
(Intercept)	4.447412	0.052678	84.426	< 0.001 ***
Host Plant: A. incarnata	0.083382	0.014422	5.782	< 0.001 ***
Pupa Age (days)	-0.08653	0.025533	-3.389	< 0.001 ***
Wasp Age (days)	-0.03392	0.001563	-21.708	< 0.001 ***
Pupa Mass (grams)	0.100492	0.04012	2.505	0.0123 *
Zero hurdle model				
(Intercept)	-0.62494	0.94002	-0.665	0.506
Host Plant: A. incarnata	0.39835	0.27579	1.444	0.149
Pupa Age (days)	0.14494	0.5035	0.288	0.773
Wasp Age (days)	-0.03108	0.02652	-1.172	0.241
Pupa Mass (grams)	1.60326	0.73128	2.192	0.0283 *

B. Total females emerged

	Estimate	Std. Error	z-value	p-value
Count model				
(Intercept)	4.288606	0.05733	74.805	< 0.001 ***
Host Plant: A. incarnata	0.067032	0.015517	4.32	< 0.001 ***
Pupa Age (days)	-0.01234	0.028832	-0.428	0.669
Wasp Age (days)	-0.03069	0.001666	-18.426	< 0.001 ***
Pupa Mass (grams)	0.153826	0.043356	3.548	< 0.001 ***
Zero hurdle model				
(Intercept)	0.07283	0.88103	0.083	0.934
Host Plant: A. incarnata	0.18488	0.25327	0.73	0.465
Pupa Age (days)	-0.63352	0.44128	-1.436	0.151
Wasp Age (days)	-0.03976	0.02466	-1.612	0.107
Pupa Mass (grams)	1.03719	0.67756	1.531	0.126

C. Proportion surviving to emergence

	Estimate	Std. Error	z-value	p-value
(Intercept)	-0.869987	0.219673	-3.960	< 0.001 ***
Host Plant: A. incarnata	0.227	0.0598	3.800	< 0.0014 **
Pupa Age (days)	-0.841653	0.092173	-9.131	< 0.001 ***
Wasp Age (days)	0.053173	0.007137	7.450	< 0.001 ***
Pupa Mass (grams)	0.460010	0.158059	2.910	0.00361**
Total wasps oviposited	0.033598	0.001034	32.502	< 0.001 ***

D. Developmental time

	Estimate	Std. Error	t-value	p-value
(Intercept)	14.66651	1.0118	14.495	< 0.001 ***
Host Plant: A. incarnata	-0.0322	0.25953	-0.124	0.901
Pupa Age (days)	0.77981	0.44907	1.737	0.0838
Wasp Age (days)	0.03513	0.02761	1.272	0.205
Pupa Mass (grams)	1.64496	0.73006	2.253	0.0251 *
Total Wasps Emerged	-0.02426	0.00379	-6.403	< 0.001 ***

E. Natal host diet

	Estimate	Std. Error	z-value	p-value
Count model				
(Intercept)	4.116816	0.078393	52.515	< 0.001 ***
Mother's Host: A. incarnata	0.189253	0.036619	5.168	< 0.001 ***
Host Plant: A. incarnata	0.070726	0.034251	2.065	0.0389 *
Pupa Age (days)	0.07087	0.045368	1.562	0.118
Wasp Age (days)	-0.02325	0.002363	-9.842	< 0.001 ***
Pupa Mass (grams)	0.275479	0.060926	4.522	< 0.001 ***
Mother's Host: A. incarnata * Host Plant: A. incarnata	-0.0614	0.046892	-1.39	0.190
Zero hurdle model				
(Intercept)	-1.70764	1.47138	-1.161	0.246
Mother's Host: A. incarnata	-0.17352	0.69898	-0.248	0.804
Host Plant: A. incarnata	-0.37495	0.61181	-0.613	0.540
Pupa Age (days)	-0.54719	0.95337	-0.574	0.566
Wasp Age (days)	0.01053	0.05147	0.205	0.838
Pupa Mass (grams)	2.53676	1.17629	2.157	0.0310 *
Mother's Host: A. incarnata * Host Plant: A. incarnata	2.00224	1.00196	1.998	0.0457 *

F. Adult lifespan

	Estimate	Std. Error	t-value	p-value
(Intercept)	7.6316	0.4873	15.66	0.004 **
Host Plant: A. incarnata	-0.2872	0.6892	-0.417	0.717

Figures

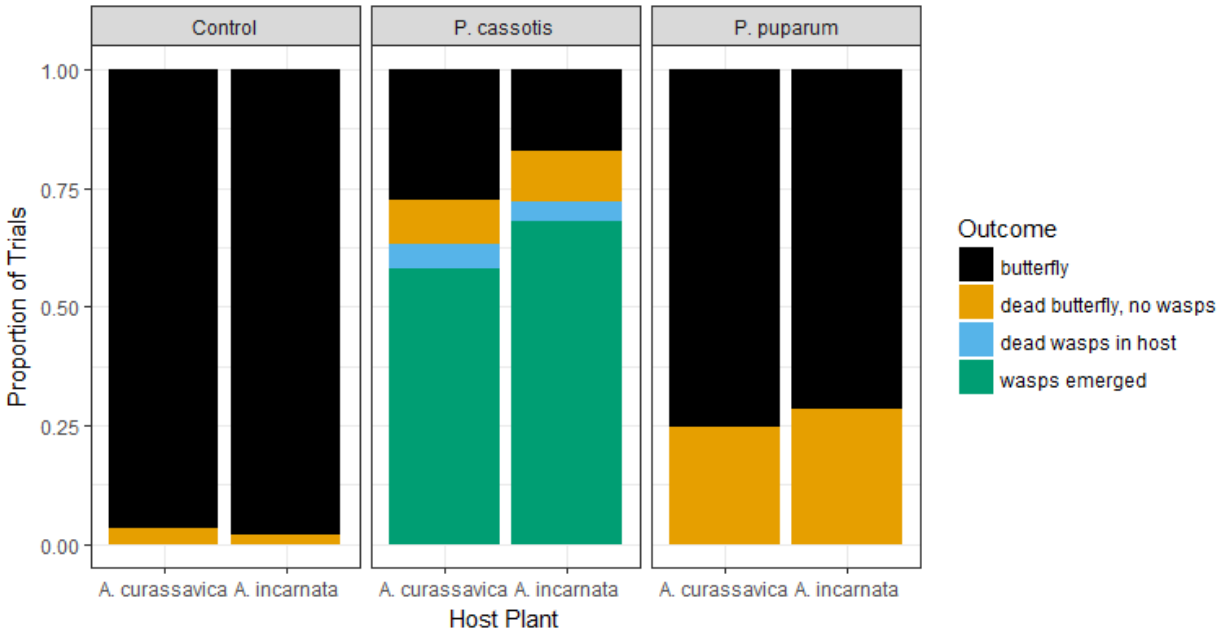


Figure 1. Outcomes of all monarch trials, regardless of wasp behavior, when exposed to either one *P. cassotis* female, one *P. puparum* female, or no wasps (control). Monarchs were reared on either high (*A. curassavica*) or low (*A. incarnata*) cardenolide plants. Both wasps were capable of killing monarchs, but *P. puparum* was incapable of developing in monarchs reared on either host plant. Monarchs reared on *A. curassavica* were more likely to survive trials with *P. cassotis.*

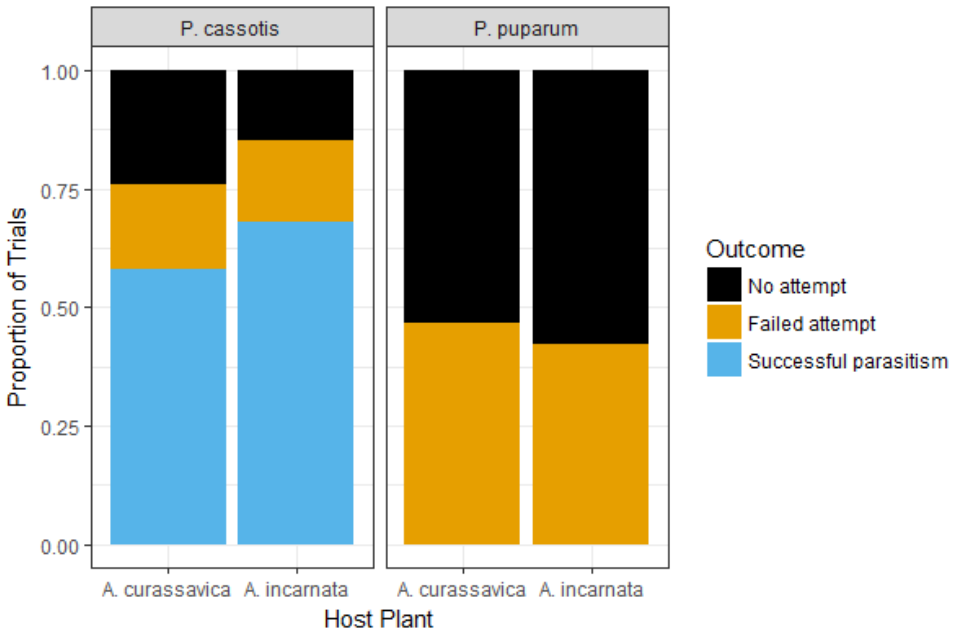


Figure 2. Oviposition behavior and success or failure of both parasitoids when paired with monarchs reared on either high (*A. curassavica*) or low (*A. incarnata*) cardenolide plants. *P. cassotis* was more likely to attempt parasitism of hosts reared on *A. incarnata.*

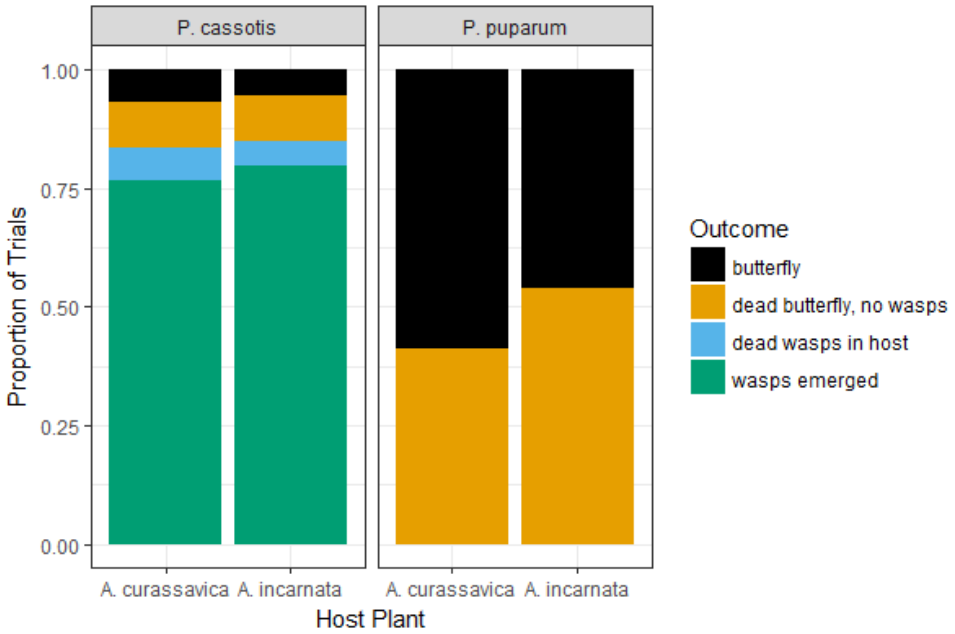


Figure 3. Outcome of monarch trials in which parasitism was attempted. Monarch host plant had no effect on the likelihood of butterfly or wasp emergence when paired with either parasitoid.

