Source and Sink Relations Mediate Depletion of Intrinsic Cycad Seed Carbohydrates by *Aulacaspis yasumatsui* Infestation

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Abstract. Ubiquitous *Aulacaspis yasumatsui* Takagi infestations of Guam's *Cycas micronesica* K.D. Hill trees cause direct herbivory of most exposed organ surfaces, including developing naked ovules and seeds. The nonstructural carbohydrates of infested vs. noninfested seeds were quantified to understand more fully the influences on seed quality for propagation purposes. Two studies compared seeds from healthy trees with those of unhealthy trees suffering from whole-tree *A. yasumatsui* infestations. The sugars fructose, glucose, maltose, and sucrose were in greater concentrations and herbivory reduced these free sugars by a greater percentage in sarcotesta tissue than in sclerotesta and gametophyte tissues. Starch concentration was greatest in gametophyte tissue, but herbivory reduced starch by a greater percentage in sarcotesta tissue. A third study was used to manipulate seeds of unhealthy infested trees such that some seeds were uninfested and some seeds were infested and revealed the nonstructural carbohydrates of the uninfested seeds were greater than those of the infested seeds in patterns that were similar to those when the entire tree was protected from herbivory. The combined results indicated that both source and sink relations were involved in the reductions of seed carbohydrates by *A. yasumatsui* herbivory. The reduction in seed resource pool by the herbivore feeding may be one of the mechanisms that results in reduced germination percentage and increased seedling mortality.

Cycas micronesica was threatened by the 2003–07 invasions of *Aulacaspis yasumatsui* Takagi and two other specialist insect pests to the islands of Guam and Rota (Marler, 2012; Marler and Muniappan, 2006; Moore et al., 2005). This unique gymnosperm was a common member of commercial and home landscapes and was the most abundant tree species in Guam's forests at the time (Donnegan et al., 2004). The invasions generated unexpected interactions among the nonnative herbivores (Marler, 2013b) and substantial responses by the host plant (Marler, 2013a; Marler and Dongol, 2016b; Marler et al., 2016b). The rapid epidemic tree mortality (Marler and Lawrence, 2012) propelled the species to designations of Endangered status under the International Union for Conservation of Nature (Marler et al., 2010) and Threatened status under the U.S. Endangered Species Act (ESA) (U.S. Fish & Wildlife Service, 2015).

Several conservation projects were initiated during the ensuing years, including establishment of an ex situ germplasm collection on the island of Tinian (Anonymous, 2014; Marler et al., 2016a). Seeds were collected throughout Guam habitats beginning 2006, and the subsequent Tinian nursery observations revealed that the Guam plants with the greatest infestation levels of *A. yasumatsui* directly on the collected seeds provisioned seeds with inferior germination and increased seedling mortality compared with seeds with minimal levels of infestation (unpublished data). This held true for subpopulations suffering from heavy *A. yasumatsui* infestations and subpopulations where the infestation level was moderate. For example, the two Guam subpopulations with the greatest level of direct seed infestations of *A. yasumatsui* exhibited germination of 7%. In contrast, the Guam subpopulation with the least level of seed infestation exhibited germination of 43%. These observations illuminated a need to more fully understand this conservation threat.

The healthy *C. micronesica* seed is a powerhouse of nonstructural carbohydrate resources (Marler and Dongol, 2016a), and a reduction of these seed resources during direct seed infestations of *A. yasumatsui* may occur. Visual observation of infested ovules and seeds (Fig. 1) illuminates the potential for depletion of resources. A greater understanding of the consequences to seed re-

sources caused by direct infestations of this pest is clearly needed. Our objectives were to use observational and experimental data to determine the influence of seed infestations by *A. yasumatsui* on nonstructural carbohydrate relations of various *C. micronesica* seed tissues. We predicted free sugars and starch would decline following *A. yasumatsui* herbivory and the relative declines of carbohydrates in the exposed sarcotesta tissues of the integument would exceed those in the other seed tissues.

