ANALYSIS OF THE FEMALE REPRODUCTIVE SECRETIONS OF *CYCAS REVOLUTA*

by

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Abstract

Gymnosperms produce sexual fluids for reproduction. Cycads ovules produce both pollination drops and a fluid from their megagametophytes. The latter pools in the archegonial chamber and mediates fertilization. Megagametophyte fluid from *Cycas revoluta* was analyzed by various techniques and, where suitable, was compared to the pollination drop of *Taxus* x *media*, yew, a commonly analyzed gymnosperm ovule secretion. Osmolarity was found to be 672 ± 66 mOsm from four individual plants, which was similar to the osmolarity of yew pollination drops. Of the 661 proteins revealed by mass spectrometry using an Orbitrap Fusion, 372 were of plant origin, and 220 were of bacterial origin. These results differ from yew in that the proteins in *Cycas* are almost entirely degradome, as opposed to *Taxus* in which are almost entirely secretome. The presence of bacteria in cycads suggests that the reproductive system of *Cycas revoluta* is not a closed system. In *Cycas revoluta*, the fluid that causes pollen tubes to burst and provides a medium in which sperm can swim has solutes that are released by the megagametophyte, indicating that the female controls major events prior and during fertilization.

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1. Introduction

Gymnosperms utilize fluids for their reproduction. Pollination drops have been studied widely, as successful pollination depends on them. In addition to pollination drops, cycads secrete sexual fluids within their archegonial chamber for the delivery of spermatozoids from the pollen tubes to the egg. These internal fertilization processes are difficult to study, and the composition of the sexual fluid in cycads was previously unknown. An understanding of the mechanisms behind sexual fluids is essential in studying the evolution of reproductive processes in gymnosperms. As cycads are the most ancient extant seed plant, their reproductive characteristics are of great interest.

1.1 Gymnosperms

There are five extant clades of seed plants (Stevenson 1992). Of these, four clades are gymnosperms. They include cycads, *Ginkgo*, Gnetales and conifers. Gymnosperms differ from angiosperms in their reproductive characteristics. Gymnosperms have naked ovules and thus pollen is directly received. Angiosperms have ovules enclosed by an ovary and thus pollen must grow for some distance before arriving at an ovule (Tomlinson and Takaso 2002). Additionally, the presence of archegonia in gymnosperms separates them from angiosperms.

1.2 Cycads

Cycads are thought to be at least 250 million years old, which dates them back to the time of the dinosaurs (Foster and Gifford 1989). The peak of cycad diversity and abundance occurred during the Jurassic-Cretaceous periods. Subsequent extinction

caused a drop in abundance (Crisp and Cook 2011; Nagalingum *et al*. 2011). However, decline has not been continuous. Nagalingum *et al*. (2011) discovered that there was a large radiation of cycad species within the last 10 million years, with six genera diverging almost simultaneously across the globe. Xiao and Möller (2015) stated that this rapid radiation was in the late Miocene. This led to the diversity of extant species known today. Cycads are found in sub-tropical regions of North America, and both sub-tropical and tropical regions of South America, Asia, Africa, and Australia (Norstog and Nicholls 1997).

The three living cycad families are the Cycadaceae, the Stangeriaceae, and the Zamiaceae (Stevenson 1990). It is thought that there over 300 species of cycads in about a dozen genera (Hill *et al*. 2003, Hill *et al*. 2004). Osborne *et al*. (2012) stated that there are 331 species in about 10 genera. Cycadaceae are monogeneric, and *Cycas* currently represents 107 species (Osborne *et al*. 2012). *Cycas* is the most ancient extant group of seed plants (Norstog and Nicholls 1997).

Cycas has a limited distribution from Australia to Japan (Chamberlain 1919). It ranges between the tropics and the subtropics, with species richness concentrated around the Tropic of Cancer and the Tropic of Capricorn. Often cycads occur in small patches or even as individuals. *Cycas* is now geographically divided between the Western Hemisphere and the Eastern Hemisphere (Hill *et al*. 2004). Though many cycads are red listed, *Cycas revoluta* populations are doing well.

1.3 Study species

Cycas revoluta, sago palm, and *Taxus* x *media*, hybrid yew, were studied as they are classics in gymnosperm reproduction, and both are referenced widely in the literature. Yew has one of the largest conifer pollination drops and it is also easily accessible.

1.4 Pollination to fertilization

Cycas revoluta

Cycas revoluta is dioecious. It is predominately wind-pollinated, although insect pollination does occur (Kono and Tobe 2007). Cycad pollen grains are captured by a pollination drop. This drop is known to contain sugars and amino acids (Tang 1987). Cycads show a diurnal rhythm of secretion and retraction of their pollination drops (Tang 1987). Once retracted, the pollen grain is brought into the micropylar canal. (Chamberlain 1910). The pollen grain germinates in a previously created hollow within the nucellus that is known as the pollen chamber (Figure 1, Figure 2). Unlike other seed plants, the pollen tubes of *Cycas* grow away from the egg as the pollen grain branches within the nucellus.

Ikeno and Hirase (1897) determined that each pollen tube in *Cycas revoluta* contains two large oval spermatozoids. These spermatozoids are released from the pollen tube and enter the archegonial chamber. This is located at the apex of the megagametophyte. The archegonial chamber is approximately 2 mm in depth and 5 mm in width. Fluid pools in the archegonial chamber. The source of this fluid has been variously interpreted. Chamberlain (1919, 1935) suggested that in some cycads the fluid

was either produced by archegonial cells, by pollen tubes, or by a combination of both. In studies of *Bowenia*, it was suggested that fluid released by the neck cells combined with fluid released from pollen tubes as they burst (Lawson 1926). According to Brough and Taylor (1940), the pollen tubes were the sole origin of the fluid in *Macrozamia*. Takaso *et al*. (2013) observed that the fluid was secreted by the female gametophyte of *Cycas revoluta*.

Once released from the pollen tubes, the spermatozoids swim through the aqueous environment towards the neck cells. It was suggested that the neck cells surrounding the archegonium separate due to accumulating turgor pressure, thereby allowing the sperm to enter the archegonium (Norstog 1972, Takaso *et al*. 2013). Norstog and Nicolls (1997) proposed that cycads utilize their flagella to propel them in a counterclockwise screwing motion through the archegonial neck cells and into the egg. Takaso *et al*. (2013) observed that four neck cells separated in a schizogenous manner to allow spermatozoids entry. Once a spermatozoid has successfully entered the egg its nucleus fuses with the egg nucleus and fertilization is complete (Chamberlain 1919).

