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Phylogenetic and functional signals in gymnosperm ovular secretions

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• **Background and Aims** Gymnosperms are either wind-pollinated (anemophilous) or both wind- and insect-pollinated (ambophilous). Regardless of pollination mode, ovular secretions play a key role in pollen capture, germination and growth; they are likely also involved in pollinator reward. Little is known about the broad-scale diversity of ovular secretions across gymnosperms, and how these may relate to various reproductive functions. This study analyses the sugar and amino acid profiles of ovular secretions across a range of ambophilous (cycads and Gnetales) and anemophilous gymnosperms (conifers) to place them in an evolutionary context of their possible functions during reproduction.
• **Methods** Ovular secretions from 13 species representing all five main lineages of extant gymnosperms were sampled. High-performance liquid chromatography techniques were used to measure sugar and amino acid content. Multivariate statistics were applied to assess whether there are significant differences in the chemical profiles of anemophilous and ambophilous species. Data were compared with published chemical profiles of angiosperm nectar. Chemical profiles were placed in the context of phylogenetic relationships.
• **Key results** Total sugar concentrations were significantly higher in ovular secretions of ambophilous species than wind-pollinated taxa such as Pinaceae and Cupressophyta. Ambophilous species had lower amounts of total amino acids, and a higher proportion of non-protein amino acids compared with anemophilous lineages, and were also comparable to angiosperm nectar. Results suggest that early gymnosperms likely had ovular secretion profiles that were a mosaic of those associated with modern anemophilous and ambophilous species. *Ginkgo*, thought to be anemophilous, had a profile typical of ambophilous taxa, suggesting that insect pollination either exists in *Gingko*, but is undocumented, or that its ancestral populations were insect-pollinated.
• **Conclusions** Chemical profiles of ovular secretions of ambophilous gymnosperms show a clear signal of pollinator-driven selection, including higher levels of carbohydrates than anemophilous taxa, lower levels of amino acids, and the presence of specific amino acids, such as β-alanine, that are known to influence insect feeding behaviour and physiology.

Key words: Amino acids, floral nectar, *Ginkgo*, gymnosperms, ovular secretions, pollination, sugars.

INTRODUCTION

In most gymnosperms, pollen lands on an ovular secretion in which the grain immediately hydrates (Nepi *et al.*, 2009). As the ovular secretion recedes, the pollen grain is pulled inside the ovule, where it germinates and achieves fertilization. Ovular secretions are present in nearly all major extant and probably most extinct gymnosperm taxa and they are a crucial part of reproduction (Gelbart and von Aderkas, 2002; Labandeira *et al.*, 2007; Little *et al.*, 2014). In addition to hydrating pollen, these secretions induce germination and promote pollen tube growth (Wagner *et al.*, 2007; Nepi *et al.*, 2009). Most gymnosperms are commonly considered wind-pollinated (anemophilous; Owens *et al.*, 1998). *Ginkgo biloba*, the only living ginkgophyte, is considered to be anemophilous (Friis *et al.*, 2011 and references therein), as are all studied species of Pinaceae and Cupressophyta (i.e. all conifers *sensu lato*), which together represent the majority of gymnosperm species (Owens *et al.*, 1998; Lu *et al.*, 2011). On the other hand there are numerous gymnosperm species in which insect pollination (entomophily) is reported (Porsch, 1910; Pearson, 1929; van der Pijl, 1953; Bino *et al.*, 1984*a, b*; Norstog and Fawcett, 1989; Kato and Inoue, 1994; Kato *et al.*, 1995; Donaldson, 1997; Norstog and Nicholls, 1997; Wetschnig, 1997; Wetschnig and Depisch, 1999; Terry, 2001; Terry *et al.*, 2005; Labandeira *et al.*, 2007; Procheş and Johnson, 2009; Marler, 2010; Bolinder *et al.*, 2016), although a contribution by wind cannot be excluded. In some gymnosperms a mixed pollination mode (ambophily) has been documented experimentally (reviewed for cycads in Marler and Lindström, 2014*b*; for *Cycas micronesica* in Terry *et al.*, 2009; Hamada 2013; for *Cycas revoluta* in Kono and Tobe, 2007; for gnetophytes in *Gnetum parvifolium* in Gong *et al.*, 2015; and for *Ephedra fragilis* in Celedón-Neghme *et al.*,

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2016). The typical pollination mode in Gnetophyta is ambophily. In some *Ephedra* species, studies provide evidence from computer simulations, wind tunnel experiments and field observations [*E. trifurca* (Niklas and Kerchner, 1986; Niklas *et al.*, 1986; Niklas and Buchmann, 1987; Buchmann *et al.*, 1989; Niklas, 2015) and *E. nevadensis* (Niklas, 2015)] that functional traits are compatible with wind pollination. However, these traits do not preclude insect pollination (Niklas, 2015), and there are several species in which an important role of insects in pollination has been demonstrated (Ren *et al.*, 2009; Friis *et al.*, 2011; Peñalver *et al.*, 2012; Terry *et al.*, 2014; Gong *et al.*, 2015; Rydin and Bolinder, 2015; Bolinder *et al.*, 2016). Thus, apart from *Ginkgo* and conifers, which are considered strictly anemophilous, the gymnosperms can be considered ambophilous.

Although the relative contributions of insects and wind to the pollination of ambophilous gymnosperms remain unclear, ovular secretions are thought to serve as a possible attractant and reward to insects (Labandeira *et al*., 2007, 2016; Ren *et al.*, 2009; Friis *et al.*, 2011; Bolinder *et al.*, 2016). The ovular secretions of entomophilous and ambophilous gymnosperms would thus be considered functionally analogous to angiosperm floral nectar, implying similar roles for the sugars, amino acids and proteins present in them (Nepi *et al.*, 2009). Thus we hypothesize that pollination drops of entomophilous and ambophilous gymnosperms may have evolved as a co-evolutionary response to feeding insects, comparable to angiosperm floral nectar and insects. This is bolstered by fossil evidence from 'preangiospermous' pollination syndromes, which involved insect–seed plant interactions during the Mesozoic (Labandeira *et al*., 2007, 2016; Ren *et al.*, 2009; Peris *et al.*, 2017), before the angiosperm radiation and at a time when gymnosperms were at their most diverse. The importance of gymnosperm ovular secretions in mediating interactions with pollinators is supported by some Gnetophyta that have strobili in which sterile ovules are regularly associated with male organs, forming morphologically bisexual, flower-like structures (Bino *et al.*, 1984*a*; Endress, 1996). Remarkably, these sterile ovules of functionally staminate plants produce secretions that resemble those produced by ovules of ovulate plants, and several insects have been reported feeding on secretions of both staminate and ovulate plants (Kato *et al.*, 1995). Drops from sterile ovules of gnetophytes have never been analysed before.

