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Pollen has higher water content when dispersed in a tricellular state than in a bicellular state

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ABSTRACT

Pollen is generally dispersed in a sexually immature and somewhat dehydrated, metabolically quiescent state. Yet, in some species, pollen at anthesis is well-hydrated and metabolically active, and in 30 % of angiosperms pollen is dispersed after having formed its sperm cells. Pollen water content and sexual maturity may be correlated, either because both are subject to trade-offs between dispersal viability and post-pollination performance, or because the traits display developmental linkages. We inferred relative water content of sexually immature ("bicellular") and sexually mature ("tricellular") pollen of 30 species of angiosperms using a hydration index (*HI*) that ranges from zero to one, based on how near fresh pollen volume is to its minimal (dehydrated) or maximal (hydrated) volume. Tricellular pollen had 30 % higher *HI* than bicellular pollen ($P < 0.005$), after controlling for initial pollen size (larger pollen had higher *HI*; $P < 0.05$). A literature survey of 344 species indicated that the tricellular and hydrated states were strongly associated, although all four trait state combinations were present ($P < 0.0001$). Our results suggest that a common mechanism for the repeated origins of tricellular pollen has been via the loss of controlled pollen dehydration, which enables either accelerated or extended pollen development.

Keywords: desiccation stress, evolution of development, pollen dispersal, pollen germination, pollen hydration, spore dormancy, trade-off

Introduction

> "...trinucleate pollen, in which the generative nucleus has divided already, can be looked upon as a less dormant dispersal organ than the slower developing binucleate grain."
>
> Hoekstra & Bruinsma 1975

Evolution is constrained by trade-offs, which occur when the fitness effects of two traits (or sets of traits) are negatively correlated (Stearns 1992). One commonly reported trade-off is between viability and fecundity. Pollen can be subject to such a trade-off, when traits that affect male gametophyte viability during dispersal cause opposing effects on the probability of fertilization after pollination. Two such characters of dispersing pollen are its water content and its degree of sexual maturity, as measured by the presence or absence of sperm cells.

Before pollen is dispersed, it generally undergoes a controlled process of dehydration within the anther (Firon *et al*. 2012). Pollen in a partially dehydrated state has reduced metabolic activity and is longer-lived and better able to tolerate further desiccation during dispersal (Hoekstra & Bruinsma 1978; Dafni & Firmage 2000; Hoekstra *et*

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al. 2001; Firon *et al*. 2012). Consequently, pollen must undergo controlled re-hydration on the stigma after pollination (Heslop-Harrison 1979; Vieira & Feijó 2016), a process that can slow germination speed and hence reduce competitive ability (Nepi *et al*. 2001; Williams 2012). In a minority of species, pollen fails to undergo dehydration and is dispersed in a well-hydrated and metabolically-active condition, rendering it vulnerable to rapid desiccation and cell death (Heslop-Harrison 1979; Aylor *et al*. 2005; Franchi *et al. 2002)*. Such pollen has reduced longevity, but it can germinate rapidly and thus has high competitive ability.

Pollen sexual maturity at dispersal is thought to be subject to the same viability-fecundity trade-off, but that may or may not be because it is developmentally linked with pollen water content. In most angiosperms and all gymnosperms, pollen dispersing from an anther is developmentally immature – its two or more sperm cells have not yet been formed. In angiosperms, such pollen is described as "bicellular," consisting of a vegetative cell and a sperm precursor cell, the generative cell. In bicellular pollen, the generative cell becomes detached from the pollen cell wall and is completely surrounded by the cytoplasm of the vegetative cell before pollen dispersal. In about 30 % of angiosperms, pollen is dispersed in a much more advanced developmental state, in which the generative cell has divided to form two sperm cells. Such pollen is described as "tricellular" (Brewbaker 1967; Williams *et al*. 2014). Tricellular pollen is generally short-lived relative to bicellular pollen (Dafni & Firmage 2000). Hoekstra & Bruinsma (1975) found that respiration rates of tricellular pollen in 12 species were about three times higher than in six species with bicellular pollen. Since tricellular pollen in some species had high water content, it was hypothesized that tricellular "...pollen, in which the generative nucleus has divided already, can be looked upon as a less dormant dispersal organ than the slower developing bi[cellular] grain" (Hoekstra & Bruinsma 1975).

