Dracula's mistress: removal of blood-red floral nectar results in secretion of more nectar

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Background and aims – Flowers of *Jaltomata quipuscoae* (Solanaceae) secrete blood-red nectar that serves as an energy reward and possible attractant to pollinators. The purposes of this study were to determine whether simulated pollinator visits (manual removal of nectar) stimulates replenishment of nectar, and report the pattern of nectar presentation during the lifespan of the flower.

Methods – For the nectar replenishment experiments flowers were paired: each pair of flowers was selected to be on the same plant and at the same developmental stage. From all 62 flowers nectar was removed and discarded (not measured) at time zero. Then, over a period of eight hours, the nectar of one flower was measured four times, i.e., every two hours, while nectar of the paired control flower was measured only at the end of the eight-hour period. In the nectar dynamics experiment five sets of flowers received different treatments: flowers were unmanipulated for zero, one, two, three or four days and then nectar was removed once every day. The volume of nectar produced and concentration of sugar in the nectar were recorded at each extraction for both studies.

Key results – In the nectar replenishment study significantly higher nectar volume and consequently significantly higher total sugar content was present in the experimental nectar-extracted flowers. In the nectar dynamics study, nectar was produced starting on day one or two, continuously through the life of the open flowers until one or two days before the corolla senesced. Delay of nectar removal from different flower sets for zero, one, two, three or four days resulted in a linear increase in nectar volume and total nectar sugar production, and had little or no effect on the cumulative (life of the flower) nectar production. Floral longevity, seven to ten days, was not affected by a single removal of nectar each day.

Conclusions – The floral nectary of *J. quipuscoae* responded to nectar removal by secreting more nectar, and thus more total sugar (not a higher concentration of sugar) than was secreted by control flowers. In flowers from which nectar was not removed, nectar volume and thus total sugar secreted continued to accumulate linearly, suggesting that reabsorption of nectar either does not occur or is slow relative to the rate of secretion. The more we (or pollinators) take, the more the flowers make: the volume of nectar and sugar production increase if nectar is removed frequently but not if nectar is removed infrequently.

Keywords – Coloured nectar; *Jaltomata*; nectar removal; nectar replenishment; Solanaceae.

INTRODUCTION

Floral nectar is offered by flowers to pollinators as an energetic reward (Corbet 2003). Patterns of nectar presentation vary: some species produce nectar continuously from the beginning to the end of anthesis and other species produce nectar for much shorter periods of time (Cruden et al. 1983; Galetto & Bernardello 1992; Bernardello et al. 1994). Replenishment of nectar that has been removed by pollinators would presumably be adaptive, promoting repeated visits that would serve both female (ovule fertilization) and male (pollen dissemination) functions (Castellanos et al. 2002; Ornelas et al. 2007). Low levels of replenishment and or nectar larceny (Castro et al. 2008) reduce the nectar offering. Regulation of the nectar offering influences pollinator behaviour, and has the potential to reduce both geitonogamy and pollen discounting and promote outcrossing (Heinrich & Raven 1972; Harder & Barrett 1996).

A meta-analysis of studies involving fifteen plant species concluded that simulating pollinator visits with nectar removal generally results in increased nectar production (Ordano & Ornelas 2004). However, it appears that this response to nectar removal is species-specific and varies depending on factors such as habitat, type of pollinator, altitude and region (Ordano & Ornelas 2004; Ornelas et al. 2007; Luo et al. 2014). Studies have presented contradictory results about the nectar replenishment patterns of various species. For example, a study involving *Macleania bullata* Yeo, a tropical plant that experiences frequent nectar robbing, concluded that nectar removal results in increased nectar production without altering the nectar sugar concentration (Navarro 1999). Other removal studies have concluded that nectar removal had no effect on the volume or sugar concentration of nectar produced (Galetto & Bernardello 1992, 1993; Nepi et al. 2011), and that the total amount of sugar in the nectar of experimental nectar-removed flowers may be lower than in controls (Galetto & Bernardello 1992; Bernardello et al. 1994). These conflicting results indicate that species differ in their genetic ability to replenish nectar.