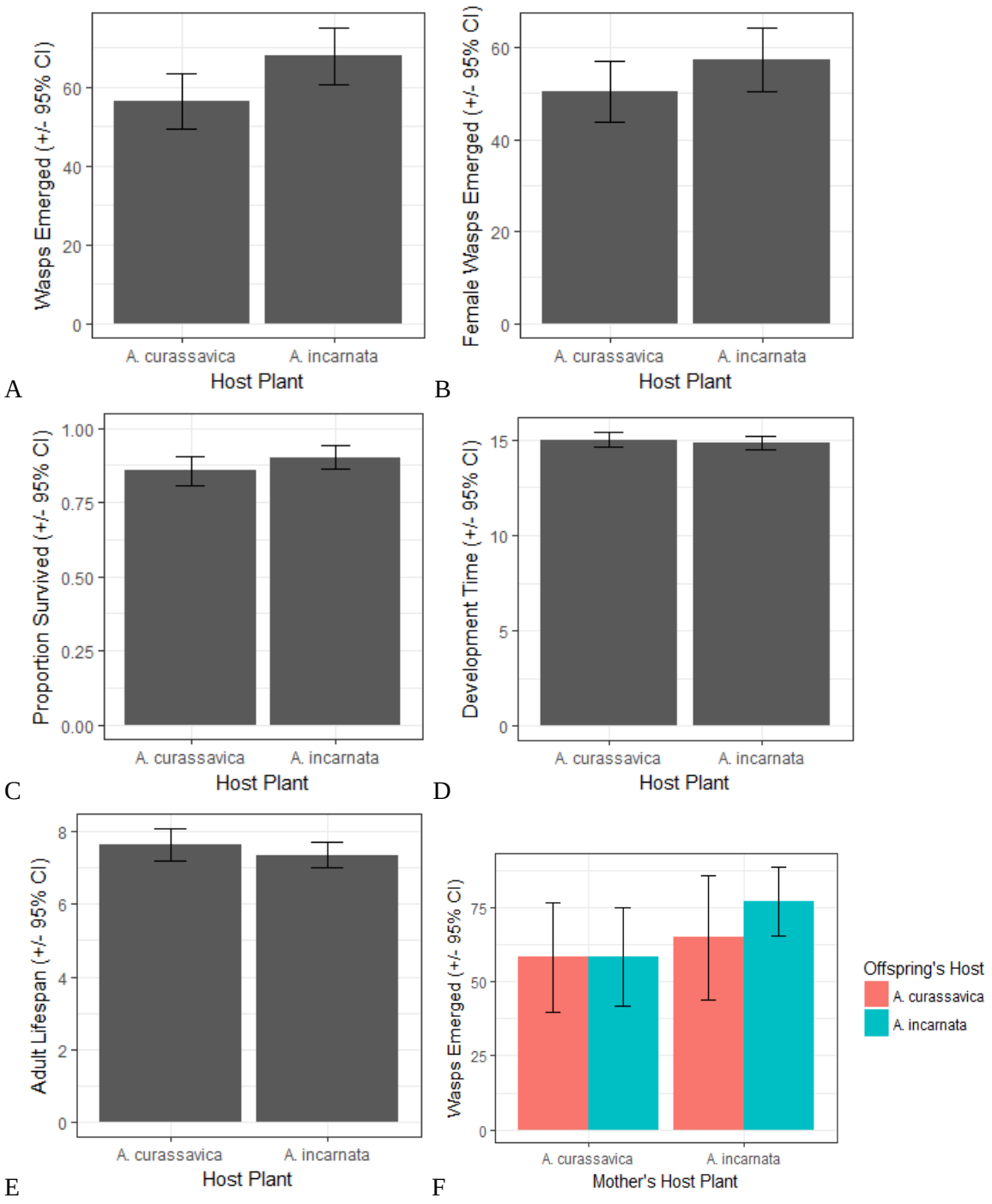


Figure 4. Brood-level and latent effects of monarch host plant on *P. cassotis* performance. Mean ± 95% confidence interval for (A) total brood size, (B) females per brood, (C) proportion of

wasps successfully emerged from host, (D) developmental time to adulthood, (E) mean lifespan when provided no food or water, and (F) brood size based on focal and parental host diet. Models accounting for additional factors (e.g. pupa age, wasp age, pupa mass) indicate that *P. cassotis* performed significantly better on lower toxicity hosts as measured by total brood size, females per brood, proportion of wasps successfully emerged, and brood size in the next generation (A,B,C,F). These models also indicate that host plant had no effect on development time or adult lifespan (D,E).

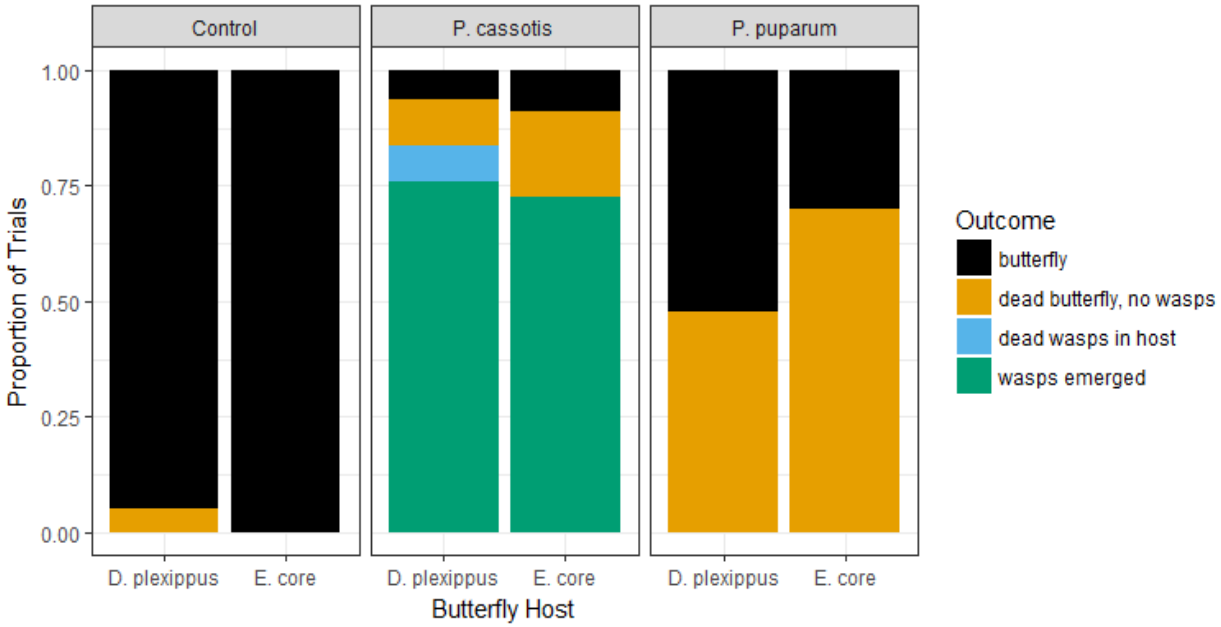


Figure 5. Outcomes of monarch and *E. core* trials in which parasitism was attempted, when exposed to either one *P. cassotis* female, one *P. puparum* female, or no wasps (control). Each species of parasitoid performed similarly on *E. core* as they did on *D. plexippus*, despite a lack of sequestered cardenolides in *E. core* hosts.

Chapter 4:
The Many Roles of Behavior in Defense Against Predation and Parasitism of Insect Pupae

Summary

Complete metamorphosis is regarded as a major innovation in insect evolution because it allowed for the exploitation of different ecological niches by the juvenile and adult forms within a species. However, insect pupae are relatively immobile and are often presumed to be highly vulnerable to natural enemies. Previous reviews have considered morphological and physiological aspects of pupal defense, but the role of behavior in pupal defense has largely been ignored. In this review, I bring together a diverse literature which shows that insect pupae likely benefit from a variety of behaviors performed before pupation (by the larva or pre-pupa), behaviors of the pupa itself, and behaviors of conspecific and heterospecific individuals. Some of these behaviors include the construction of protective enclosures and devices, behavioral enhancement of crypsis and mimesis, evasive movements, the use of biting mandibles and "gin-traps," and intraspecific interactions including mutualisms and host manipulation by parasitoids. All the behaviors described here at least plausibly function in defense against would-be natural enemies of insect pupae, yet the adaptive potential of many of these behaviors remain untested. Given the diverse behaviors collated here, it seems possible that the often-presumed cost of increased mortality by natural enemies during the pupal stage may be lower than expected because of these often-overlooked behavioral defenses. If so, this decreased cost of the evolution of a pupal stage may help to further explain the evolutionary success of the holometabolous insects. More complete investigations of these behaviors and their incorporation into existing models will likely improve our understanding of the predator-prey interactions and insect population dynamics in natural and agricultural systems.

Introduction

The evolution of holometaboly is widely considered an innovation that has spurred the insects' evolutionary success, as the Endopterygota comprises 80-90% of all insect species (Berlese 1913, Poyarkoff 1914, Imms 1938, Hinton 1948, Helsop-Harrison 1958, Wigglesworth 1973, Hinton 1977, Price 1984, Truman & Riddiford 1999, Yang 2001, Mayhew 2007, Bernays 1986, see Erezyilmaz 2006 for historical review). The primary hypothesis for endopterygotes' success is that morphological and life-history differences between larval and adult forms allow parents and offspring to exploit different habitats and food sources, which reduces intraspecific competition and allows for relatively rapid lifecycles (Hinton 1977, Price 1984, Truman & Riddiford 1999). Stage-specific selection pressures led to the extreme specialization of larvae for feeding and growth, and adults for dispersal and reproduction. For example, holometabolous larvae lack external wingbuds, which allows them to burrow inside of foods, which are largely inaccessible to hemimetabolous nymphs and winged adult insects.

Although a holometabolous lifestyle probably contributed to the diversification of the Endopterygota, the transitionary pupal stage is often thought to be particularly vulnerable to natural enemies and unpredictable changes in the abiotic environment. During this period of dramatic bodily reorganization from larval to adult forms, pupae cannot feed or mate and the pupae of most species are sessile or have very limited mobility. This has led many to describe them as "quiescent," "resting," and "death-like" (Lubbock 1890, Hinton 1946, van Emden 1957, Belles 2011, Gullan & Cranston 2009, Engel 2015). Several widely-used, but non-peer-reviewed resources even describe pupae as "defenseless" (e.g. Wikipedia.org, NewWorldEncyclopedia.org).

The many books, reviews, and textbook chapters on insect defenses delve deeply into the strategies and fascinating examples of larval, nymphal, and adult defenses, but pupal defenses are typically given little recognition (Lederhouse 1990, Hunter 2000, Gentry & Dyer 2002, Gullan & Cranston 2009, Matthews & Matthews 2010, Zvereva & Kozlov 2016). Pupae are certainly not defenseless (Hinton 1955), yet this area of study has probably lagged because of the difficulty of studying the often cryptic or hidden pupal stages, compared to the more active and apparent larval and adult stages. It is possible that our limited knowledge of pupal defenses may be a byproduct of their success at avoiding detection by potential enemies. To assume that pupal defenses are

rare simply because few researchers have observed and described them is flawed logic, especially if a primary purpose of defense traits is to avoid detection by potential enemies.

Insects use a variety of strategies to defend themselves from natural enemies including crypsis, mimesis, chemical defenses, aposematism, shelter building, startle-inducing traits, direct combat, and the enlistment of allied conspecifics and heterospecifics (Evans & Schmidt 1990). One way to organize our understanding of insect defenses is to categorize each defensive trait as either passive or active: passive defenses are said to be the result of constitutive morphological or physiological traits, while active defenses are defined as behavioral responses to attacks (Matthews & Matthews 2010). However, some defenses are difficult to categorize using this schema because many "passive" defenses rely on appropriately coordinated behaviors to function. For example, moths with cryptic forewings and conspicuous hindwings will often remain motionless and camouflaged unless threatened by a predator, at which point, they will reveal the starkly contrasting hindwings, which presumably startle the predator and allow the moth to escape (Stevens 2005). An alternative approach is to categorize defensive traits by their function, as Gross (1993) did in his account of insect defenses against parasitoids. Accordingly, a defense can function in three ways: 1) By reducing the likelihood that the prey item is found and contacted by enemies, 2) By reducing the probability of successful attack once encountered, or 3) In the case of attack by parasites, to suppress and outlive the invaders (Gross 1993).

The primary literature is rich with examples of pupal defenses, but all existing summaries of the defense of insect pupae have been embedded in reviews of partially overlapping topics and have primarily focused on the role of morphology in pupal defense. Conspicuous in its absence, however, is a summary dedicated to the role of behaviors in defense of insect pupae. Over 60 years ago, Hinton published an extensive review of the morphological and device-based defenses, such as camouflage and cocoons, but downplayed or ignored behaviors required for such defenses to function, such as pupation site choice and cocoon construction (1955). More recently, Gross (1993) summarized morphological and behavioral defenses against parasitoids across all lifestages of insects, though he only considered those traits that defended the host once it had been found and approached (i.e. he excluded defensive behaviors that occur either before host recognition or after oviposition by the parasitoid). While pupal morphology received nearly a full page of coverage in this review, the role of behaviors in pupal defense against parasitoids contributed only six sentences (Gross 1993). Like Hinton's account, many of the examples of

morphological defenses that Gross presented rely on behaviors, which were largely ignored. Around the same time, Brakefield and colleagues (1992) published a review of stage-specific defenses of butterflies. Despite the limited taxonomic scope, these authors more fully acknowledged the behaviors involved in the defense of butterfly chrysalides. Chiefly, they recognized that, in addition to wiggling and jerking behaviors of the pupa itself, the behavior of pupation site choice by the larva (or pre-pupa) and interactions with associated heterospecific species (in this case, lycaenids and ants) are important components of pupal defense for some taxa.

The primary literature on the role of behavior in pupal defense is quite broad, though it is often descriptive in nature, lacking conceptual integration, and rife with untested hypotheses. Therefore, the objective of this review is to more deeply and explicitly examine the role of behavior in the defense of insect pupae. I address several specific questions: What types of behaviors are performed, at what point in the life cycle, and who performs them? Against which enemies do these behaviors function and how effective are they in preventing injury or mortality?

This review highlights defenses against vertebrate and invertebrate predators, insect parasitoids, and cannibalism. It does not mention defenses against koinobiont parasitoids that attack earlier lifestages because this pupal mortality is delayed and defensible only by an earlier life stage. It also does not address defenses that protect pupae from any non-animal parasites, such as bacteria, fungi, and protozoans, which are typically the responsibility of the immune system (Beckage 2011), except for rare phenomena such as kin grooming in social insect colonies (Tragust *et al.* 2013).

The behaviors are divided into three categories: (I) behaviors performed before pupation, (II) behaviors performed during the pupal stage (including those of pharate adults), and (III) behaviors performed by conspecific or heterospecific organisms. The examples presented here will demonstrate that behaviors performed before the life stage in question are much more important for pupal defense than defense of larval or adult stages. Because many behavioral defenses rely on coordinated morphological and physiological traits, I will reconsider several of the morphological and device-based defenses described by previous authors using a behavioral perspective (see Hinton 1955, Gross 1993, Brakefield 1992).

