Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

Citation: C.F. Crane (2019) Megagametophyte Differentiation in *Zephyranthes drummondii* D. Don and *Zephyranthes chlorosolen* (Herb.) D. Dietr. (Amaryllidaceae). *Caryologia* 72(4): 105-119. doi: 10.13128/caryologia-670

Published: December 23, 2019

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Megagametophyte Differentiation in *Zephyranthes drummondii* D. Don and *Zephyranthes chlorosolen* (Herb.) D. Dietr. (Amaryllidaceae)

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Abstract. Megagametophyte differentiation was examined in cleared ovules from emergent buds and open flowers of *Zephyranthes drummondii* D. Don and *Z. chlorosolen* (Herb.) D. Dietr., two highly apomictic species that exhibit the *Antennaria* type of megasporogenesis and hemigamy. Stages from binucleate megagametophytes through early endosperm divisions were sampled. A large central vacuole appears after the megasporocyte divides mitotically, and this vacuole persists until the endosperm becomes cellular. Upon cellularization of the egg and antipodal apparati, a central column of cytoplasm develops longitudinally across the central vacuole, and both polar nuclei move into it before moving in unison to the chalazal end. The mature megagametophyte is organized conventionally, with one egg, two synergid, two polar nuclei, and three antipodal cells. Endosperm development is helobial, but there are few divisions in the chalazal chamber of the endosperm. The behavior of fertilized and unfertilized ovules was also studied in response to pollination. Although synergids degenerate autonomously, pollination accelerates synergid degeneration in unfertilized as well as fertilized ovules relative to unpollinated flowers of the same age. Tabulation of numerical abnormalities suggests that progressive imprinting is involved in megagametophyte differentiation; the data do not support a strictly zonal specification of nuclear fate, but instead a role for nuclear polarization before mitotic divisions. This study demonstrated the value of cleared ovules in gathering statistically and temporally meaningful observations of megagametophyte differentiation, relating in particular to the movement of polar nuclei and the response of the megagametophyte to pollination.

Keywords. Megagametophyte, embryo sac, apomixis, hemigamy, polar nuclei.

INTRODUCTION

Zephyranthes drummondii D. Don and *Z. chlorosolen* (Herb.) D. Dietr. are two congeneric amaryllid species that share apomictic reproduction and a flowering response to rainfall, hence the common name "rain lilies". The buds differentiate within the bulb for several months and then emerge

in response to wetting of the roots. The flowers open at sunset, most frequently on the fourth day following rainfall. The flowers remain open for one or two days, depending on temperature. In nature, self-pollination usually occurs soon after the anthers dehisce inside the bud at mid-morning on the day of anthesis. The incongruous combination of large, showy, sweetly scented flowers and self-pollination has motivated several studies of reproduction in these species and the related *Habranthus tubispathus* (L'Her.) Traub (Pace, 1913; Brown, 1951; Coe, 1953). These studies indicate obligate apomixis by mitotic megasporogenesis, i.e., the *Antennaria* type (first described in *Antennaria alpina* (L.) Gaertn. by Juel, 1900), and hemigamy (synonym: semigamy; Battaglia, 1945), which is development of the zygote after plasmogamy but without fusion of egg and sperm nuclei within the cytoplasm of the egg cell. Various details of the stages from the free-nuclear embryo sacs through fertilization remain to be documented, such as the path taken by the polar nuclei to reach the chalazal end of the central cell and whether the central cell experiences triple fusion to initiate the endosperm.

Many apomictic species require pollination for seed set, but the mechanisms vary. In *Potentilla* (Gustafsson, 1946, p. 31), the embryo can begin to develop autonomously in unpollinated flowers, and a sperm nucleus fertilizes only the central cell, thus initiating endosperm development. The exact fate of the other sperm nucleus is rarely known; in the *Ranunculus auricomus* species complex it has been reported also to fertilize the central cell frequently (Nogler, 1972), leading to expectedly 6n endosperm with the 2:1 maternal to paternal genome ratio usually found in sexually produced endosperm. Facultative double fertilization of the central cell has also been indicated with flow cytometry in apomictic *Crataegus* (Talent and Dickinson, 2007). In most apomictic panicoid and eragrostoid Poaceae (Brown and Emery, 1957; Voight and Bashaw, 1972), there is but one polar nucleus, and fusion with only one sperm would produce the 2:1 maternal:paternal ratio. Nevertheless, Bashaw and Hanna (1990) reported frequently observing a sperm in the central cell of the panicoid grass *Cenchrus ciliaris* L., but none near the egg, possibly indicating that both sperms usually enter the central cell but only one fuses with the lone polar nucleus. In most species with nucellar (adventitious) embryos, such as apomicts in the genus *Citrus*, the megagametophyte is reduced and sexual fertilization is more or less unaffected; the nucellar embryos then outcompete the sexually produced embryo (Gustafsson, 1946, p. 35; Nygren, 1967, p. 559). As defined above, nearly obligate hemigamy is known in nature only in certain species of *Rudbeckia* (Asteraceae; Battaglia, 1945) and *Habranthus* and *Zephyranthes* (sister genera in the Amaryllidaceae). Also, a dominant, incompletely penetrant hemigamous mutant *Se* has been recovered in cotton (Turcotte and Feaster, 1969); unlike hemigamy in *Zephyranthes*, it readily produces maternal haploid, paternal haploid, and hybrid sectors in chimeric embryos or maternal-paternal twin embryos. Similar behavior has been observed at ca. 1% frequency in wild-type *Theobroma cacao*, where it has been exploited as a source of haploids (Lanaud, 1988).

In contrast to broadly descriptive classical studies, modern research (mostly in *Arabidopsis*) has taken advantage of a battery of transposon insertion mutants, a finished genome sequence, fluorescent reporter molecules, and informatic tools, to accumulate a body of concepts and literature dealing with signaling and gene interactions during fertilization in sexual species (Zhou and Dresselhaus, 2019). No form of apomictic reproduction is understood in comparable detail, although apomictic behaviors can spur insights into aspects of sexual reproduction. For example, the facultative double fertilization of the central cell in *Crataegus* (Talent and Dickinson, 2007) suggests that the polar nuclei briefly remain attractive or receptive to a second sperm nucleus after fusing with the first one, that the central cell can attract both sperm nuclei, and that the egg more strongly attracts one and only one sperm nucleus (else triploids and haploids result); once framed, all three of these hypotheses can be tested experimentally in an amenable species. Unfortunately, to date there apparently is no reported *Arabidopsis* mutant that exactly duplicates the hemigamous behavior seen in *Zephyranthes*.

Previous embryological studies of apomictic *Zephyranthes* and *Habranthus* (Pace, 1913; Brown, 1951; Coe, 1953) have produced limited evidence about megasporogenesis, because this stage occurs within the bulb where the bud length is not visible without destroying the plant and each plant produces zero to five buds per year. The evidence in favor of the *Antennaria* type is mostly negative: dyads expected from first-meiotic restitution (the *Taraxacum* type) or complete omission of the first meiotic division (the *Blumea* type [Chennaveeriah and Patil, 1971] or the syndrome in *Elymus rectisetus* (Nees in Lehm.) A. Love et Connor [Crane and Carman, 1987]) have not been observed, while enlarging, vacuolate, undivided megasporocytes are frequent. The positive evidence is a single image of a mitotic metaphase in an enlarged, vacuolate megasporocyte in *Habranthus tubispathus* (Brown, 1951). Indirect evidence is the occurrence of the ordinary, monosporic *Polygonum* type in sexual *Zephyranthes candida* (Ao et al., 2016), which militates against the occurrence of the *Ixeris* type (mei-

otic first-division restitution in the tetrasporic *Fritillaria* type) in *Zephyranthes*. The present study took advantage of the readily accessible later stages in bud development to understand the maturation of the megagametophyte in two apomictic species of *Zephyranthes*, with particular interest in three processes shared with related sexual species: the movements of polar nuclei, the senescence of the megagametophyte with and without fertilization, and the regulation of nuclear fates as the megagametophyte matures. These are universal aspects of angiosperm reproduction that are easily observed in apomictic *Zephyranthes*.