It is not clear how pollen cell number (a reflection of pollen developmental advancement) and pollen water content are mechanistically related. Pollen dehydration imposes developmental arrest on maturing pollen, and shifts in the timing of that developmental arrest might prevent or enable pollen cell cycle progression to the tricellular condition before dispersal (Williams *et al*. 2014). Hence, even if there are cell cycle blocks other than controlled pollen dehydration that regulate the timing of sperm cell formation, the evolution of tricellular pollen would be favored in species that disperse pollen in a hydrated state (Williams *et al*. 2014).

The issue of potential correlated evolution of pollen cell number and hydration status seemed to be settled by several broad surveys on pollen hydration status (Nepi *et al*. 2001; Franchi *et al*. 2002). These studies rejected the hypothesis that species with tricellular pollen have higher water content than those with bicellular pollen, because both types of pollen exhibited a full range of hydration states (Nepi *et al*. 2001; Franchi *et al*. 2002).

An innovation of the Nepi *et al*. approach was the development of a quantitative measure of pollen hydration status, in which fresh pollen size was compared to its fully hydrated size: the larger the difference between fresh pollen volume and hydrated volume, the more dehydrated the fresh pollen must have been when it was collected (Nepi *et al*. 2001). The Nepi *et al*. method works well to identify pollen that is either highly hydrated or highly dehydrated, but as the authors acknowledged, intermediate values were difficult to interpret. The authors cautioned that researchers should not take their hydration index as a strict quantitative measure of water content, but instead to use it in combination with other methods to categorize pollen as either "partially hydrated" or "partially dehydrated." A weakness of the Nepi *et al*. method is that initial (fresh) pollen size and dehydrated size are not taken into account. For example, a small pollen grain with a fresh size 50 % of its hydrated size, is likely to be near its minimal possible dried size, and hence to have a very low water content. In contrast, a large pollen grain with a fresh size 50 % of its hydrated size might still be much larger than its minimal possible dehydrated size, indicating substantial water content.

In this study, we explore the hypothesis that pollen water content and pollen cell number are positively correlated. First, we tested the prediction that tricellular pollen has higher water content, and hence higher metabolic activity, than bicellular pollen (Hoekstra & Bruinsma 1975). To accomplish this, we modified the Nepi *et al*. method to provide a more precise quantitative assessment of hydration status, by scaling fresh pollen size to both its dried and its fully hydrated sizes, instead of just to its fully hydrated size. In testing the prediction, we controlled for initial (fresh) pollen size, which can affect the hydration index because larger pollen has a smaller surface to volume ratio and is less subject to evaporation. Next, we reasoned that if tricellular pollen is generally more hydrated than bicellular pollen, then reports that have characterized pollen as hydrated would be more common from species with tricellular pollen than from those with bicellular pollen. To test this prediction, we expanded our dataset to include a large literature of categorical descriptions of pollen hydration state (dehydrated versus hydrated) and asked if hydration status was independent of pollen cell number.

Materials and methods

A total of 30 more or less unrelated species were chosen haphazardly based on their likely pollen cell number (to achieve equal numbers of each type), similarity in habit (most were ruderal) and summer-flowering period (Tab. 1). Thirteen species had tricellular and 17 bicellular pollen (based on Brewbaker 1967; Williams *et al*. 2014). Accepted species names were taken from the Plant List (2013)

and photo vouchers are included in Figures S1-S4 in supplementary material. For each species, fresh pollen was collected from one male-phase flower from each of five plants at the time of anther dehiscence (with some exceptions, Tab. 1). Pollen from each flower was immediately placed in either: 1) immersion oil, for measuring fresh volume; 2) water, for measuring fully hydrated volume; or 3) a dry vial, for measuring fully dehydrated volume. For fully dehydrated volume, pollen was incubated for 48 or more hours at 80 °C and then viewed in immersion oil. For fully hydrated volume, pollen was allowed to expand in water for one or more hours and viewed in water. Pollen was photographed on its median focal plane (largest area) using the appropriate objective for each species (10x-40x), and volumes of 25 pollen grains for each treatment were estimated from measurements of long and short axes of pollen. We did not differentiate equatorial and polar axes of pollen, but determined the consistent pollen shape for each species/treatment by viewing multiple focal planes. We used formulas for spheroids based on pollen shape (Tab. 1; Sun & Liu 2003).

For *Ipomopsis purpurea*, dry pollen had a peroblate spheroid shape, hence we measured the minor axis perpendicular to the field of view on a subset of pollen grains by subtracting the height of the focal plane of the bottom of the pollen grain from that of the top of the grain. That minor axis length, S, was then divided by the diameter of the same grain measured in median view, to calculate a correction factor that was applied to dry pollen grains such that, S = 0.4532 x average diameter in median view.