Some of the behaviors described here are common across entire orders or families of insects, while others are unique, occurring at the tips of evolutionary trees. Some likely function

against a broad suite of enemies, while others may deter only certain types of enemies. Some may have evolved and are maintained by selection pressures imposed by natural enemies, while others may be exaptations or pre-adaptations with unrelated evolutionary origins (Gould & Lewontin 1979, Gould and Vrba 1982). The defensive function of many of the behaviors described remains hypothetical, but I point them out with the hope that they will inspire more rigorous and informative tests of these hypotheses, which will be valuable to our understanding of the co-evolution and ecology of insects in natural and agricultural systems, and may help explain the evolutionary success of holometabolous insects.

Natural enemy-induced pupal mortality

Weather, natural enemies, competition, disease, host defenses, and many other factors cause insect deaths, and their relative importance varies with the developmental stage of the insect. The primary sources of pupal mortality are thought to be predation, parasitism, and abiotic factors such as weather (White 1986 and citations therein, Cornell *et al.* 1998). Because most pupae have relatively limited mobility and represent relatively large amounts of biomass, one might expect them to be a particularly valuable resource to natural enemies and be susceptible to natural enemy-induced mortality. On the other hand, because pupae do not feed or mate, they may be less conspicuous to visually and acoustically-oriented natural enemies than the more active larval and adult lifestages. Pupae may also be less conspicuous to chemically-oriented natural enemies because they are a source of fewer potential kairomones such as pheromones and waste products. Finally, because pupae do not feed, they are exempt from the dangers of bottom-up defenses of plants and prey items. Given these conflicting predictions, what does the empirical literature tell us about the role of natural enemies in pupal mortality?

Observational and experimental studies have shown that many types of vertebrate and invertebrate natural enemies consume pupae. These enemies range in size from many times larger (e.g. birds) to many times smaller (e.g. ants and gregarious parasitoids) than the pupal prey item. A sampling of documented vertebrate natural enemies of pupae includes small mammals (Frank 1967, Hanksi 1992, Hastings *et al.* 2002), birds (Stefanescu 2000, Schuler 1990), reptiles (Sales *et al.* 2012, Abensperg-Traun & Steven 1997), and primates (Raubenheimer & Rothman 2013). Of these, insectivorous and omnivorous mammals such as shrews are known to cache and consume large numbers of sawfly and moth pupae and likely contribute to the prevention of

outbreaks of potential forest pests (Hanski 1992). Invertebrate enemies include many types of predators, including spiders, beetles, wasps, and cannibals, as well as insect parasitoids (Frank 1967, Tschinkel & Willson 1971, Hinton 1955, Gross 1993). Though rarer, species of aquatic pupae are consumed by fish, amphibians, birds, and many aquatic invertebrates (Merritt & Cummins 2008). Some aquatic pupae are even attacked by specialized parasitic wasps (Bennett 2008, Hirayama *et al.* 2014). Finally, entomophagy by humans, including the consumption of various types of pupae, is practiced in many cultures and is gaining popularity as a potential solution to issues of food security and as a protein source with a smaller environmental footprint than other livestock (Raubenheimer & Rothman 2013, van Huis *et al.* 2014). Because each population of insects has evolved with different suites of enemies as pupae and because each of those enemies differs in size, sensory modalities, and diet breadth, it seems likely that a variety of defenses have evolved and that the functionality of any given defense is highly context dependent.

Lists of natural enemies of a given species of pupa can be compiled from anecdotal observations and from studies that involve placing pupae in the field and recording their fates some time later. However, because of their small temporal and/or spatial resolution, these methods have likely led to the omission of some enemies and an inability to estimate the frequency of interactions and mortality attributable to different enemies. Generalist predators are especially likely to go undetected because they may consume insect pupae only rarely, but may consume many if they are able to learn the appropriate search behaviors. Parasitoids likely kill more pupae, but could also go undetected if naturally parasitized pupae are cryptic and difficult to collect in the field (Hawkins *et al.* 1997). As an example, pupae of the chemically defended monarch butterfly (*Danaus plexippus*) have been observed to be consumed by predatory wasps, mantids, ants, and chipmunks (Rayor 2004, Rafter *et al.* 2013, Oberhauser *et al.* 2015, McCoshum *et al.* 2016, K. Oberhauser *personal communication*). However, even for this uniquely well-studied butterfly, more than 125 years passed between the first report of monarch parasitism by a gregarious pupal parasitoid, *Pteromalus cassotis*, and the "rediscovery" of this interaction in 2008 by placing and recovering pupae in the field (Gillette 1888, Oberhauser *et al.* 2015, Stenoien 2015). We now know that this host-parasitoid interaction occurs across most of the U.S. and that rates of parasitism are highly variable in space and time, but can reach 100% within some patches (Oberhauser *et al.* 2015, Stenoien *et al.* 2015). This example illustrates how

lists of enemies are only a starting point in understanding pupal mortality, even for well-studied species.

The most comprehensive approach to the study of stage-specific mortality has been led by Hawkins, Cornell, and colleagues, who conducted meta-analyses on all available life table data for phytophagous holometabolous insects (Cornell & Hawkins 1995, Hawkins *et al.* 1997, Cornell *et al.* 1998). Emerging from the idiosyncrasies of dozens of studies, they found that the causes, but not the overall risk of mortality change with age. Across development, natural enemies caused the most mortality, especially during the later larval instars and pupal stage (Cornell & Hawkins 1995, Cornell *et al.* 1998). They estimated that natural enemies accounted for ~75% of total pupal mortality. There were no clear patterns in the causes of mortality for different orders of insects (Cornell *et al.* 1998), but it was clear that parasitoids kill more herbivores than do predators or pathogens, and this pattern becomes stronger with age (Hawkins *et al.* 1997). Hunter (2000) also studied the survival of phytophagous insects, but with a focus on the role of gregariousness and repellent defenses. He also found that the overall risk of mortality is roughly constant between across stages of solitary species, but that mortality risk increases with age for the gregarious species. He estimated that there is over 60% pupal mortality for gregarious species, while only about 45% for solitary species and hypothesized that this has to do with the retention of chemical defenses into the pupal stage, but conceded that neither gregariousness nor chemical defenses fully explained these patterns.

Despite these pioneering efforts, many questions regarding pupal mortality remain. Unfortunately, economically important species were over-represented in the studies by Hawkins and Cornell and findings from phytophagous species cannot necessarily be extrapolated to non-phytophagous species. Another limitation of their methods is that many koinobiont parasitoids and disease agents may infect young larvae, but do not cause mortality until later in life, which decouples the timing of cause and effect of mortality and over-represents mortality in the later stages of development. Still another limitation of life table data is that mortality due to parasitoids can be masked by predation of an infected host, but predation cannot be masked by parasitism. Finally, the importance of natural enemies relative to other sources of mortality remains contentious (Peterson 2009, Schneider 2011). Given the huge taxonomic and ecological diversity of insect pupae, filling these gaps in knowledge will not be easy to do, but would greatly improve our understanding of insect ecology and the evolutionary tradeoffs of a holometabolous lifestyle.

Diversity of pupal forms

The first holometabolous insects evolved approximately 350-400 million years ago (Misof *et al.* 2014, Tong *et al.* 2015) and radiated to the estimated 5.5 million species present today (range of estimates: 2.6-7.8 million, Stork *et al.* 2015). Today, the Endopterygota includes 11 orders (see Peters *et al.* 2014 for phylogeny), though most species diversity exists within just four orders: the Coleoptera, Hymenoptera, Diptera, and Lepidoptera. Few have speculated on the form and behavioral repertoire of the ancestral state of insect pupae, but it may have been similar to that of present day Raphidioptera and Megaloptera, both of which have active pupae capable of walking and with movable mandibles (Peters *et al.* 2014). However, the more derived orders of the Holometabola, especially the Lepidoptera, Coleoptera, Hymenoptera, and Diptera, have pupae that are capable of little or no locomotion and very limited movement, typically restricted to the abdominal segments. The aquatic pupae of mosquitos and midges, also restricted to movement of the abdomen, are uniquely active swimmers and an interesting counterexample to this pattern.

Pupae can first be differentiated by whether they are exarate, with appendages free and extended, or obtect, with appendages folded in and fixed to the body. Exarate pupae can be further differentiated by the presence (decticious) or absence (adecticious) of articulated mandibles. Exarate decticious pupae exemplify the most primitive pupal form, and include the Neuroptera, Megaloptera, Mecoptera, Raphidioptera, most Trichoptera, and relatively few Lepidoptera (Hinton 1946, Stehr 1987). The mandibles of decticious pupae are typically used to aid the emergence from a cocoon or pupal case, though the unusually mobile pupae of the Megaloptera and Raphidioptera also use them in defense (Hinton 1946, Contreras-Ramos 1997, Grimaldi & Engel 2005). Exarate adecticious pupae include the Strepsiptera, Siphonaptera, most Coleoptera and Hymenoptera, and many Diptera, suggesting that the loss of mandibles has evolved independently several times (Peters *et al.* 2014). The exarate adecticious dipterans, members of the Cyclorrhapha, an unranked taxon within the infraorder Muscomorpha, are especially unique in that they pupate inside of the barrel-shaped exoskeleton of the final larval instar. This uniquely derived pupal form is called “coarctate” and the pupa is said to reside inside of a “puparium”. Finally, obtect pupae have evolved multiple times and include nearly all Lepidoptera, several families of Coleoptera, most species within the Chalcidoidea (order Hymenoptera), and more primitive dipterans, including mosquitoes and crane flies (Hinton 1946, Grimaldi & Engel 2005).

While morphological features are used to distinguish between different types of pupae, it is also important to consider how development influences pupal behaviors. First, the duration of the pupal stage varies widely, from just two or three days for several dipteran families and small parasitic hymenoptera (Danks 2006) up to multiple years, as in two swallowtail butterflies that undergo pupal diapause, *Papilio zelicaon* and *Papilio alexanor* (Sims 1980, Nakamura & Ae 1977). Given this inter-specific variation, I expect that pupal duration could be predictive of pupal mortality and perhaps even investment in pupal defenses. Within a species, pupal developmental rates depend on temperature and whether pupal diapause has been induced. Second, although the shedding of the last larval skin or spinning of a cocoon is often thought of as the transition between larva and pupa, there are also less apparent changes that occur before and during the pupal stage. The transitions between larval, pupal, and adult forms are most obvious at ecdysis (emergence and separation from the shed cuticle), but the recognition of apolysis (separation of the cuticle from the epidermis) lends a more accurate depiction of insect development and can inform the study of pupal behaviors (See Hinton 1946, Hinton 1971, Wigglesworth 1973, Hinton 1973, Whitten 1976, and Hinton 1976 for further discussion). A particularly important term is "pharate," which refers to any life stage that remains within (sometimes visibly) the cuticle of the preceding stage (Hinton 1946, 1958). For example, during the last moments before ecdysis to the pupal stage, a pharate pupa is enclosed in the larval skin. Similarly, after apolysis but before ecdysis to the imago, a pharate adult resides within the pupal cuticle. In several taxa with "active pupae" (Raphidioptera, Megaloptera, Tricoptera), these are actually pharate adults behaving within the pupal cuticle (Hinton 1946, Stehr 1987). In this review, I include any behaviors performed by a larva, pharate pupa, pupa, or pharate adult that are thought to protect the pupa or pharate adult before ecdysis to the imago. Pharate adults are included because of the difficulty of knowing whether apolysis has occurred and therefore whether a pupa or pharate adult is the actor within a pupal exoskeleton.

Proactive defensive behaviors performed before pupal ecdysis

Insects can proactively invest in pupal defenses by, for example, choosing appropriate pupation sites and building pupation shelters. Most proactive defensive behaviors performed

before the molt to the pupal stage aim to reduce the probability of being located by an enemy, though the construction of some pupation shelters may also ultimately reduce the probability of successful attack even after being encountered. These proactive defensive behaviors often require significant investments of larval resources, such as endogenously produced silk and/or time and energy spent seeking a pupation site. Presumably, these upfront costs are offset by the delayed benefits of increased likelihood of pupal survival, though the efficacy of some examples mentioned in this section remain to be tested.

Construction of protective chambers and devices

Within almost all of the orders that make up the Endopterygota (except Raphidioptera and Strepsiptera), some or most species construct devices which completely or partially cover the insect during its pupal stage. Some authors have considered these devices to be morphological characters (e.g. Wrona and Dixon 1991), but they are more accurately described as end-products of behavioral repertoires, examples of niche construction, or extended phenotypes (Dawkins 1982, Hansell 2005). These approaches focus on the role of behaviors, rather than objects, in defense of pupae. In support of this idea, analyses of aquatic black fly cocoons have shown that the behavioral traits of cocoon formation are more phylogenetically conserved and informative than the traits of the cocoon itself (Stuart & Hunter 1995, 1998, Stuart & Currie 2002).

Cocoons, excavated chambers, and other devices are typically constructed by the larva or pharate pupa which will later use the device and can serve as a refuge from natural enemies (Hinton 1958, Berryman & Hawkins 2006). Pupal shelters may serve to reduce the probability of detection by enemies by concealing the pupa, blending in with the background (crypsis), or resembling a discrete non-edible object (mimesis) (Endler 1981, Ruxton *et al.* 2004). These terms are often applied to visual cues, but work equally well with chemical, acoustic, or vibrational cues. If a pupa in a self-constructed shelter is detected, the shelter could serve a secondary function, reducing the probability of successful attack (Hansell 2005). It should be noted that many insects build enclosures that are inhabited by and provide protection to developing larvae and diapausing larvae, in addition to pupae.

