The Ecology and Evolution of Cycads and Their Symbionts

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

<table>
<thead>
<tr>
<th>Citation</th>
<th>Salzman, Shayla. 2019. The Ecology and Evolution of Cycads and Their Symbionts. Doctoral dissertation, Harvard University, Graduate School of Arts &amp; Sciences.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citable link</td>
<td><a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:42013055">http://nrs.harvard.edu/urn-3:HUL.InstRepos:42013055</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA">http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA</a></td>
</tr>
</tbody>
</table>
The ecology and evolution of cycads and their symbionts

A DISSERTATION PRESENTED
BY
SHAYLA SALZMAN
TO
THE DEPARTMENT OF ORGANISMIC AND EVOLUTIONARY BIOLOGY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE SUBJECT OF BIOLOGY

HARVARD UNIVERSITY
CAMBRIDGE, MASSACHUSETTS
AUGUST 2019
The ecology and evolution of cycads and their symbionts

ABSTRACT

Interactions among species are responsible for generating much of the biodiversity that we see today, yet coevolved associations with high species specificity are rare in nature and have sometimes been considered to be evolutionary dead ends. The plant order Cycadales is among the most ancient lineages of seed plants, and the tissues of all species are highly toxic. Cycads exhibit many specialized interactions, making them ideal for analyzing the causes and consequences of symbiotic relationships. In Chapter 1, I characterize the pollination mutualism between Zamia furfuracea cycads and their Rhopalotria furfuracea weevil pollinators. I find that pollination in this New World species pair closely mirrors that of an Old World cycad and its thrips pollinators, and that this strict, pair-wise interaction is ancestral to the group. Molecular phylogenetics and fossil evidence suggest that it represents one of the earliest insect/plant pollination mechanisms, arising long before the evolution of visual signaling commonly employed by flowering plants. In Chapter 2, I identify systematic patterns in the gut bacteria of cycad herbivores. I survey the gut microbial communities of five insect species feeding on a diversity of tissues of cycads and collected world-wise, and discover a set of core bacteria that is shared amongst cycad herbivores and not found in their non-cycad feeding relatives. Some of these microbes have known anti-cancer and nitrogen-fixing capabilities, and may function to facilitate herbivory of these toxic plants. This is the first report of a core-microbiome amongst distantly related organisms that feed on a common plant family. Finally in Chapter 3, I synthesize all published literature on cycad feeding Lepidoptera and evaluate their diets and ecology in a phylogenetic framework. Cycad feeding has independently evolved multiple times from angiosperm-feeding ancestors, representing a striking shift in host plant morphology and chemistry. As most cycad specialists are warningly colored and many are known to sequester cycad toxins, this presents an ideal system to further explore potential mechanisms of plant/insect coevolution.
“I consider cycads to be the Rosetta Stone for plant biology due the the wealth of information stored within them”

Knut Norstog

“Cycads are the coolest plants in the world”

Me
# Contents

Abstract .......................................................... iii
Acknowledgements .............................................. vii
Introduction ....................................................... 1

1 An ancient obligate pollination mutualism in cycads .......... 7
   1.1 Introduction ............................................... 7
   1.2 Materials and methods .................................... 10
       1.2.1 Experimental design ............................... 10
       1.2.2 Study species ....................................... 10
       1.2.3 Volatile collection .................................. 11
       1.2.4 Gas chromatography mass spectrometry .......... 12
       1.2.5 Electrophysiological analysis: GC-EAD .......... 13
       1.2.6 Weevil expulsion from pollen cones .............. 14
       1.2.7 Behavioral analysis: Pit-tests of 1,3-octadiene concentrations ................................................. 15
       1.2.8 Behavioral analysis: Weevil movement in relation to 1,3-octadiene concentrations .......................... 16
       1.2.9 Phylogeny .............................................. 19
   1.3 Results .................................................. 22
   1.4 Discussion .............................................. 30

2 Cycad-feeding insects share a core gut microbiome .......... 32

3 Ecology and Evolution of Cycad-Feeding Lepidoptera ....... 44
   3.1 Introduction ............................................... 44
   3.2 Lepidopteran Cycad Herbivores ......................... 47
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3 Aposematism and Defensive Ecology</td>
<td>59</td>
</tr>
<tr>
<td>3.4 Evolutionary Origins of Cycadivory</td>
<td>64</td>
</tr>
<tr>
<td>3.5 Hostplant Use</td>
<td>68</td>
</tr>
<tr>
<td>3.6 Management of Cycadivorous Species</td>
<td>72</td>
</tr>
<tr>
<td>3.7 Discussion</td>
<td>74</td>
</tr>
<tr>
<td>3.8 Conclusions</td>
<td>80</td>
</tr>
<tr>
<td>Concluding Remarks</td>
<td>82</td>
</tr>
<tr>
<td>A Supplement Chapter 1</td>
<td>85</td>
</tr>
<tr>
<td>B Supplement Chapter 2</td>
<td>88</td>
</tr>
<tr>
<td>C Supplement Chapter 3</td>
<td>92</td>
</tr>
<tr>
<td>C.1 Evaluating hostplant records</td>
<td>92</td>
</tr>
<tr>
<td>C.2 Cycadivorous Lepidoptera species synonyms</td>
<td>101</td>
</tr>
</tbody>
</table>

**Bibliography**                                                   | 102  |
Acknowledgements

As with a child, it takes a village to raise a PhD thesis and the student completing it. And as such, this work would have been impossible without the kindness, support, and guidance of many people. First and foremost I thank my advisors, Drs. Naomi Pierce and Robin Hopkins. Dr. Pierce has been a guiding force in my personal, professional, and educational life since before even the start of the degree program and in every sense of the word, is truly an excellent mentor. Dr. Hopkins embraced my interest and desire to join her lab group with open arms and has significantly guided the scope and direction of this work as with all students she works with. Both Dr. Pierce and Dr. Hopkins have been endlessly supportive of me personally and my independent research program. Together, they have challenged me, encouraged me, and made me laugh. I cannot thank either of them enough for being such excellent examples of scientist, mentors, and human beings.

Similarly I thank my thesis committee, Drs. David Haig, Brian Farrell, and Dennis Stevenson. Their perspectives and advice are clearly visible in the following work. I have been so lucky to have such a brilliant and supportive team to work with. Outside of campus, I have had the invaluable support and guidance from Drs. William Tang, Irene Terry, Chelsea Specht, Angélica Cibrián-Jaramillo, and Francisco Barona-Gómez. Drs. Thiago André and Amanda Mortati have been more than collaborators and field guides, they are both brilliant scientists and mentors, excellent friends, and inspirational examples of humanity.
My office mate and the administrative assistant in the Pierce lab, Maggie Starvish, has been my on campus family. She has kept me sane and laughing as well as kept my research supplies and reimbursements coming. She is always available to discuss all things Harvard, kitty, or moral. She is an integral part of both my time at Harvard and the ability to accomplish research. I am thankful for her presence on a daily basis.

Many people assisted in the actual data collection or analysis that deserve extra acknowledgement. Dr. Melissa Whitaker has had her hand in almost every thing I have done and has established herself as not just a collaborator, but a mentor and friend as well. Her guidance has been priceless and life changing. Dr. Damon Crook at the USDA was an off campus collaborator for the entirety of my thesis and provided the opportunity for a complete line of work. Dr. James Crall provided a unique perspective and direction to this thesis. The staff at the Ernst Mayr library of the Museum of Comparative Zoology, especially Mary Sears, deserves thanks for their diligence and detail in hunting down obscure references. Dr. Steven Worthington at the Harvard Institute for Quantitative Social Science assisted with statistical analyses. Most of this work was done at the cycad conservation garden, Montgomery Botanical Center, where their dedicated and knowledgeable staff have helped in endless ways. At Montgomery Botanical Center, I thank Dr. Patrick Griffith, Dr. Michael Calonje, Claudia Calonje, Vicky Murphy, Tracy Magellan, Dr. Joanna Tucker Lima, and Xavier Gratacos for their dedication to both scientific research and to these incredible plants.

I thank all the current and former members of the Naomi Pierce and Robin
Hopkins labs including Jon Sanders, Lori Shapiro, Leonora Bittleston, Jack Boyle, Chris Baker, Marjorie Lienard, João Tonini, Wei-Ping Chan, Richard Childers, Mark Cornwall, Kadeem Gilbert, Evan Hoki, Zhenyang Wang, Matt Farnitano, Federico Roda, Ben Goulet, Franchesco Molina-Henao, Austin Garner, Henry North, Heather Briggs, Sevan Suni, Callin Switzer, and Diana Bernal-Franco. I specifically thank Rachel Hawkins of the Pierce lab for her detailed help with museum collections and protocol as well as her endlessly cheerful attitude and persistent intellectual curiosity.

Finally, I thank my family. My parents, Randy Salzman, Jeanne Liedtka, Debra Brown, and Jim Brown have been endlessly supportive of this work both professionally and personally. As have my brothers Quinn Warner and Holland Brown and my extended family, Ginger and Dan Schaible. Most importantly, I thank my husband Rory Maher and daughter Laurel Maher. Rory has supported me and my dreams for almost two decades, providing much needed emotional support, child care, laughter, and stress relief. He is truly my rock and everything I do in life I do better because of and with him. Laurel has kept me present and always aware of the most important things in life. They are both a blessing in my life and my work and I am endlessly grateful for them.
Dedicated to Rory Maher without whom none of this would have been possible
Introduction

Relationships are endlessly fascinating to us, and the causes and consequences of organismal interactions have long captivated naturalists. Darwin highlighted the importance of species interactions as agents of natural selection (Darwin, 1859), even writing an entire book on coevolution of orchids and their pollinators (Darwin, 1862). More than a century later, Ehrlich and Raven, 1964 described the coevolution of insects and plants as being responsible for generating the wealth of biodiversity as we know it. Despite their intrigue, strictly coevolved systems that involve a high degree of specialization by both partners are not common in nature and have sometimes been argued to lead to evolutionary dead ends (Vamosi, Armbruster, and Renner, 2014). A considerable body of work, however, suggests that specialized lineages may be able to thrive and diversify under natural selection (Day, Hua, and Bromham, 2016 and see Futuyma and Moreno, 1988). Controversy still exists regarding the potential evolutionary trajectory of these interactions as well as the degree to which they are mediated by third party associations such as other insects or microbes whose roles have been less tractable to study until now.

The order Cycadales is among the most ancient lineages of seed plants, with a fossil record dating back over 265 my (Gao and Thomas, 1989). These dioecious gymnosperms dominated the terrestrial landscape (Taylor, Taylor, and
Introduction

Krings, 2009) and exhibited specialized insect pollination during the Mesozoic era, well before the rise of flowering plants (Cai et al., 2018). While each of the ten living genera have persisted through geological time, the 350 modern species of cycad are reported to have arisen during synchronous global radiations in each genus ca. 10-15 million years ago (Nagalingum et al., 2011; Salas-Leiva et al., 2013; Condamine et al., 2015). Throughout this time, cycads have maintained specialized relationships with insects (Cai et al., 2018; Terry et al., 2012a), making it a particularly suitable system in which to address the mechanisms underlying the origin and maintenance of specialized species interactions.

The unique nature of toxicity (Vega and Bell, 1967; De Luca et al., 1980; Moretti, Sabato, and Gigliano, 1981; Moretti, Sabato, and Gigliano, 1983) exhibited by these plants may be responsible for both their evolutionary longevity and the specialized nature of their species relationships. All cycads produce two classes of carcinogenic and neurotoxic compounds, BMAA and MAM (Vega and Bell, 1967; De Luca et al., 1980; Moretti, Sabato, and Gigliano, 1981; Moretti, Sabato, and Gigliano, 1983). B-methylamino-L-alanine (BMAA) is a non-protein amino acid that can be misincorporated into proteins, altering their structure and function (Dunlap, Banack, and Rodgers, 2013). BMAA also disrupts glutamate receptor function with toxic effects reported in plants (Brenner et al., 2000), insects (Goto, Koenig, and Ikeda, 2012) and mammals (Spencer et al., 1987; Kisby, Moore, and Spencer, 2013). BMAA is the source of ongoing medical research as it has been shown to produce symptoms similar to amyotrophic lateral sclerosis (ALS) and Alzheimer’s disease (Coz, Banack, and Murch, 2003).
Introduction

The other class of cycad toxins is methylazoxymethanol (MAM). The carcinogenic and neurotoxic (Morgan and Hoffman, 1983; Laquer and Spatz, 1968) effects of MAM result from its spontaneous degradation into formaldehyde and methyl-diazonium, a potent methylating agent (Morgan and Hoffman, 1983). MAM toxicity has been described in yeast, bacteria, insects, plants, and mammals (Morgan and Hoffman, 1983). The presence of these two potent toxins makes cycad tissue a distinctive food source and only a handful of specialized insects are able to feed on cycad tissues.

Indeed, it appears that most species interactions involving cycads are highly specialized. For instance, the parasitic butterfly genus *Eumaeus* obligately feeds on *Zamia* cycad leaves and can do great damage to the plants. These butterflies in turn sequester *Zamia* plant toxins into their own tissues for defense against natural enemies (Rothschild, Nash, and Bell, 1986). Early work done by Norstog, Stevenson, and Niklas, 1986 and Tang, 1987b gave rise to an avalanche of cycad insect pollination studies until the paradigm shifted from a belief in strictly wind pollination for the group (Chamberlain, 1919) to an understanding that the system involved obligate insect pollination (Terry et al., 2012a). Norstog and Fawcett, 1989 first identified the specific and codependent nature of the pollinator relationship by showing that the *Rhopalotria* weevil pollinators live their entire lifecycle within the pollen cone tissue, staying faithful to their host plant species and even going dormant during the 10 months of the year when the plant does not have a pollen cone.

In Chapter 1, I investigate the mechanism of pollination mutualism between
Introduction

Rhopalotria furfuracea and Zamia furfuracea first described by Norstog, Steven- son, and Niklas, 1986. Specialized coleopteran pollination has existed in the Cy- cadales since before the rise of flowering plants (Cai et al., 2018), yet the mech- anism by which cycads entice pollination services from their coleopteran brood site mutualists remains unknown. I characterize a push-pull pollination mech- anism between a New World cycad and their weevil pollinators that mirrors the mechanism between a distantly related Old World cycad and their thrips pollinators (Terry et al., 2007). The behavioral convergence between weevils and thrips, combined with molecular phylogenetic dating and a meta-analysis of thermogenesis and coordinated patterns of volatile attraction and repulsion suggest that a strict pollination mutualism strategy is ancestral in this ancient, dioecious plant group. As such, it represents one of the earliest insect/plant pol- lination mechanisms, arising long before the evolution of visual floral signaling commonly employed by flowering plants.

Chapter 2 was published as an article in the Biological Journal of the Lin- nean Society (Salzman, Whitaker, and Pierce, 2018). In this chapter I identify a third potential level of specialization in the gut bacterial community of cy- cad feeding insects. I investigate the gut microbial communities of five insect species from across a diversity of cycad genera, feeding tissues, and geography, and uncover a set of core bacteria that is shared amongst cycad herbivores and not found in their non-cycad feeding relatives. The presence of a set of core bacter- aria, including a bacterium with known anti-cancer and nitrogen-fixing ca- pabilities, suggests that they are important in helping cycad herbivores detoxify their poisonous host plants. This is the first reporting of a set of core bacteria
shared amongst herbivores of a group of phylogenetically and chemically related plants.

Chapter 3 is a review of cycad feeding Lepidoptera. Cycads are chemically defended gymnosperms with highly specialized insect associations that include both pollinators and herbivorous parasites. Among parasitic species, the larvae of many moths and butterflies (order: Lepidoptera) feed exclusively on cycads, despite the presence of neurotoxic and carcinogenic compounds. The phylogenetic distribution of cycadivorous Lepidoptera suggests that cycad feeding has evolved independently in at least 7 lepidopteran families, with 36 substantiated reports of cycadivorous species across 12 genera. All cycad-feeding species appear to have evolved from angiosperm-feeding ancestors, such that shifts to cycads may represent particularly striking changes in phytochemistry relative to host switches within angiosperms, yet very little is known about the biology of these species. This review synthesizes published information about the ecology and evolution of cycadivory among Lepidoptera while highlighting issues relevant for species conservation and management and identifying outstanding questions and areas for future research.

It is thanks to the pioneering work of Knut Norstog, Pracilla Fawcett, Dennis Stevenson, Willie Tang, and Irene Terry that cycad mutualisms have gained attention and my work stands on the backs of these giants. Chapter 1 was done in collaboration with Damon Crook of the USDA who assisted in EAD-FID and GCMS and James Crall at Harvard who assisted with video analysis of weevil behavior. Chapters 2 and 3 were done in close collaboration with Melissa Whitaker while she was a postdoctoral fellow first at Harvard and later at ETH.
Introduction

Zurich. All of the unpublished chapters are written in the format for submission for peer-review publication. I conclude with a few remarks on future directions arising from this work.
Chapter 1

An ancient obligate pollination mutualism in cycads

Note: Supplemental materials included in Appendix A.

1.1 Introduction

 Highly specialized coevolved systems are uncommon in nature and are sometimes considered evolutionary dead ends (Vamosi, Armbruster, and Renner, 2014). However, the plant order Cycadales is one of the oldest living lineages of seed plants (Chaw et al., 2000) and most species form obligate brood site pollination mutualisms with insects (Terry et al., 2012a; Tang, 2006). Here, we investigate whether unrelated pollination mutualists exhibit matching behavioral responses to conserved plant behaviors representing a shared pollination strategy. The push-pull pollination mechanism of the Australian cycad, Macrozamia lucida is characterized by a daily peak in plant volatile production whereby thrips pollination mutualists are initially attracted to lower levels of a plant volatile
Chapter 1. An ancient obligate pollination mutualism in cycads

and later repelled by high levels of the same compound (Terry et al., 2007). Across the Cycadales, reproductive cones undergo a daily cycle of thermogenesis (Tang, 1987a) and respiratory processes that similarly culminate in a peak of volatile production (Terry et al., 2016). By analyzing a distantly related New World cycad species that, like most cycads, is associated with a coleopteran pollination mutualist, we investigate whether this characteristic cycle of thermogenesis and coordinated volatile production is broadly indicative of an ancient and conserved pollination strategy.

Specialized insect pollinators, mostly beetles, live and feed solely within the male pollen cone tissue of Cycadales (Terry et al., 2012a; Tang, 2006; Hall et al., 2004; Donaldson, 1997; Norstog and Fawcett, 1989). Successful cycad reproduction requires these brood site mutualists to leave the host pollen cone and transfer pollen to a female ovulate cone. Over the course of a day, reproductive cones produce a predictable peak in respiration, followed by a peak in thermogenesis, and finally a peak in volatile production, each separated by tens of minutes (Terry et al., 2016). Both pollen and ovulate cones of an individual species produce similar thermogenic and volatile profiles (Tang, 1987a; Pellmyr et al., 1991; Terry et al., 2004; Azuma and Kono, 2006; Proches and Johnson, 2009), yet across all species, pollen cones produce a higher temperature peak (Tang, 1987a) and more concentrated volatile compounds (Terry et al., 2012a; Pellmyr et al., 1991; Terry et al., 2004; Proches and Johnson, 2009). Pollinator exit from pollen cones has been observed to coincide with thermogenic and volatile peaks (Terry et al., 2007; Pellmyr et al., 1991; Terry, 2001; Azuma and Kono, 2006; Wallenius et al., 2012; Norstog, Stevenson, and Niklas, 1986). The specific behavioral mechanism
Chapter 1. An ancient obligate pollination mutualism in cycads

by which these brood site mutualists are repelled from their host pollen cone and enticed into ovulate cones has been described in *Macrozamia lucida* (Terry et al., 2007) where *Cycadothrips chadwickii* thrips (Order: Thysanoptera) are attracted ("pulled") by lower concentrations of the dominant plant volatile compound, -myrcene, and repelled ("pushed") by higher concentrations of the same compound. The daily plant volatile cycle induces pollinator movement between pollen and ovulate cones enabling pollination and seed set (Terry et al., 2007). This push-pull pollination mechanism differs from most pollination syndromes because it has a repulsive component that expels pollinators from pollen cones at a certain point in the cycle in addition to the attractive mechanisms commonly seen in the visual and chemical cues of flowers. Furthermore, the same chemical cue is used for attraction and repulsion.