Images were photographed on a Zeiss Axioplan II light microscope using a Zeiss Axiocam HRc digital camera, and measurements were done using Zeiss Axiovision 4.8 software. The programs Zerene Stacker (Zerene Systems, Richland, WA) and Photoshop 7.0 (Adobe, San Jose, CA, USA) were used to produce photomicrographs of pollen from images at multiple focal planes. Temperature and humidity were recorded at the time of collection.

Our modified hydration index can be calculated for each flower by comparing the mean volume (V) of fresh pollen to its mean minimal fully dried and mean maximal fully hydrated sizes (Fig. 1). The hydration index:

$$HI = \frac{(mean\ Fresh\ V - mean\ Dried\ V)}{(mean\ Hydrated\ V - mean\ Dried\ V)}$$

can range from 0, when fresh pollen is fully dehydrated, to 1, when fresh pollen is fully hydrated. For each species, the least squared (LS) means of each treatment (dry, fresh, hydrated) were calculated from an ANOVA in which plant and plant x treatment were treated as random effects (one flower per plant). LS means were then used in the *HI* formula for each species, and *HI* was set to zero or one if measurement error resulted in $HI < 0$ or > 1, respectively. Outliers were identified as being > 3.5 standard deviations away from the treatment mean (Sokal & Rohlf 1981) and were removed prior to calculating means ($N = 24$ of 9613 datapoints).

Analysis of Covariance (ANCOVA) was used to test for a difference in mean *HI* of species with bicellular versus tricellular pollen. The mean log(10) volume of fresh pollen (calculated as above) was used as the covariate and the response variable was *HI*. All statistical analyses were run in JMP 12.1 (SAS Institute 2017).

We also reanalyzed the Nepi *et al.* (2001) data, using the volumes of fresh pollen and hydrated pollen they provided along with pollen cell numbers from Williams *et al.* (2014). We used fresh volume divided by hydrated volume as the response variable and log(10) fresh pollen volume as a covariate, as above.

Finally, we surveyed the literature to test for an association between pollen cell number (bi- vs. tricellular)

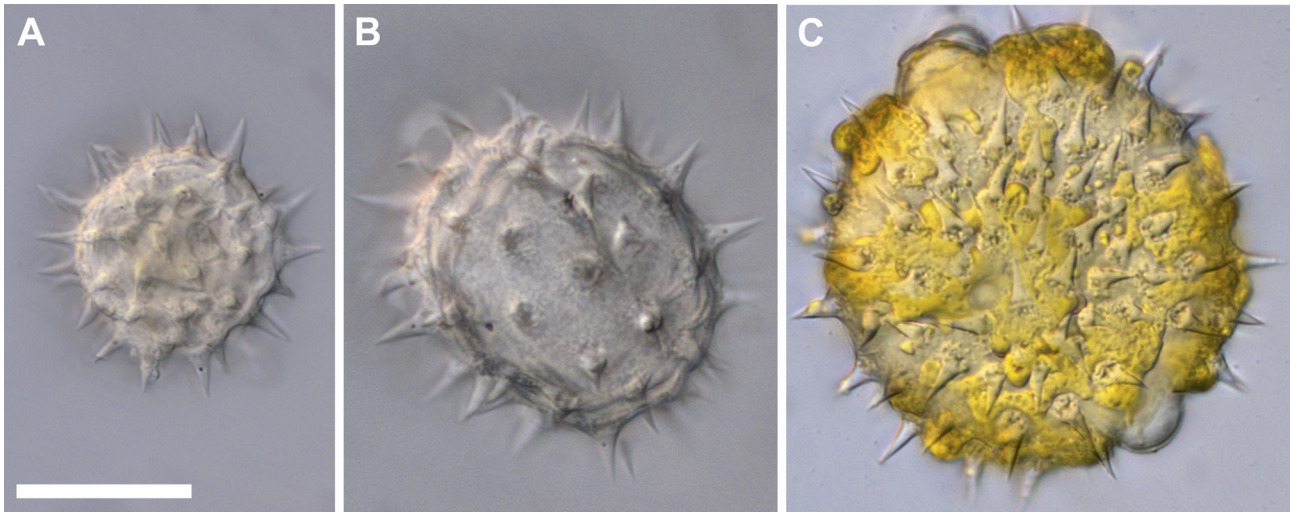

Figure 1. Range of potential sizes of pollen of *Helianthus annuus*. **A.** Minimal, fully oven-dried size; **B.** Fresh size at anthesis; **C**. Maximal, fully-hydrated size. The mean hydration index of *H. annuus* was *HI* = 0.25, reflecting the fact that its fresh pollen was nearer to its fully dried than to its fully hydrated size. Scale bar = 20 µm in all.

