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Free and glycosylated sterol bioaccumulation in developing *Cycas micronesica* seeds

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ABSTRACT

The bioaccumulation of free and glycosylated forms of stigmasterol and β-sitosterol were determined from *Cycas micronesica* K.D. Hill seeds throughout seed ontogeny. Per-seed pool of the four compounds increased linearly from 2 to 24 months, indicating no developmental period elicited a major shift in the rate of bioaccumulation. The slopes were not homogeneous, signifying a change in relative sterol profile concomitant with seed maturation. This shift was in favour of the glucosides, as their rate of accumulation exceeded that of the free sterols. Stigmasterol content exceeded that of β-sitosterol, but ontogeny did not influence the ratio of these dominant sterols. The quantity and quality of sterol exposure during consumption of foods prepared from gametophytes by humans is strongly influenced by age of harvested seeds. Results are critical for a further understanding of the link between human neurodegenerative diseases and historical consumption of foods derived from the seed gametophyte tissue.

1. Introduction

Cycads are primitive gymnosperms characterised by slow growth, long life, and abundant secondary chemistry (Norstog & Nicholls, 1997). Cycad seed chemistry has been the focus of decades of research due to the demonstrated link between foods prepared from cycad seeds and neurodegenerative diseases (Borenstein et al., 2007; Whiting, 1963). Our interest has been a group of sterols because free sterol (Kim et al., 2008) and derived steryl glucoside (Shaw et al., 2007; Tabata et al., 2008) ingestion in mammalian model systems has revealed their neurodegenerative properties. Additionally, a second health hazard attributed to dietary intake of sterols is accumulation of lipid-containing plaques on arterial walls (Sudhop & von Bergmann, 2004).

Various compounds have been identified in cycad tissues as candidate neurotoxins then scrutinised in relation to human health; but their physiological role in these ancient plants remains unstudied (Brenner, Stevenson, & Twigg, 2003). We have begun to address this paucity of information by determining that concentration of sterols was greatest in young *Cycas micronesica* seeds, and declined log–log linearly throughout seed development and maturation (Marler, Lee, Chung, & Shaw, 2006). This nonlinear decline in concentration was dependent on intact import/export relations, as evidenced by a stable concentration in detached seeds during extended storage (Marler, Lee, & Shaw, 2007b). However, absolute amounts of accumulated sterols (and the other metabolites previously studied) in developing cycad seeds have not been reported. Therefore, we are currently unable to discern whether the decline in sterol concentration during seed ontogeny is due to waning total pool per seed throughout development (the initial sterol pool is metabolised or exported during seed ontogeny); total pool per seed is unchanged throughout development (sterol biosynthesis or net import ceases early in seed development or turnover is balanced and increased seed size elicits a dilution that accounts for the decrease in concentration); or total pool per seed increases throughout development (positive net biosynthesis or import that fails to keep up with the concurrent increase in seed size).

The aim of this study was to answer the following questions that define chemistry of foods prepared from cycad seeds: (1) does seed age influence bioaccumulation of major free and glycosylated sterols; and (2) does a qualitative change in the sterol profile occur during seed development?

2. Materials and methods

2.1. Field methods

The study site was located in northwest Guam, centred at 13°38′43″N, 144°51′30″E. Habitat characteristics were described in Marler, Lee, and Shaw (2007a; Dededo #1 site). *C. micronesica* plants were tagged and dated to record emergence of reproductive events during 2002–2003. Seeds were harvested beginning two months after emergence, and were harvested through late 2004 in a manner that generated a maximum of 24 months in seed age. Pollination of *C. micronesica* ovules occurs at about one month, and the transition to mature brown colour occurs as early as 17

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months. Therefore, our age range included samples from one month after pollination to several months past the mature brown stage that evinces readiness for food harvest. To eliminate the possibility of creating artifacts by eliciting a change in seed chemistry from experimental seed removal, seeds were harvested exclusively from plants that had not been harvested on any prior date. The number of seeds for each age represented ($n = 37$) ranged from 20 seeds for two month harvests to eight seeds for all ages above six months. These were homogenised into one tissue sample per plant. Seeds were transported to the University of Guam where they were stored at −40 °C until we had time to process them.