The Mexican cycad *Zamia furfuracea* (Zamiacea), which lineage separated over 150 million years ago from *Macrozamia lucida*, has an obligate pollination mutualism with *Rhopalotria furfuracea* (Coleoptera: Belidae) weevils (formally R. mollis) (Norstog, Stevenson, and Niklas, 1986). *Rhopalotria furfuracea* complete their entire development within the plants’ disposable pollen cone parenchyma tissue (Norstog and Fawcett, 1989) and the plants do not set seed without pollinators (Norstog, Stevenson, and Niklas, 1986). Specialized coleopteran pollination is widespread in extant Cycadales (Terry et al., 2012a; Tang, 2006) and has existed in the lineage since the mid-Mesozoic (Cai et al., 2018), well before the rise of flowering plants (Niklas, Tiffney, and Knoll, 1983) or the first evidence of possible thrips pollination (Peñalver et al., 2012). While push-pull pollination has also been hypothesized for Coleoptera-pollinated cycads (Valencia-Montoya
et al., 2017; Suinyuy, Donaldson, and Johnson, 2013), the mechanism by which the plants manipulate their behavior to carry out pollination has not yet been explored.

1.2 Materials and methods

1.2.1 Experimental design

The purpose of this study was to determine the insect and plant behaviors responsible for the specific brood-site pollination mutualism seen in Zamia furfuracea cycads and their weevil pollinators, Rhopalotria furfuracea, and to contrast that with the behaviors seen in other cycad pollination systems, including Macrozamia lucida cycads and their thrips pollinators, Cycadothrips chadwickii (Terry et al., 2007). Plant volatile analysis was used to identify the volatile components produced by pollen and ovulate cones. Physiological analysis of R. furfuracea weevils was used to identify the electro-antennally active plant compounds, and behavioral responses of R. furfuracea to plant compounds. Field experiments matched plant volatiles corresponding to weevil behavior in order to confirm laboratory experiments. Finally, thermogenic and volatile patterns across cycad genera were mapped onto a dated phylogeny to provide an evolutionary context.

1.2.2 Study species

Zamia furfuracea is an endangered dioecious gymnosperm native to Veracruz, Mexico (Calonje, Stevenson, and Osborne, 2019). Both pollen and ovulate cones
are known to undergo a daily synchronous thermogenic peak at the time that they are reproductive, with a higher temperature spike in pollen cones (2.5 °C above ambient, ovulate cones 0.8 °C above ambient) (Tang, 1987a). Pollen cones produce two main volatile components, the hydrocarbon 1,3-octadiene and the alcohol linalool (Pellmyr et al., 1991).

*Rhopalotria furfuracea* is an obligate pollination mutualist with *Z. furfuracea* (Norstog and Fawcett, 1989; Norstog, Stevenson, and Niklas, 1986). It is required for *Z. furfuracea* reproduction (Norstog, Stevenson, and Niklas, 1986) and its entire lifecycle is tied to that of its host plant (Norstog and Fawcett, 1989). *R. furfuracea* adults feed, breed, and lay eggs in *Z. furfuracea* pollen cones and larvae feed and develop inside of microsporphylls, pupating inside of the microsporophyll stalk (Norstog and Fawcett, 1989). *R. furfuracea* visit ovulate cones of *Z. furfuracea* but do not feed or lay eggs on them. Late instar larvae go into diapause for 10 months out of the year when plants are not reproductive, remaining inside of the stalks of the dried, dead microsporphylls (Norstog and Fawcett, 1989).

**1.2.3 Volatile collection**

*Zamia furfuracea* plant volatiles collections were performed using headspace collection methods (Schiestl and Marion-Poll, 2002) on three pollen dehiscing male cones and two receptive female cones in situ. Accession information for all plants is found in Table 1.1. Cones remained attached to the plant and covered in oven bags (Reynolds Consumer Products, Lake Forest IL) that were tied shut at the bottom of the cone. Low-flow vacuum air samplers (Gilian model
Chapter 1. An ancient obligate pollination mutualism in cycads

LFS-113DC) calibrated to 0.1 L/min pulled headspace air and volatiles for 45 min through filters made with 300 mg Porapak Q adsorbant mesh 80-100 75 cc (Sigma Aldrich, Saint Louis MO, part number 20331) held into glass pastuer pipettes size 5.75” (VWR International, Randor PA, part number 14672-200) with plugs of glass wool (Sigma Aldrich, Saint Louis MO, part number 20411). Prior to volatile collection, filters were soaked in HPLC grade dichlormethane (Sigma Aldrich, Saint Louis MO, part number 650463) for 48 hours and then prewashed ten times with full flow throughs of dichloromethane and dried with charcoal purified air pushed through at 0.4 L/min. After volatile collection, samples were eluted with 500 ul HPLC grade dicholomethane (Sigma Aldrich, Saint Louis MO, part number 650463) pushed through with charcoal purified air.

<table>
<thead>
<tr>
<th>Cone sex</th>
<th>Accession</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pollen</td>
<td>981474*A</td>
<td>Fully open, releasing pollen</td>
</tr>
<tr>
<td>Pollen</td>
<td>20010214*M</td>
<td>Fully open, releasing pollen</td>
</tr>
<tr>
<td>Pollen</td>
<td>981743*A</td>
<td>Fully open, releasing pollen</td>
</tr>
<tr>
<td>Ovulate</td>
<td>951468*A</td>
<td>Receptive, open at top</td>
</tr>
<tr>
<td>Ovulate</td>
<td>20010214*D</td>
<td>Receptive, open at top</td>
</tr>
</tbody>
</table>

1.2.4 Gas chromatography mass spectrometry

Initial chemical analyses were conducted using a combined Agilent Technologies 6890 network gas chromatograph and 5973 mass-selective detector. The GC
Chapter 1. An ancient obligate pollination mutualism in cycads

was equipped with a DB-5 column (J W Scientific Inc., Folsom CA); 30 m x 0.25 mm I.D.; film thickness, 0.25 m; splitless mode). Helium was the carrier gas at a constant flow rate of 0.7 ml/min. The injector temperature was 250°C. Oven temperature was held at 40°C for 1 min, programmed to 170°C at 10°C/min and held for 5 min. Volatile peaks were manually integrated. Volatiles were identified based on their mass spectra (NIST version 2.0, 2002), Kovats indices (Piji, 1960; Kovats, 1965) and by comparison of the retention indices and mass spectra with those of available authentic synthetic compounds and a computerized data library (NIST version 2.0, 2002). An authentic standard of 1,3-octadiene was obtained from ChemSampco Co. (Dallas, TX, Catalog number 7015.90). Authentic standards of (+/-) linalool were obtained from Sigma Aldrich Co. (Saint Louis, MO, Catalog number L2602-5G). Differences in volatile production between pollen and ovulate cones was determined using a weighted least squares model to account for the variation between groups. Similarly, differences in 1,3-octadiene increases over time in pollen cones was determined using a weighted least squares model to account for the variation between groups.

1.2.5 Electrophysiological analysis: GC-EAD

The physiological response of *Rhoplaotria furfuracea* weevils to host plant *Zamia furfuracea* volatile compounds was determined using Gas Chromatography - Electroantennograph Detection (GC-EAD). The coupled GC-EAD system used was as previously described by Crook et al., 2008 with a few modifications. Samples of aerations or standards were injected (two microliters), splitless, onto a
Chapter 1. An ancient obligate pollination mutualism in cycads

Hewlett Packard (Agilent Technologies, Santa Clara CA) 6890 gas chromatograph with a DB-5MS-DG column (J W Scientific Inc., Folsom CA, 0 m x 0.25 mm ID, 0.25 m film thickness) and a 1:1 effluent splitter that allowed simultaneous FID and EAD detection of the separated volatile compounds. Helium was the carrier gas (2.5 ml/min). Oven temperature was held at 50 C° for 1 min, programmed to 280 C° at 10 C°/min and held for 15 min. Injector temperature was 280 C°. The GC outlets for the EAD and FID were 300 C°. The column outlet for the EAD was held in a water-cooled humidified air stream (20 C°) flowing at 20 ml/min over the antennal preparation of adult Rhopalotria furfuracea attached to an EAG probe (Syntech, Hilversum, the Netherlands). Whole heads of an adult beetle were removed so that both antennae could be used for recording. Tips of both antennae were cut off to make a clean opening for conducting gel (Spectra 360, Parker Laboratories, Fairfield, NJ) to form an uninterrupted connection to the EAG probe. The EAG probe was connected to an IDAC-232 serial data acquisition controller (Syntech). Signals were stored and analyzed on a PC equipped with the program EAD (version 2.6, Syntech). GC-EADs using whole Zamia furfuracea plant volatile collections were performed on 6 males and 4 females and GC-EADs of the 1,3-octadiene standard were performed on 3 males and 3 females.

1.2.6 Weevil expulsion from pollen cones

Pollen dehiscing male cones were collected and videotaped throughout the course of the day in conjunction with hourly plant volatile collections to determine the correlation between plant volatile emission and weevil expulsion from male
Chapter 1. An ancient obligate pollination mutualism in cycads

cones. Cones were placed into clear plastic containers 20.5 cm x 17 cm x 17 cm. One square side of the container was mesh to allow airflow and volatile dissipation and the cone was placed closest to this side. Cones used in video analysis were the same stage as those used for volatile collection, open and releasing pollen. These boxes were placed on top of a white sheet of paper set on a clear plastic sheet that was suspended over a 29.6 cm x 29.6 cm LED light panel (BK3301, US Solid State LLC, Shreveport, LA). This entire set up was covered with black cloth so that the only source of light was the even backlite LED panel. Cones were videotaped for 11 hours using a Sony Handycam HDR-CX260V. Weevil expulsion from the cone was manually counted at 30-minute intervals from the resulting videos.

1.2.7 Behavioral analysis: Pit-tests of 1,3-octadiene concentrations

The behavioral response of Rhopalotria furfuracea weevils to the Zamia furfuracea plant volatile compound 1,3-octadiene was determined in pit-tests (Fig. A.1) using differing amounts of the compound along with a control of dicholomethane carrier solvent. Pits were constructed of 100x15 mm petri dishes with 1.5 cm circular holes cut into them and hot glued onto 5 cm deep plastic cups. These were set into larger glass cups so that all dishes were completely level. 10 ul of 1,3-octadiene standards (1 ng/ul, 10 ng/ul, 100 ng/ul, 1ug/ul, 10 ug/ul and 65.5 ug/ul) were applied to 1 cm by 4 cm pieces of filter paper (Whatman plc number 4, GE Healthcare Life Sciences, Chicago IL) handled with forceps and dropped into the pits. For each trial 1,3-octadiene concentrations and control
were run simultaneously. *R. furfuracea* weevils were taken directly from *Z. furfuracea* pollen cones so as to be well fed. Five to ten weevils were placed at the edge of the petri dish which was then closed and placed in the dark at room temperature (22 C\(^{\circ}\)) for 30 minutes, at which time the number of weevils inside of the pits was counted and transformed into proportions (weevils in pits/total weevils). A likelihood ratio test was used to determine whether variances between the groups should be accounted for. No significant difference was found between a weighted least squares model that allowed for different variances between groups and an ordinary least squares model (p=0.1044). Therefore, the ordinary least squares model with fewer parameters was used to determine significant differences in proportions of weevils attracted to baits between the different amounts of 1,3-octadiene.

### 1.2.8 Behavioral analysis: Weevil movement in relation to 1,3-octadiene concentrations

To determine whether the high concentration of 1,3-octadiene acts as a repellent, arenas were constructed in which 80-100 *Rhopalotria furfuracea* weevils were introduced to either 10,000 ng or 650,000 ng 1,3-octadiene and recorded for 30 minutes. Arenas were constructed of white acrylic sides and bottom with a clear acrylic top (Figure A.2). Dimensions were 14.5 cm x 9.5 cm and 3.5 cm tall. In order to allow airflow and the dissipation of volatile compounds, windows were cut into the sides and covered with white mesh fabric (13.5 cm x 1 cm holes in the long sides and 8.2 cm x 1 cm holes in the short sides). Openings 2 cm x 5 cm were cut in the bottom of the arena for sample and control. These were covered with
Chapter 1. An ancient obligate pollination mutualism in cycads

fine white mesh fabric from the bottom. 10 ul of the 1,3-octadiene concentration was placed onto 1 cm x 4 cm filter paper (Whatman plc number 4, GE Healthcare Life Sciences, Chicago IL) and set under the opening in the bottom of the arena. A blank filter paper piece was placed under the other opening as a control. This entire setup was placed on a clear acrylic panel suspended over a 29.6 cm x 29.6 cm LED light panel (number BK3301, US Solid State LLC, Shreveport, LA) and encased in a black box covered with black fabric so as to be backlit. Weevils were allowed to feed freely on Z. furfuracea pollen cones prior to trials so as to be well fed. Videos were recorded using a Sony Handycam HDR-CX260V.

Raw videos were converted from AVCHD to .mov, then processed in Matlab. The locations of the outer arena edges, and the left and right bait locations were manually located for each trial using custom scripts. Next, a background image was computed separately for each trial by calculating the median intensity at each pixel. This median-averaging method meant that weevils that were immobile are considered part of the background and excluded from further analysis.

For each trial, we analyzed a subset of 4000 frames. For each analyzed frame, weevils were separated from the background by subtracting the frame’s pixel intensity values from the computed background image. This intensity-differential image was then turned into a binary map of weevils using an intensity threshold, which was subsequently digitally eroded and dilated to remove digital noise. After this segmentation step, image regions that were within a size threshold (i.e., between 150 and 800 pixels, manually calibrated) and located within the arena were considered separate weevils. For each identified weevil, distance was calculated to the nearest point on each bait. If weevils were located within
Chapter 1. An ancient obligate pollination mutualism in cycads

the edges of the baits, the distance to that bait was set to zero. For each sampled frame, a mean distance to bait and to control was calculated across all the weevils tracked for that frame.

Weevil movement in relation to 1,3-octadiene amount was determined by the change in asymmetry towards the bait (distance to bait box – distance to control box) from the start of the trial to the end. *Rhopalotria furfuracea* often play dead when startled and take upwards of 10 minutes to begin behaving again. Therefore, the ‘starting’ asymmetry towards bait was determined for each trial by subtracting the average distance to the control from the average distance to the bait over the first 15 minutes of video (frame < 3000). Weevils were given 10 minutes to make a choice and the ‘ending’ asymmetry towards bait was determined beginning at 25 minutes (frame > 5000). Autocorrelation of the change in asymmetry was analyzed using the acf function in R separately for each trial. We then computed the average autocorrelation function, which showed a substantial diminishment in autocorrelation (correlation < 0.1) at a lag of 500 of the sampled frames. We then subsampled our raw tracking data over 500 sampled frames (or roughly one frame every 2.5 minutes) to minimize the impacts of autocorrelation. The ‘starting’ asymmetry towards bait value for the trial was then subtracted from the ‘ending’ asymmetry values of the subsampled frames.

A likelihood ratio test was used to determine if variances between the groups should be accounted for, and no significant difference was found between a weighted least squares model that allowed for different variances between groups and an ordinary least squares model (p=0.402). Therefore, the ordinary least
squares model with fewer parameters was used to determine the significance of change in asymmetry towards bait for the two amounts of 1,3-octadiene.

1.2.9 Phylogeny

Dated molecular phylogenetic analyses have been performed in the Cycadales with conflicting results (Condamine et al., 2015; Salas-Leiva et al., 2013; Nagalingum et al., 2011). The data set from Salas-Leiva et al., 2013 was used to construct a dated phylogeny using the methods and ‘traditional’ fossil calibrations suggested in Condamine et al., 2015. The Salas-Levia et al. data set was chosen over Condamine et al. due to its use of markers from more gene regions (5 verses 3) and the completeness of its matrix (100% gene coverage for all taxa versus 51% coverage). The two data sets were not combined because the represented taxa did not overlap completely and preference was given to a complete data matrix. Molecular dating was performed using BEAST v 1.10.0 (Suchard et al., 2018). Fossil calibrations are based on Hermsen et al., 2006 and were defined as uniform priors as follows: stem node of Bowenia (lower=33.9, upper = 265.1 million years (MY)), stem node of Lepidozamia (lower= 33.9, upper = 265.1 MY), stem node of Dioon (lower = 56, upper = 265.1 MY). Methods followed Condamine et al., 2015 except for an unpartitioned analysis and the use of a random starting tree and a random local clock model for determining tree likelihood as suggested by Salas-Leiva et al., 2013. Literature used to determine the presence of pollinators and thermogenic and volatile patterning can be found in Table 1.2.
Table 1.2: Sources for pollinators and daily patterns of thermogenesis and volatile expression across the Cycadales. *Thermogenesis has been tested twice in Stangeria eriopus. Tang, 1987a found a very small thermogenic peak at 4:30 hrs. Proches and Johnson, 2009 did not take temperatures overnight and provide only one example of their temperature measurements in supplemental material that shows a small peak in late afternoon. **Suinyuy, Donaldson, and Johnson, 2013 finds a significant pattern in volatile emissions in one population but not in another of Encephalartos villosus.
<table>
<thead>
<tr>
<th>Cycad genus</th>
<th>Coleoptera pollinators</th>
<th>Other pollinators</th>
<th>Pollinator</th>
<th>Citations</th>
<th>Thermogenic</th>
<th>Volatile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lepidozamia</td>
<td>Entylidae</td>
<td></td>
<td>Hall et al., 2004</td>
<td>Tang, 1987a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bowenia</td>
<td>Curculionidae</td>
<td></td>
<td>Wilson, 2002</td>
<td>Tang, 1987a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrocycas</td>
<td>Entylidae</td>
<td></td>
<td>Chavez and Genaro, 2005</td>
<td>Tang, 1987a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stangeria</td>
<td>Nitidulidae</td>
<td></td>
<td>Proches and Johnson, 2009*</td>
<td>Tang, 1987a*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ceratozamia</td>
<td>Entylidae</td>
<td></td>
<td>Tang et al., 2018</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Farrera et al., 2000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Skchez-Rolonda, 1993</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrozamia</td>
<td>Languriidae</td>
<td>Thysanoptera</td>
<td>Terry, 2001</td>
<td></td>
<td>Wallenius et al., 2012</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Curculionidae</td>
<td></td>
<td>Terry et al., 2005</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Terry, 2007</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Encephalartos</td>
<td>Boganiidae</td>
<td></td>
<td>Donaldson, 1997</td>
<td>Suinyuy et al., 2012</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Entylidae</td>
<td></td>
<td>Suinyuy, Donaldson, and Johnson, 2009</td>
<td>Suinyuy, Donaldson, and Johnson, 2013**</td>
<td>Tang, 1987a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Curculionidae</td>
<td></td>
<td>Suinyuy, Donaldson, and Johnson, 2015</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zamia</td>
<td>Belidae</td>
<td></td>
<td>Tang, 1987b</td>
<td>This study</td>
<td>Tang, 1987a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Entylidae</td>
<td></td>
<td>Valencia-Montoya et al., 2017</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Norstog, Stevenson, and Niklas, 1986</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tang et al., 2018</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dioso</td>
<td>Belidae</td>
<td></td>
<td>Tang et al., 2018</td>
<td>Tang, 1987a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Entylidae</td>
<td></td>
<td>Vovides, 1991</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycas</td>
<td>Entylidae</td>
<td>Cosmopterigidae</td>
<td>Tang et al., 2018</td>
<td>Tang, 1987a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nitidulidae</td>
<td></td>
<td>Qian et al., 1997</td>
<td>Tang, 1987a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Curculionidae</td>
<td></td>
<td>Tang, Obergrieler, and Yang, 1999</td>
<td>Tang, 2018</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kono and Tobe, 2007</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Terry et al., 2009</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Marler and Niklas, 2011a</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.3 Results

We identify the chemical communication mechanism underlying the mutualism between *R. furfuracea* and *Z. furfuracea*. Using head-space collection methods, plant volatile compounds were collected from *Z. furfuracea* pollen and ovulate cones to determine the plant volatile profile and the physiological response of *R. furfuracea* to host plant volatiles. *Zamia furfuracea* pollen and ovulate cones produce two major compounds: the hydrocarbon 1,3-octadiene and the alcohol linalool (Figure 1.1). Electroantennograph detection demonstrated that *Rhopalotria furfuracea* male and female weevils are physiologically capable of perceiving only 1,3-octadiene (Figure 1.1, n=10), explaining why prior attempts to elicit behavioral responses in *R. furfuracea* using linalool were unsuccessful (Pellmyr et al., 1991).
Chapter 1. An ancient obligate pollination mutualism in cycads

Figure 1.1: Zamia furfuracea produce two main volatile components, 1,3-octadiene and linalool, yet Rhopalotria furfuracea only physiologically respond to 1,3-octadiene. Gas chromatography-flame ionization detector (GC-FID) of Z. furfuracea plant pollen cone volatiles is shown at the top with R. furfuracea weevil electroantenograph detection (EAD) on the bottom. Scale bar is 1 mV for EAD channel. Red arrow denotes a positive physiological response to 1,3-octadiene that is not seen for linalool (n=10). Weevil response to 1,3-octadiene was confirmed with a standard (n=6).