Taxus x *media*

This dioecious conifer is wind-pollinated. Airborne non-saccate pollen grains are captured by aqueous pollination drops that protrude from the micropylar end of an ovule, where it is captured (Foster and Gifford 1989; Williams 2009). The drop then retracts, which delivers the pollen grain to the nucellus (Chamberlain 1935; von Aderkas *et al*., 2018).

Once the pollen grain reaches the nucellus, it sheds its exine and elongates. The tube cell and the generative cell develop. The generative nucleus divides into the spermatogenous cell nucleus and the sterile nucleus. At this stage, the microgametophyte has a tube nucleus, a sterile nucleus, and a spermatogenous cell, which will go on to form two male gametes that are equal in size (Pennell and Bell 1986; Anderson and Owens 1999, 2001).

In the receptive megagametophyte, neck cells open to allow the pollen tube to pass into the egg (Pennell and Bell 1988). Upon contact, the tube bursts and the contents are released (Foster and Gifford 1989). One male nucleus fuses with the egg (Pennell and Bell 1988); the other degenerates (Dupler 1917).

1.5 Comparing *Taxus* and *Cycas*

In terms of reproduction, there are numerous gamete-related differences between *Taxus* and *Cycas*. Cycads and yew differ in the size of their gametes. Cycads have the largest egg ever observed in plants; it can be one fifteenth of an inch in diameter (Chamberlain 1919, Foster and Gifford 1989). Takaso *et al*. (2013) measured *Cycas revoluta* spermatozoids that were 180 μm wide, which were large enough to be seen with the naked eye. By comparison, a male gamete of *Taxus* can be around 1 μm across (Anderson and Owens 1999), which is two orders of magnitude smaller than a cycad male gamete. *Cycas* sperm is unlike conifer, Gnetalean, and other seed plant gametes, because it is highly flagellated. The only other order of plants that shares this peculiarity is *Ginkgo*, another extant ancient clade of early seed plants (Bhatnagar and Moitra 1996). *Taxus* male gametes do not have flagellated sperm (Anderson and Owens 2000).

Both cycads and yew have non-saccate pollen grains. The pollen enters through retraction of their sugar and protein loaded pollination drops (Norstog and Nicholls 1997; Prior and von Aderkas 2014; Nepi *et al*., 2017). The pollen grains each travel through the micropyle, but their development begins to differ once they arrive in the pollen chamber.

The development of pollen tubes differs between cycads and yew by the direction in which they grow. *Cycas* pollen tubes grow away of the archegonium, whereas *Taxus* pollen tubes grow towards the archegonium (Chamberlain 1935; Norstog and Nicholls 1997). Yew exhibits siphonogamy, meaning pollen tubes enter the archegonium directly (Foster and Gifford 1989). *Cycas revoluta* pollen tubes do not enter the archegonia directly; instead, they burst in a fluid-filled archegonial chamber (Ikeno and Hirase 1897). The spermatozoids then squeeze through the neck cells to enter the archegonia (Norstog and Nicholls 1997). The spermatozoids swim into the archegonia, which is called zooidogamy. Of the two types, zooidogamy is the plesiomorphic characteristic (Williams 2009).

1.6 Apoplastic fluid

The apoplast is the extracellular space in a plant tissue. In plants, sugars, amino acids, and other energy-rich compounds are off-loaded from inside cells, so that they can be transported into the other cells or tissues. Proteins are also secreted or transported out of cells, often to serve in constitutive defense, or in substrate enzymatics of extracellular solutes. The medium into which those substances are secreted is water (Nobel 1991). A gymnosperm apoplast is a place for biological processes (Coulter *et al*., 2012). Fluid

originates from various structures. Pollination drops originate from the diploid nucellus (Poulis *et al*., 2005), whereas archegonial sexual fluids originate from the haploid megagametophyte (von Aderkas *et al*., 2018).

Gymnosperm pollination drops are not pure water. Coulter (2005) found that *Taxus* x *media* pollination drops had an osmolarity of 637.81 ± 104.82 mOsm using a Clifton osmometer. Pollination drops can contain sugars (Ziegler 1959; Seridi-Benkaddour and Chesnoy 1988; Tang 1993), calcium (Fujii 1903), malic and citric acid (Ziegler 1959), galacturonic acid (Seridi-Benkaddour and Chesnoy 1988), and amino acids (Ziegler 1959). There are many different proteins present in gymnosperm pollination drops, which have been identified using proteomic techniques (O'Leary *et al*. 2004, 2007; Prior and von Aderkas 2014). Contents can vary according to pollination syndrome. Nepi *et a*l. (2017) found that anemophilous species, such as *Taxus*, had lower sugar concentrations in their pollination drops in comparison to species pollinated by both wind and insects, i.e. ambophilous species such as cycads.

Pollination drops originate from the nucellus (Poulis *et al*. 2005; Coulter *et al*. 2012). In cycads, the nucellus degenerates to form the pollen chamber (Roberts *et al*. 2012). As a result, cycad pollination drops contain proteins from both intact cells (the secretome) and those released from cell lysis of the degrading nucellus (the degradome). As the nucellus degenerates, proteins are washed into the pollination drop. By comparison, O'Leary *et al*. (2004) found that in *Taxus*, the nucellus does not degrade during the formation or secretion of the pollination drop, which means that all proteins found in a drop constitute a secretome only.

Cycads produce apoplastic sexual fluid that pools into their archegonial chambers, which are specialized structures for sperm reception. Sexual fluids are not evident in cycad fossil records, though it is thought that they are a primitive trait as their ovular features have conserved through time (von Aderkas *et al*. 2018). There has been very limited description of cycad archegonial fluid composition, and chemical analysis has not been done.

1.7 Functions

Fluids have a variety of essential roles in the reproductive biology of gymnosperms. They are necessary for capturing microgametophytes, for delivery of microgametophytes into ovules, and for germination. Enzymes involved in carbohydrate-modification have been identified in some plants: in these cases they are thought to aid in pollen development (Poulis *et al*. 2005). In some plants, fluids provide a nectar reward to pollinators (Nepi *et al*. 2017). Fluids are important for ovule defense, as the pollination drop is the barrier between the inside of the ovule and the environment (Prior and von Aderkas 2014).