MATERIALS AND METHODS

The ovules of maturing flowers in *Zephyranthes* are particularly suited for clearing studies. At this stage, the ovules are easily removed from the ovary before fixation, and the cleared ovules are readily pipetted onto a microscope slide. The ovules are flat and thus present the embryo sac in sagittal optical section. The embryo sac is very large and thus the egg apparatus and polar nuclei are not close to the plane of overlying and underlying nucellar cells. The nucellus is free of birefringent calcium oxalate crystals at the stages examined, facilitating differential interference-contrast microscopy. Finally, the refractive index of methyl salicylate is close to optimal to resolve nuclei and yet see through many cell layers, which has not been the case in other species like *Nothoscordum bivalve* (L.) Britton in N.L.Britton & A.Brown (Crane, 1978) and *Elymus rectisetus* (Nees) A.Love & Connor (Crane and Carman, 1987).

Flowers were examined in *Z. drummondii* D. Don and *Z. chlorosolen* (Herb.) D. Dietr. at stages from emergence from the bulb through 48 hours post-anthesis. *Zephyranthes drummondii* was collected from two sites in Austin, Texas: at the intersection of 27th Street and Speedway, and at a hilltop on the east side of Interstate 35 just south of its interchange with U.S. 183. *Zephyranthes chlorosolen* was collected along the entrance ramp of U.S. 183 onto southbound Interstate 35. Excised ovules of both species were fixed overnight in FPA50, which is 37% formalin: glacial propionic acid: 50% ethanol, 1:1:18 v:v:v (Herr, 1971), and dehydrated through 70%, 95%, and absolute ethanol. The ovules were infiltrated with methyl salicylate in three steps: 2:1 absolute ethanol: methyl salicylate, 1:2 absolute ethanol: methyl salicylate, and pure methyl salicylate. The dehydration and infiltration steps were minimally one hour. Ovules were viewed under differential interference contrast (Nomarski) as whole mounts in methyl salicylate with cover slips at the side to support an overlying cover slip. Variations of this method have been used subsequently by Young et al. (1979), Stelly et al. (1984), and Zeng et al. (2007); a recent application appeared in Kwiatkowska et al. (2019).

Buds of *Z. drummondii* were collected during the spring of 1976 at stages from emergence through early endosperm development. Buds of *Z. chlorosolen* were collected in triplicate in seven specific groups from 7 July 1976 through 10 July 1976 after rainfall on 4 July 1976, in order to survey development in pollinated and unpollinated flowers before, during, and shortly after the usual time of self-pollination. The *Z. chlorosolen* groups are detailed in Table 1 (below). All the flowers in the first six rows of Table 1 were emasculated at sunset one or two days before opening. Pollinations were performed within an hour with pollen from opening flowers elsewhere in the population. The stigma was removed from unpollinated flowers to prevent pollination. The stigma was slightly exserted above the anthers in the three flowers of the seventh row in Table 1, and these flowers were self-pollinated upon anthesis. After a delay of 48 to 72

Table 1. Batches of *Z. chlorosolen* flowers used in this study.

Code[a]	Emasculation date	Pollination status	Date of anthesis	Date picked	Age relative to anthesis when picked
-2e+72	7 July	Unpollinated	9 July	10 July	+1 day
-2pol+72	7 July	Pollinated	9 July	10 July	+1 day
-1e+48	7 July	Unpollinated	8 July	9 July	+1 day
-1pol+48	7 July	Pollinated	8 July	9 July	+1 day
-1e+72	7 July	Unpollinated	8 July	10 July	+2 days
-1pol+72	7 July	Pollinated	8 July	10 July	+2 days
0pol+48	8 July	Pollinated	8 July	10 July	+2 days

[a]Codes consist of number of days relative to anthesis (0, -1, -2), pollination status (pollinated or emasculated and unpollinated, and the approximate number of hours after pollination or emasculation when the flower was picked for fixation.

hours, picked flowers were brought indoors, with ovule excision and fixation commencing immediately for the first flower processed. The other two flowers per treatment were held at 4C until earlier flowers had been processed. About 30 of the 50 to 80 total ovules were randomly sampled per flower.

Mature embryo sacs of *Z. chlorosolen* were classified as normal or abnormal on the basis of nuclear and cell count. A normal embryo sac consisted of one egg, two synergids, three antipodals, and a binucleate central cell whose nuclei ultimately fused. Abnormal embryo sacs differed in count, usually as a result of non-division of a nucleus at an earlier stage. Synergids were classified as having a filiform apparatus, which usually coincided with a micropylar-end position of their nucleus and a chalazal-end vacuole. The egg did not have a filiform apparatus and had a chalazal or lateral nuclear position and a micropylar-end vacuole. Free nuclei in the central cell were classified as polar nuclei. Sometimes the antipodal cells resembled a second egg apparatus in nuclear and vacuolar positions, such that one antipodal had a nucleus closer to a polar nucleus. Abnormalities were tabulated in an attempt to discern if there was a pattern of successive determination of nuclear fates. Synergid and egg degeneration were also followed in relation to ageing and pollination. Degeneration was indicated by cytoplasmic collapse, nuclear shrinkage, and general loss of visible cellular content.

RESULTS

Gametophytic maturation in Zephyranthes drummondii

Most fertile ovules had reached the four-nucleate stage as the bud emerged from the neck of the bulb at the soil surface, but a few were still binucleate. The embryo sac was discoid at this stage, and its micropylar end directly abutted the nucellar epidermis. There was a large central vacuole, which persisted throughout further development until the endosperm cellularized in fertilized ovules. A tapering cytoplasmic strand, narrowest at the middle, traverses the vacuole after the first mitosis, but this strand disappears before the second mitosis. A prominent hypostase was fully developed at the chalazal end of the ovule, and it persisted well into endosperm development.

Four-nucleate embryo sacs (Fig. 1A) and eight-nucleate embryo sacs lacked any visible cytoplasmic strands that span the central vacuole. In most ovules, the last mitosis occurred on the third day before flower opening. Mitosis at the chalazal end of a four-nucleate embryo sac was observed to precede mitosis at the micropylar end,

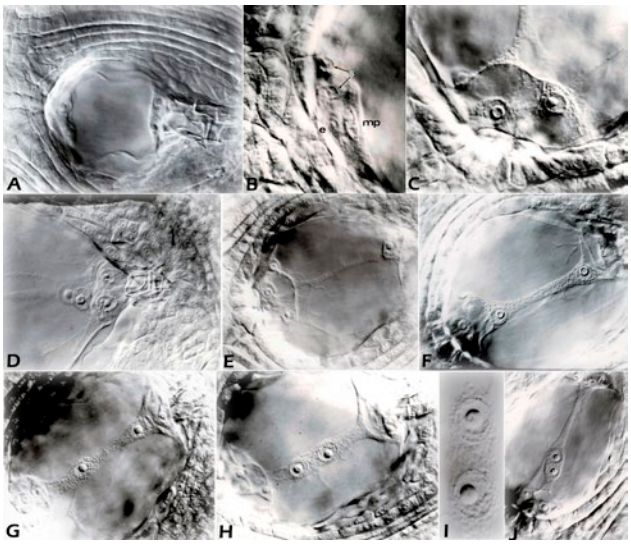

Figure 1. Early development and migration of polar nuclei in *Z. drummondii*. The micropylar end is to the left or down in each picture. A. Tetranucleate embryo sac with nuclei side by side at each end. B. Flattened nuclei soon after telophase at the micropylar end, indicating orthogonal spindles; e, predicted egg nucleus; mp, predicted micropylar polar nucleus; s, predicted synergids, based on frequently occurring positions in the mature egg apparatus. C. More mature, fully cellular egg apparatus with egg nucleus (at left) unusually close to the micropylar end of the egg cell. The filiform apparatus of a synergid (right) appears feltlike or fibrillar. D. Mature antipodal apparatus in contact with the hypostase, whose walls are thickened and birefringent. The migrated but unfused polar nuclei lie immediately to the left. E. Inception of the central column. There are also smaller, variously oriented cytoplasmic strands that appear (with light microscopy) to have intruded into a previously uninterrupted central vacuole. The egg apparatus of this embryo sac appeared in C. F. The central column has reached full thickness. G. The micropylar polar nucleus has entered the central column first. H. Both polar nuclei have entered the central column. I. Striations between the approaching polar nuclei are possibly cytoskeletal elements. J. The polar nuclei can meet near the egg apparatus, or move there temporarily after meeting at the center.

