Stigmatic exudate in the Annonaceae: Pollinator reward, pollen germination medium or extragynoecial compitum?

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Abstract Although “dry-type” stigmas are widely regarded as ancestral in angiosperms, the early-divergent family Annonaceae has copious stigmatic exudate. We evaluate three putative functions for this exudate: as a nutritive reward for pollinators; as a pollen germination medium; and as an extragynoecial compitum that enables pollen tube growth between carpels. Stigmatic exudate is fructose dominated (72.2%), but with high levels of glucose and sucrose; the dominance of hexose sugars and the diversity of amino acids observed, including many that are essential for insects, support a nutritive role for pollinators. Sugar concentration in pre-receptive flowers is high (28.2%), falling during the peak period of stigmatic receptivity (17.4%), and then rising again toward the end of the pistillate phase (32.9%). Pollen germination was highest in sugar concentrations <20%. Sugar concentrations during the peak pistillate phase therefore provide optimal osmolarity for pollen hydration and germination; subsequent changes in sugar concentration during anthesis reinforce protogyny (in which carpels mature before stamens), enabling the retention of concentrated exudate into the staminate phase as a pollinator food reward without the possibility of pollen germination. Intercarpellar growth of pollen tubes was confirmed: the exudate therefore also functions as a suprastylar extragynoecial compitum, overcoming the limitations of apocarpy.

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INTRODUCTION

The earliest angiosperm flowers are likely to have relied on pollen grains as a nutritive reward for pollinators (Friis et al. 2011). Although the secretion of sugar-rich floral nectar is widespread in angiosperms—with the independent evolution of specialized nectary tissues associated with many different organs (Bernardello 2007)—the earliest floral nectar is likely to have been stigmatic (Lloyd and Wells 1992; Endress 1994). Stigma types have long been classified as either “wet” or “dry”, with the former characterized by a fluid secretion when receptive, whereas the latter has a proteinaceous extracellular pellicle layer overlying the stigma surface and generally lacks any free-flowing secretion (Heslop-Harrison and Shivanna 1977). Although there are many reports of early-divergent angiosperms with visible stigmatic exudate (e.g. Endress 1990, 2001), the functional interpretations of this have recently been reappraised: many lineages that were previously regarded as having wet stigmas are now regarded as dry, including those in the “ANITA” grade (Amborellaceae: Thien et al. 2003; Hydatellaceae: Prychid et al. 2011; Cabombaceae: Galati et al. 2016; Nymphaeaceae: Heslop-Harrison and Shivanna 1977; Illiciaceae: Koehl 2002; Schisandraceae: Lyew et al. 2007; Trimeniaceae: Bernhardt et al. 2003) as well as the Chloranthales (Hristova et al. 2005) and magnoliids (Heslop-Harrison and Shivanna 1977; Pontieri and Sage 1999). Variation in stigma type is evident within the Magnoliidae: although the Magnoliaceae (Heslop-Harrison and Shivanna 1977) and Eupomatiaceae (Endress 1984)
similarly possess dry stigmas, other families in the order are clearly wet and often with copious stigmatic exudate (e.g. Myristicaceae: Sharma and Shivanna 2011; Himantandraceae: Prakash et al. 1984; and Annonaceae: Lora et al. 2011). The previous interpretation of wet stigmas as the plesiomorphic condition among angiosperms has consequently been reversed, and wet stigmas can be interpreted as the derived condition within the Magnoliales.

An association is furthermore recognized between stigma type and the occurrence of self-incompatibility mechanisms, with self-incompatibility more common in lineages with dry stigmas (e.g. Illiciaceae: Saururaceae: Pontieri and Sage 1999; Trimeniaceae: Bernhardt et al. 2003; Koehl et al. 2004; Chloranthaceae: Hristova et al. 2005; and Schisandraceae: Lyew et al. 2007), raising the possibility that self-incompatibility may be the ancestral state in angiosperms (Allen and Hiscock 2008). Among early-divergent angiosperms, the Annonaceae and related magnoliids are self-compatible (Eupomatiaceae: Pang and Saunders 2014).

Stigmatic exudate is an extracellular aqueous solution of sugars, lipids and proteins, supplemented with phenols, amino acids, reactive oxygen/nitrogen species and calcium ions (Suárez et al. 2012; Rejón et al. 2014). As with other nectars, the main component of stigmatic exudate is a combination of sugars, including simple sugars that can be digested easily and metabolized rapidly by animals (Simpson and Neff 1983) and which therefore serve as an excellent energy source for floral visitors (Galetto et al. 1998). Sugars are furthermore key phagostimulants that trigger sugar-sensitive cells in various insect groups (including beetles: Mitchell and Schoonhoven 1974; Mitchell and Gregory 1979; Mitchell and Harrison 1984; Mitchell 1985; Petanidou et al. 2006): identification of the presence and composition of amino acids in stigmatic exudate therefore enables invaluable inferences on the possible role of the exudate as a nutritive reward to pollinators.