Previous studies on ovular secretion composition have been sporadic and relatively unsystematic; most of them focused on assessing carbohydrates (Fujii, 1903; Tison, 1911; McWilliam, 1958; Ziegler, 1959; Owens *et al.*, 1987; Seridi-Benkaddour and Chesnoy, 1988; Carafa *et al.*, 1992; von Aderkas *et al.*, 2012). To date, sugar and amino acid profiles have only been simultaneously analysed in eight species from three of the five major gymnosperm lineages (Ziegler, 1959; Bino *et al*., 1984a; Tang, 1987; Seridi-Benkaddour and Chesnoy, 1988; Carafa *et al.*, 1992). It is a challenge to compare and interpret these data because of the different techniques applied and the qualitative nature of some studies. Here, we test our hypothesis by investigating the sugar and amino acid composition of ovular secretions of both wind-pollinated and insect- and wind-pollinated species across all major clades of gymnosperms. Other nutritional components, such as lipids, could be present in gymnosperm ovular secretions, although they have never been reported. Thus we restricted the current research to carbohydrates and amino acids to obtain data comparable with those in the literature. Most studies about the chemical composition of both pollination drops and nectar focus on just these two classes of solutes. In particular we addressed the following questions: (1) are the chemical profiles of ovular secretions different in ambophilous and anemophilous gymnosperms? (2) Are these secretions different in ovulate and staminate individual of gnetophytes species? (3) Are the chemical traits of ovular secretions related to gymnosperm phylogeny? Moreover, we compared the chemical profiles of gymnosperm ovular secretions with angiosperm floral nectar to more robustly test our hypothesis that ambophilous gymnosperm secretions should bear the signal of insect-driven selection and thus display at least some convergence in their chemical traits with angiosperm nectar.

MATERIALS AND METHODS

Plant material

We collected 31 samples of ovular secretions using glass microcapillary tubes or micropipettes for 13 gymnosperm species (Table 1). When possible, we pooled ovular secretions of different individuals for a given sample (Table 1), representing at least 80–100 ovular secretions. Collections were made directly in the field or in greenhouses for all species except *Larix* × *marschlinsii*, *Pseudotsuga menziesii* and *Zamia furfuracea*, which could only be accessed by cone dissection. For these three species, ovule-bearing complexes were laid individually in closed Petri dishes lined with moistened Whatman No. 1 filter paper. Secretions that appeared shortly afterwards were then collected with glass microcapillary tubes. Once full, microcapillaries were voided into Eppendorf vials containing ethanol (70 % v/v). Prior to analysis, samples were air-dried in a Speedvac centrifuge (Jouan RC 1010) to eliminate the ethanol and diluted 1:50 with distilled water.

Twelve nectar samples from three angiosperm species [*Cucurbita pepo* (two male flower samples, three female flower samples; Nepi *et al*., 2011, 2012*a*), *Cerinthe major* (four samples; Nocentini *et al.*, 2012) and *Gentiana lutea* (three samples; Rossi *et al.*, 2014)] were also analysed to provide a comparison between gymnosperm ovular secretions and angiosperm floral nectar (Supplementary Data Tables S1–S3). The sugar and amino acid profiles of gymnosperm ovular secretion and those of flower nectar were determined using the same method (see below). The published nectar data show only the mean values across the high-performance liquid chromatography (HPLC) measurements.

Sugar analysis

Samples were analysed for sugar content using isocratic HPLC. The sample and standard solutions containing glucose, fructose and sucrose (20 µL) were injected into a Waters 600 E pump system. The mobile phase was deionized water. The flow rate was set at 0.5 mL min^{-1} and column temperature at 85–90 °C. Sugars were separated in a Waters Sugar-Pak

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TABLE 1. *Details of sample collection and pollination mode of the gymnosperm species used*

Species	Site of collection	Number of samples	Number of individuals	Pollination
Gingkophyta				
Gingko biloba (Gb)	University of California, Davis, CA, USA	3	4	Wind
Cycadophyta				
Zamia furfuracea (Zf)	Montgomery Botanical Center, Miami, FL, USA	1	17	Wind and insect
Cupressophyta				
Taxus baccata (Tb)	University of Victoria, Campus, Victoria, BC, Canada	3	13	Wind
Cephalotaxus koreana (Ck)	Arnold Arboretum, Harvard University, Boston, MA, USA	1	3	Wind
Chamaecyparis lawsoniana (Cl)	Dorena Genetic Resource Center, USDA Forest Service, Cottage Grove, OR, USA	3	30	Wind
Juniperus communis (Jc)	Greve in Chianti, Firenze, Italy	4	10	Wind
Juniperus oxycedrus (Jo)	Campiglia M.ma, Livorno, Italy	3	14	Wind
Pinaceae				
Larix × *marschlinsii* (Lm)	University of Victoria, Campus, Victoria BC (Canada)	2	3	Wind
Pseudotsuga menziesii (Pm)	University of Victoria, Campus, Victoria, BC, Canada	2	8	Wind
Gnetophyta				
Ephedra minuta (Em)	University of California, Davis, CA, USA	1	30	?
Ephedra fragilis m (Efm)	University of Siena Botanical Garden, Siena, Italy	4	2	Wind and insect
Gnetum gnemon f (Ggf)	Munich Botanical Garden, Munich, Germany	1	1	Wind and insect
Gnetum gnemon m (Ggm)	Kampong, Coral Gables, FL, USA	1	1	Wind and insect
Welwitschia mirabilis f (Wmf)	University of California, Davis, CA, USA	1	3	Wind and insect
Welwitschia mirabilis m (Wmm)	University of Washington, WA (USA)	1	1	Wind and insect

Pollination mode is as reported in the literature.

m, staminate individual(s); f, ovulate individual(s). *Ephedra minuta* is monoecious.

I (6.5–300 mm) column and identified with a Waters 2410 refractive index detector. The concentration of each single sugar was calculated by comparing the area under the chromatogram peaks with standards using the software Clarity (DataApex).

The total sugar concentration (TSC) was calculated by summing the concentrations of the three main sugars. Relative percentages for each sugar were also calculated as (Cs/TSC) × 100, where Cs is the concentration of a single sugar.

Amino acid analysis

Amino acid analysis was performed by gradient HPLC with an AccQtag system column (15 mm × 4.6 mm) maintained at 37 °C and a Waters 470 scanning fluorescence detector (excitation at 295 nm, detection at 350 nm). An AccQtag system buffer and a 6:4 acetonitrile–water solution were used in the gradient as the mobile phase at a flow rate of 1.0 ml min^{-1}. The selected volume of each reconstituted sample was amino acid derivatized (Cohen and Micheaud, 1993) with AQC fluorescent reagent and 0.02 M borate buffer (pH 8.6), according to AccQtag protocol (Waters Corp.). In addition to 19 of 20 protein-associated amino acids (tryptophan is not detectable with this method), standards for nine non-protein amino acids [β-alanine, citrulline, α-aminobutyric acid (AABA), β-aminobutyric acid (BABA), γ-aminobutyric acid (GABA), hydroxyproline, ornithine and taurine] were also used. The concentration of each individual amino acid was calculated by comparing the area under the chromatogram peaks with standards using the software Clarity (DataApex).

The total amino acid concentration (TAC) was calculated by summing the concentration of all the amino acids detected in each sample. Relative percentages of amino acids were also calculated as (Ca/TAC) × 100, where Ca is the concentration of a single amino acid. Variability across multiple samples of the same species was determined using the coefficient of variation (CV) for sugar and amino acids, calculated as σ/μ, where σ is the standard deviation and μ is the mean. The repeatability of the analytical procedures for both sugars and amino acids was assessed by replication of randomly chosen samples. Variability among the replicates was <4 %, thus ensuring a high repeatability.

Statistical analyses

All statistical analyses were performed with PAST (ver. 3.06) (Hammer *et al.*, 2001). Principal components analysis (PCA) was applied in order to interpret relationships in ovular secretion chemical variation among species, and to determine the strongest source of variation in the data set. In addition to the absolute and relative abundances of each sugar and amino acid, we analysed TSC, TAC and the different amino acid classes (essential protein amino acids, non-essential protein amino acids, non-protein amino acids). The normality of distribution of data was assessed by the Shapiro–Wilk *W* test. Since data were not normally distributed, non-parametric statistics were applied to compare differences between anemophilous and ambophilous species (Mann–Whitney *U* test).