but it is not known if this is generally the case. The post-telophase daughter nuclei at the micropylar end already occupied the positions expected of nuclei in the egg apparatus (Fig. 1B), and their flattened shape indicated that the two mitotic spindles had been perpendicular to each other. The prospective egg nucleus and micropylar polar nucleus were already larger than the prospective synergid nuclei on the second day before flower opening. Meanwhile, the divisions at the chalazal end of the embryo sac were more difficult to see because of the thick, birefringent walls of the hypostase and the presence of up to 20 cell layers in the light path. Nevertheless, the last mitotic spindles there appeared to be mutually perpendicular as they were at the micropylar end,

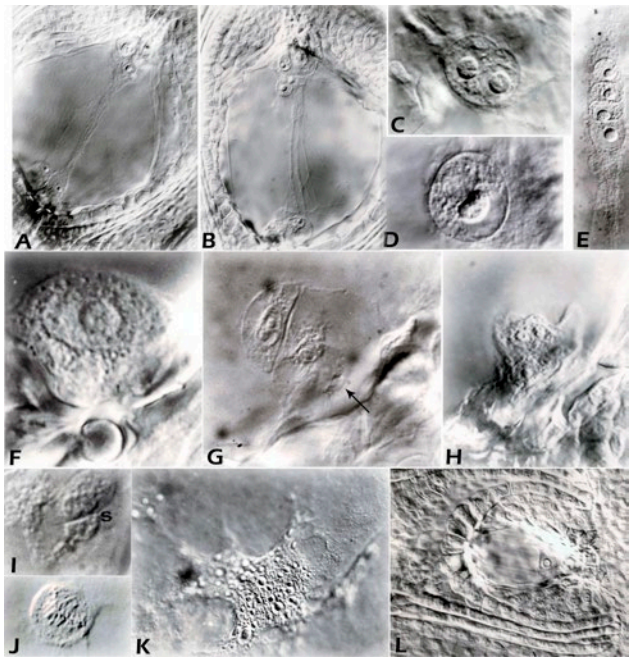

Figure 2. Later development in *Z. drummondii*, except J, which is from *Habranthus robustus* pollinated with *Z. macrosiphon*. A, B. Dissipation of the central column, which splits into parallel strands. C. Appressed polar nuclei next to antipodals. D. Completely fused polar nuclei with fused nucleoli. E. Persistent central column in abnormal embryo sac with four polar nuclei and no synergids. F. Egg cell during or shortly after plasmogamy with a sperm cell. G. Bicellular embryo; one of two sperm-derived daughter nuclei (arrow) lies below the maternal nucleus in *cb*. H. Zygote shrinkage or degeneration. I. Both sperm-derived nuclei (s) have been walled off from *cb* in the same embryo as G. J. Intranuclear metaphase of endosperm nucleus in the micropylar chamber of the helobial endosperm. K. Highly endopolyploid, multinucleolate nucleus in failing endosperm. L. Sterile ovule with uninucleate embryo sac whose nucleus resembles a polar nucleus and occupies the chalazal position of migrated polar nuclei.

and a candidate chalazal polar nucleus was evident farthest from the hypostase before cellularization.

Cellularization occurred by the day before flower opening. The filiform apparatus began to develop (Fig. 1C) in the synergids. The egg nucleolus developed a nucleolar vacuole (Fig. 1C). After cellularization, one of the antipodal nuclei was larger than the other two (Fig. 1D), just as the egg nucleus was larger than the synergid nuclei, and this distinction was even more evident in unfertilized ovules post-anthesis.

The polar nuclei migrated on the day before opening, after the initial cellularization of the egg and antipodal apparati. Before migration, both polar nucleoli had formed a nucleolar vacuole. Thin strands of cytoplasm began to intrude into the central cell vacuole (Fig. 1E), and the central strand soon spanned the vacuole. The central strand continued to thicken and developed a granular appearance (Fig. 1F). The polar nuclei migrated into the central strand; in Fig. 1G the micropylar nucleus has moved first. The polar nuclei approached each other in the strand (Fig. 1H), and faint striations indicated the presence of cytoskeletal elements (microtubules and/or microfilaments) between them (Fig. 1I). Although the polar nuclei could meet relatively near the egg apparatus (Fig. 1J), they finally moved in unison to the chalazal end of the central cell (Fig. 1D). Then the central cytoplasmic strand began to separate into separate strands and disappear (Figs. 2A and 2B; also Fig. 1D) on the day of flower opening. Over time the polar nuclei became appressed (Fig. 2C) and then fused, as indicated by fusion of their nucleoli (Fig. 2D). In one abnormal case with extra polar nuclei in lieu of synergids, all four polar nuclei became trapped within the middle of the central strand and maintained their distinctness through three days past flower opening (Fig. 2E). The abnormality with four trapped polar nuclei was seen several times also in cleared ovules of *Hippeastrum xjohnsoni* (*H. reginae* (L.) Herb. x *H. vittatum* (L'Her.) Herb.).

Fertilization occurred on the second day post-opening. Figure 2F possibly depicts plasmogamy of the egg and sperm cells. Later on the smaller sperm nucleus was visible within the egg, and it could divide before being walled off from cb of the bicellular embryo (Figs. 2G and 2I). Some eggs partially collapsed after plasmogamy (Fig. 2H), but most maintained an expanded, semicircular shape leading to elongation toward the chalaza. The initial division plane was usually transverse. The pollen tube was sometimes visible. In one ovule that had been punctured during excision and handling, where the surrounding synergid debris had been lost, there was an apparent pore at the hooked pollen tube tip where sperms had been released.

Karyogamy appeared to be necessary for endosperm development. In one abnormal instance, karyogamy was incomplete on the tenth day after floral opening, and the partially decondensed, distended sperm nucleus was still appressed to the polar fusion nucleus, which had not divided. In another ovule, the sperm and polar fusion nucleus were close to each other, and both were degenerating. Further evidence for the necessity of karyogamy in hemigamous *Zephyranthes* and *Habranthus* comes from the results of interspecific pollinations, which often result in an increased frequency of endosperm failure and empty seeds. In *H. tubispathus* x *Z. candida* (Lindl.) Herb., for example, all the seeds were empty in spite of seed setting of more than 95%. The primary endosperm nucleus divided transversely and thus produced a small chalazal cell covering the antipodals and a far larger

micropylar cell containing the rest of the volume of the central cell. Free-nuclear divisions ensued in both cells, but no more than five nuclei were observed in the chalazal cell. Abundant free-nuclear mitoses in the micropylar cell resulted in a multinucleate shell that laid down cell walls centripetally as it began to fill in the central vacuole. The first mitoses in the micropylar cell were synchronized and resulted in 2, 4, 8, 16, or 32 nuclei at a time before synchrony broke down, whereas mitoses in the chalazal cell were not synchronized. The early endosperm nuclei were large enough to hold an entire spindle apparatus within the old nuclear membrane (Fig. 2J). The nuclei of mature, fully cellular endosperms were smaller and spherical. Lobate, multinucleolate endosperm nuclei sometimes appeared, but they seemed to be associated with endosperm failure. They appeared to be under traction by attached spindle fibers (Fig. 2K; this example is from *Habranthus robustus* Herb. ex Sweet pollinated with *Z. carinata* Herb.).

The fate of unfertilized embryo sacs was also examined. Neither the egg nucleus nor the fused polar nuclei ever divided. Both synergids usually degenerated one or two days before the egg did. The degenerating egg apparatus lost evident vacuoles as the nuclei faded out. The cytoplasm collapsed, i.e., the egg shrank and its boundary became crenulate. The antipodals degenerated at the about same time as the synergids, and they could be crushed by proliferating nucellar cells near the incompressible hypostase. The central cell nucleus was usually the last nucleus to degenerate in the embryo sac.

From five to 20% of the ovules were sterile, lacking a mature embryo sac. Although both integuments were of normal size, the nucellus was smaller. Sterile ovules fell into four main types: those with a uninucleate embryo sac in a hypodermal position, those with a slightly enlarged megasporocyte in a hypodermal or subhypodermal position, those with no enlarged cells at all, and those with the megasporocyte surrounded by thickened cell walls within the hypostase. Sterile ovules were most frequent toward the base of the ovary, but they could occur anywhere. Figure 2L shows a uninucleate embryo sac with an enlarged nucleus and prominent nucleolar vacuole.