Irrespective of its potential as a nutritive reward, the original function of stigmatic exudate is likely to have been for pollen adhesion and as a germination medium, with sugar concentrations providing suitable osmolarity for pollen hydration and meeting the energy demands for pollen-tube growth (Zhang and Croes 1982; Potts and Marsden-Smedley 1989). Lipids in the stigmas are similarly likely to be important for pollen adhesion and hydration and pollen-tube growth (Lush et al. 1998; Zinkl et al. 1999; Wolters-Arts et al. 2002).

The copious stigmatic exudate produced in the Annonaceae and related magnoliids often greatly exceeds the volume necessary for pollen germination and as a pollinator reward. This suggests an alternative function, possibly as an “extragynoecial compitum” to enable intercarpellary growth of pollen tubes and hence overcome the limitations of apocarpy (unfused carpels) by allowing pollen tubes to potentially fertilize ovules in any carpel in the flower (Endress 1980, 1982; Du and Wang 2012; Wang et al. 2012). Extragynoecial pollen-tube growth is generally classified into three types—suprastylar, infrastylar and extrastylar—based on location within the carpel (Wang et al. 2012). Suprastylar pollen-tube growth, which occurs in the exudate layer covering the stigma (Wang et al. 2012), has been observed in several disparate early-divergent angiosperm lineages, viz.
Amborellaceae, Nymphaeaceae, Himantandraceae, Eu-
pomatiaceae, Annonaceae, Menispermaceae and Lar-
dizabalaceae (Endress 1995; Igersheim and Endress
1997; Endress and Igersheim 2000). The formation of an
extragynoecial compitum is likely to have been a key
evolutionary innovation in apocarpous angiosperms
(Endress and Igersheim 2000).

The three possible functions of stigmatic exudate—
as a pollinator reward, as a pollen germination medium
and as an extragynoecial compitum—have all been
suggested for the early-divergent angiosperm family
Annonaceae. Many previous studies include observa-
tions that visitors to Annonaceae flowers consume
stigmatic exudate and that the exudate is therefore
likely to function as a pollinator reward (Gottsberger
1970, 1988, 1989; Armstrong and Marsh 1997;
Nagamitsu and Inoue 1997; Momose et al. 1998;
Roubik et al. 2005), although the detailed chemical
composition of the exudate has never been deter-
mined. Extragynoecial pollen-tube growth has been
suggested for the Annonaceae (Deroin 1991; Endress
and Igersheim 1997; Igersheim and Endress 1997),
although intercarpellary pollen tube growth has only
been demonstrated in Annona cherimola Mill. (Lora
et al. 2011, 2016).

The present study aims to investigate all
three possible functions of stigmatic exudate in
Annonaceae flowers. The flowers are predominantly
beetle-pollinated, invariably apocarpous and mostly
hermaphroditic, with autogamy prevented in the
hermaphroditic-flowered species by protogyny rein-
forced by a sexually non-functional interim phase
separating the pistillate and staminate phases (Pang
and Saunders 2014). We aim to assess the nutritive
role of the exudate of selected Annonaceae species—Uvaria
macrophylla Roxb. (Figure 1A) and U. grandi-
flora Roxb. ex Hornem. (Figure 1B)—by determining sugar and
amino acid composition and concentration and corre-
lating this with the food preferences of the observed
pollinators. We furthermore aim to assess the role of

Figure 1. Flowers of study species
(A) Mature flowers of Uvaria macrophylla. (B) Mature flowers of U. grandi-
flora. (C) Mature flowers of Goniothalamus
tapisoides. (D) Stigmas of G. tapi-
soides. (E) Mature flowers of G. paralle-
lichenius. (F) Stigmas of G. parallel-
lichenius.
the exudate as a medium for pollen grain germination, identifying the optimal sugar concentration for germination and monitoring temporal changes in concentration in relation to the sexually functional floral stages during anthesis. This will enable a test of whether changes in sugar concentration reinforce the effectiveness of the non-sexual interim phase separating the pistillate and staminate phases, potentially allowing persistence of the exudate into the staminate phase as a nutritive reward for pollinators while preventing pollen grain germination late in anthesis. We also assess the possible function of the exudate as an extragynoecial compitum—using *Goniothalamus tapisoides* Mat-Salleh (Figure 1C, D) and *G. parallelivenius* Ridl. (Figure 1E, F) as study taxa—using epifluorescence microscopy techniques to identify possible intercarpellary growth of pollen tubes. These results are interpreted in an evolutionary context, with character-state transitions from the dry to wet stigma conditions considered either in the ancestor of the Magnoliidae-Laurales lineage or independently in the two orders.