Table 1. Collection information for study taxa. Unless otherwise noted, all plants were sampled in 2016 from wild plants near Knoxville, TN or from cultivated material on the University of Tennessee, Knoxville campus (UTK). "Temp. (°C)" lists average temperature and collection time over all samples. Photo vouchers of all material are available in Figures S1-S4 in supplementary material.

Taxon	Family	Date	*N* (plants)	Temp. (°C)	Location description	Coordinates (Lat, Long)	Pollen volume formulas[A]			Pollen cell #
							Dry	Fresh	Hydrated	
Abelmoschus esculentus L.	Malvaceae	28-Jul	5	30.2 °C 10:06 am	UTK Botanical Garden	(35.944008, -83.938261)	Sph	Sph	Sph	2
Campsis radicans L. Seem.	Bignoniaceae	14-Jul	6	37 °C 1:20 pm	Papermill drive NW, Knoxville	(35.934391, -84.015430)	Sph	Sph	Sph	2
Cichorium intybus L.	Asteraceae	13, 25-Jul	6	28.8 °C 9:20 am	Lot nr Lomas dr & Middlebrook pike, Knoxville	(35.94579, -84.02509)	Sph	Sph	Sph	3
Convolvulus arvensis L.	Convolvulaceae	11-Jul	6	31 °C 10:30 am	Lot nr Lomas dr & Middlebrook pike, Knoxville	(35.94579, -84.02509)	Sph	Sph	Sph	2
Datura stramonium L.	Solanaceae	29-Jul	4	31 °C 6:10 pm	UTK Railroad track South of Soccer Stadium	(35.945362, -83.935633)	Sph	Sph	Sph	2
Dianthus barbatus L.	Caryophyllaceae	11-Aug	5	30.4 °C 12:18 pm	UTK Botanical Garden	(35.944008, -83.938261)	Sph	Sph	Sph	3
Elodea canadensis Michx.	Hydrocharitaceae	25-May	5	31 °C 3:24 pm	UTK water tank SW side Hesler building	(35.956579, -83.926599)	Sph	Sph	Sph	3
Escholzia californica Cham.	Papaveraceae	30-May 2017	6	28 °C 12:52 pm	UTK Botanical Garden	(35.944008, -83.938261)	Pr-Sph	Sph	Sph	2
Foeniculum vulgare Mill.	Apiaceae	7-Jun	5	30 °C 3:30 pm	UTK SW side Hesler building	(35.956579, -83.926599)	Pr-Sph	Pr-Sph	Pr-Sph	3
Geranium cv Rozanne	Geraniaceae	5-Jul	5	26 °C 9:30 am	Linville, NC	(36.071117, -81.870448)	Sph	Sph	Sph	3
Helianthus annuus L.	Asteraceae	16-Aug	6	33 °C 12:22 pm	Forks of the River Wildlife Area	(35.953735, -83.858724)	Sph	Sph	Sph	3
Helianthus tuberosus L.	Asteraceae	20-Jul	5	31.2 °C 10:15 am	UTK Botanical Garden	(35.944008, -83.938261)	Sph	Sph	Sph	3
Hemerocallis fulva (L.) L.	Xanthorrhoeaceae	20-Jun	5	23.9 °C 10:00 am	Roadside I-40 & Lovell road, SE corner	(35.904709, -83.143395)	Pr-Sph	Pr-Sph	Pr-Sph	2
Hibiscus mosheutos L.	Malvaceae	10-Aug	5	30.6 °C 10:45 am	Experimental farm, UTK	(35.89756, -83.95693)	Sph	Sph	Sph	2
Hibiscus syriacus L.	Malvaceae	8-Jul	5	29.4 °C 10:30 am	Woodland and Kennesaw st., Knoxville	(35.938059, -83.964367)	Sph	Sph	Sph	2
Impatiens cv Divine orange bronze leaf	Balsaminaceae	21-Jul	4	30.5 °C 9:36 am	UTK Botanical Garden	(35.944008, -83.938261)	Sph	Sph	Sph	2
Ipomoea purpurea (L.) Roth	Convolvulaceae	26-Jul	6	29.2 °C 9:31 am	Lot nr Lomas dr & Middlebrook pike, Knoxville	(35.94579, -84.02509)	Ob-Sph	Sph	Sph	2
Iris ensanata Thunb. cv Caprician butterfly	Iridaceae	5-Jul	5	24 °C 9:00 am	Linville, NC	(36.071117, -81.870448)	Pr-Sph	Pr-Sph	Pr-Sph	2
Juncus sp.	Juncaceae	23-Jul	5	31.6 °C 11:14 am	UTK Botanical Garden	(35.944008, -83.938261)	Sph	Sph	Sph	3
Lagerstroemia indica L.	Lythraceae	13-Aug	5	32 °C 11:26 am	South Knoxville	(35.951013, -83.914475)	Pr-Sph	Pr-Sph	Sph	2
Lilium sp.	Liliaceae	19-Jul	3	30 °C 9:50 am	UTK Botanical Garden	(35.944008, -83.938261)	Pr-Sph	Pr-Sph	Pr-Sph	2
Oenothera biennis L.	Onagraceae	25-Aug	4	25 °C 9:12 am	S Knoxville Vestal area	(35.911607, -83.936834)	Sph	Sph	Sph	2
Oenothera speciosa Nutt.	Onagraceae	26-May	5		UTK S-facing hillside Hesler building	(35.956563, -83.926710)	Sph	Sph	Sph	2
Opuntia sp.	Cactaceae	31-May	2	30 °C 12:50 pm	UTK SW side Hesler building	(35.956579, -83.926599)	Sph	Sph	Sph	3
Origanum vulgare L.	Lamiaceae	7-Jun	5	30 °C 3:51 pm	UTK SW side Hesler building	(35.956579, -83.926599)	Pr-Sph	Sph	Sph	3
Passiflora incarnata L.	Passifloraceae	19-Aug	6	27.5 °C 9:00 am; 1:55 pm	UTK along railway S of soccer stadium	(35.945362, -83.935633)	Sph	Sph	Sph	2
Passiflora lutea L.	Passifloraceae	26-Jul	3	32 °C 11:00 am	Lot nr Lomas dr & Middlebrook pike, Knoxville	(35.94579, -84.02509)	Pr-Sph	Pr-Sph	Sph	2
Rudbeckia fulgida Aiton	Asteraceae	19-Jul	5	30.8 °C 10:42 am	UTK Botanical Garden	(35.944008, -83.938261)	Sph	Sph	Sph	3
Utricularia gibba L.	Lentibulariaceae	7-Jun	5	30 °C 4:40 pm	UTK water tank on SW side Hesler building	(35.956579, -83.926599)	Pr-Sph	Sph	Sph	3
Zea mays L. (subsp. *mays*)	Poaceae	20-Jul	6	29.5 °C 10:32 am	UTK Botanical Garden	(35.944008, -83.938261)	Sph	Sph	Sph	3