2.2. Analytical and statistical methods

Tissue was lyophilised, dry weight of each seed was determined, and the free sterols stigmasterol (SS) and β-sitosterol (BSS) and derived steryl glucosides stigmasterol β-D-glucoside (SG) and β-sitosterol β-D-glucoside (BSSG) were quantified as described by Marler, Lee, and Shaw (2005b). Total bioaccumulation of the two free sterols and their derived steryl glucosides was calculated from seed dry weight to determine cumulative pool on a per seed basis.

We calculated two synthetic variables to more fully understand sterol metabolism during *C. micronesica* seed development. The relationship of free to derived sterols was defined as (SS + BSS)/(SG + BSSG). The relationship of stigmasterol variants to β-sitosterol variants was defined as (SS + SG)/(BSS + BSSG).

We used the REG procedure (SAS software version 12.3) to determine the relationship of bioaccumulation of the four metabolites with age, and the two calculated variables with age. The bioaccumulation as seeds matured was linear for all four metabolite pools. We then used the GLM procedure to test for homogeneity of slopes among the four populations.

3. Results and discussion

3.1. Changes in bioaccumulation and sterol profile

The free sterols SS and BSS occurred at less than 5 mg per seed at two months following emergence of the reproductive event from the stem apex (Fig. 1). Thereafter, free sterols increased in a linear pattern (SS: slope = 0.92, $P \leqslant 0.0001$, $R^2 = 0.74$; BSS: slope = 1.14, $P \leqslant 0.0001$, $R^2 = 0.79$) until reaching the predicted 22 mg per seed for SS and 30 mg per seed for BSS by 24 months. The pool of derived steryl glucosides SG and BSSG at two months was also less than 5 mg per seed, and increased in a linear pattern (SG: slope = 1.31, $P \leqslant 0.0001$, $R^2 = 0.90$; BSSG: slope = 2.07, $P \leqslant 0.0001$, $R^2 = 0.77$) until reaching the predicted 30 mg per seed for SG and 47 mg per seed for BSSG by 24 months. The slopes were not consistent for the four populations ($P \leqslant 0.0001$), therefore the rate of bioaccumulation of these metabolites was not homogeneous within the age limits of the study.

The ratio free/glucoside sterol was <1 initially, indicating the glucoside pool exceeded the free sterol pool. This ratio further decreased as seeds developed between 2 and 24 months in age (Fig. 2; slope = −0.007, $P \leqslant 0.0328$, $R^2 = 0.13$). This result was consistent with the greater slopes for the derived glucoside metabolites depicted in Fig. 1. The ratio of stigmasterol variants to β-sitosterol variants was not influence by seed age ($P \leqslant 0.0769$). An overall mean of 0.65 indicated stigmasterol accounted for a greater proportion of the sterol profile than did β-sitosterol.

3.2. Historical context

The link between ingestion of foods prepared from cycad seeds and neurodegenerative diseases (Borenstein et al., 2007; Whiting, 1963) establishes the urgency of defining causal agents of neurodegeneration. Therefore, several teams have been using various mammalian models to study the neurodegenerative properties of sterol compounds which are present in cycad seeds (Kim et al., 2008; McDowell et al., 2007; Shaw et al., 2007; Tabata et al., 2008; Valentino et al., 2006). The importance of more fully understanding sterol biology is accentuated by the puzzling contrast between these reported toxicity characteristics versus the conversely reported health benefits of sterol dietary intake (Bradford & Awad, 2007; Moreau, Whitaker, & Hicks, 2002; Oomah & Mazza, 1999).

We have mentioned that prior studies of *Cycas* seed chemistry have been of limited utility because response variables were restricted to concentration of various compounds (Marler et al., 2006), such that the pattern of changes in bioaccumulation of compounds has remained unreported. We have now addressed this deficiency to reveal that *C. micronesica* seed sink activity for sterol and steryl glucoside compounds or the net biosynthesis of these compounds in seeds is consistently sustained throughout their development up to 24 months. The pace of bioaccumulation in seeds throughout the two years was stable, as evidenced by the raw data conforming to a linear model without transformation. This contrasts with major shifts in sterol relations at the embryo developmental stage of buckwheat (*Fagopyrum esculentum* Moench) seed ontogeny (Horbowicz & Obendorf, 1992) or at the final stages of tomato (*Lycopersicon esculentum* Mill.) fruit maturation (Fraser et al., 1995). The results indicate that the previously