### RESULTS

#### Stigmatic exudate chemistry

Sugar concentrations in the stigmatic exudate of pre-, mid- and late-pistillate anthetic phase flowers of *Uvaria macrophylla* (Table 1) differed significantly (*P* < 0.001, one-way analysis of variance (ANOVA), Tukey post hoc). Concentrations were high immediately prior to the onset of the pistillate phase (28.2 ± 1.4%), declined during the peak period of stigmatic receptivity (17.4 ± 1.4%), but were subsequently elevated again toward the end of the pistillate phase (32.9 ± 4.9%). The stigmatic exudate of *Uvaria grandiflora* is fructose dominated (72.2%), although with high levels of glucose (19.3%) and sucrose (8.4%).

Total amino acid concentrations in the exudates of *U. grandiflora* and *U. macrophylla* were 114.5 µg/mL and 103.5 µg/mL, respectively (Table 2). Nine essential and 13 other amino acids were recorded from the exudate of *U. grandiflora*: the most abundant of these was arginine (43 µg/mL; 37.5% of total amino acid content), followed by glutamic acid (20.4 µg/mL; 17.8%). Analysis of the stigmatic exudate of *U. macrophylla* revealed a similar range of amino acids, with eight essential and 13 other amino acids recorded: arginine was again found to be the most abundant (35.8 µg/mL; 34.6%), with phenylalanine (15.3 µg/mL; 14.8%) as the second-most abundant amino acid.

#### Pollen germination under differing sugar concentrations

*Uvaria macrophylla* pollen failed to germinate in the absence of sucrose (Figure 2), and showed varying degrees of germination success at different sucrose concentrations. The optimal sucrose concentration for pollen germination ranged from 5%–20%, with an average of 8.7% germination (*P* < 0.001, ANOVA, Tukey post hoc). Very low levels of pollen germination were recorded at sucrose concentrations of 0% and over 20%, with levels approaching zero in concentrations exceeding 30% (Figure 2).

#### Intercarpellary growth of pollen tubes

*Goniothalamus tapisoides* stigmas project outward to occupy much of the enclosed floral chamber (Figure 1D; S in Figure 3A). Pollen grain germination is apparent, with pollen tubes (arrowed in Figure 3B) passing through the stigmatic exudate to reach other stigmas (Figure 3B, C). Pollen germination was similarly observed in *G. parallelivenius* (Figure 3E), with

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**Table 1. Sugar concentration in the stigmatic exudate of *Uvaria macrophylla*, sampled immediately before, during and toward the end of the pistillate anthetic phase**

| Anthetic phase                  | Time (Day of anthesis) | Sample size (Flowers) | % Sugar concentration (±SE)* |
|---------------------------------|------------------------|-----------------------|------------------------------|
| Immediately prior to pistillate phase | 17:00–17:30 hours (day 1) | 8                      | 28.2 ± 1.4^a                 |
| Peak pistillate phase           | c. 21:00 hours (day 1)  | 12                    | 17.4 ± 1.4^b                 |
| Toward end of pistillate phase  | c. 06:30 hours (day 2)  | 8                      | 32.9 ± 4.9^a                 |

* Superscript letters indicate significant differences between anthetic phases (one-way analysis of variance, Tukey Honestly Significant Difference post hoc comparison, *P* < 0.001).
intercarpellar growth of pollen tubes again achieved via the stigmatic exudate (Figure 3E, F). Pollen tube growth in *G. tapisoides* primarily occurred toward the base of the stigmas (Figure 3C, D), whereas in *G. parallelivenius* it was observed across the top of the stigmas (Figure 3E, F).

DISCUSSION

**Stigmatic exudate as a nutritive reward for pollinators**

Although sucrose is generally the dominant sugar in floral nectar (Nepi et al. 2009), the concentration of fructose in the stigmatic exudate of *Uvaria grandiflora* exceeds that of sucrose and glucose. Hexose sugars (including glucose and fructose) were found to contribute over 90% of the total sugar composition in the exudate; these simple sugars are easily metabolized by animals (Simpson and Neff 1983), suggesting that the exudate might function as a nutritive reward for pollinators. This is consistent with our observations that the species is predominantly beetle pollinated, since the nectar of beetle-pollinated flowers is typically hexose-rich (Steenhuisen and Johnson 2012).