The genus *Jaltomata* Schlecht. (Solanaceae) consists of approximately seventy species ranging from Arizona to Bolivia (Mione et al. 2015). This genus is interesting for many reasons: the fruits of most species are eaten by people, the corolla forms are remarkably diverse (rotate, campanulate, short-tubular, long-tubular), bees and hummingbirds have been recorded on flowers of different species, habitats range from desert to rain forests, and altitudes range from near sea level to over 4000 m (Mione & Leiva González 1997; Mione 1999, Mione et al. 2000, 2001, 2004, 2007, 2016, 2019; Kostyun & Moyle 2017; Mione & Anderson 2017; Wu et al. 2019). One of the more striking features is deeply coloured floral nectar, ranging from orange to red or purple: coloured nectar is regularly produced by at least fifteen *Jaltomata* species, a subset of the South American species of Peru and Bolivia (Mione & Anderson 1996; Hansen et al. 2007; Leiva González et al. 2016). This study employed a recently discovered wild species, *J. quipuscoae* Mione & S.Leiva, that produces blood-red nectar (Mione et al. 2015). Red nectar in the genus *Jaltomata* is highly UV-absorptive (Mione et al. 2018). While taking into consideration that UV light is not more conspicuous to pollinators than other wavelengths that nonhuman animals see, it is possible that UV-absorptive nectar enhances the foraging signal (Thorp et al. 1975; Frohlich 1976).

The purposes of this study were to determine if nectar removal from the flower of *J. quipuscoae* results in removal-enhanced nectar replenishment (RENR, Luo et al. 2014), and to report patterns of nectar presentation based on daily observations during the lifespan of the flower. Consideration of Ordano & Ornelas' (2004) meta-analysis led us to predict that nectar removal from flowers of *J. quipuscoae* would likely result in increased nectar production. Given the importance of the caloric reward of floral nectar to pollinators, we also investigated the sugar concentration of nectar, and the total quantity of nectar-sugar produced over the lifespan of flowers.

MATERIALS AND METHODS

Study species

Jaltomata quipuscoae is a wild, herbaceous perennial of Department Arequipa, Peru, growing to 70 cm high. Flowers are solitary, hermaphroditic, protogynous, and in the greenhouse are 4.0–4.7 cm in diameter during the hermaphroditic phase. Numerous flowers are open on a plant at a given time, most of which are in the hermaphroditic phase because the pistillate phase usually lasts only one day. The broadly campanulate corolla is green when it opens but soon darkens to purple and remains purple for the life of the flower (fig. 1A). Pollinators have not been studied, but hummingbirds visit *J. calliantha* S.Leiva & Mione, a similar and closely related species of northern Peru (T. Mione & S. Leiva González, unpubl. res.). Nectar of both *J. calliantha* and *J. quipuscoae* aggregates (pools) on the floral corona (Plourd & Mione 2016), a structure present in flowers of only three *Jaltomata* species. In *J. calliantha*, hand-pollination does not affect the volume of nectar produced (Plourd & Mione 2016). Nectar darkens with age (Mione et al. 2019) but not when oxygen is experimentally excluded (N. Glagovich & T. Mione, unpubl. res.). Ripe berries are green, and in the greenhouse drop at maturity. Seeds were collected (*Mione et al. 798*) from native wild plants at the type locality by Victor Quipuscoa, T. Mione, S. Leiva González and Leon Yacher.