In each cycad ovule, there are two archegonia; in each pollen tube two spermatozoids (Norstog and Nicholls 1997). Cycads may development numerous pollen tubes within a single ovule. Brough and Taylor (1940) identified up to twenty pollen tubes in a single ovule of the cycad *Macrozamia*. In *Cycas revoluta*, numerous pollen tubes release multiple sperm into the archegonial chamber (Takaso *et al*. 2013).

Hori and Miyamura (1997) observed that *Cycas revoluta* pollen tubes released spermatozoids following the addition of sucrose solution. Takaso *et al*. (2013) observed that pollen tubes of *C. revoluta* would not release spermatozoids following the addition of water. They confirmed that a solution with a high osmotic potential equivalent to that of 10 % to 15% sucrose was required (Takaso *et al*. 2013). The pollen tubes release the spermatozoids only after contact with the sexual fluid in the archegonial chamber, which originated from the megagametophyte (Takaso *et al*. 2013).

It is not uncommon for more than one sperm to enter the egg of a cycad (Chamberlain 1919). Brough and Taylor (1940) and Takaso *et al*. (2013) observed that up to five spermatozoids could enter the archegonia through the neck cells of *Macrozamia* and C*ycas revoluta*, respectively.

In yew, two male gametes form in each pollen tube. However, the number of archegonia present in each megagametophyte ranges from one to eight (Anderson and Owens 2001). Although Hofmeister (1862) proposed that multiple fertilizations may be possible in *Taxus*, Pennell and Bell (1987) argued that this was unlikely. Upon the pollen tube's delivery of two male gametes into the archegonium of *T. brevifolia*, the second male gamete degenerates following successful fertilization of the first. Direct pollen delivery is more sexually selective. Polyspermy, or multiple fertilization events, do not occur in yew.

Two systems of gamete delivery have been described, one of which depends on water (i.e. zooidogamy). How does this compare with other plants? Mosses and ferns reproduce in external water. Their reproductive system is open to the elements: their

sperm must travel in external water, i.e. rainwater, puddles, in search of archegonia to fertilize. Cycads and *Ginkgo* were thought to reproduce by a closed system in that they were thought to supply their own needs for fluid in which the sperm must swim.

I hypothesized that the fluid originating from the megagametophyte in *Cycas revoluta* was not pure water. To investigate this, I analyzed osmolarity and utilized shotgun proteomics, two methods widely used to study gymnosperm reproductive fluids.

2. Materials and Methods

2.1 Collection of fluids

The *Taxus* x *media* Rehder (hybrid yew) pollination drop samples were located on the University of Victoria campus, Victoria, British Columbia, Canada. Samples were used from February 2008 and were also collected in March 2019. The 2019 collection included pollination drops taken directly from around 30 female trees on two separate occasions. Disposable micropipette tips that held 50 µL were used to tap each pollination drop directly from the exposed ovules on the trees (Figure 3). The micropipette contents were pooled into a 1.5 mL cryotube and refrigerated at -20 °C until analysis.

The *Cycas revoluta* Thunb. sexual fluid was collected by Patrick von Aderkas from both planted and wild plants found on Iriomote Island, Okinawa Prefecture, Japan. Female plants were manually pollinated in May. Ovules were removed before and during fertilization in August. The ovules were transversely cut below the archegonia. The portion of the ovule containing the integument, nucellus, and pollen tubes were then separated from the female gametophyte. Fluid was removed as it appeared on the surface

of the megagametophyte. Samples were frozen immediately and frozen at -20 °C until sample analysis.

2.2 Measurement of osmolarity

Osmolarity was determined by measuring freezing-point depression. With a Pressure-Lok Precision Analytical Syringe, approximately 10 µL of mixed sample was loaded into the well of a silver sample holder on the cooling module. A cover slip was placed on top to prevent evaporation. The sample was viewed under an Olympus BHT microscope. The sample was flash frozen to -40 °C with a calibrated Clifton Nanolitre Osmometer. The sample was then heated until crystals were seen to dissolve. At the point when only a single crystal remained, the osmolarity was recorded.

The sample holder was removed from the stage and cooling module, and was placed into the Branson 200 Ultrasonic Cleaner with detergent for ten minutes. The sample holder was then rinsed twice with deionized water and 70% ethanol, then left to air dry before repeating the procedure. The Clifton Nanoliter Osmometer was calibrated using 3422 milliosmoles of 10% NaCl and deionized H_2O solution. The syringe was rinsed with sample prior to each procedure.

2.3 Protein analysis

Samples from four *Cycas revoluta* individuals were obtained from storage for analysis. Using 20 µL of megagametophyte fluid from each individual cycad, Bradford assays were run to identify the presence of proteins in the solution. Next, *Taxus* x *media* and *Cycas revoluta* samples were thawed and processed as follows. Along with 20 µL of

either cycad or yew sample, 5 μL of 1x PBS (phosphate-buffered saline) pH 7.4 buffer, 1 μL of 1 M Dithiothreitol, and 7 μL of SDS-PAGE (sodium dodecyl sulfate) were added to each vial. Samples of 1 μg, 5 μg, 10 μg, and 20 μg of BSA (Bovine Serum Albumin) were prepared as well. The samples were heated in the Eppendorf Thermomixer for 10 min at 99 °C. Next they were run though the Eppendorf 5415D centrifuge, and afterwards gas was gently released from each tube. An SDS-PAGE Gel (NuPAGE 4 - 12 % Bis-Tris Gel) was loaded with the samples. BLUelf Prestained Protein Ladder was used for reference. The gel ran in the Invitrogen Mini Gel Tank with 20x MES (2-ethanesulfonic acid) buffer for 25 min at 200 V. The gel was fixed for 10 min with a 40 % ethanol/10 % acetic acid solution, stained in Coomassie Brilliant Blue for 3 h. It was then kept in 10 % acetic acid destain for 5 h. The gel was imaged using AlphaImager HP. The gel was kept in destain solution afterwards.

The *Cycas revoluta* sample lane from the SDS-PAGE gel was cut into 16 equal pieces at the University of Victoria Genome Proteomics and was further processed. Shotgun proteomics using an Orbitrap Fusion and trypsin for digestion was performed. The proteins were BLASTed against published from SwissProt. Further sorting was done using Scaffold Proteome. Rigorous selection was applied: I used 95 % protein probabilities for both proteins and peptides with a minimum of two peptides. The proteins were sorted into three categories using Microsoft Excel: those of plant origin, bacterial origin, or other. The proteins that were categorized as "other" were excluded from further analysis, because generally they included common contaminant proteins, such as keratin.