Essential amino acids are those that cannot be synthesized by an organism and hence must be obtained through their diet. Ten amino acids are essential for insects (Table 2; Haydak 1970), all of which have previously been recorded in floral nectar (Baker and Baker 1975; Gottsberger et al. 1984). The stigmatic exudates of *U. grandiflora* and *U. macrophylla*

| AA                        | U. grandiflora (µg/mL) | U. macrophylla (µg/mL) | Stimulation of insect sugar-sensitive cells |
|---------------------------|------------------------|------------------------|--------------------------------------------|
| 1. Arginine               | 43.0                   | 35.8                   |                                            |
| 2. Phenylalanine          | 5.4                    | 15.3                   | Yes¹                                      |
| 3. Histidine              | 3.3                    | 4.1                    |                                            |
| 4. Leucine                | 2.2                    | 1.6                    | Yes¹                                      |
| 5. Lysine                 | 1.5                    | 0.0                    |                                            |
| 6. Valine                 | 0.9                    | 1.5                    | Yes¹                                      |
| 7. Isoleucine             | 0.7                    | 1.0                    | Yes¹                                      |
| 8. Threonine              | 1.2                    | 0.3                    |                                            |
| 9. Methionine             | 0.2                    | 0.1                    | Yes¹                                      |
| 10. Serine                | 7.4                    | 9.4                    | Yes³                                      |
| 11. Asparagine            | 0.2                    | 6.9                    | Yes³                                      |
| 12. Ornithine             | 5.2                    | 5.6                    | –                                          |
| 13. Glycine               | 3.2                    | 4.8                    | Yes³                                      |
| 14. Taurine               | 0.2                    | 4.1                    | –                                          |
| 15. Alanine               | 4.6                    | 3.2                    | Yes³                                      |
| 16. Glutamic acid         | 20.4                   | 2.9                    | –                                          |
| 17. Tyrosine              | 0.8                    | 2.5                    | –                                          |
| 18. Aspartic acid         | 9.8                    | 2.3                    | –                                          |
| 19. γ-aminobutyric acid   | 3.3                    | 1.5                    | –                                          |
| 20. Citrulline            | 0.4                    | 0.4                    | –                                          |
| 21. Cystine               | 0.2                    | 0.1                    | –                                          |
| 22. α-aminobutyric acid   | 0.4                    | 0.1                    | –                                          |
| Total AAs                 | 114.5                  | 103.5                  |                                            |

¹Shiraishi and Kuwabara (1970); ²Mitchell and Gregory (1979)
contain nine and eight of these essential amino acids, respectively (Table 2): only tryptophan is absent from both, with lysine furthermore absent from *U. macrophylla*. The diversity of essential amino acids in these exudate samples suggests that the exudate may function as a nutrient-rich food source for insects.

Nine of the amino acids present in the stigmatic exudate of *U. grandi flora* and *U. macrophylla* (phenylalanine, leucine, valine, isoleucine, methionine, serine, asparagine, glycine and alanine; Table 2) have previously been reported to be sugar-cell stimulants for beetles and flies (Shiraishi and Kuwabara 1970; Mitchell and Gregory 1979). Of these, phenylalanine is the second- and fourth-most abundant amino acid in the stigmatic exudates of *U. macrophylla* and *U. grandi flora*, respectively (Table 2). Phenylalanine is reported to have a significant phagostimulatory effect on honeybees (Inouye and Waller 1984), consistent with our observations that bees also visit *U. grandi flora* flowers. It has been suggested that even a small quantity of free amino acids may influence the pollinators’ perception of taste (Baker 1977) and may also have a phagostimulatory...
effect, although other nectar components may also contribute to taste, including lipids and phenolics (Baker and Baker 1982). Amino acid concentrations in the stigmatic exudate of Uvaria macrophylla (103.5 μg/mL) and U. grandiflora (114.5 μg/mL) were similar to those previously reported for species pollinated by long-tongued bees, moths, hummingbirds and bats (Gottsberger et al. 1984), although there is unlikely to be any correlation between amino acid composition and specific pollinator guilds.