Nectar replenishment study

Pairs of flowers on the same plant were tagged prior to anthesis. Once we were certain that the corollas of the two flowers opened on the same day, we waited one full day, and nectar removal experiments were done on day two, the second day both flowers were open (the first day of the hermaphroditic phase). The results of the nectar dynamics study (see following) suggested that we could have selected any one or more days of days two through six as representative of nectar production; we selected day two. Nectar of both flowers of the pair was removed with microcapillary tubes (Galetto & Bernardello 2005; fig. 1B) and discarded (not measured) at the beginning of the experiment at 10 am. For the experimental flower of the pair, nectar was removed and measured every two hours throughout the eight-hour period. For the control

flower, nectar was removed and measured at the end of the eight-hour period. After each nectar removal, plants were watered in order to ensure that water was not limiting (when soil moisture is reduced lower nectar volumes generally result, see Boose 1997 and Gallagher & Campbell 2017). Two similar experiments were conducted from October 2017 to January 2018 with 20 pairs of flowers, and from April to December of 2018 with 11 pairs of flowers. We had no data suggesting that variables were confounding in our first experiment, but were concerned that proximity of a tagged flower (from which the nectar was measured) to non-utilized flowers to which the plant was allocating resources for nectar production, and / or height of a flower on the plant, could potentially affect nectar production. Thus, to eliminate potentially confounding variables, in the second experiment we selected pairs of flowers on the same plant that were at approximately the same height on a plant, and excised open flowers (open flowers produce nectar) on the same branches as our chosen flowers. The first experiment was the same as the second except the flowers of a pair were not necessarily at the same height and nearby open flowers were not excised. To consider sugar concentration we divided sugar content by volume (mg / µl); we did not use raw refractometer readings because for each pair of flowers there was one refractometer reading for the control and four refractometer readings for the experimental (every two hours). For paired t-tests (all two-tailed), differences between pairs of measurements were normally distributed.

Nectar dynamics study

Each set of flowers received a different treatment: flowers were unmanipulated for zero, one, two, three or four days and then nectar was removed once a day on subsequent days (Bernardello, personal communication). Four to five flowers
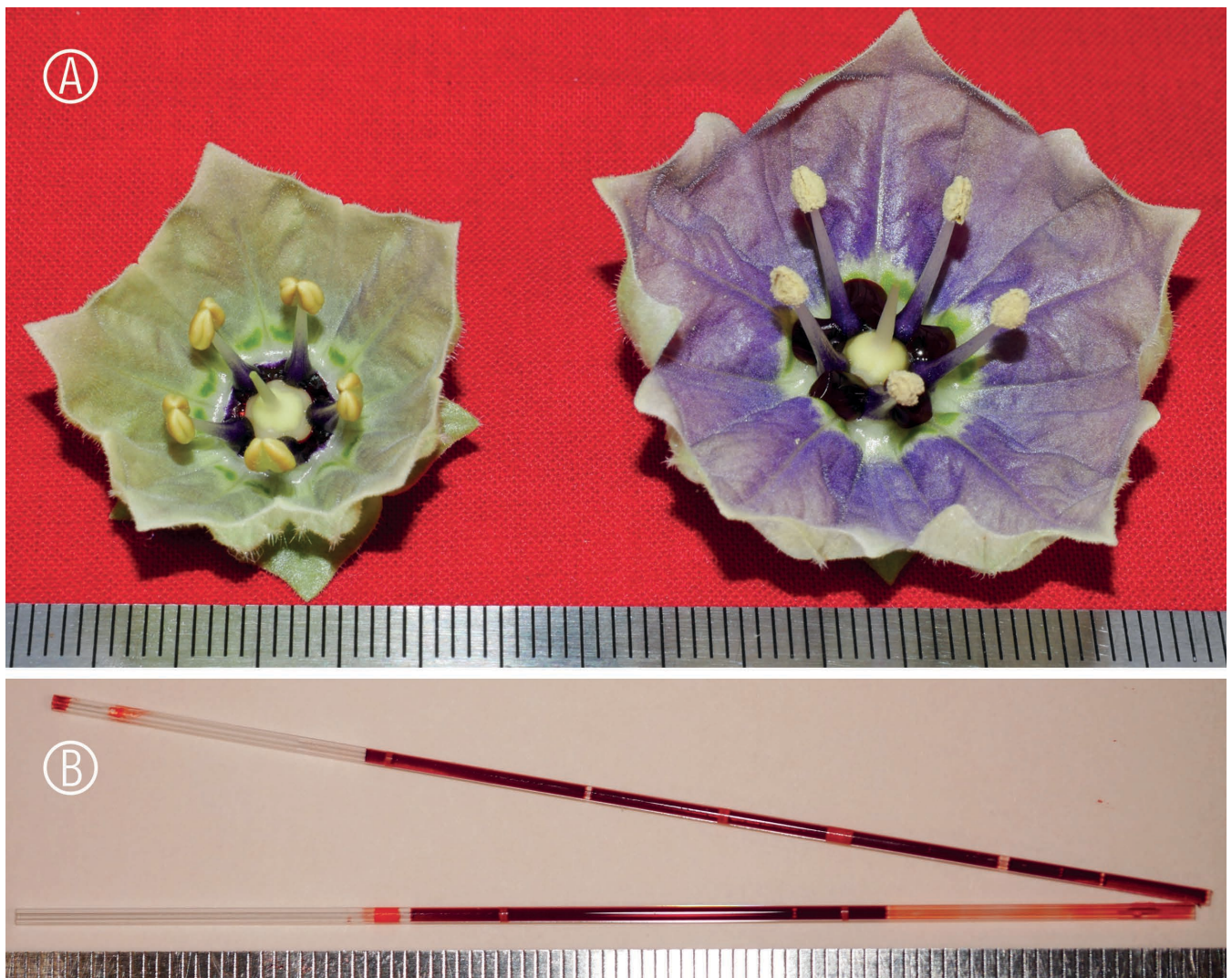