The plant proteins were further categorized as follows. Gene ontology annotations were used to identify protein function under three general categories, including biological processes, cell components, and metabolic functions. The cell component proteins were sorted into two categories: intracellular and plasma origin or extracellular origin. Further sorting was done to identify categories that had the most proteins present, as well as those categories which were lacking proteins entirely.

2.4 Statistical analyses

Statistical analyses were performed in Microsoft Excel and RGui. A single-factor ANOVA (analysis of variance) was run with the osmolarity readings of twenty samples from four *Cycas revoluta* individuals. All data were represented as the mean ± standard deviation of the mean. The significance level for analyses was set at $p\text{-value} < 0.05$.

3. Results

3.1 Osmolarity

The *Cycas revoluta* megagametophyte fluid was found to have a mean of 672 ± 66 mOsm (N = 4) (Table 1). Within the species, a highly significant variance was found in osmolality readings from the four individual cycads studied (p=0.00578). There were clear individual differences in osmolarity. All samples ranged between 570 – 747 mOsm. Individual 1 had osmolarity readings that varied greatly with a range of 173 mOsm, while individual 2 – 4 each had a maximum range of 17 mOsm between sample readings. Megagametophyte fluid from individual 4 had the highest osmolarity of the four individuals analyzed, with a mean of 734 ± 7 mOsm (Table 1).

3.2 Proteomics

Proteins were present in the megagametophyte fluid of *Cycas revoluta*. The Bradford assay showed that the *C. revoluta* megagametophyte fluid had a mean of 0.398 ± 0.095 µg/µL. Sexual fluid from both *Taxus* x *media* and *C. revoluta* showed distinct protein bands on the SDS-PAGE (Figure 4). The *C. revoluta* lane appeared fuller than the *T. media* lane on the gel. There were approximately eight distinct bands in the yew pollination drop lane, and approximately fourteen distinct bands in the cycad megagametophyte fluid lane. The yew and cycad bands ranged from approximately 6 – 57 kDa and 4 – 80 kDa, respectively.

Shotgun proteomics revealed 661 proteins in the *Cycas revoluta* megagametophyte fluid. Each of the three categories of biological processes, cell component, and metabolic functions, included hundreds of proteins (Figure 5). The distribution of protein origin between plant and bacteria, respectively, were as follows: 372 and 220 (Figure 6). There were many different types of plant proteins present in the fluid (Table 2).

Degradome proteins made up more than 95 % of the proteins in the sexual fluid. Most of these proteins originated from the cytoplasm or the cellular organelles (Table 3). The secretome accounted for very little of the fluid contents, as just 5% (18 proteins) were found in the apoplast (Table 3).

The identified plant proteins in the sexual fluid represent a wide range of cytoplasmic processes. Most of the proteins in the megagametophyte fluid were involved in metabolism and other cellular processes (Table 4). Of the plant proteins involved in biological processes, less than 4 % of them (18 proteins) were identified as contributing to reproductive processes (Table 4). Of the few reproductive plant proteins, a third of them (6 proteins) could be categorized as extracellular proteins.

The proteins in the fluid did not indicate the presence of strong defense mechanisms or of involvement in chemotaxis, but they may have been involved in other stress responses, such as response to climate (Table 4, Table 5). There were 5 proteins listed that were involved in immune response (Table 4). None of the proteins were

involved in chemotaxis (Table 5). Many heat shock proteins were identified, as were proteins involved in cold response, such as the 14-3-3-like protein (Table 2).

There were also 220 bacterial proteins present in the sample. From these proteins, 76 different bacteria species were identified as contributing proteins to the megagametophyte fluid (Table 6). Included in the list of bacteria were those that are often found in soils, such as the Proteobacteria.

Figure 1. Labelled diagram of a *Cycas revoluta* ovule cross section.

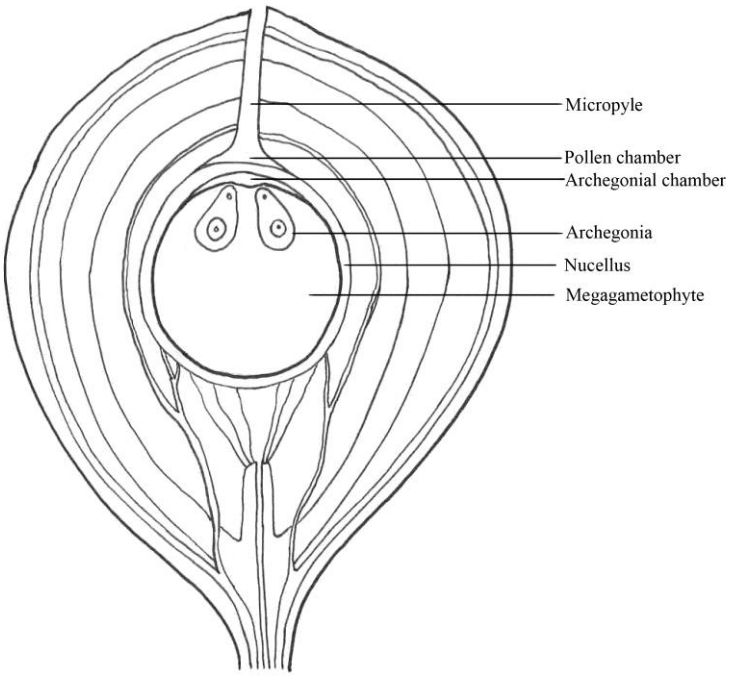
Micropyle
Pollen chamber
Archegonial chamber
Archegonia
Nucellus
Megagametophyte

Figure 2. Labelled diagram of a *Cycas revoluta* upper ovule cross section.