Materials and Methods

Preliminary observations. Extensive *C. micronesica* seed toxicology studies were ongoing when the *A. yasumatsui* invasion occurred. The armored scale immigrated into our research site in western Guam in Feb. 2005, and our ability to continue meeting the objectives for these studies was destroyed by the invasion. At this time, we had stored preinvasion seed tissue from several ontogeny and storage studies (Marler and Shaw, 2009, 2010; Marler et al., 2006). These samples were frozen at -40°C and lyophilized in Dec. 2003 and Jan. 2004 before storage and were used to provide a look at the preinvasion seed carbohydrate status.

We were also monitoring a population of female trees with megastrobili that were 3 months in age when the *A. yasumatsui* infestations were initiated. The trees which provided the preinvasion stored seed tissue, and these postinvasion infested trees were growing in the same habitat with coralline soils formed in slope alluvium, loess, and residuum overlying limestone (Clayey-skeletal, gibbsitic, nonacid, isohyperthermic Lithic Ustorthents) (Young, 1988). We continued to monitor these scale-infested seeds until they began abscising at 19 months in age. Seeds were harvested from these trees at this time to provide a look at the postinvasion seed carbohydrate status.

Ten seeds were harvested from each of 10 postinvasion trees on 18 June 2007. Each seed was separated into outer soft integument tissue (sarcotesta), inner hard integument tissue (sclerotesta), and gametophyte tissue. The samples from each of the three seed tissues were combined for each tree into a composite sample to provide 10 replications of each tissue. The tissue was frozen at -40°C then lyophilized.

We used stored sarcotesta, sclerotesta, and gametophyte tissue samples from seeds that were 19 months in age to ensure seeds of the same age were compared for pre- and postinvasion trees. Tissue from 10 stored samples that met these requirements was included. Methods for carbohydrate quantification followed Marler and Dongol (2016a). All tissue samples were milled to pass through 20-mesh screen. Soluble sugar extraction was conducted using hot-water extraction with acetonitrile (80°C ; Schlöter et al., 2005). The concentrations of four free sugars (the hexoses fructose and glucose, and the disaccharides sucrose and maltose) were

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Fig. 1. Healthy *Cycas micronesica* naked ovule (A) and ovule infested with *Aulacaspis yasumatsui* (B). Healthy 11-month-old *C. micronesica* seed (C) and seed of same age infested with *A. yasumatsui* (D). Upper white bar 1 cm for panels A and B, lower white bar 5 cm for panels C and D.

determined by HPLC-RI (Thermo Scientific RI-150, AS3000 autosampler, P2000 pump). Starch was quantified using the anthrone colorimetric method following removal of sugars by ethanol then hydrolysis of starch to glucose using sulfuric acid at 95 °C (Clegg, 1956).

No statistical test was applied to these observational data because there were too many confounding variables. The means and standard errors were compared for preinvasion (13 months before initial scale infestation) and postinvasion (16 months after initial scale infestation) samples to provide a preliminary determination of seed carbohydrate responses to *A. yasumatsui*.

Whole-tree protection. Several plots throughout Guam were established in 2007 for protection from *A. yasumatsui* threat by using root drenches of imidacloprid every 3 to 4 months (Marler, 2018). A site on the east coast of Guam containing the same soil series

described in the preceding text was used to determine seed carbohydrate status with and without plant protection. Synchronized reproductive events that emerged in Nov. 2011 were used to select the experimental female trees. Twelve healthy trees within the protected plot were selected, and 12 nearby unprotected trees with a synchronized megastrobilus were also selected. The trees ranged in height from 2.0 to 2.4 m. Seed set was greater for the healthy trees, so developing seeds were thinned on 24 Feb. 2012 to 15 seeds per megastrobilus among all of the replications to ensure homogeneous seed load throughout seed maturation. Determination of pollinated seeds based on increased size is an unambiguous endeavor at this age (Marler and Dongol, 2011).

Seed harvests occurred in June 2013 when seeds were 19 months in age. Four of the unprotected trees died during the study, so the protected tree closest to each of these trees

was also removed from the study. Ten of the healthiest seeds were harvested from each of the eight remaining trees per treatment. The healthy seeds from the protected trees had received whole-tree insecticide protection for 6 years, and the infested seeds from outside the plot were harvested from trees that had been damaged by *A. yasumatsui* for 7 years.