Proline is commonly reported as the most abundant amino acid in floral nectar (Nepi et al. 2009); it is rapidly metabolized and is therefore hypothesized to be particularly important for enabling initial lift in insect flight, which is very energy-demanding (Carter et al. 2006). The absence of proline from the exudate of both Uvaria species (Table 2) is therefore surprising. This possibly reflects the diversity of food sources utilized by the non-specialist pollinating beetles, and it is significant that pollen grains are rich in proline; beetles might therefore compensate for the lack of proline in the exudate by directly consuming pollen. However, it is also known that proline disperses from the pollen grain to the stigmatic exudate after pollen deposition (Gottsberger et al. 1984), and hence post-pollination exudate may be nutritionally more rewarding for pollinators.

**Stigmatic exudate as a medium for optimizing pollen germination**

The stigmatic exudate that forms immediately after the onset of the pistillate phase in Uvaria macrophylla flowers has a relatively high sugar concentration (28.2 ± 1.4%; Table 1). Pollen germination is unlikely under these conditions, since our in vitro pollen germination experiments revealed a significant decrease in germination level in sugar concentrations exceeding 20% (Figure 2). Previous studies have demonstrated that increased sugar concentrations can change water potential and may damage the pollen membrane by increasing its permeability (Zhang and Croes 1982). However, if the exudate functions as a nutritive reward for pollinators, this elevated sugar concentration might promote the attraction of beetles at the onset of the pistillate phase, thereby maximizing opportunities for successful pollen transfer as the flower enters its peak pistillate phase.

The sugar content in the exudate subsequently becomes relatively diluted during the peak pistillate phase (17.4% ± 1.4%; Table 1), achieving a concentration that is close to the optimal conditions identified in the in vitro pollen germination study (5%–20%; Figure 2). The change in sugar concentration in the stigmatic exudate is presumably due to increased water transportation via the xylem vessels as overall exudate volume was observed to increase during the pistillate phase. However, sugar concentration subsequently increases toward the end of the pistillate phase (32.9% ± 4.9%; Table 1), probably due to evaporation as exudate volume is visibly reduced over time. The sugar concentration in this late-pistillate-phase exudate is therefore similar to the early-formed exudate, and is unlikely to promote pollen germination.

The stigmatic exudate clearly optimizes conditions for pollen germination, and by restricting the period during which sugar concentrations are suitable for pollen germination, germination is presumably restricted to periods when the ovules are most receptive. The increased sugar concentration in the exudate toward the end of the pistillate phase possibly serves to reinforce protogyny: even in Annonaceae species in which the non-sexual interim phase that typically separates the pistillate and staminate phases is brief or absent, the retention of the stigmatic exudate while the stamens are dehiscing (providing a food reward to pollinators) does not necessarily undermine the effectiveness of protogyny for avoiding autogamy.

Although U. macrophylla pollen can germinate on simple sucrose solution, the germination level is relatively low (c. 13%; Figure 3). Similar results were obtained from other Annonaceae species (c. 14% in Pseuduvoria mulgraveana: Pang et al. 2013), although much higher levels of germination have been reported for other species in the family, viz. c. 30% in Xylophia championii (Ratnayake et al. 2007), c. 40% in Annona squamosa (Kishore et al. 2012), and as high as c. 90% in Uvaria semecarpifolia (Attanayake 2010); pollen germination in Annona cherimola can furthermore reach 43.6% under natural conditions (Lora et al. 2010). This suggests that other chemicals may be essential for optimizing pollen germination and/or pollen-tube growth. Boric acid, calcium, calmodulin (a calcium-modulated protein), endo-1,3-β-glucanase and arabinogalactan proteins are all believed to be essential for pollen germination and pollen-tube growth (Potts and Marsden-Smedley 1989; Wu et al. 2001; Golovkin and Reddy 2003; Rejón et al. 2013). Non-protein amino
acids—including canavanine, α, γ-diaminobutyric acid, homoarginine and lathyrine—can promote or inhibit pollen germination and pollen-tube growth depending on the concentrations of the specific amino acid (Simola 1967), and other non-protein amino acids such as γ-aminobutyric acid (GABA) play a role in pollen tube growth in Arabidopsis (Palanivelu et al. 2003). GABA and arginine—a structural analogue of canavanine (Tsikas and Wu 2015)—have both been identified from the stigmatic exudate of the two Uvaria species studied (Table 2), with arginine the most abundant amino acid in both. It is therefore possible that these two amino acids are also important for pollen germination and pollen-tube growth in the study species.