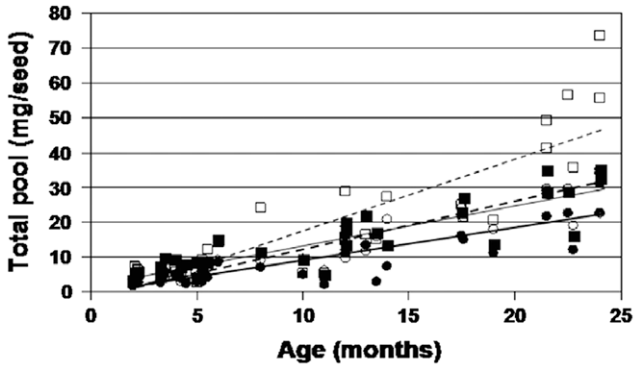

Fig. 1. The influence of *Cycas micronesica* seed age from 2 to 24 months on total pool of free sterols stigmasterol (closed circle, solid bold line) and β-sitosterol (closed square, solid line), solid lines; and derived steryl glucosides stigmasterol β-D-glucoside (open circle, dashed bold line) and β-sitosterol β-D-glucoside (open square, dashed line), dashed lines.

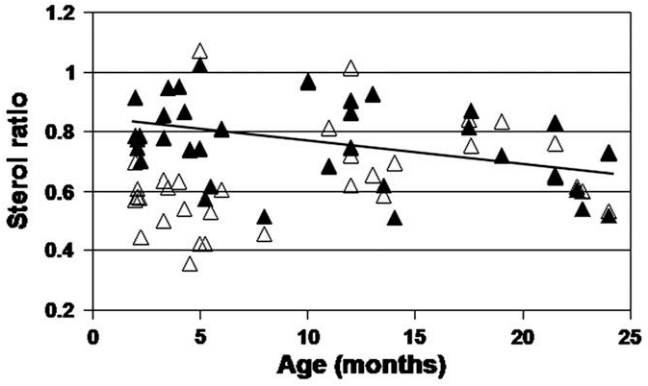

Fig. 2. The influence of *Cycas micronesica* seed age on the ratio free/glucoside sterols (closed triangle, bold line) and β-sitosterol/stigmasterol (open triangle).

reported nonlinear decline in concentration of these metabolites as *C. micronesica* seeds increase in age (Marler et al., 2005b, 2006) is not a result of the initial pool being net metabolised or exported. Rather, the decline in concentration results from a sustained rate of sterol bioaccumulation that does not keep pace with the concurrent accumulation of dry weight throughout seed ontogeny.

3.3. Age of reproductive structures and sterols

A few reports provide a parallel for discussion of sterol relations in developing reproductive structures exploited for human consumption. Davis and Poneleit (1974) described sterol relations that concur with our results for developing *Zea mays* L. kernels throughout 90 days after pollination. Sterol and steryl glucoside concentration declined concomitant with an increase in bioaccumulation as kernels increased in age. Similarly, the same pattern occurred for total sterol pool in fenugreek (*Trigonella foenum-graecum* L.) seeds and pods up to 90 days after flowering (Brenac & Sauvaire, 1996). In contrast, Horbowicz and Obendorf (1992) reported an increase in concentration of free sterols during buckwheat seed development to 20 days after pollination and a concurrent increase in total accumulation per seed. Free and esterified sterols increased in concentration as olive (*Olea europaea* L.) fruits developed up to 30 weeks after flowering (Stiti, Triki, & Hartmann, 2007), and concentration of free and bound sterols increased in tomato fruit tissue until reaching the mature stage (Fraser et al., 1995). These reports did not include measured bioaccumulation, but the increase in fruit size concurrent with increased concentration as fruits developed inferred a greater increase in sterol bioaccumulation than in concentration. These few reports universally corroborated our results of an increase in bioaccumulation of sterol variants as reproductive structures developed. However, only two of the five studies reported a concurrent decline in sterol concentration as we have reported for *Cycas* (Marler et al., 2006).