Because a physiological response does not equate with attraction, we analyzed the behavioral response of R. furfuracea to different amounts of 1,3-octadine. Using pit-tests (Figure A.1, Figure 1.2.A inset), we show that R. furfuracea are positively attracted to 1,3-octadiene and that weevil attraction to increasing amounts of 1,3-octadiene is nonlinear (Figure 1.2.A). Weevils are more attracted to the intermediate amount of 10,000 ng 1,3-octadiene than to all lower quantities (p<0.012
for all lower amounts, Table 1.3) as well as to the highest quantity (p=0.0102 for 650,000 ng). The decrease in attraction suggests but does not show that weevils are repelled (i.e. “pushed”) by high concentrations.

**Table 1.3:** Sequential Bonferonni adjusted p-values for the proportion of weevils attracted to pits containing different amounts of 1,3-octadiene. 10,000 ng 1,3-octadiene is significantly more attractive than all amounts except for 100,000 ng. The highest amount, 650,000 ng 1,3-octadiene, is not significantly different from any amount except the most attract amount, 10,000 ng.

<table>
<thead>
<tr>
<th>ng 1,3-octadiene</th>
<th>10</th>
<th>100</th>
<th>1,000</th>
<th>10,000</th>
<th>1e+5</th>
<th>6.5e+5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.0044</td>
<td>0.4994</td>
<td>1.0</td>
</tr>
<tr>
<td>10</td>
<td>1.0</td>
<td>1.0</td>
<td>0.0087</td>
<td>0.6635</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1.0</td>
<td>0.0051</td>
<td>0.4994</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,000</td>
<td></td>
<td>0.0110</td>
<td>0.7492</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1e+5</td>
<td></td>
<td></td>
<td>0.7492</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.5+e5</td>
<td></td>
<td></td>
<td>0.0102</td>
<td>0.6635</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

We therefore tested whether intermediate (10,000 ng) and high (650,000 ng) quantities of 1,3-octadiene elicited different movement responses in weevils. The push-pull hypothesis predicts that weevils should move towards 10,000 ng 1,3-octadiene and move away from 650,000 ng 1,3-octadiene. We set up enclosed arenas (Figure A.2, Figure 1.2.B inset) with 80 weevils, exposing them to either 10,000 ng of 1,3-octadiene plus a control, or 650,000 ng plus a control, and videotaped their movements over a period of 30 minutes. We measured the change in asymmetry towards the 1,3-octadiene bait (distance from bait box – distance from control box) from the beginning of the video to the end, and determined
that the change in asymmetry is significantly different between the two concentrations (p=0.0056). As predicted, *Rhopalotria furfuracea* move towards 10,000 ng 1,3-octadiene, but move away from 650,000 ng 1,3-octadiene (Figure 1.2.B). Together, these experiments demonstrate that weevil attraction preference is for an intermediate amount of 1,3-octadiene, and that their movement can be induced by varying the quantities of plant compounds, consistent with the push-pull pollination observed in *Macrozamia lucida* (Terry et al., 2007).
Figure 1.2: Weevil behavior follows push-pull pollination where they are attracted to lower concentrations of 1,3-octadiene and repelled by higher amounts. (A) Weevils are preferentially attracted to mid-level amounts of 1,3-octadiene in pit-tests (shown in inset and Figure A.1) and less attracted to more concentrated amounts. Attraction index is the proportion of weevils found in pits after 30 minutes. (B) Weevils are attracted to and move towards 10,000 ng 1,3-octadiene and are repelled and move away from 650,000 ng in behavioral arenas (shown in inset and Figure A.2). Attraction and repulsion are determined by a change in asymmetry towards the volatile compound over the course of the trial. Values above the grey line show a change in orientation away from the 1,3-octadiene bait, and below the line a shift towards the bait. Amounts of 1,3-octadiene are shown on the x-axis.
Finally, we determined the pattern of *Z. furfuracea* volatile release and its correlation to *R. furfuracea* movement in the field. We hypothesized that there would be an increase in volatile production and *R. furfuracea* activity around 19:00-20:00 after the thermogenic peak at 18:30 (6). We collected volatiles from reproductive pollen and ovulate cones (n=5, Table 1.1) at hourly intervals throughout the day and identified a typical diurnal pattern in volatile production in *Z. furfuracea* whereby cones change the ratio of the two major volatile compounds over the course of the day, approaching 100% production of 1,3-octadiene in the evening (Figure 1.3.A). Pollen cones consistently produce a greater quantity of volatile compounds (p=0.0007) than ovulate cones (Figure 1.3.B) and show a large increase in production of 1,3-octadiene in the evening (Figure 1.3.C, p=0.0137) that coincides with *R. furfuracea* expulsion from pollen cones around 20:00 (Figure 1.3.D).
Chapter 1. An ancient obligate pollination mutualism in cycads

Figure 1.3: An increase in production of 1,3-octadiene by *Zamia furfuracea* is positively correlated with an exodus of *R. furfuracea* weevils from the pollen cone. (A) The percent composition of the daily volatile profile shifts towards 100% 1,3-octadiene after 20:00 hrs in both pollen and ovulate cones (n=5). (B) Pollen cones produce more volatile emissions throughout the day than ovulate cones (p=0.0007). Total peak area from GCMS analysis is shown on the y-axis. (C) The percent increase in 1,3-octadiene production in pollen cones rapidly increases after 20:00 hrs (p=0.0137). The smoothed conditional mean is shown (method=loess) with one standard error at 0.95 confidence in A, B, C. (D) Weevils housed in pollen cones are repelled from the cones at 20:00 hrs. Each colored line represents an individual pollen cone and numbers are raw counts of weevils seen in video recordings.
Chapter 1. An ancient obligate pollination mutualism in cycads

Figure 1.4: Bayesian molecular dated phylogeny of Cycadales with all known information on pollinators and thermogenic and volatile patterns. The number of species in each genus is noted in parentheses. Grey bars show the 95% confidence interval for dating at the nodes. Recently reported fossil evidence of specialized beetle pollination in cycads (Cai et al., 2018) as well as the estimated timing of the rise of flowering plants are noted. Citations for metadata are provided in Table S3.
1.4 Discussion

*Rhopalotria furfuracea* weevils and *Cycadothrips chadwickii* thrips have converged on the same behavioral response to an ancient plant pollination mechanism. Both pollinators utilize the pollen cone as a larval development site, as do all known cycad pollinators (Terry et al., 2012a; Tang, 2006; Pellmyr et al., 1991; Terry et al., 2004; Azuma and Kono, 2006). Both insects show differential behavioral responses according to the quantities of one host plant volatile compound: attraction to intermediate amounts and repulsion to higher amounts. The increase in volatile production causes expulsion of pollinators from pollen cones, a pattern that has been observed in other cycad genera as well (Terry et al., 2007; Terry et al., 2004; Terry, 2001; Wallenius et al., 2012; Norstog, Stevenson, and Niklas, 1986). In both plants described here, as well as all cycad genera so far tested, the production of these compounds changes throughout the day (Figure 1.4) and is known to be caused by thermogenesis (Terry et al., 2016), an ancestral trait among the Cycadales (Tang, 1987a).

The Cycadales are the basal gymnosperm lineage (Ran et al., 2018) and have an extensive fossil record stretching back 265 MY (Hermsen et al., 2006), placing them amongst the most ancient extant seed plants. Specialized beetle pollination has existed in this group well before the rise of flowering plants (Niklas, Tiffney, and Knoll, 1983) from at least the mid-Mesozoic (Figure 1.4) (Cai et al., 2018) and perhaps as early as 245 MY (Klavins et al., 2005). Stereotypical sequential timing of thermogenesis in pollen and ovulate cones as well as specialized brood site pollination have been documented to occur across the Cycadales (Table 1.2). These observations, in combination with our discovery here of convergence in
Chapter 1. An ancient obligate pollination mutualism in cycads

define the behavioral responses of both thrips and weevils to volatiles released by
the cones of Old World (*Macrozamia*) and New World (*Zamia*) lineages support
the hypothesis that the push-pull system found in cycads represents the most
ancient insect/plant pollination mechanism yet documented. These dioecious
plants give us insight into the earliest forms of insect pollination prior to the
prolific diversification of visual floral signaling and reward systems employed
by angiosperms. Odor attraction has been thought to precede color attraction in
angiosperms (Piji, 1960) and the coupling of thermogenesis with strong odors as
seen in cycads has been suggested as an exaptation leading to early angiosperm
pollination syndromes (Thien, Azuma, and Kawano, 2000). The hypothesis that
plant volatiles attractive to pollinators are derived from herbivore deterrent sec-
ondary compounds (Pellmyr and Thien, 1986) is also supported in that attractive
volatile compounds in cycad push-pull pollination maintain a repellent compo-
nent for their specialized mutualists. Early insects found rewards such as pol-
lination drops (Tang, 2006; Labandeira, Kvaček, and Mostovski, 2007), ovular
exudates found in cycads and other gymnosperms that predate composition-
ally similar angiosperm nectar, and high concentrations of starch in cone tissues
(Tang, 2006). As they overcame the deterrent quality of host plant compounds
and found brood sites in plant reproductive tissues, plant volatiles became the
basis of chemical plant-insect communication, with signals for attraction and
repulsion mediated in part by circadian cycles of thermogenesis.
Chapter 2

Cycad-feeding insects share a core gut microbiome

Published article: Cycad-feeding insects share a core gut microbiome

The following pages contain a reproduction of the paper. Supporting material is included as Appendix B.
Chapter 2. Cycad-feeding insects share a core gut microbiome

Biological Journal of the Linnean Society, 2018, 123, 728–738. With 3 figures.

Cycad-feeding insects share a core gut microbiome

SHAYLA SALZMAN*, MELISSA WHITAKER and NAOMI E. PIERCE

Department of Organismic and Evolutionary Biology, Harvard University, 26 Oxford Street, Cambridge, MA 02149, USA

Received 29 September 2017; revised 31 January 2018; accepted for publication 31 January 2018

Five insect species including three species of weevils (Coleoptera) and two species of lycaenid butterflies (Lepidoptera) that feed exclusively on the carcinogenic and neurotoxic tissues of cycads were found to share a core set of bacterial phylotypes, including the bacterium *Raoultella ornithinolytica*, which has known anti-cancer and nitrogen-fixing capabilities. The other shared bacteria belong to lineages that include insect-associated and extremophilic taxa. The presence of *Raoultella ornithinolytica* and an unknown Enterobacteriaceae was in contrast to a set of non-cycad-feeding relatives of these insects, none of which contained this same set of shared bacterial phylotypes. Given the considerable phylogenetic distance between the cycadivorous insect species as well as the fact that shared microorganisms are not found in their non-cycad-feeding relatives, our data suggest that this core set of shared bacteria are important in helping cycad feeders detoxify their poisonous host plants.


INTRODUCTION

The plant order Cycadales comprises ten genera with ~350 species found across the tropics (Calonje, Stevenson & Stanberg, 2017). These dioecious gymnosperms are often obligately insect-pollinated and in many cases provide a brood site for pollinators that feed and develop on their tissue (Norstog, Stevenson & Niklas, 1989; Stevenson, Norstog & Fawcett, 1998; Terry et al., 2012; Brookes et al., 2015; Valencia-Montoya et al., 2017). Cycads are among the most ancient lineages of seed plants with a fossil record extending back over 250 My (Mamay, 1969; Gao & Thomas, 1989) and while they are currently the most endangered plant order in the world (IUCN, 2017), they were once a dominant component of the Mesozoic flora (Friis, Chaloner & Crane, 1987; Thomas & Spicer, 1987). Cycads produce many secondary metabolites (De Luca et al., 1982; Khabazian et al., 2002), including the two highly toxic compounds found in species throughout the order: methylazoxymethanol (MAM) (De Luca et al., 1980; Moretti, Sabato, Gigliano, 1983) and β-methylamino-l-alanine (BMAA) (Vega & Bell, 1967). Whereas non-cycadivorous insect herbivores do not encounter these plant compounds in their diets, insects that have specialized on cycads must be capable of contending with both toxins concurrently, and each compound acts in very different ways.

MAM has both carcinogenic and neurotoxic effects (Laqueur & Spatz, 1968; Morgan & Hoffmann, 1983). This compound occurs in the plants in a non-toxic form in which the toxic agent, MAM, is attached to a glycoside. While MAM-glycosides are found in all cycad genera (De Luca et al., 1980; Moretti, Sabato & Gigliano, 1981, 1983), the non-toxic storage form may differ depending on the glycoside attached to MAM. The two most common MAM-glycosides are cycasin, in which the glycoside is β-D-glucose (Nishida, Kobayashi & Nagahama, 1955), and macrozamin, in which the glycoside is a disaccharide of glucose and xylose (Lythgoe & Riggs, 1949). In both cases, toxicity results from cleavage of the glycoside from MAM by the activity of endogenous glucosidase enzymes (Laqueur & Spatz, 1968; Schneider et al., 2002) that are produced in the digestive tracts of mammals and insects (Conchie & MacDonald, 1959; Terra & Ferreira, 1994). Once cleaved, MAM spontaneously degrades into formaldehyde and methyl-diazonium, a potent methylating agent (Morgan & Hoffmann, 1983). MAM-induced genetic alterations have been described in plants, mammals, yeast, bacteria and insects (Morgan &

*Corresponding author. E-mail: shaylasalzman@fas.harvard.edu
Chapter 2. Cycad-feeding insects share a core gut microbiome

Hoffmann, 1983). MAM-glycosides have been found in all cycad genera and in all plant tissues that have been tested, including seeds, leaves, pollen and ovulate cones, roots and stems (Cooper, 1941; Riggs, 1954; De Luca et al., 1980; Moretti et al., 1981, 1983; Blagrove, Lilley & Higgins, 1984; Rothschild, Nash & Bell, 1986; Yagi & Tadera, 1987; Bowers & Larin, 1989; Lindblad, Tadera & Yagi, 1990; Nash, Bell & Ackery, 1992; Vovides et al., 1993; Castillo-Guevara & Rico-Gray, 2003; Yagi, 2004; Prado, 2011; Nair & Staden, 2012; Prado et al., 2014, 2016).

The second class of cycad toxins, BMAA, has received considerable attention by virtue of its implication as the causative agent of amyotrophic lateral sclerosis-parkinsonism-dementia, a human neurobiological disease that is endemic to the island of Guam and is also referred to as Guam’s dementia (Cox, Banack & Murch, 2003). BMAA was first isolated from cycads by Vega & Bell (1967) in response to an extremely detailed account of cycad consumption and toxic effects in humans and cattle (Whiting, 1963). BMAA’s toxicity arises from its disruption of glutamate receptor function. Glutamate receptors have deep homology and are found across plants and animals (Lam et al., 1998; Chiu et al., 1999; Lacombe et al., 2001). BMAA works as a neurotoxin in insects (Goto, Koenig & Ikeda, 2012) and mammals, causing convulsions and neural degeneration (Spencer et al., 1987) as well as abnormalities in brain development (Kisby, Moore & Spencer, 2013). In Arabidopsis, BMAA-induced glutamate receptor blockage affects signal transduction causing hypocotyl elongation and inhibiting cotyledon opening (Brenner et al., 2000). Moreover, as a non-protein amino acid, BMAA can be incorporated into proteins, fundamentally altering their structure and function (Dunlap et al., 2013). It is unknown how the cycad protects itself from this endogenous toxin. BMAA has been found in all cycad genera and in all tested tissues, including leaves, cones, seeds, pollen and roots (Dossaji & Bell, 1973; Duncan, Kopin & Crowley, 1989; Norstog & Fawcett, 1989; Vovides et al., 1993; Pan et al., 1997a, b; Banack & Cox, 2003).

Given the highly toxic nature of these compounds and their presence throughout plant tissues, only specialized insects are able to utilize cycads as a food source. Many of these are pollinating herbivores, including species from several genera of beetles that feed on pollen cone parenchyma tissue and coevolve with their host cycads (Donaldson, Nanni & Bösenberg, 1995; Stevenson et al., 1998; Terry et al., 2012; Tarnawski, unpublished & Johnson, 2015). In addition to pollinating herbivores, several folivorous Lepidoptera are obligate cycad feeders for either their entire larval development or for their first few instars (e.g. Sihvonen, Staude & Mutanen, 2015). Relatively little is known about potential toxin tolerance mechanisms in cycadivorous insects, which could include methods to detoxify, sequester and/or avoid plant defensive chemicals. One study focused on BMAA avoidance in the pollinating weevil, Rhopalotria furfuracea (previously R. mollis), which feeds on pollen cone parenchyma tissue of Zamia furfuracea (Norstog & Fawcett, 1989).

In this case, staining experiments demonstrated that plants sequester BMAA in specialized plant cells (idioblasts) that are able to pass through the insect gut intact. Interestingly, these idioblast cells were found intact in the pollen cones on which the weevils feed, but burst open in ovulate cones, which the weevils visit but never eat. The authors suggested that this plant mechanism restricts pollinator herbivory to the expendable pollen cone tissue. No known or proposed mechanism exists for BMAA tolerance or avoidance in leaf or ovulate cone feeders where the toxin is not sequestered in plant idioblasts.

The only other investigation into cycad toxin tolerance mechanisms focused on MAM tolerance in a leaf-feeding moth. A β-glucosidase enzyme was found to be localized in the guts of the larvae of the ‘echo moth’, Siericrtia echo, feeding on leaves of Zamia integrifolia, and this insect was shown to produce cycasin when fed the toxic MAM (Teas, 1967). Echo moths are aposematically coloured as both larvae and adults, and are thought to sequester and utilize the host plant’s cycasin for protection. Teas (1967) hypothesized that this endogenous β-glucosidase enzyme rehydrolyses the toxic MAM with a glycoside, reverting the compound into its non-toxic form, cycasin. Laqueur & Spatz (1968) suggested that this enzyme could be of microbial origin, yet this remains untested, and it is unknown whether the enzyme is present in the guts of other cycadivorous insects.

