Pollen transfer efficiency of *Apocynum cannabinum* (Apocynaceae): A comparative perspective

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**Abstract**—Pollen transfer efficiency (PTE), the percentage of removed pollen delivered to conspecific stigmas, has been implicated in the morphological evolution, population dynamics, and lineage diversification of flowering plants. Pollinia, the aggregated contents of pollen sacs, present in Apocynaceae subfamilies Asclepiadoideae (milkweeds), Secamonoideae, and Periplocoideae and orchids (Orchidaceae), are the pre-eminent example of a plant trait that elevates PTE (to ca. 25%). However, comparison of species with pollinia to “average” flowers (PTE ca. 1%) may over-estimate the gains from pollinia. We hypothesize that elevated PTE evolved in Apocynaceae prior to pollinia. We measured PTE and pollen to ovule ratio, a possible correlate of PTE, in *Apocynum cannabinum*, a milkweed relative with pollen tetrads (instead of pollinia) and simple bands of style head adhesive (instead of complex pollinium-carrying translators), comparing them to reports of other species collated from the literature. PTE of *A. cannabinum* is 7.9%, in the 24th percentile of reports for 36 milkweed species, but more than twice the highest PTE reported for a species with monads (3.4%). The bands of style head adhesive are functionally equivalent to the translators of milkweeds. The pollen to ovule ratio of *A. cannabinum*, at 19.8, is in the 94th percentile of ratios reported for milkweeds (mean 9.6%). Our results are consistent with the hypothesis that floral novelties of Apocynaceae that evolved prior to pollinia also promote aggregated pollen transport and elevated PTE.

**Keywords:** floral function; pollen ovule ratio; pollen transfer efficiency; pollinium; pollination; tetrad

**Introduction**

Pollen transfer efficiency (PTE, the percentage of pollen removed from a flower that is subsequently deposited on conspecific stigmas) is one of the primary components of male reproductive fitness in plants, since only those pollen grains that are delivered to conspecific stigmas may father the next generation (Harder 2000; Harder & Johnson 2008; Thomson 2006). All else being equal, the more efficient pollen donors in a population will have greater siring success. Thus, to the extent that plant traits influence pollen transfer efficiency, selection for male fitness via PTE can shape the evolution of plant traits (Kobayashi et al. 1997; Ren & Tang 2010; Thomson 2006). Low PTE can contribute to pollen-limitation, and thus limit female reproductive output (seed production) at the population level (Harder & Aizen 2010). Since low female reproductive output can contribute to the extinction of populations and species (Groom 1998), differences in PTE among species may produce different species-specific probabilities of population persistence over ecological time scales and different rates of lineage accumulation over evolutionary time scales (Armbruster & Muchhala 2009). Thus, plant traits that result in significantly greater PTE could potentially be key innovations that facilitate species accumulation (Armbruster & Muchhala 2009; Livshultz et al. 2011). Given that plants at low population densities are more likely to experience pollen limitation, and are at higher risk of reproductive failure due to mate-finding Allee effects (Gascoigne et al. 2009; Ghazoul 2005a; Ghazoul 2005b), both at the species and population levels, both selection on individuals within populations and differential extinction of populations may produce a pattern of higher PTE in plant species that typically live at low population densities than in species that live at high densities (Livshultz et al. 2011). The importance of pollination success for plant demography remains to be determined empirically, however, since other factors such as maternal resources, seedling mortality, and clonality may be of greater importance in population persistence.

The observed pollen transfer efficiency in animal-pollinated plants is an emergent property of the interaction among the pollinator community (Thomson 2006; Wilson & Thomson 1991; Young et al. 2007), demographic variables such as population size and density (Ghazoul 2005b; Kunin 1997), the larger plant community which may reduce or facilitate pollination of the target species (Flanagan et al. 2011; Ghazoul 2006), and plant traits such as floral morphology and reward (Castellanos et al. 2003; Kobayashi et al. 1997; Ren & Tang 2010). Pollinia, formed by the aggregation of the pollen contents of anther sacs into single masses, are the pre-eminent example of a plant trait that contributes to elevated PTE (Cruden 2000; Harder 2000; Harder & Johnson 2008; Johnson et al. 2005; Livshultz et al. 2011). Species with pollinia have pollen transfer efficiencies more than an order of magnitude higher than those measured in plants with monads (Harder & Johnson 2008). Pollinia have evolved in only two families of flowering plants,
Orchidaceae and Apocynaceae, but other forms of pollen aggregation (tetrads, polyads, massulae, viscin threads) have evolved at least 39 times among flowering plants (Harder & Johnson 2008). The effect of these alternate forms of pollen aggregation on PTE is largely unknown, since pollen transfer efficiency has been reported for only a single species with pollen tetrads (Harder & Johnson 2008; Wilson 1995). Furthermore, the rarity of pollinia among flowering plants and the fact that the vast majority of species disperse their pollen as monads, suggest that the fitness benefits of aggregated pollen dispersal outweigh the costs only under certain, relatively rare, circumstances (Harder & Johnson 2008; Harder & Thomson 1989).

Apocynaceae are an ideal group for the investigation of the effect of pollen aggregation and other modifications of floral morphology on PTE in a phylogenetic context (Livshultz et al. 2011). Within Apocynaceae, pollinia have evolved at least three times independently: once in the common ancestor of subfamilies Secamonoideae and Asclepiadoideae (the milkweeds) (Livshultz et al. 2007; Straub et al. 2014), and at least twice within subfamily Periplocoideae (Ionta & Judd 2007). Pollen tetrads have likely evolved at least five times independently within the family: at least once in subfamily Periplocoideae (Livshultz et al. 2007; Straub et al. 2014), and at least once within each of the tribes Apocyneae (Livshultz et al. 2007), Alyxiaceae (van der Ham et al. 2001), Melodineae (Van De Ven & Van Der Ham 2006), and Tabernaemontaneae (van der Weide & van der Ham 2012).