Pollination response and senescence in Zephyranthes chlorosolen

The experimental layout appears in Table 1. Observations centered on timing of fertilization relative to pollination, synergid degeneration, sequence of degeneration of cells in unfertilized embryo sacs, timing of first division in the egg versus the endosperm, frequency of spermatic division in the egg, and numerically abnormal embryo sacs. In total, 796 ovules were examined. They were classified as unfertilized normal, fertilized normal, unfertilized abnormal, or sacless, depending on presence of an embryo sac, number of components in the embryo sac, and evidence of fertilization such as dividing endosperm or presence of a pollen tube at the micropyle or presence of a sperm nucleus within the egg. There were 524 unfertilized normal, 122 fertilized normal, 63 unfertilized abnormal, and 87 sacless ovules in all. Table 2 gives the numbers of instances for all conditions of embryo sacs and their components versus treatment. Supplemental Table 1 gives the same information divided among the 21 individual flowers sampled.

No embryo or endosperm developed in emasculated, unpollinated flowers. Pollen tubes reached the ovules about 48 hours after pollination, but their growth rate depended on the time of pollination, and their arrival continued over a period of many hours. Thus in the "Egg" rows of Table 2, most of the fertilizations occurred between 48 and 72 hours after pollination in buds pollinated the day before natural anthesis, whereas most of the fertilizations had occurred less than 48 hours after pollination in flowers pollinated upon opening. No pollen tubes reached the ovules after pollination two days before opening. In spite of the slower growth rate, pollen tubes reached the ovules sooner after early pollination, as evidenced by the presence of multicellular embryos only after early pollination. At the times sampled, 110 ovules had received one pollen tube, seven had received two, and one had received three; the number was not noted or pollen tubes were not seen in the other evidently fertilized ovules. If only the early pollinated buds collected 72 hours later are considered, these totals became 65, two, and one. When more than one pollen tube had reached an ovule, they followed different paths toward the embryo sac. No instances of two sperm nuclei were seen in undivided egg cells.

The synergids degenerated in both fertilized and unfertilized ovules, but pollination accelerated degeneration (Table 2, "Synergid" rows). At least one of the synergids had begun to degenerate in every fertilized embryo sac, whereas both synergids were intact in a substantial minority of embryo sacs in unpollinated flowers of the same age. Synergids were more degenerated in the unfertilized ovules of pollinated flowers than they were in unpollinated flowers. Although the two synergids generally did not degenerate exactly synchronously, the combination of intact and fully degenerated synergids was relatively uncommon and appeared mostly in fertilized ovules. The independence of degeneration was tested with the chi-squared test. For ovules collected from

Table 2. Classification of *Z. chlorosolen* embryo sacs and their components, merged by time of emasculation, pollination, and collection.

Treatment[a]	-2e	-2pol	-1e	-1pol	-1e	-1pol	0pol			
Hours post	72	72	48	48	72	72	48			
Unfertilized	96	91	94	91	96	22	33			
Fertilized	0	0	0	6	0	70	47			
Abnormal	9	8	15	4	3	7	17			
Sacless	8	13	12	3	9	10	32			
Total	113	112	121	104	108	109	129			
Frac.abnorm.[b]	0.08	0.071	0.124	0.038	0.028	0.064	0.132			
Frac.sacless	0.071	0.116	0.099	0.029	0.083	0.092	0.248			
Frac.(ab+sa)	0.15	0.188	0.223	0.067	0.111	0.156	0.38			
	unf	unf	unf	unf	fert	unf	unf	fert	unf	fert
Egg										
OK	95	89	87	83	5	91	20	59	33	47
Embryo	0	0	0	0	0	0	0	8	0	0
Deging	1	0	2	5	1	4	1	3	0	0
Deg	0	2	5	3	0	1	1	0	0	0
Synergids										
OK-OK	22	16	19	16	0	10	4	0	0	0
OK-+/-OK	4	4	11	8	0	6	2	0	3	0
OK-deging	10	2	2	4	0	7	0	1	1	3
OK-deg	9	13	4	2	0	3	0	9	1	11
+/-OK-+/-OK	3	5	17	11	0	7	0	0	2	1
+/-OK-deging	5	6	6	8	1	5	1	1	4	1
+/-OK-deg	9	7	10	14	2	12	2	9	1	5
Deging-deging	9	10	3	4	0	11	5	2	6	2
Deging-deg	8	4	7	8	1	16	0	14	8	9
Deg-deg	17	24	15	16	2	19	8	34	7	15
Polar nuclei										
OOP	5	8	0	1	0	0	0	0	0	1
Deging	9	10	2	3	0	11	0	1	3	6
Ch appress	63	54	69	69	3	12	2	0	4	7
Ch fusing	14	11	19	13	2	28	6	5	10	5
Ch fused	5	5	4	4	1	45	14	9	15	17
Mult. PEN	0	0	0	0	0	0	0	3	0	5
>=2 endosp	0	0	0	0	0	0	0	50	0	6
1 mc + 1 ch	0	3	0	1	0	0	0	1	1	0
Antipodals										
3: all OK	75	72	59	68	4	47	12	18	9	13
3: all+/-OK	5	1	3	2	0	7	1	7	1	2
3: all deging	2	0	1	2	0	2	3	9	5	4
3: all deg	1	1	0	0	0	3	2	16	4	13
2+/-OK 1deging	3	6	7	7	0	11	3	4	5	4
1+/-OK 2deging	3	1	5	2	0	11	1	9	2	2
5 any	0	1	0	2	0	0	0	0	0	0
4 any	3	4	3	2	1	2	0	1	0	1
2 any	3	3	14	5	0	8	0	3	5	4
1 any	1	0	1	1	1	5	0	3	2	3
none seen	0	2	1	0	0	0	0	0	0	1
Code										
UUUU	16	13	11	9	0	6	3	0	0	0
UDUU	59	56	50	55	5	44	9	13	10	13

Treatment[a]	-2e	-2pol	-1e	-1pol	-1e	-1pol	0pol			
UDUD	9	7	19	11	0	28	7	51	20	27
UDDU	1	7	2	0	0	2	0	0	1	0
UDDD	4	1	0	0	0	7	0	2	1	6
UUUD	2	2	4	5	0	3	1	0	0	0
UUDU	2	1	1	2	0	0	0	0	0	0
UUDD	2	0	0	1	0	1	0	0	1	0
DDUU	1	2	4	8	1	3	0	2	0	0
DUUU	0	0	3	0	0	0	0	0	0	0
DDUD	0	0	0	0	0	2	2	1	0	0

[a]Treatment and "hours post" refer to pollination versus emasculation and the number of hours after either when picked. [b]Left column abbreviations are: Frac.sacless, fraction of all ovules that lacked an embryo sac; Frac.abnorm, fraction of all embryo sacs that were abnormal; Frac.(ab+sa), sum of Frac.sacless and Frac.abnorm.; deg, degenerated; deging, degeneration in progress; OK, intact; OOP, out of position; ch, chalazal; mc, micropylar; mult. PEN, multinucleolate primary endosperm nucleus; appress, appressed; any, any condition. The UD codes follow a system given in the Results section in reference to Table 2.

unpollinated flowers 24 hours after natural opening (the first and third columns of Table 2, "Synergid" rows), there were 76 in which neither synergid had greatly degenerated, 55 in which only one synergid had degenerated, and 59 where both had degenerated. This gave a probability of 0.5447 of not having degenerated versus 0.4553 of having degenerated. If degeneration occurred at random, one would expect 56.373 with neither synergid degenerated, 94.241 with one degenerated, and 39.387 with both degenerated. The resulting chi-squared value was 32.94 with two degrees of freedom, and thus the synergids did not degenerate independently.

The polar nuclei had failed to migrate in six embryo sacs, and they were out of their usual chalazal-end position in 15 more embryo sacs (Table 2, "Polar nuclei" rows). Eighteen of these instances occurred in flowers emasculated two days before opening, even though the flowers were collected at the same age as those emasculated one day before opening. Most polar nuclei were appressed at 24 hours after natural opening. While the polar nuclei were aligned along the long axis of the embryo sac during migration, they assumed other alignments upon reaching the chalazal end, and about a 20:4 ratio of perpendicular to parallel alignments was observed relative to the long axis. Most polar nuclei had begun to fuse or had completely fused, as indicated by complete fusion of their nucleoli, at 48 hours past natural opening. Pollination accelerated nuclear and nucleolar fusion of polar nuclei in both fertilized and unfertilized ovules. The primary endosperm nucleus developed multiple nucleoli before dividing. The large number of multinucleate endosperms after pollination one day early further indicated the earlier time of fertilization in that group.

