— Novel Ideas and Pilot Projects —

PRELIMINARY STUDIES ON ORNITHOPHILOUS FLORAL VISITORS IN THE AUSTRALIAN ENDEMIC *PASSIFLORA HERBERTIANA* KER GAWL. (PASSIFLORACEAE)

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Abstract—The pollination biology of the Australian endemic species *Passiflora herbertiana* (*Passiflora* subgenus *Decaloba,* supersection *Disemma,* section *Disemma*) was investigated in a single population growing in the Witches Falls section of Mount Tamborine National Park, Queensland. Three native honeyeaters were observed at the flowers, including Lewin's Honeyeater (*Meliphaga lewinii*), the Noisy Miner (*Manorina melanocephala*), and the Eastern Spinebill (*Acanthorhynchus tenuirostris*). Visitation began at 07:30 and ended by 15:30 each day. The most frequent visitor was Lewin's Honeyeater. Flowers typically began anthesis in the afternoon, with a small number of flowers opening in the early morning. Flowers remained open between four and five days, even after successful pollination. Both the age of the flower and the amount of sun exposure were determined to affect perianth colour change from pale yellow to salmon-pink. Andromonoecy was observed infrequently in the population; most plants exhibited bisexual flowers, but a small number of individuals exhibited both hermaphroditic and male flowers with short styles held permanently erect. Controlled hand pollinations indicated that *P. herbertiana* is self-compatible but is not autogamous. Pollen tubes required at least 48 hours to reach the most apical ovules within the ovary. These data provide new insights into the evolution of ornithophily in the Old World *Passiflora.*

Keywords: Australia, Disemma, honeyeater, ornithophily, Passiflora herbertiana, Passifloraceae

INTRODUCTION

Passiflora L. is a genus of ca. 525 vines, lianas and small trees distributed throughout Mexico, Central and South America, with an additional 24 species endemic to Asia, Southeast Asia, and the Austral Pacific. Insect pollination is thought to be ancestral for *Passiflora* (MacDougal 1994), but hummingbird and bat pollination syndromes have evolved independently in multiple clades (Kay 2001; Krosnick et al. 2013). For example, subgenus *Decaloba* (DC.) Rchb. includes at least four unrelated lineages of hummingbird-pollinated species. These species have tube-shaped flowers with bright red, yellow, or pink colouration, produce large quantities of dilute sucrose-rich nectar, have horizontal to erect floral orientation at anthesis, and lack floral scent. While most species of *Passiflora* are self-incompatible, a few species are self-compatible (MacDougal 1994; Koschnitzke & Sazima 1997; Amela Garcia & Hoc 1998; Kay 2003).

The Australian native species *Passiflora herbertiana, P. cinnabarina,* and *P. aurantia* (supersection *Disemma* (Labill.) J. M. MacDougal & Feuillet, section *Disemma* (Labill.) J. M. MacDougal & Feuillet) exhibit many of the same floral features as New World hummingbird-pollinated species (e.g. *P. murucuja* L. and *P. tulae* Urb.). The Australian species are known to be self-compatible in cultivation, and two species (*P. herbertiana* and *P. aurantia*) exhibit floral colour change with age. No studies have been performed to document avian visitors to the Australian species in their native ranges. Thus, these species provide a unique opportunity to examine the evolution of ornithophilous pollination syndromes and self-compatibility in Australia. As a first step towards understanding these species in greater detail, an investigation into the reproductive biology of *P. herbertiana* was conducted. The objectives were to 1) document floral phenology, 2) determine types of floral visitors, 3) examine floral nectar constituents and 4) perform controlled hand pollinations to determine the nature of self-compatibility in this species.

MATERIALS AND METHODS

Study site

Fieldwork was carried out between June 30 and July 9, 2011 at a large population of *P. herbertiana* growing in the

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Witches Falls section of Mount Tamborine National Park, Queensland, Australia (27°93'S, 153°18'E; elevation 559 meters). The population consisted of ca. 150 individual plants, most being non-reproductive juveniles growing in the dense shade of the rainforest canopy. Three mature plants of *P. herbertiana* were chosen along the forest margin. The selected individuals were mature lianas ca. 3-10 meters long, each climbing up into nearby trees and displaying numerous flowers in various stages of maturity, and located ca. 35 meters apart from one another (plant 1, *SK 735*; plant 2, *SK 734*; plant 3, *SK 736*; vouchers at MO).