Perhaps the simplest form of a constructed chamber is an open space formed by the excavation of a soft substrate such as soil or rotten wood. Many Mecoptera and Megaloptera, and some Coleoptera and Lepidoptera, excavate such chambers, and some groups of moths further

stabilize their soil pupation chambers with silk or other secretions (Danks 2002). It has been hypothesized that these chambers provide protection via visual, chemical, and structural concealment compared to a hypothetical conspecific placed on the surface of the substrate. There may be tradeoffs, however, because while escaping surface level enemies, underground pupation potentially exposes these insects to a suite underground enemies, including ants, nematodes, fungi, bacteria, and burrowing mammals. A recent field test suggests that underground chambers of *Manduca sexta* function primarily to provide open space, preventing soil from deforming the metamorphosing individual (Sprague & Woods 2015). This study found no evidence that the compacted walls of underground chambers were sufficient to prevent mortality due to underground natural enemies, but they did not measure the effect of natural enemies on pupae placed above ground. The potential value of these chambers in a fuller suite of field-realistic conditions and in other taxa remains to be investigated.

Caddisflies (Trichoptera) and bagworms (Lepidoptera: Psychidae) reside within self-constructed cases throughout the larval phase, progressively expanding these cases as they molt and grow. In both groups, the larval cases undergo unique final modifications and also serve as pupal cases. Caddisflies such as the fixed retreat makers (suborder Annulipalpia) construct a dome of rock fragments at the end of the last larval instar, which is presumed to keep predators and other enemies out (Wiggins 2015). At the end of their larval stage, bagworms tightly affix the anterior (previously open) end of the larval case to a substrate, thereby completing the pupal enclosure, of which a defensive function is likely, though I'm unaware of such tests (Davis 1964, Rhainds *et al.* 2009).

Many pupae reside in shelters made entirely or partially of silk, a strong, lightweight, elastic, and water-resistant polypeptide-based material produced only by arthropods. Silk is stored as a liquid inside of the organism and is "spun" into thread as it is exposed to the environment (Craig 1997). Although lepidopteran caterpillars are the best-known silk spinners, cocoon-making is a taxonomically widespread trait with multiple evolutionary origins (Sutherland *et al.* 2010). Holometabolous larvae produce silk in either the labial glands (Lepidoptera, Hymenoptera, Trichoptera, Diptera, Siphonaptera), or in the Malpigian tubules (Neuroptera, Coleoptera) (Sutherland *et al.* 2010). Between species, cocoons vary in their thickness, density, coloration, and incorporation of other materials from the environment. In many species, cocoons vary intraspecifically in size, shape, thickness, texture, density, shape, and toughness, and those of

diapausing insects are often more robust than those of non-diapausing insects (Danks 1987, Table 6).

Detailed accounts of cocoon construction behaviors have been made for several species, including parasitic wasps (Wilson & Ridgway 1974, Fulton 1940, Cross & Simpson 1972), blackflies (Stuart & Hunter 1995), noctuids (Shorey *et al.* 1962), and the famous saturniid silk moths (Van der Kloot & Williams 1953, Lounibos 1975). In general, cocoons are constructed from the outside in, as the larva slowly encloses itself into the cocoon by repeatedly drawing out lengths of silk and attaching them to the substrate or existing silk. The process can often be broken into behavioral phases such as site preparation, the construction of outer scaffolding and attachments to the substrate, spinning of the outer and inner portions of the cocoon, and impregnation with various substances (e.g. Cross & Simpson 1972, Lounibos 1975, Stuart & Hunter 1995). The weaves used vary, but a few common patterns are shared across many taxa, including zig-zags, figure-eights, and the stacking of inverted U-shaped strands of silk (Hansell 2005, Fulton 1940). Except for larval case bearers' pupal enclosures and those species that use the same structure as a larval feeding retreat and pupation site, pupal cocoons are constructed in one continuous behavioral sequence which is often stereotyped and inflexible (Fulton 1940, Van der Kloot & Williams 1953). The duration of cocoon construction represents a significant investment of time, typically lasting one to two days, or even longer at cooler, yet ecologically relevant, temperatures (Van der Kloot & Williams 1953, Cross & Simpson 1972, Zhao *et al.* 2005, Shorey *et al.* 1962). Cocoons may also require large investments in silk, evidenced by some silk moths, which generate threads up to 1300 meters in length (Downing 2006).

Because cocoons provide a barrier between the pupa and the outside world, they are often assumed to have a general defensive function against biotic and abiotic threats. Owing to the strength, elasticity, and arrangement of the silk, the cocoons of a model organism of silk production, *Bombyx mori*, have been described as having "optimum microstructure and superior mechanical properties" which aid in the prevention of damage posed by exterior impacts and attacks (Zhao *et al.* 2005, p. 9200). *Bombyx mori* cocoon silk also contains anti-microbial proteins, providing direct defense against bacterial and fungal infections (Pandiarajan *et al.* 2011).

The presence of a cocoon has been experimentally demonstrated to protect some pupae from natural enemies. Fleas (Siphonaptera) construct pupal cocoons from silk and locally

collected debris and spend up several months within these cocoons as pre-pupae, pupae, and pharate adults. Silverman & Appel (1984) found that ant predators (*Iridomyrmex humilis*) readily carry flea eggs, larvae, and naked pupae (cocoons experimentally removed) into their nest, but ignore or discard pupae inside intact cocoons. Because the cocoons are permeable to water and air, they hypothesize that these cocoons afford physical protection rather than visual or chemical camouflage or concealment (Silverman & Appel 1984). *Lymantria dispar* cocoons provide protection against a parasitic wasp, *Brachymeria intermedia* (Rotheray & Barbosa 1984); pupae with intact cocoons were more responsive to approaching parasitoids, spinning and arching in ways that increased handling times and sometimes entangled the parasitoid in the silk, compared to those with experimentally removed cocoons. Furthermore, larger cocoons can afford greater protection to pupae by preventing oviposition by parasitoids with ovipositors of insufficient length (Hinton 1955). Some insects create larger or thicker cocoons by simply using more materials to achieve a similar design to related individuals or species. Limacodid larvae, for example, spin such densely woven cocoons that few parasitoid species can pierce through to reach the pupa inside (Gauld & Bolton 1988). A thicker cocoon can also be achieved by suspending a densely woven cocoon within a more loosely constructed silken web. This strategy is enacted by the moth *Ortholepis betulae* (Pyralidae), preventing parasitoids without long enough ovipositors from reaching the pupa within (Cole 1959). Some bagworms and moths construct cocoons with enough space inside to allow movement of the pupa within (Gross 1993 and citations therein, Cole 1959). For the moth pupae tested, this space, combined with the hard cuticle and mobility of the abdominal segments, often results in glancing blows by ichnuemonids' ovipositors, increasing handling times and potentially deterring the parasitoids (Cole 1959). When the cocoons were experimentally compressed, however, the pupae could not move with the same freedom nor find refuge in empty space within this cocoon and were easily parasitized (Cole 1959).

Finally, instraspecific variation in cocoons is evident in many species of diapausing parasitoids, which often make "tougher" cocoons than their non-diapausing counterparts, though the extent to which biotic *vs.* abiotic threats are mitigated by tougher cocoons is unclear (Godfray 1994 and citations therein). Similarly, the cocoons of a bivoltine megachilid bee, *Lithurgus corumbae*, vary with seasonality. The first generation produces single-layered cocoons, which are tended by the mother, while the second generation, which does not receive parental care,

produces double-layered cocoons. Double-layered cocoons may provide additional physical protection from environmental fluctuations and predators, thermal benefits that lead to an increased rate of development, or both (Mello & Garófalo 1986).

Despite these apparent benefits, in some cases, cocoons may hinder defense or cause other tradeoffs. For example, some parasitoids may find cocoons easier to handle than a hardened, naked pupa because the rough surface allows for more secure footing (Cole 1959). The construction of a self-enclosing shelter also requires that the insect is able to escape the shelter upon eclosion, which is accomplished by chemically dissolving the silk, cutting the silk with mandibles or "cocoon-cutters" on adult wings, or through built-in escape hatches or one-way trap doors (Hinton 1946, Hinton 1955).

One particularly interesting innovation in cocoon design and construction is the suspension of the cocoon on a long silken cord. Hinton (1955) referred to these as "pensile cocoons," though they are now commonly referred to as "suspended cocoons" (Zitani & Shaw 2002). This form is constructed by few species of moths (Urodidae, some Saturniidae) and parasitic wasps (Braconidae: Meteorinae, Ichneumonidae: Campopleginae and few others), and has long been hypothesized to provide a refuge from various enemies (Hinton 1955, Quicke 1997, Zitani & Shaw 2002). Some spiders suspend their eggs in similar cocoons (Scheffer 1905). Generalist ant predators are capable of destroying these egg cocoons when placed on vegetation, but they do not attack suspended cocoons, apparently because they are unable to venture down the suspension lines (Hieber 1992).

Moths of the family Urodidae (~80 known species), build "filigreed" ellipsoid-shaped cocoons woven into a widely-spaced open mesh cage, some using bright orange or red silk (Heppner 2008). The pupa inside is plainly visible and there is often a hole at the bottom of the orb through which the larval exoskeleton is discarded and the eclosing adult escapes. Non-silk materials are incorporated by some species and the length of the suspension cord varies. One relatively large Urodid, *Urodus isthmiella*, is reported to use a silken cord over 32 cm long (Busck 1910). At least some of these pupae are reported to thrash violently when disturbed, a motion that travels up the suspension thread and could small deter potential enemies from descending to the pupa below (Busck 1910).

The genus *Meteorus* (Braconidae) is named for the resemblance of its pensile cocoons to meteors and is comprised of 300+ species of parasitic wasps whose hosts are lepidopteran or

coleopteran larvae (Yu 2017). After emerging from its host, a typical *Meteorus* larva moves to twig or leaf, spins a silk pad, suspends itself on a silk thread, builds a cocoon at the bottom of the thread, and pupates within. Most species produce silken threads approximately 3 cm in length, though lengths vary from 1-45 cm in length. Zitani and Shaw (2002) hypothesized that *Meteorus* cocoons evolved in response to predation by ants, and Shirai and Mateo (2009) tested this hypothesis by comparing predation of suspended vs. manipulated non-suspended *Meteorus pulchricornis* cocoons in the presence of ants (*Crematogaster matsumurai*). After 12 hours, >75% of the non-suspended cocoons sustained damage by the ants, while none of the suspended cocoons were damaged. Suspended *Meteorus* cocoons do not provide protection from all enemies, however, as they are successfully attacked by hyperparasitoids (Zitani & Shaw 2002).

Many insects incorporate materials other than silk into their cocoons. These items typically come from the local environment, such as vegetation, or are produced by the constructing insect, such as fecal shields and urticating larval spines. The most commonly incorporated items are pieces of living or dead vegetation, such as twigs and leaves, which could have a mimetic or camouflaging effect. The incorporation of plant materials is especially common among moths. For example, although some saturniid larvae excavate underground pupation chambers, many others wrap themselves in the leaves of their host plant or in leaf litter before spinning a cocoon within. Of those that pupate on the host plant, they often first use silk to attach the leaves to the plant, preventing the cocoon from falling to the ground (Tuskes *et al.* 1996). Bagworms (Psychidae) build cocoons of dead vegetation, though as larval casebearers, they append silk and local plant materials to their portable case with each molt, eventually sealing the anterior portion to a substrate, transforming it into a pupal case (Rhainds *et al.* 2009). At least ten other lepidopteran families also construct larval cases, though the extent to which these families retain and modify the structure for use as a pupal case is unclear (Rhainds *et al.* 2009). One particularly interesting example of incorporation of vegetation comes from an unidentified caterpillar (possibly *Negritothripa* sp. in the Nolidae) in Borneo which builds its cocoon using a small amount of silk and fragments of dried resin collected from the trunk of the tree *Vatica rassak* (Symondson *et al.* 2015). A series of photographs during cocoon construction showed that the aposematically-colored larva built two walls on either side of its body, collecting additional pieces of resin as needed, and eventually pulled these walls together to form the completed cocoon, white in color and rough in texture due to the fragmentation patterns of the resin. The

construction of a resin-based cocoon on large patch of the same resin may contribute to chemical camouflage and chemical defense. Analyses of one such cocoon revealed a complex chemical mixture including many sequiterpines and triterpines known to function against non-specialist herbivores and fungi (Symondson *et al.* 2015, Gershenzon and Dudareva 2007).

Another strategy employed in cocoon defense by some moths is to incorporate and redeploy urticating hairs (setae, modified setae, or spines) from the last larval stadium into or near the cocoon (Battisti *et al.* 2011). To achieve this, larvae must either remove the hairs from their larval integument and weave them into the silk as the cocoon is being constructed, or possess hairs that naturally disassociate from the larval integument during or after pupation. Hinton (1955) mentions the use of urticating hairs in the cocoons of at least five moth families and described the behavior of some tropical Lasiocampidae which construct a loose tangle of silk and setae both above and below the cocoon on a twig. These defensive hairs often contain deterrent compounds and can persist in an active state, ready to release these compounds if disturbed, for long periods of time in the soil or leaf litter (Demolin 1971, Battisti *et al.* 2011). This strategy is likely most effective against vertebrate enemies, and examples of human contact with such cocoons causing "cocoon dermatitis" and "lepidopterism" abound in the medical literature (Caffrey 1918, Mulvaney *et al.* 1998, Shenefelt 1991, Balit *et al.* 2004). Interestingly, evidence of the effectiveness of setae for larval defense is mixed, leading Battisti *et al.* (2011) to hypothesize that the fitness benefits of setae may be greater for pupae than for larvae.