In the milkweed flower, pollinia are one of four functionally integrated floral structures that together constitute the highly efficient pollen transfer mechanism. The others are the gynostegium, lignified anther guide-rails, and translators (Fishbein 2001; Livshultz et al. 2007). The gynostegium consists of the adnate androecium and gynoecium (Fig. 1A, B). The anther guide-rails are formed by the adpressed lignified margins of adjacent anthers (Fig. 1B, a-g). Together these two structures function to trap and guide the appendages of flower visitors first to the stigma (Fig. 1B, sc) and then to the pollen for export (Fig. 1B, ps). They are hypothesized to increase the precision of pollen transfer (Fallen 1986) and may also increase PTE. The gynostegium and anther guide-rails evolved prior to pollinia in the most recent common ancestor of the APSA clade (Livshultz et al. 2007), the lineage within which milkweeds evolved, and function together with solitary pollen grains in ca. 700 species classified within the paraphyletic subfamily Apocyneoidae (Endress et al. 2014; Livshultz et al. 2007). Lignified anther guide-rails also evolved independently in tribe Tabernaemontaneae where they function without a gynostegium (Simões et al. 2010; Simões et al. 2007). Translators are structures comprised of hardened, molded style-head secretion that function to attach pollen to a pollinator. Each flower has five translators, each removed as a unit with a load of attached pollen. Translators likely evolved independently in milkweeds and in Periplocoideae (Straub et al. 2014), and possibly also in the apocynoid genera Apocynum and Forsteronia (Leggett 1872; Nilsson et al. 1993), although no functional evidence for the presence of translators in either of these genera has been presented until now.

Comparative study of Apocynaceae species has the potential to elucidate the individual contribution of each of the four structures (gynostegium, guide-rails, translators, pollinia) that constitute the milkweed pollination mechanism to overall PTE. It may also provide an appropriate context for understanding the selective pressures that drove the evolution of the extremely efficient pollen transfer mechanism of milkweeds (Livshultz et al. 2011).

Low pollen to ovule ratio, a trait that has been correlated with high PTE in xenogamous species (Cruden 2000; Cruden & Jensen 1979; Erbar & Langlotz 2005; Harder & Johnson 2008), has been reported in Apocynaceae species from across the phylogeny (range 20–315 outside milkweeds (11 species with monads or tetrads), 2.7–22 within milkweeds (46 species with pollinia)) (Cruden 1977; Darrault & Schlindwein 2005; de Moura et al. 2011; Erbar & Langlotz 2005; Herrera 1991; Lin & Bernardello 1999; Raju & Ramana 2009; Raju et al. 2005; Tanaka et al. 2006; Wyatt et al. 2000). This points to the possibility that efficiency gains from pollinia may not be as large as they appear when milkweeds are compared to “average” xenogamous or facultatively xenogamous flowers, which have mean pollen to ovule ratios of 5859 and 797, respectively (Cruden 1977). The plesiomorphic flower of Apocynaceae, with style-head secretion that glues pollen to floral visitors (Fallen 1986), a basal collar on the style-head that traps pollen (Simões et al. 2007), and tight packing of the anthers and style-head within a narrow-tubed salverform corolla (Fallen 1986), may already possess an elevated PTE as compared to the average non-Apocynaceous xenogamous flower.

As a first step toward the comparative study of the evolution of floral function in Apocynaceae, we here report the PTE of Apocynum cannabinum L. This is the first measurement of PTE in a species of Apocynaceae outside subfamily Asclepiadoideae, and only the second report of PTE in an animal pollinated plant with pollen dispersed in tetrads (Harder & Johnson 2008; Wilson 1995). We also present the first evidence that the adhesive bands in Apocynum flowers function as translators and report which orders of insects remove them.

**Materials and Methods**

**Study taxon**

The genus *Apocynum* belongs to Apocynaceae tribe Apocyneae, a well-supported monophyletic lineage of 23 genera (Endress et al. 2014; Livshultz 2010; Livshultz et al. 2007; Middleton & Livshultz 2012). Within Apocynaceae, the species of *Apocynum* are highly unusual in their morphology, biogeography, and ecology (Livshultz et al. 2011). All four *Apocynum* species are erect perennial herbs or shrubs with pollen dispersed in tetrads (Woodson 1930). Floral morphology of *Apocynum* has been extensively studied and illustrated (Lipow & Wyatt 1999; Nilsson et al. 1993; Omlor 1996; Rosatti 1989; Safwat 1962; Semblad et al. 1998; Woodson 1930). Translators (or “glands” or “plates”) have been reported in three *Apocynum* species based on morphological criteria (Demeter 1922; Leggett 1872; Nilsson
et al. 1993; Omlor 1996; Safwat 1962), but never confirmed by functional analysis. Species of *Apocynum* are components of herb-dominated communities in temperate Asia, Europe, and North America (Rosatti 1989). In contrast, all other species of Apocynaceae are woody lianas with pollen monads occurring in tropical to sub-tropical forests of Asia and Australasia (Livshultz et al. 2007; Middleton 2007). Translators have never been reported in any species of Apocynaceae outside *Apocynum*.

*Apocynum cannabinum* L. is a common component of early successional herbaceous communities across temperate North America (Rosatti 1989). A significant agricultural weed in some regions (Schultz & Burns 1979), it forms large clonal colonies via underground roots (Johnson et al. 1993; Omlor 1996; Safwat 1962), but never confirmed by functional analysis. Species of *Apocynum* are components of herb-dominated communities in temperate Asia, Europe, and North America (Rosatti 1989). In contrast, all other species of Apocynaceae are woody lianas with pollen monads occurring in tropical to sub-tropical forests of Asia and Australasia (Livshultz et al. 2007; Middleton 2007).
Apocynum cannabinum is an obligate out-crosser with a late-acting self-incompatibility system, similar to that of Asclepias and other milkweed genera (Lipow & Wyatt 1999).