Reproductive phenology of Passiflora herbertiana

Nineteen floral buds across all three plants were tagged and covered with mesh bags for observation and controlled pollinations. Flowers were examined three times per day (07:00, 12:00, 16:00) to determine patterns of anthesis.

Assessment of self-compatibility - controlled pollination experiment

Pollen tube germination and rates of ovule fertilization were used to assess self-compatibility in *P. herbertiana.* Hand-pollinations were carried out on 15 bagged flowers across all three plants. Seven flowers were self-pollinated and eight were outcrossed with pollen from the other two vines in the population. Outcrossed flowers were emasculated while in bud to prevent contamination of selfed pollen. As soon as floral buds opened enough to expose the stigmas, they were assumed receptive and pollination was performed. The stigmas of out-crossed flowers were evenly dusted with pollen using a single anther removed from the donor flower. In selfed individuals, pollinations were performed once the anthers had begun dehiscing, and pollen from a single anther was divided among all stigmas. The remaining anthers were left on the flower to serve as potential pollen donors in other pollinations. Flowers were collected 48 hours after pollination, fixed in FAA for 24 hours, and transferred to 70% ethanol for long term storage. Pollen tube growth was examined with aniline blue staining following Kay (2003) and Bernhardt (1982) on a ZEISS Primostar iLED microscope using a fluorescence LED module at 455 nm and filter set 67.

Analysis of nectar concentration and constituents

Nectar concentration was assessed for 16 flowers sampled across all three plants using a hand-held refractometer. Samples were collected at 16:00 each day using 50 μL microcapillary tubes, and concentrations were recorded at ambient temperature (18°C to 22°C). Twelve samples were collected for analysis of sugar constituents using High Performance Liquid Chromatography (HPLC) with 20 or 50 μL microcapillary tubes, quantified, blotted on filter paper, and stored with silica gel. Samples were dissolved by rinsing filter paper 3-5 times with 4 ml of Milli-Q Direct purified water (Millipore, Billerica, MA) heated to 95°C. Samples were then diluted to 50 ml and analyzed using a Shimadzu Prominence twin pump HPLC system with a Shodex Asahipak NH2P-50 4E column (Shimadzu, Columbia, MD). Each day of analysis, calibration curves of known sugar amounts were constructed using standard solutions from 400 ppm (0.4 g/L) to 2000 ppm (2.0 g/L) of glucose, fructose and sucrose (working concentration 100 μg/g). Data were integrated using SHIMADZU LCSolutions software and peak areas were regressed against sugar weight. For each sample, percentages of glucose, fructose and sucrose of the total sugar weight were calculated.

Floral visitors

Observations of floral visitors were conducted between 06:00 and 17:00 each day for the 10 days of the study. Animals were identified using field guides and confirmed with photographs from the site. Videos were taken to document visitor behaviour (see Appendix 1).

RESULTS

Reproductive phenology

Floral buds take ca. 21 days to develop to maturity from initiation along the shoot tip. Most flowers opened overnight (N = 15), but a small number opened over the course of the day (N = 4). In night-opening flowers, the afternoon before full anthesis (ca. 16:00) sepals split to 1-2 cm at the apex (Fig. 1A), at which point the styles and anthers became visible. This was defined as the start of