The suggestion that microbes may play a role in toxin tolerance in Siericrtia echo moths is particularly interesting given the growing evidence that many herbivorous insects rely on symbiotic gut bacteria to mediate the challenges associated with plant-based diets (Douglas, 2013), including degrading plant secondary metabolites (Boone et al., 2013; Ceja-Navarro et al., 2015) and countering specialized plant defences (Chu et al., 2013). It is possible that cycadivorous insects rely on gut bacteria to ameliorate their highly toxic diets, and given the similar plant defensive chemistry that all cycad herbivores are exposed to, even distantly related insects may have converged upon similar bacterial associations. To investigate these possibilities, we used 16S amplicon sequencing to characterize and compare the gut bacterial communities of five species of cycadivorous insects from two different orders, and to identify and investigate bacterial phylotypes that are shared across these phylogenetically distinct insect species.
MATERIAL AND METHODS

We collected three Curculionidae (Coleoptera) weevil species and two Lycaenidae (Lepidoptera) butterfly species that feed on a variety of cycad species and tissues (Table 1, Fig. 1). Insects were either immediately flash frozen whole in liquid nitrogen or dissected and the gut preserved in ethanol, and all samples were stored at –80 °C. Whole insect samples were surface sterilized for 5 s in 10% bleach and rinsed in PBS prior to DNA extraction using the Powersoil DNA isolation kit and protocol (MoBio Laboratories, Carlsbad, CA, USA) with the addition of 60 µg proteinase K to the lysis buffer. DNA concentration was assessed using a Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and samples with low DNA yields were concentrated using the MoBio protocol.

Extracted DNA was sent to Argonne National Laboratories (Lemont, IL, USA) for library preparation and sequencing of the V4 region of the 16S rRNA gene. Library preparation used barcoded primers 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′) and the methods from Caporaso et al. (2012). Libraries were pooled, and 150-bp paired end reads were sequenced on an Illumina MiSeq sequencer.

Raw sequences were preprocessed using previously published methods (Whitaker et al., 2016). Because the 16S universal primers we used are known to also amplify organellar DNA, we compared chloroplast abundance across samples before removing non-target sequences, which included chloroplasts and mitochondria as well as the common laboratory contaminants, Staphylococcaceae and Escherichia. We then applied a filtering method requiring bacterial operational taxonomic units (OTUs) to be represented by at least ten sequences in the data set and at a minimum relative abundance of 0.05% per sample in order to be included in the analysis.

Core microbiomes were calculated using the filterfun function in the phyloseq package in R, requiring bacterial OTUs to be present in all replicates within a species (species cores) or all cycad herbivore replicates (cycad herbivore core, hereafter ‘shared OTUs’). The taxonomic assignments of all core OTUs were further checked using NCBI BLAST and Seqmatch from the Ribosomal Database project (Cole et al., 2014). Shared bacterial OTUs were further analysed using the Oligotyping pipeline v2.1 (Eren et al., 2013) with a minimum substantive abundance of 10 and the smallest number of entropy positions needed to properly decompose oligotypes. For comparison, we searched for these shared OTUs in published surveys of the gut microbiomes of six species of non-cycadivorous ‘outgroup’ insects: two Lycaenidae (Lepidoptera) and four Curculionidae (Coleoptera) (details in Supporting Information S1). For these comparisons, we consider only samples that passed the filtering requirements and were included in analyses.

Table 1. Cycad herbivore sampling included two cycad genera and multiple insect species, families and orders, feeding tissues and localities; sample numbers consider only samples that passed the filtering requirements and were included in analyses.

<table>
<thead>
<tr>
<th>Herbivore Order</th>
<th>Herbivore</th>
<th>Host plant</th>
<th>Feeding tissue</th>
<th>Life stage</th>
<th>Number</th>
<th>Preservation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coleoptera: Belidae</td>
<td>Rhipaloctoides furfuracea (O’Brian &amp; Tang)</td>
<td>Zamia furfuracea (Aiton)</td>
<td>Pollen cone</td>
<td>Adult</td>
<td>6</td>
<td>Whole flash frozen</td>
</tr>
<tr>
<td>Coleoptera: Erythidae</td>
<td>Eubulus sp. (Kirsh)</td>
<td>Zamia aff. portoricensis (Urban)</td>
<td>Subterranean stem</td>
<td>Late instar</td>
<td>6</td>
<td>Whole flash frozen</td>
</tr>
<tr>
<td>Lepidoptera: Rhopalocera</td>
<td>Chilades pandava (Horsfield)</td>
<td>Cycas revoluta (Thunberg)</td>
<td>Leaf</td>
<td>Late instar</td>
<td>7</td>
<td>Gut dissected in ethanol</td>
</tr>
</tbody>
</table>
Chapter 2. Cycad-feeding insects share a core gut microbiome

RESULTS

Raw sequences clustered into 1789 unique OTUs across the 31 samples. As expected, chloroplast 16S sequences were found in high abundance in caterpillars of both leaf-feeding Lepidoptera, *Eumaeus atala* and *Chilades pandava*, but were unexpectedly prevalent in cone-feeding weevils, *R. furfuracea*, as well (median 60% of the total sequences, 18% inter-quartile range). After abundance filtering and removing non-target OTUs, the filtered dataset lost one *Eum. atala* sample due to small library size but retained 177 taxonomic OTUs across the remaining 30 samples. This filtered dataset was used in all subsequent analysis.

Two additional samples were omitted following rarefaction for diversity analyses due to small library sizes, one *R. furfuracea* and one *Eum. atala*. The remaining samples clustered in a characteristic pattern in non-metric multidimensional scaling (NMDS) ordinations and hierarchical clustering (Fig. 2B, C). The gut microbiota of all five cycad-feeding insects were remarkably conserved, clustering mainly by species except for an overlap between *R. furfuracea* and *C. pandava* in NMDS ordinations of Bray–Curtis distances (stress 0.13; Fig. 2B). *Rhopalotria furfuracea* and *C. pandava* also grouped by similarity in hierarchical clustering (Fig. 2C). Taxonomy plots demonstrate a compositional similarity between these two species, driven by the dominance of Alicyclobacillaceae and Comamonadaceae in the gut bacterial communities of both insects (Fig. 2A).

The five most abundant bacterial families in our dataset were Alicyclobacillaceae, Enterobacteriaceae, Moraxellaceae, Comamonadaceae and Enterococcaceae (Fig. 2A). Bacterial OTUs found in all samples of a species are reported briefly in Table 2 and in detail in Supporting Information S2. Most significantly, the microbiota of all five cycad-feeding species showed significant overlap for five OTUs that were present in all replicates (Table 2). The greengenes taxonomic assignments, however, did not match NCBI BLAST results for any of these five shared OTUs. Only one of processed and analysed raw 16S sequences of gut bacteria in the same way that we did with the cycadivorous insects.

For diversity analyses, libraries were first rarified to 10000 sequences, retaining any library with at least 5000 sequences. The similarity of each sample’s bacterial community composition was compared using the Bray–Curtis dissimilarity metric and hierarchical clustering based on weighted UniFrac distances where OTU abundances were averaged across replicates within a species. All sequence data are deposited in the EMBL-EBI database.
the shared OTUs could be identified to species using NCBI BLAST (OTU 5: *Raoultella ornithinolytica* in the Enterobacteriaceae). The four remaining OTUs could only be identified to the family level using NCBI BLAST search (OTUs 4 and 249 in the Enterobacteriaceae, OTU 3 in the Alicyclobacillaceae and OTU 85 in the Rickettsiaceae; Table 2). Unidentified bacteria are not surprising when exploring novel environmental samples, and the four OTUs that could be identified only to family belong to bacterial families that include insect-associated and extremophilic bacteria. Of these five shared OTUs, OTU 4 (Enterobacteriaceae) and OTU 5

![Figure 2](https://academic.oup.com/biolinnean/article-abstract/123/4/728/4920834)

**Figure 2.** (A) The relative abundance of the 20 most abundant bacterial families per sample shows that Enterobacteriaceae and Alicyclobacillaceae are dominant in the guts of several species. (B) In ordinations based on Bray–Curtis distances (stress 0.13), gut bacterial communities generally cluster according to species, except for a striking similarity in the gut communities of *Rhopalotria furfuracea* and *Chilades pandava*. (C) Hierarchical clustering of insect species by bacterial community composition also highlights the similarity between *R. furfuracea* and *C. pandava*. 

(Raoutella ornithinolytica) were found solely in cycad herbivores. None was found in any of the outgroup weevils. However, OTUs 3, 85 and 249 were found in both outgroup butterflies.

Oligotyping results for shared OTUs are presented in Figure 3 (data in Supporting Information S3). Twenty-one unique oligotypes were found for Raoutella ornithinolytica (OTU 5). Pharaxanotha floridana had the most consistent composition of Raoutella ornithinolytica oligotypes, with Eubulus sp. showing a similar although less constrained pattern (Fig. 3). In contrast, Eum. atala was dominated (> 93%) by one Raoutella ornithinolytica oligotype across all replicates. Twenty unique oligotypes were found for OTU 4 (Unknown Enterobacteriaceae). Again, P. floridana and Eub. sp. showed consistent patterns of OTU 4 oligotype composition, whereas OTU 4 oligotype composition was highly variable in the remaining species (Fig. 3). Only three oligotypes were recovered for OTU 249 (Unknown Enterobacteriaceae), one of which dominated all samples. Finally, oligotyping analysis found no entropy peaks for either OTU 85 (Unidentified) or OTU 3 (Unknown Alicyclobacillaceae).

**DISCUSSION**

Our results show that five cycad-feeding insect species from two orders share a core set of bacterial OTUs in their gut microbiota, despite being generally distinct in overall bacterial community composition. While the functions of these OTUs remain unknown, our results are consistent with the hypothesis that gut bacteria may mediate herbivory of cycads, and they identify specific bacterial phylotypes as candidates for future functional study.

In terms of the entire community of gut microbiota, we found that each insect harbours a distinctive species-specific bacterial assemblage, with the exception of R. furfuracea weevils and C. pandava butterflies, whose communities of microbiota were surprisingly similar. For example, chloroplasts were found in high abundance in all Lepidoptera, as well as R. furfuracea, but were not found in the other Coleoptera. In ordination plots based on community dissimilarity metrics, the gut bacterial communities of R. furfuracea weevils clustered more closely with C. pandava than with P. floridana, the other cone-feeding coleopteran (Fig. 2B). Larvae and adults of R. furfuracea feed only...
Figure 3. Oligotyping analysis of the two OTUs that were unique to cycad herbivores. *Eumaeus atala* was dominated by one oligotype for OTU 5 (*Raoultella ornithinolytica*). OTU 4 (Unknown Enterobacteriaceae) displayed highly constrained compositional patterns within *Eubulus* sp. and *Pharaxanotha floridana*. OTU 85 (Unidentified) and OTU 3 (Unknown Alicyclobacillaceae) were each represented by a single oligotype across all samples, and OTU 249 (Unknown Enterobacteriaceae) was dominated by a single oligotype across all species, so oligotyping results for those three OTUs are not shown here.
on the microsporophyll of the pollen cone (Fawcett & Norstog, 1993), which is developmentally a modified leaf (Gifford & Foster, 1989), whereas P. floridana larvae feed on cone peduncle tissue (Fawcett & Norstog, 1993), which is developmentally a modified stem (Gifford & Foster, 1989). It is possible that the bacterial compositional similarities we observe between R. furfuraceae and C. pandava reflect developmentally related chemical or nutritional similarities in the plant tissues on which they feed. Further research on the chemical composition of various cycad tissues will be necessary to test this hypothesis.

Ordination plots demonstrated that the microbiota of P. floridana, and to some extent those of Eub. sp., were more similar to each other than they were to those of the remaining three species. In fact, whether looking at the OTU (Fig. 2A) or sub-OTU level (oligotype; Fig. 3), P. floridana samples exhibited highly conserved bacterial community compositions across replicates. It is unclear if these conserved assemblages are a product of limited environmental exposure, vertical transmission or selection by the host. Pharaonotha floridana beetles feed on pollen for their first two instars, and then spend the remainder of their larval development within the peduncle of the pollen cone, a fairly closed environment (Fawcett & Norstog, 1993). Further sampling of Pharaonotha adults, which feed exclusively on cycad pollen (Fawcett & Norstog, 1993), would help to elucidate whether these insects harbour consistent bacterial communities during all life stages. This would enable us to assess the relative contributions of host selection versus environmental exposure in determining gut bacterial community assemblages in these beetles.

Four of the five shared OTUs found in the guts of cycad-feeding insects were unidentifiable to genus. The one that could be identified, however, offers some insight into potential activities of the gut bacterial community. OTU 5 was identified as *Raoultella ornithinolytica*, a bacterium that has been shown to fix nitrogen in the guts of wild *Ceratitis capitata* fruit flies (Behar, Yuval & Jurkevitch, 2005) and to elicit cytotoxicity and apoptotic and necrotic death of mammalian cancer cells due to the activity of a protein–polysaccharide complex (Fiolka et al., 2013). Oligotyping of *Raoultella ornithinolytica* revealed consistent strain-level compositional patterns within *Eum. atala*, *P. floridana*, and *Eub. sp.* (Fig. 3). The leaf- and cone-feeding *Eumaeus* larvae are more vagile than the cone- and stem-feeding *Pharaonotha* and *Eubulus* larvae, such that we might expect *Eum. atala* to be exposed to a greater diversity of bacteria in the environment. It is therefore somewhat unexpected that the *Eum. atala* caterpillars were dominated by only one oligotype. *Raoultella ornithinolytica* warrants further functional research to assess whether this bacterium provides critical benefit to cycadivorous insects, such as detoxification of their poisonous cycad host plants.

OTU 4 was identified with equal confidence to two bacterial genera, *Pantoea* and *Klebsiella*, and in our analysis it remains an unidentified member of the family Enterobacteriaceae. Oligotyping revealed conserved strain-level compositional patterns in *P. floridana* and *Eub. sp.* for this OTU (Fig. 3). As these are the two least mobile insects in our dataset, it is unclear if this pattern arises from limited exposure to environmental bacteria, or from selection on the part of the host or gut environment. While it is unknown whether this bacterium might contribute to host nutrition or fitness, we can make some inferences based on its similarity to *Pantoea* and *Klebsiella*. *Pantoea* is a known gut symbiont in many insects, and it has been shown to confer nutritional benefits including nitrogen fixation, toxin degradation and hydrolysis of proteins (Sood & Nath, 2002; MacCollom et al., 2009). Additionally, ingested *Pantoea* and *Klebsiella* have both been shown to colonize the guts of insects and be subsequently vertically transmitted (Lauzon et al., 2009). Future work should focus on isolating and identifying this OTU and on characterizing its metabolic capabilities and symbiotic potential.

Of the remaining shared OTUs, two were initially identified by greengenes taxonomic assignment as *Buchnera* (OTU 249) and *Wolbachia* (OTU 85), both known insect symbionts. However, these assignments were not supported by the NCBI BLAST database, and both OTUs displayed little oligotypic variation. OTU 3 (Unidentified Alicyclobacillaceae) showed no oligotypic variation across samples, despite being represented by a large number of reads (16383). Bacteria in this family are found in extreme environments, often growing at extreme temperatures or pH (Vos et al., 2009). With no information about the function of these bacteria or their symbiotic potential as well as their presence in the ‘outgroup’ butterfly samples, it is impossible to determine whether they have a functional relationship with their cycadivorous insects.

To our knowledge, this survey represents the first report of gut bacterial associations that are conserved among a phylogenetically and geographically disparate set of herbivores that share a common toxic host plant. By comparing the composition of gut bacterial communities of five species of cycad-feeding insects, we found that each insect harbours a distinctive species-specific bacterial assemblage, with the exception of *R. furfuraceae* weevil larvae and *C. pandava* caterpillars that host similar communities of microbiota. Most importantly, we found that all five of the insect species share a core set of bacterial OTUs, which we believe warrant future functional study. We identified...
Chapter 2. Cycad-feeding insects share a core gut microbiome


REFERENCES


Chapter 2. Cycad-feeding insects share a core gut microbiome


Chapter 2. Cycad-feeding insects share a core gut microbiome


SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**S1.** Non-cycadivorous lycaenid and curculionid samples that passed filtering requirements and were analysed for the presence of shared bacterial OTUs found in cycad herbivores.

**S2.** Species core microbiome OTU and assigned taxonomy are listed with OTUs present in more than one species highlighted in grey. Species’ microbiome cores were determined by OTU presence in 100% of samples of that species after filtering and are represented by their OTU number.

**S3.** Oligotyping results
Chapter 3

Ecology and Evolution of Cycad-Feeding Lepidoptera

Note: Supplemental materials included in Appendix C.

3.1 Introduction

Lepidoptera (butterflies and moths) have long been used to test theories about the evolutionary origins and consequences of ecological traits, and their larval associations with host plants have served as a scientific cornerstone of studies of coevolution. An extensive literature on the physiological, morphological, behavioral, genetic, and ecological mechanisms of plant-butterfly interactions has developed over the last half century, largely in response to Ehrlich’s & Raven’s seminal 1964 paper describing macroevolutionary patterns of host use among butterflies (Ehrlich and Raven, 1964). In observing that related butterfly groups tend to feed on related plant groups, Ehrlich & Raven proposed a step-wise process that is now commonly referred to as "escape and radiate"
coevolution (Thompson, 1989), in which plants diversify after evolving novel defenses that allow them to ‘escape’ from herbivores, and herbivores diversify after evolving novel counter-adaptations to plants’ defenses. Thirty years later, advances in molecular phylogenetics provided the necessary tools to examine trait variation from an evolutionary perspective, and phylogenetically explicit analyses of hostplant associations have since been conducted for numerous lepidopteran groups (see Mitter, Davis, and Cummings, 2017 and references therein), with cross-taxon analyses presented in several other papers (e.g., Janz and Nylin, 1998; Janz and Nylin, 2008; Fordyce, 2010; Menken, Boomsma, and Van Nieukerken, 2010). While these studies report different patterns at different taxonomic scales and for different lepidopteran lineages, most find general support for Ehrlich’s & Raven’s hypothesis that butterflies’ hostplant associations are significantly phylogenetically conserved, at least at higher taxonomic levels. Moreover, in cases where closely related butterflies use distantly related host plants, those plants often share phytochemical similarities rather than phylogenetic relatedness, underscoring the importance of plant defensive chemistry in determining the food preferences, dietary breadth, and host transitions of Lepidoptera. However, though phylogenetic and phytochemical conservatism appear to be widespread they are far from ubiquitous.

Several explanations have been proposed for cases in which lepidopterans switch to plants that are phylogenetically and phytochemically dissimilar to their ancestral host, but a common challenge in identifying the factors underlying such host shifts is defining a "major" host shift in a biologically relevant way, including determining the appropriate taxonomic scale at which to assess
host shifts. This limitation is borne out of the fact that the overwhelming majority of previous studies have focused on Lepidoptera that feed on just a few angiosperm families, with comparatively little investigation into transitions to non-angiospermous diets (but see Braby, 2000; Pierce et al., 2002; Kaliszewska et al., 2015; Cong et al., 2016; Whitaker et al., 2016). This is a missed opportunity, as transitions to gymnosperms and other non-angiosperm plants likely represent some of the most drastic host switches in terms of phylogenetic, morphological and (potentially) phytochemical dissimilarity. We therefore suggest that studying the causes and consequences of transitions away from angiosperms—in this case, to a group of gymnosperms called cycads—could yield novel insight into the study of plant-insect interactions.