One or two extra antipodal cells occurred in 18 embryo sacs, but the divisions that produced them were not seen (Table 2, "Antipodal" rows). Fertilization, especially endosperm development, accelerated antipodal degeneration. The unfertilized ovules of pollinated flowers also had more degenerated antipodals than did ovules in unpollinated flowers of the same age. Asynchronous degeneration was more likely to be observed in unpollinated flowers.

The condition of each embryo sac was encoded in Table 2 in four letters, all either U for intact or D for degenerating and degenerated. The letters respectively denoted the egg, synergids, polar nuclei, and antipodals, and any instance of degeneration merited a "D" for synergids and antipodals. Completely intact embryo sacs were most common at 24 hours past natural opening, and pollination two days early did not affect them. Later pollination accelerated synergid and antipodal degeneration. Completely intact embryo sacs were never seen in fertilized ovules, since at least one synergid had degenerated. Antipodal degeneration usually began soon after synergid degeneration had begun. In 20 ovules, the entire egg apparatus was degenerating before the polar nuclei or antipodals began to degenerate. The egg and the polar nuclei were about equally likely to be the last intact component in senescing embryo sacs. However, all possible degeneration sequences were observed multiple times.

Endosperm division clearly preceded egg division (Table 2, ninth column). The egg usually divided after at least eight nuclei existed in the micropylar chamber of the helobial endosperm. In four out of 122 fertilized ovules, the egg degenerated without dividing; this is a typical frequency of mature seeds with plump endosperm but no embryo in *Z. chlorosolen*. The number of sperm nuclei was tabulated in 83 fertilized egg cells. Three of them had experienced division of both sperm

Table 3. Component counts in numerically abnormal embryo sacs in *Z. chlorosolen*[a].

row	N	total	eggs	syn	cent mc	cent ch	antip	inf mc	inf ch
1	7	8	1	2	0	3	2	1	2
2	1	8	0	2	0	3	3	2	1
3	1	8	1	1	0	3	3	2	1
4	1	8	1	1	0	4	2	2	2
5	0	8	1	0	0	4	3	3	1
6	2	7	1	2	0	1	3	0	1
7	1	6	1	0	0	2	3	1	1
8	3	6	1	2	0	2	1	1	1
9	1	6	0	2	0	2	2	1	1
10	6	5	0	0	0	2	3	1	1
11	1	5	1	0	0	1	3	0	1
12	2	5	1	2	0	0	2	0	0
13	1	5	1	0	1	2	1	2	1
14	2	5	1	2	0	1	1	1	0
15	4	4	0	0	0	1	3	0	1
16	2	4	0	0	0	2	2	0	2
17	3	4	1	0	0	2	1	1	1
18	1	4	1	2	0	1	0	1	0
19	2	3	0	0	0	1	2	0	1
20	4	3	0	0	0	3	0	1	2
21	1	3	0	0	0	2	1	1	1
22	1	3	1	0	1	1	0	1	1
23	3	2	0	0	0	1	1	0	1
24	3	2	1	0	0	1	0	1	0
25	1	1	0	0	0	1	0	0	1

[a]syn, synergids; cent mc, free nuclei in micropylar half of the central cell; cent ch, free nuclei in chalazal half of the central cell; antip, antipodal cells; inf mc, inferred to come from the micropylar end of the central cell; inf ch, inferred to come from the chalazal end of the central cell.

and egg nuclei. The remainder contained only one sperm nucleus.

There were 63 embryo sacs that differed from the conventional organization of one egg, two synergids, two polar nuclei, and three antipodals. These are described and enumerated in Table 3, whose columns give the row number in the table, the number of instances observed, the total number of nuclei, the counts of eggs, synergids, free nuclei in the micropylar part of the central cell, free nuclei in the chalazal part of the central cell, and antipodals. Because most of the embryo sacs were examined after the polar nuclei had met and migrated into the chalazal end of the central cell, the last two columns give a best guess as to the numbers of free nuclei at each end of the central cell prior to migration. Most abnormal embryo sacs contained fewer than eight nuclei. The most common abnormalities were misdifferentiation of an antipodal nucleus as a third polar nucleus (row 1), and absence of the egg apparatus (rows 10 and 15). There were 13 embryo sacs with only one antipodal cell, and in four of these (rows 8 and 17) there appeared to be only two chalazal-end nuclei prior to cellularization. The number of functioning polar nuclei ranged from one to four; the latter occurred when both expected synergid nuclei instead became polar nuclei and behaved as in Fig. 2E. Although no instances were observed in *Z. chlorosolen*, four polar nuclei and no synergids (row 5) were seen multiple times in *Hippeastrum xjohnsonii*.

Eight embryo sacs could not be described as combinations of egg, synergids, polar nuclei, and antipodals. In the first, the antipodal nuclei were not walled off from the central cell in an otherwise normal embryo sac. Instead, they remained separate from the two fused polar nuclei. In the second and third, the synergids lacked a filiform apparatus and the polar nuclei were still separate at the ends of the central cell 24 hours after normal anthesis. In the fourth, only the cell walls of the egg apparatus persisted, and all nuclei had degenerated. In the fifth, there was but one antipodal, and it was unusually large. The two polar nuclei flanked this antipodal, and the egg apparatus had degenerated. In the sixth, a transverse partition near the micropylar end divided the sac into two cells, each with four free nuclei seemingly randomly arranged near the partition. In the seventh, the synergid and egg nuclei occupied the same, egglike cell. In the eighth, the egg nucleus had been walled off partially, but then the egg nucleus had migrated to the chalazal end of the central cell as a polar nucleus. The embryo sac was otherwise normal.

DISCUSSION

Development of the embryo sac from the megasporocyte in apomictic *Zephyranthes* is similar to development from the surviving megaspore in *Ornithogalum caudatum* (Tilton and Lersten, 1981). The biggest differences are the larger size and more discoid shape of the embryo sac and its central vacuole in *Zephyranthes*, such that the four-nucleate stage has two nuclei side-by-side at each end, rather than arranged linearly as is usual in *Ornithogalum*. A second difference is the stated movement of only the micropylar polar nucleus through the central column to the chalazal end in *Ornithogalum*, versus migration of both polar nuclei into the column in *Zephyranthes* before migration of both to the chalazal end. Otherwise, Tilton and Lersten (1981) observed the initiation, thickening, and splitting of the column

much as in *Zephyranthes*, although they did not specifically order these stages in time. Tilton and Lersten mentioned other examples of the central column from various taxa, including *Crocus*, *Hordeum*, and in general any species with helobial endosperm. They also cited the notion, from a 1902 paper by Ikeda, that at least one polar nucleus moves through the column. More recently, Zeng et al. (2007) and Hu et al. (2009) also observed in rice the migration of polar nuclei through a transient, de-novo formed cytoplasmic column, where they met at the center and then moved to the micropylar end of the central cell. Tilton and Lersten (1981) suggested that in *Ornithogalum* the second sperm nucleus might reach the fused polar nuclei via the central column, but in *Zephyranthes drummondii* this column has dissipated prior to arrival of the pollen tube at a synergid, and therefore the sperm must take some path through the peripheral cytoplasm of the central cell.

Embryo sac development in emergent buds of *Z. drummondii* and *Z. chlorosolen* is more rapid than in the related *Zephyranthes candida*, at four days in the former and up to seven days in the latter. As in *Z. drummondii*, Ao (2018) reported (on the basis of 10-µm paraffin sections) the formation of a central cytoplasmic column that spanned the central cell and conveyed the micropylar polar nucleus to the chalazal end. However, Ao (2018) reported that the column persisted until fertilization and that sometimes the polar nuclei and sperm nucleus fused within the chalazal part of it, as depicted in his Figure 2D. Ao did not distinguish a stage where both polar nuclei moved into the column and met near its midpoint. Ao (2018, 2019) also noted that the antipodal cells developed callosic walls when the polar nuclei approached them, that the antipodal cells later fused when the callose had disappeared, and that the antipodal nuclei ultimately fused into one. In contrast, in *Z. drummondii* no callose or cellular fusion was observed in the antipodal apparatus before it degenerated. Also, Ao (2019) noted a nuclear endosperm in *Z. candida*, and this in conjunction with the claimed fusion of antipodal cells and claimed increase in antipodal nuclei to nine is consistent with a misinterpretation of a helobial endosperm where the small chalazal chamber has been mistaken for fused antipodal cells.