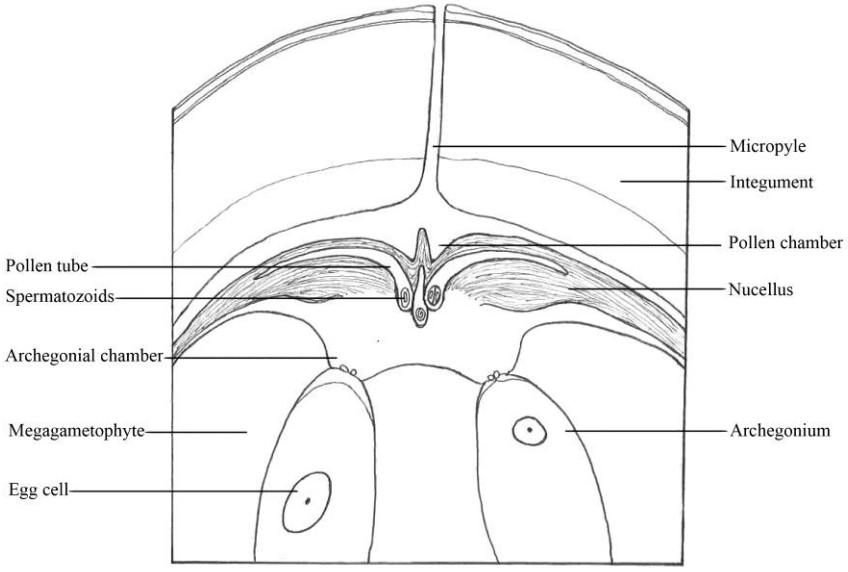
Micropyle
Integument
Pollen chamber
Pollen tube
Spermatozoids
Nucellus
Archegonial chamber
Megagametophyte
Archegonium
Egg cell

Figure 3. *Taxus* x *media* pollination drop at the University of Victoria, Victoria, British Columbia in March 2019.

Figure 4. *Taxus* x *media* pollination drop (YPD) and *Cycas revoluta* megagametophyte fluid (CMF) samples run on SDS-PAGE in 20x MES buffer. BLUelf Prestained Protein Ladder (MW) and BSA (1 µg, 5 µg, 10 µg, and 20 µg, from left to right).

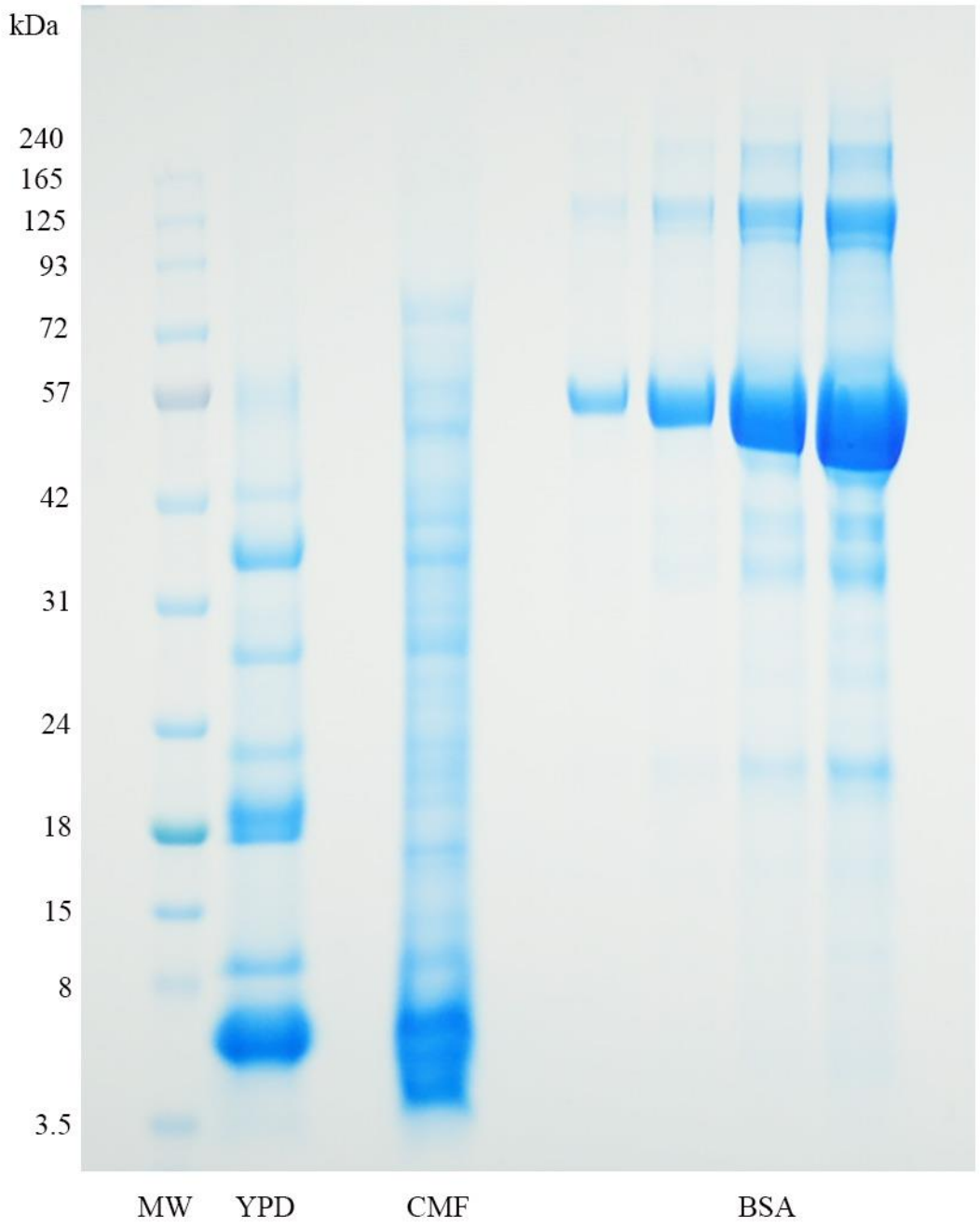
kDa
240
165
125
93
72
57
42
31
24
18
15
8
3.5
MW
YPD
CMF
BSA

Figure 5. Gene ontology of plant proteins in *Cycas revoluta* megagametophyte fluid.