Many types of pupae remain in contact with the larval cuticle, rather than detaching completely from it. As an extreme example, coarctate dipterans pupate within the 3rd instar larval cuticle. Midges in the genus *Forcipomyia* are terrestrial and, as larvae, are covered in setae that collect water droplets from humid environments. The setae are impregnated with an unknown hygroscopic substance (Hinton 1955), and the droplets deter ants from attacking *Forcipomyia* larvae, either because they are chemically repulsive or sticky. Upon molting to the pupal stage, the larval cuticle remains attached and retains its ability to form droplets in humid environments and experiments have shown that pupae with the larval cuticle experimentally removed are more likely to be carried into ant nests than those with their larval cuticle still attached. Hinton (1955) observed that vigorous cleaning behaviors performed by ants (*Lasius niger*) were identical whether contacting a larva or the larval cuticle attached to a pupa. Lastly, Hinton (1955) mentions dermestid beetle pupae that do not fully shed their spine-covered larval cuticles, and suggests that

pupae may benefit from the spines of the larval cuticle, even if only posteriorly attached to the pupa, as in some lycaenids.

Many moths impregnate their cocoons with liquid excretions from the Malpigian tubules (Lounibos 1975). One common component of these excretions is calcium oxalate, a common plant defense compound (Ohnishi *et al.* 1968, Francesci & Nakata 2005). These secretions wet the cocoons, which crosslinks sericin silk proteins, thereby tanning the cocoon and catalyzing a transformation into a tough and inflexible object presumed to prevent desiccation and thwart attacks by natural enemies (Lounibos *et al.* 1975 and citations therein).

Fecal materials are used as pupal coverings, both in the cocoons of some insects and by some cocoon-less coleopterans. Insects that incorporate fecal material into their cocoons include species within the lepidopteran families Pyralidae, Choreutidae, Sessidae, and Mimallonidae (Aiello and Solis 2003) and at least one bee, *Bombus attratus* (Mello 1982). Many beetle species use fecal material as larval and, sometimes, pupal coverings. Both Passalidae and some Scarabaeidae use fecal matter in the construction of their cocoon chambers. In both of these families, the use of fecal material is probably due to its mechanical properties and availability in the local environment, and may or may not afford any additional protection (Schuster & Schuster 1985, Sanchez *et al.* 2010). Many leaf beetles (Chrysomelidae) employ fecal shields as larvae, and some of these species retain the shield throughout the pupal stage as well (Olmstead 1994). Some flea beetles (Chrysomelidae: Alticini) use feces and debris to construct puparia, many case-bearing beetles (Chrysomelidae: Camptosomata) pupate within their larval case after sealing it to a substrate, and many, but not all, tortoise and leaf-mining beetles (Chrysomelidae: Cassidinae) retain their larval fecal shields as pupae (Olmstead 1994 and citations therein). Several studies have tested the effectiveness of larval fecal shields against natural enemies, most demonstrating moderate to strong defensive properties against at least a subset of possible invertebrate natural enemies (see reviews by Olmstead 1994, Müller and Hilker 2003). The shields function as camouflage, as a chemical deterrent, or as a physical shield against most mandibulate insect predators. The most successful natural enemies of case bearing larvae are parasitoids and predators with sucking mouthparts, both of which may be better at avoiding detrimental contact with the fecal shield than mandibulate predators. While the defensive function of larval fecal shields has been demonstrated, their efficacy in pupal defense is often assumed, but remains untested (Olmstead 1994, Weiss 2006).

There are many potential benefits of using materials available in the environment in the construction of pupal shelters. Clearly, the use of certain objects from the environment can aid in defense, especially if they are more sturdy or repellent than silk or aid in camouflage. Some insects are unable to produce silk, so are limited to the use of other objects. Even for silk producers, there may be an energetic advantage to using silk to bind large materials because it descreases the silk required to cover the body.

Thus far, most examples of device-based defenses enhance existing physical defenses, such as being better able to prevent intrusion due to tougher cocoons and deterring attacks via spines and chemicals. However, not all methods may function directly against predators. One hypothesized method of indirect pupal protection gained via unique cocoon construction is to appear to have already been consumed by a parasitoid. Hinton (1955) listed four lepidopteran families known to adorn their cocoons with small protrusions that, at least to human observers, resemble the cocoons of braconid parasitoid wasps. The methods of false cocoon construction are apparently similar across families and begin with the creation of pellets by the larva from endogenously produced "dried bubbles." Two or three pellets are often grouped to form a single false cocoon, which is either pushed outward from the interior of the cocoon or attached to the outside before the larva crawls inside (Hinton 1955). At least one ichneumonid parasitoid (*Hyposoter parogyiae*) also creates "false cocoons" (Finlayson 1966). After feeding on its larval lepidopteran host, the mature larva emerges from the ventral side of the host and spins a false cocoon with an open end. The parasitoid larva then re-enters the host larval skin, where it spins its true cocoon. Finlayson (1966) admitted that an attempted explanation based on the available evidence would be "pure conjecture," but suggested that potential hyperparasites might be lured to attack the false cocoon instead of the true cocoon or aid in attachment of the host and pupa to the surface.

Many parasitoids do not spin cocoons on the surface of the host, but their presence can be inferred from exit holes left in the host integument or host cocoon. *Aidos amanda* (Lepidoptera: Zygaenoidea) seem to leverage this signal by creating false exit holes in their cocoons that resemble the exit holes of parasitic Hymenoptera (Epstein 1995). In this species, cocoon construction takes three to four days and the creation of false exit holes was apparent within the first 24 hours of cocoon spinning. The holes were reinforced and eventually sealed from a recessed point within the cocoon. In addition to their resemblance to parasitized cocoons, Epstein

(1995) suggested that the cocoons may also resemble nests of vespid wasps. Experimental studies are still needed to determine if false parasitized cocoons suffer less predation or parasitism than similar cocoons lacking false exit holes (Epstein 1995).

Given the huge variety in cocoon morphology, different methods of cocoon construction lead to different interactions with the abiotic environment. Therefore, methods of cocoon construction could indirectly reduce the risk of natural enemies through the harnessing of solar radiation because increased temperatures within the cocoon accelerate development though the vulnerable pupal stage, which may ultimately reduce the likelihood of detection and consumption by natural enemies. Lyon and Cartar (1996) experimentally placed pupae of two arctic moths, *Gynaephora rossii* and *G. groenlandica* (Lymantriidae), in their natural environments, either with or without their cocoons. Both species orient to the sun similarly, but *G. groenlandica* has a unique double-layered cocoon with a pale outer layer and dark inner layer, while *G. rossii* has a simpler, single-layered brown cocoon. The double-layered cocoon trapped heat and accelerated pupal development of *G. groenlandica* by at least ten days, on average. The single-layered brown cocoons of *G. rossi* did not accelerate development over naked conspecifics. *G. groenlandica* experienced shortened pupal duration, but also increased rates of mortality to avian predators, probably because they were more conspicuous than naked pupae. It remains to be seen whether the potential benefits of faster development, such as increased reproductive success short arctic growing seasons, outweigh the costs of increased predation (Lyon & Cartar 1996).

Choice of pupation site

Because most pupae are immobile for periods of days to months, the decision of where to pupate is important for avoiding both biotic and abiotic causes of mortality. At a macro level, pupae can be found almost anywhere in their natural environments, including in vegetation, soil, and even underwater. Pupae have fewer location constraints than other life stages because they have no need to forage or find mates. However, the safety of a pupation site selected by a final instar larva is limited by dispersal distance, the ability to burrow into substrates, and perceptive ability, each of which are shaped by evolutionary history. On a finer scale, larvae also choose pupation sites based on characteristics of the microhabitat, such as the location within a plant, background color, three-dimensional shape of the site, and orientation, each of which could enhance or impede detection by enemies. Finally, in addition to characteristics of the macro- and

microhabitat, the local density of conspecifics has been shown to influence pupation decisions in some species, inducing dispersal, aggregation, and even delaying pupation in some species.

One potential benefit of pupation site choice is a reduction in the probability of detection or subsequent attack by potential enemies. One method to accomplish this is to choose a site where enemies are unable or unlikely to forage, thereby finding a refuge of enemy-free-space (Jeffries & Lawton 1984, Berryman & Hawkins 2006). As an example of enemy-free-space, suspended cocoons, such those of *Meteorus* species, may afford protection from ants, which are unable to descend the silken cord, and birds, which are unable to manipulate the freely hanging object (Shirai and Mateo 2009). Similarly, *Zygaena filipendulae* typically pupate on grasses or thin stems of various forbs, which are too slender to hold the weight of an insectivorous bird (Žikić *et al.* 2003). Hinton noted that when these insects pupate on a fence, however, they were readily pecked open (Hinton 1955). Given the diversity of predatory foraging strategies and the ongoing co-evolution of predators and prey, the discovery of a location free of all potential enemies may be impossible or at least transient in evolutionary time. In addition to choosing sites inaccessible to enemies, larvae can also leverage pupation site choice to hide from enemies by minimizing the cues available for exploitation by enemies. Many lepidopteran caterpillars undergo an extended period of wandering before choosing an appropriate pupation site, often in a protected location and usually away from larval host plants (Douglas & Douglas 2005). This behavior is thought to distance hosts from kairomones emanating from host plants and frass, which many predators and parasitoids exploit while foraging (Vet & Dicke 1992). Codling moth (*Cydia pomonella*) larvae, for example, travel from the fruit to spin a cocoon and pupate under the bark or in a protected place at base of the host tree (Blomefield & Giliomee 2012).

There are several documented examples of differential predation based on pupa location. One example comes from two species of swallowtail butterflies. *Papilio glaucus* is not chemically defended and pupates at low densities in the leaf litter. *Battus philenor* is chemically defended from some bird predators, but not small mammals, and often pupates exposed on tree trunks and cliffs at densities approximately 2.5 times that of *P. glaucus*. West and Hazel (1982) tested the hypothesis that these pupation site preferences have evolved in response to differential susceptibility to bird predation by experimentally placing pupae of both species at both locations at their approximate natural densities and recording their survival. Indeed, each species attained its highest survival in its “typical” pupation site and there was evidence of density-dependence,

probably resulting from search image formation by small mammals and conditioned taste aversion by some birds when preying upon *B. philenor* (West & Hazel 1982).

Another example comes from an observational study by Waldbauer and Sternberg (1967), who demonstrated that *Cecropia* moth larvae in an urban area spun their cocoons and pupated in either trees or shrubs. Those pupae on shrubs remained well hidden and survived at much higher rates than those on trees, which were almost certainly eaten by woodpeckers and occasionally blue jays. Not only were the pupae on shrubs less visible, they likely also benefitted by being out of the typical foraging habitat of large insectivorous birds. Most pupae were within 30 cm of the ground, many of them being surrounded by stems and nearly covered by grass and fallen leaves. Based on host plant records, the authors assumed that cocoons found in the trees contained larvae that had fed on those trees, while those on shrubs contained larvae that had dispersed from nearby trees. They suggest that this differential survival is a new phenomenon borne of the urban environment and that there is to be strong selection against spinning cocoons on trees in these environments.

Wheelright *et al.* (2017) recently published a thorough investigation of the likelihood of pine sawfly pupal mortality based on several characteristics of pupation site choice, including plant species, branch thickness, and location within, above, or below a branch or fork. They found that over half of all pupae failed to survive the pupal stage and that predators were approximately twice as likely to kill pupae as were parasitoids. Cocoons placed on thin branches and on the underside of branches were more likely to escape predation (mostly by birds), but cocoons were equally likely to be parasitized, regardless of site characteristics.

A study by Lucas and colleagues (2000) tested the hypothesis that larvae of *Coleomegilla maculata* (Coleoptera: Coccinellidae), which are predators of aphids, choose molting and pupation sites that reduce their susceptibility to intraguild predation (IGP). They found that molting and pupating larvae were more susceptible to IGP than mobile larvae and molting or pupating at sites near an aphid colony were most likely to result in death due to IGP. They also found that molting larvae usually remained on the plant, near their aphid prey, yet exposed to IGP. Unlike molting larvae, 90% of larvae left the plant to pupate. The authors suggest that the benefit of remaining close to the aphid resource is more important when choosing a molting site, while the risk of IGP is more important when choosing a pupation site. They suggest that the key

difference is the duration of exposure to the risk of IGP, which is approximately 100 times greater for pupae than for molting larvae.

Pupation site choice can also be leveraged to maximize the effect of morphologically derived visual camouflage. In order to achieve effective camouflage, larvae must also be able to locate and pupate in the appropriate microhabitat. I've discussed how some moth larvae and case-bearing insects use materials from the environment to strengthen their cocoons and cases. If those items are collected from the local environment, this strategy might also help the cocoon achieve visual and chemical crypsis through resemblance to the background.