The minute flowers, ca. 3-4 mm long, are produced in dichasial inflorescences (Fig. 1A). Each flower has five greenish sepal s and a white-to-greenish urceolate corolla, with five short corolla lobes that diverge at anthesis (Fig. 1A). The five nectaries are situated around the ovary, opposite the corolla lobes and alternating with the stamens (Fig. 1B, n), each nectary positioned below a minute corolline corona lobe located in the lower half of the corolla tube. The five anthers form a tightly closed cone around the style-head (the apex of the gynoecium) via appression of their lignified margins (Fig. 1A, B). The ovoid style-head has a narrow collar (Fig. 1B, c), situated ca. 1/3 of the distance from its apex to its base, and sits atop the two half-inferior ovaries (Fig. 1B, o). The gynostegium is formed by adhesion of each anther (Fig. 1B, a) via an adaxial patch of hairs (Fig. 1B, r) in the shape of an inverted V (termed a “retinacle”) to the style-head collar (Fig. 1B, c). The two pollen sacs (Fig. 1B, ps) are on the adaxial side of the anther, above the retinacle. The five closely packed anthers and the distal surface of the style-head, the “style-head apex” (Fig. 1B, sa), form a closed chamber that retains the pollen, which remains in tetrads at maturity. We confirm that the vast majority of pollen tetrads are retained within the pollen sacs at and after anthesis (Lipow & Wyatt 1999), and are not shed onto the apex of the style-head as has been reported (Rosatti 1989). The location of the pollen sacs, inside a closed pollen chamber distal to the retinacle and style-head collar, separate from the stigmatic surface (Fig. 1B, st) of the style head, which is situated below the collar (Fallen 1986; Lipow & Wyatt 1999), prevents both pollen loss from the anthers (e.g. via wind agitation) and autonomous pollen transfer to the stigma. The only way pollen can exit the flower is by way of removal by an animal visitor, and the only way pollen tetrads can move to the stigma is via the action of an animal pollinator. A rectangular band of yellowish style head adhesive (Fig 1C, D, E, ab), with an extremely viscous and gummy texture at anthesis, is formed in each of the five alternating staminal zones of the style-head apex, distal to the style-head collar. At anthesis, the bands of style-head adhesive are often found attached to the anthers, below the proximal pollen sacs of two adjacent anthers, spanning the narrow space in between them, and unattached to the style-head. These adhesive bands have been termed “translators” (Nilsson et al. 1993), but will be referred to as “adhesive bands” in this paper since a “translator” is defined by function (removal as a single unit with a load of pollen) and not by structure.

Pollen transfer in Apocynum (and most other species in the paraphyletic subfamily Apocynoideae) has been hypothesized to occur via trapping and guidance of insect appendages by the appressed lignified anther guide-rails (Fig. 1B, ag) as the insect reaches toward the base of the flower for nectar (Fig. 1B, n). Once the insect appendage is trapped between the bases of the anther guide-rails, the insect pulls up to escape and is guided first to the stigma, depositing any attached pollen (from previous floral visits), then to the adhesive band, and finally to the pollen in the pollen sacs (Fallen 1986; Rosatti 1989; Waddington 1976). Species of five insect orders have been recorded as floral visitors of A. cannabinum, but only butterflies (10 species) have been reported as carrying pollen attached to their proboscises (Waddington 1976). Our observations of appendages characteristic of Hymenoptera attached to pollen clumps deposited on stigmas, indicate that other insect orders are also likely legitimate pollinators (Fig. 1C, D, i). Some insects cannot escape once trapped by the anther guide-rails (Bailey 1874).

Floral development

Late stage floral development was followed on June 22-30, 2010 in one population, Lemon Hill, Philadelphia. Six buds from each of fourteen haphazardly selected inflorescences (84 buds total) were marked with an oil paint marker on the pedicel, and their development was followed on a daily basis until they abscised, the corolla withered, and/or they began developing into fruits. Developing fruits were confirmed on July 2, 2010. Censuses were conducted daily between 6 and 10 AM.

To determine whether flowers that abscised in the closed hydrated state were more likely to have been pollinated than those that abscised after the corolla withered, we made bulk collections of closed hydrated flowers and closed withered flowers, and scored each flower for the presence or absence of pollen clumps on the stigma.

Sampling for estimation of population pollen transfer efficiency

Inflorescences were collected from three populations, referred to as populations A, B, and C, in Burlington County, New Jersey on July 16, 2009. Two of these populations were located in the vegetation margin between a paved road and a planted agricultural field; the third was located in the vegetation margin between a paved road and a mown lawn. Insects, including Hymenoptera and Lepidoptera, were observed visiting flowers in all three populations on the day of collection. One to three inflorescences were collected from each flowering stem in the population and preserved in 70% ethanol (added to each sample within 24 hours of collection) and stored at 4°C.

Inflorescences from 6 flowering stems were selected haphazardly from each population, and all post-anthesis flowers with turgid, unwithered corollas (Fig. 1A) were placed in a 50 mL Falcon tube with 70% ethanol; each sample was therefore limited to flowers that had closed 0-3 days prior to the collection date (Fig. 2). Five post-anthesis flowers were selected haphazardly from each sample for quantification of pollen removal and deposition. Flowers that showed evidence of florivory were excluded. In total, pollen removal and deposition were quantified for 90 flowers (3 populations × 6 flowering stems/population × 5 flowers/flowering stem).

Pollen and ovule counts

We used two ultra-fine forceps to disperse the pollen in a drop of Caliber’s fluid (Dafni 1992) on a microscope slide; basic fuchsin stained the pollen pink. After the dissection, a drop of fluid was applied to the tips of each forceps and released into the drop on the slide to remove any pollen clinging to the forceps. A cover slip was added and the slide
scanned systematically under 100× magnification with a compound microscope, counting all tetrads using a thumb tally counter. Ovules were counted by dissecting out the placenta of one ovary and using two ultra-fine forceps to tear it up and release the ovules into a drop of water on a microscope slide. A cover slip was added, the slide scanned systematically under 50× magnification with a compound microscope, and all ovules were counted.

**Pre-anthesis pollen content** of flowers from each of the same 18 sampled flowering stems was estimated by counting all the tetrads from one undehisced anther from one bud and multiplying by 5, the number of anthers. The largest bud with undehisced anthers (the one chronologically closest to the post-anthesis flowers) was selected for counting.

**Pollen to ovule ratio** was calculated for 11 of the 18 flowering stems by also counting all the ovules in one of the two ovaries from the same bud and multiplying by 2. The number of tetrads (four pollen grains in each) was multiplied by 4 to obtain the number of pollen grains, which was then divided by the number of ovules in the flower.