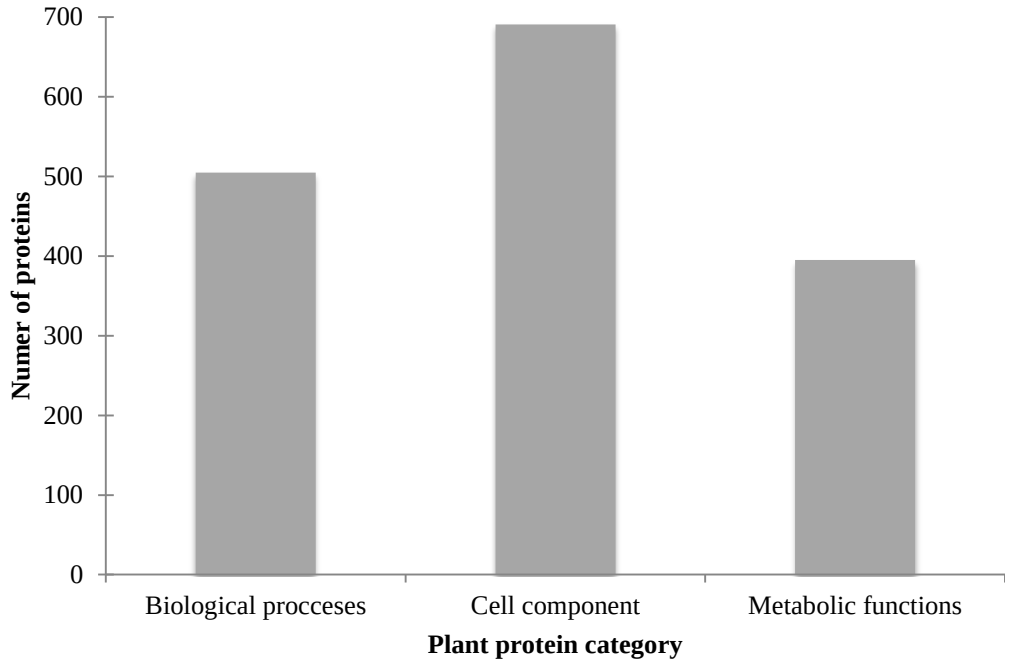
700
600
500
400
300
200
100
0
Numer of proteins
Biological procceses
Cell component
Metabolic functions
Plant protein category

Figure 6. Number of bacterial and plant proteins found in *Cycas revoluta* megagametophyte fluid.

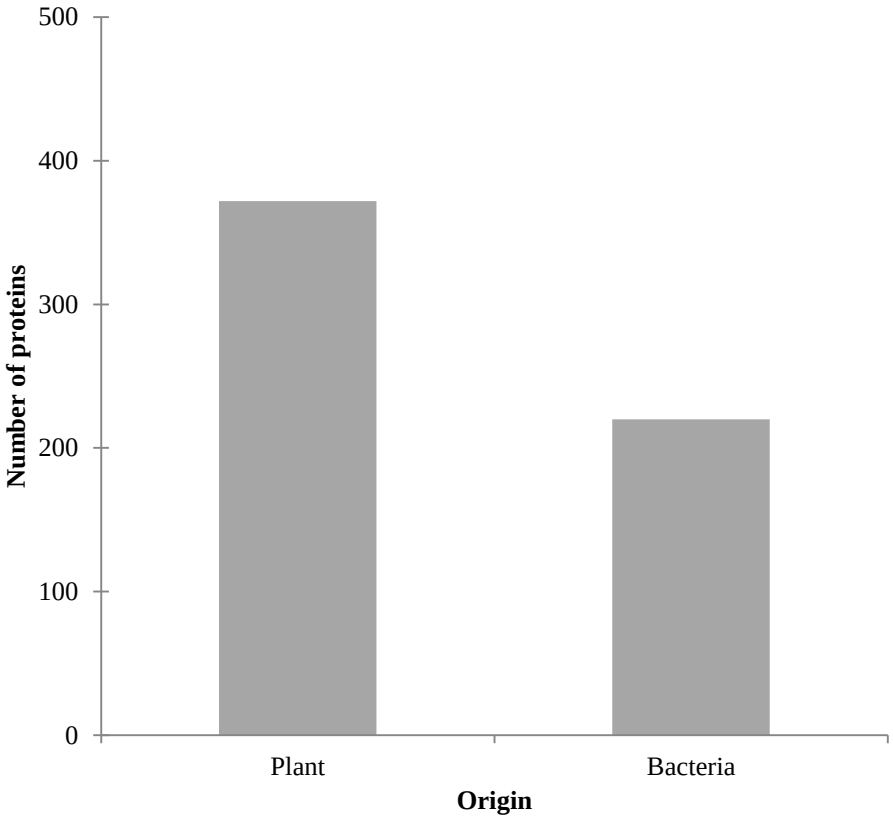
500
400
300
200
100
0
Number of proteins
Plant
Bacteria
Origin

Table 1. Osmolarity of megagametophyte fluid from *Cycas revoluta* individuals (N = 4, n = 20).

Plant	n	Osmolarity (mOsm)
1	7	646 ± 80
2	3	615 ± 4
3	3	642 ± 9
4	7	734 ± 7

Table 2. Plant proteins present in *Cycas revoluta* megagametophyte fluid. Listed in alphabetical order.

Protein
ABC transporter
Aconitate hydratase
Actin
Adenosylhomocysteinase
ADP-ribosylation
Aldo-keto reductase
Alpha-glucan phosphorylase
Aminopeptidase
Antigen
Arginine--tRNA ligase
Aspartate aminotransferase
ATP synthase
ATP-dependent Clp protease
ATPase
Auxin
Calmodulin
Calreticulin
Carbamoyl-phosphate synthase
Catalase
Cell division cycle protein
Chaperone protein ClpB1
Chaperonin CPN60-2
Class I heat shock protein
Clathrin
COP9 signalosome complex
Cullin-1
Cullin-associated NEDD8-dissociated protein
Cytosolic oligopeptidase
Diphosphoinositol-pentakisphosphate kinase
DNA damage-binding protein
DNA-directed RNA polymerase
Elongation factor
Endoplasmin
Enolase
Eukaryotic initiation factor
Eukaryotic peptide chain release factor
Eukaryotic translation initiation factor
Exportin
Fructose-1,6-bisphosphatase
Fructose-bisphosphate aldolase

Glucose-6-phosphate isomerase
Glutathione S-transferase
Glyceraldehyde-3-phosphate dehydrogenase
GTP-binding protein
Guanosine nucleotide diphosphate dissociation inhibitor
Heat shock protein
Histone chaperone ASF1A
Histone H4 variant
Hsp70-Hsp90 organizing protein
Ilityhia
Importin
Inositol hexakisphosphate
Isocitrate dehydrogenase
Isoflavone reductase
Isoleucine
L-arabinokinase
L-ascorbate peroxidase
Lactoylglutathione lyase
Leucine aminopeptidase 2
Leukotriene A-4 hydrolase homolog
Linoleate 9S-lipoxygenase B
Luminal-binding protein
Malate dehydrogenase
Mediator of RNA polymerase II transcription
Monodehydroascorbate reductase
NADP-dependent malic enzyme
Nascent polypeptide-associated complex subunit alpha-like protein
Nuclear pore complex protein NUP133
Nucleoside diphosphate
Organellar oligopeptidase
Peroxisomal isocitrate dehydrogenase
Phosphoenolpyruvate carboxylase
Phosphoglucomutase
Polygalacturonase-2
Proteasome subunit
Protein argonaute
Puromycin-sensitive aminopeptidase
Putative lactoylglutathione lyase
Pyrophosphate--fructose 6-phosphate 1-phosphotransferase
Pyruvate decarboxylase 2
Pyruvate kinase