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We reconstructed the ancestral state of ovular secretion components in order to map variations in chemical traits along the phylogeny of seed plants, considering a tree topology, based on Leslie *et al.* (2012) and Xi *et al.* (2013), that is consistent with most recent phylogenetic analyses of seed plants (e.g. Wickett *et al.*, 2014). Trait mapping and ancestral state reconstructions were inferred with least-squares parsimony using Mesquite (Maddison and Maddison, 2015). Figures were exported from PAST and Mesquite and composed in Adobe Illustrator CS5 (Adobe Systems, San Jose, CA, USA).

RESULTS

Sugar content of ovular secretions

Total sugar concentration varied by one order of magnitude among species, taxonomic groups and pollination types (Table 2, Fig. 1). In multiple samples of the same species the absolute concentrations of the more abundant sugars as well as the total sugar concentrations had a CV between 0.1 and 0.6. Anemophilous species had a significantly lower TSC than ambophilous species ($Z = -2.000$, $P = 0.045$; U test; Fig. 1). The exceptions to this overall pattern occurred in *Zamia furfuracea* and *Ginkgo biloba*: the former is insect-pollinated but with low TSC, whereas the latter, traditionally classified as wind-pollinated, had high TSC (Table 2). Floral nectar of the representative entomophilous angiosperms *Cucurbita pepo*, *Cerinthe major* and *Gentiana lutea* were all in the range of TSC of ovular secretions of ambophilous gymnosperms (Fig. 1).

Generally, the most abundant sugars were fructose and glucose, with the one notable exception of *Larix* × *marschlinsii*, in which sucrose was most abundant (Table 2). Relative percentages of sugars had a generally lower CV (≤0.3) than those of absolute concentrations. There were no significant differences in the relative percentages of the three sugars between anemophilous and ambophilous species. However, there were significant differences in the concentrations of fructose and sucrose: lower concentrations of both sugars were found in wind-pollinated species ($Z = -2.143$, $P = 0.032$ and $Z = -2.173$, $P = 0.029$, respectively; U test). Ovular secretions from sterile ovules of staminate plants had lower TSC than secretions from fertile ovules of female plants in *Gnetum gnemon* and *Welwitschia mirabilis*, although the relative percentages of each main sugar were similar (Table 2).

Although other sugars (melezitose, xylose) and polyalcohols (xylitol, sorbitol) were sometimes present, they were not abundant, representing <1 % of TSC.

Amino acid content of ovular secretions

The TAC varied by two to three orders of magnitude among species, taxonomic groups and pollination type. *Ginkgo biloba*, *Zamia furfuracea* and all gnetophytes had amino acid-poor ovular secretions (Table 3). Cupressophyta had a TAC greater than that of Gnetophyta but lower than that of Pinaceae. Anemophilous species had a significantly higher TAC than ambophilous species ($Z = 2.71$, $P = 0.006$, Fig. 1). The floral nectars of the representative entomophilous angiosperms *Cucurbita pepo*, *Cerinthe major* and *Gentiana lutea* were in the range of total amino acid concentrations of ovular secretions of ambophilous gymnosperms (Fig. 1).

The percentages of the amino acid classes were significantly different in anemophilous versus ambophilous species. In both groups the most abundant class of amino acids was non-essential protein amino acids (Fig. 2). Ambophilous species had lower percentages of non-essential protein amino acids

TABLE 2. *Absolute concentrations (mg/ml) and relative percentages of sugars in ovular secretions*

	Sucrose	Fructose	Glucose	TSC	Fructose/ Glucose ratio	Sucrose (%)	Fructose (%)	Glucose (%)
Gingkophyta								
Gingko biloba	11.12 ± 3.8	144.3 ± 45.6	192.04 ± 2.7	347.46 ± 80.8	0.75 ± 0.1	3.2 ± 1.5	41.5 ± 5.0	55.30 ± 4.0
Cycadophyta								
Zamia furfuracea	1.16	9.81	9.02	19.99	1.09	5.8	49.1	45.12
Cupressophyta								
Cephalotaxus koreana		4.26	27.40	31.66	0.16		13.5	86.5
Chamaecyparis lawsoniana		10.9 ± 4.7	11.40 ± 4.5	22.30 ± 8.8	0.96 ± 0.2		48.9 ± 5.3	51.1 ± 5.3
Juniperus communis		45.49 ± 26.3	8.30 ± 3.9	53.79 ± 30.3	5.48 ± 2.6		84.6 ± 5.2	15.4 ± 5.2
Juniperus oxycedrus		29.82 ± 13.8	1.75 ± 0.7	31.57 ± 14.3	17.04 ± 4.0		94.5 ± 1.1	5.5 ± 1.1
Taxus baccata	2.7 ± 1.0	32.70 ± 9.7	4.90 ± 1.7	40.30 ± 12.3	6.67 ± 0.8	6.7 ± 0.7	81.1 ± 1.8	12.2 ± 1.2
Pinaceae								
Larix × *marschlinsii*	52.6 ± 28.7	26.52 ± 13.3	24.11 ± 12.7	103.23 ± 54.7	1.16 ± 0.03	50.9 ± 1.0	25.7 ± 0.8	23.4 ± 0.1
Pseudotsuga menziesii	1.05 ± 0.1	23.07 ± 7.2	25.62 ± 8.5	49.74 ± 15.6	0.90 ± 0.02	2.1 ± 0.8	46.6 ± 0.1	51.3 ± 0.9
Gnetophyta								
Ephedra fragilis, m	17.14 ± 7.1	262.18 ± 94.9	295.70 ± 90.3	575.02 ± 116.9	0.89 ± 0.4	3.0 ± 1.0	45.6 ± 12.3	51.4 ± 13.3
Ephedra minuta	52.14	184.09	259.04	495.27	0.71	10.5	37.2	52.3
Gnetum gnemon, f	151.06	698.08	44.90	894.04	15.55	16.9	78.1	5.0
Gnetum gnemon, m	30.20	139.00	8.90	178.10	15.62	17.0	78	5.0
Welwitschia mirabilis, f	35.91	520.27	150.76	706.94	3.45	5.1	73.6	21.3
Welwitschia mirabilis, m	3.20	82.04	25.16	110.40	3.26	2.9	74.3	22.8

For *Gnetum gnemon* and *Welwitschia mirabilis*, ovular secretions from functional ovules of ovulate individuals (f) and from sterile ovules of staminate individuals (m) were analysed.