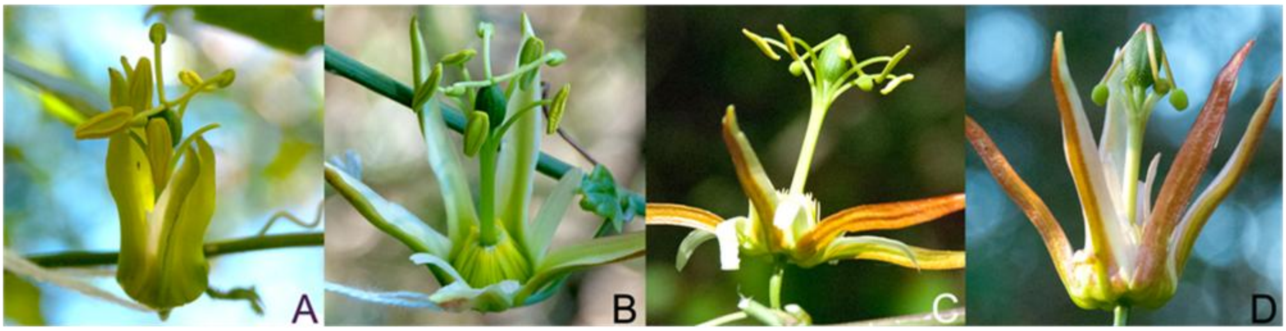

FIGURE 1. Anthesis pattern of *Passiflora herbertiana* over five days. (A) Onset of anthesis: Sepals open 1-2 cm in the afternoon, styles initially held at ca. 20° angle from androgynophore. (B) Next morning: perianth begins to open; anthers start to dehisce, styles will eventually become level with stamens. (C) 48-72 hours: perianth becomes fully expanded; the styles continue descent downward. (D) Last day of anthesis (stamens removed): perianth begins to close; styles fully extended downward, eventually curling around the ovary.

anthesis or "0 hours." The three styles are initially held at a 20° angle relative to the androgynophore. By 07:00 the next morning, the sepals are nearly expanded and at least one anther is dehiscing; the petals are still oriented towards the centre of the flower (Fig. 1B). Throughout the first full day of anthesis, the styles gradually bend downward at a 45° angle, eventually becoming aligned with the stamens. Day-opening flowers begin with a 1-2 cm split at 07:00; by 15:00 that same day, flowers are completely open and anthers are dehiscing. Day-opening flowers follow the same stylar movement patterns as night-opening flowers. All flowers remain open both day and night for 48-72 hours. Sepals and petals open wider each day, eventually becoming perpendicular to the androgynophore (Fig. 1C). Stigmas gradually bend down towards the ovary while the stamens remain at the same general position. All flowers remain open up to five days, with most flowers closing on day four or five. By the end of the last day, the stigmas bend down and curl around the ovary, the anthers shrivel and the perianth closes (Fig. 1D).

Three flowers were observed with styles 4-6 mm shorter than normal flowers (Fig. 2); in these flowers styles remained fully upright throughout anthesis. No pollinations were performed with short-styled individuals. Both sun exposure and flower age are important factors contributing to floral colour in *P. herbertiana*. Regardless of age, flowers in full shade remain pale yellow, while those in full sun are salmon-pink. In partial shade, colour and flower age are more closely correlated, where yellow (Fig. 2A) changes to salmon pink or reddish (Fig. 2B) during the final days of anthesis.

FIGURE 2. Short style morph in *Passiflora herbertiana.* (A) Young flower with short, erect styles that lack normal movement patterns. Note anther dehiscence. (B) Older flower with short styles that remain in original position and anthers that have released all available pollen (photo credit: R. Van Raders).

Nectar concentration and nectar constituents

The mean sugar concentration of nectar in *P. herbertiana* was 19.49% ± 1.85 (1 S.D., $N = 16$). HPLC analysis indicated that all samples consisted of 100% sucrose with no detectable peaks of glucose or fructose.

Self-compatibility

Evidence for compatible pollen transfer in *P. herbertiana* was considered to be pollen tube germination and growth through stylar tissue followed by fertilization of ovules. All selfed and outcrossed flowers exhibited pollen germination on the stigma and growth through stylar tissue (Fig. 3A-B). Pollen tubes in *P. herbertiana* require longer than 48 hours to reach all ovules within the ovary. All 15 hand-pollinated flowers showed pollen tubes present the entire length of the style (Fig. 3B), but only six had fertilized ovules and these were present at the apex of the ovary. Fertilization was visible (Fig. 3C) in both hand-outcrossed and -selfed flowers. The mean number of ovules per ovary was 476.27 ± 107.23 (1 S.D., $N = 17$). Three outcrossed flowers had fertilized ovules visible (3-20 ovules; 0.71-4.75% fertilization), as did three self-pollinated flowers (2-55 ovules; 0.34-11.96%).