Ras-related protein
RecA
RuBisCO large subunit-binding protein
RuvB-like protein 1
Serine hydroxymethyltransferase 4
Serine/threonine-protein phosphatase
SKP1-like protein 1A
T-complex protein
Transketolase
Translationally-controlled tumor protein homolog
Triosephosphate isomerase
Tubulin
Ubiquitin carboxyl-terminal hydrolase
Ubiquitin-activating enzyme
Ubiquitin-conjugating enzyme
Ubiquitin-NEDD8-like protein RUB1
UDP-glucose 4-epimerase
UDP-glucuronic acid decarboxylase
UDP-xylose 4-epimerase
Ureidoglycolate hydrolase
UTP--glucose-1-phosphate uridylyltransferase
V-type proton ATPase
Valine-tRNA ligase
Villin-3
Xyloglucan endotransglucosylase/hydrolase
14 kDa zinc-binding protein
14-3-3-like protein
2,3-bisphosphoglycerate-independent phosphoglycerate mutase
26S proteasome non-ATPase
40S ribosomal protein
5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase
5-oxoprolinase
60S ribosomal protein
70 kDa heat shock-related protein

Table 3. Distribution of plant proteins present in various cellular components of *Cycas revoluta* megagametophyte fluid.

Cellular component	N
cytoplasm	146
intracellular organelle	116
organelle part	80
membrane	77
nucleus	64
plasma membrane	52
organelle membrane	38
Golgi apparatus	36
ribosome	28
extracellular region	18
mitochondrion	18
cytoskeleton	7
endosome	7
endoplasmic reticulum	4

Table 4. Distribution of plant proteins in *Cycas revoluta* megagametophyte fluid involved in biological processes.

Biological process	N
cellular process	133
metabolic process	104
response to stimulus	69
biological regulation	49
developmental process	26
multicellular organismal process	25
reproduction	18
localization	17
multi-organism process	14
growth	6
immune system process	5
viral process	3
rhythmic process	1
cell killing	0
locomotion	0
pigmentation	0

Table 5. Distribution of the functions of plant proteins in *Cycas revoluta* megagametophyte fluid.

Function	N
molecular function	147
binding	124
catalytic activity	76
structural molecule activity	28
enzyme regulator activity	9
transporter activity	5
antioxidant activity	4
molecular transducer activity	1
protein tag	1
chaperon regulation	0
chemoattractant activity	0
chemorepellent activity	0
electron carrier activity	0
transport	0
motor activity	0
nutrient reservoir activity	0
transcription regulation	0
translation regulation	0

Table 6. List of bacteria species identified by proteins found in *Cycas revoluta* megagametophyte fluid.

Species	
Acidovorax sp.	Methylococcus capsulatus
Actinobacillus pleuropneumoniae	Mycoplasma pulmonis
Actinobacillus succinogenes	Neorickettsia sennetsu
Alkalilimnicola ehrlichii	Nitrosococcus oceani
Azotobacter vinelandii	Pectobacterium atrosepticum
Bdellovibrio bacteriovorus	Pectobacterium carotovorum
Beutenbergia cavernae	Photorhabdus luminescens
Bifidobacterium longum	Proteus mirabilis
Blochmannia floridanus	Pseudoalteromonas haloplanktis
Bordetella bronchiseptica	Pseudomonas aeruginosa
Buchnera aphidicola	Pseudomonas entomophila
Burkholderia multivorans	Pseudomonas savastanoi
Carboxydothermus hydrogenoformans	Pseudomonas stutzeri
Citrobacter freundii	Psychromonas ingrahamii
Citrobacter koseri	Raoultella planticola
Cronobacter sakazakii	Rhodospirillum centenum
Cryptococcus neoformans	Ruthia magnifica
Edwardsiella ictaluri	Saccharophagus degradans
Enterobacter cloacae	Salmonella agona
Enterobacter sp.	Salmonella arizonae
Enterococcus hirae	Salmonella berta
Erwinia amylovora	Salmonella choleraesuis
Erwinia tasmaniensis	Salmonella paratyphi
Escherichia coli	Salmonella typhi
Escherichia fergusonii	Salmonella typhimurium
Geobacter bemidjiensis	Serratia marcescens
Geobacter metallireducens	Serratia proteamaculans
Granulibacter bethesdensis	Shigella flexneri
Haemophilus influenzae	Sodalis glossinidius
Haemophilus parasuis	Spirochaeta aurantia
Haemophilus somnus	Synechococcus sp.
Idiomarina loihiensis	Syntrophus aciditrophicus
Klebsiella aerogenes	Thermosynechococcus elongatus
Klebsiella pneumoniae	Tolumonas auensis
Kosmotoga olearia	Vibrio cholerae
Lactobacillus plantarum	Wigglesworthia glossinidia brevipalpis
Leuconostoc citreum	Xenorhabdus nematophila
Macrococcus caseolyticus	Yersinia pseudotuberculosis

4. Discussion

The hypothesis was supported: the fluid originating from the megagametophyte in *Cycas revoluta* was not pure water. The osmolarity analysis along with results from shotgun proteomics provided ample evidence that the megagametophyte fluid was rich in solutes. The results of this analysis provide the first observations about the contents of the sexual fluid in the archegonial chamber of *C. revoluta* at the time of fertilization.

4.1 Solute-filled fluid

The osmolarity reading above zero was the initial indication that there were solutes in the sexual fluid of *Cycas revoluta*. Bold (1980) proposed that this fluid had an estimated solute concentration of 0.6 M. No methods were given and the book was no peer reviewed.The specific solutes involved had not been determined, though there were a few possibilities. Both pollination drops and the cycad megagametophyte fluid are ovular secretions involved in reproduction. It was known from Coulter (2005) that *Taxus* x *media* pollination drops had an osmolarity of 638 ± 105 mOsm, which is quite similar to what I found in cycad megagametophyte fluid. There the similarity ends. Previous studies have identified proteins, carbohydrates, and other classes of compounds in gymnosperm pollination drops (Ziegler 1959; Seridi-Benkaddour and Chesnoy 1988; Tang 1987, 1993; O'Leary et al., 2004, 2007; Prior and von Aderkas 2014). What I found in cycads were proteins, but of a very different nature.