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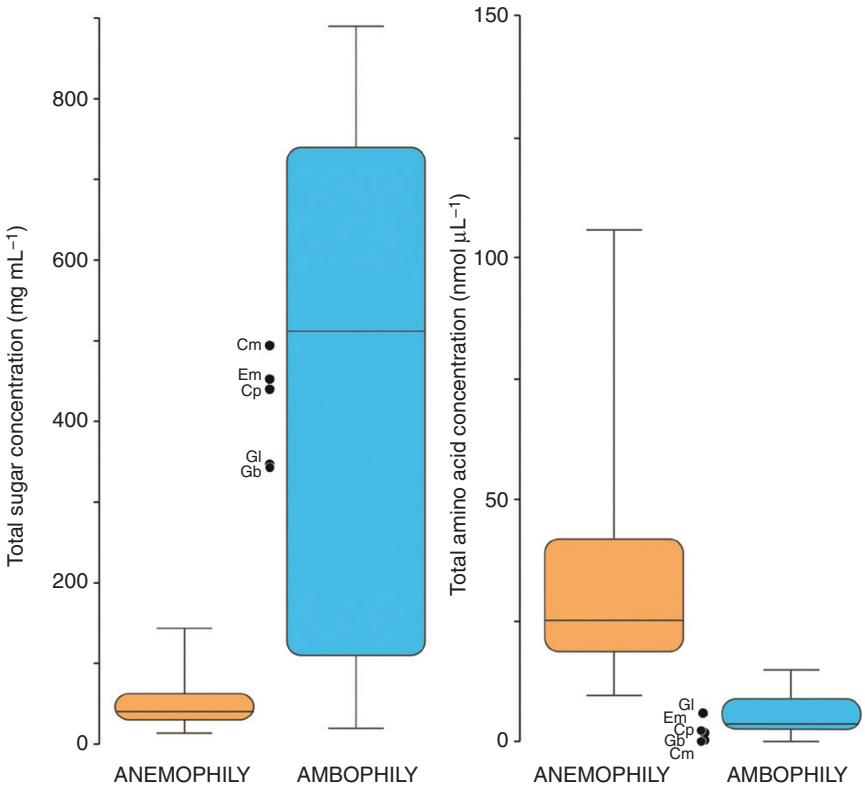

FIG. 1. Total sugar concentration and total amino acid concentration in wind-pollinated and wind- and insect-pollinated gymnosperms and in floral nectar of representative entomophilous angiosperms: *Cucurbita pepo* (Cp); *Cerinthe major* (Cm); and *Gentiana lutea*(Gl). *Ginkgo biloba* (Gb) and *Ephedra minuta* (Em) are plotted separately to highlight their ambophilous-like concentrations.

and higher percentages of non-protein amino acids than anemophilous species ($Z = -2.85$, $P = 0.004$ and $Z = 2.79$, $P = 0.005$, respectively; Fig. 2). In most of the wind-pollinated species non-protein amino acids were not detected (Table 3). The floral nectar of representative entomophilous angiosperms had proportions of the different amino acid classes that were similar to those in ambophilous gymnosperms (Fig. 2).

Among the protein amino acids, serine, glutamic acid, glycine, histidine, alanine and proline were the most commonly abundant across gymnosperm ovular secretions (Table 3) (Supplementary Data Table S4). In multiple samples of the same species the CV of the absolute concentrations and relative percentages of these amino acids was in the range 0.1–0.7. Proline was frequently the most abundant amino acid in both anemophilous and ambophilous species, reaching as high as 90 % of TAC in wind-pollinated *Juniperus* species (Supplementary Data Table S4). Proline was also typically the most abundant amino acid in the floral nectar of the representative entomophilous angiosperms, ranging from 11 to 42 % of TAC (Supplementary Data Table S3).

β-Alanine was either the most abundant or among the more abundant of the non-protein amino acids in ambophilous species. Levels reached 52.5 and 63.4 % of TAC in staminate ovular secretions of *Zamia furfuracea* and *Ephedra fragilis*, respectively (Supplementary Data Table S4). This amino acid was also present, albeit only in trace amounts, in some wind-pollinated species. In *Ephedra minuta* and *Ginkgo*, β-alanine accounted for 24 and 33 % of TAC, respectively, levels comparable to those found in ambophilous species. The same amino acid ranged from 4 to 43 % in the floral nectar of the representative angiosperm species (Supplementary Data Table S3). Hydroxyproline was typically found in both staminate and ovulate ovular secretions of *Welwitschia mirabilis*, in which it was also the most abundant amino acid (Table 3).

Integrating sugar and amino acid profiles

Multivariate analyses of all sugar and amino acid values discriminate major taxonomic groups and pollination modes (Fig. 3). Ambophilous gymnosperms occupied overlapping multivariate space with each other and the chemical profiles of angiosperm floral nectars, especially *Gentiana* (Fig. 3).

The first two axes of the PCA performed on absolute concentrations explained 71 % of the total variance. The loading of these two axes was dominated by the variation in concentrations and percentages of the sugars, absolute values of proline and percentage of β-alanine (Supplementary Data Table S5). Notably, *Ginkgo* and *Ephedra minuta* clustered with the ambophilous species (Fig. 3).

Mapping ovular secretion contents onto a phylogenetic framework of seed plants (Leslie *et al.*, 2012; Xi *et al.*, 2013) provided a preliminary inference of the ancestral states of the sampled species. The least-squares parsimony reconstruction predicted that *Ginkgo biloba* and the common ancestor

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TABLE 3. *Absolute concentrations (nmol μL^{-1}) of amino acids in the ovular secretions*

	Asp	Ser	Glu	Gly	**His**	**Arg**	**Thr**	Ala	Pro	**Val**	**Lys**	**Ile**	**Leu**	*HPro*	Gln	*β-Ala*	*GABA*	TAC	Prot	NoProt	Ess	NoEss
Gb		0.19 ± 0.09			0.15 ± 0.06				2.01 ± 0.93	0.11 ± 0.03			0.20 ± 0.08		0.20 ± 0.09	1.44 ± 0.47		4.35 ± 1.19	2.90 ± 1.29	1.44 ± 0.47	0.51 ± 0.18	2.4 ± 1,1
Zf								0.03								0.03		0.06	0.03	0.03		0.03
Ck		9.05	8.69	0.30	0.20		0.18	1.46	6.43	0.80		0.07	0.05					27.47	27.47		1.50	25.97
Cl		1.06 ± 0.51		0.05 ± 0.03	0.12 ± 0.05		0.03 ± 0.3	0.15 ± 0.08	5.19 ± 1.01	0.06 ± 0.051		0.10 ± 0.06	0.14 ± 0.04	0.19 ± 0.06	2.21 ± 0.77	0.02 ± 0.04		10.04 ± 1.74	9.74 ± 1.68	0.30 ± 0.06	0.51 ± 0.2	9.23 ± 1.49
Jc		0.46 ± 0.16	0.11 ± 0.04	0.28 ± 0.1			1.69 ± 0.58	0.18 ± 0.06	20.61 ± 5.30			0.13 ± 0.04	0.12 ± 0.06					23.58 ± 4.90	23.58 ± 4.90		1.94 ± 0.50	21.64 ± 5.40
Jo		1.64 ± 0.72	1.17 ± 0.41	0.12 ± 0.08			0.53 ± 0.21	0.08 ± 0.08	20.24 ± 6.30			0.08 ± 0.05	0.55 ± 0.26					24.42 ± 6.3	24.42 ± 6.3		1.17 ± 0.18	23.25 ± 6.39
Tb	0.70 ± 0.35	0.93 ± 0.47		0.12 ± 0.06	0.75 ± 0.34	0.11 ± 0.07	0.11 ± 0.08	1.23 ± 0.48	9.15 ± 2.98	0.13 ± 0.06		0.06 ± 0.05	0.03 ± 0.05	0.04 ± 0.05	3.31 ± 0.97	0.04 ± 0.04	0.12 ± 0.08	17.33 ± 5.77	17.04 ± 5.69	0.29 ± 0.11	1.57 ± 0.87	15.47 ± 5.11
Lm	5.73 ± 2.90	9.89 ± 4.60	26.35 ± 8.62	0.98 ± 0.38	25.03 ± 10.63	1.37 ± 0.70	2.19 ± 1.12	9.55 ± 4.53	6.02 ± 3.65		0.22 ± 0.09		0.68 ± 0.36					89.69 ± 5.98	89.69 ± 5.98		30.68 ± 13.41	59.01 ± 7.43
Pm	4.96 ± 2.23	11.70 ± 2.75	12.26 ± 4.55		4.60 ± 1.78	1.34 ± 0.56	2.65 ± 1.41	5.88 ± 2.47	30.27 ± 8.83	1.90 ± 0.65	1.83 ± 0.56	2.39 ± 0.63						82.36 ± 27.9	82.36 ± 27.9		15.14 ± 5.81	67.22 ± 22.09
Efm		0.63 ± 0.16						0.50 ± 0.12								1.96 ± 0.43		3.09 ± 072	1.13 ± 0.29	1.96 ± 0.43		1.13 ± 0.29
Em		0.17							2.07	0.03						0.71		3.00	2.29	0.71	0.05	2.24
Ggf	5.44	73.18	6.37	34.78	27.55			0.02	0.04									14.74	14.74		2.76	11.98
Ggm	0.45	2.32		0.16	0.39	0.48	0.19	0.69	0.22	0.27	0.39	0.22	0.29		0.34	0.24	0.32	7.24	6.40	0.84	2.23	4.17
Wm, f								1.92	2.11					2.45		0.51		6.99	4.03	2.96		4.03
Wm, m		1.62			1.79	2.06		0.22	0.66	0.06	0.13		0.03	3.07	0.21	0.20	0.16	10.23	6.8	3.43	4.09	2.71

