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Steryl glucoside concentration declines with *Cycas micronesica* seed age

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Abstract. Neurotoxins contained in the seeds of *Cycas micronesica* K.D. Hill have been implicated in the Guam neurological disease cluster, amyotrophic lateral sclerosis–parkinsonism dementia complex (ALS–PDC). Some of these neurotoxins remain in the washed cycad seed flour that was historically an important part of the Chamorro diet. Of these, variant steryl glucosides have been identified by us as a possible etiological factor in the disease. *In vitro* and *in vivo* animal studies have strongly supported a role for these molecules in some forms of neurodegeneration. As part of a series of studies, we have now determined the concentrations of several steryl glucosides and their sterol precursors as affected by the age of *C. micronesica* seeds. The concentration of these molecules declined with seed age from 2.0 to 30.5 months. Following log-transformation of both axes, the decline was linear. Similarly, concentration of all but one of the molecules declined with age when samples were restricted to gametophyte tissue. Factors suspected of influencing phenotypic plasticity must be addressed when interpreting plant physiology data. Our results confirm for the first time that tissue age must be documented and reported in cycad seed biochemistry studies to remove ambiguities from results. Past studies in this important area of research have failed to account for the potential impact of seed age, rendering previous outcomes and interpretations of cycad neurotoxins in their impact on ALS–PDC ambiguous.

Keywords: ALS–PDC, cycad, *Cycas micronesica*, neurotoxin, seed age, sterol, steryl glucoside.

Introduction

The neurological diseases known as amyotrophic lateral sclerosis–parkinsonism dementia complex (ALS–PDC) have been documented for centuries and heavily studied for decades on the island of Guam and elsewhere in the Western Pacific (Kurland 1988; Kurland *et al*.1994). To date, the strongest epidemiological correlate for ALS–PDC on Guam is consumption of the seed of the endemic cycad *Cycas micronesica* K.D. Hill (see Kurland 1993; Marler *et al.* 2005*a*; Shaw *et al*. 2006). That consumption of washed cycad flour from Guam can induce behavioural and histopathological features of ALS–PDC in various mammalian species has been known since 1964 in studies with rhesus monkeys (Dastur 1964), more recently in mice (see Shaw *et al*. 2006 for a summary), and most recently in rats (Valentino *et al*. 2006).

Despite this, a review of the research addressing cycad tissue toxicity is an exercise in frustration. The retrospective study reveals a series of predictable steps done time and time again: discovering a 'new' cycad metabolite, chasing that toxin until its association with ALS–PDC is disproved, and then temporarily abandonning further study of the cycad hypothesis. A few toxins that were dead ends in this pattern include azoxyglycosides, such as cyasin and its active toxin methylazoxymethano (MAM), and the plant amino acids β-*N*-oxalylamino-L-alanine (BOAA) and β-methyl-amino-alanine (BMAA) (see Shaw *et al*. 2006). However, the Guam cycad and other cycad species produce an abundance of secondary metabolites (Norstog and Nicholls 1997). Demonstrating that exposure to one of these metabolites is not associated with the onset of ALS–PDC in no way infers that cycad flour consumption is not causal.

We have continued to pursue the role of several isolated cycad metabolites in persistent attempts to identify causal components of the cycad seed tissues that are clearly neurotoxic. We have found that the most injurious to date are various steryl glucosides that while common to most plants, express at extremely high levels in cycad seeds

Abbreviations used: ALS–PDC, amyotrophic lateral sclerosis–parkinsonism dementia complex; BSS, β-sitosterol; BSSG, β-sitosterol β-D-glucoside; SG, stigmasterol β-D-glucoside; SS, stigmasterol.

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(Shaw *et al*. 2006). A series of *in vitro* studies using primary neural cell cultures (Khabazian *et al*. 2002), a fused hybrid of motor neurons and neuroblastoma cells (NSC-34 cells) (Ly *et al*. 2006), and two types of neuronal organotypic slice (K Andreassen, C Mathews, personal communication) demonstrate neuronal cell death following exposure to cycad steryl glucosides in the low micromolar range. Additionally, *in vivo* studies in mice show highly significant and progressive spinal motor neuron loss following steryl glucoside feeding over a period of 10 weeks (Wilson and Shaw 2005; Wilson *et al.* 2006).