**Pollen removal** was estimated by subtracting the pollen remaining in a post-anthesis flower from the calculated pre-anthesis pollen content of a bud from that same flowering stem. The amount of pollen remaining in each post-anthesis flower was assessed in two ways: first by scoring, and then by counting. Scoring was accomplished under a dissecting microscope (20-30×) by carefully dissecting out the five anthers and laying them, pollen sacs up, on a slide. The pollen content of each of the 10 pollen sacs (2 per anther) was estimated as 0 (no pollen remaining), 0.33 (some pollen remaining), 0.67 (some pollen removed) and 1 (no pollen removed). Late flower buds (pre-anthesis but with anther sacs already dehisced) were used as the reference point for the four scores. The estimated pollen contents of the 10 pollen sacs were summed and divided by two to obtain the number of full anther equivalents of pollen remaining in the flower; this estimate was multiplied by the estimated pre-anthesis pollen content to obtain the pollen remaining. In a few flowers, clumps of tightly packed pollen were found outside the pollen sacs but inside the chamber formed by the attachment of the anthers to the style-head. Each of these clumps was broken up, and counted and added to the total of pollen remaining.

Pollen remaining was both estimated and counted for 18 flowers, one from each of the sampled flowering stems. Linear regression showed that the estimate closely predicted the count (Fig. 3A). Thus, pollen remaining was scored but not counted for the remaining 62 flowers. The linear regression equation derived from the 18 flowers that were both scored and counted was used to calculate pollen remaining for all 90 flowers. Pollen clumps found inside the anther chamber were always counted and the count added to the estimate.

**Pollen deposition.** For each of the 90 post-anthesis flowers, the number of pollen clumps on the stigma (Fig. 1B-D, p) was counted, the presence of pollen tubes noted, and the number of pollen tetrads in all clumps counted (Fig. 1E). Solitary pollen tetrads on the stigma were not counted since their presence was likely an artefact of the process of dissecting the flower. Pollen tubes were never observed from any solitary pollen tetrad on the stigma even though both compatible and incompatible pollen germinates on the stigmas of *Apocynum cannabinum* (Lipow & Wyatt 1999).

**Pollen transfer efficiency (PTE)** was calculated per flower, following the design of Harder & Johnson (2008), and defined as $PTE = \frac{\text{pollen deposited on stigma}}{\text{pre-anthesis pollen content} \times \text{post-anthesis pollen content}}$. For each population, and for the species, PTE was calculated by pooling pollen export and import for all flowers using the formula:

$$PTE = \frac{1}{\Sigma(\text{pollen deposited on stigma})/\Sigma(\text{pre-anthesis pollen content} \times \text{post-anthesis pollen content})}.$$

**Adhesive band removal and deposition.** The number of missing adhesive bands (from 0 to 5) was scored for each of the 90 flowers scored for pollen removal and deposition. The proportion of pollen clumps deposited with adhesive bands was not recorded for these 90 flowers. Instead, post-anthesis flowers from one population were dissected until 20 flowers with deposited pollen clumps were observed. For each of these 20 flowers, the pollen clumps and attached stigmatic regions were dissected out with minimal disturbance, placed on slides with Calberla’s fluid (Dafni 1992), topped with a coverslip, and examined with 100× magnification for presence of a yellowish mass of adhesive attached to the clump of tetrads (Fig. 1E, ab).
Adhesive band removal was also scored for virgin flowers presented to single insect visitors in the mid-morning to early afternoon of July 2 and 4, 2010. Inflorescences at the Lemon Hill population were covered with fine mesh cloth for 48 hours, and then uncovered and observed until one insect probed for nectar; some insects revisited individual flowers multiple times before moving on to a different inflorescence. After the insect departed the inflorescence, all open flowers were immediately collected into 70% alcohol. The flowers were transferred to 100% alcohol for ca. 10 minutes prior to dissection to harden the adhesive bands so that they would not stretch and break when the anthers were pulled apart, as we observed when flowers preserved in 70% ethanol were dissected. The number of bands remaining in each flower was scored, and any partial or broken bands noted. Voucher specimens of plants and insect visitors to *A. cannabina*um are deposited in the collections of the Academy of Natural Sciences of Drexel University.

**Statistical analyses**

We used the Pearson *χ²* test of goodness of fit to test if flowers that abscised in the closed hydrated state were more likely to have been pollinated than those that abscised after the corolla withered, linear regression to estimate the correlation between pollen estimates and pollen counts, and Shapiro-Wilk *W* to test for normality of distributions. For normally distributed data (*pollen tetrad removed*), we used 1-way ANOVA and Student’s T-tests to test for statistical significance. For data that are not normally distributed (*pollen tetrad and pollen clumps deposited, adhesive bands removed, pollen transfer efficiency*), we used the Kruskal-Wallis *χ²* test. All statistical analyses were conducted with the Analyse-it Standard Edition plug-in (Analyse-it Software, Ltd.) for Microsoft Excel (Microsoft Corporation).

**Comparative data from other species**

Pollen to ovule ratios of Apocynaceae species (Appendix I) were taken from Erbar & Langlitz (2005) citing the work of Ali & Ali (1989); Christ et al. (2001); Crueden (1977); Lohne et al. (2004); Torres & Galetto (1999); Wyatt et al. (2000) and supplemented with counts from de Araujo et al. (2011); Darrault & Schlindwein (2005); de Moura et al. (2011); Herrera (1991); Lin & Bernardello (1999); Raju & Ramana (2009); Raju et al. (2005). Pollen transfer efficiencies (Fig. 4, Appendix II) were taken from Harder & Johnson (2008) and articles cited by them (Aizen & Raffaele 1996; Broyles & Wyatt 1995; Freitas & Paxton 1998; Galen & Stanton 1989; Harder & Thomson 1989; Hei & Suzuki 2001; Kunze & Liese 1991; Lipow & Wyatt 1998; Ollerton et al. 2003; Paw 1998; Snow & Roubik 1987; Tanaka et al. 2006; Vieira & Shepherd 2002; Webb & Bawa 1983; Wilson 1995; Wyatt 1976; Young & Stanton 1990) and additional articles located by searches in Google Scholar on the phrases “pollen transfer efficiency” and “pollination efficiency” (Castellanos et al. 2003; Conner et al. 1995; Ren & Tang 2010; Shuttleworth & Johnson 2006; Shuttleworth & Johnson 2008; Shuttleworth & Johnson 2009; Thostesen & Olesen 1996; Wolff et al. 2008; Young et al. 2007). Studies were included if they reported both removal and deposition of pollen grains. When pollen transfer efficiencies were reported for multiple populations of a species, the populations with the lower values were excluded if evidence was presented that the pollination environment for that population was less than optimal (e.g. due to absence of the most effective pollinator [Shuttleworth & Johnson 2008] or due to pollinator behaviour [Young et al. 2007]). The pollen transfer efficiency for the species was then calculated as the mean of all the other populations. For *Impatiens capensis* Meeth. (Balsaminaceae), the pollen transfer efficiency measured by Young et al. (2007), 0.64% for nectar collecting *Apis mellifera*, almost an order of magnitude higher than that measured by Wilson & Thomson (1991), 0.088% for nectar collecting *Bombus impatiens*, was included.