For *Gnetum gnemon* and *Welwitschia mirabilis*, ovular secretions from functional ovules of ovulate individuals (f) and from sterile ovules of staminate individuals (m) were analysed. Non-essential protein amino acids are in Roman characters, essential protein amino acids are in bold characters and non-protein amino acids are in italics. Amino acids with concentration <2 % of total amino acid concentration in all species are not reported.

Prot, total protein amino acids concentration; NoProt, total non-protein amino acids concentration; Ess, total essential amino acids concentration; NoEss, total non-essential amino acids concentration. B-Ala, β-alanine; HPro, hydroxyproline. All other amino acids are indicated with standard abbreviations. Abbreviations of taxa are as in Table 1.

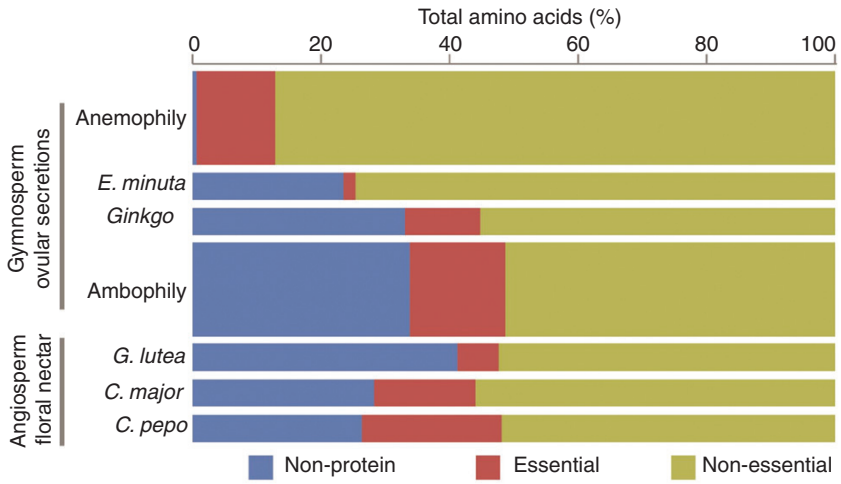

FIG. 2. General amino acid profile of ovular secretions of insect- and wind-pollinated gymnosperms and floral nectar of the representative entomophilous angiosperms *Cucurbita pepo*, *Cerinthe major* and *Gentiana lutea*. *Ginkgo biloba* and *Ephedra minuta* are plotted separately to highlight their ambophilous-like profiles.

of Gnetophyta would have higher sugar concentrations compared with those of the Pinaceae and Cupressaceae (Fig. 4A, B). *Ginkgo* and the common ancestor of Gnetophyta had the highest concentrations of fructose and non-protein amino acids (Fig. 4A, B). A moderate level of fructose was inferred for the common ancestor of gymnosperms, similar to that of secretions from staminate plants of the entomophilous *Welwitschia* (Fig. 4A). Proline concentrations were highest among the anemophilous Pinaceae and Cupressaceae, with the ancestral state for extant gymnosperms predicted to reflect this higher proline content (Fig. 4B). We consider low non-protein amino acid concentrations to be typical of the anemophilous Pinaceae and Cupressophyta, and high non-protein amino acid concentrations to be typical of *Ginkgo* and Gnetophyta. The common ancestor of gnetophytes was predicted to be in the range of concentrations found in *Ephedra minuta* (Fig. 4A). Despite being ambophilous, *Zamia furfuracea* did not share concentration levels of sugars and proline with those of the ambophilous Gnetophyta, and was more comparable to anemophilous Pinaceae and Cupressophyta (Fig. 4A, B).

Staminate and ovulate secretions of *Gnetum gnemon* and *Welwitschia mirabilis* had contrasting concentrations of fructose and non-protein amino acids (Fig. 4A). The ovulate secretions of both species had higher fructose concentrations than those of staminate secretions, and the staminate secretions had higher concentrations of non-protein amino acids than those of ovulate secretions.

DISCUSSION

Our results show profound differences between the chemical profiles of ovular secretions of anemophilous and ambophilous gymnosperms: the former have lower sugar and higher amino acid content than the latter. In addition, there are significant differences in some specific solutes. These differences are probably linked to their different functions in gymnosperm reproduction and are likely due to the evolution of the interactions with insects.

Chemistry of ovular secretion and its ancestral function: interaction with pollen

The ovular secretion was used to capture prepollen or pollen in seed plant ancestors (Little *et al.*, 2014) and has been demonstrated in the fossil record [300-million-year-old pollination drop of a callistophytalean seed fern, *Callospermarion pusillum*, late Carboniferous (Rothwell, 1977)]. This original function appears to have been maintained throughout much of the evolutionary history of gymnosperms, with extant species bearing ovular secretions that have a chemical composition suited for pollen hydration, germination and pollen tube growth (Nepi *et al.*, 2012*b*). Ovular secretions can thus be considered to be a culture medium for captured pollen grains, providing them with both an optimal osmolarity as well as initial nutritional substrates for pollen metabolism. An optimal osmolarity is particularly important for pollen hydration, germination and tube growth (Shivanna, 2003), and is largely due to carbohydrates, the most abundant solutes. Pollen development also shows stage-specific responses to carbohydrate concentrations. For example, *in vitro* studies of *Brassica* show that pollen germination is optimal at sugar concentrations around 10–15 % (Shivanna, 2003). Reducing sugar concentration to 5 % reduces pollen germination; however, the lower concentrations improve *in vitro* pollen tube growth in *Brassica* (Shivanna, 2003). In gymnosperms there are few studies of *in vitro* germination of pollen. Some gymnosperm pollen, such as that of pine, will readily germinate on standard pollen germination medium [i.e. Brewbaker and Kwack medium (Brewbaker and Kwack, 1963); Varis *et al.*, 2010], whereas larch and Douglas-fir pollen require high concentrations of carbohydrates to germinate *in vitro* (Fernando *et al.*, 1998; Dumont-Béboux *et al.*, 1999, 2000), levels that are much higher than those that we found in their ovular secretions. *Ephedra* pollen is reported to germinate at high sugar concentration (Bhatnagar and Moitra, 1996), more comparable to the TSC found in secretions of *Ephedra* species here.