**Stigmatic exudate as an extragynoecial compitum**

Annonaceae flowers are apocarpous, with Goniothalamus tapisoides flowers containing (14–)20–50 carpels, each with a single ovule (R.M.K. Saunders, pers. observ.), and G. parallelivenius flowers having 8–56 carpels (Saunders 2002), each containing 5–10 ovules (Mat-Salleh 1993). Intercarpellary growth of pollen tubes via stigmatic exudate is clearly demonstrated here for both species, supporting the interpretation of the exudate as a suprastylar extragynoecial compitum (Wang et al. 2012). The evolutionary advantages of intercarpellary growth of pollen tubes via an extragynoecial compitum in apocarpous angiosperms—enabling pollen tubes to reach ovules in any carpel in the flower, irrespective of which stigma the pollen grain was deposited—are well documented (e.g. Armbruster et al. 2002). The apparently ubiquitous presence of stigmatic exudate in the Annonaceae, albeit in variable quantities, suggests that its function as an extragynoecial compitum may represent a key evolutionary innovation for the family, enabling flowers to overcome the limitations of apocarpy.

The difference in the location of intercarpellary pollen tube growth in two study species (toward the base of the stigmas in G. tapisoides: Figure 3C, D; and toward the top of the stigmas in G. parallelivenius: Figure 3E, F) is explained by structural differences in carpel morphology. The stigmas of G. tapisoides are divergent (Figure 1D), drawing the exudate toward the base of the stigmas despite the pendent orientation of the flowers, whereas those of G. parallelivenius are appressed (Figure 1F), restricting the exudate to the apex of the stigmas.

The function of the stigmatic exudate as an extragynoecial compitum is likely to be particularly important in species that produce pollen polyads. Pollen tetrads are characteristic of Goniothalamus species, and pollen aggregation is widely reported in other Annonaceae genera in tribes Bocageeeae (Tsou and Fu 2007), Annonae (Walker 1971; Tsou and Fu 2002; Lora et al. 2014; Li et al. 2015) and Miliuseae (Su and Saunders 2003). Pollen aggregation is likely to be promoted under conditions in which pollinator visits are limited: pollen transfer by the pollinator would necessarily involve multiple pollen grains that would potentially enable multiple ovules to be fertilized (Harder and Johnson 2008; Li et al. 2015); however, for species with a single ovule per carpel (such as G. tapisoides), pollen aggregation is only advantageous in conjunction with an extragynoecial compitum.

**The role of stigmatic exudate in Annonaceae flowers**

Our data strongly support the inference that the stigmatic exudate in Annonaceae flowers functions in all three ways suggested: as a pollinator reward; as a pollen germination medium; and as an extragynoecial compitum. The dominance of hexose sugars in the Uvaria exudate and the complex array of its constituent amino acids, including those that are essential for insect nutrition, support a nutritive role for the exudate, corroborating numerous field observations of insects apparently consuming the secretion (Gottsberger 1970, 1988, 1989; Armstrong and Marsh 1997; Nagamitsu and Inoue 1997; Momose et al. 1998; Roubik et al. 2005).

There is furthermore evidence for limited specificity of the exudate chemistry in relation to floral visitors: in addition to the dominance of hexose sugars (common in beetle-pollinated flowers: Steenhuisen and Johnson 2012), the exudate contains phenylalanine, an amino acid that is known to have a phagostimulatory effect on bees (Inouye and Waller 1984) that are also observed to visit the flowers. The sugar concentration in the exudate during the peak pistillate phase (17.4% ± 1.4%) furthermore provides the optimal osmolarity for pollen hydration and germination, as well as presumably providing necessary nutrition for pollen-tube growth. Subsequent changes in sugar concentration during anthesis reinforce the effectiveness of protogyny, allowing the retention of concentrated stigmatic exudate (32.9% ± 4.9%) beyond the functional end of the pistillate phase and thereby enabling the continued
presentation of exudate as a pollinator food reward without the possibility of pollen germination during the staminate phase. Our experiments on *Goniothalamus* demonstrate the role of the stigmatic exudate as a suprastylar extragynoecial compitum, allowing intercarpellar growth of pollen tubes and effectively overcoming the limitations of apocarpy by potentially allowing the microgametophyte to reach any ovule in any ovary, irrespective of which carpel the pollen grain was deposited on.

Experimental constraints on the number of species included in our study inevitably preclude unequivocal conclusions on how widespread the three functions are across the Annonaceae. Although it seems likely that stigmatic exudate functions as a pollinator reward and as a pollen germination medium throughout the family, it is possible that the role of the exudate as an extragynoecial compitum may be restricted to those species with particularly abundant exudate, associated with numerous carpels per flower.