**RESULTS**

**Flower development**

Seventy-eight of the 84 buds followed developed without obvious damage. Six that showed evidence of chewing or premature wilting were excluded from analysis. The most common developmental sequences are summarized in Fig. 2. In 73 of the 78 intact flowers (93%), the corolla lobes were reflexed on the first day of anthesis (Fig. 2, day 1). In five (5) flowers (7%), anthesis was not observed; they were buds on day 0, and the lobes were already closed when censused on day 1. Of the 73 flowers observed at anthesis on day 1, 61 (83%) were closed on day 2, while 12 (17%) were still partially open, the lobes parallel or not quite closed. Two of these 12 were still not quite closed on day 3 but closed on day 4. Thus, anthesis lasts <24 hours for 7% (5 of 78) flowers, ca. 24 to <48 hours for 78% (61 of 78) of flowers, >24 but <96 hours for 15% (12 of 78) flowers.

Once the lobes closed, the corolla remained turgid and well-hydrated for two days (2 flowers, 3%), three days (46 flowers, 59%), or four days (30 flowers, 38%) (Fig. 2, day 2-4/5). During these days when the lobes were closed but the corolla still turgid, many flowers began to nod due to geotropic curvature of the pedicel, corollas changed colour from white/cream to yellowish and finally brownish, and pedicels changed from pale green to greenish-yellow. Finally, on day 4 or 5, the whole flower was gone (59%, 46 of 78 flowers), presumably abscised in the 24 hours between censuses (Fig. 2, 46 flowers abscised), or the corolla withered, became dry and shrivelled (Fig. 2, day 5/6-7). Once the corolla shrivelled, either the flower was easily dislodged with a touch within one or two days (36%, 28 flowers) (Fig. 2, 28 flowers abscised), or fruit initiation was detected within four days of withering (4%, 3 flowers) (Fig. 2, day 8), or neither abscission nor fruit initiation were detectable after six days with a withered corolla (1%, 1 flower).

The three flowers that initiated fruit were each at anthesis on day 1, closed and nodding with turgid corollas on days 2-4, and with withered corollas on day 5. Unlike the flowers that abscised, however, the pedicels were noticeably green on day 5. The flowers remained in this state, nodding, withered corolla, green pedicel, until day 8, when the base of the calyx was noticeably enlarged (Fig. 2, day 8). On day 10, nine days after anthesis, a pair of initiating fruits was visible as the

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*LIVSHULTZ ET AL.*

*J Poll Ecol 22(4)*
enlarging ovaries pushed the withered corolla away from the receptacle (Fig. 2, day 10).

We collected 22 flowers with a hydrated corolla and 25 flowers with a withered corolla that all fell with a gentle touch. Of the 22 hydrated flowers, 14 were pollinated, seven were not, and one was damaged by herbivory and not counted. Of the 25 withered flowers, 20 were pollinated, four were not pollinated, and one was excluded due to herbivory. The pollination rate for flowers with hydrated versus withered corollas was not significantly different (Pearson $\chi^2 = 1.68$, $DF = 1$, $P = 0.19$).

**Pollen and ovules**

Buds contained an average of $1536 \pm 28$ S.E. ($N = 18$ buds) tetrads or $6144 \pm 112$ S.E. monads ($N = 18$ buds) and $328 \pm 19$ S.E. ($N = 11$ buds) ovules. The pollen to ovule ratio (monads per ovule) is $19.8 \pm 1.3$ S.E. ($N = 11$ buds).

**Pollen removal.** Estimates and counts of tetrads remaining in post-anthesis flowers were highly correlated ($r^2 = 0.89$, $N = 18$ flowers) (Fig. 3A). The regression equation $y = 196.7 + 0.816x$ was used to estimate pollen remaining.

Pollen removal from the 10 pollen sacs within a flower was typically very heterogeneous. Both full and empty sacs were found within a single flower. The two adjacent sacs of two adjacent anthers were usually similar, i.e. either both full or both empty, while the two pollen sacs within a single anther were often different, i.e. one full and one empty.

The average post-anthesis flower had $612 \pm 274$ S.D. tetrads removed (Fig. 3B). The distribution was not significantly different from normal (Shapiro-Wilk $W = 0.98$, $DF = 89$, $P = 0.30$). The average number of tetrads removed per flower varied among populations (Tab. 1) from $508 \pm 269$ S.D. (population A) to $744 \pm 295$ S.D. (population B).
TABLE 1. Comparison of three populations of *Apocynum cannabinum* for pollen removal, deposition, transfer efficiency, and adhesive band removal. Means or distributions that are not significantly different (*P > 0.05*) are designated with the same superscript letter. Six (6) flowering stems and 5 flowers per flowering stem were sampled per population. Significance of differences between populations was tested with one-way ANOVA (tetrads removed) or with the non-parametric Kruskal-Wallis test (all other parameters).