The separation of seeds into three tissue categories was conducted as previously described. The fresh tissue was immediately placed in a forced draft oven at 75 °C and dried to a constant weight. The dry weight of gametophyte, sarcotesta, and sclerotesta tissue was determined for each 10-seed composite sample. Carbohydrate quantification was as previously described. The total pool of each carbohydrate was calculated for each tissue category by multiplying the concentrations by the corresponding tissue weight.

The two sources of variation were tree protection (two treatments) and seed tissue category (three categories). An exploratory analysis of the concentration and total pool data indicated many differences in variances due to treatment and tissue. Therefore, the Proc MIX function in SAS (SAS Institute, Cary, NC) was used for analysis, and the model allowed for differences in treatment variances and differences in tissue category variances while assuming that the measurements on the three tissues were correlated. Treatment design was a two-way factorial in a split plot design with treatment as the whole plot treatment and tissue as the subplot treatment. Means separation for significantly different means was conducted with Tukey's honestly significant difference test.

Seed-only protection. Twenty unprotected *C. micronesica* female trees with synchronized emergent reproductive structures were selected from the west coast experimental site in Nov. 2011. The naked ovules were protected every 2 weeks with horticultural oil spray to keep the *A. yasumatsui* infestation to a minimum but to allow natural pollination. Determination of seed set was unambiguous by 25 Feb. 2012, and the number of developing seeds per sporophyll was standardized to two by thinning individual seeds and unpollinated ovules. Moreover, when a tree exhibited an odd number of sporophylls, one sporophyll was removed to enable an even number of sporophylls for each of the two treatments.

Each sporophyll was covered by an individual sleeve constructed from lucite screen with 130 holes per cm and affixed to the base of the sporophyll. The sleeve for half of the sporophylls on each tree was left open to enable direct *A. yasumatsui* infestations on the seeds. The sleeves for the other half of the sporophylls on each tree were fully closed to exclude incoming *A. yasumatsui* crawlers. The few scale individuals that were observed inside of the closed sleeves were treated with Pyriproxyfen. This insect growth regulator is absorbed by the plant tissue but it is not systemic. A follow-up spray of horticultural oil suspension was applied to the closed sleeves after 2, 4, and 6 weeks to ensure

mortality of the few initially enclosed *A. yasumatsui* individuals. Inspections for the duration of the study confirmed there were no *A. yasumatsui* individuals infesting the maturing seeds within the enclosed sleeves.

Six of these unhealthy trees died during the study. Moreover, most of the seeds on six of the 14 live trees were aborted due to compromised plant health. Protected and unprotected seeds were among the aborted seeds. These trees were excluded from the study. The remaining eight trees ranged in height from 2.2 to 2.6 m, and all seeds were harvested and separated into protected vs. unprotected seeds for each tree.

The separation of seed tissues and tissue preparation and chemical analyses were as described for the whole-tree protection study. These data met prerequisites for parametric approaches. The concentration and total seed pool data were subjected to a 2×3 factorial with two levels of seed protection and three tissue categories using the Proc GLM function in SAS. Means separation for significant variables was as described earlier.

Results

Preliminary observations. *Cycas micronesica* seed tissue carbohydrate concentrations either declined after the *A. yasumatsui* invasion or remained unchanged among the tissue categories (Fig. 2). The pre-invasion gametophyte concentrations of glucose, maltose, and sucrose were about double those of the postinvasion gametophyte tissue. Post-invasion gametophyte starch concentration was approximately two-thirds that of pre-invasion gametophyte tissue. The gametophyte fructose concentration was not influenced by the scale invasion. All five carbohydrates declined following the invasion within sarcotesta tissue, with fructose concentration declining relatively more than the other carbohydrates. Sclerotesta tissue revealed the least amount of change in carbohydrate concentrations. The disaccharides maltose and sucrose declined after the invasion, but the other three carbohydrates were not influenced by the invasion.

The sum of all five seed carbohydrates after the *A. yasumatsui* invasion was 60% of the sum before the invasion for sarcotesta, 66% of the sum for gametophyte, and 79% of the sum for sclerotesta. These observational results confirmed that nonstructural carbohydrates declined relatively more in sarcotesta tissue than in the other tissues and illuminated that a manipulative experiment was needed to understand more fully the seed carbohydrate responses to direct herbivory by *A. yasumatsui*.