Cycads (order: Cycadales) are a basal, pantropical group of dioecious gymnosperms with a fossil record extending back over 265 million years (Gao and Thomas, 1989). They possess an arsenal of distinctive chemical defenses that are themselves deserving of review, and yet a number of insects use cycads as larval and adult food plants. The great majority of cycad-feeding (cycadivorous) insects belong to a handful of lepidopteran families that exhibit varying degrees of dietary specialization and belong to multiple feeding guilds. However, the biology of cycadivorous Lepidoptera has never been reviewed, and the majority of relevant studies have concentrated on just a few focal species without examining broader ecological or evolutionary patterns. The aims of this review are therefore to 1) assemble an authoritative list of cycadivorous Lepidoptera and distinguish verified from unverified records, 2) discuss key ecological and evolutionary implications of cycad feeding, 3) summarize conservation and pest
management concerns, and 4) highlight important data gaps and areas for future study.

3.2 Lepidopteran Cycad Herbivores

Cycadivory occurs in seven Lepidopteran families (Table 3.1), including the butterfly families Nymphalidae and Lycaenidae. Among nymphalid butterflies, larvae of two species in the Australasian genus *Taenaris*—*T. onolaus* and *T. butleri*—have been reported to feed on *Cycas* in Papua New Guinea (Parsons, 1984; Parsons, 1999). In addition to larval cycad feeding, some adult *Taenaris* butterflies imbibe cycad juices: *T. onolaus* and *T. catops* have been observed visiting fermenting cycad seeds, feeding on exudates from wounded cycad leaves, and even probing the fresh frass of cycadivorous beetle larvae with their probosces (Parsons, 1984). This behavior is particularly remarkable in *T. catops*, the larvae of which feed on palms (Arecaceae) and are not known to be cycadivorous.

Three genera of lycaenid butterflies—*Luthrodes*, *Eumaeus*, and *Theclinesthes*—include species that are obligate cycad herbivores. The *Luthrodes - Chilades* clade is comprised of two sister genera that have historically been lumped together (typically under name *Chilades*). Here we follow Talavera et al., 2013 and treat them as separate genera. Thus, we consider the cycadivorous lycaenid species that are typically referred to in the literature as *Chilades* to be properly placed in *Luthrodes*: *L. pandava*, *L. peripatria*, and *L. cleotas*. *Luthrodes pandava* is widespread across southern and southeast Asia and the larvae are often serious pests of *Cycas*. *Luthrodes cleotas* also occurs in southeast Asia and feeds on *Cycas* (Parsons,
Chapter 3. Ecology and Evolution of Cycad-Feeding Lepidoptera

1999), but less is known about its life history. The third species, *L. peripatria*, is endemic to Taiwan and its taxonomic status is unclear: some authors treat it as a full species (Hsu, 1989; Talavera et al., 2013) while others consider it a subspecies of *L. pandava* (Wu et al., 2010; Ravuiwasa, Tan, and Hwang, 2011). The larvae of *L. peripatria* historically fed only on *Cycas taitungensis*, also endemic to Taiwan, though it now accepts the ornamental species *Cycas revoluta* (Ravuiwasa, Tan, and Hwang, 2011) that has been introduced to Taiwan in large numbers since the 1990s (Wu et al., 2010).

The neotropical lycaenid genus *Eumaeus* is comprised of six species distributed from Peru to the Caribbean (Lamas, 2004), with *E. atala* extending into southeastern Florida and some (perhaps dubious) records of rare strays of *E. toxea* into southern Texas (Kendall, 1984). All six *Eumaeus* species are obligate cycad herbivores, utilizing cycads in the neotropical genera *Zamia*, *Dioon*, and *Ceratozamia* (Koi and Daniels, 2015; Hammer, 1996; Contreras-Medina, Ruiz-Jiménez, and Luna Vega, 2003; Ramírez-Restrepo, Koi, and MacGregor-Fors, 2017; Comstock, 1948; Koi and Daniels, 2017). Larvae of several *Eumaeus* species have been observed feeding on plants’ fresh male and female reproductive cones in addition to stem and leaf tissue (Farrera et al., 2000; Cascante-Marín and Araya, 2012; González, 2004; Ruiz-García et al., 2015; Bosque and Rosales-Robles, 2015; Koi and Daniels, 2017), and we know of a single report of *E. childrenae* adults feeding on cycad exudates (Murillo, 1902). Finally, *Theclinesthes* is a mostly Australian genus of six species, of which one species, *T. onycha*, feeds on cycads in the genera *Cycas* and *Macrozamia* in eastern Australia (Braby, 2000).

Among moths, 23 species from 8 genera have been recorded on cycads but
Chapter 3. Ecology and Evolution of Cycad-Feeding Lepidoptera

this is likely an underestimation, as many cycad-feeding moths remain poorly collected and understudied. An entire tribe of Geometrid moths, the Diptychini, consists of 17 cycadivorous species in 3 genera (Sihvonen, Staude, and Mutanen, 2015). Colloquially called "the cycad moths," these are the best studied of the cycadivorous moths and are the only cycadivorous Lepidoptera known from Africa. The hostplants of all Diptychini larvae are Encephalartos and Stangeria cycads for the first 3 instars, but larvae in later instars often switch to angiospermous host plants (Donaldson, 1991; Staude, 1994; Staude and Sihvonen, 2014). Hostplant species for Diptychini moths are therefore separated into primary (cycad) and secondary (non-cycad) hosts in Table 3.1.
### Table 3.1: Larval hostplant records for cycadivorous Lepidoptera. Species synonyms are given in the Supplementary Materials. *Introduced plant species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cycad Hosts</th>
<th>Other Hosts</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nymphalidae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Taenaris Hübner, [1819]</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>T. butleri</em> (Oberthür, 1880)</td>
<td><em>Cycas</em> (species unknown)</td>
<td></td>
<td>Parsons, 1984; Parsons, 1999</td>
</tr>
<tr>
<td><em>T. onolaus</em> (Kirsch, 1877)</td>
<td><em>Cycas</em> (species unknown)</td>
<td></td>
<td>Parsons, 1984; Parsons, 1999</td>
</tr>
<tr>
<td><strong>Lycaenidae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Eumaeus Hübner, [1819]</em></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 3.1 – continued from previous page

<table>
<thead>
<tr>
<th>Species</th>
<th>Cycad Hosts</th>
<th>Other Hosts</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. childrenae</em> (Gray, 1832)</td>
<td><em>Dioon edule, D. merolae,</em></td>
<td></td>
<td>Comstock, 1948;</td>
</tr>
<tr>
<td></td>
<td><em>Ceratozamia matudae,</em> C. mexicana,*</td>
<td></td>
<td>Contreras-Medina, Ruiz-Jiménez, and Luna Vega, 2003; Ramírez-Restrepo, Koi, and MacGregor-Fors, 2017; Bosque and Rosales-Robles, 2015</td>
</tr>
<tr>
<td></td>
<td><em>C. norstogii,</em> <em>C. robusta,</em> <em>C. chimalapensis,</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Zamia fischeri,</em> <em>Z. soconus-cencis,</em> <em>Cycas</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>revoluta</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. godartii</em> (Boisduval, 1870)</td>
<td><em>Zamia acuminata,</em></td>
<td></td>
<td>Cascante-Marín and Araya, 2012</td>
</tr>
<tr>
<td></td>
<td><em>Z. fairchildiana,</em> <em>Z. manicata,</em> <em>Z. stevensonii</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. minyas</em> (Hübner, [1809])</td>
<td><em>Zamia encephalartoides,</em> <em>Z. skinneri</em></td>
<td></td>
<td>González, 2004; Clark, Clark, and Grayum, 1992</td>
</tr>
<tr>
<td><em>E. toxana</em> (Boisduval, 1870)</td>
<td>Unknown</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Continued on next page
### Table 3.1 – continued from previous page

<table>
<thead>
<tr>
<th>Species</th>
<th>Cycad Hosts</th>
<th>Other Hosts</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Luthrodes Druce, 1895</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. cleotas</em> (Guérin-Méneville, [1831])</td>
<td><em>Cycas</em> (species unknown)</td>
<td></td>
<td>Parsons, 1999</td>
</tr>
<tr>
<td><em>L. pandava</em> (Horsfield, [1829])</td>
<td>&gt;85 species of <em>Cycas</em></td>
<td></td>
<td>Burkill, 1918; Khew, 2015; Marler, Lindström, and Terry, 2012</td>
</tr>
<tr>
<td><em>L. peripatria</em> (Hsu, 1980)</td>
<td><em>Cycas taitungensis</em>, <em>Cycas revoluta</em></td>
<td></td>
<td>Wu et al., 2010</td>
</tr>
<tr>
<td><em>Theclinesthes</em> (Röber, 1891)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>T. onycha onycha</em> (Hewitson, 1865)</td>
<td><em>Cycas megacarpa</em>, <em>C. ophiolitica</em>, <em>C. media</em></td>
<td></td>
<td>Forster and Machin, 1994; Wilson, 1993</td>
</tr>
</tbody>
</table>

Continued on next page
Table 3.1 – continued from previous page

<table>
<thead>
<tr>
<th>Species</th>
<th>Cycad Hosts</th>
<th>Other Hosts</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. onycha capricornia</td>
<td>Macrozamia spiralis,</td>
<td>M. communis, M. pauliguilie</td>
<td>Forster and Machin, 1994</td>
</tr>
<tr>
<td>1978</td>
<td></td>
<td>mi</td>
<td></td>
</tr>
<tr>
<td><strong>Geometridae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zerenopsis Felder, 1874</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cephalartos hildebrandtii</td>
<td>the wild, Diospyros lycioides</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>in captivity</td>
<td></td>
</tr>
<tr>
<td>Z. flavimaculata</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Staude and Sihvonen, 2014</td>
</tr>
<tr>
<td>2014</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z. kedar (Druce, 1896)</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Staude and Sihvonen, 2014</td>
</tr>
</tbody>
</table>

Continued on next page
Table 3.1 – continued from previous page

<table>
<thead>
<tr>
<th>Species</th>
<th>Cycad Hosts</th>
<th>Other Hosts</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z. meraca (Prout, 1928)</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Staude and Sihvonen, 2014</td>
</tr>
<tr>
<td>Z. tenuis (Butler, 1878)</td>
<td>Encephalartos hildebrandtii</td>
<td>Adansonia digitata</td>
<td>Staude and Sihvonen, 2014</td>
</tr>
<tr>
<td>Veniliodes Warren, 1894</td>
<td></td>
<td></td>
<td>Continued on next page</td>
</tr>
</tbody>
</table>
### Table 3.1 – continued from previous page

<table>
<thead>
<tr>
<th>Species</th>
<th>Cycad Hosts</th>
<th>Other Hosts</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. inflammata</em> Warren, 1894</td>
<td>Primary hosts: <em>Stangeria eriopus</em>, <em>Encephalartos villosus</em></td>
<td>Secondary hosts: <em>Apodytes dimidiata</em>, <em>Diospyros lycioides</em></td>
<td>Staude, 2001; Staude, 2001</td>
</tr>
<tr>
<td><em>V. setinata</em> (C. &amp; R. Felder, 1875)</td>
<td><em>Stangeria eriopus</em></td>
<td></td>
<td>Staude, 2001</td>
</tr>
<tr>
<td><em>C. apicisecta</em> Prout, 1915</td>
<td><em>Stangeria eriopus</em> &amp; <em>Encephalartos tegulaneus</em> in the wild, <em>E. vollosus</em> in captivity</td>
<td></td>
<td>Staude, 2001</td>
</tr>
</tbody>
</table>

Continued on next page
<table>
<thead>
<tr>
<th>Species</th>
<th>Cycad Hosts</th>
<th>Other Hosts</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. curlei</em> Staude, 2001</td>
<td><em>Stangeria eriopus</em>, <em>Encephalartos friderici-guilielmi</em></td>
<td></td>
<td>Staude, 2001</td>
</tr>
<tr>
<td><em>C. grandis</em> Prout, 1922</td>
<td><em>Encephalartos gratus</em></td>
<td></td>
<td>Staude, 2008</td>
</tr>
<tr>
<td><em>C. mayeri</em> Staude, 2001</td>
<td><em>Encephalartos friderici-guilielmi</em></td>
<td></td>
<td>Staude, 2001</td>
</tr>
</tbody>
</table>

**Erebidae**

*Seirarctia* Packard, 1864

*Seirarctia echo* (Smith, 1797) | *Zamia integrifolia* | *Sabal palmetto*, *Diospyros* spp.**, *Quercus* spp.*, *Croton* spp.*, *Lupinus* spp.*, many other woody plants, lettuce | Packard, 1890; Wagner, 2005 |

**Cosmopterigidae**

Continued on next page
<table>
<thead>
<tr>
<th>Species</th>
<th>Cycad Hosts</th>
<th>Other Hosts</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anatracnyntis Meyrick, 1915</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. badia (Hodges, 1962)</td>
<td><em>Zamia integrifolia, Cycas revoluta, C. circinalis</em></td>
<td>Dozens of species, including both angiosperms and gymnosperms</td>
<td>Hua, Salzman, and Pierce, 2018; Dawidowicz and Rozwałka, 2017; Bella and Mazzeo, 2006</td>
</tr>
<tr>
<td>A. sp</td>
<td><em>Cycas micronesica</em></td>
<td>Unknown</td>
<td>terry2009cone; Marler and Muniappan, 2006; Marler and Niklas, 2011b</td>
</tr>
<tr>
<td><strong>Tineidae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Dasyes rugosella</em> (Stainton, 1859)</td>
<td><em>Cycas micronesica</em></td>
<td>Dozens of plant species, mushrooms</td>
<td>Marler and Muniappan, 2006; Robinson and Nielsen, 1993</td>
</tr>
<tr>
<td><em>Erechthías sp.</em></td>
<td><em>Cycas micronesica</em></td>
<td>Unknown</td>
<td>Marler and Muniappan, 2006</td>
</tr>
<tr>
<td><strong>Blastobasidae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undetermined</td>
<td><em>Zamia pumila</em></td>
<td>Unknown</td>
<td>Terry et al., 2012b</td>
</tr>
</tbody>
</table>
In addition to these obligate cycad herbivores, a number of facultative cycad Lepidoptera exist. *Seirarctia echo* (Erebidae) occurs in the southeastern United States where the larvae are highly polyphagous, feeding on leaves of the cycad *Zamia integrifolia* as well as plants in the families Arecaceae, Euphorbiaceae, Fabaceae, Fagaceae, and Ebenaceae. In captivity, they have even been reared on lettuce (Asteraceae) (Packard, 1890). One undetermined leaf-mining *Erechthias* moth (Tineidae) has been found feeding and pupating in the leaves of *Cycas micronesica* in Guam (Marler and Muniappan, 2006). Larvae of *Dasyses rugosella* (Tineidae) have been observed feeding on dead Cycas stems in India, Sri Lanka, Thailand, Indonesia, and Guam (Robinson, Tuck, and Shaffer, 1994; Marler and Muniappan, 2006). Colloquially called "yam moths," *D. rugosella* are best known as pests of stored yams in West Africa (Iheagwam and Ezike, 1989; Ashamo, 2005), and are broad generalists on decaying vegetable matter (Robinson and Nielsen, 1993). Larvae of *Anatrachyntis badia*, another highly polyphagous and cosmopolitan moth species, have been found in pollen cones of *Zamia integrifolia* in Florida, USA (Hua, Salzman, and Pierce, 2018) and feeding on leaves of *Cycas revoluta* and *C. circinalis* in Italy (Bella and Mazzeo, 2006). An undetermined *Anatrachyntis* species pollinates *Cycas micronesica* in Guam and feeds on pollen cones as larvae (Marler and Niklas, 2011b; Terry et al., 2009). Finally, larvae of an undetermined microlepidopteran in the family Blastobasidae have been found feeding in copious numbers on pollen cones of *Zamia pumila* in the Caribbean (Terry et al., 2012b).

Many records exist for lepidopteran species feeding on cycads which are likely to be erroneous or require further confirmation. We discuss these in the
Chapter 3. Ecology and Evolution of Cycad-Feeding Lepidoptera

Supplementary Materials. The species listed in Table 1 have been identified by experts, confirmed by multiple sources, supported with photographic evidence, and in many cases their larvae have been reared in captivity on cycads. However, while we feel that this paper serves as an authoritative list of cycadivory among Lepidoptera, it is likely not an exhaustive account of all cycadivorous species considering that new records of cycad-insect associations are still being reported (e.g., Hua, Salzman, and Pierce, 2018), particularly among cone-feeding microlepidoptera.

3.3 Aposematism and Defensive Ecology

Cycads produce several toxic compounds in their leaves and other tissues, including steryl glycosides, β-Methylamino-L-alanine (BMAA), and methylazoxymethanol acetate (MAM) (Morgan and Hoffman, 1983; Laquer and Spatz, 1968; Spencer et al., 1987; Kisby, Moore, and Spencer, 2013). These compounds are toxic to most animals and are therefore presumed to serve as anti-herbivore defenses, though MAM is the only compound for which experimental evidence exists for insect deterrence (Bowers and Larin, 1989; Castillo-Guevara and Rico-Gray, 2003; Prado et al., 2014). MAM is mutagenic and neurotoxic (Laquer and Spatz, 1968; Morgan and Hoffman, 1983) but occurs in plant tissues in a non-toxic form in which the toxic agent is attached to a glycoside. When ingested by insects or other animals, endogenous glucosidase enzymes cleave the glycoside from MAM (Laquer and Spatz, 1968; Rothschild, Nash, and Bell, 1986), which then spontaneously degrades into formaldehyde and methyldiazonium. Early
work by Teas (1966-7) showed that larvae of *Seirarctia echo* are able to chemically modify dietary MAM into its non-toxic, glycosylated form and accumulate MAM-glycosides in their tissues after feeding on cycad leaves (Teas, Dyson, and Whisenant, 1966; Teas, 1967). It is presumed that other cycadivorous species are capable of similar chemical modifications though it has never been explicitly tested in other species. Possible mechanisms of resistance to BMAA and steryl glycosides have not been investigated for any cycadivorous lepidopteran, though there is evidence that cycadivorous weevils are able to avoid BMAA by consuming only pollen cone parenchyma tissue where BMAA is sequestered in specialized cells that the weevils excrete in their frass (Vovides et al., 1993; Norstog, Stevenson, and Niklas, 1986).

Even so, cycadivorous Lepidoptera appear to tolerate all cycad toxins and several species are brightly colored, diurnal, and gregarious—traits commonly associated with chemically defended Lepidoptera (Bowers, 2003). Indeed, several studies have shown that some cycadivorous species sequester MAM-glycosides into their larval and adult tissues. Rothschild, Nash, and Bell, 1986 found that *Eumaeus atala* larvae, pupae, and adults contained MAM-glycosides in surprisingly high amounts relative to their hostplants, and Castillo-Guevara and Rico-Gray, 2003 detected MAM-glycosides in the eggs, larvae, pupae and adults of what is likely to be *Eumaeus toxea* in Mexico (identified by the authors as *E. minyas*, although the range of this species is not thought to extend to Mexico). Nash, Bell, and Ackery, 1992 quantified MAM-glycosides in dried museum specimens of adult butterflies, including some specimens that were over 70 years old. The authors detected MAM-glycosides in *Eumaeus minyas* (male
and female), *Luthrodes cleotas* (male and female), *Taenaris butleri* (male and female), *Taenaris catops* (male), and *Taenaris onolaus* (female) but did not detect MAM-glycosides in *Theclinesthes onycha* (either gender), female *Taenaris catops*, or male *Taenaris onolaus*. They concluded that MAM-glycosides were not detectable from the latter two because of the advanced age of the museum specimens, but that *Theclinesthes onycha* probably do not sequester MAM-glycosides.