The historical reliance on cycad seeds as a food source by the Chamorro population made use of the starch-rich gametophyte tissue (Whiting 1963). Preparation began by collecting mature brown seeds directly from plants and from the ground. The gametophyte tissue was extracted, sliced, washed to remove acute toxins; then dried and pulverised into flour. Our phenology observations indicate the age of seeds harvested in this manner would be impossible to determine. For example, the naked *C. micronesica* ovule is light green until pollination. Thereafter the seeds morph from this light green to dark green, bronze, and finally brown before abscission (see Marler *et al.* 2005*a*). The onset of brown seed exterior may occur as early as 17 months, yet seeds may remain on plants more than 30 months before abscission. Moreover, cycad seed embryo development frequently occurs after abscission, and germination does not occur until embryo development is suitable (Jones 1993; Norstog and Nicholls 1997; Whitelock 2002). Thus, brown seeds harvested from the ground could easily have been in storage in the forest seed bank for several months, adding even more time to seed age.

Biochemistry of the brown cycad seeds historically used to make flour for consumption cannot be legitimately studied by ignoring the topic of seed development, as brown seeds harvested from plants for food preparation could be any age from approximately 17 months to 30+ months. Yet to date no questions have been asked about the influence of seed age on the concentration of putative neurotoxins, despite the decades of research on various cycad seed toxins. More disturbing is the fact that only one report among the long list of publications on this subject met the minimum requirement of documenting and reporting the age of seeds that comprised research samples (Marler *et al.* 2005*b*). In that report, 12-month-old seeds from one experiment exhibited greater sterol and steryl glucoside concentration than 17- or 19-month old seeds from other experiments.

We use this steryl glucoside toxin group as the model for the present paper. Our objectives were to define the influence of seed age on four sterol and steryl glucoside compounds that we have previously identified and believe to be one source of neurotoxicity related to ALS–PDC. Confirmation of the proposition that seed age influences toxin concentrations will impart ambiguity to past publications and improve future protocols in this important field of research.

Materials and methods

The study site included the forests in the Andersen Air Force Base overlay of the Guam National Wildlife Refuge in northern Guam. As part of ongoing phenology and ontogeny studies, we began tagging female cycad plants in June 2002 as megasporophyll emergence occurred. This allowed us to assign accurate age to all harvested seeds upon subsequent plant visits.

We used repeated visits to ultimately develop a set of samples defined by a range in seed age. In selecting the individuals to sample, we used several criteria. Three soils are present throughout the study site, yet nothing is known about the relationship between soil characteristics and cycad seed chemistry. Thus, we restricted our sampling to plants growing in the Ritidian-Rock outcrop complex (clayey-skeletal, gibbsitic, non-acid, isohyperthermic Lithic Ustorthents) (Young 1988). Nothing is known about the relationship between herbivory history and cycad seed toxins, thus we only sampled plants devoid of obvious signs of herbivory history. Nothing is known about the relationship between crop load and seed toxins, thus we avoided plants with minimal or copious seed set. Instead, all sampled plants carried a moderate seed load (range of 76–97 seeds). Nothing is known about the relationship between plant size and seed toxins, thus we restricted sampled plants to medium-sized individuals 2.5–3.5 m tall. Nothing is known about the relationship between developmental shade level and seed toxins, thus we selected plants that were typical of partial shade and partial sun exposure. We obtained hemispherical photographs of each plant's microhabitat and digitally determined percent visible sky on each image (Marler *et al.* 2005*b*). Habitat shade level ranged from 44 to 58% visible sky. Nothing is known about the relationship between prior harvest of seeds and subsequent chemistry of remaining seeds on a plant, thus we restricted seed harvests to plants that had no prior seed harvests. Other variables measured were basal stem circumference (76 ± 5 cm) and total leaf number (59 ± 4).

Harvests continued through February 2005 to provide a range of samples from 2 to 30.5 months in age. The number of seeds that were collected from each individual to create a composite sample varied with seed age. For seeds greater than 6 months old, 8 seeds were harvested. The number of seeds required to create a representative sample was greater for younger seeds, as dry weight of each seed's megagametophyte tissue was minimal. This number ranged from 20 seeds for 2-month-old seeds to 12 seeds for 6-month-old seeds. In all cases, seeds that were evenly spaced horizontally throughout the megasporophyll complex were selected for inclusion.