The inception, thickening, and eventual dissipation of a central column differ markedly from the description of polar-nuclear migration in *Arabidopsis thaliana*. There the polar nuclei are depicted as traveling through a peripheral cytoplasmic shell surrounding the central vacuole, and they migrate while the egg and antipodal apparati form cell walls (Yadegari and Drews, 2004). In rice, the photographs of Zeng et al. (2007) and Hu et al. (2009) were not suitable to detect incipient cell walls, since the nuclei were stained preferentially, but it is possible that the migration began before cellularization began.

The egg apparatus appears to develop complete cell walls in *Zephyranthes*. This is supported by Fig. 1C, which is the egg apparatus from the embryo sac whose pre-migration central cell appears in Fig. 1E. A *Zephyranthes* egg cell tends to maintain its hemispherical shape even when plasmolysis pulls the central cell cytoplasm away from it, which is consistent with a rigid egg-cell wall. Also, Tilton and Lersten (1981) noted the appearance of a complete cell wall around the egg in *Ornithogalum caudatum*, but commented that the chalazal end of the wall might be reticulate at ultrastructural magnification.

Synergid degeneration appears to be autonomous in *Zephyranthes*, but pollination accelerates degeneration in unfertilized as well as fertilized ovules. Degeneration of one synergid is positively correlated with degeneration of the other synergid. Although up to three pollen tubes were observed at the micropyle, there is no evidence in this study that intact synergids attracted pollen tubes. It is possible that penetration necessarily destroyed the receiving synergid. The situation is concordant with the conclusions of Leydon et al. (2015) and cited references therein, that synergids can degenerate autonomously, that approach of a pollen tube promotes synergid degeneration, and that discharge of a pollen tube is observed only in a degenerated synergid.

The embryo-sac abnormalities observed in *Zephyranthes* are not consistent with a random assignment of function at the eight-free-nucleate stage, since combinations such as three egg cells or one synergid and three micropylar-end polar nuclei were not observed. On the other hand, there was insufficient evidence to support a rigidly progressive imprinting of nuclear fate, with consistent defaults for undivided nuclei from each of the three rounds of mitosis. For example, embryo sacs with only one synergid were rare (two out of 63 abnormals), and thus the nuclei that normally become synergid nuclei tended to share the same fate, either becoming synergids or becoming extra, functioning polar nuclei. For this reason, one might conclude that the commitment to becoming synergid nuclei has already happened at the four-nucleate stage of the embryo sac and that it is distinct from the commitment to divide. Yet the combination of no synergids and three polar nuclei was not observed, so the usual fate of an undivided synergid precursor is unclear. For another example, an undivided micropylar end nucleus (one having skipped the second and third mitotic divisions) tended to function

as a polar nucleus, moving to the chalazal end of the central cell upon cellularization. For that matter, even an undivided megasporocyte nucleus could develop the chalazal position and single, large, vacuolated nucleolus typical of migrated polar nuclei (Fig. 2L). In contrast, if the second round of mitosis were skipped, each daughter of the third round could form a polar nucleus and one of something else. Furthermore, failure to wall off one antipodal resulted in an extra free nucleus that was not observed to become appressed to or fuse with the legitimate polar nuclei. Thus very careful, systematic evaluation of a much larger sample of properly timed, abnormal embryo sacs, with attention to the filiform apparatus, nuclear position, and cellular and nucleolar vacuolization, will be needed to establish if the spectrum of abnormalities can support the inference of checkpoints and default fates in differentiating embryo sacs.

The spectrum of abnormalities varies among plant taxa. Yudakova (2009) observed a 10- to 100-fold higher frequency of extra eggs and polar nuclei in embryo sacs of the *Hieracium* type in *Poa chaixii* and *Poa pratensis* than in embryo sacs of the *Taraxacum* (or possibly *Elymus rectisetus*) type in *Poa badensis*. Yudakova (2009) also stated that, "No significant cases of extra polar nucleus formation from the synergid nuclei were recorded among numerous analyzed embryo sacs of the studied bluegrass species, while female gametophytes with additional egg cells instead of synergids occurred in all studied plants." This situation markedly contrasts with the greater frequency of expected synergid nuclei becoming extra polar nuclei in *Zephyranthes* and *Hippeastrum*. In semifertile hybrids of *indica* × *japonica* rice, Zeng et al. (2007) observed a wide range of abnormalities over stages from degenerating megaspores through mature embryo sacs. At maturity, frequent abnormalities included small size, a misplaced (lateral) egg apparatus, absence of the egg apparatus, misplaced (lateral) antipodals, and migration of polar nuclei to the chalazal end (which would be normal in *Zephyranthes*). If the egg apparatus was absent, the micropylar-end polar nucleus was either present or absent. Otherwise, Zeng et al. (2007) did not detail numerical abnormalities in the manner of Table 3.

The failure to observe multiple egg cells in *Zephyranthes* (Table 3) could reflect observational bias, since the filiform apparatus develops over time and is not always clearly visible in whole mounts, and the position of the nucleus varied from lateral to chalazal-end within egg cells. An indirect line of evidence comes from twin embryos. Spontaneous diploid-haploid twins are relatively easily found in *Lilium* (Cooper, 1940) and in grasses, where they are a source of spontaneous haploids apart from *indeterminate gametophyte* mutants. Within *Zephyranthes*, identical (maternal) twins are about 10 times more frequent in *Z. pulchella* J.G. Smith than in *Z. chlorosolen*. An extra egg would be the most facile source of such twins, although postzygotic cleavage is also possible, and experimental distinction would be difficult in an apomictic species. An extra egg could account for frequent maternal haploid production from *indeterminate gametophyte* (*ig*) mutants in maize and rice (Evans, 2007; Zhang et al., 2015), although *ig* maize also produces androgenetic haploids when used as a female parent with other maize genotypes (Kindiger and Hamann, 1993).

Yudakova (2009) followed the four-zone hypothesis of Enaleeva (2002) to account for abnormalities in *Poa* embryo sacs. From the micropylar end, the consecutive zones are synergid, egg, central, and antipodal. Yudakova (2009) conjectured that the zones were established in response to signals from adjacent nucellar cells and that each zone determined the fate of its contained nuclei. Abnormalities would arise when a free nucleus was situated out of its proper zone, and a piece of evidence for this was the prevalence of an extra egg rather than polar nucleus in place of a synergid, since the synergid and central zones are not adjacent. However, in *Zephyranthes* and *Hippeastrum* the relative abundances are reversed, with a tendency of both prospective synergid nuclei to function as extra polar nuclei. Also, in *Zephyranthes*, the egg and synergid nuclei can begin at the same distance from the micropylar extremity of the embryo sac (Fig. 1B). Furthermore, in rice (Zeng et al., 2007) and *Habranthus tubispathus* (Pace, 1913), an otherwise normal egg apparatus sometimes appears on the side of the embryo sac rather than at the micropylar end. A lateral egg apparatus would require that the zones would respond to particular cells or cell groups in the nucellus and that free nuclei would move in response to some attraction from those nucellar cells. Alternatively, a lateral egg apparatus could easily arise from a misoriented spindle of the first mitotic division, if the nuclei move only slightly thereafter.

Nuclear movement seems to occur only at particular times in the differentiation of embryo sacs, first after the division of the megasporocyte (which functions as the surviving megaspore in apomictic *Zephyranthes*), then upon migration of the polar nuclei, then upon fertilization, and finally during the first few rounds of mitosis in the developing endosperm. The nuclei appear to remain nearly stationary apart from these four episodes. The nature of the fertilization block is particularly interesting in *Zephyranthes*, since the sperm nucleus can approach within its own diameter the egg nucleus with-

out fusing with the egg nucleus. Also remarkable is how the other sperm nucleus moves or is moved across more than 100 micrometers of the peripheral cytoplasm of the central cell to reach the fused polar nuclei.

Karyogamy of the sperm and fused polar nuclei appears to be necessary for endosperm development in *Z. drummondii* and *Z. chlorosolen*, as evidenced by an invariably undivided central cell nucleus in unfertilized ovules and the similar types of endosperm failures in interspecific crosses of these species and interspecific crosses of sexual *Z. traubii* (W. Hayw.) Moldenke. However, this necessity contrasts with the situation described by Tandon and Kapoor (1962) in *Zephyranthes* cv. 'Ajax' (*Z. candida* (Lindl.) Herb. x *Z. citrina* Baker), where 4n chromosome counts were reported for 366 out of 430 endosperm metaphases after self-pollination. This suggests that hemigamy also operates in the central cell, assuming that this seed-propagatable cultivar is apomictic like its *Z. citrina* parent (Howard, 1996). Apomictic *Zephyranthes* possibly vary in their requirement for karyogamy for endosperm inception. If so, there is a very unanswered question as to how the usual maternal-paternal imprinting mechanism (endosperm balance number) is circumvented in *Z. citrina* and *Z. pulchella*. The problem merits further investigation with flow cytometry and paternal markers in all of these species.