[A]Formulas for pollen volume were based on shape (Sun & Liu 2003): 1) "Sph" (globose/spheroidal): $V = \pi^*[(L + S)/2]^3/6$; 2) "Pr-Sph" (prolate spheroid): $\pi^*L^*S^2/6$; and 3) ""Ob-Sph" (oblate spheroid): $V = \pi^*L^{2*}S/6$; where L and S are longest and shortest observed diameters of the pollen grain. For prolate spheroids, the minor axis perpendicular to the field of view is assumed to be equal to that of S, whereas in the oblate spheroid of *Ipomoea purpurea*, the length of the perpendicular minor axis was estimated empirically (see text).

and pollen hydration status (hydrated vs. dehydrated). Because direct measurement of pollen water content by weight is difficult to do confidently outside the lab, most studies have inferred relative water content using indirect methods that involve observation of pollen volume changes, fresh pollen morphology, or sensitivity to desiccation, as measured by viability over time (Payne 1981; Nepi *et al.* 2001; Hoekstra 2002; this study).

Based on the method of observation, studies that infer pollen to be more or less dehydrated at dispersal use the terms, "dehydrated, partially-dehydrated, orthodox, or desiccation-tolerant," whereas pollen inferred to be relatively hydrated at dispersal is described as, "hydrated, partially-hydrated, recalcitrant, or desiccation-sensitive." We took such descriptions of pollen hydration status at face value, or categorized them ourselves using cut-off points suggested by Nepi *et al.* (2001) for pollen volume changes or by Hoekstra *et al.* (2001) and Hoekstra (2002) for water content by weight. For the 30 species here, we categorized the pollen of 23 species with *HI* < 25 % as partially dehydrated. The other seven species had *HI* > 35 % and were clearly strongly hydrated in the fresh state. We took pollen cell numbers from Williams *et al.* (2014) and scored all gymnosperms as "bicellular," taking bicellular to mean, "sperm cells not formed before pollen dispersal."