On each harvest date, seeds were transported to the University of Guam for processing. We developed two groups of tissue for analysis. One group was comprised exclusively of gametophyte tissue. This is the tissue that was historically ingested by Guam residents as processed flour. We experienced difficulty collecting enough of this haploid gametophyte tissue for samples younger than 4 months old, thus the age range for this dataset was 4–30.5 months. The other group comprised complete seeds, inclusive of gametophyte, sclerotesta, and sarcotesta tissue. Tissue was combined into one composite sample for further handling. The age range for the tissue set comprised of entire seeds was 2–30.5 months.

Tissue was frozen, lyophilised, and analysed by Reversed Phase HPLC analysis as described by Marler *et al*. (2005*b*). The data were analysed with General Linear Models using the SAS statistical package (SAS Institute 1996) in order to define the significance and model the influence of seed age on two phytosterol glucosides and two sterol precursors.

Results and discussion

The concentration of β-sitosterol β-D-glucoside (BSSG), stigmasterol β-D-glucoside (SG), β-sitosterol (BSS), and

stigmasterol (SS) in samples comprised of all seed tissues declined with seed age. The relationship was non-linear in every case, with a steeper decline in metabolite concentration per month within the youngest seed range. The raw data were defined by a double logarithmic curve (Steel *et al*. 1996). Thus, $\log_{10}$ transformations for both axes allowed the age-defined decline in steryl glucoside and sterol concentrations to be fitted with highly significant linear functions (Table 1, Fig. 1).

The concentration of BSSG, BSS, and SS in gametophyte tissue also declined with seed age, and the response was described by a double logarithmic model (Table 1, Fig. 1). The *y*-intercepts of gametophyte-only samples were greater than those of samples comprised of total seed tissue (Table 1). The slopes for the lines of the samples restricted to gametophyte tissue were more negative than those of samples comprised of total seed tissue (Table 1). Seed age did not influence the concentration of SG in gametophyte tissue.

The vast literature in biology reveals plant organ structure and function are strongly influenced by organ developmental stage and plant ontogeny (e.g. Raven *et al*. 1999). The extensive literature in seed production and seed science indicates maturity and physiology are under strict control of seed developmental stage (e.g. Bewley and Black 2004). The informative literature on seed storage technology reveals seed physiology and chemistry are under direct control of storage conditions and duration (e.g. Desai 2004). The practical literature covering plant propagation principles reveals that knowledge of pre-harvest and post-harvest seed maturity and physiology indices are necessary for successful propagation (e.g. Hartmann *et al*. 2001). This immense knowledge base in so many pertinent disciplines collectively indicates that cycad seed chemistry is undoubtedly under the influence of seed developmental stage. Yet with the exception of one article (Marler *et al.* 2005*b*), the decades of research on chemistry of cycad seeds has failed to endorse the need to document seed age during this costly research that is so critical for increasing our knowledge of the pathogenesis of human neurodegenerative diseases on Guam.

The results reported herein validate that age of cycad seed exerts a strong influence on the concentration of the neurotoxic steryl glucosides we have been studying. Clearly, future studies of all previously or yet to be identified cycad seed toxins must report sample age for methods to be complete enough to allow repeatability of experiments.

A more disturbing conclusion from this research is that failure to report seed sample age in past publications adds a source of ambiguity that mars interpretation. For example, differences attributed to inter-species comparisons by Yagi (2004) may be little more than disparity in unreported seed age among the samples. Similarly, differences attributed to inter-organ (including seeds) comparisons by Banack and Cox (2003) may be entirely obscured and controlled by unreported age of seeds. In this light, the failure to document age of research samples throughout the history of this field of research renders such data meaningless.

Steryl glucosides may serve a signal transduction role, especially as mediators in stress responses that are expressed during developmental and transitional stages (e.g. Sakaki *et al*. 2001; Kim *et al.* 2002; Kunimoto *et al*. 2002). Steryl glucosides may also serve as donors or precursors of substrates during biosynthesis of structural or functional components of plant development (e.g. Cantatore *et al*. 2000; Peng *et al*. 2002; He *et al*. 2003). As previously discussed (Marler *et al*. 2005*a*), the loading of immature seed tissues with these compounds conforms well to these proposed functions. Early storage pools could certainly serve as substrate donors for long-term development of tissue throughout the lengthy seed maturation process.