C. micronesica is the only native gymnosperm in the Mariana Islands (Marler, Lee, & Shaw, 2005a), so a comparison of our data with sterols in reproductive structures of any other gymnosperm may be of value for determining the physiological role of these compounds in seeds of ancient spermatophyte taxa. Unfortunately, we have found no reports to address this need.

3.4. Age of cycad organs and non-sterol metabolites

We have found no published reports for any other cycad species that is analogous to ours, where the intent was to determine the influence of seed age on secondary compounds. However, several reports include concentration of secondary compounds in various cycad organs of contrasting age as an ancillary result within a study. Methylazoxymethanol β-D-glucoside (cycasin) concentration of young leaves exceeded that of older leaves for *Zamia* (Rothschild, Nash, & Bell, 1986) and *Encephalartos hopei* (Yagi, 2004). Macrozamin increased temporarily after *Cycas revoluta* leaf emergence, then declined to the point that it could not be detected in older leaves (Yagi, Taderu, & Kobayashi, 1983). Concentration of BMAA was greater in immature male cone tissue than in mature cone tissue, but an inconsistent pattern occurred for glutamic acid in the same tissue (Banack & Cox, 2003). These studies were based on extremely small numbers of replications and insufficient description of sampling methods. Clearly, much remains to be vetted in order to clarify changes in secondary compounds throughout the ageing process of cycad organs.

3.5. Changes in sterol profile

The steady increase in steryl glucoside pool exceeded that of free sterols (Fig. 1), which altered the profile of these compounds in *C. micronesica* seeds in favour of the derived glucosides as seeds matured (Fig. 2). Qualitative changes during the late stages of fenugreek seed development corroborated our results, in that free/glycosylated sterol ratio decreased (Brenac & Sauvaire, 1996). However, the fenugreek seeds retained a predominance of free sterols throughout, so quantitative characteristics were in contrast to our results. Other reports convey an opposing pattern, with an increase in the proportion of free sterols during plant development. For example, mature *Nicotiana tabacum* seeds contained about twice the amount of free form of stigmasterol and sitosterol in the seeds than the glycosylated forms (Bush & Grunwald, 1972). Concentration of free sterols further increased in young plants after germination as steryl glucosides decreased in the same tissue. Similarly, free sterols increased with tobacco leaf maturation while steryl glucosides remained stable, leading to an increase in the ratio of free/glycosylated forms during maturation (Grunwald, 1975).

Throughout two years of *C. micronesica* seed development, no major changes in the ratio of stigmasterol to β-sitosterol occurred (Fig. 2). The stigmasterol content slightly exceeded that of the β-sitosterol content throughout. Comparison of these results to the sterol literature yields a confusing outcome. A first group of species retained a similar sitosterol/stigmasterol ratio throughout organ development. For example, sitosterol accounted for 95% of the sterol pool in developing olive fruits, with no reported changes in the relative proportion of sterol variants as fruits matured to 30 weeks after flowering (Stiti et al., 2007). Sitosterol was the major sterol in *Z. mays* seeds and its ratio to stigmasterol remaining stable throughout 60 days of seed development (Davis & Poneleit, 1974). Sitosterol accounted for 70% of the total sterol profile for buckwheat seeds, stigmasterol was present in trace amounts, and the sterol variants exhibited a similar general increase as seeds matured (Horbowicz & Obendorf, 1992).

A second group of species revealed a shift in the sterol profile in favour of more stigmasterol during organ development. For example, sitosterol content of *Sorghum bicolour* leaves was more than twice that of stigmasterol content when plants were seven days old, but was less than that of stigmasterol content when plants were 66 days old (Heupel, Sauvaire, Le, Parish, & Nes, 1986). During fenugreek seed and pod ontogeny stigmasterol was absent in the early stages but increased in relation to total sterols during the later stages (Brenac & Sauvaire, 1996). In contrast, sitosterol was dominant throughout but its prevalence declined as the pods matured.