Metabolites for pollen nutrition are required to develop pollen tubes, which according to Nygaard (1977) requires an

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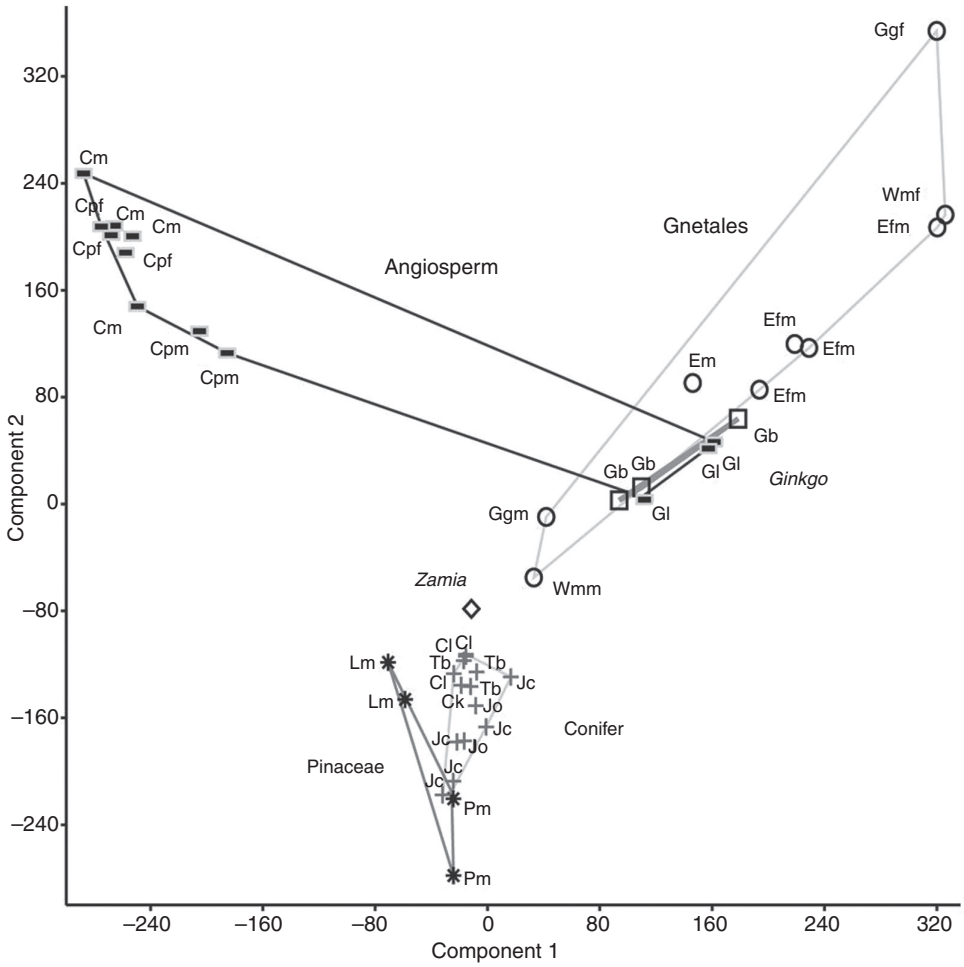

FIG. 3. Principal components analysis of sugar and amino acid contents in the ovular secretions of 13 species of gymnosperms and floral nectar of representative entomophilous angiosperms *Cucurbita pepo*, *Cerinthe major* and *Gentiana lutea*. Taxon abbreviations are as noted in Table 1.

exogenous source of carbohydrates, in particular fructose. In a set of experiments on *Pinus mugo* pollen cultures, exogenous fructose is metabolized more readily than glucose. In our analyses of different gymnosperm ovular secretions, fructose tended to be the dominant sugar compound, which may reflect its importance in pollen tube development.

Among the amino acids, proline is frequently the most abundant in the studied species (up to almost 90 % of TAC in the two *Juniperus* species). According to Shivanna (2003), pollen grains can use proline either directly as a substrate during germination or in the synthesis of hydroxyproline-rich wall proteins of pollen tubes (Shivanna, 2003). Uptake experiments demonstrated that mature and germinating pollen take up proline rapidly by means of a specific transporter (Schwacke *et al.*, 1999). Ovular secretions of *Welwitschia mirabilis* also have a high content of hydroxyproline that presumably can be utilized during pollen germination and tube growth.

The chemical environments of ovular secretions may be responsible for prezygotic selection against heterospecific pollen, as shown in crosses between *Larix* × *marschlinsii* and *Pseudotsuga menziesii* (von Aderkas *et al.*, 2012), two species that share similar chemical profiles in our analyses. These two species differ significantly in their overall osmolarity: the TSC of *Larix* × *marschlinsii* is double that of *Pseudotsuga menziesii*. *Larix* × *marschlinsii* is the only species in which we found that sucrose is more abundant than either glucose or fructose. This difference is linked to the presence of apoplastic invertase enzymes that are active in post-pollination prefertilization drops of Douglas-fir, but not larch (von Aderkas *et al.* 2012).

Modification of ovular secretion chemistry in ambophilous gymnosperms

High sugar concentrations in ovular secretions of ambophilous gymnosperms have been well documented (Bino *et al.*, 1984a, b; Carafa *et al.*, 1992; Kato *et al.*, 1995; Labandeira *et al.*, 2007; Nepi *et al.*, 2009). These concentrations are comparable to those of the floral nectar of the three representative angiosperm species. In addition, conversion of the total sugar content from mg/ml to % w/w (Galetto and Bernardello, 2005, page 278) allows us to show that our results have similar ranges to the previously published range for floral angiosperm nectar (Nicolson and Thornburg, 2007). It is likely that