Butterfly larvae do not build cocoons. Instead, most species spin a small pad of silk, sometimes with support girdles, to which the obtect pupa ("chrysalis") is attached. The extreme inter-specific variation in the color and shape of butterfly chrysalides suggests that selection acts strongly on the appearance of exposed pupae. One way to reduce the strength of this selection pressure on appearance is to minimize visibility through concealment. For example, pierids typically pupate in sites with angles of visibility of $<90^{o}$ (Baker 1970). If the angle of visibility is sufficiently narrow, such as that achieved by a chrysalis formed in a notch or gap, this not only reduced detectability, but also increased the handling time of bird predators, often enough to cause the bird to abandon its efforts (Baker 1970). Further, many butterflies, especially papilionids and pierids, exhibit developmental plasticity where the color of the chrysalis (typically green or brown) depends upon cues perceived as final instar larvae, such as surface color, surface texture, photoperiod, temperature, and humidity (Wood 1867, Poulton 1887, Poulton 1892, Ohtaki & Ohnishi 1967, Clarke and Sheppard 1972, Hazel & West 1979, Smith 1980a, Smith 1980b, see Roff 1996 for review of environmentally-cued polymorphisms). Although the coloration of the exposed pupa is a developmentally determined morphological trait, if there is heterogeneity in the types of pupation surfaces in the environment, achieving crypsis or mimesis depends upon the appropriate corresponding behavioral trait of pupation site choice by the larva. Stable and predictable environments where larvae can develop consistent preferences for a particular type of site are expected to result in the evolution of monotypic pupae, while pupal color polymorphisms are expected to evolve in changing or unpredictable environments where the larvae cannot reliably pupate on a single surface type (Clarke & Sheppard 1972, Wiklund 1975). Following this prediction, the color of polymorphic pupae is often predictable based on season (Wiklund 1975, Stefanescu 2004). Temperate winter habitats of

diapausing butterfly pupae are often mostly brown woody stems, while summer habitats are typically composed of both green foliage and brown woody stems. As expected brown diapausing pupae predominate in the winter and a mix of green and brown pupae are formed in the summer (Stefanescu 2004). The tendency of pupae to match the color of the substrate has been observed in several species with polymorphic pupal coloration and a selective advantage of this trait has been experimentally demonstrated in at least two pierids (*Pieris rapae* and *Pieris brassicae*: Baker 1970) and three papilionids (*Papilio machaon*: Wiklund 1975, *Battus philenor*: Sims & Shapiro 1983, and *Papilio polyxenes*: Hazel *et al.* 1998) by placing pairs of pupae at known locations in the field. Interestingly, in each of these experiments, the selective advantage of color matched pupae was evident against either green or brown backgrounds, but not both. In many cases, this conditional advantage seems to depend upon the macrohabitat pupation site (eg. leaf litter *vs.* above ground) as well as diurnal and seasonal changes in the identity, foraging strategies, susceptibility to sequestered plant toxins, and color perception of local communities of natural enemies (Wiklund 1975, Sims & Shapiro 1983, Hazel 1998).

Solitary pupation

Not only do insects choose where to pupate based on site characteristics, they may also choose when and where to pupate based on the local density of conspecifics. Species with solitary larvae almost always have solitary pupae. While some species with gregarious larvae remain in groups through the pupal stage, many other gregarious species disperse from the group before pupation (Vulinec 1990). One explanation for these patterns is that the evolutionary transition from gregarious larvae to solitary is simpler, and therefore more probable, than the transition from solitary larvae to grouped pupae. The transition from solitary larvae to grouped pupae would require either an active mechanism, such as signaling between post-feeding larvae, or a passive mechanism that results in inadvertent aggregation, such as individuals reacting similarly to a set of environmental conditions. Alternatively, to transition from gregarious larvae to solitary pupae simply requires a relaxation of the cues or the response to cues that provide group cohesion and a roughly equal probability that suitable pupation sites could be in any direction. Another potential explanation for the high frequency of solitary pupae is that pupae are often incapable of, and gain no benefit from, group defense behaviors of larvae and adults, such as shared vigilance, mobbing,

and coordinated motion which function as startling, confusing, or aposematic displays (Krause & Ruxton 2002).

What evidence is there that solitary pupation serves a defensive function? Because solitary larvae are expected to remain solitary as pupae, I'll only mention examples of grouped larvae that disperse away from conspecifics before pupating. As previously mentioned, many lepidopteran caterpillars undergo an extended period of wandering before choosing a pupation site, often in a protected location and usually away from larval host plants (Douglas & Douglas 2005). When larvae disperse from groups, they presumably reduce their detectability by simultaneously disassociating themselves from cues related to the food source, such as visible feeding damage and herbivore-induced plant volatiles, and cues related to conspecifics, such as visibly conspicuous groups and volatiles from excrement. *Nymphalis antiopa* (Nymphalidae) caterpillars feed gregariously on trees, but disperse during the final instar. Besemer and Meeuse (1938) found that after feeding gregariously as larvae, 45 individuals dispersed to cover 2.5 hectares before pupation, an extreme example of over-dispersion that should minimize the association of each larva with relevant kairomones. The wandering phase of many Lepidopteran larvae is well known, but is surprisingly poorly studied, perhaps because finding dispersed pupae in natural environments can prove very difficult.

Post-feeding larval dispersal behaviors of blowflies (Calliphoridae) have garnered more attention than most taxa because of their importance in forensic entomology. The presence of wandering calliphorid larvae or pupae near a corpse can be used to estimate the time of death, assuming that practitioners understand the dispersal capacity of these organisms under various conditions (Greenberg 1990). Gomes and colleagues (2006) reviewed the literature on this topic, which showed that dispersal distance and depth into the soil are influenced by species identity, as well as temperature, humidity, degree of soil compactness, and the presence of other species. Among those species studied, dispersal distances were typically within 6-10 m laterally (though some reached ~30 m), and depth in the soil was typically 2-15 cm with a maximum of 23 cm (Gomes 2006). Dispersing larvae serve as prey for a variety of birds, small mammals, and insects (Putman 1983), and it is often assumed that dispersal is an adaptation that reduces the risk of predation and parasitism, but very few studies have actually tested this hypothesis. The authors of one study that suggested that dispersal behaviors are shaped by natural enemy-induced morality because they found *Calliphora vomitoria* and *Lucilia caesar* disperse exclusively at night, which

exempts them from the diets of the majority of insectivorous birds and insects, which are diurnal (Kocárek 2001).

Many insects perform cannibalism under various circumstances and pupae are especially vulnerable (Elgar & Crespi 1992). The wandering of larvae away from food sources and conspecifics may also serve to avoid cannibalism (Bogner & Eisner 1992). Many species of tenebrionid beetles and some moth pupae (e.g. *Utetheisa ornatrix*) are at risk of cannibalism as pupae and typically wander away from conspecific larvae before pupation (Tschinkel & Willson 1971, Tschinkel 1981, Weaver & McFarlane 1990, Bogner & Eisner 1992). In addition, many tenebrionids delay pupation if exposed to mechanical stimulation which mimics that of nearby conspecifics (Tschinkel & Willson 1971, Tschinkel & van Belle 1976). By delaying metamorphosis to the vulnerable pupal stage, these individuals retain some capacity for self-defense and defense of pupation chambers (Tschinkel 1978). Once a sufficient period without mechanical stimulation has passed, pupation commences. Recently, it was demonstrated that stimulation of *Gnatocerus cornutus* by conspecific and heterospecific beetles had the same effect in delaying pupation, suggesting that the delay may function as a defense against heterospecific predation as well as conspecific cannibalism (Ozawa *et al.* 2015).

Gregarious pupation

Although grouped pupae are less common than solitary pupae, what defensive benefits, if any, accrue to individuals that pupate communally? Oft-cited benefits of group living include increased foraging efficiency and decreased risk of predation and parasitism (Krause & Ruxton 2002). Pupae do not feed, but gregariousness could still decrease their risk of predation and parasitism in several ways, including the increased efficacy of existing defenses when deployed as a group, encounter effects, predator dilution effects, the selfish herd, and the enhanced recruitment of allied heterospecifics. These mechanisms could act alone or in concert to protect pupae, though disentangling their effects may be difficult.

One mechanism by which sessile individuals may benefit from group membership is through the increased efficacy of defenses when deployed as a group. I've already discussed the construction of protective chambers and devices by solitary pupae, and in doing so, described the suspended cocoons of the genus *Meterous*. Interestingly, at least two species of gregarious *Meteorus* parasitoids construct *shared* suspended cocoons; Broods of *M. townsendi* suspend their

cocoons on threads up to 3 meters in length and *M. komensis* builds suspended cocoons with precise, radially symmetric architecture, evenly covered with raised projections from which the adult wasps emerge (Huddleston 1980, Zitani & Shaw 2002). *M. komensis* pupae are attacked by a hyperparasitoid, but because of the spatial arrangement of pupae inside of the communal cocoon, only some of the pupae inside are accessible to the probing hyperparastioid ovipositors. These examples lead to many interesting questions: Why does *M. townsendi* suspend its communal cocoons on such incredibly long threads? How do *M. komensis* build such a precisely organized structure? Are selfish-herd dynamics at play, driving competition amongst the *M. komensis* larvae for central pupation locations (Hamilton 1971)?

Two additional examples of group pupation structures are built by pergid and argid sawflies. Upon completion of feeding as larvae, members of the genus *Perga* descend from their host plant, join, and move *en masse* across the ground "like a single organism," which, from a distance resembles a "gigantic planarian" (Wheeler & Mann 1923, p.9). The best descriptions of this behavior are for *P. affinis* (Froggatt 1891, Froggatt 1918, Carne 1962). In this species, groups of 100 to more than 1000 larvae, each of which may reach up to 7cm in length, rove in a compact ellipsoid formation. They seek the soft soil near the base of a tree during the time of greatest solar insolation, which supports high metabolic activity for digging a subterranean pupation chamber. Once at a suitable site, they begin writhing downward to break the crust of the soil. Once an entry point through the crust has been achieved, the other larvae abandon their individual efforts and join at the site of the breach. They jointly excavate down ~10 cm, often under rough bark or a root. Once inside the subterranean pupation chambers, the larvae spin individual cocoons, which are stacked in a honeycomb-like pattern. The cocoons incorporate soil and frass, and are covered with regurgitated eucalyptus oil, which likely has a defensive function (Froggatt 1918, Costa 2006). It seems the main benefit afforded by these gregarious behaviors is social facilitation; when considering the effort required to break through the crust of the soil, group effort results in a greater likelihood of success in per capita terms than if each fended for itself. Because these larvae have no specialized digging morphology and the crust of the ground is so hard and dry, it is likely that individuals or smaller groups desiccate before succeeding in excavating a chamber (Costa 2006). Any potential defensive function of underground group pupation remains to be seen. One experiment by Fletcher (2009) did not demonstrate any effect of group size on pupal survival, though this study was short in duration and did not expose pupae to natural hazards such

as hard crusted soil or natural enemies. Therefore, the question remains: Do the benefits of group membership extend beyond the excavation phase to include defense, such as through predator dilution effects or eucalyptus-derived chemical defenses?

The sawfly subfamily Dielocerinae (Argidae) constructs oval-shaped, silken structures on the trunks of their host trees in which they pupate. The earliest account of these structures is from Curtis (1844), who coined the term "compound nidus" to describe these structures with a rough silken shell which typically measure 5-15cm across (Curtis 1844, Dias 1976). To build the structure, it seems that a few of the larvae first create the outer silken shell, then all of the larvae build individual pupal cells and cocoons within. A single compound nidus may hold a few dozen pupae, though Dias (1976) reported that groups of >500 larvae sometimes fuse and build structures that cover >300 cm^2. Interestingly, even in the smaller chambers, relatedness between individuals is often low, suggesting that there are often no benefits to kin, and that there are direct benefits of this behavior to individuals (Boraschi 2005).

Several more taxa also construct group pupation structures: *Phenylpera distigma*, a weevil with gregarious larvae, spins communal silken pupation structure on the underside of a leaf. After the loose shelter has been built, each larvae spins its own denser spherical cocoon. Many species of moths spin communal cocoons that are used by larvae as feeding retreats and pupae as cocoons. Many species in the genus *Hylesia* (Saturniidae, ~200 spp.) pupate in groups of a few to ~300 individuals. These species construct large shelters by silking leaves together and, before pupation, these shelters are reinforced with copious amounts of silk and have been described as "tough, dry, and leathery" (Wolfe 1988, Costa 2006). *Eucheira socialis* (Pieridae) create a group pupation structure described by Westwood (1834, p.38) as the "most perfectly formed nest of any lepidopterous insect yet described." To build such a structure, several to several hundred larvae (average in one study = 112) employ a unique mode of silk deposition to reinforce their larval shelter, resulting in a thick intertwined matrix akin to Tyvek (Kevan & Bye 1991). The indigenous Tarahumara people of present-day Mexico have used these shelters for carrying water and storing goods for hundreds of years (Costa 2006). While these chambers are certainly impressive and can easily be assumed to serve protective functions, there is little more than natural history descriptions to support such claims. However, in most cases, by being a member of a group, these insects are able to construct shelters with features that they simply could not produce on their own. Furthermore, because as the surface area to volume ratio

decreases as structures increase in size, each additional larva can contribute more thickness to the shared outer layer, rather than spreading this investment over the relatively high surface area of an individual cocoon.

Aside from building group pupation structures, chemically defended pupae may be able to leverage their combined noxiousness for defense. For example, many midges in the genus *Forcipomyia* retain their deterrent larval cuticles as pupae. The larval cuticle is attached posteriorly, and therefore only potentially protects individuals against attacks from behind. However, these midges arrange themselves in a cycloalexic group formation, each with their larval cuticle facing outward, such that the alternatively-oriented cuticles of the others in the group might decrease the likelihood of attack on any individual from the front or side (Hinton 1955). Groups of chemically defended individuals might also gain protection from group membership through predator learning. Chemically defended *Eumaeus atala florida* and *Eumaeus minyas* (Lycaenidae) pupae pupate in groups and provide rare examples of aposematically colored pupae, further suggesting that predator learning is important in their defense (DeVries 1977, Rothschild *et al.* 1986). I'm unaware of any experimental tests of the hypothesis that chemically defended pupae benefit from group membership, but it is plausible that a predator may only sample one unpalatable member before abandoning the group.