Whole-tree protection. A significant interaction in fructose concentration occurred because the scale invasion reduced fructose to a greater degree in sarcotesta than in the other tissues (Table 1). Fructose concentration was greatest in the sarcotesta tissue and was similar for gametophyte and sclerotesta tissues. The main effects of invasion and tissue were both significant for glucose con-

centration, but the interaction was not significant due to a similar reduction in glucose among the tissues following the invasion. The invasion reduced the overall glucose concentration from 14.5 to 10.6 mg·g⁻¹ (mean of the three tissues). A significant interaction in maltose concentration occurred because the scale invasion did not influence concentrations in gametophyte or sclerotesta tissue but drastically reduced concentration in sarcotesta tissue. Maltose concentration of sarcotesta tissue was almost triple that of the other two tissues before the invasion, but maltose concentration was similar among the tissues after the invasion. A significant interaction in sucrose concentration occurred because the invasion reduced sucrose in gametophyte and sarcotesta tissues but did not influence sucrose in sclerotesta tissue. Sucrose concentration was greatest in sarcotesta and least in gametophyte tissue.

Starch concentration greatly exceeded that of the free sugars, and was greatest in gametophyte and similar in the other two tissue categories (Table 1). A significant interaction occurred for starch concentration because the invasion greatly reduced starch in gametophyte, slightly reduced starch in sarcotesta, and did not significantly influence starch in sclerotesta.

The sum of all five seed carbohydrates after the *A. yasumatsui* invasion was 63% of the sum before the invasion for sarcotesta, 78% of the sum for gametophyte, and 108% of the sum for sclerotesta. These results corroborated the preliminary observational data to show that nonstructural carbohydrate concentration declined relatively more in sarcotesta tissue than in the other tissues.

A significant interaction occurred for total pool of fructose, glucose, maltose, and sucrose, and these sugars exhibited similar relative patterns among the tissues. The pre-invasion pool size and the magnitude of postinvasion reduction in pool size followed the order sclerotesta < gametophyte < sarcotesta for each of the sugars (Table 1). The substantial amplitude of preinvasion differences of these sugars among the tissue categories was muted by the invasion, as sugar pool size converged. The pool size of sucrose exceeded that of the other three sugars in every invasion × tissue category.

A significant interaction occurred for total starch pool because the invasion caused a drastic reduction in starch of gametophyte tissue, a moderate reduction in starch of sarcotesta tissue, and no change in starch of sclerotesta tissue (Table 1). The majority of seed starch was located in the gametophyte tissue before and after the invasion.

The pre- and postinvasion differences in carbohydrate pools were much greater than the differences in carbohydrate concentration. Therefore, the influence of direct infestation of seeds by *A. yasumatsui* was mostly a consequence of a reduction in seed biomass accumulation, and secondarily a consequence of reduced concentrations.

Seed only protection. The sugar and starch concentrations of clean vs. infested seeds

from unhealthy infested female *C. micronesica* trees (Table 2) were generally below those of seeds from the whole-tree protection study (Table 1). However, the patterns of significance and the rankings of the means were remarkably similar for the two manipulative studies, despite the contrast in whole-tree health. The primary difference in patterns was more overlap among the groups of means for starch concentration data but not for total pool data.

The sum of all five seed carbohydrates after the *A. yasumatsui* invasion was 65% of the sum before the invasion for sarcotesta, 72% of the sum for gametophyte, and 103% of the sum for sclerotesta. These results again corroborated the previous studies to show that non-structural carbohydrates declined relatively more in sarcotesta tissue than in the other tissues.

Discussion

We used a three-tiered approach to show that free sugars and starch declined in *C. micronesica* seeds as a result of direct infestations by *A. yasumatsui*. This was borne out by comparing field-collected seeds before the invasion of *A. yasumatsui* vs. after the invasion. The pattern held true for seeds on pesticide-protected trees exhibiting minimal *A. yasumatsui* damage vs. unprotected trees following 7 years of *A. yasumatsui* infestation. However, it also held true for infested seeds vs. uninfested seeds on trees that were suffering from chronic *A. yasumatsui* damage to exposed leaves, stems, and reproductive structures.