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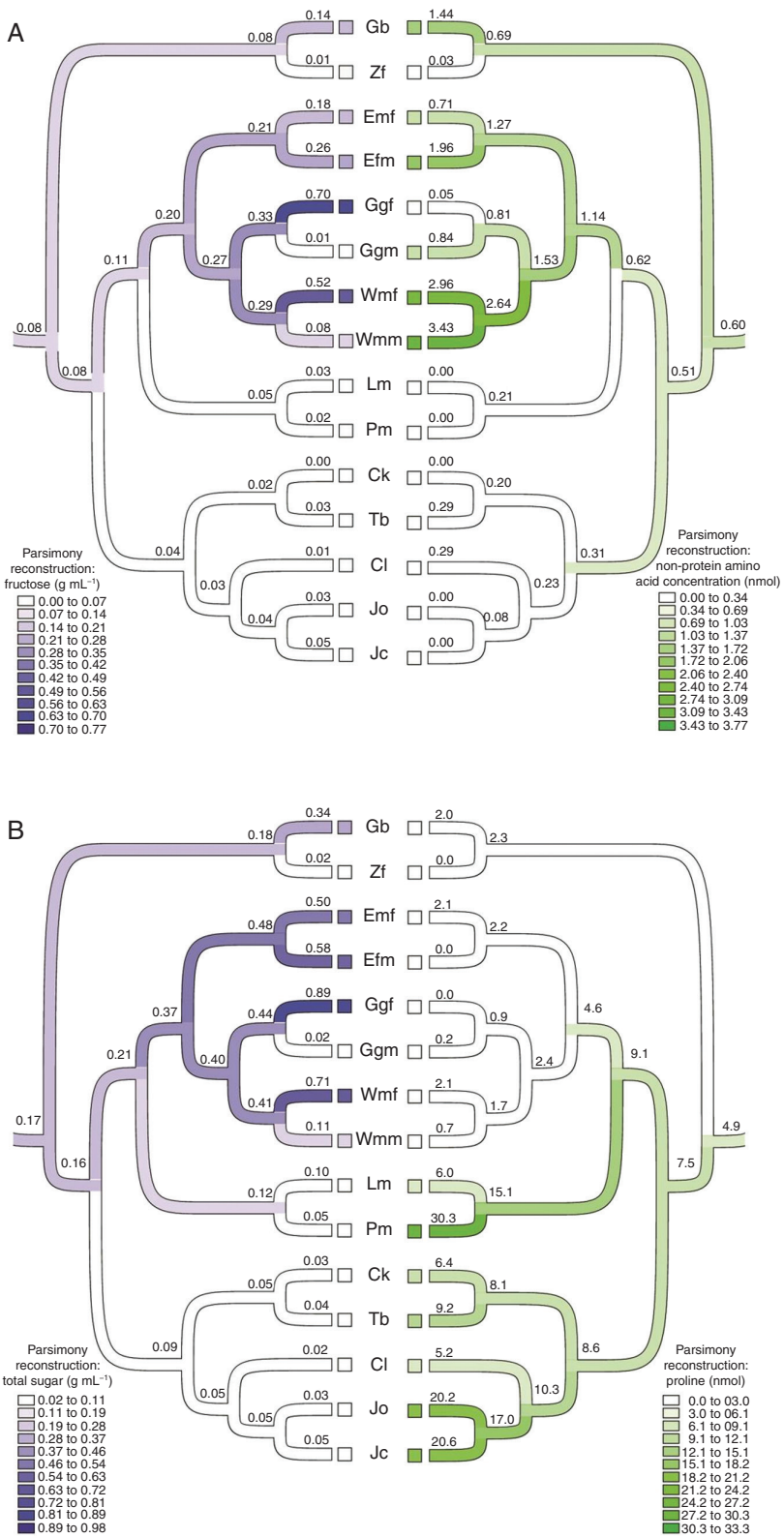

FIG. 4. Least squares parsimony reconstruction of absolute concentration of fructose versus absolute concentration of non-protein amino acids (A) and absolute concentration of total sugars (TAC) versus absolute concentration of proline (B). Phylogeny is based on Leslie *et al.* (2012) and Xi *et al.* (2013). Taxon abbreviations are as noted in Table 1.

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both pollination drops and nectar are consumed by pollinators since sugars, satisfying energetic needs of actively flying insects (Nicolson and Thornburg, 2007). Insect metabolism also requires input from amino acids and lipids to accomplish other bodily functions. Amino acids were found in the pollination drops of ambophilous gymnosperms (although at a lower concentration than in anemophilous gymnosperms; see below), but the presence of lipids was never reported (contrary to their presence in angiosperm nectar; Nicolson and Thornburg, 2007), although they cannot be excluded. These two substances can be obtained by insects feeding on other plant secretions or tissues/organs.

Not all ambophilous gymnosperms have high sugar levels. We found that ovulate secretions of *Zamia furfuracea* have a low carbohydrate content, confirming the low amounts recorded in other cycads (e.g. *Zamia pumila* and *Ceratozamia robusta*; Tang, 1987, 1993; Norstog *et al.*, 1986). Unlike many gnetophytes that are pollinated by insects that feed on pollination drops, cycads are pollinated by insects that feed mainly on pollen and/or reproductive and vegetative tissues of the plants fulfilling their metabolic needs (Kato *et al.*, 1995; Peñalver *et al.*, 2012; Marler and Lindström, 2014a, *b*; Terry *et al.*, 2014). Thus it is reasonable to assume that the nutritional needs of these insects do not exert the same kind of selection on the chemistry of their pollinations drops as in other insect-pollinated taxa.

The ovular secretions of ambophilous gymnosperms have higher sucrose concentrations than those of wind-pollinated species. The latter either lack sucrose or only have trace amounts of it (with the noted exception of *Larix* × *marschlinsii*). Sucrose is a potent phagostimulant for insects that induces specific chemoreceptors (Schoonhoven *et al.*, 2005). This is probably why sucrose is the most common sugar in angiosperm nectar, where it was present in almost 90 % of the 765 species studied by Baker and Baker (1983). In this respect, gymnosperms differ from angiosperms: sucrose is never the dominant sugar in their ovular secretions. This may be related to differences in their pollinators. To be absorbed and metabolized, sucrose requires invertase in the insect gut. Sucrose-rich fluid can be exploited as a nutritional resource only by animals that possess this enzyme (Nicolson, 2007). Extant Diptera, especially flies, are known to prefer hexose-rich sugary secretions, probably because they have a low invertase activity in their gut or lack it altogether (Yang and Davies, 1968; Baker and Baker, 1983). Flies are among the more common pollinators feeding on ovular secretions of extant gnetophytes (Bino *et al*., 1984; Kato *et al.*, 1995; Wetschnig and Depisch, 1999; Labandeira *et al.*, 2007; Ickert-Bond and Renner, 2016). There is fossil evidence that suggests their involvement in insect–plant associations with cycads, seed ferns, pentoxylaleans, ginkgoopsids (Czekanowskiales) and gnetaleans, beginning in the Early Permian and increasing during the Middle Jurassic (Mamay, 1976; Labandeira *et al.*, 2007; Ren *et al.*, 2009; Taylor *et al.*, 2009; Labandeira 2010; Wang *et al.*, 2012; Peñalver *et al.*, 2012, 2015).

All ambophilous gymnosperms examined have low concentrations of amino acids, similar to those of the floral nectars of the three representative angiosperms and other entomophilous angiosperm species (Gardener and Gillman, 2002; Nicolson and Thornburg, 2007; Nepi *et al.*, 2009). Amino acids are known to affect the taste of sugary solutions (Gardener and Gillman, 2002); high amino acid concentrations in solution can alter the taste of nectar, making it unpleasant for insects attracted by sweet-tasting solutions (Gardener and Gillman, 2002; Schoonhoven *et al.*, 2005; Nicolson, 2007; Nicolson and Thornburg, 2007).

Amino acid profiles of ovular secretions of ambophilous gymnosperms are characterized by larger percentages of non-protein amino acids, which are present in very low amounts or completely absent in wind-pollinated species, especially β-alanine. β-Alanine is not involved in protein synthesis. Its ecological function, therefore, is not directly related to its nutritional value. β-Alanine is now established as a key component of glial-cell-based recycling of the neuroreceptor histamine in the retina of the fly *Drosophila*, via interaction with the products of the genes *tan* and *ebony* (Gavin *et al.*, 2007; Chaturvedi *et al.*, 2014). Furthermore, the genes *tan* and *ebony* are linked to several traits in *Drosophila*, such as aggression and pigmentation (Takahashi, 2013), that are adaptive. This compound, and similar non-protein amino acids such as GABA and taurine, are also present in the floral nectar of the representative angiosperms included here. They were reported to frequently account for about a quarter of the total amino acid concentration in angiosperm floral nectar (Nepi, 2014). They can affect the physiology of the nervous system of insects, regulating nectar intake by phagostimulation, and promoting muscle function during flight. Thus, they may improve aspects of dispersal by stimulating flying activity of insects (Nepi, 2014).