STUDY LOCATION

The *Z. drummondii* flowers were collected at latitude 30°17'37.5"N, longitude 97°44'11.5"W (30.293736, -97.736538). The *Z. chlorosolen* flowers were collected at latitude 30°20'16.4"N, longitude 97°42'04.4"W (30.337887, -97.701208). Both populations have been destroyed by subsequent building and road construction.

ACKNOWLEDGEMENTS

I performed the experiments in partial fulfillment of the requirements for the Ph.D. degree at the University of Texas at Austin. I thank my now deceased major professor, Walter V. Brown, for stimulation and tolerance of this and related projects during my graduate research. I thank Stefan Kirchanski for suggesting methyl salicylate as a clearing agent. I thank Edwin T. Bingham, David A. Sleper, John G. Carman, David M. Stelly, and Stephen B. Goodwin for support over subsequent decades. Finally, I thank my wife, Yan M. Crane, for reformatting the photomicrographs and for her steadfast support.

DECLARATION

The author declares no conflicts of interest regarding this research.

REFERENCES

Ao, C.Q. 2019. The endosperm development and the variations of structures of embryo sacs: unraveling the low fecundity of *Zephyranthes candida* (Amaryllidaceae). Plant Biosystems. 153: 673-678.

Ao, C.Q. 2018. Double fertilization in *Zephyranthes candida*, with special notes on the second fertilization and the behavior of the primary endosperm nucleus. Phyton. 58: 135-138.

Ao, C.Q., Wang, L.Y., Sun, H., Lin, J.T., Chai, Y., and Chen, C.C. 2016. Megasporogenesis and megagametogenesis in *Zephyranthes candida* (Amaryllidaceae), with special notes on the behavior of the synergids, the central cell and the antipodal cells. Phyton. 56: 91-101.

Bashaw, E. C., and Hanna, W. W. 1990. Apomictic reproduction. In: Chapman GP, ed. *Reproductive versatility in the grasses.* Cambridge, UK: Cambridge University Press, 100–130.

Battaglia, E. 1945. Fenomeni citologici nuovi nella embriogenesi (semigamia) e nella microsporogenesi (doppio nucleo di restituzione) di *Rudbeckia laciniata* L. Nuovo Giorn. Bot. Ital. N. S. 52:34-38.

Brown, W. V. 1951. Apomixis in *Zephyranthes texana* Herb. American Journal of Botany 38: 697-702.

Brown, W. V., and Emery, W. H. P. 1957. Apomixis in the Gramineae, tribe Andropogoneae: *Themeda triandra* and *Bothrochloa ischaemum*. Botanical Gazette 118: 246-253.

Chennaveeriah, M.S., and Patil, R.M. 1971. Apomixis in *Blumea*. Phytomorphology 21: 71-76.

Coe, G. E. 1953. Cytology of reproduction in *Cooperia pedunculata*. American Journal of Botany 40: 335-343.

Cooper, D. C. 1943. Haploid-diploid twin embryos in *Lilium* and *Nicotiana*. American Journal of Botany 30(6): 408-413.

Crane, C. F. 1978. Apomixis and crossing incompatibilities in some Zephyrantheae. Ph.D. dissertation, The University of Texas at Austin.

Crane, C. F., and Carman, J. G. 1987. Mechanisms of apomixis in *Elymus rectisetus* from eastern Australia and New Zealand. American Journal of Botany. 74: 477-496.

Enaleeva, N.K. 2002. On the cytological mechanism of "normal" and "abnormal" cell differentiation in angi-

osperm megagametophytogenesis. Reproduktivnaya biologiya, genetika i selektsiya (Reproductive Biology, Genetics, and Selection). Saratov: Izd-vo un-ta, 2002, p. 47–54.

Evans, M.M.S. 2007. The indeterminate gametophyte1 gene of maize encodes a LOB domain protein required for embryo sac and leaf development. The Plant Cell. 19: 46-62.

Gustafsson, A. 1946. Apomixis in higher plants. I. The mechanism of apomixis. Lunds Universitets Arsskrift 42: 1-67.

Herr, J. M. 1971. A new clearing-squash technique for the study of ovule development in some angiosperms. American Journal of Botany. 58: 785-790.

Howard, T. 1996. Two new *Zephyranthes* species from Mexico. Herbertia. 51: 38-41.

Hu, C. Y., Zeng, Y. X., Lu, Y. G., Li, J. Q., and Liu, X. D. 2009. High embryo sac fertility and diversity of abnormal embryo sacs detected in autotetraploid indica/japonica hybrids in rice by whole-mount eosin B-staining confocal laser scanning microscopy. Plant Breeding 128: 187-192.

Juel, H.O. 1900. Vergleichende Untersuchungen über typische und parthenogenetische Fortpflanzung bei der Gattung *Antennaria*. Kongliga Svenska Vetenskaps-Akademiens Handlingar 33: 1-59.

Kindiger, B., and Hamann, S. 1993. Generation of haploids in maize: a modification of the indeterminate gametophyte (*ig*) system. Crop Science. 33: 342–344.

Kwiatkowska, M., Kadluczka, D., Wedzony, M., Dedicova, B., and Grzebelus, E. 2019.

Refinement of a clearing protocol to study crassinucellate ovules of the sugar beet (*Beta vulgaris* L., Amaranthaceae). Plant Methods 15: 71.

Lanaud, C. 1988. Origin of haploids and semigamy in *Theobroma cacao* L. Euphytica 38: 221-228.

Leydon, A., Tsukamoto, T., Dunatunga, D., Qin, Y., Johnson, M., and Palanivelu, R. 2015. Pollen tube discharge completes the process of synergid degeneration that is initiated by pollen tube-synergid interaction in *Arabidopsis*. Plant Physiology. 169: 485-496.

Nogler, G. 1972. Genetik der Aposporie by *Ranunculus auricomus* s.l. W. Koch. II. Endospermzytologie. Berichte Schweiz. Botanische Gesellschaft. 82:54-63.

Nygren, A. 1967. Apomixis in the angiosperms. In Ruhland, W., ed., Handbuch der Pflanzenphyiologie, vol. 18: 551-596.

Pace, L. 1913. Apogamy in *Atamosco*. Botanical Gazette. 56: 376-394.

Stelly, D.M., Peloquin, S.J., Palmer, R.G, and Crane, C.F. 1984. Mayer's hemalum-methyl salicylate: A stain-clearing technique for observations within whole ovules. Stain Technology 59: 155-161.

Talent, N., and Dickinson, T.A. 2007. Endosperm formation in aposporous *Crataegus* (Rosaceae, Spiraeoideae, tribe Pyreae): parallels to Ranunculaceae and Poaceae. New Phytologist 173: 231-249.

Tandon, S.L, and Kapoor, B.M. 1962. Contributions to the cytology of endosperm in some angiosperms -- I. *Zephyranthes Ajax* Sprenger. Caryologia. 15: 21-41.

Tilton, V. R., and Lersten, N. R. 1981. Ovule development in *Ornithogalum caudatum* (Liliaceae) with a review of selected papers on angiosperm reproduction. III. Nucellus and megagametophyte. New Phytologist 88: 477-504.

Turcotte, E.L, and Feaster, C.V. 1969. Semigametic production of haploids in Pima cotton. Crop Sci. 9: 653-655.

Voight, P.W., and Bashaw, E.C. 1972. Apomixis and sexuality in *Eragrostis curvula*. Crop Science 12: 843-847.

Yadegari, R., and Drews, G.N. 2004. Female gametophyte development. The Plant Cell. 16: S133-S141.

Young, B.A., Sherwood, R.T, and Bashaw, E.C. 1979. Cleared-pistil and thick-sectioning techniques for detecting aposporous apomixis in grasses. Canadian Journal of Botany. 57: 1668-1672.

Yudakova, O.I. 2009. Abnormalities of female gametophyte development in apomictic bluegrass forms. Russian Journal of Developmental Biology 40: 150–156.