Figure 1 – A. Flowers of *Jaltomata quipuscoae*. On the left, during the pistillate phase (anthers undehisced) the corolla is green and begins to turn purple. On the right, hermaphroditic phase (anthers dehisced) flowers have turned purple. Nectar darkens with age, appearing orange-red during the pistillate phase and then darkening to purple. These flowers were grown in the same greenhouse at the same time. **B**. Microcapillary tubes containing floral nectar of *J. quipuscoae,* in which 1 mm equals 0.38 µl. Photographs by T. Mione and I. Diaz. Units along bottom are mm in both photos.

Table 1 – Nectar replenishment study.
Summary statistics for two nectar removal experiments.

	experiment 1 n = 20		Experiment 2 n = 11	
	removal every two hours	control	removal every two hours	control
nectar volume (µl)				
mean	14	8.5	37	15
SEM	1.8	1.3	3.7	2.4
paired *t*-test P	0.0016		< 0.0001	
nectar sugar content (mg)				
mean	4.1	3.2	12	6.1
SEM	0.48	0.43	2.4	1.7
paired *t*-test P	0.013		0.0008	
sugar concentration (mg/µl)				
mean	0.34	0.45	0.28	0.38
SEM	0.03	0.05	0.034	0.063
paired *t*-test P	0.0103		0.0316	

were used per set for a total of 21 flowers. Data were collected between noon and 2:00 pm from 12 December 2017 to 19 February 2018. The mean temperature in the greenhouse during data collection was 18.3°C (range 15.6 to 22.8°C), with cooler, unrecorded temperatures at night. Twenty-one values were used in all regressions. For the nectar volume and nectar sugar content regressions, log transformation of the Y values (plus one) satisfied assumptions of normality

(Anderson-Darling test) and homoscedasticity. However, P-values were unchanged and so graphs (fig. 5A, B) are presented with the original data.