“Dry” stigmas, which lack a significant fluid secretion when receptive, are likely to be plesiomorphic within angiosperms (Hristova et al. 2005). In contrast, the stigmas of most species in the Laurales (Endress and Igersheim 1997) and Magnoliales (Myristicaceae: Sharma and Shivanna 2011; Himantandraceae: Prakash et al. 1984; Annonaceae: Lora et al. 2011, confirmed in the present study) are classified as “wet”, although in the latter order the stigmas in the Magnoliaceae and Eupomatiaceae are dry (Heslop-Harrison and Shivanna 1977) and data are unavailable for the Degeneriaceae. This implies either a transition from the dry to wet conditions in the ancestor of the Magnoliaceae-Laurales lineage, or parallel transitions independently in the two clades (Figure 10A in Endress and Doyle 2009). Since copious stigmatic exudate is not a prerequisite for pollen adhesion, hydration or germination, it can be speculated that the main drivers for this change were possibly the combined selective advantages of a suprastylar extragynoecial compitum enabling intercarpellar growth of pollen tubes, and the presentation of sugar- and amino acid-rich stigmatic nectar as a nutritional reward for pollinators. In this context, it is significant that many other early-divergent angiosperms with extragynoecial pollen-tube growth exhibit infrastylar growth (Williams et al. 1993; Igersheim and Endress 1997; Lyew et al. 2007), in which the pollen tube grows down the style and exits the ovary through the receptacle to enter another carpel and which therefore do not require a stigmatic exudate that connects adjacent stigmas (Wang et al. 2012).

**MATERIALS AND METHODS**

**Field sites and species sampled**

Four Annonaceae species were studied over two flowering seasons at three sites in Hong Kong and one site in Brunei Darussalam. *Uvaria macrophylla* (Figure 1A) was studied at San Tau, Lantau Island, Hong Kong (22°17′06.5″N; 113°55′30.9″E) and South Lantau Country Trail, Lantau, Hong Kong (22°14′55.0″N; 113°56′36.8″E) between June and August 2015/2016. *Uvaria grandiflora* (Figure 1B) was studied at Mo Tat Wan, Lamma Island, Hong Kong (22°12′25.3″N; 114°08′43.8″E) from June to August 2015/2016. *Goniothalamus tapisoides* (Figure 1C, D) and *G. parallelevinicus* (Figure 1E, F) were studied in Jalan Rampayoh Timur, Labi, Belait District, Brunei Darussalam (04°22′05.2″N, 114°27′48.9″E and 04°22′03.6″N, 114°27′37.3″E, respectively) in May 2013/2014 (permit 53/JPH/BOT/02 PT.3, issued in 2013 and renewed in 2014 by the Forestry Department, Ministry of Industry and Primary Resources, Bandar Seri Begawan, Brunei Darussalam, with export permit BioRIC/HOB/TAD/51-22).

Both *Uvaria* study species are pollinated by small beetles, although *U. grandiflora* is also visited by bees (C.-C. Pang and J.Y.Y. Lau, pers. observ.). Anthesis in *U. macrophylla* extends over 4 d, with the pistillate phase (c. 13 h duration) followed by a non-sexual interim phase (c. 11 h) and then the staminate phase (c. 48 h) (C.-C. Pang, unpubl. data). Floral phenological data are unavailable for *U. grandiflora*, although field observations indicate that it is similarly protogynous.

*Goniothalamus tapisoides* is pollinated by small curculionid beetles and is predominantly xenogamous (Lau et al. 2016). The flowers are protogynous, with anthesis extending over c. 23 h, from the early morning (c. 06:00 hours) of the first day of anthesis until early morning (c. 05:00 hours) the next day, and with the pistillate phase (c. 13 h duration) followed by a non-sexual interim phase (c. 3 h) prior to the onset of the staminate phase (c. 7 h) (Lau et al. 2016). Corresponding phenological data is not available for *G. parallelevinicus*, although field observations indicate that it is also protogynous and pollinated by small beetles (J.Y.Y. Lau, pers. observ.).
Stigmatic exudate chemistry
The overall sugar concentration of stigmatic exudate in *Uvaria macrophylla* was determined using samples from 28 flowers from 21 individuals, with the exudate extracted from flowers using a micropipette. Three different anthetic stages were assessed, viz. (i) immediately prior to the onset of the pistillate phase, c. 06:30 hours on the 1st day of anthesis (*n* = 8); (ii) during the peak pistillate phase, c. 21:00 hours on the same day (*n* = 12); and (iii) toward the end of the pistillate phase, c. 06:30 hours on the 2nd day of anthesis (*n* = 8). As the volume of stigmatic exudate in each flower was inadequate for direct measurement, 2 µL of exudate were diluted with distilled water, giving a final solution of 20 µL. Sugar concentration was then measured using an Atago Palette PR-100 digital refractometer (Atago Co. Ltd., Tokyo, Japan), which has an accuracy of ±0.2%. Measurements were made in the field immediately after the removal of flowers, thereby avoiding inaccuracies due to exudate evaporation; days with heavy precipitation were furthermore avoided to prevent possible dilution of exudate with rainwater.