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<td>Tetrad removed (mean per flower)</td>
<td>508 ± 269 S.D.</td>
<td>744 ± 295 S.D.</td>
<td>584 ± 201 S.D.</td>
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<tr>
<td>Pollen clumps deposited (mean per flower)</td>
<td>0.40 ± 0.56 S.D.</td>
<td>1.0 ± 0.79 S.D.</td>
<td>0.60 ± 0.68 S.D.</td>
</tr>
<tr>
<td>Tetrads deposited (mean per flower)</td>
<td>25 ± 45 S.D.</td>
<td>75 ± 67 S.D.</td>
<td>44 ± 56 S.D.</td>
</tr>
<tr>
<td>Pollen transfer efficiency (mean % per flower)</td>
<td>5.6 ± 13.3 S.D.</td>
<td>11.6 ± 13.7 S.D.</td>
<td>8.4 ± 12.9 S.D.</td>
</tr>
<tr>
<td>Population pollen transfer efficiency (%)</td>
<td>4.9</td>
<td>10.1</td>
<td>7.6</td>
</tr>
<tr>
<td>Adhesive bands removed (mean per flower)</td>
<td>1.4 ± 1.2 S.D.</td>
<td>2.2 ± 1.3 S.D.</td>
<td>2.1 ± 0.86 S.D.</td>
</tr>
</tbody>
</table>

Populations A and B were significantly different from each other (1-way ANOVA, *F* = 6.56, *DF* = 2, *P* = 0.0022, difference between means = 236 tetrads, 95% CI 74 to 399, *P* < 0.05), but neither was significantly different from population C (Tab. 1).

**Pollen deposition.** Pollen was deposited below the style head collar in distinct clumps producing a tangle of pollen tubes (Fig. 1B-D). Forty-three (48%) of 90 post-anthesis flowers had no deposited pollen clumps, 36 (40%) had one deposited pollen clump, 10 (11%) had two deposited pollen clumps, and one (1%) had three deposited pollen clumps. The average post-anthesis flower had 48 ± 60 S.D. tetrads deposited, but the distribution is highly left skewed (Shapiro-Wilk *W* = 0.80, *DF* = 89, *P* < 0.0001) (Fig. 3C). Flowers with one pollen clump deposited had significantly more tetrads removed (709 ± 249 S.D.) than flowers with no deposited pollen clumps (511 ± 259 S.D.) (*t* = 3.45, *DF* = 75.5, 1-tailed *P* = 0.0005) (Fig. 3D).

The average number of clumps and tetrads deposited per flower varied among populations (Tab. 1): 0.40 ± 0.56 S.D. clumps and 25 ± 45 S.D. tetrads (population A) to 1.0 ± 0.79 S.D. clumps and 75 ± 67 S.D. tetrads (population B). Populations A and B were significantly different from each other for both number of clumps (Kruskal-Wallis test *χ*² = 10.49, *DF* = 2, *P* = 0.0053, mean rank difference = 19, *P* = 0.0041) and number of tetrads deposited (Kruskal-Wallis test *χ*² = 10.81, *DF* = 2, *P* = 0.0045, mean rank difference = 21, *P* = 0.0028), but neither was significantly different from population C (Tab. 1).

**Pollen transfer efficiency per flower varied from 5.6 ± 13.3% S.D.** (population A) to 11.6 ± 13.7% S.D. (population B). It was significantly different between these two populations (Kruskal-Wallis test *χ*² = 6.40, *DF* = 2, *P* = 0.041, mean rank difference = 16, *P* = 0.035) but neither was significantly different from population C (Tab. 1).

Population pollen transfer efficiency varied from 4.9% (population A) to 10.1% (population B) (Tab. 1). Species-level pollen transfer efficiency is 7.9% when all 90 flowers are pooled, 7.5% when the three populations are averaged. The species-level pollen transfer efficiency falls in the 24th percentile of pollen transfer efficiencies reported for 36 species of milkweeds in subfamily Asclepiadoideae (mean = 26%, median = 20%, Fig. 4).

**Adhesive band removal** varied from zero (seven flowers, 8%) to five (three flowers, 3%) bands missing per flower, with most flowers missing either one (29 flowers, 32%) or two adhesive bands (31 flowers, 34%). Significantly more tetrads had been removed from flowers with two missing adhesive bands (660 ± 241 S.D.) than from flowers with one band missing (465 ± 219 S.D.) (*t* = 3.28, *DF* = 58, 1-tailed *P* = 0.0009) (Fig. 3E).

Population A had significantly fewer bands removed per flower than both populations B and C (Kruskal-Wallis test *χ*² = 10.91, *DF* = 2, *P* = 0.0043; A versus B mean rank difference = 17.9, *P* = 0.0135; A versus C, mean rank difference = 19.1, *P* = 0.0075) while populations B and C are not significantly different (Tab. 1).

Of 78 virgin flowers visited by bumblebees (*Bombus* sp.), 11 had adhesive bands missing (10 with four of five bands remaining, one with only one band remaining), as did two of 22 virgin flowers visited by small bees (one with three and one with four bands remaining). Two flowers visited by butterflies (*Pieris rapae*) did not have missing adhesive bands. One flower visited by a wasp had three intact bands remaining along with fragments of broken bands in the other positions.

**Adhesive band deposition.** Of 20 pollen clumps dissected out from stigmas, 19 had yellowish adhesive bands attached (Fig. 1B, ab). In the 20th flower, the adhesive band along with a part of the pollen clump was not attached to the stigma but stuck in the hairs of the retinacle and on the stamen filaments, below the attachment of the retinacles to the style head collar (Fig. 1A). Among the pollinated flowers, four were discovered...
with insect appendages still attached to the pollen clumps, the adhesive bands sandwiched between the appendage and the pollen (Fig. 1C, D).

**DISCUSSION**

Selection for increased pollen transfer efficiency (PTE) may have been an important force in shaping the evolution of flowers. Milkweeds and orchids (Orchidaceae), the two plant lineages with pollinia, have average PTE around 25% (Harder & Johnson 2008), versus 1.3% in species with monads (Fig. 4). However, such broad comparisons can confound efficiency gains due to pollinia with those resulting from other floral novelties. Flowers of *Apocynum cannabinum* (Apocynaceae), like those of milkweeds, have gynostegia and lignified anther guide-rails, but they produce pollen tetrads rather than pollinia and simple bands of style head adhesive instead of complex pollinium-carrying translators. To provide a phylogenetically appropriate comparison to quantify efficiency gains from the evolution of pollinia, we measured PTE in *A. cannabinum*. We also tested the frequently proposed hypothesis (Nilsson et al. 1993) that the adhesive bands in flowers of *Apocynum cannabinum* are translators functionally similar to those found in flowers of milkweeds and Periplocoideae. We compiled reports of pollen to ovule ratios, a correlate of PTE (Harder & Johnson 2008), from Apocynaceae to investigate when elevated PTE may have evolved in the family.