Results

Hydration indices ranged from zero to 1 in tricellular pollen and zero to 0.58 in bicellular pollen. In the ANCOVA, the pollen cell number x pollen size interaction effect was non-significant ($P = 0.37$) and was removed from the model. Under the same-slopes model (Fig. 2, Tab. 2), there was a significant difference in *HI* between species with tricellular and bicellular pollen ($P = 0.003$), after accounting for the effect of initial (eg. fresh) pollen size. *HI* increased with fresh pollen size [$P = 0.042$; $R^2 = 0.30$; common slope = 0.133 ± 0.063 *SE* * log10(initial size)]. The mean hydration index of species with tricellular pollen was 30 % higher than in species with bicellular pollen (LS mean ± *SE* was: HI_{Tri} = 0.36 ± 0.07 versus HI_{Bi} = 0.06 ± 0.06; Fig. 2). Temperature at collection time was not significantly different between species with bicellular (mean ± *SE* = 29.46 ± 0.67 °C) and tricellular pollen (mean ± *SE* = 30.18 ± 0.75 °C)($F_{1,27}$ = 0.51, $P = 0.48$).

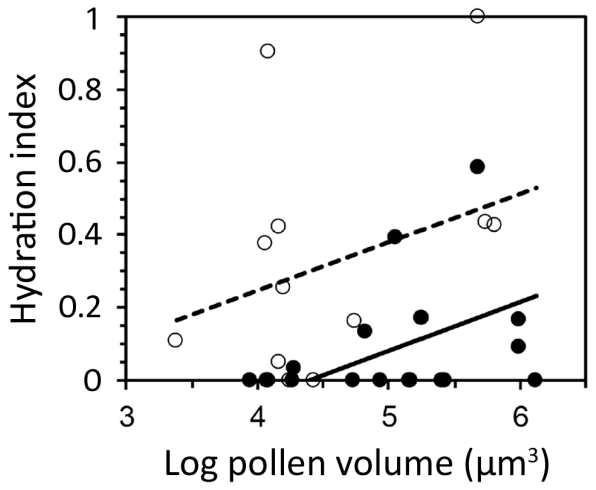

Figure 2. Relationship between pollen cell number and hydration index. Regression lines from ANCOVA. The covariate was pollen volume on a log base 10 scale (x-axis). Dashed line and unfilled circles, tricellular pollen; solid line and filled circles, bicellular pollen.

Table 2. ANCOVA for the effect of pollen cell number on hydration index. Same slopes model $R^2 = 0.30$ ($N = 30$).

Source	DF	Sum of Squares	*F* ratio	P
Pollen cell number	1	0.56726075	10.3416	0.0034
Initial size (fresh)	1	0.24952868	4.5491	0.0422

In the reanalysis of the Nepi *et al.* (2001) data, the interaction between pollen cell number and pollen size was non-significant ($P = 0.51$) and was dropped from the model. In the same slopes model, after controlling for a significant positive effect of pollen size on *HI* ($P < 0.001$), *HI* was 11 % higher in species with tricellular than in those with bicellular pollen, a marginally non-significant difference ($P = 0.076$).

In the survey of 344 seed plants from 24 sources on pollen hydration (Tab. 3), independence of pollen cell number and hydration status was rejected ($\chi^2 = 42.41$; $DF = 1$; $P < 0.0001$). Though the majority of species had bicellular, dehydrated pollen, such pollen was only slightly over-represented in the survey (Tab. 3). The largest deviations occurred in species with tricellular pollen, in which hydrated pollen was

Table 3. Pollen hydration status versus pollen cell number in 344 angiosperms. *Dehydrated* indicates mature pollen described in the literature as, "dehydrated, desiccation-resistant, orthodox, or partially-dehydrated," whereas *Hydrated* indicates pollen described as, "desiccation-sensitive, hydrated, partially-hydrated, or recalcitrant." Shown in parentheses are the percentage deviations from expected under a hypothesis of independence. Nine species with conflicting reports on pollen hydration status and four with conflicts on pollen cell number were not included in the analysis.