Floral visitors

Three avian species were observed at *P. herbertiana*: Noisy Miners (*Manorina melanocephala* Latham; Fig. 4A), Lewin's Honeyeaters (*Meliphaga lewinii* Swainson; Fig. 4B), and Eastern Spinebills (*Acanthorhynchus tenuirostris* Latham; Fig. 4C). Regardless of species, visits were 30-60 seconds long and consisted of the bird probing the nectar chamber at the base of the flower several times. Lewin's Honeyeaters spent 2-4 minutes total on an individual vine, visiting multiple flowers. Eastern Spinebills targeted a flower from a distance, probed that flower, and immediately left. Noisy Miners perched on a vine for several minutes before visiting a flower, quickly probed it, and departed. All species appeared to establish some contact with the androgynophore while probing the flower. Frequency of floral visitation (Fig. 5) varied among the three bird species. Lewin's Honeyeaters were most frequent, followed by Eastern Spinebills, and then Noisy Miners. Visitation began at approximately 07:30 and continued until 15:30; no preference for mornings or afternoons was observed.

DISCUSSION

This is the first study to document ornithophilous floral visitation in Australian native *Passiflora.* This study highlights the presence of floral features that may be associated with pollination in *P. herbertiana*, including the production of dilute, sucrose-rich nectar and changes in floral colour. These data also confirm early self-compatibility in *P. herbertiana*. New insights into the reproductive biology of *P. herbertiana* include the presence of day- and night-opening flowers, andromonoecy, and an extended 4-5 day anthesis.

Floral phenology

Passiflora herbertiana has two flowering events per year (Krosnick 2006); the largest occurs in December–February, with a smaller flowering event in May–July. In the current study, onset of anthesis took ca. 18 hours (Fig. 1A-B); it is possible temperature played a role in the length of onset. Staggered anthesis (night versus day-opening flowers) may limit gene flow among ramets (Borchsenius 2002), or may result from daily variations in temperature. Onset of anthesis appeared to be correlated with observed visits of pollinators (Fig. 5), as pollinators did not visit flowers before 07:30 or after 15:30.

Passiflora herbertiana exhibits perianth colour change throughout anthesis, which may signal decreased availability of nectar rewards and may be associated with fertilization of

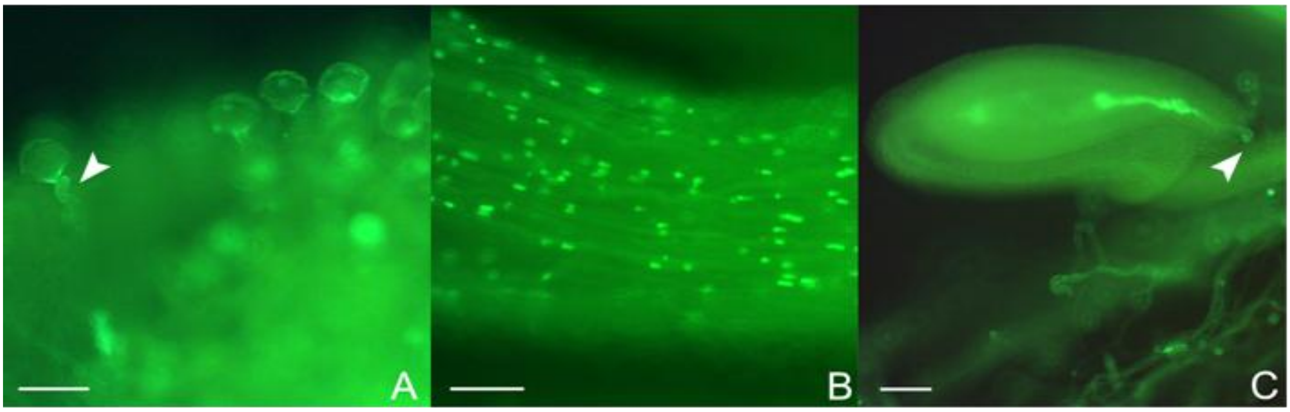

FIGURE 3. Assessment of self-compatibility in *Passiflora herbertiana.* (A) Pollen grains germinating on the style. Arrow indicates pollen tube. (B) Example of pollen tubes germinating through the centre of the style in a self-pollinated individual. (C) View of a fertilized ovule in a self-pollinated individual. Arrow indicates pollen tube penetrating the micropyle and reaching the nucellus. Scale bars: a, c = 50 µm; b = 100 µm.

FIGURE 4. Floral visitors to *Passiflora herbertiana.* (A) Noisy Miner. (B) Lewin's Honeyeater. (C) Eastern Spinebill.

ovules (Delph & Lively 1989; Weiss 1995). Anthocynanins are often responsible for floral colour change (Weiss 1995), and provide UV protection to plant tissues (Mazza et al. 2000). Flowers exposed to full sun were salmon-coloured while those in shade were pale yellow. In full sun, anthocyanins may accumulate more quickly as a protective response. Most flowers received indirect sunlight exposure and accumulated pigmentation gradually as the flower aged (Fig. 2). Thus, colour change may serve a dual purpose as both a UV sunscreen and a signal to pollinators in *P. herbertiana*.