The sugar composition of stigmatic exudate was assessed using pistillate-phase *Uvaria grandiflora* flowers, with exudate (10 µL) pooled from six flowers due to the limited volume in each flower. The aggregated samples were preserved in 50 µL 80% ethanol and stored at 0°C prior to analysis. Sugar composition was determined using a Dionex high-performance liquid chromatography (HPLC) system (Dionex Corp., Sunnyvale, California, USA) with a CarboPac PA-1 (4 × 250 mm) column and an ED40 electrochemical detector together with a pulsed amperometric cell. A 10 µL sample loop was used and isocratic elution at 1 mL/min with 10 mmol/L NaOH. Authentic sugar standards enabled comparison with the HPLC peaks generated from the exudate samples.

The amino acid composition of the exudate (10 µL samples from both *Uvaria grandiflora* and *U. macrophylla*) was similarly determined using exudate pooled from six bagged pistillate-phase flowers from six individuals, and again preserved in 50 µL 80% ethanol. Flowers were bagged prior to the onset of the pistillate phase to avoid pollen contamination as previous studies have shown that germinating pollen can release free amino acids (Linskens and Schrauwen 1969). The samples were commercially processed at the South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, China. Each sample was centrifuged at c. 8,000 × g for 5 min, with 1 mL of the supernatant transferred to an Eppendorf tube and ethanol added to achieve a final concentration of 80% alcohol. The samples were then vortexed and incubated for 15 min at ambient temperature, and then centrifuged at c. 8,000 × g for 20 min. The supernatant was vacuum dried and 200 µL of 0.1 mol/L HCl added, then filtered through a 0.22 µm membrane and injected into a Sykam S433 amino acid analyser (Sykam GmbH, Eresing, Germany) (fitted with a 150 × 4.6 mm column).

Pollen germination under different sugar concentrations
The optimal conditions for pollen germination were assessed *in vitro* using *Uvaria macrophylla* pollen immersed in a graded series of artificial sucrose concentrations (Dafni 1992). Pollen was collected from five staminate-phase flowers from five different individuals, and incubated in 10 µL of each sucrose solution on cavity slides within closed Petri dishes for 24 h at ambient temperature. The percentage of pollen grains germinating was calculated from 100 randomly selected pollen grains, with 19 replicates for each sucrose concentration and four replicates for each flower.

Intercarpellary growth of pollen tubes
Possible intercarpellary growth of pollen tubes was investigated using *Goniothalamus parallelivenius* and *G. tapisoides*. Thirty flower buds were bagged prior to anthesis, and pollen from staminate-phase flowers used for hand pollination of pistillate-phase flowers borne on other individuals. The latter flowers were collected 1 d after pollination, since previous studies of *Annona cherimola* (which similarly exhibits 2 d anthesis) have demonstrated ovule fertilization within 24 h of pollination (Lora et al. 2010, 2011). The samples were fixed in formalin-acetic acid-alcohol (FAA) overnight and then transferred to 70% ethanol for long-term storage. Carpels and part of the attached receptacle were excised and immersed in 5% KOH at ambient temperature until they became translucent. The specimens were then rinsed with distilled water and stained with aniline blue (0.1% in 0.03 mol/L K2PO4) overnight, with the carpels then squashed with glycerine under a cover slip (Kho and Baer 1968; Du and Wang 2012). The preparations were examined with a Carl Zeiss LSM 710
NLO confocal laser scanning microscope with green fluorescent protein (GFP) filter.

**Statistical analysis**

Sugar concentration of stigmatic exudate from different anthetic phases of *Uvaria macrophylla* was analyzed using one-way ANOVA. A similar analysis was performed to evaluate the effects of sucrose concentration on the pollen germination. Differences among groups (i.e. anthetic phases for the former and sucrose concentration for the latter) were further tested using Tukey Honestly Significant Difference (HSD) post hoc comparisons. All analyses were performed in R (R Core Team 2014), using the agricolae package for Tukey HSD post hoc comparison.

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**AUTHOR CONTRIBUTIONS**

R.M.K.S. conceived the study. J.Y.Y.L. performed the fieldwork and experiments with assistance from co-authors. All authors analyzed the data and discussed interpretation. J.Y.Y.L. and R.M.K.S. wrote the manuscript.

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