Group pupation may also aid in other forms defensive signaling. Kojima (2015) showed that *Trypoxylus dichotomus* (Coleoptera, Scarabaeidae) are able to accelerate or delay pupation in order to synchronize their pupation with conspecifics and that this plasticity is probably costly (lower pupal weight) to the actors. He did not test the adaptive significance of this behavior, but hypothesized that groups of pupae may be better able to deter invertebrate predators by collectively vibrating in a way that mimics vibrations produced by moles and deters invertebrate intruders (Kojima *et al.* 2012). Kojima acknowledged that synchronous pupation may provide other benefits, such as to synchronize adult emergence and therefore increase mating opportunities.

Individual pupae and other sessile organisms organized in groups can also gain protection through encounter effects and predator dilution effects (Turner & Pitcher 1986, Wrona and Dixon 1991, Fels *et al.* 1995, Johannesen *et al.* 2014). Encounter effects influence the probability of encounter with or detection by an enemy. A group of prey is expected to be proportionally more difficult to detect than individuals, especially by random-search predators (Hassell 1978).

Considering the foraging behavior of enemies is essential for predicting encounter effects, though if traits that function in hiding (e.g. camouflage, mimesis) are more effective when employed by a group than an individual hiding, these traits are also relevant. A study by Sakakibara (2004) showed that *Eurema blanda arsakia* (Lepidoptera: Pieridae) are gregarious in all stages and pupate on the underside of twigs on host plant. When choosing pupation sites, they exhibit aggressive behaviors toward each other, which results in relatively uniformly spaced pupae. The author described the pupae as conspicuous, but, because of their grouping, resembling plant seeds or other plant parts. Although palatable to solider bugs and probably to birds, very few pupae were attacked (Sakakibara 2004). The effect of grouping was not tested, but could lend credence to the hypothesis that their mimesis of plant parts, and therefore survival, is enhanced by their proximity to conspecific pupae.

Once a group is encountered, the predator dilution effect is a statistical phenomenon that occurs if the enemy is unlikely to kill the entire group (Foster & Treherne 1981, Fels *et al.* 1995). As group size increases, each individual becomes less likely to be consumed before the enemy is satiated or egg-depleted. Dilution effects also depend upon the traits of the encountered enemy. For example, if a predator is large or starved, or a parasitoid has a large egg load, these enemies may kill more prey before becoming satiated or egg-limited, weakening the dilution effect. An entertaining and relevant example comes from a study of gull "predation" on croutons (Fels *et al.* 1995). Of course, croutons are not animals, but they do "behave" like a group of immobile pupae in that they cannot actively defend themselves. This study demonstrated the advantage of being a member of even a small group due to the predator dilution effect.

Based on models, Turner and Pitcher (1986) proposed that neither encounter nor predator dilution effects could function without the other, and that the two terms should be combined and renamed "attack abatement." Gallepp (1974) was the first to propose that individual caddisflies, which provide rare examples of solitary larvae that aggregate before pupation, might benefit from membership within groups through encounter and dilution effects. Since then, *Potamophylax cingulatus* and *Rhyacophila vao* have been shown to benefit from the predator dilution effect when paired with chironomid larvae and planaria, respectively (Otto & Svensson 1981, Wrona & Dixon 1991). However, the study by Wrona and Dixon (1991), demonstrated that one effect can function in the absence of the other. As group size increased, so too did encounter rates between *R. vao* and the planaria, yet the predator dilution effect was still strong enough to provide a net

benefit of group membership (Wrona & Dixon 1991). A more recent demonstration of the dilution effect functioning in the absence of reduced predator encounter rates comes from Coccinellid pupae, which suffer mortality from cannibals and intraguild predators and sometimes aggregate in groups of 2-5 in the field (Roberge *et al.* 2016). Gregarious pupation did not affect the probability that intraguild predators and cannibals locate pupae, but mortality was higher for isolated pupae than for grouped pupae, whether in the presence of intraguild predators or cannibals.

We have seen that delaying pupation allows tenebrionids to achieve solitary pupation, yet the same technique could hypothetically be applied to synchronize pupation within groups of individuals of varied maturity in order to amplify group size and increase the dilution effect. For example, imagine that an organism is susceptible to enemies during a limited window of time, such as the time between pupal ecdysis and hardening of the cuticle. If those enemies can only handle a limited number of prey during the sensitive period, pupating as part of a group forces the enemies into a time-limited, rather than egg-limited situation. *Acromis sparsa* (Chrysomelidae, Cassinidae) are known to delay pupation until younger members of the group are ready to pupate, which would enhance the predator dilution effect, if present (unpublished work of Trillo, described in Costa 2006, p.497).

A third mechanism by which gregarious pupation could benefit individuals is through the selfish-herd effect, which is borne out when predators are more likely to attack peripheral group members, creating a benefit to those positioned in the middle of the group. This effect would be most likely to occur in pupal groups where some individuals may be more accessible to natural enemies than others due to their spatial arrangement within the structure. The suspended cocoons of *Meteorus* pupae and compound nidus shelters of Argid sawflies satisfy these criteria and would be interesting systems in which to test for the selfish-herd effect (Zitani & Shaw 2002, Dias 1976).

The final example of enhanced defense by pupal group membership involves allied heterospecifics. Ant-associated (Myrmecophilous) Lycaenid larvae and pupae produce sugary secretions and vibratory signals that are used in communication and collaboration with ants. In return, the ants provide protection from natural enemies. Atsatt (1981) showed that larger groups of these larvae and pupae are more likely to be tended by ants and larger groups require fewer

ants per capita to yield the same level of defense. Therefore, they aggregate in pursuit of enemy-free-space, which is found in close proximity to the ants that protect them (Costa 2006).

Defensive behaviors performed by pupae

This section covers the "fight or flight" responses performed by insect pupae when they come into contact with natural enemies. Although it has long been recognized that most insect pupae are capable of at least some movement (Lubbock 1890), the mobility of most pupae is limited to the abdominal segments. Still, many unique defensive adaptations using only these muscles have evolved across the Endopterygota. These behaviors include directional movement, non-directional thrashing or jerking, articulation of specialized defensive organs, and sound production, allowing the pupa to escape, startle, deter, or injure potential attackers. One difficulty in studying these behavioral responses is the experimenter's ability to recreate realistic stimuli under laboratory conditions. For example, Cole (1959) was clever enough to realize that although stimulating a pupa with a horsehair brush elicited little or no response, contact from the antennae or tarsi of even a dead parasitoid wasp elicited a vigorous response from pupae of *Aglais urticae* (Nymphalidae). Future experiments will be most informative if they employ live natural enemies and several variations of realistic, controlled stimuli that mimic relevant natural enemies.

Evasive movements

When detected by predators, a common strategy among animal prey is to flee, and many pupae leverage the limited mobility of their abdominal segments to attempt to escape or evade potential natural enemies. Perhaps the most mobile insect pupae are the many species of nematoceran dipterans with aquatic pupae, including mosquitos and midges. These pupae often reside at the air-water interface, where they perform gas exchange, but also dive under the surface of the water periodically, especially when disturbed (Burrows & Dorosenko 2014). Mosquito larvae and pupae are attacked by a variety of predators (Chapman 1985), and predator avoidance was one of the earliest hypotheses to explain diving behaviors (Romoser 1975), though during rain, diving may also prevent the disruption of gas in the ventral air space and being washed out of container habitats (Romoser & Lucas 1999). An experiment by Rodriguez-Prieto *et al.* (2006) mimicked aerial bird predation by inserting a stick into a trough containing many *Culex pipiens* pupae. They found that upon the stimulus, pupae closer to the surface fled deeper into the water,

that the density of conspecifics had a negative effect on the distance fled by pupae and that shorter intervals between "attacks" resulted in far more pupae at depths greater than 5cm (Rodriguez-Prieto *et al.* 2006).

Another apparent defensive behavior performed by mosquito pupae is to seek out and rest in concave menisci, which are formed by emergent vegetation or at the edge of a container. Shuey *et al.* (1987) determined that pupae of three mosquito species are able to orient to concave menisci by halting swimming motions upon contact with a vertically oriented object under water, and then floating to the surface, increasing the likelihood of entering the meniscus. Importantly, pupae in menisci were less likely to dive in response to a nearby disturbance than conspecifics in open water, suggesting there may be less risk of predation in a meniscus than in open water (Shuey *et al.* 1987).

Probably the most commonly observed behavior of terrestrial pupae is bending, thrashing, wriggling, or rotational movements, caused by contraction of the abdominal muscles. These movements are performed by many coleopterans (e.g. Hodek *et al.* 2012), but most experimental studies of the effectiveness of wiggling as a defense come from lepidopterans. Whether hanging from a cremaster or hidden in the substrate, many lepidopteran pupae will wriggle spontaneously or when presented with tactile stimulation, as if by a parasitoid or predator (Askew 1971, Roever 1964). Some butterfly chrysalides are capable of such violent movements that would-be parasitoids are unable to maintain a foothold and are thrown into the air. This has been shown for to occur in interactions between an *Apechthis* parasitoid (Ichnuemonidae) and *Nymphalis urticae* (Askew 1971).

For pupae positioned inside of a loose cocoon wiggling has been shown to induce spinning, which may cause parasitoids to be knocked from the pupa, especially if the host has also spun a web around itself (Cole 1959, Askew 1971). Gypsy moth pupae (*Lymantria dispar*) spin a thin cocoon and provide such an example: stimulation by a parasitoid induced repeated spinning and the simultaneousand repeated arching of the posterior segments, causing most parts of the pupa to come into contact with the thin cocoon, thereby disrupting *Brachymeria intermedia* parasitoids before oviposition (Rotheray & Barbosa 1984). These pupae, when inside intact cocoons, responded much earlier to parasitoid attacks and were greatly extended handling times over pupae experimentally removed from their cocoons. In lab settings, increased handling times still often result in parasitism, but in more natural settings, throwing a parasitoid from a host

could prevent parasitism by causing the parasitoid to be unable to relocate the host, inducing the parasitoid to give up, or subjecting it to natural enemies of its own. It is possible that these thrashing movements function as an evasive movement when facing small predators or parasitoids, but as a startle-inducing behavior when facing enemies large enough to secure the whole body of a pupa.

Most butterfly chrysalides are soft and flexible for several hours after pupation, but they become harder, more slippery, and exhibit a reduced range of motion as they age. This morphological change is hypothesized to defend against pupal parasitoids because it can impede a secure foothold and prevent oviposition if the parasitoid cannot puncture the hardened pupal skin. Several ichnuemonid pupal parasitoids experience much lower rates of success against older, hardened chrysalides than younger, softer conspecifics (Cole 1959). Similarly, *Pteromalus puparum* has been reported to only infect Pierid and Nymphalid host pupae within 24-48 hours of pupal ecdysis, though unclear if oviposition and development of offspring after this point is impossible or simply rarely attempted (Takagi 1985, Barron *et al.* 2003). It seems plausible that this shift from a soft to hard cuticle coincides with a shift from behavioral to morphological defenses for some butterflies such as Danaids, which lack mobility once hardened. Some other butterfly pupae, have both a hard cuticle and mobility, and are able to deflect the ovipositors of attacking parasitoids through their combined effects. There may be a tradeoff, however, because mobility requires thin and soft tissues between the abdominal segments, which some parasitoids adaptively exploit (Cole 1959).

Startle-inducing behaviors

Startle involves an abrupt behavioral change in the prey item which then evokes an instantaneous response in the predator. Sudden movements of a previously motionless pupa may not only be evasive, as previously described, but could also startle a predator and cause it to abandon the prey item. Some insect pupae may also startle or confuse natural enemies through the production of sounds or vibrations. For example, *Eumaeus atala florida* has brightly colored (reddish-orange) pupae and stridulates in response to tactile stimulation. This behavior is thought to be a type of startle-inducing response or aposematic sound (Rothschild *et al.* 1986). Because most insect sounds are produced by percussive movement of the insect's body, the independent effects of startle-inducing movements and sounds may sometimes be difficult to separate

experimentally. Regardless, studies of the effectiveness of movements and acoustic or vibratory signals as startle-inducing defenses against a variety predators and parasitoids would be informative, and would complement studies of startle-inducing behaviors and aposematic sounds produced by insect larvae, nymphs, and adults (Rowe & Halpin 2013).

Fighting back

Instead of attempts to evade or startle natural enemies, a third behavioral strategy when encountered by a natural enemy is to fight back. The fully mobile exarate decticious pupae of the Megaloptera (alderflies, dobsonflies, fishflies, 300 known species) and Raphidioptera (snakeflies, 260 known species) are quite capable of fighting back. They are able to use their sclerotized mandibles against enemies, sometimes for months before emergence of the adult (Hinton 1946). Many mecopteran pupae (scorpionflies, 600 species) also have functional mandibles, though it's not clear if they employ them in defense.