Floral rewards and associated pollinators

Nectar produced by *P. herbertiana* is comprised exclusively of sucrose. This is consistent with hummingbird-pollinated *Passiflora mathewsii* (Mast.) Killip and *P. murucuja* (Krosnick unpublished data), both of which have sucrose-dominant nectar. Hummingbirds require sucrose-rich nectar (Baker & Baker 1982, 1983; Lotz & Schondube 2006) due to their high energetic needs. Passerines were thought to prefer hexose sugars (Baker & Baker 1982, 1983), but recent studies (Dupont et al. 2004; Johnson & Nicolson 2008) indicate they are varied in their preferences. Honeyeaters have been shown to be nearly as efficient at assimilating sucrose as hummingbirds (Lotz & Schondube 2006), suggesting that the Australian honeyeaters observed in the present study should have little difficulty in utilizing nectar from *P. herbertiana*.

Honeyeaters are the dominant floral visitors in Australia, and they have been described as both generalists and opportunists: they visit many species of flowers, including non-natives (Ford et al. 1979; Recher 1981). Little information is known about nectar preferences and patterns of visitation in Eastern Spinebills, Lewin's Honeyeaters, or Noisy Miners. Mitchell and Paton (1990) suggest that even though honeyeaters are a diverse lineage, they place similar selective pressures on nectar traits including nectar volume and sugar content. The three species observed visiting *P. herbertiana* are not at all closely related based on the most recent Melaphagid phylogeny (Joseph et al. 2014), yet exhibit similar preferences for the dilute, sucrose-dominant nectar in *P. herbertiana*.

Self-compatibility

Floral morphology and phenology in *P. herbertiana* are consistent with outcrossing, yet this species is clearly self-