**Pollen transfer efficiency** of *Apocynum cannabinum* is 7.9%. This is in the 24th percentile of pollen transfer efficiencies reported for 36 species of milkweeds in subfamily Asclepiadoideae (mean = 26%, median = 20%, Fig. 4). It is more than twice as high as any pollen transfer efficiency reported for animal-pollinated angiosperms with monads (mean = 1.3%, median = 0.64%, range = 0.07-3.4%, N = 14 species), but less than that reported for *Drosera tracyi* (13%), the only other species with tetrads where pollen transfer efficiency has been measured (Fig. 4, Appendix II) (Harder & Johnson 2008; Wilson 1995).

These results are consistent with the following hypotheses: 1) Apocynaceae flowers without pollinia have elevated pollen transfer efficiency conferred by other floral novelties such as the gynostegium and style head adhesive; 2) evolution of pollinia in milkweeds resulted in even higher pollen transfer efficiency (Fig. 4); and 3) intermediate levels of pollen aggregation (tetrads) elevate pollen transfer efficiency (Fig. 4). Any statistical test of these statements requires much more data on pollen transfer efficiency in other species of Apocynaceae and in other angiosperm families. Outside the orchids and milkweeds, where pollinia greatly simplify the task, pollen transfer efficiency has rarely been measured in a way that permits comparison across species (Harder & Johnson 2008), despite the fact that selection for pollen transfer efficiency may drive selection on many plant traits and may be an important factor in plant demography and population dynamics. Of particular interest for Apocynaceae, is comparison of flowers of *Apocynum*, which have been proposed as a model for the early ancestors of milkweeds (Livshultz et al. 2011), to other species in the APSA clade with pollen in monads and with undifferentiated style head adhesive, proposed as appropriate models for the ancestral flowers from which early milkweeds diverged.

**Translators**

The pollen translators of milkweeds and Periplocoideae develop via restricted secretion and hardening of the style-head adhesive present in all Apocynaceae (Kunze 1993; Kunze 1994; Omlor 1996; Safwat 1962; Schick 1982). Each of the five translators is removed as a discrete unit along with the
contents (pollinia or tetrads) of the adjacent pollen sacs of two adjacent anthers. In milkweeds, the translator is usually broken and remains attached to the insect when the pollinium is deposited, although pollinia can be deposited with all or part of the translator still attached (Kunze 1991). To our knowledge, the mode of pollen deposition has never been reported for any species of Periplocoideae.

The five adhesive bands of *Apocynum* (Fig. 1C, D), also illustrated in Nilsson et al. (1993); Omlor (1996); Safwat (1962), function in fundamentally the same way as translators of milkweeds but with less precision. The adhesive bands are removed as discrete units. Virgin flowers visited by insects usually had zero (87 flowers) or one (11 flowers), rarely up to four (two flowers) adhesive bands removed. Fragmentation and possible partial removal of adhesive bands was detected in only one flower, visited by a wasp, bringing into question whether wasps are legitimate pollinators of *Apocynum*.

The two adjacent pollen sacs of two adjacent anthers are frequently either both full or both empty in post-anthesis flowers, similar to the situation in milkweeds where pollinia are either removed or not. Removal of two adhesive bands from a flower is linked to significantly more pollen removal than removal of only one adhesive band (Fig. 3E). On average, removal of a second adhesive band resulted in removal of 195 additional tetrads, 660 ± 241 S.D. versus 465 ± 219 S.D. (Fig. 3E), about 63% of the pollen content of an average anther (307 tetrads) with considerable scatter around the mean (Fig. 3E). Overall, however, pollen removal per flower fits a continuous, normal distribution (Fig. 3B). This indicates that both removal of pollen without an adhesive band and removal of an adhesive band with less than the full pollen contents of two adjacent anther sacs occur frequently, making this pollen transfer mechanism less precise than that of milkweeds where removal of one translator always results in removal of two (Asclepiadoideae) or four (Secamonoideae) pollinia.

The pattern of pollen deposition (Fig. 3C) is likewise similar but less precise than that of milkweeds where each flower is either unpollinated or receives at least one pollinium, each with sufficient pollen to fertilize all ovules in the flower (Wyatt et al. 2000). Almost half of *Apocynum* flowers (43, 48%) received no pollen clumps while 24 flowers (27%) received 82 or more tetrads (Fig. 3C), sufficient to pollinate all ovules in an average flower, 328 ± 19 S.E. ovules. The largest single deposited pollen clump we discovered had 158 tetrads; and butterflies have been reported to carry clumps of up to 123 tetrads (Waddington 1976).

Nineteen of 20 pollen clumps dissected out from stigmas were deposited with adhesive bands attached (Fig. 1E). Given the structural simplicity of these bands in *Apocynum*, it was not possible to determine whether some of the adhesive remained on the pollinator or not. When an insect appendage was left behind with the pollen clump (Fig 1C, D), the adhesive band was clearly visible attaching the pollen clump to the pollinator (Fig. 1D, ab).

Confirmation that simple bands of style-head adhesive function as translators in *Apocynum cannabinum* makes it more likely that similar structures in *Forsteronia affinis* (Meeschiteae) (Nilsson et al. 1993) are also translators. This would imply multiple independent origins of simple translators. A careful survey of species from across the Apocynaceae to better document the occurrence of these minute and easily overlooked structures is required, along with additional functional studies.