Since several of the species that sequester MAM-glycosides are brightly colored, their coloration may be considered aposematic. Aposematism and chemical defense are exceedingly rare traits among lycaenid larvae (Rothschild, Nash, and Bell, 1986; DeVries, 1977; Fiedler, 1996), which typically rely on crypsis and ant association for protection against natural enemies (Pierce et al., 2002). *Eu- maeus* provide a striking exception in that they are gregarious and warningly colored in all lifestages, are known to sequester plant chemicals, and have larvae that do not associate with ants (Atsatt, 1981), whereas other cycadivorous lycaenid larvae commonly associate with ants (Wilson, 1993; Eastwood and Fraser, 1999; Tan and Sin Khoon, 2012) and most are not warningly colored. The adults of *Theclinesthes onycha* and *Luthrodes pandava* are typical of other Polyommatine butterflies with blue, brown, or purple uppersides and gray mottled undersides. The larvae of both species are polymorphic, with three main color forms: green, red, and mixed. This variation in coloration is common among Polyommatine larvae but it is not known if or how larval coloration affects survival and predation risk. Larvae of *Luthrodes cleotas* are cryptically colored but adults have much larger orange spots on their hindwings than do their congeners, and it is possible that they are aposematic, particularly given that adults
have been shown to sequester MAM-glycosides (Nash, Bell, and Ackery, 1992).

Larvae and adults of Dyptichini moths are brightly colored with gregarious larvae and diurnal adults, but it is unclear whether they sequester plant toxins at any life stage (Donaldson and Basenberg, 1995 suggest that Z. lepida sequester MAM-glycosides, but do not provide experimental evidence). Seirarctia echo larvae are warningly colored and covered with protective hairs. This species sequesters MAM-glycosides when feeding on cycads (Teas, 1967; Teas, Dyson, and Whisenant, 1966), but it remains unknown how feeding on non-cycad host-plants affects their palatability and predation risk. Finally, Anatrachyntis moths and the other microlepidoptera are not aposematic in any lifestage and many species spend their entire development concealed inside plants’ pollen cones, where they may avoid some cycad toxins (Vovides et al., 1993; Norstog and Fawcett, 1989) (see Hostplant Use section below). It is completely unknown whether leaf-mining Erechthias and detritivorous Dasyses encounter cycads’ defensive compounds while feeding.

Unfortunately, records of predators and parasitoids are lacking for nearly all cycadivorous species. Natural enemies of Lepidoptera generally include birds, small reptiles, spiders, mantids, reduviid bugs, ants, and parasitic wasps and flies, though direct observations of attacks on larvae and adult butterflies are exceedingly rare in nature (Molleman, Whitaker, and Carey, 2010). The best-studied cycadivorous species with regard to defensive ecology is Eumaeus atala in southeastern Florida. Both native and non-native ants have been observed consuming E. atala eggs and pupae (Smith-Cavros, 2002), but are thought to avoid adult butterflies (Bowers and Larin, 1989). Some assassin and ambush
bugs (Reduviidae) will attack *E. atala* larvae (Koi and Hall, 2019) although published records are scarce. Unconfirmed reports exist of native and non-native reptiles attacking *E. atala* larvae and adults. Starlings, peacocks, and other non-native birds have been reported to attack caterpillars, though it’s possible that only naïve birds will attempt to eat *E. atala*, as adult butterflies were shown to be distasteful to grey jays (Bowers and Farley, 1990).

Interestingly, all of the predators mentioned thus far are visual hunters, whereas many parasitoid wasps and flies use olfactory cues to locate their hosts. There are no reports of parasitoids using *E. atala* as hosts, a conspicuous absence given that parasitoids are typically significant natural enemies of lycaenid larvae. It may be that *E. atala* larvae utilize non-visual cues to advertise their chemical defensive status to potential parasitoids. Alternatively, parasitoids may simply not occur in the same places, or they occur but are sufficiently rare that they remain undetected. Given the history of this butterfly in Florida (see Conservation & Management section below), it is also possible that a historical parasitoid occurred in Florida prior to the temporary extirpation of *E. atala*, but was not reintroduced along with its host. Proper evaluations of these possibilities would require a better understanding of parasitization of *E. atala* populations across its full geographic range.

Among other cycadivorous species, Manners, 2015 reports that "high levels of parasitism" sometimes occur in *Theclinesthes onycha* larvae in Australia, and provides photographs of larvae parasitized by braconid wasps. Ruiz-García et
al., 2015 monitored survival and development of *Eumaeus toxea* larvae in Oaxaca, Mexico and observed *Dasydactylus* beetles preying on molting *E. toxea* larvae but did not report finding any parasitoids. The only published records of parasitization among cycadivorous moths come from *Zerenopsis lepida*: Staude and Sihvonen, 2014 reared a single parasitoid fly (Tachinidae) from a late instar larva in South Africa, and Sommerer, 2014 reared 15 *Z. lepida* larvae and found more than 50 percent had been parasitized by *Charops* sp. (Ichneumonidae) or *Drino* sp. (Tachinidae). Aside from these scattered records we know relatively little about the natural enemies of cycadivorous Lepidoptera in the wild.

### 3.4 Evolutionary Origins of Cycadivory

To evaluate evolutionary origins of cycadivory and relationships among cycadivorous Lepidoptera, cycadivory was mapped on to a phylogenetic tree constructed by combining a Lepidoptera phylogeny (Regier et al., 2013) including butterflies and moths with a heavily sampled butterfly phylogeny (Espeland et al., 2018) (Figure 3.1). Both phylogenies were downloaded as .nex files from published sources and brought into R (version 3.5.1) (R Core Team, 2018) where the butterfly clade from Espeland et al., 2018 was substituted in place of the less sampled clade from Regier et al., 2013 using the R packages ape (Paradis and Schliep, 2018), GEIGER (Harmon et al., 2008), and ggtree (Yu et al., 2017; Yu et al., 2018). In cases where cycadivorous species were not represented as tips on the tree, the represented tip of the closest relative was identified using published
phylogenies of families or genera (Zahiri et al., 2012; Sihvonen et al., 2011; Talavera et al., 2013; Wahlberg et al., 2009; Sihvonen, Staude, and Mutanen, 2015).

A visual inspection of the Lepidoptera phylogeny suggests that cycadivory has evolved independently in multiple lepidopteran lineages, with several origins likely within single families and potentially even single genera. For example, a poorly resolved phylogenetic hypothesis based on morphological data for *Taenaris* does not place the cycadivorous species within a monophyletic clade or closely related to each other (Parsons, 1999), suggesting multiple origins of cycadivory in the genus. Similarly, an unpublished molecular phylogeny that includes some species of *Luthrodes* does not place the two included cycadivorous species as sister taxa (Wu, 2009). Conversely, cycadivory appears to be an ancestral trait in *Eumaeus* butterflies (6 species) and Diptychini moths (17 species). Given that both of these clades are warningly colored and obligately cycadivorous, it seems likely that cycad feeding or defensive traits (or both) have led to limited radiations in these groups. Dated phylogenetic hypotheses for all genera would be required to understand the general evolutionary significance of cycadivory and why some lineages have diversified while others are represented by just one or two species nested within otherwise non-cycadivorous clades.

It is possible that ancient lepidopteran herbivores fed on cycad species that are now extinct, as cycads were widespread and abundant from the late Triassic through the Cretaceous (Taylor and Taylor, 1993; Niklas, Tiffney, and Knoll, 1983), during which time the Lepidoptera originated and diversified (Espeland et al., 2018; Misof et al., 2014). However, cycads subsequently experienced high rates of extinction and low diversity until the late Miocene, when all extant
cycad species arose (Nagalingum et al., 2011; Condamine et al., 2015; Salas-Leiva et al., 2013). Given the evolutionary history of cycads and phylogenetic placement of cycadivorous Lepidoptera, it is likely that transitions to cycadivory among extant cycadivorous Lepidoptera occurred within the last 15 to 20 million years. Indeed, at least in lyceanid butterflies the evolutionary origins of cycadivory appear to be somewhat recent. Talavera et al., 2013 dates the split between Luthrodes and its sister genus Chilades at ~6 MY. Notably, the cycadivorous species of Luthrodes included in the analysis are derived, placing the evolution(s) of cycadivory in this lineage as even younger. Similarly, an unpublished molecular clock analysis in Theclinesthes puts the origin of the genus at 2-3 MY (Eastwood, 2006). Finally, Espeland et al., 2018 places the split between Eumaeus and Calycopis at ~18 MY, making the origin of Eumaeus even younger as this analysis did not include the sister genus Theorema.
Chapter 3. Ecology and Evolution of Cycad-Feeding Lepidoptera

Figure 3.1: Phylogenetic placement of cycadivorous Lepidoptera. Genera containing cycadivorous species are shown by red tips, with the butterfly clade in black and moths in grey. Warning coloration is indicated symbolically, along with the feeding guild and whether the species is facultatively or obligately cycadivorous (black and green, respectively). The number of cycadivorous species and the total number of species in the genus are given in parentheses.

Improved phylogenetic estimates for extant cycadivorous Lepidoptera would
be useful for reconstructing and comparing historical diet evolution among cycadivorous lineages. Research in this area is hindered by incomplete or erroneous hostplant records for many species (see Appendix C). Some have speculated that monocot-feeding may be an evolutionary precursor to cycadivory because non-cycadivorous *Taenaris* feed on monocots (Schneider et al., 2002), though there is little evidence from other groups to support this as a broad pattern. Among lycaenids, close relatives of cycadivorous species feed on dicots in the families Fabaceae, Amaranthaceae, Proteaceae, Sapindaceae, Myrtaceae, and Euphorbiaceae (Dunn and Dunn, 1991; Braby, 2000). Cycadivorous moths and their close relatives exhibit a broad range of hostplant preferences that includes both monocots and dicots. Improved knowledge of the evolutionary histories of cycadivorous lineages would provide a framework for testing hypotheses about evolutionary precursors to cycadivory and host breadth among extant species.

### 3.5 Hostplant Use

Based on the records reported here, cycadivorous Lepidoptera utilize 7 of the 10 recognized cycad genera (Calonje, Stevenson, and Osborne, 2019). Absent among accepted hostplant genera are *Lepidozamia, Bowenia* and *Microcycas*. These are all small genera (*Lepidozamia*: 2 species; *Bowenia*: 2 species; *Microcycas*: 1 species) but are fairly well studied and co-occur with other cycad species that are known lepidopteran foodplants, making their omission all the more curious.
All cycadivorous butterflies appear to be obligate cycad specialists while cycadivorous moths exhibit a broader range of dietary preferences. *Seirarctia echo* is the only confirmed facultative cycad folivore, accepting leaves from a wide variety of hostplants from several plant families. The ecological causes and consequences of feeding on cycad versus non-cycad plants are completely unexplored in this species. Diptychini moths are facultatively polyphagous in their 4th-6th instars but all species are obligate cycad specialists for the first 3 instars. Donaldson and Basenberg, 1995 found no significant differences in survival rate, developmental duration or pupal mass between 4th instar *Z. lepida* larvae reared on angiosperm versus cycad hosts. Staude and Sihvonen, 2014 have suggested that some Diptychini moths may not require cycads even in their early stages, as they collected a single final-instar *Z. tenuis* larva feeding on the leaves of a baobab tree (*Adansonia digitata*, Malvaceae) on Misali Island, Tanzania, where no cycads were found. The remaining cycadivorous moth species are either highly polyphagous (e.g. *Dasyses rugosella*) or their host breadth is unknown (e.g. *Erechthias* sp.).

Whereas not all cycadivorous Lepidoptera are specialists of cycads, their larvae are specialized on particular plant tissues and can therefore be categorized into discrete feeding guilds. These guilds include leaf chewers, leaf miners, ovulate cone feeders, pollen cone feeders, and detritivores, and the larvae in each of these guilds likely experience qualitative and quantitative differences in exposure to cycad toxins. For example, pollen cone feeders may experience reduced exposure to toxins since pollen cones generally have lower toxin concentrations than leaves or ovulate cones, and at least one cycad toxin, BMAA, appears to
be sequestered in specialized cells in the pollen cones that can pass through the guts of other insects intact (Vovides et al., 1993; Norstog and Fawcett, 1989). Detritivorous species feed on decaying cycad pollen cones and stems that may also harbour lower concentrations of toxins. In contrast, *Eumaeus* butterfly larvae feed on both ovulate and pollen cones as well as leaves (González, 2004; Cascante-Marín and Araya, 2012), and some evidence suggests that *Z. lepida* moths also feed on ovulate cones in addition to leaves (Donaldson, 1991).

Among cycad specialists, it appears that larvae can accept diverse cycad species and hostplant breadth is expanding for several species, particularly as exotic cycads are planted as ornamentals in gardens worldwide. The Caribbean species *Eumaeus atala*, for example, historically fed only on Caribbean cycads in the genus *Zamia*, but have been observed laying eggs and feeding on cultivated Central American cycad species that are outside of the native range, as well as some species of African, Australian, and Asian cycads. The ability to feed on non-native cycads has been observed in other *Eumaeus* species as well (Bosque and Rosales-Robles, 2015), and increased hostplant breadth has been reported for *Luthrodes pandava*, a widespread species that feeds on numerous native and exotic cycads across Asia and the Middle East (Wu et al., 2010; Tiple et al., 2009; Fric et al., 2014; Feulner et al., 2014).

Contemporary host use may challenge the species status of *Luthrodes peripatria*, which some authors consider to be a subspecies of *Luthrodes pandava*. The natural range of *L. pandava* is widespread across southern Asia (excluding Taiwan), whereas *L. peripatria* is endemic to Taiwan and has historically fed on a single cycad species restricted to southeastern Taiwan, *Cycas taitungensis* (Shen,
Chapter 3. Ecology and Evolution of Cycad-Feeding Lepidoptera

Hill, and Chen, 1994). In the past 30 years, L. pandava has been introduced to Taiwan along with several exotic Cycas species. As both Luthrodes species accept native and non-native Cycas species as hostplants, expanded hostplant use and range overlap likely provide opportunities for interbreeding. Further assessment of the population structure, introgression, and species status of L. pandava and L. peripatria would be fruitful (but see Wu et al., 2010).

Hostplant specialization may promote divergence in the Australian species Theclinesthes onycha, for which two subspecies are recognized, T. onycha onycha and T. onycha capricornia. T. o. onycha feeds only on Cycas species distributed from Queensland to southern New South Wales, whereas T. o. capricornia feeds only on Macrozamia cycads found in central Queensland. They overlap in their distributions in a narrow region in central Queensland, though microhabitat preferences may maintain allopatry even within this contact zone. Patterns of hostplant use and mate choice are not well described within the contact zone, though Eastwood, 2006 found considerable genetic differentiation in the mitochondrial genes of each subspecies, suggesting that there is little to no gene flow between them.

Careful analysis of hostplant use, species relationships, and reproductive barriers would also be useful for the two pairs of sympatric species of Eumaeus butterflies in Central and South America. Eumaeus childrenae and E. toxea co-occur in some parts of their ranges in Mexico, where they are easily distinguished based on wing pattern. These species are likely quite diverged and they utilize different cycad genera as hostplants throughout much of their range, though detailed studies of host use in areas of sympatry and allopatry have not
been carried out. *Eumaeus toxana* and *E. minyas* both occur in South America and according to published records their ranges overlap in Peru. However, it is difficult to glean even basic natural history information for these two species due to widespread mistakes in species identifications in the published literature. *Eumaeus minyas* is commonly confused with several other *Eumaeus* species, especially *E. toxana* and the isthmus species *E. godartii*, but also *E. toxea* and even *E. atala*. Credible accounts of the distributions and range limits for these two species are needed, with *E. toxana* being particularly under-collected and poorly studied.

Among Caribbean species, the ranges of *Eumaeus atala* and *Seirarctia echo* overlap in southern Florida but there are very few records of both species occurring in the same place, suggesting that there is some displacement at a relatively fine spatial scale. Since *S. echo* is broadly polyphagous, hostplant competition is unlikely to be a sufficient explanation for this displacement. Furthermore, the range of *E. atala* does not occupy the entire range of its hostplants in Florida, and a better understanding of the factors that determine the range boundaries of these species would be very valuable for the management of local butterfly and cycad populations.

### 3.6 Management of Cycadivorous Species

With 75% of the 355 extant cycad species threatened with extinction, cycads are the most imperiled plant order in the world (Calonje, Stevenson, and Osborne, 2019; Gilbert, 2010; Baillie, Hilton-Taylor, and Stuart, 2004). The lepidopteran
Chapter 3. Ecology and Evolution of Cycad-Feeding Lepidoptera

herbivores of cycads therefore present something of a conservation conundrum: obligate cycad specialists almost certainly warrant formal protection, yet they are natural enemies of endangered plants. Conservation efforts that prioritize plants over insects (or vice versa) could have unintended negative consequences (e.g. Wu et al., 2010); instead, conservationists should prioritize the co-existence of these interacting species (Marler, Lindström, and Terry, 2012). However, co-management of multiple interacting species typically requires extensive knowledge of the natural history and population dynamics of each target species.

The lycaenid butterfly *Eumaeus atala* has served as a flagship species for local conservation efforts in Florida, USA. Once common across the southeastern parts of the state, overharvesting of Florida’s cycad populations in the early 1900’s led to the extirpation of *E. atala* from Florida. In 1959 a small breeding population was discovered near Miami (Rawson, 1961), and subsequent grassroots conservation efforts have led to the dramatic recovery of Florida’s *E. atala* populations. However, overzealous reintroductions of *E. atala* have the potential to be catastrophic for local cycads, including both native species and ornamental exotics. Like many cycadivorous Lepidoptera, *E. atala* larvae are prodigious feeders and a single clutch can decimate a large cycad, which are exceedingly slow to grow and reproduce. The African cycad moths are also of serious conservation concern due to habitat loss and large-scale poaching of cycads from the wild (Staude and Sihvonen, 2014), and one species, *Callioratis millari*, is considered critically endangered in South Africa (Mecenero et al., 2013).

Conservation of cycads and their herbivores is made more challenging by the emerging pest status of some cycadivorous species. *Luthrodes pandava* is already
Chapter 3. Ecology and Evolution of Cycad-Feeding Lepidoptera

considered a terrible pest of *Cycas* plants in India, Taiwan (Wu et al., 2010), Singapore (Marler, Lindström, and Terry, 2012), Papua New Guinea (Tennent, 2014) and Guam (Moore et al., 2005), and has recently been introduced in Egypt (Fric et al., 2014) and possibly the United Arab Emirates (Feulner et al., 2014). While there are no reports of *L. pandava* in Australia or the neotropics, its introduction would likely present a significant threat to cultivated and perhaps native cycads in these regions. The invasiveness of this species is exacerbated by its broad hostplant use: *L. pandava* larvae have been recorded feeding on 85 species of *Cycas* in a tropical botanical garden in Thailand (Marler, Lindström, and Terry, 2012) and can feed on other cycad genera as well. *Theclinesthes onycha* are also prone to localized population outbreaks in Australia (Rathie, 2004), and even *Eumaeus atala* have been known to reach destructive population sizes at highly localized scales in southeastern Florida. While occasional outbreaks may represent natural population cycles, the increasing availability of non-native cycads that are sold as garden ornamentals could lead to novel population dynamics even within species’ native ranges, with unknown consequences for local cycad populations.

3.7 Discussion

Cycadivorous Lepidoptera comprise a ‘component community’ of distinct lineages with varying degrees of specialization and diverse feeding ecologies, and therefore present numerous opportunities for comparative studies of ecoevolutionary dynamics (e.g., Farrell, 2001). With improved molecular phylogenies
and natural history information researchers can begin to identify the factors that have led to adaptation and the consequences of specific adaptations on diversification in cycadivorous lineages. Until then, we speculate here on some of the salient questions regarding cycad-Lepidoptera interactions.