Both studies

Experiments were done in the greenhouse to exclude floral visitors. Five plants were used. The nectar volume (µl) and sugar concentration (as a percent) were recorded at each removal. The percent sugar concentration was measured using two Bellingham and Stanley refractometers, model 45-81 for concentrations ranging from 0 to 50%, and model 45-82 for concentrations ranging from 45 to 80% (Bellingham and Stanley, Kent, United Kingdom). The sugar content of each sample was calculated as follows: mg of sugar equals 10 (C) (V) (D) where C is the refractometer reading (g sucrose / 100 g solution), V is the volume of the nectar in µl / 1000, and $D = 0.0037291 (C) + 0.0000178 (C^2) + 0.9988603$ (Prys-Jones & Corbet 1987). Statistical analyses were done with Prism 8.2.1 (GraphPad Software, Inc., San Diego, CA, USA; GraphPad 2019).

Figure 2 – Total nectar volume produced by experimental flowers from which nectar was discarded at time zero and then measured every two hours, and control flowers from which nectar was discarded at time zero and measured at the end of the eight-hour experiment. The scale for the Y axes differs in the two graphs.

RESULTS

Nectar replenishment study

Flowers from which nectar was removed every two hours over the eight-hour period produced significantly more nectar than those that only had nectar removed at the end of the eight-hour period (table 1, fig. 2). As well, over the eight hours of the study the experimental flowers produced significantly more sugar than the control flowers (table 1, fig. 3). In the experimental flowers, cumulative sugar content rose during the experiment, indicating a fairly constant rate of secretion (fig. 4). For both replenishment experiments sugar concentration was significantly higher in the control flowers than in the experimental flowers (table 1).
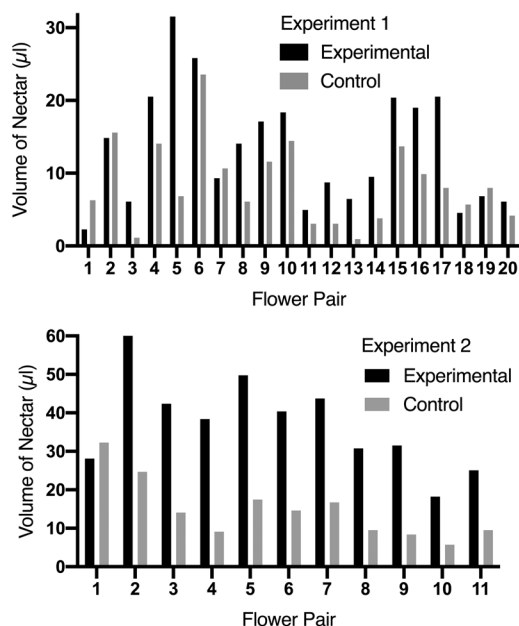
Table 2 – Summary statistics for regressions in nectar dynamics study (21 flowers per regression).

	nectar volume	sugar content	cumulative sugar	cumulative volume	floral longevity
figure	5A	5B	5E	graph not presented, similar to 5E	5F
P	< 0.0001	< 0.0001	0.3925	0.3582	0.8501
95% confidence interval of slope	9.694–15.10	7.288–10.61	-1.6–3.985	-16.8–6.368	-0.29–0.35
r^2 (after log transformation)	0.8289 (0.74)	0.8700 (0.86)	0.0387	0.0446	0.0019

Nectar dynamics study

The nectar volume and sugar content rose linearly in sets of flowers that were unmanipulated for zero, one, two, three or four days (fig. 5A–D). After the flowers of a set were left undisturbed for a given number of days, nectar was removed once every day. And once nectar removal began, flowers of all treatments had similar nectar volumes and sugar contents (fig. 5C, D). During the first five days, in different sets of flowers for each treatment, mean sugar content rose linearly to 36 mg per flower, and nectar volume increased linearly to 50 µl (fig. 5A, B), which was approximately the volume at which nectar dripped (out of the troughs) down the corolla. Delay of removal of nectar for up to four days had little or no effect on the cumulative sugar and nectar production (the graph for cumulative volume is similar in appearance to fig.