The three-tiered approach including two manipulative studies allow us to tease apart some of the underlying causal mechanisms of how *A. yasumatsui* herbivory reduced *C. micronesica* seed resources. The pre- vs. postinvasion observations provided an initial look at the overall influence of whole-tree damage to seed resources and showed that the hexoses, disaccharides, and starch declined as a result of *A. yasumatsui* access to leaves, stems, and reproductive structure surfaces. However, these interpretations were complicated by the fact that different trees, different times of pollination/fertilization, and different years were involved. The whole-tree protection study removed some ambiguity by using synchronized megastrobili emergence and seed maturation among all replications in one locality and season. These results confirmed that direct infestation of *A. yasumatsui* on stems, leaves, and reproductive structures of *C. micronesica* trees caused a decline in nonstructural carbohydrates in mature seeds. However, the role of source vs. sink relations could not be determined from this study, and the declines in seed carbohydrates could have been a consequence of declines in whole-tree health and not due to direct seed infestations per se. The seed-only protection on unhealthy infested trees provided the evidence that both source and sink behaviors were involved in this response of a native cycad tree to a nonnative

Fig. 2. The concentration of nonstructural carbohydrates in gametophyte (Gam), sarcotesta (Sar), and sclerotesta (Scl) tissue of 19-month-old seeds from *Cycas micronesica* trees on Guam 13 months before the invasion of *A. yasumatsui* (shaded bars) and 16 months after initial *A. yasumatsui* infestation (open bars). (A) Fructose, (B) Glucose, (C) Maltose, (D) Sucrose, (E) Starch. Mean $\pm$ SE, $n = 10$.

specialist pest. Uninfested seeds on unhealthy infested trees exhibited carbohydrate concentrations that were in excess of infested seeds on the same trees, verifying that sink activity was damaged in seeds suffering from direct *A. yasumatsui* infestations. The infested and uninfested seeds on these experimental trees were competing for non-structural resources from the same whole plant pool. Additionally, the concentrations and total pools of the carbohydrates in the seed-only protection study (Table 2, direct herbivory of leaves and stems allowed) were less than those in the whole-tree protection study (Table 1, no herbivory of leaves and stems allowed), verifying source activity was also damaged by infestations of this non-native pest.

The patterns of relationships among organs and carbohydrates were similar for the initial observational data (Fig. 2) and the whole-plant protection study (Table 1), but the absolute values were slightly higher in the observation study for several carbohydrates. These minor differences may have been due to the two methods of water extraction from the fresh tissue in that we used freezing and lyophilizing for the initial observational study and drying at 75 °C for the manipulative study. A more likely explanation was that the preinvasion female trees had never experienced nonnative pest infestations, but the protected trees in the manipulative study suffered from *A. yasumatsui* damage for 1 year before the imidacloprid treatments were initiated in 2007. Moreover, the postinvasion samples in the observation study were collected from female trees that had experienced *A. yasumatsui* infestations for only 16 months because the infestations were initiated when the sampled seeds were 3 months in age. In contrast, the unprotected samples in the manipulative study were collected from trees

Table 1. The concentrations and total pool size of nonstructural carbohydrates in gametophyte, sarcotesta, and sclerotesta tissue of mature seeds of healthy *Cycas micronesica* trees protected from *Aulacaspis yasumatsui* infestations by 6 years of imidacloprid insecticide applications and unprotected trees following 7 years of *A. yasumatsui* infestations. $n = 8$.