The pollen to ovule ratio of *Apocynum cannabinum*, 19.8 ± 1.3 S.E., is lower than that reported for any species of Apocynaceae with pollen in monads (range 31-204, N = 9 species) but in the 94th percentile of ratios reported for milkweeds of subfamily Asclepiadoideae (mean = 9.6 ± 0.7 S.E., range = 2.7-21.9, N = 46 species), consistent with the angiosperm-wide trend of lower pollen to ovule ratios in species with more highly aggregated pollen dispersal (Harder & Johnson 2008). Interestingly, both the highest [315 for *Periploca aphylla* (Wyatt et al. 2000)] and among the lowest [20 for *Decalepis hamiltonii* (Raju & Ramana 2009)] ratios in Apocynaceae are reported for species of Periplocoideae. *Periploca* has translators that carry loose tetrads (Verhoeven & Venter 2001) while *Decalepis* has translators that carry four pollinia of aggregated tetrads (Raju & Ramana 2009; Verhoeven & Venter 1998). A possible interpretation of this is that the pollination mechanism of *Periploca* may result in less aggregated pollen deposition (i.e. a few tetrads deposited in each of many flowers) than that of other Apocynaceae and thus select for greater pollen production via increased pollen competition (Harder & Johnson 2008; Queller 1984). A corollary of this hypothesis is that multiple paternity should be more frequent in fruits of *Periploca* than of other Apocynaceae. Unfortunately, pollen deposition and paternity has never been studied in any species of Periplocoideae. Further studies are also necessary to understand why Apocynaceae overall have such low pollen to ovule ratios and to what extent these are linked to aggregated pollen deposition and/or elevated pollen transfer efficiency.

**Population variation**

All measures of pollination success including pollen removal and deposition, translator removal, and pollen transfer efficiency were significantly higher in population B than in population A with population C intermediate (Tab. 1). The population level pollen transfer efficiency varied two fold between population A (4.9%) and B (10.1%). Large differences among populations in pollen transfer efficiency (1.9% versus 13-16%) were detected in the South African milkweed *Xysmalobium undulatum* and attributed to the low abundance of the more effective pollinator (pompadul wasps) at the site with low pollen transfer efficiency (Shuttleworth & Johnson 2008). Even the same species of pollinator can produce significantly different pollen transfer efficiencies with different behaviours (Young et al. 2007). Likewise, population density and the larger plant community can affect pollen transfer efficiency (Ghazoul 2005a; Ghazoul 2005b; Ghazoul 2006).

In spite of the diversity of factors other than floral morphology that impact on pollen transfer efficiency, the pollen transfer efficiency measured for population A (4.9%) was still higher than the highest measured for any angiosperm with monads, 3.4% for *Campsis grandiflora* (Bignoniaceae) pollinated by vespid wasps (Ren & Tang 2010). Thus, while
pollen transfer efficiency is an emergent property of the interaction of floral morphology with many ecological factors, the role of floral morphology (which is likely much more constant among populations than the other parameters) can be elucidated by studying multiple populations of a species and/or the same population at multiple time points. Furthermore, even cursory studies of multiple species with a shared floral trait can produce a distribution that shows how the trait performs on average across a diversity of habitats, pollinator assemblages, population densities, etc. (Fig. 4).

Pollinators of Apocynum cannabinum.

Species of Hymenoptera, Diptera, Coleoptera, and Hemiptera have been recorded as floral visitors to Apocynum cannabinum (syn. Apocynum sibiricum Jacq.), but only species of Lepidoptera have been previously reported as carrying pollen (Waddington 1976). Our observations, including the deposition of pollen clumps with attached appendages that could not have come from butterflies (Fig. 1C, D) and removal of translators by bumblebees and small bees after visits to virgin flowers, suggest that Apocynum cannabinum has a more generalized pollination system than previously reported, although the importance of each of these different groups of insects to the male and female fitness of A. cannabinum remains to be determined.

Flower development and sampling for pollen transfer efficiency

Understanding late stage flower development (from anthesis to fruit initiation) and how it is modified by pollination is important for unbiased sampling of flowers for estimation of population level pollen transfer efficiency. In milkweeds, pollen transfer efficiency has typically been estimated by scoring the standing population of open flowers for estimation of population level pollen transfer efficiency. In milkweeds, pollen transfer efficiency has typically been estimated by scoring the standing population of open flowers for pollen removal and deposition (Ollerton et al. 2003; Shuttleworth & Johnson 2008; Wyatt 1976). This provides an unbiased sample of pollinated and unpollinated flowers only if anthesis of flowers is neither abbreviated nor extended by pollination; something that has not been reported in any study that measured pollen transfer efficiency in milkweeds. Our preliminary studies of several Apocynaceae species show that anthesis may be either extended or truncated by pollination (Livshultz, unpublished), potentially a widespread phenomenon across flowering plants (Fung & Thomson 2017).

Because anthesis of Apocynum cannabinum flowers is brief (ca. 24 to 48 hours, Fig. 2), we sampled recently closed flowers (still turgid) to estimate pollen transfer efficiency since the ratio of pollen deposition to pollen removal is likely to increase through the life-span of a one-day flower. The vast majority (97%) of flowers whose development was followed in our census were in this closed, turgid state for three to four days (Fig. 2, day 2-4/5), including both flowers that ultimately abscised without change of corolla hydration and flowers whose corollas ultimately withered prior to either abscission or fruit initiation (Fig. 2). Turgid flowers that abscised with a gentle touch were not significantly more likely to be pollinated than flowers with withered corollas that abscised with a gentle touch. We thus have no evidence that the standing population of closed turgid flowers was either depleted or enriched for pollinated flowers.

Conclusions

The measured pollen transfer efficiency of Apocynum cannabinum, 7.9%, fits neatly with predictions from floral morphology. It is less efficient than the average Apocynaceae species with pollinia (milkweeds, 26%), but more efficient than an average flower with monads (1.25%) (Fig. 4). The elevated efficiency of Apocynum cannabinum is likely due to the combined function of floral structures shared by Apocynum and milkweeds: gynostegium, lignified anther rails, aggregated pollen, and translators (Fig. 1). These structures occur in various combinations among species of Apocynaceae, making them an ideal group for comparative study of functional floral morphology.

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Appendices

Additional supporting information may be found in the online version of this article:

Appendix I. Literature sources for pollen: ovule ratios of Apocynaceae.

Appendix II. Literature sources for pollen transfer efficiencies (PTE) in Fig. 4.

References


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