Our confirmation that younger cycad seeds contain greater concentrations of steryl glucosides than older seeds also conforms well to the observed epidemiological patterns of ALS–PDC. The peak in ALS–PDC incidence followed World War II and an ongoing decline in the risk of acquiring the disease began in the early 1950s (see Duncan 1993; Kurland 1993; Zhang *et al*. 1996). This pattern is consistent with the assertions that cycad seeds became more of a dietary staple during the War (Whiting 1963; Duncan 1993). A greater reliance on forest resources for survival assuredly led to greater pressures on this limited source of famine food, and many families that were concerned with survival were possibly less discretionary in restricting seed harvests to the desired dark brown seeds. Prior to our confirmation in the present paper that younger seeds contain more putative toxins, epidemiologists have been unable to consider the fact that this likely consumption of younger seeds during World War II led to a direct increased risk of exposure to cycad toxins. Moreover, many families during this time may have been unconcerned or unable to thoroughly complete the prolonged traditional leaching process during seed flour

Table 1. Summary statistics for regressions of log-transformed data for four steryl glucosides as affected by seed age

Sterols and steryl glucosides are β-sitosterol β-D-glucoside (BSSG), stigmasterol β-D-glucoside (SG), β-sitosterol (BSS), and stigmasterol (SS)

Variant	Slope	*y*-intercept	r^2	*P*
Total seed tissue				
BSS	−0.476	0.273	0.56	0.001
SG	−0.160	−0.140	0.14	0.027
BSSG	−0.305	0.168	0.21	0.005
SS	−0.441	0.003	0.32	0.001
Gametophyte tissue				
BSS	−0.648	0.442	0.50	0.001
BSSG	−0.548	0.522	0.40	0.001
SS	−0.839	0.514	0.54	0.001