A third group of species revealed a shift in the relative sterol profile in favour of more sitosterol as organs developed. For example, the sterol profile of *Cucurbita maxima* plants changed during germination and early seedling growth, with the seed's initial stigmasterol pool declining more rapidly and disappearing sooner than the seed's sitosterol pool (Garg & Nes, 1985). Therefore, shoot age exerted substantial control over the general proportion of these two major sterols. As young tobacco plants developed, stigmasterol increased while sitosterol content was stable (Bush & Grunwald, 1972). The net result was a considerable decline in the sitosterol/stigmasterol ratio as plants developed. Grunwald (1975) reported more sitosterol than stigmasterol in immature tobacco leaves, but sitosterol declined as stigmasterol increased with leaf maturation such that they were present in essentially equal amounts in mature leaves. The decrease in sitosterol/stigmasterol ratio during tomato fruit development led Fraser et al., (1995) to indicate the ratio may be useful as a marker for fruit maturity. The high sitosterol/stigmasterol ratio of young mung bean hypocotyl tissue decreased as tissue aged (Geuns, 1973).

The confusing nature of this literature survey is underscored by species that exhibited contrasting sterol profile changes among studies or among organs within studies. For example, Fenner, Patterson, and Koines (1986) reported the ratio of stigmasterol to

sitosterol remained constant in soybean (*Glycine max*) shoot and root tissue throughout many ontogenetic changes. In contrast, Travis and Berkowitz (1980) reported sitosterol accounted for most of the free sterols, and declined with soybean root development but increased with hypocotyl development. Stigmasterol accounted for a smaller proportion of the sterols, but increased with root development and remained stable with hypocotyl development. Therefore, the ratio of sitosterol/stigmasterol increased with maturation of hypocotyls but greatly decreased with maturation of roots.

Nes et al. (1977) have demonstrated the utility of using sterol profiles for phylogenetic implications of ferns and spermatophytes. Therefore, a comprehensive sterol profile for various gymnosperms taxa may lead to a greater understanding of evolutionary development of various biosynthetic pathways during early spermatophyte development. Since a majority of the world's foods are derived from spermatophyte species, this may shed light on the origins of many of the pathways that generate health benefits and risks in contemporary taxa. Fischer and Höll (1991) reported an absence of stigmasterol and a predominance of sitosterol in leaves of the gymnosperm *Pinus sylvestris* throughout three years of development. Sato et al. (2007) also reported sitosterol as the predominant sterol variant in the gymnosperm *Larix kaempferi* (Lamb.) Carr., and free sterols exceeding glycosylated sterols. These reports are in contrast to our results for the more ancient gymnosperm genus *Cycas*. Clearly, much remains to be studied in order to understand sterol relations of these ancient spermatophyte taxa.

4. Conclusions

The vast majority of foods prepared from plants are derived from spermatophyte species. Members of the *Cycas* genus are considered the most basal clade of the cycads and may be the oldest extant lineage of spermatophytes (Brenner et al., 2003). Therefore, continued research on this genus may clarify many aspects of evolutionary and contemporary plant physiology (Brenner, Stevenson, & Twigg, 2003). Some of their traits, including biochemical traits, may be of ancient origin even for the most derived species in the genus.

We have shown here that the literature on secondary compounds and the ageing process of plant organs used for human consumption is defined by highly contrasting results. The inconsistent patterns of sterol changes during tissue development reported for non-cycad taxa and the conflicting patterns of non-sterol metabolite changes during tissue development reported for cycad taxa preclude a generality from emerging. For our model species, seed age remains the only independent factor we have studied to date that exerts unambiguous control over sterol and steryl glucoside phenotype. Our earlier studies revealed foods derived from younger seeds would generate exposure upon ingestion to higher concentrations of sterols and steryl glucosides than would foods made from older seeds (Marler et al., 2006). The historical pattern of disease incidence on Guam is consistent with the assertions that cycad seeds became more of a dietary staple during World War II. For example, consumers were possibly less discretionary in restricting seed harvests to the desired mature dark brown seeds and may have been unconcerned or unable to thoroughly complete the traditional leaching process during seed flour preparation. Incompletely leached flour made from younger seeds may have contained a variety of toxins that worked in synergy. These issues also conform ideally to the aetiological patterns of the diseases (Shaw et al., 2007). The present paper expands the known effects of seed age to include an increase in bioaccumulation that remains linear for up to two years, and a concurrent change in the chemical profile in favour of the derived glucosides. Therefore, foods derived from younger seeds not only contain more of these lipid-soluble neurotoxins than foods prepared from older seeds, they also contain a greater proportion of free sterols relative to the corresponding glucosides.

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