**Is cycad-feeding adaptive?** Herbivory is considered an adaptive trait among insects when evolutionary shifts to herbivory are associated with increased diversification in phytophagous lineages relative to their aphytophagous sister groups (Mitter, Farrell, and Wiegmann, 1988; Wiens, Lapoint, and Whiteman, 2015). Evolutionary transitions to feeding on plants that contain defensive secondary compounds that deter other herbivores are claimed to promote diversification of Lepidoptera through escape and radiation (e.g., Braby and Trueman, 2006). If cycadivory has similarly promoted diversification in lepidopteran lineages, then it might be considered an adaptive trait. Based on the phylogenetic pattern shown in Figure 3.1, *Eumaeus* butterflies and Diptychini moths exhibit modest radiation following their transition to cycad-feeding, whereas other cycadivores remain as only one or two species at the tips of otherwise angiosperm-nivorous clades. Why have some cycadivorous lineages diversified while others have not?

That the largest clades of cycadivorous Lepidoptera are also aposematic suggests that defensive ecology may play a role in diversification: perhaps it is not cycadivory *per se* that leads to diversification in some lineages, but rather the subsequent evolution of aposematism. This explanation is consistent with the cryptic coloration of cycadivorous species that have not radiated, though
a few exceptions must be considered. *Luthrodes cleotas* and *Seirarctia echo* are both known to sequester cycad toxins and could be considered warningly colored; why have these species not diversified? Cycadivorous *Taenaris* species are also warningly colored but do not appear to have radiated (though even non-cycadivorous *Taenaris* are considered aposematic so this situation may be more complicated). It may be that evolutionary trade-offs or constraints have limited diversification in these groups, that other cycadivorous relatives once existed but have gone extinct, or that cycadivory has evolved too recently for diversification to have yet taken place. Indeed, cycadivorous species of *Luthrodes* and *Theclinesthes* appear to be very young, and it would be interesting to compare their ages to those of *Eumaeus* butterflies and *Diptychini*. Among generalists, cycadivory is not expected to significantly influence speciation rates (at least for detritivorous moths), though *Seirarctia echo* and *Erechthias* sp. may be exceptions given that they possess adaptations for feeding on cycads’ fresh leaf tissue of cycads.

*Is there evidence of coevolution between cycads and their lepidopteran herbivores?* All cycadivorous Lepidoptera must possess adaptations to circumvent or tolerate cycad-specific defenses, and the selective value of cycad defensive traits against herbivores seems clear. Do cycadivorous Lepidoptera exert selective pressure on their host cycads to become more toxic? While there is little debate about the importance of plant defensive traits for herbivore fitness (Janz
and Nylin, 1998; Futuyma and Agrawal, 2009), the importance of insect herbivores as selective agents is less clear as most plants seem able to tolerate intermediate levels of herbivory without a significant reduction in fitness (Cornell and Hawkins, 2003). Evidence of reciprocal adaptation between pairs of plants and herbivores has been relatively scarce (Farrell, 1993), and the step-wise selection scenario initially envisaged by Erlich & Raven appears to be extremely asymmetrical: shifts to chemically novel hosts lead to bursts in diversification in many herbivore groups, but escape from herbivores through chemical novelty seems to have had little impact on diversification rates in most plant groups (Farrell, 1998; Wheat et al., 2007) (but see Berenbaum and Zangerl, 1998 and Edger et al., 2015).

Yet damage inflicted by folivorous Lepidoptera can be so extreme that just a few generations can kill a large cycad, which are exceedingly slow to grow and reproduce. Selective pressures exerted by specialist herbivores may therefore be especially severe for cycads relative to other plants groups, raising the possibility that some lepidopteran herbivores could select for escalated chemical defenses and perhaps influence the diversification of their cycad hosts. Previous work has identified diverse secondary compounds in cycads (Pan et al., 1997; Snyder and Marler, 2011; De Luca et al., 1982) that appear to be evolving (De Luca et al., 1982), but phylogenetically explicit comparisons of cycad defensive chemistries (toxins, antinutritive compounds, and volatile organic compounds) would be required to look for evidence of phytochemical escalation and coevolution sensu Ehrlich & Raven.
Of course, the phylogenetic distribution of cycadivory in Lepidoptera suggests repeated, independent colonizations of cycads from distantly related angiosperm hosts, and co-speciation with cycads is reasonably plausible only among *Eumaeus* butterflies and Diptychini moths. Attention should therefore be focused on assessing coevolution between *Eumaeus* with the new world cycad genera *Zamia*, *Dioon*, and *Ceratozamia*, and between the African Diptychini moths with cycad genera *Encephalartos* and *Stangeria*. All cycad genera likely diversified within the last 10-20 MY (Nagalingum et al., 2011; Condamine et al., 2015; Salas-Leiva et al., 2013; Calonje et al., 2019). While we do not have age estimates for *Eumaeus* we know that it is less than 18MY old (Espeland et al., 2018) and likely much younger, though dated phylogenetic analyses are needed to properly evaluate the possibility of co-diversification in any lineage pair.

*How does cycadivory evolve?* Identifying evolutionary and ecological precursors to cycadivory could help explain the repeated transitions to cycads among Lepidoptera. For example, did the host plants of ancestral species somehow facilitate shifts to cycad feeding, either through chemical similarity or other features? From the data gathered and presented here, there is no evidence that cycadivory has evolved from a single, shared host lineage. The ancestors of cycadivorous taxa likely fed on diverse angiosperms including both monocots and dicots, though improved phylogeographic and life history information will be required to infer the most likely ancestral food plants of cycadivorous lineages. Hypotheses regarding what the ancestors of cycadivorous species ate prior to their transitions to cycads may suggest as yet unknown chemical similarities
between cycads and some angiosperm groups. Or, if no chemical similarities are found, it raises questions about the origins of potentially novel adaptations for overcoming cycads’ defenses.

Lepidoptera are known to employ numerous adaptations for feeding on chemically defended host plants. These include behavioral adaptations (Dussourd and Denno, 1991), physiological mechanisms (Hartmann et al., 2005), and perhaps even associating with symbiotic gut bacteria (e.g. Salzman, Whitaker, and Pierce, 2018, Chapter 2), though this is considered rare in Lepidoptera (Whitaker et al., 2016; Hammer et al., 2017). Increased host breadth and feeding on select plant tissues can also minimize an insect’s exposure to plant defensive compounds. Even Diptychini moths – which we consider to be obligate cycad specialists – often switch to feeding on angiospermous plants and thereby potentially reduce their exposure to cycad defenses. It is presently unknown which specific adaptations might be required for cycadivory, or how widely specific adaptations are shared across and within feeding guilds, e.g., among specialized folivores. *Sierarctia echo* are capable of modifying dietary MAM into its non-toxic form (Teas, 1967), but it is unknown whether other herbivores actively detoxify MAM using a similar mechanism. Moreover, no adaptations have been identified to date that would enable herbivores to cope with BMAA, steryl glycosides, or other defensive compounds, let alone complex phytochemical mixtures. Finally, herbivores need to locate and discriminate between potential host plants, and while previous work has described chemical cues used by the insect pollinators of cycads (Terry et al., 2005), no work has addressed chemical communication between cycads and lepidopteran herbivores.
Different lepidopteran lineages may also experience different evolutionary constraints in their ability to feed on cycads. Among butterflies, the Nymphalidae appear to be particularly constrained in their ability to colonize new host-plant families (Hamm and Fordyce, 2015), whereas the Lycaenidae exhibit enormous trophic diversity that includes both phytophagous and aphytophagous diets (Pierce et al., 2002). Indeed, Ehrlich & Raven were able to identify few phylogenetic patterns in lycaenids’ host use and were puzzled by their ‘bewildering array’ of host plant affiliations. The only published lycaenid genome demonstrates significant expansion in detoxification and digestion enzymes (Cong et al., 2016), which, if shared broadly across the family might explain why lycaenid butterflies seem predisposed to trophic innovation, including repeated colonization of cycads over the last 20 MY. Even so, feeding on chemically defended host plants and sequestration of host plant defensive chemicals is rare among lycaenids (Fiedler, 1996).

### 3.8 Conclusions

Cycadivorous Lepidoptera are remarkably diverse in their defensive strategies, life histories, and hostplant relationships, providing numerous opportunities for research. Despite several decades of research on a handful of focal species, many cycadivorous Lepidoptera remain understudied, undersampled, and undescribed. Additional systematic surveys of herbivore diversity and host breadth, studies of predator and parasitoid pressures in natural habitats, and investigations into insects’ adaptations to cycad toxins would be valuable, along with
genus- and tribe-level phylogenies of cycadivorous groups and their sister taxa. Future studies should consider other insect groups as well, as cycadivory has been reported among larvae and adults of non-pollinating beetles (Coleoptera) (Marler and Muniappan, 2006); bees (Hymenoptera) (DeVries, 1983; Ornduff, 1991; Terry, 2001; Valencia-Montoya et al., 2017); leaf-mining larvae of an unidentified fly (Diptera) (DeVries, 1983); termites (Blattodea) (Marler, Yudin, and Moore, 2011); and phloem-feeding scale insects, aphids, and mealybugs (Hemiptera). In summarizing what is known about the phylogenetic placement of cycadivorous Lepidoptera, along with their hostplant relationships, defensive ecology, and management, we hope to encourage more research on the insect herbivores of cycads and other gymnosperms.
Concluding Remarks

In this thesis I have used the plant order Cycadales as a backdrop to study symbiosis. I choose cycads because of their long evolutionary history and many understudied and highly specific species interactions, but also because I fell in love with these plants the first time that I heard about them in Chelsea Specht’s undergraduate plant morphology course at UC Berkeley. Dr. Specht is the academic granddaughter of Knut Norstog, whose wonderful quote opens this thesis and guides my work. Dr. Norstog believed that we should view the living cycads as the Rosetta Stone for plant biology, and I would add, symbiosis biology and evolutionary biology.

The Neotropical genus *Zamia* maintains multiple specialized organismal relationships. *Rhopalotria* weevils have an obligate brood site pollination mutualism with *Zamia* in which the entire genus of weevil feeds solely on *Zamia* male cone parenchyma tissue, and each *Zamia* species is pollinated by a single *Rhopalotria* species (Tang, 1987b; Norstog and Fawcett, 1989). A phylogenetic analysis of these beetles and their hosts will enable us to assess whether the cospeciation that we expect to find between them also reflects the kind of co-evolution we might expect from such an exquisitely co-evolved system. Beetles in the genus *Pharaxanotha* also feed on and pollinate *Zamia*, although less is known about their specificity (Fawcett and Norstog, 1993; Tang et al., 2018),
and they would also be fruitful candidates for further phylogenetic analysis, in part so that their histories might be compared with those of *Rhopalotria* and their hosts. In contrast to these specialized pollination mutualisms, *Zamia* cycads also possess a number of specialized insect herbivores such as those found in the lycaenid butterfly genus *Eumaeus*. This thesis found yet a third layer of specialization by identifying key gut bacterial species shared by unrelated and geographically distant cycad herbivores (Chapter 2, Salzman, Whitaker, and Pierce, 2018). *Zamia* and its multiple specialized organismal relationships represent a natural simplified interaction network.

The simplicity of the network and specialization of the interactions allows for evolutionary analysis of an entire network of specialized interacting partners that could ultimately provide insight into the the causes and consequences of specialized species interactions. Assessing the population structure of *Rhopalotria* weevils that are shifting host plants could elucidate the potential for specialized species interactions to lead to lineage diversification. Understanding the mechanisms of host plant perception and acceptance in these populations will also illuminate how insect host preference might contribute to partner fidelity as well as lineage diversification.

This work has already spurred multiple lines of research. The work presented in Chapter 1 prompts the obvious question: Is there evidence of coevolution between pollinators and plants? In work done during my thesis and continued in my postdoc, I am undertaking systematic surveys of plant volatiles and insect response to look for reciprocal evolution of those phenotypes. The results found in Chapter 2 instigated a project with Drs. Angélica Cibrián-Jaramillo,
Concluding Remarks

and Francisco Barona-Gómez, investigating the potential for the gut bacterial communities to aid in detoxification of cycad toxins and identifying emergent bacterial gene clusters unique to the core bacteria found in cycad herbivores. Chapter 3 introduces the concept of cycadivorous lineages as excellent candidate systems for comparative life history strategies and has lead to phylogenetic analyses of lyceanid cycad herbivores with Dr. Melissa Whitaker.

As a graduate student, I have had the great good fortune to study a system that I love, investigating questions that intrigue me, and working with collaborators who inspire me. I look forward to continuing my research on this beautiful system with these incredible researchers during my postdoctoral studies.
Appendix A

Supplement Chapter 1
FIGURE A.1: Schematic of pit test
FIGURE A.2: Schematic of behavioral arena
Appendix B

Supplement Chapter 2

Published article: Cycad-feeding insects share a core gut microbiome supporting material

The following pages contain a reproduction of the paper supporting material.
Appendices

Supplementary Material 1: Non-cycadivorous lycaenid and curculionid samples that passed filtering requirements and were analyzed for the presence of shared bacterial OTUs found in cycad herbivores.

<table>
<thead>
<tr>
<th>Outgroup herbivore</th>
<th>Order</th>
<th>#</th>
<th>Host plant</th>
<th>Locality</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jalmenus daemeli (Lucas)</td>
<td>Lepidoptera:</td>
<td>3</td>
<td>Acacia</td>
<td>Queensland, Australia</td>
<td>Whitaker et al. 2016</td>
</tr>
<tr>
<td></td>
<td>Lycaenidae           &amp;</td>
<td></td>
<td>(von Martius)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lycaenidae           &amp;</td>
<td></td>
<td>(Wight &amp; Arn.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypothenemus hampei (Ferrari)</td>
<td>Coleoptera:</td>
<td>4</td>
<td>Coffea arabica</td>
<td>Chiapas, Mexico</td>
<td>Ceja-Navarro et al. 2015</td>
</tr>
<tr>
<td></td>
<td>Curculionidae</td>
<td></td>
<td>(Linnaeus)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypothenemus eruditus (Westwood)</td>
<td>Coleoptera:</td>
<td>4</td>
<td>Cecropia</td>
<td>Chiapas, Mexico</td>
<td>Ceja-Navarro et al. 2015</td>
</tr>
<tr>
<td></td>
<td>Curculionidae</td>
<td></td>
<td>(Loefl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypothenemus crudiae (Panzer)</td>
<td>Coleoptera:</td>
<td>4</td>
<td>Cecropia</td>
<td>Chiapas, Mexico</td>
<td>Ceja-Navarro et al. 2015</td>
</tr>
<tr>
<td></td>
<td>Curculionidae</td>
<td></td>
<td>(Loefl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scolytodes maurois</td>
<td>Coleoptera:</td>
<td>5</td>
<td>Cecropia</td>
<td>Chiapas, Mexico</td>
<td>Ceja-Navarro et al. 2015</td>
</tr>
<tr>
<td>(Blandford)</td>
<td>Curculionidae</td>
<td></td>
<td>(Loefl)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Supplementary Material 2: Species core microbiome OTU and assigned taxonomy are listed with OTUs present in more than one species highlighted in grey. Species’ microbiome cores were determined by OTU presence in 100 percent of that species samples after filtering and are represented by their OTU#.

<table>
<thead>
<tr>
<th></th>
<th>Chilades pandava</th>
<th>Eumaeus atala</th>
<th>Rhopalotria furfuracea</th>
<th>Pharaxanotha floridana</th>
<th>Eubulus sp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown Amycolabacillaceae</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Unknown Rickettsiaceae</td>
<td>85</td>
<td>85</td>
<td>85</td>
<td>85</td>
<td>85</td>
</tr>
<tr>
<td>Unknown Enterobacteriaceae</td>
<td>249</td>
<td>249</td>
<td>249</td>
<td>249</td>
<td>249</td>
</tr>
<tr>
<td>Enterobacteriaceae Raoultella ornithinolytica</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Unknown Enterobacteriaceae</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Comamonadaceae Curvibacter</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Comamonadaceae Lampropedia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>36</td>
</tr>
<tr>
<td>Comamonadaceae Comamonas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>432</td>
</tr>
<tr>
<td>Enterococcaceae Enterococcus</td>
<td></td>
<td>15</td>
<td></td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>Streptococcaceae Lactococcus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Alcaligenaceae Achromobacter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>Unknown Blattabacteriaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>240</td>
</tr>
</tbody>
</table>

89
### Appendix B. Supplement Chapter 2

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
<th>Count</th>
<th>ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphingobacteriaceae</td>
<td>Sphingobacterium</td>
<td></td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Moraxellaceae</td>
<td>Acinetobacter</td>
<td></td>
<td>763</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Pseudomonadaceae</td>
<td>Pseudomonas</td>
<td></td>
<td>9</td>
<td>443</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1045</td>
</tr>
<tr>
<td>Rhodobacteraceae</td>
<td>Rhodobacter</td>
<td></td>
<td>212</td>
<td>65</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Pantoea</td>
<td></td>
<td>487</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Serratia</td>
<td></td>
<td>1526</td>
<td>654</td>
</tr>
<tr>
<td>Sphingobacteriaceae</td>
<td>Novosphingobium</td>
<td></td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>Weeksellaceae</td>
<td>Weeksella</td>
<td></td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Weeksellaceae</td>
<td>Chryseobacterium</td>
<td></td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>Flavobacteriaceae</td>
<td>Flavobacterium</td>
<td></td>
<td>251</td>
<td></td>
</tr>
<tr>
<td>Xanthomonadaceae</td>
<td>Stenotrophomonas</td>
<td></td>
<td>1555</td>
<td>6</td>
</tr>
<tr>
<td>Microccaceae</td>
<td>Arthrobacter</td>
<td></td>
<td>279</td>
<td></td>
</tr>
<tr>
<td>Brucellaceae</td>
<td>Ochrobactrum</td>
<td></td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Erwinia</td>
<td></td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td></td>
<td>642</td>
<td></td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td></td>
<td>Alcaligenacea</td>
<td>112</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alcaligenes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Porphyromonadaceae</td>
<td>Dygonomonas</td>
<td></td>
<td>78</td>
<td>77</td>
</tr>
<tr>
<td>Porphyromonadaceae</td>
<td>Parabacteroides</td>
<td></td>
<td>159</td>
<td></td>
</tr>
<tr>
<td>Bacteroidaceae</td>
<td>Bacteroides</td>
<td></td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>Weeksellaceae</td>
<td>Wautersiella</td>
<td></td>
<td>167</td>
<td></td>
</tr>
<tr>
<td>Hyphomicrobiaceae</td>
<td>Devosia</td>
<td></td>
<td>132</td>
<td></td>
</tr>
<tr>
<td>Cytophagaceae</td>
<td>Leadbetterella</td>
<td></td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Erysipelotrichaceae</td>
<td>Erysipelothrix</td>
<td></td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Rhizobiaceae</td>
<td>Agrobacterium</td>
<td></td>
<td>30</td>
<td></td>
</tr>
</tbody>
</table>
Supplementary Material 3: Oligotyping results

Seven entropy peaks were necessary to decompose oligotypes for OTU5 (*Raoultella ornithinolytica*). Twenty one unique oligotypes were found and represented 16,154 of the 16,383 sequence reads (98.60%). *Eubulus sp.* and *Eumaeus atala* are most strongly represented in this OTU (34.73% and 33.03%) with *Pharaxanotha floridana* close behind (22.11%). *Rhopalotria furfuracea* is represented by 9.50% and *Chilades pandava* by only 0.64%. *P. floridana* has the most consistent composition of oligotypes across all species with *Eub. sp.* showing a similar although less constrained pattern (Figure 3). *Eum. atala* is completely dominated (>93%) by one oligotype across all samples.

Six entropy peaks were necessary to decompose oligotypes for the 16,383 sequence reads for OTU 4 (Unknown Enterobactereaceae). The resulting 20 unique oligotypes represent 16,223 reads (99.02%). *P. floridana* and *Eub. sp.* represent the majority of sequences in this OTU (60.65% and 28.57% respectively) with relatively low abundances in *R. furfuracea* (7.71%) and *Eum. atala* (2.77%) and very few in *C. pandava* (0.30%). *P. floridana* and *Eub. sp.* show consistent patterns of oligotype composition, with *P. floridana* again more constrained (Figure 3). OTU 4 oligotypes are varied across the remaining species.