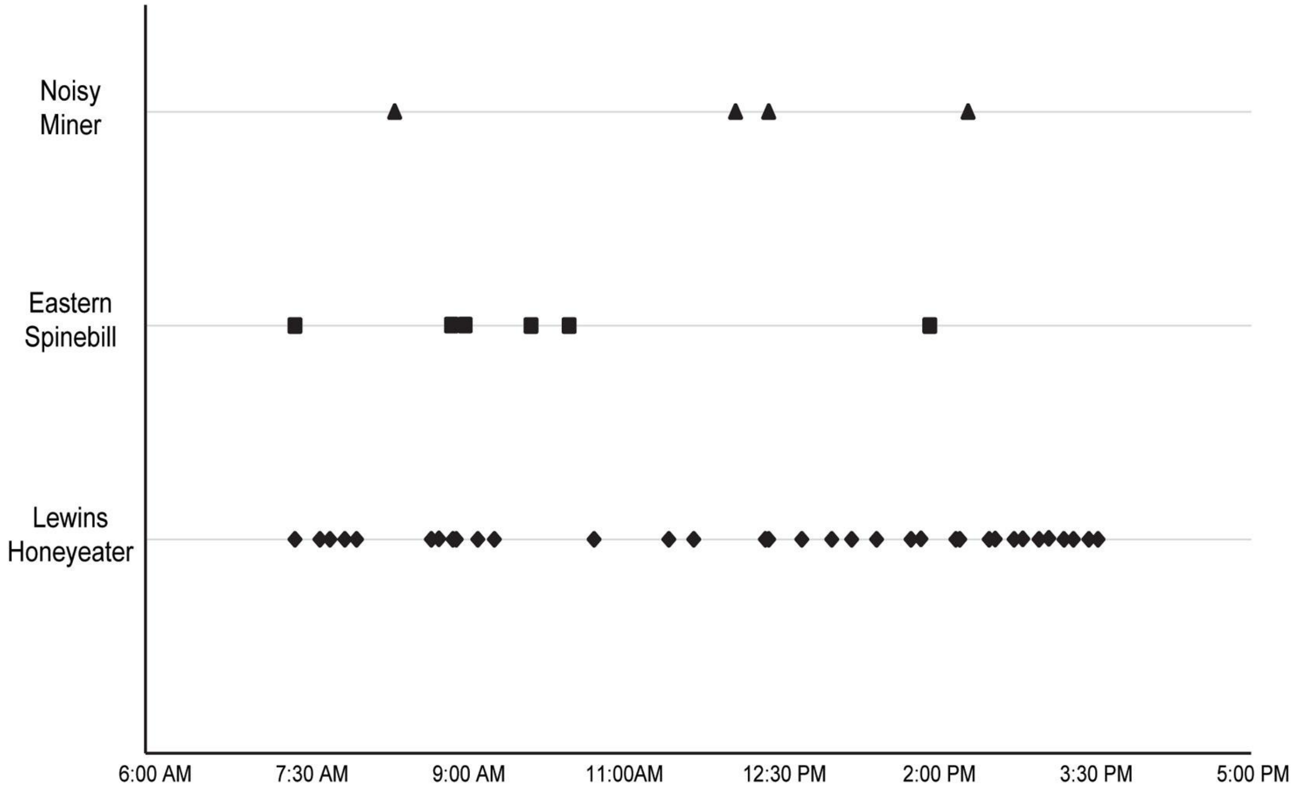

FIGURE 5. Visitation patterns of birds on *Passiflora herbertiana.* Floral visits generally began by 7:30 and stopped by 15:30 each day. Lewin's Honeyeaters were most frequent with visits documented fairly consistently throughout the day. Eastern Spinebills were the next most frequent, followed by Noisy Miners.

compatible. Pollinator-mediated selfing may provide an alternative when other pollen sources are lacking (Barrett 2002). Late-acting self-incompatibility was not examined in this study, and if present could have a significant effect by eliminating self-fertilized progeny.

Reproductive biology

Similar to *P. aurantia* and *P. cinnabarina,* flowers of *P. herbertiana* remain open for three to five days (Krosnick 2006). Most *Passiflora* typically open for just one day, but some hummingbird-pollinated species in the Andean supersection *Tacsonia* have flowers that remain open for up to three days (MacDougal 1994). This may be an adaptation to infrequent visits from pollinators, or may result from slow rates of pollen tube growth due to cooler temperatures at higher elevations. In June–July, *P. herbertiana* was just beginning to undergo fertilization of ovules at 48 hours post-pollen transfer (Fig. 3), but this might occur more quickly during December–February when ambient temperatures are higher. Stigmas in other *Passiflora* have been shown to be receptive quite early during anthesis (MacDougal 1994).

Styles in *P. herbertiana* are notable in their movement patterns. As the end of anthesis approaches, the styles in most *Passiflora* return to their original vertical orientation, regardless of whether or not pollination has occurred (MacDougal 1994). In *P. herbertiana,* the styles continue downward (Fig. 1C-D); this movement might promote self-pollination as the flower ages. Moreover, short-styled floral morphs were observed (Fig. 2) that lacked any stylar movement. This phenomenon has been noted sporadically across *Passiflora* (MacDougal 1994). Andromonecious plants were documented in *P. incarnata* (Dai & Galloway 2012); in that study, male flowers appeared hermaphroditic but had short, erect styles that never bent downward, making self-pollination nearly impossible. More studies are needed in *P. herbertiana* to examine the effect short-style morphs may have on population dynamics.

Conclusion

The similarities observed in NW hummingbird-pollinated *Passiflora* and Australian *P. herbertiana* provide an example of convergence of both floral form and function in response to similar pollination syndromes. Many details remain to be examined for *P. herbertiana,* including the frequency and significance of andromonecy, seasonal variation in pollen tube growth rates, timing of stigma receptivity, and rates of floral nectar production during anthesis. Floral visitation is not the same as effective pollination, and field studies specifically designed to examine pollen transfer during floral visits are needed to address interactions between *P. herbertiana* and its visitors. To understand the evolution of reproductive syndromes in the Australian *Passiflora,* studies are also needed for *P. cinnabarina* and *P. aurantia.* All three species are relatively widespread, and studies across their entire range should reveal new plant-pollinator interactions in addition to those documented here. It will be especially interesting to see if the *Passiflora*-honeyeater association described for *P. herbertiana* is supported in the other Australian species.

APPENDICES

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

APPENDIX I. This video shows footage of Lewin's Honeyeaters visiting various *P. herbertiana* flowers at the field study location (Witches Falls section of Mount Tamborine National Park, Queensland, Australia).

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