Treatment	Seed tissue	Fructose	Glucose	Maltose	Sucrose	Starch
		Concentrations (mg·g ⁻¹)				
Protected	Gametophyte	7.75 c ^z	11.25 ^y	7.13 b	25.25 c	549.40 a
	Sarcotesta	28.00 a	22.88	18.88 a	53.25 a	127.89 c
	Sclerotesta	11.63 bc	8.75	8.50 b	43.63 b	84.85 de
Unprotected	Gametophyte	8.00 c	6.75	6.63 b	12.62 d	432.93 b
	Sarcotesta	14.63 b	13.13	7.38 b	44.13 b	79.23 e
	Sclerotesta	8.13 c	7.88	8.25 b	51.25 b	93.76 d
Among-treatments <i>P</i>		<0.001	0.003	<0.001	<0.001	<0.001
Among-tissues <i>P</i>		<0.001	<0.001	<0.001	<0.001	<0.001
Treatment $\times$ tissue <i>P</i>		<0.001	0.803	<0.001	<0.001	<0.001
		Total pool size (mg)				
Protected	Gametophyte	126.80 b	187.14 b	116.19 b	416.77 b	9039.53 a
	Sarcotesta	312.59 a	217.88 a	259.38 a	730.41 a	1754.63 c
	Sclerotesta	46.89 c	47.41 d	45.62 c	233.95 c	455.89 d
Unprotected	Gametophyte	47.54 d	50.57 d	46.61 c	90.44 d	3066.86 b
	Sarcotesta	78.89 c	72.99 c	45.18 c	270.38 c	482.05 d
	Sclerotesta	44.45 d	43.52 d	46.42 c	288.30 c	527.79 d
Among-treatments <i>P</i>		<0.001	<0.001	<0.001	<0.001	<0.001
Among-tissues <i>P</i>		<0.001	<0.001	<0.001	<0.001	<0.001
Treatment $\times$ tissue <i>P</i>		<0.001	<0.001	<0.001	<0.001	<0.001

^zMeans within columns with same letters are not significantly different according to Tukey's honest significant difference.

^yNo means separations for glucose concentration due to nonsignificant interaction.

Table 2. The concentrations and total pool size of nonstructural carbohydrates in gametophyte, sarcotesta, and sclerotesta tissue of 19-month-old seeds on unhealthy *Cycas micronesica* trees infested with *Aulacaspis yasumatsui*. Clean seeds were protected by lucite screen and remained pest-free; infested seeds were exposed to direct *A. yasumatsui* infestations. n = 8.

Treatment	Seed tissue	Fructose	Glucose	Maltose	Sucrose	Starch
		Concentrations (mg·g ⁻¹)				
Clean	Gametophyte	6.75 c ^z	8.63 ^y	5.88 b	20.87 c	492.64 a
	Sarcotesta	20.63 a	12.08	14.87 a	46.65 a	114.79 c
	Sclerotesta	6.75 c	7.25	7.24 b	38.63 b	78.84 de
Infested	Gametophyte	5.75 c	5.88	5.88 b	10.13 d	358.88 b
	Sarcotesta	11.25 b	10.25	5.88 b	36.38 b	72.94 e
	Sclerotesta	7.88 c	7.75	7.63 b	37.13 a	82.88 cd
Among-treatments <i>P</i>		<0.001	<0.001	<0.001	<0.001	<0.001
Among-tissues <i>P</i>		<0.001	<0.001	<0.001	<0.001	<0.001
Treatment × tissue <i>P</i>		<0.001	0.143	<0.001	<0.001	<0.001
Treatment	Seed tissue	Total pool size (mg)				
		Fructose	Glucose	Maltose	Sucrose	Starch
Clean	Gametophyte	85.25 b	110.25 b	73.63 b	261.07 b	6108.59 a
	Sarcotesta	264.87 a	153.66 a	191.78 a	591.93 a	1459.16 c
	Sclerotesta	34.41 c	37.14 d	37.19 c	197.79 c	403.19 d
Infested	Gametophyte	36.17 d	37.63 d	38.01 c	63.41 d	2255.86 b
	Sarcotesta	64.68 c	59.35 c	34.62 c	210.92 c	422.92 d
	Sclerotesta	41.77 d	41.44 d	41.03 c	197.34 c	441.26 d
Among-treatments <i>P</i>		<0.001	<0.001	<0.001	<0.001	<0.001
Among-tissues <i>P</i>		<0.001	<0.001	<0.001	<0.001	<0.001
Treatment × tissue <i>P</i>		<0.001	<0.001	<0.001	<0.001	<0.001

^zMeans within columns with same letters are not significantly different according to Tukey's honest significant difference.

^yNo means separations for glucose concentration due to nonsignificant interaction.

that had experienced *A. yasumatsui* infestations for 7 years.