Zeng, Y. X., Hu, C. Y., Lu, Y. G., Li, J. Q., and Liu, X. D. 2007. Diversity of abnormal embryo sacs in *indica/japonica* hybrids in rice demonstrated by confocal microscopy of ovaries. Plant Breeding 126: 574-580.

Zhang, J., Tang, W., Huang, Y., Niu, X., Zhao, Y., Han, Y., and Liu, Y. 2015. Down-regulation of a *LBD*-like gene, *OsIG1*, leads to occurrence of unusual double ovules and developmental abnormalities of various floral organs and megagametophyte in rice. Journal of Experimental Botany 66: 99-112.

Zhou, L.Z., and Dresselhaus, T. 2019. Friend or foe: Signaling mechanisms during double fertilization in flowering seed plants. Current Topics in Developmental Biology 131: 453-496.

Supplemental Table 1. Classification of *Z. chlorosolen* embryo sacs and their components, itemized in columns by individual flower. Codes appear as in Table 2, except FIXD, which is time of day when ovules were fixed.

COND	-2e72	-2e72	-2e72	-2p72	-2p72	-2p72	-1e48	-1e48	-1e48	-1p48	-1p48	-1p48	-1e72	-1e72	-1e72	-1p72	-1p72	-1p72	0p48	0p48	0p48								
FIXD	20:00	22:00	1:00	21:00	0:00	2:00	22:00	22:00	23:00	22:00	23:00	-	20:00	23:00	1:00	21:00	23:00	1:00	23:00	-	-								
UNFE	33	33	30	28	30	33	32	31	31	33	31	27	31	33	32	11	8	3	12	7	14								
FERT	0	0	0	0	0	0	0	0	0	0	1	5	0	0	0	21	22	27	7	23	17								
ABNO	5	3	1	5	3	0	5	6	4	0	0	4	1	2	0	1	6	0	9	4	4								
SACL	4	1	3	7	4	2	2	4	6	2	1	0	4	1	4	4	4	2	15	8	9								
TOTA	42	37	34	40	37	35	39	41	41	35	33	36	36	36	36	37	40	32	43	42	44								
FRAB	0.119	0.081	0.029	0.125	0.081	0	0.128	0.146	0.098	0	0	0.111	0.028	0.056	0	0.027	0.15	0	0.209	0.095	0.091								
FRSC	0.095	0.027	0.088	0.175	0.108	0.057	0.051	0.098	0.146	0.057	0.03	0	0.111	0.028	0.111	0.108	0.1	0.062	0.349	0.19	0.205								
FRAS	0.214	0.108	0.118	0.3	0.189	0.057	0.179	0.244	0.244	0.057	0.03	0.111	0.139	0.083	0.111	0.135	0.25	0.062	0.558	0.286	0.295								
	unf	unf	unf	unf	unf	unf	unf	unf	unf	unf	unf	fert	unf	fert	unf	unf	unf	unf	fert	unf	fert	unf	fert	unf	fert	unf	fert	unf	fert
Egg																													
INTA	33	33	29	27	29	33	30	31	26	31	29	1	23	4	29	31	31	9	18	8	21	3	20	12	7	7	23	14	17
EMBR	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	6	0	0	0	0	0	0
DEGI	0	0	1	0	0	0	1	0	1	2	1	0	2	1	1	2	1	1	1	0	1	0	1	0	0	0	0	0	0
DEGE	0	0	0	1	1	0	1	0	4	0	1	0	2	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Synergids																													
ITIT	5	10	7	3	7	6	14	2	3	6	3	0	7	0	0	2	8	0	0	3	0	1	0	0	0	0	0	0	0
ITPI	1	3	0	1	3	0	2	5	4	5	1	0	2	0	0	4	2	1	0	1	0	0	0	2	0	0	0	1	0
ITDN	3	6	1	0	2	0	0	1	1	2	0	0	2	0	2	2	3	0	0	0	1	0	0	1	0	0	1	0	2
ITDG	0	6	3	5	4	4	0	1	3	0	0	0	2	0	1	0	2	0	0	0	7	0	2	0	2	1	7	0	2
PIPI	1	1	1	1	2	2	3	6	8	2	6	0	3	0	3	0	4	0	0	0	0	0	0	0	2	0	0	0	1
PIDN	4	1	0	6	0	0	4	2	0	3	4	0	1	1	0	3	2	1	1	0	0	0	0	3	0	0	0	1	1
PIDG	4	2	3	1	4	2	1	6	3	6	6	1	2	1	6	4	2	0	2	2	2	0	5	0	1	0	2	1	2
DNDN	6	3	0	3	3	4	1	1	1	1	0	0	3	0	3	6	2	5	1	0	0	0	1	0	1	3	1	3	0
DNDG	5	1	2	2	1	1	3	4	0	4	3	0	1	1	9	5	2	0	8	0	3	0	3	3	1	2	6	3	2
DGDG	4	0	13	6	4	14	4	3	8	4	8	0	4	2	7	7	5	4	9	2	9	2	16	1	2	1	6	5	7
Polar nuclei																													
OOPO	2	0	3	4	0	4	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
DEGI	3	5	1	4	3	3	2	0	0	2	1	0	0	0	3	6	2	0	0	0	0	0	1	2	1	1	5	0	0
CAPP	17	23	23	14	25	15	22	27	20	25	26	0	18	3	4	3	5	1	0	1	0	0	0	2	0	1	6	1	1
CFUS	7	4	3	3	2	6	7	4	8	4	3	1	6	1	11	7	10	2	0	2	4	2	1	4	0	2	1	4	4
CFUD	4	1	0	1	0	4	1	0	3	2	0	0	2	1	13	17	15	8	4	5	2	1	3	4	4	2	1	9	12
MPEN	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	1	0	4	0	0
END2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	15	0	15	0	20	0	0	0	6	0	0
MCCH	0	0	0	2	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
Antipodals																													

3INT	24	29	22	22	22	28	17	22	20	20	26	1	22	3	16	12	19	4	2	7	11	1	5	5	1	0	4	4	8
3PIP	1	3	1	1	0	0	1	0	2	1	0	0	1	0	0	3	4	1	3	0	1	0	3	0	0	0	1	1	1
3DGN	1	0	1	0	0	0	1	0	0	1	0	0	1	0	1	1	0	2	5	1	3	0	1	4	0	0	3	1	1
3DEG	1	0	0	1	0	0	0	0	0	0	0	0	0	0	2	1	0	1	7	0	3	1	6	1	2	2	10	1	1
2PDN	1	0	2	2	1	3	2	3	2	3	3	0	1	0	6	2	3	2	1	0	0	1	3	1	1	2	1	2	2
1PDN	2	1	0	1	0	0	4	0	1	2	0	0	0	0	1	7	3	1	3	0	2	0	4	1	1	0	0	1	1
5ANY	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4ANY	2	0	1	0	2	2	0	1	2	1	1	0	0	1	2	0	0	0	0	0	1	0	0	0	0	0	0	0	1
2ANY	0	0	3	1	2	0	5	5	4	3	1	0	1	0	3	3	2	0	0	0	1	0	2	0	0	2	3	3	1
1ANY	1	0	0	0	0	0	1	0	0	1	0	0	0	1	0	4	1	0	0	0	0	0	3	0	1	1	1	1	1
UNSE	0	0	0	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Status																													
UUUU	3	8	5	2	6	5	7	2	2	1	2	0	6	0	0	1	5	0	0	3	0	0	0	0	0	0	0	0	0
UDUU	22	18	19	15	18	23	9	23	18	18	23	1	14	4	17	11	16	4	1	4	9	1	3	4	1	2	4	4	8
UDUD	5	2	2	5	1	1	7	6	6	7	3	0	1	0	9	14	5	5	19	1	11	1	21	6	4	4	14	10	9
UDDU	0	1	0	4	1	2	2	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0
UDDD	1	2	1	0	0	1	0	0	0	0	0	0	0	0	3	3	1	0	0	0	1	0	1	0	1	1	5	0	0
UUUD	0	0	2	1	0	1	4	0	0	3	0	0	2	0	0	0	3	0	0	0	0	1	0	0	0	0	0	0	0
UUDU	0	2	0	0	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
UUDD	2	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0
DDUU	0	0	1	1	1	0	0	0	4	2	2	0	4	1	1	1	1	0	1	0	1	0	0	0	0	0	0	0	0
DUUU	0	0	0	0	0	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DDUD	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	2	0	0	0	0	1	0	0	0	0	0	0