Hydration status[B]	Pollen cell number[A]		Totals
	Bicellular	Tricellular	
Dehydrated	179 (+17.3 %)	32 (-44.8 %)	211
Hydrated	69 (-27.5 %)	64 (+71.1 %)	133
Totals	248	96	344

Notes: A Pollen cell numbers are from Newcomer 1938; McConchie *et al.* 1982; Heslop-Harrison *et al.* 1984; Pacini *et al.* 2014; Williams *et al.* 2014 and Williams, unpublished. B Pollen hydration status was taken from: Bassani *et al.* 1994; this study; Chamberlain 1935; Chichiricco *et al.* 2009; Dafni & Firmage 2000; Dawkins & Owens 1993; Franchi *et al.* 2002; Franchi *et al.* 2011; Fernando *et al.* 2005; Friedman 1987; Gabarayeva & Grigorieva 2012; Halbritter & Weber 2017; Hoekstra *et al.* 1992; Hoekstra 2002; Jett *et al.* 1993; Blackmore *et al.* 1987; Nepi *et al.* 2001; Owens *et al.* 1994; Pacini 1996; Pacini & Hesse 2004; Payne 1981; Platt-Aloia *et al.* 1986; Williams 2012; JH Williams, AR Moffatt & JA Edwards unpubl. res.; and this study.

71 % more frequent than expected and dehydrated pollen 45 % less frequent (Tab. 3). Of the two underrepresented combinations, nine percent of species had tricellular, dehydrated pollen (eg. *Arabidopsis* and other Brassicoids) and 20 % had bicellular, hydrated pollen (eg. *Oenothera speciosa*, some gymnosperms).

Discussion

In this study, tricellular pollen had a 30 % higher hydration index, and hence a higher inferred water content, than bicellular pollen over a range of pollen sizes. Hoekstra & Bruinsma (1975) hypothesized that tricellular pollen must in general maintain a higher water content than bicellular pollen because of their finding of higher metabolic activity in tricellular than in bicellular pollen. However, Nepi *et al. (*2001) and Franchi *et al*. (2002) suggested that pollen cell number (bicellular vs. tricellular) and hydration status (hydrated vs. dehydrated) were developmentally independent, based on the observation that all four combinations of trait states can easily be found in nature and that both bicellular and tricellular pollen exhibited the full range of hydration indices.

Nepi *et al*. (2001) cautioned that their hydration index calculation was best used in concert with other methods to categorize pollen at anthesis as more or less hydrated or more or less dehydrated, rather than as a quantitative measure of relative water content. But that may be overly cautious. A statistical analysis of their data indicated that pollen size effects might have obscured the significance of their data. In our study we were able to address the issue of pollen size effects in two ways. First, we developed a hydration index that is biologically more realistic. In the Nepi *et al*. method, fresh pollen with an *HI* of 50 % does not necessarily refer to an intermediate water content. But when dried pollen size is subtracted from fresh and hydrated pollen size before calculating HI, fresh pollen size is more accurately placed on a scale from its fully dehydrated to its fully hydrated state.

Importantly, the more accurate quantitative assessment of the *HI* of fresh pollen allows one to detect real functional consequences of pollen size on desiccation rate. Larger pollen is less subject to desiccation because its surface to volume ratio becomes smaller with increasing volume. As such, one might expect larger pollen to be able to maintain a higher water content for a given exposure to the elements. Our metric allows this biological effect of pollen size to be detected, and in fact, both our result and the reanalysis of the Nepi *et al*. (2001) data showed a strong positive correlation between *HI* and fresh pollen size. In sum, we removed pollen size artifacts from our *HI* metric to better estimate relative water content and we used pollen size as a covariate to remove functional effects of pollen size that might obscure differences in *HI* between bicellular and tricellular pollen. Our results support the prediction of Hoekstra & Bruinsma (1975) that tricellular pollen has quantitatively higher water content than bicellular pollen.

We did not measure pollen water content by weight, although Nepi *et al*. (2001) provided such data on a number of species. An instructive case was for *Helianthus annuus* and *H. tuberosus*, which have fresh pollen water contents of 16.7 % and 43.7 % by weight, respectively (Nepi *et al*. 2001). The former and latter values reflect strongly-dehydrated versus strongly-hydrated conditions (Hoekstra *et al*. 2001). The extremely divergent hydration states of these same two species is well described by our metric (*HI* equal to 25 % versus 91 %, respectively) compared to the Nepi *et al*. *HI* values of 47 % versus 98 %, respectively.

Implications for the evolution of pollen dispersal states

Our experimental results, the reanalysis of the Nepi *et al*. (2001) data, and our literature survey of 344 seed plant species together indicate that bicellular pollen tends to be dehydrated, tricellular pollen tends to be hydrated, and on a quantitative basis, tricellular pollen is more hydrated than bicellular pollen of similar size. The association between pollen cell number and hydration state could be due to either parallel viability-fecundity trade-offs, or to a developmental bias in which the evolution of one trait is facilitated by the evolution of the other, irrespective of the selective environment (Maynard-Smith *et al*. 1985).