The documented declines in carbohydrates following infestations of *A. yasumatsui* on *Cycas* L. plants are not restricted to seeds. A reduction in stem carbohydrates after chronic *A. yasumatsui* herbivory was correlated with capacity to develop adventitious roots (Marler, 2018). A reduction in whole-plant carbohydrates during lethal *A. yasumatsui* infestations preceded plant mortality (Marler and Cascasan, 2018). This collective body of information indicates that a primary mechanism that this pest uses to reduce tree and seed health and increase plant mortality is uninterrupted depletion of nonstructural carbohydrates.

Future directions. Our revelations that reduced seed resources may partly explain poor germination and seedling performance of infested seeds do not exclude other causal factors. For example, epigenetic regulation of gene expression is heavily involved in seed-to-seedling transition (Xu et al., 2018), and direct herbivory of seed integument tissues by the nonnative *A. yasumatsui* may directly alter these epigenetic phenomena. The study of the epigenetic basis of cycad seed development may allow for a better understanding of how *A. yasumatsui* herbivory negatively influences *Cycas* plants.

Our prediction that the exposed sarcotesta tissue would decline to a greater degree in carbohydrate resources during *A. yasumatsui* herbivory than the other seed tissues was confirmed for relative percentage reductions. However, the absolute loss in biomass was greatest in the gametophyte because starch was stored in much greater quantities than the sugars in all tissues, and most of the starch was in the gametophyte. The relative percentage reductions among the various seed tissues may not reflect the most influential facet of how this armored scale reduces seed quality. For example, the absolute decline in

gametophyte starch may have a much greater impact on seed viability because this haploid tissue is constructed to support embryo growth and development. Germination studies will be required to determine the direct relationship between gametophyte starch and seedling germination and growth.

The outer sarcotesta tissue was the site of direct seed herbivory by the Hemiptera pest, and the size differential between sarcotesta depth and scale body size confirmed that the direct feeding was restricted to sarcotesta tissue. The role of direct photosynthesis by ovule and seed epidermis may also be a consequential factor in the carbon relations of maturing *C. micronesica* seeds. The exposed ovules and seeds of this species may remain green for more than 1 year of development (Marler and Dongol, 2016a), and *A. yasumatsui* infestations on these photosynthetic surfaces may impair the ability of seeds to act as their own carbon source. To our knowledge, no studies have been conducted to date on any *Cycas* species to quantify photosynthesis or respiration flux associated with the green surfaces of the naked seeds. Manipulative studies that physically exclude light energy from developing seeds may improve our understanding of the role of exposed *Cycas* seeds as carbon source. Carbon labeling studies would provide unambiguous evidence of the relative roles of concurrent carbon fixation by leaves, stored carbon in stems and roots, and direct seed photosynthesis in meeting the nonstructural carbohydrate needs of developing *Cycas* seeds.

Conservation implications. The decline in seed resources that we have documented after direct *A. yasumatsui* infestation of seeds may partly explain why infested seeds exhibit reduced germination percentages and increased seedling mortality compared with uninfested seeds. These findings indicate that practitioners attempting to conserve genetic

diversity of *Cycas* populations threatened by *A. yasumatsui* should collect a greater number of seeds from subpopulations that exhibit direct seed infestations than from subpopulations that have relatively clean seeds. This approach may enable a more even population structure of the resulting ex situ germplasm collections by taking into account the lower germination percentage and the greater seedling mortality from the heavily damaged subpopulations.

These results also underpin our assertions that *A. yasumatsui* continues to be the single primary threat to *C. micronesica* (Marler and Lindström, 2017). The enduring plant mortality is proceeding unabated due to failure to adequately establish efficacious biological control of the nonnative armored scale. For example, four of 12 unprotected trees in our first study and six of 20 trees in our second study died during the 16-month plan of work. Large sums of federal conservation funds are currently being expended to rescue unhealthy *C. micronesica* trees from military construction sites and transplant them to alternative recipient sites (Marler and Lindström, 2017). The primary threat of *A. yasumatsui* herbivory is not being addressed by any of these expensive conservation actions, and the transplanted trees will likely eventually die from this primary threat if this it continues to be ignored by conservation funding agencies. A redirection of these federal conservation funds toward mitigation of the primary threat would enable a more intelligent approach to species conservation, and if successful, the species could be delisted from the ESA.

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