Three entropy peaks decomposed oligotypes for the 522 reads for OTU 249 (Unknown Enterobactereaceae). The resulting three oligotypes represented 464 reads (88.89%). All species are fairly evenly represented in the oligotyping (*C. pandava*, *Eum. atala*, and *R. furfuracea* each 15.52%, *Eub. sp.* 28.66% and *P. floridana* 24.79%). One oligotype dominated across all samples with no species showing a consistent pattern regarding the remaining two oligotypes.

Oligotyping analysis found no entropy peaks for either OTU 85 (Unknown Rickettsiaceae) (1,092 reads) or OTU 3 (Unknown Alicyclobacillaceae) (16,383 reads) suggesting that these two OTUs are represented by one oligotype each across all herbivore samples or that rare oligotypes are not present in enough abundance in the dataset to be distinguished from sequencing noise.
Appendix C

Supplement Chapter 3

C.1 Evaluating hostplant records

In preparing this review, we found it challenging to confirm the accuracy of published hostplant records, even for relatively well-studied organisms such as butterflies. In the words of Robinson et al., 2010, "erroneous hostplant records are cumulative." Through repeated citation these errors propagate throughout the literature, such that the original errors become increasingly difficult to find and correct. In an effort to confirm as many records of cycadivory as possible, we have traced back every citation (when possible) until original errors were found. We successfully located original sources in all cases unless otherwise noted.

Table C.1 shows unconfirmed hostplant association(s) with the original source and citing authors. An explanation for each record follows. In compiling this list we noticed a few common errors that were made by multiple recorders. For example, many authors list Cycas circinalis as a larval hostplant even in areas where C. circinalis does not occur (e.g., Brazil). We gather that Cycas circinalis
Appendix C. Supplement Chapter 3

was something of a catchall name for cycads prior to improved knowledge of cycad taxonomy and distributions. Additionally, many authors attempt to identify Lepidoptera based on larval morphology alone. Doing so is especially error-prone for moths and lycaenid species, as these larvae are exceedingly difficult to identify even by trained experts. Such records therefore require additional scrutiny.

Importantly, not all records listed here are taken to be erroneous. Several records simply lack necessary data and are therefore provisionally listed here pending further confirmation.
### Table C.1: Unverified host plant records

<table>
<thead>
<tr>
<th>Species</th>
<th>Host(s)</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nymphalidae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. <em>Opsiphanes invirae</em></td>
<td><em>Cycas circinalis</em></td>
<td>Schneider et al., 2002; Robinson et al., 2010; Peña and Espeland, 2015</td>
</tr>
<tr>
<td>2. <em>Elymnias agondas</em></td>
<td><em>Cycas circinalis, C. revoluta</em></td>
<td>Robinson et al., 2010; Merrett, 1993; Peña and Espeland, 2015</td>
</tr>
<tr>
<td>3. <em>Faunis eumeus</em></td>
<td><em>Cycas revoluta</em></td>
<td>Robinson et al., 2010; Easton and Pun, 1997</td>
</tr>
<tr>
<td><strong>Lycaenidae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. <em>Eumaeus atala</em></td>
<td><em>Manihot</em></td>
<td>Comstock, 1948; Ehrlich and Raven, 1964</td>
</tr>
<tr>
<td>5. <em>Eumaeus childrenae</em></td>
<td><em>Amaryllis</em></td>
<td>Ehrlich and Raven, 1964; Schneider et al., 2002; Contreras-Medina, Ruiz-Jiménez, and Luna Vega, 2003</td>
</tr>
<tr>
<td>6. <em>Euchrysops cnejus</em></td>
<td><em>Cycas</em></td>
<td>Robinson et al., 2010; Cayabyab, 1993; Io, 1994</td>
</tr>
<tr>
<td>7. <em>Acytolepis puspa</em></td>
<td><em>Cycas rumphii</em></td>
<td>Robinson et al., 2010; Ceylon Department of Agriculture, 1924; Vane-Wright and Jong, 2003</td>
</tr>
<tr>
<td>9. <em>Chilades kimurae</em></td>
<td><em>Cycas revoluta</em></td>
<td>Wakabashi and Yoshizaki, 1967</td>
</tr>
<tr>
<td>10. <em>Luthrodes pandava</em></td>
<td>Non-cycad plants in the families Fabaceae and Sapindaceae</td>
<td>Tiple et al., 2009; Schneider et al., 2002; Ehrlich and Raven, 1964; Nitin et al., 2018</td>
</tr>
<tr>
<td><strong>Pyralidae</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Explanations

1. The neotropical nymphalid species *Opsiphanes invirae* is often claimed to feed on *Cycas circinalis*. Authors typically cite Ackery, 1988 or Penz, Aiello, and Srygley, 1999, although the latter source also highlights this hostplant record as dubious. The original source is a catalog of Brazilian insects and their host plants (Silva et al., 1968) that states that *O. invirae* larvae feed on "palmeira de jardim," which translates simply to "garden palm." How this was interpreted to mean *Cycas circinalis* is unknown, though hostplants for *O. invirae* do include several palm species. In due diligence, we sought all original sources for the Silva et al., 1968 publication, many of them obscure Brazilian agricultural articles from the turn of the century and were successfully able to access 16 of the 21. None of these mentioned cycads as a host plant.

2. Merrett, 1993 reports finding a single pupa of another nymphalid butterfly, *Elymnias agondas*, on *Cycas circinalis* in a field study in New Guinea. He then attempted to rear *Elymnias agondas* larvae on cycads but observed 100% mortality among larvae. We suspect that the pupa fed on palms and merely wandered onto the cycad to pupate. Interestingly, this species co-occurs with and is thought to be involved in mimetic relationships with *Taenaris catops*, which feeds on cycad exudates as an adult.

3. Easton and Pun, 1997 reared larvae of the nymphalid *Faunis eumeus* on *Cycas revoluta* and several palm species in Macau, China, noting that these
are not the normal hostplants for this species. The authors report nothing about the performance of larvae fed on cycad versus non-cycads, so we assume that larvae were able to develop. Even so, considering that this species does not normally feed on cycads we do not consider it to be naturally cycadivorous, though further study would be helpful.

4. The larvae of *Eumaeus atala* have been said to feed on introduced *Manihot* species (cassava) by Comstock, 1948 and Ehrlich and Raven, 1964 (citing "Comstock unpubl."). Comstock does not provide any citations or details about the observation so the basis of this hostplant association is unclear, but given that cassava is widely cultivated throughout the Caribbean yet no other records exist for *E. atala* feeding on them, we classify this as an erroneous record.

5. Ehrlich and Raven, 1964 note that the larvae of *Eumaeus childrenae* feed on Amaryllis (Amaryllidaceae), citing only "Comstock unpubl." Schneider et al., 2002 also mention that *E. childrenae* feed on Amaryllis , citing Draudt, 1921. However, Comstock, 1948 highlights this as a likely dubious report:

"Dr. M. Draudt, in Seitz, says that "the carmine, black-belted [*E. childrenae*] larva lives gregariously on an Amaryllis standing in water." His reference does not give the specific name of the plant and the designation of "black-belted" does not tally with the [*E. childrenae*] larva reared by us, unless the sparse black hairs crossing the center of the segments gives an impression of black bars."
Based on the physical description of the larva and the fact that no further observations have been recorded since 1921, it is likely that Draudt misidentified the larva found feeding on *Amaryllis* as *Eumaeus childrenae*.

6. The lycaenid butterfly *Euchrysops cnejus* presents a somewhat tricky case. Vane-Wright and Jong, 2003 state that the larvae of this species accept *Cycas* (species not given) in Sulawesi, but the authors provide only a single citation (Watson et al. 1995). However, Watson, Ooi, and Girling, 1995 do not mention *Euchrysops cnejus* feeding on *Cycas*, so perhaps this record is based on the authors’ own field observations. Cayabyab, 1993 also claim to have found *E. cnejus* on *Cycas revoluta* but provide no details, vouchers, or photographs. *Euchrysops cnejus* is quite polyphagous among lycaenids, so it is certainly possible that the larvae accept cycads as larval foodplants. On the other hand, *E. cnejus* larvae are nearly identical to *Luthrodes pandava* larvae, so it is also possible that the authors confused these two species. Finally, Chou, 1994 Monograph of Chinese Butterflies also lists *Cycas* as a foodplant of *E. cnejus* but we are unable to further evaluate the this record.

7. The lycaenid species *Acytolepis puspa* was reported to feed on *Cycas* (species not given) by Vane-Wright and Jong, 2003, citing Fukuda et al., (1992) and Fiedler, unpubl. We were unable to find a publication by Fukuda et al. (1992). However, (Fukuda et al., 1984 does not list cycads as a food source for *Acytolepis puspa*, nor do more recent accounts of this species’ larval foodplants by the same author (Igarashi and Fukuda, 2000). We are therefore unable to trace the original source for this hostplant record. A second publication, a list of agricultural pests in India published in 1924, states
that *Cyaniris puspa* (a former synonym for *Acytolepis puspa*) feeds on *Cycas circinalis* and *Cycas rumphii* (Ceylon Department of Agriculture, 1924), but does not provide citations or details. We conclude that further documentation is needed regarding hostplant use in this species.

8. A single record exists for *Everes lacturnus* (Lycaenidae) feeding on *Cycas* (species not determined) from a survey of butterflies found on the Kerala University Campus in India (Antony, Prasad, and Kalesh, 2016). Given the difficulty of identifying Polyommatine larvae and that this is an isolated record, we suspect the larva was misidentified.

9. Wakabashi and Yoshizaki, 1967 reported feeding by "*Chilades kiamurae*" on *Cycas revoluta* in Japan, providing extensive photographic evidence of larval feeding and female oviposition on the plants. However, *Chilades kiamurae* is not a recognized species (Talavera et al., 2013), and though it is sometimes considered a subspecies of *Luthrodes mindora*, *L. mindora* does not occur in Japan. Based on the photographic evidence provided by Wakabayashi & Yoshizaki, we suspect that the larvae they observed feeding on cycads were most likely to be *Luthrodes pandava*, a confirmed cycadivorous species.

10. The lycaenid species *Luthrodes pandava* has been recorded feeding on numerous native and introduced cycads across its large range, and several authors claim that *L. pandava* larvae have been found feeding on non-cycad hostplants as well. Tiple, Khurad, and Dennis, 2011 note that they were
Appendix C. Supplement Chapter 3

able to rear *L. pandava* larvae on non-cycad hosts in captivity. We tentatively list these non-cycad host records here due to the extreme difficulty of identifying juvenile stages of *Luthrodes* larvae, and the high likelihood of authors confusing *L. pandava* larvae with other co-occurring lycaenid species. However, it may indeed be true that *L. pandava* feed on both cycad and non-cycad hosts, in which case they would provide the only example of polyphagy among cycadivorous butterflies. We encourage efforts to confirm the host breadth of this species through continued captive rearing efforts combined with expert identification and molecular sequencing.

11. Larvae of an unidentified moth were found feeding on *Zamia amblyphyladia* and *Z. portophylla* male cones in Puerto Rico (Franz and Skelley, 2008). The authors placed the moth in the family Pyralidae but did not provide details about the method of identification, and we wonder if they may have mistaken the larvae of Blastobasidae moths, which have been confirmed on these same plant populations by Terry et al., 2012a, who provide photograph evidence and means of identification. Further study is needed to confirm the identity of this species.

12. A single record exists of *Cryptoblabes plagioleuca* (Pyralidae) larvae feeding on *Cycas rumphii* in Fiji (Hinckley, 1964). Further reports of this hostplant association have not been published.

13. Several authors claim that the moth *Orvasca subnotata* (Erebidae) feeds on *Cycas circinalis* in Borneo. However, Holloway, 1999 points out that this moth is often misidentified, cautioning that:
"The possibility that this species has been confused with others means that lists of host records need to be treated with some caution and may particularly overlap with those for *Somena similis*. No larval description has been located apart from those of Moore (1883), who may have confused *Somena* species and *subnotata*.

14. *Cryptoptila immersana* (Tortricidae) is a highly polyphagous leaf roller that Common, 1990 records from *Bowenia* in Australia, though he does not provide any supporting evidence. Further confirmation of this record would be particularly valuable, as this would present the first and only record of a lepidopteran feeding on *Bowenia* cycads.

15. The Natural History Museum’s HOST database lists *Cycas revoluta* as a hostplant for *Oiketicus kirbyi* (Psychidae) (Robinson et al., 2010) but we can find no original reports of this hostplant association.
C.2 Cycadivorous Lepidoptera species synonyms

### Table C.2: Species synonyms

<table>
<thead>
<tr>
<th>Accepted Species Name</th>
<th>Synonyms / Previous Names</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lycaenidae</strong></td>
<td></td>
</tr>
<tr>
<td><em>E. atala</em> (Poey, 1832)</td>
<td><em>E. floridana, Eumenia atala</em></td>
</tr>
<tr>
<td><em>E. childrenae</em> (Gray, 1832)</td>
<td><em>E. deborah, Eumaea debora, Eumenia childrenae</em></td>
</tr>
<tr>
<td><em>E. toxea</em> (Godart, [1824])</td>
<td><em>Eumenia toxea, erroneously as E. minyas</em></td>
</tr>
<tr>
<td><em>E. godartii</em> (Boisduval, 1870)</td>
<td><em>E. costaricensis, erroneously as E. minyas</em></td>
</tr>
<tr>
<td><em>E. minyas</em> (Hübner, [1809])</td>
<td><em>E. minijas</em></td>
</tr>
<tr>
<td><em>E. toxana</em> (Boisduval, 1870)</td>
<td><em>E. minyas obsoleta, E. giganteus, E. sara, Eumenia toxana, erroneously as Eumaeus minyas</em></td>
</tr>
<tr>
<td><em>L. cleoatas</em> (Guérin-Méneville, [1831])</td>
<td><em>Chilades cleotas, Polygonimus cleotis</em></td>
</tr>
<tr>
<td><em>L. pandava</em> (Horsfield, [1829])</td>
<td><em>Chilades pandava, Lycaena pandava, Edales pandava, Catochrysops pandava, Catochrysops nicola, Lampides pandava, Catochrysops bengalia, Euchrysops pandava, Euchrysops insularis</em></td>
</tr>
<tr>
<td><strong>Geometridae</strong></td>
<td></td>
</tr>
<tr>
<td><em>Z. lepida</em> (Walker, 1854)</td>
<td><em>Z. leopoldina, Deiopeia lepida</em></td>
</tr>
<tr>
<td><em>Z. tenuis</em> (Butler, 1878)</td>
<td><em>Z. fulva</em></td>
</tr>
<tr>
<td><em>C. abraxas</em> Staude, 2001</td>
<td><em>C. boisduvalii</em></td>
</tr>
<tr>
<td><em>V. setinata</em> (Felder &amp; Rogenhofer, 1875)</td>
<td><em>Durbana setinata</em></td>
</tr>
</tbody>
</table>


Azuma, H and M Kono (2006). “Estragole (4-allylanisole) is the primary compound in volatiles emitted from the male and female cones of Cycas revoluta”. In: Journal of Plant Research 119.6, pp. 671–676.


BIBLIOGRAPHY


Bowers, MD and S Farley (1990). “The behaviour of grey jays, Perisoreus canaden-
sis, towards palatable and unpalatable Lepidoptera”. In: Animal Behaviour 39.4, pp. 699–705.


Castillo-Guevara, C and V Rico-Gray (2003). “The role of macrozamin and cy-
casin in cycads (Cycadales) as antiherbivore defenses”. In: Journal of the Torrey Botanical Society 103, pp. 206–217.

Ceylon Department of Agriculture (1924). “A preliminary list of the pests of cultivated plants in Ceylon”. In: Bulletin of the Department of Agriculture, Ceylon 67, pp. 1–68.


Chaw, SM et al. (2000). “Seed plant phylogeny inferred from all three plant genomes: monophyly of extant gymnosperms and origin of Gnetales from conifers”. In: Proceedings of the National Academy of Sciences 97.8, pp. 4086–4091.


Common, IFB (1990). Moths of Australia. CSIRO PUBLISHING.


Cong, Q et al. (2016). “Complete genomes of Hairstreak butterflies, their speciation, and nucleo-mitochondrial incongruence”. In: Scientific reports 6, p. 24863.

Contreras-Medina, R, CA Ruiz-Jiménez, and I Luna Vega (2003). “Caterpillars of Eumaeus childrenae (Lepidoptera: Lycaenidae) feeding on two species of cycads (Zamiaceae) in the Huasteca region, Mexico”. In: Revista de Biología Tropical 51.1, pp. 201–204.


BIBLIOGRAPHY


— (1862). On the various contrivances by which British and foreign orchids are fertilized by insects and the good effects of intercrossing. John Murray, London.


Feulner, GR et al. (2014). “First UAE and Arabian records of Chilades pandava, the Cycad Cupid butterfly, an introduced oriental species (Lepidoptera: Lycaenidae) hosted by the ornamental sago plant Cycas revoluta”. In: Tribulus 22, pp. 48–57.


Forster, PI and PJ Machin (1994). “Cycad host plants for ’Lilioceris nigripes’ (Fabricius) (Coleoptera: Chrysomelidae) and ’Theclinesthes onycha’ (Hewitson) (Lepidoptera: Lycaenidae)”. In: The Australian Entomologist 21.3, p. 99.


Gao, Z and BA Thomas (1989). “A review of fossil cycad mega-sporophylls, with new evidence of Crossozamia pomel and its associated leaves from the


BIBLIOGRAPHY


Menken, SBJ, JJ Boomsma, and EJ Van Nieukerken (2010). “Large-scale evolutionary patterns of host plant associations in the Lepidoptera”. In: Evolution 64.4, pp. 1098–1119.


BIBLIOGRAPHY


Pierce, NE et al. (2002). “The ecology and evolution of ant association in the Lycaenidae (Lepidoptera)”. In: Annual review of entomology 47.1, pp. 733–771.


Robinson, GS et al. (2010). *HOSTS-A database of the world’s lepidopteran hostplants*. URL: [http://www.nhm.ac.uk/hosts](http://www.nhm.ac.uk/hosts).


BIBLIOGRAPHY

Sevastopulo, DG (1941). “On the food plants of Indian bombyces (Heterocera)”. In: Journal of the Bombay Natural History Society 41.

Shen, CF, Tsou CH Hill KD, and CJ Chen (1994). “Cycas taitungensis, sp. nov. (Cycadaceae), a new name for the widely known cycad species endemic in Taiwan”. In: Botanical Bulletin Academia Sinica 35.2, pp. 133–140.


Silva, AGdeA et al. (1968). “Quarto catálogo dos insetos que vivem nas plantas do Brasil, seus parasitos e predadores”. In: Seus parasitos e predadores. Parte 2.


Teas, HJ (1967). “Cycasin synthesis in Seirarcta echo (Lepidoptera) larvae fed methylazoxymethanol”. In: Biochemical and Biophysical Research Communications 26.6, pp. 686–690.


Wilson, GW (1993). “The relationships between Cycas ophialitica K. Hill (Cycadaceae), the butterfly Theclinesthes onycha (Lycaenidae), the beetle Lilioceris nigripes (Coleoptera: Chrysomelidae) and the ant Iridomyrmex purpureus”. In: Proceedings 1993 Postgraduate Student Association Symposium. Ed. by A Dekkers, G Wilson, and G Harris, 53–7.


Wu, LW et al. (2010). “Elucidating genetic signatures of native and introduced populations of the cycad blue, Chilades pandava to Taiwan: a threat both to Sago Palm and to native Cycas populations worldwide”. In: Biological Invasions 12.8, pp. 2649–2669.