The tricellular state of pollen has arisen independently from bicellular ancestors many times within angiosperms, with only rare instances of transitions from the tricellular to the bicellular state (Williams *et al*. 2014). The evolution of pollen water content has not been studied at a similar scale, but it was long held that all pollen was dispersed in a partially dehydrated state (Franchi *et al*. 2002). Hydrated pollen has now been found in several gymnosperms (Franchi *et al*. 2002; Fernando *et al*. 2005; Franchi *et al*. 2011), several early-divergent angiosperms (JH Williams, AR Moffatt & JA Edwards unpubl. res.), and in many eudicots and monocots (references in Tab. 3).

High pollen water content and the tricellular condition are thought to have similar effects on pollen performance speed after pollination. Hydrated, tricellular pollen should reach female flowers in a metabolically-active and sexually-mature state, effectively skipping most of the re-hydration process, the need to re-activate quiescent metabolic machinery, and the process of forming sperm cells after pollination. Such pollen would have high competitive ability and could facilitate rapid fertilization in species with time-limited reproduction.

Bicellular, dehydrated pollen was also over-represented in our survey, as well as being the most common trait set (Tab. 3), and such pollen requires a longer reproductive process, due to the extra time needed to re-hydrate, re-activate metabolic processes and then to develop sperm cells before fertilization. Bicellular, dehydrated pollen is less

differentiated, less metabolically active and has a greater longevity than tricellular, hydrated pollen. Longevity is advantageous when the pollination process is uncertain, as it seems to be in so many species (Knight *et al*. 2005; Burd 2016).

The two over-represented combinations of trait states in Table 3 may represent the evolving poles of a classic macroevolutionary trade-off (Stearns 1992). In the putatively ancestral bicellular-dehydrated combination, increased fitness in the dispersal stage occurs at the expense of reduced performance after pollination; whereas in the putatively derived tricellular-hydrated combination, decreased viability in the dispersal stage is associated with an increase in early pollen performance after pollination. Life history stage trade-offs depend on there being a benefit at one stage and a cost at the other, and benefits and costs here depend on the pollination environment. The over-representation of species with tricellular-hydrated pollen suggests there have been repeated shifts to more rapid and reliable pollination systems and/or to more intense pollen competition, such that dispersal costs have become reduced or are outweighed by the advantages of faster post-pollination performance.

The fact that all four combinations of trait states were found indicates some degree of evolutionary and developmental dissociation between the two traits. The appearance of evolutionary dissociation may reflect the unidirectional nature of contingency in development. It is likely that the origin of the tricellular state is contingent on the prior evolution of the hydrated state, but there is no mechanism that would cause pollen hydration state to be contingent on pollen cell number. Hence, hydration state should be less constrained to evolve than pollen cell number. This seems to be the case, since hydration states can vary considerably between closely-related species (Franchi *et al*. 2002; this study), whereas pollen cell number is generally not variable at such shallow levels of phylogeny (Williams *et al*. 2014). These data suggest that pollen water content evolves faster than pollen cell number and are consistent with high water content as a precursor to the origin of tricellular pollen. A likely scenario is that bicellular, dehydrated pollen fails to undergo dehydration, resulting in a lengthened period of pollen development within the anther, which eventually allows cell cycle mutations to have effects before pollen dispersal, causing the tricellular stage to be reached prematurely.

An alternate route to evolving the tricellular state involves the acceleration of development of bicellular pollen to reach the tricellular stage without the loss of controlled dehydration (Williams *et al*. 2014). Yet, species with tricellular, dehydrated pollen were the least common (Tab. 3), perhaps because the tricellular condition reduces pollen longevity despite dehydration, since gamete cells are terminally-differentiated and presumably shorter-lived than generative cells. The tricellular-dehydrated combination may also be an easily lost intermediate step in the evolution of the tricellular, hydrated condition. It is not known whether or not species with hydrated pollen can re-evolve the actively dehydrated state.

In conclusion, pollen hydration status and pollen cell number are traits that show a developmental and likely also an evolutionary linkage through their effects on pollen performance. Bicellular pollen has given rise to tricellular pollen many times during the course of angiosperm history. Today, species with tricellular pollen tend to disperse pollen in a somewhat more hydrated and metabolically active condition than those with bicellular pollen. That linkage may reflect a contingency in evolution – hydration might allow time for the evolution of cell cycle regulatory modifications that affect the timing of sperm cell formation. On a more ecological scale, pollen water content likely reflects conditions such as average pollination speed and reliability (Williams 2012) – the shorter and less variable the average duration of dispersal, the less pollen has to rely on dehydration as a mechanism for ensuring longevity during dispersal.

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