4.2 Degradome contents

The strong presence of proteins in the fluid was evident from both the SDS-PAGE and the Bradford assay. I found that most of the proteins in the megagametophyte fluid were from the degradome, in contrast to the yew pollination drop which were all secretome (O'Leary *et al*. 2004, 2007; Prior *et al*. 2018). At the time that the cycad megagametophyte produces fluid, the nucellus has already degraded into a thin layer of cells. It is unclear where the degradome proteins in the megagametophyte fluid have come from. A possible origin of the degradome is the degradation of the outer layer of the megagametophyte. The upper epidermis may have degraded prior to or during fluid production.

4.3 Secretome function

There were only 18 proteins that were extracellular components, i.e. Apoplastic. Though small, the secretome could be involved in a number of functions. Takaso *et al*. (2013) proposed that the fluid may be involved in spermatozoid discharge. In *Ginkgo*, Wang *et al*. (2014) proposed that the fluid in the archegonial chamber likely functioned as a chemoattractant, guiding male gametophytes to the ovule. However, chemotaxis was ruled out, as I found there were zero proteins described for this purpose, according to gene ontologies. It is unlikely that the fluid was involved in ovule defense, which was indicated by the presence of numerous bacteria and thus the shortcomings of the defense mechanisms of the fluid. By comparison, the proportion of defense proteins in yew is

overwhelming (Coulter *et al*. 2012). Yew have incredible defense mechanisms, such as the presence of chitinase. The purpose of secretome in *Cycas* remains unknown.

4.4 Bacteria-rich environment

There was a strong presence of bacterial proteins in the cycad. Yew has defense mechanisms which combat their exposure to bacteria in the environment, so that bacteria cannot influence their reproduction in the ovule (Prior and von Aderka 2014). The presence of bacteria in the cycad megagametophyte fluid initially indicated that the system may be compromised; however, it should be noted that mosses and ferns are perfectly able to reproduce in fluids that contain bacteria (Ponce de León and Montesano 2017). When their male gametes are released, they are exposed to bacteria and other living organisms in dirty water such as puddles, ponds, and streams. The cycad megagametophytes were healthy and able to successfully reproduce, and thus the bacteria cannot be representative of an unhealthy individual. The presence of bacteria in the cycad ovules is likely harmless to the plant.

Soil microbial diversity has been observed to have a negative correlation with latitude, and a positive correlation with temperature (Staddon *et al*. 1998). As cycads have sub-tropical and tropical habitats, this environment can expose them to a higher bacterial load than conifers are exposed to in temperate regions (Chamberlain 1919). The Proteobacteria in the cycad ovule, such as *Escherichia*, *Salmonella*, and *Yersinia*, are known to be some of the most common bacteria found in soils (Roesch et al., 2007; Nemergut et al., 2010). The bacteria are entering the ovule through the air, the mist, or by

animal vectors. If the megagametophyte fluid has sugar in it as Takaso *et al*. (2013) considered, the bacteria are likely thriving in this environment. The presence of bacteria in the ovule indicates that there appears to be no barrier between the external environment and the internal reproductive environment.

4.5 Limitations

The potential limitations to this study were considered. Firstly, when the protein contents from the megagametophyte fluid of *Cycas revoluta* were analyzed, false positives were described. There were species listed, such as marine creatures, that were surely not present in the cycad ovule. It is not known why they were represented in our results. Another considerable limitation of my research was the accuracy of the osmolarity reading. There were a few variables that could have influenced the osmolarity reading.

Given the variability in osmolarity of *Cycas revoluta*, it would have been beneficial to analyze more individuals. Samples of the megagametophyte fluid are difficult to collect due to their location and small volume, and thus the results are limited to four individuals. Additionally, cycad pollination drops were not available to compare for our research. The inaccessible habitat, as well as the small size of the pollination drops, made it too challenging to study them. Yew was used to compare with the megagametophyte fluid as it was readily available. As a representative of conifer pollination drops, yew could inform as a comparison between conifers and the earlier land plants I studied.

4.6 Future perspectives

Our results have opened up many new questions. In the future, the composition of cycad pollination drops could be compared to both of the fluids studied here; yew pollination drops and cycad megagametophyte fluid. Conifers are known to possess defense proteins in their pollination drops (Prior and von Aderkas 2014). Defense proteins in cycad pollination drops have been identified (Prior *et al*. 2018). However, the bacteria rich environment indicates that the defense proteins identified by Prior *et al*. (2018) are inadequate.

The remaining solutes within the fluid must be considered. An analysis on the carbohydrate, phosphate, and elemental makeup of the fluid, should be carried out. These results may help to reveal the physiological mechanisms during reproduction. Before cycads, plants used fluid purely for the transportation of spermatozoids. The presence of solute-filled fluid in the cycad ovules leads to questioning why plants evolved to possess a more complex reproductive system that does not rely on external water to deliver gametes.

It is not clear how the bacteria in the cycad fluid arrived there. To understand their activity and the impact they have on cycad individuals, it would be interesting to identify the classes of bacteria. The origin of the bacteria is testable, and future research should determine how the bacteria are entering the cycad ovule. It could be from the soil, from the air, in water, or perhaps through a combination. Additionally, culturing the bacteria would uncover if they are living intruders in the plant or if they have merely moved

through the ovule as non-living stragglers. By answering these questions, the relationships unfolding between the bacteria and the cycads can be better understood.

4.7 Conclusion

Cycas revoluta is the intermediate plant between their predecessors, the mosses and ferns, and the land plants that came after them, the conifers. Their reproductive system is the link between the utilization of water through an open system and a closed system. As the first plants to evolve on land, mosses and ferns relied heavily on rainwater. The fluid that cycads produced within their ovules has been referred to the as "the sea within the seed", as they evolved to produce their own liquid rather than to release their gametes to an open environment. I found that not only do cycads contain sexual fluid full of proteins, but the cycads have been inundated with bacteria from their surroundings. These novel results indicate that cycads do not have a closed system, as was previously thought. The cycad reproductive mechanisms are unlike any plants studied before, as their reproduction is entirely dictated by the female gametophyte, and the success of the male gamete fertilizing the egg is completely reliant on the production of the female's solute-filled fluid.

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