Although many Gnetophyta are dioecious, ovular secretions can be produced on ovulate (from functional ovules) and microsporangiate cones (from sterile ovules; Endress, 1996). Ovular secretions from sterile ovules in staminate individuals likely serve to attract and reward insects (Karsten, 1892; Pearson, 1929; Lloyd and Wells, 1992; Endress, 1996; Jörgensen and Rydin, 2015; Bolinder *et al.*, 2016). All three extant gnetophyte genera have insect visitors that feed on both types of ovular secretion (Pearson, 1929; Labandeira *et al.*, 2007; Ickert-Bond and Renner, 2016). When we analysed ovular secretions of staminate and ovulate individuals of the same species (*Gnetum gnemon* and *Welwitschia mirabilis*) we found similar relative percentages of the major sugars, although TSC was higher in secretions of ovulate plants. Thus, staminate and ovulate gnetophyte plants appear to maintain similar proportions in their ovular secretions to attract the same pollinators and ensure efficient pollen transfer, comparable to the relationship between angiosperm floral nectar and specific pollinators (Baker and Baker, 1983). However, staminate gymnosperm plants appear to reduce the expense of producing ovular secretions that are not directly involved in pollen germination and growth by having a reduced sugar content. The differences between staminate and ovulate individuals in TSC and viscosity may explain the recent observation that 'pollination drops on sterile ovules of staminate strobili in *Gnetum* were much smaller in size and easier to disintegrate, and flowed more easily to the base of the collars' (Gong *et al.*, 2015).

Inference of gymnosperm pollination mode from the chemistry of ovular secretion

To date, the pollination mode of some gymnosperms is still uncertain. Several species of *Ephedra* are considered to be anemophilous, while other species are considered to be

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entomophilous (Niklas, 2015), although only facultatively (Bolinder *et al.*, 2015). Anemophily and entomophily have recently been associated with particular pollen ultrastructure in *Ephedra* (Bolinder *et al.*, 2015). *Ephedra minuta* has not been documented as either being insect- or wind-pollinated. However, our data suggest that ambophily is likely for *Ephedra minuta* based on its pollination drop profile. This is also supported by the observation of flies feeding on these drops during our collections (empidid fly, Empididae; identification by Derek Sikes, Curator of Insects, University of Alaska Museum of the North, USA, pers. comm.) (Supplementary Data Fig. S1). Empididae have also been reported in fly pollination of a number of angiosperms (reviewed in Woodcock *et al.*, 2014).

Pollination drop of Ginkgo biloba*: an evolutionary anachronism?*

A particularly surprising result of this study concerns *Ginkgo biloba*. Although this species has long been thought to be wind-pollinated (Proctor *et al.*, 1996; Ackerman, 2000), the chemical profile of its pollination drops fits with those of the insect-pollinated Gnetophyta. Both *Ginkgo* and gnetophytes have high TSC and low TAC, and a high relative abundance of β-alanine. The only published information on *Ginkgo biloba* ovular secretions reports high sugars (I. Baker, pers. comm. in Friedman, 1987). The lack of documented entomophily in *Ginkgo* may be explained by the scarcity of information about its pollination biology in native habitats such as forests in south-western China (Tang *et al.*, 2012). Alternatively, *Ginkgo* may have co-evolved with insect assemblages early in its evolution, developing ambophilous pollination, and later may have lost its ancient pollinators (Labandeira *et al.*, 2007). The persistence of pollination drops that fit the chemical profile of insect pollination could be considered an evolutionary anachronism (Barlow, 2000). The existence of a diverse herbivore community (16 taxa) on well-documented ginkgoalean foliage from the Late Triassic (i.e. Molteno *Paraginkgo*) to the present (modern *Ginkgo*) (Labandeira *et al*., unpubl. res.), findings of mimicry between a mecopteran insect and ginkgoalean leaves from the Middle Jurassic (Wang *et al.*, 2012), and ginkgoopsids as potential hosts for Eurasian, long-proboscid scorpion flies in late Middle Jurassic to the late Early Cretaceous (Ren *et al.*, 2009), false blister beetles and other insect pollinators supporting gymnosperm–insect pollination modes and host associations with ginkogaleans, cycads, conifers and bennettitalean gymnosperms during the mid-Cretaceous (Peris *et al.*, 2017) all hint at possible insect pollination in the broader ginkgoalean lineage (C. C. Labandeira, pers. comm.). Drop collectors observed both ants and flies visiting *Ginkgo* drops during collection in our study. In addition, collectors also noted a 'sweet' scent associated with the pollination drops of ovulate plants.

Conclusions

The ovular secretion mechanism for pollen capture and nourishment is of ancient origin in seed plants. It is inferred to have first appeared in seed ferns that date back to the late Devonian, and was probably present and widespread in early extant gymnosperms at least as early as the Carboniferous (Labandeira *et al.*, 2007; Little *et al.*, 2014), when modern insect lineages were not present and wind pollination is thought to have been the only way to transport pollen or pre-pollen. Later on, insects diversified and started to feed on gymnosperm ovular secretions and pollen (Ren *et al.*, 2009; Yong and Ferguson, 2015). Based on fossil evidence, it has been hypothesized that during the Middle Jurassic to Early Cretaceous interval there were several taxa of insects with specialized mouthparts (i.e. siphonate proboscides) that fed on ovular secretions and were most likely engaged in pollination mutualisms with gymnosperms, especially extinct scorpion flies (Ren *et al.*, 2009), true flies (Peñalver *et al.*, 2015), and kalligrammatid lacewings (Labandeira *et al.*, 2016).

We predict that early gymnosperm ancestors had ovular secretions that are a mosaic of what is seen among modern species: high fructose and high non-protein amino acid concentrations similar to those found in extant ambophilous species, but with similarities to those in extant anemophilous species (i.e. elevated proline concentrations). This study reinforces the antiquity of insect–plant pollination mutualisms in Gnetophyta, which have a fossil record beginning in the Triassic and reaching their highest diversity during the Early Cretaceous, suggesting a diversification episode with angiosperms (Labandeira *et al.*, 2007).

The long history of association between ovular secretions of gymnosperms and surface-fluid-feeding insects predates that of nectar-feeding insects on angiosperms. Through time, this may have resulted in a modification of the chemical profile of the ovular secretion to fit insect needs for high metabolism to sustain active flight. The chemical profile of the ovular secretions of extant Gnetophyta (and *Ginkgo*) reveals a clear impact of selection driven by insects towards higher levels of carbohydrates, lower levels of amino acids and specific sugar and amino acid profiles. In this regard, it is interesting to note that Diptera, which are the more common pollinators of entomophilous Gnetophyta, experienced limited extinction during the interval when angiosperms became ecologically dominant (Labandeira, 2010). Most probably Diptera shifted from earlier fluid-feeding on ovular secretions of gymnosperms to later nectar-feeding on angiosperms (Labandeira, 2010). Subsequently other major clades of pollinating insects arose and co-evolved with rapidly radiating angiosperms, which provided a nutritionally efficient system for consumption of surface fluid, as the chemistry of floral nectar fits the specific needs of co-evolving insect groups well (Baker and Baker, 1982; Nicolson, 2007). The results suggest that similar adaptive mechanisms occurred in ancient as well as more recent seed plants.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: concentrations and relative percentages of sugars in floral nectar of the three representative angiosperms. Tables S2 and S3: concentrations and relative percentages of amino acids, respectively, for floral nectar of the three representative angiosperms. Table S4: relative percentages of amino acids of ovular secretions of the studied gymnosperms. Table S5: loadings of PCA axes. Figure S1: an empidid fly (Empididae; Derek Sikes, University of Alaska Museum of the North, pers. comm.) visiting and feeding on *Ephedra minuta* in greenhouse conditions in Davis, CA, USA.

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