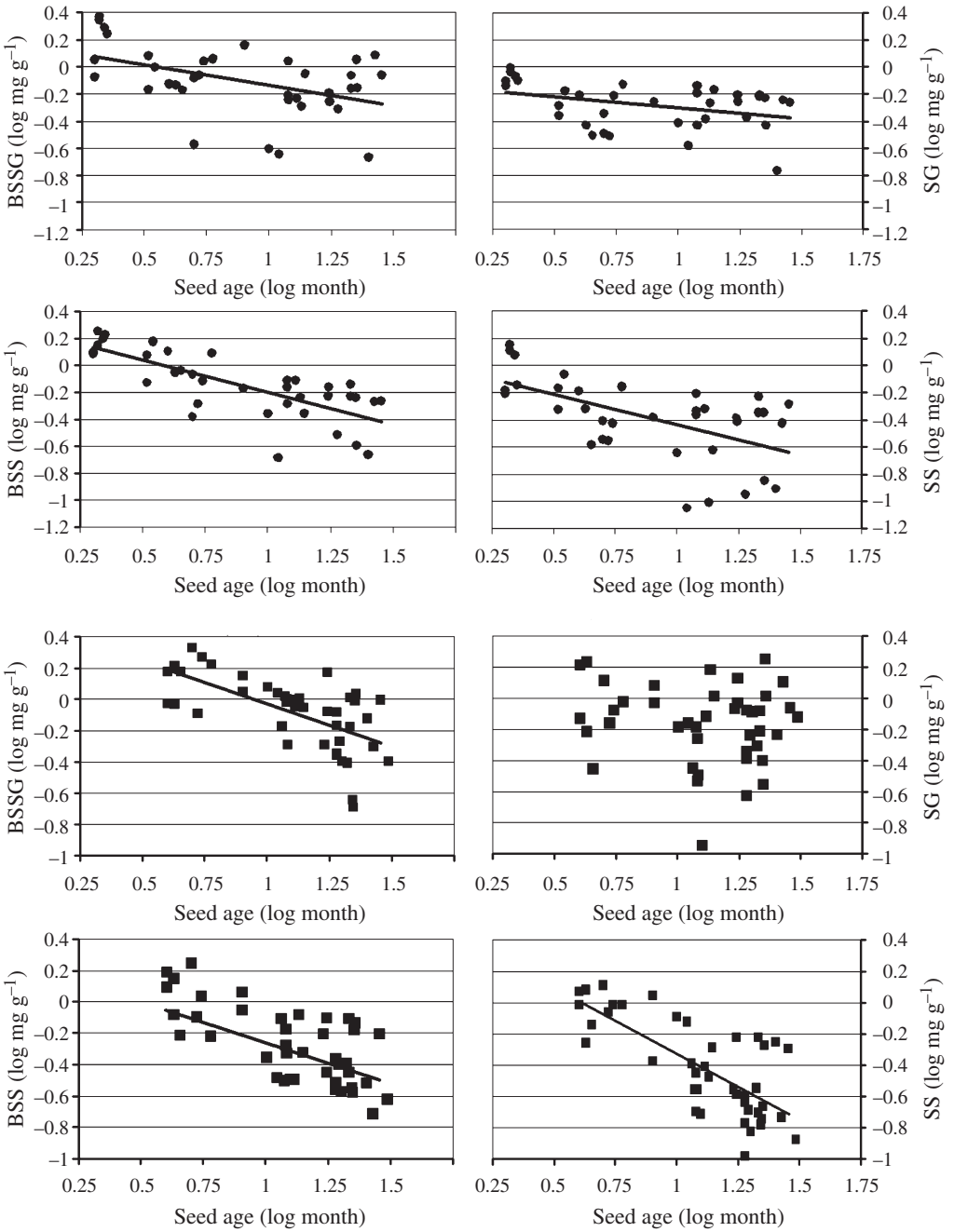

Fig. 1. The influence of *Cycas micronesica* seed age on concentration (DW basis) of β-sitosterol β-D-glucoside (BSSG), stigmasterol β-D-glucoside (SG), β-sitosterol (BSS), and stigmasterol (SS). Each point represents a composite sample from a minimum of eight seeds. Raw data seed age range was 2–30.5 months. Data are from samples derived from all seed tissues (circles) or from gametophyte tissue only (squares).

preparation (LT Kurland personal communication). A less rigorous leaching process would not have influenced ultimate concentration of lipid-soluble steryl glucoside toxins, but it may have affected a range of known and unknown water-soluble toxins. To date none of the known water-soluble toxins have proven to be strong enough to be causally linked to ALS–PDC alone based on *in vivo* models (see Shaw *et al*. 2006). However, nothing is known about potential synergistic interactions among the various toxins including steryl glucosides, and incompletely leached flour may have been a cocktail of toxins during this unfortunate ephemeral period in Guam's history.

In general, slopes were more negative and *y*-intercepts were greater for gametophyte tissue than for tissue comprised of gametophyte, sclerotesta, and sarcotesta (Fig. 1). From a functional and evolutionary perspective the very young gametophyte tissue may sustain preferential loading of steryl glucosides in preparation for the prolonged bioaccumulation of starch in this tissue. Further studies may add validity to this proposal by more fully linking loss of steryl glucosides to concomitant gains in starch within each tissue category of developing seeds. From a biochemical perspective, the more negative slopes for gametophyte tissue may indicate the enzymes responsible for cleaving the steryl glucosides into sterols and glucose may be more abundant or more fully expressed in this tissue as the embryo matures within this seed structure. This possibility is the subject of future investigations.

Sclerotesta and sarcotesta tissue contains greater concentrations of these sterols and steryl glucosides than does gametophyte tissue when seeds reach 17–19 months in age (Marler *et al.* 2005*b*). This contrasts with a greater concentration of these compounds in gametophyte tissue at very young seed age. This age-related disparity also conforms well to the more negative slopes found for gametophyte-only samples compared with samples comprised of total seed tissue.

A question that remains unanswered pertains to the influence of seed age on absolute amount of metabolites. This question was beyond the scope of the present study, since the previously reported data of relevance to our question were reported as concentration. Absolute amount of any metabolite would not be comparable to this vast literature.

In conclusion, factors known to influence phenotypic plasticity must be addressed when interpreting plant physiology data. For example, Wright and McConnaughay (2002) argue that conclusions differ dramatically throughout plant ontogeny, and studies that do not acknowledge this may under- or over-estimate the degree of plasticity. Similarly, Zotz (2000) exposes the need to report plant size, and argues that past failures to report plant size in plant physiology studies imparts ambiguities in interpretation. We previously mentioned the possibility that cycad seed age is controlling of seed biochemistry (Marler *et al.* 2005*a*). We have now answered this question, and confirm that age must be reported in cycad seed biochemistry studies for data to be accurate and interpretations to be unambiguous.

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