Adecticious pupae have evolved several unique strategies and morphologies that capitalize upon the limited movement of abdominal segments. One particularly interesting example is the use of jaw-like, pinching organs along the abdomen called "gin traps," first described by Hinton (1946). Gin traps are modified, sclerotized, telescoping edges of abdominal segments. They are arranged medially or laterally on the abdomen and occur in several Coleoptera and Lepidopteran families (Hinton 1955, Crowson 1981, Bouchard & Steiner 2004). *Manduca sexta* and *Tenebrio molitor* have been used as model organisms for the study of gin traps, though several other taxa have also been studied (Hinton 1946, Bate 1973a,b,c, Lemon & Levine 1997, Bouchard & Steiner 2004, Ichikawa *et al.* 2012). Weak tactile stimulation of short sensilla near the intersegmental "jaws" of the gin trap results in a rapid and powerful contraction and closure of the hardened plates, which have the potential to crush, pierce, or send a painful signal to small predators or parasitoids (Hinton 1946, Ichikawa 2012). At least some species are capable of closing individual gin traps or all 2-8 traps simultaneously. The rapid closure of the traps usually only lasts a fraction of a second and often causes the rest of the pupa to snap upward, if attached to a substrate, or locomote a small distance, if unattached (Hinton 1946, Eisner & Eisner 1992, Ichikawa *et al.* 2012). Many have contributed to understanding the neurobiology and mechanisms of gin trap closure, but it seems only two studies have attempted to measure the effectiveness of gin traps as a defense mechanism against true natural enemies. At

the same time as he first described three types of gin traps in six different families of beetles, Hinton (1946) used *Dermestes maculatus* and *Tenebrio molitor* to provide preliminary evidence of their effectiveness against cannibalism, a predatory beetle, and mites. Though not particularly rigorous by today's standards, these experiments stood as the only test of the effectiveness of gin traps against live enemies until a study of the operation and defensive function of gin traps a coccinellid, *Cycloneda sanguinea*, against two types of ants, *Solenopsis invicta* and *Aphaenogaster albisetosa* (Eisner & Eisner 1992). They found that individual pupae responded to encounters with ants by snapping all of their gin traps closed (observed by the "flipping" motion of the attached pupae) 55-100% of the time. Multiple flips were often observed per encounter, and most ants simply departed at the onset of flipping. A few ants were flung away from the pupae, and at least occasionally, ant antennae were caught in the gin traps.

On a related note, a few other potentially defensive behaviors of beetle pupae can be elicited via mechanical stimulation, including a dorsoventral flexion of the abdomen, circular rotations of the entire abdomen (reviewed by Bouchard & Steiner 2004). Additionally, in response to a specific experimental stimulus, *Tenebrio molitor* has been shown to perform a delayed sequence of behaviors including basic movements, vibrations, circular rotations, and wiggling movements (Ichikawa & Sakamoto 2013). While a defensive function of each of these behaviors is plausible, only the circular rotations of the abdomen have been tested and proved a partially effective deterrent against cannibalism in *Tenebrio molitor* (Ichikawa & Kurauchi 2009).

Chrysomelids' passive pupal fecal shields have already been discussed in the context of protective chambers and devices, but some members of this group use fecal shields and larval cuticles in active defenses as well, thrashing and waving the shield in response to tactile stimulation. For example, members of the genus Cassida employ this strategy, waving the last larval cuticle which contains two large spines (Hinton 1951). Some chrysomelid pupae are even able to deploy glandular chemical defenses from the retained larval cuticle, which retains its intact and functional exocrine glands (Hinton 1951). Pupae of *Chrysomela scripta, C. tremula, C. cuprea* and *Plagiodera versicolor* have each been shown to jerk suddenly in response to slight stimuli, forcing defensive salicylaldehyde secretions from the retained larval exocrine glands. This motion is achieved by contracting the dorsal longitudinal muscles of the abdomen, so that the pupa presses against the larval cuticle, and has been shown to prevent Argentine ants from feeding on *C. scripta* pupae (Wallace & Blum 1969).

Protection derived from the behaviors of others

Protection derived from conspecifics

Besides the previously mentioned numerically-derived benefits of gregarious pupation, most examples of protection of pupae by conspecifics come from the eusocial ants, bees, wasps, and termites. In eusocial insects, defensive behaviors performed by worker adults serve to protect the entire brood from natural enemies. For example, Japanese honeybees swarm invading giant hornets, leveraging their slightly elevated temperature and carbon dioxide tolerances in attempts to cook and asphyxiate the hornets before they can kill the adults and raid the brood (Sugahara & Sakamoto 2009). In many examples, eusocial workers sacrifice themselves in defense of their kin (Shorter & Rueppell 2012). The protective benefits of these behaviors to pupae are inarguable, but their selective advantage cannot be ascribed to pupae alone. Additionally, because thoroughly detailed accounts of defense mechanisms in social insects exist, I'll mention only two examples particularly relevant to pupal defense (Wilson 1971, Hermann 1981ab, Hermann 1984, Costa 2006).

Like many eusocial hymenopterans with annual lifecycles, at the beginning of a growing season, *Polistes chinensis antennalis* foundresses build nests, lay eggs, and then rear their offspring. Foraging outside of the nest leaves her offspring at risk of predation, especially cannibalism from nearby foundresses. Furuichi and Kasuya (2015) have shown that these foundresses invest most in the success of their more mature offspring. This investment comes in the form of a highly variable pulp-based cap placed over pupal cocoons. Predators were much more likely to attack pupae with smaller or non-existent pulp caps, and foundresses built the largest caps over her most mature offspring and smaller or no caps over her least mature offspring. This behavior has likely evolved because the productivity of a colony is limited by the number of individuals able to contribute, the first adults to emerge are particularly valuable to the success of the colony and because the foundress is limited in her ability to invest in pulp caps (Furuichi & Kasuya 2015).

A less convincing, but still suggestive example comes from sub-social passalid beetles. Most sub-social insects do not continue to care for their offspring past the larval stage, but passalids pupate within chambers made of tightly packed excrement and shredded wood (Schuster & Schuster 1985, Valenzuela-González 1993). These chambers are largely constructed

by any adults in the brood chamber, though older beetles are more likely to participate. Adults pile detritus on top of the pre-pupa and shape the chamber from the outside and repair the chambers if damaged. These cells have been hypothesized to defend against cannibalism, predation, and parasitism, but unfortunately, experimental evidence for any such functions is lacking (Miller 1932, Gray 1946, Schuster & Schuster 1985).

Protection derived from heterospecifics

Finally, it is interesting to consider ways in which pupae gain protection by the actions of members of another species. I present two classes of this type of defense; one in which behaviors are performed by allied species, and another derived from manipulation of hosts by parasitic insects.

Many species of lycaenid butterflies that are associated with various ant species in relationships that range from parasitism to mutualism (previously mentioned in the section on gregarious pupation, see Pierce *et al.* 2002 for review). While ants physically confront the natural enemies, behaviors of the lycaenid pupae are also important because they facilitate recruitment of the ants (Cottrell 1984, Brakefield *et al.* 1992). The pupae of myrmecophilous lycaenids are unique from most other lepidopterans in two ways; they have glands called pore cupola that produce sugar-rich secretions which are consumed by tending ants, and they stridulate, allowing for acoustic and vibrational communication with their associates (first reported by Kleeman in 1774, see Downey 1966). Research has shown that larvae and pupae use a combination of chemical, behavioral and secretory signals to maintain groups of ants that protect them from natural enemies while producing sugar rich secretions which the ants consume. Many ants also stridulate, and pupal stridulation songs are thought to simultaneously alarm and pacify the ants so that the ants are in an agitated state, ready to attack enemies, but not the pupa (Wilson 1971). Compared to muted caterpillars, stridulating caterpillars suffer lower rates of parasitism by two larval parasitoids and one larval-pupal parasitoid (Pierce & Mead 1981, Pierce *et al.* 1987). Stridulating pupae have garnered less attention than their larval counterparts, but one study has shown that pupae allowed to stridulate normally do so in response to tactile stimulation and in order to recruit ant attendants (Travassos & Pierce 2000), and another study has shown that stridulating pupae survived at higher rates than muted conspecifics (Pierce *et al.* 2002). Some

species of ants are even known to carry pupae deeper into the nest, which affords them further protection enemies foraging near the nest entrance (Brakefield *et al.* 1992).

Many parasitic flies and wasps are themselves attacked by hyperparasitoids while still in their host. As result, there is strong pressure on koinobiont insect parasitoids to evolve the ability to manipulate the behavior of their still-living hosts in ways that decrease the likelihood of hyperparasitism (Poulin 2010). Brodeur and McNeil studied a system in which parasitic wasps of (*Aphidius nigripes*) use their aphid hosts (*Macrosiphum euphorbiae*) as vehicles in their choice of pupation site, causing their hosts to leave the colony before the parasitoid pupates and mummifies the host. These authors showed that parasitized aphids wander to different microhabitats depending on whether the wasp larva inside will enter diapause as a pupa or immediately begin developing into an adult and, more importantly, that pupation in these microhabitats significantly decreases the parasitoids' likelihood of attack by a hyperparasitoid and (Brodeur & McNeil 1989, 1992).

Even more dramatically, several braconids are known to induce their partially-consumed hosts to act as bodyguards, protecting the pupae from potential predators and parasitoids as their complete their development. A few examples: After *Glyptapanteles* sp. exit their caterpillar host (*Thyrinteina leucocerae*) to pupate, the host stops feeding, remains close to the pupae, and sometimes spins silk over them. The host remains still unless disturbed, which prompts it to violently swing its head at potential predators, knocking them away (Grosman *et al.* 2008). *Cotesia melanoscela* entangles the host's (*Lymantria dispar*) prolegs as it spins its pupal cocoon, yet the host remains alive and continues to twitch when disturbed; Laboratory experiments have shown that the number of attacks by hyperparasitoids was lower for those parasitoids that remained attached to the host larva than for those whose host was experimentally removed (Gross 1993). Similarly, *Microplitis* species entangle the caudal appendages of their noctuid host, *Mythimna separata*, into their pupal cocoons. The tethered caterpillars react aggressively when disturbed, and in choice tests, significantly fewer attended *Microplitis* cocoons were attacked by a hyperparasitoid, *Gelis agilis*, than unattended cocoons (Harvey *et al.* 2011). Finally, *Dinocampus coccinellae*, a parasitoid of a lady beetle, *Coleoomegilla maculata*, also spins a cocoon beneath its host and entangles its legs and cocooned pupae under a living bodyguard were less susceptible to parasitism than those without a bodyguard or those with a dead bodyguard, though it was not

clear what behaviors, if any, were performed by the live hosts (Maure 2011). These behavioral manipulations are a classic example of an extended phenotype (Dawkins 1982).

Conclusions and future directions

Insects as prey items have long been a topic of interest to researchers in basic and applied fields for their experimental tractability, economic importance, and diverse defenses. When considering behavioral defenses, pupae have received less attention than larvae and adults, perhaps because pupae have limited behavioral repertoires or because they are cryptic and difficult to study in the field. Pupae are vulnerable to threats from predation, parasitism, pathogens, and abiotic factors. Any traits that confer increased pupal survival and fitness would be selected for, so that pupae employ diverse approaches to defense should not be a surprise. The literature reviewed here demonstrates that behavioral defenses of late-instar larvae, pupae, and allied organisms may be performed pre-emptively (such as building defensive shelters) or immediately in response to attacks by natural enemies. Importantly, insects protect themselves as pupae by investing heavily in proactive defensive behaviors, which are often performed hours, days, or weeks before a potential attack. These often-costly behaviors have largely been ignored, but can only be explained in an adaptive context if benefits are accrued by a later life stage. Despite the diverse array of behaviors described here, I have likely overlooked many in the literature, and many others undoubtedly remain undiscovered.

Each of the behavioral traits of pre-pupae, pupae, and associated organisms described here at least plausibly functions in defense against would-be natural enemies of insect pupae, yet well-designed experimental studies are necessary to move beyond adaptive "just-so" stories and improve our understanding of the effectiveness of defensive behaviors. Conducting these experiments can be difficult and time-consuming, though perhaps less-so today than in Howard Hinton's time. Controlled experiments can be difficult because they often require maintaining multiple trophic levels under lab conditions. Testing the efficacy of a behavior against many types of potential enemies further complicates the logistics of such experiments. Field studies are made difficult due to the potential difficulty in finding wild pupae, the capriciousness of nature and difficulty in interpreting results. Fortunately, improvements in video and audio recording technologies have allowed behaviorists to collect more extensive and informative data. A rarely-utilized but potentially informative technique is to lab-rear insects until they are final instar larvae

or pupae, place them in the field, and video-record interactions or infer causes and frequency of mortality from recaptured pupae. Advancements in field-based tracking methodologies, such as UV paints and radio- and radar-based tracking devices, could inform the role of pupation site choice in interactions with natural enemies (Kissling *et al.* 2014, Rice *et al.* 2015). Finally, difficulty in interpretation of these studies arises from the fact that we may never be able to clarify the evolutionary history of specific behavioral traits. Single behaviors may serve multiple adaptive functions and their current adaptive value is not necessarily indicative of their evolutionary origin or initial adaptive value (Gould & Lewontin 1979). Regardless, it is valuable to consider the functionality and ecological significance of traits in their current contexts in addition to their evolutionary history.

Despite the difficulties, behavioral defenses that protect pupae are important to recognize and study for many reasons. First, the importance of pupal mortality is obvious and has been demonstrated in lepidopteran population dynamics (e.g. Varley & Gradwell. 1960, 1970). Understanding these defenses could improve our estimates of stage-specific mortality, which could then be incorporated into existing models of predatory-prey interactions, insect population dynamics, and the community ecology of natural and agricultural systems. Secondly, further study into this topic will lead to a phylogenetically informed representation of pupal traits, which will likely reveal patterns in ecological pressures that drive the evolution of certain defenses and lend insights into the co-evolutionary history of insect pupae and their natural enemies. Finally, recognizing these behaviors may help to explain the evolutionary success of the Endopterygota. Given the diverse examples collated here, it seems possible that the often-presumed cost of increased mortality by natural enemies during the pupal stage may be lower than expected because of these often-overlooked behavioral defenses. Furthermore, the diversity of the Holometabola indicated that the study of pupal defenses, especially the role of behavior in pupal defense, is an area of entomology and behavioral ecology that is ripe for progress. My hope is that keen observations will inspire well-designed experimental studies, which are essential for beginning to quantify the significance of the myriad roles of behavior in the defense of insect pupae.

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