5E but not presented). Summary statistics for regressions are presented in table 2.

Flowers from which nectar was removed daily (after zero, one, two, three or four days of non-removal) were open for seven to ten days. Floral longevity was apparently not affected by daily removal of nectar (fig. 5F). There were five flowers from which nectar was removed once every day starting on day one; two of these had no nectar on day one
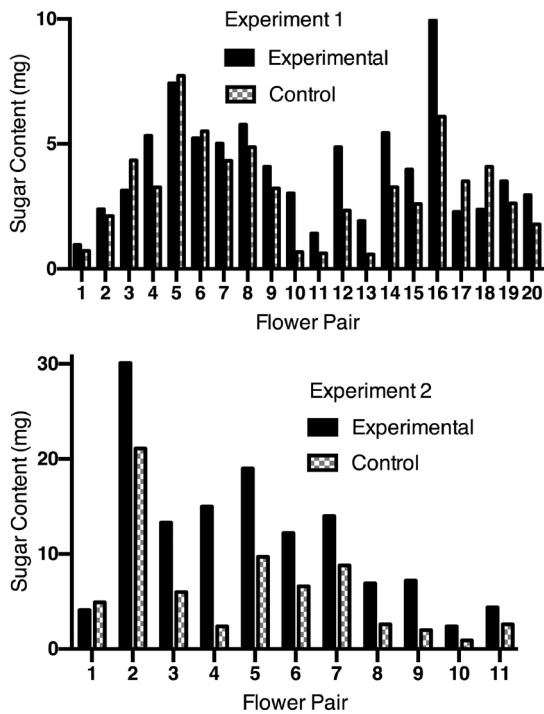

Figure 3 – Sugar produced by experimental flowers from which nectar was discarded at time zero and then measured every two hours, and control flowers from which nectar was discarded at time zero and measured at the end of the eight-hour experiment. The scale for the Y axes differs in the two graphs.
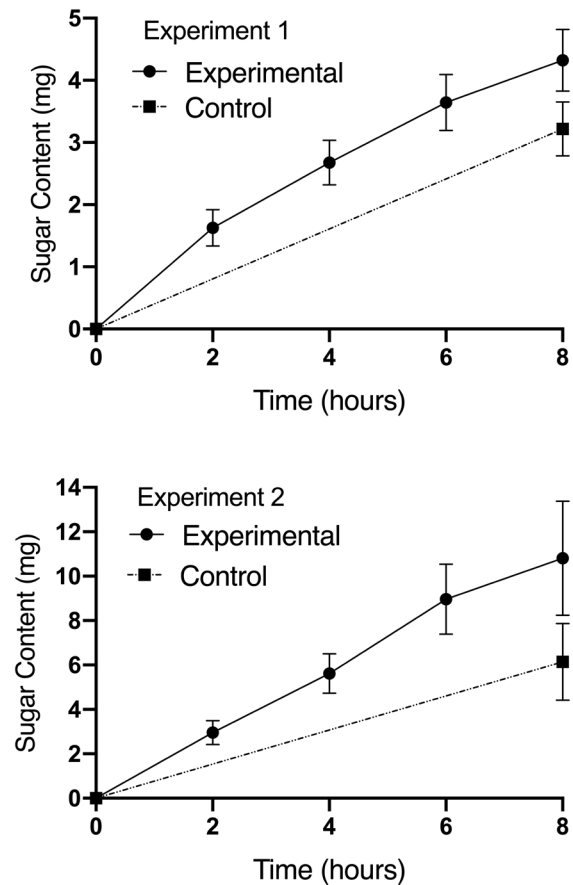

Figure 4 – Cumulative sugar content produced by experimental flowers from which nectar was discarded at time zero and then measured every two hours, and control flowers from which nectar was discarded at time zero and measured at the end of the eight-hour experiment. Plotted points are means; vertical bars are standard error of the mean.
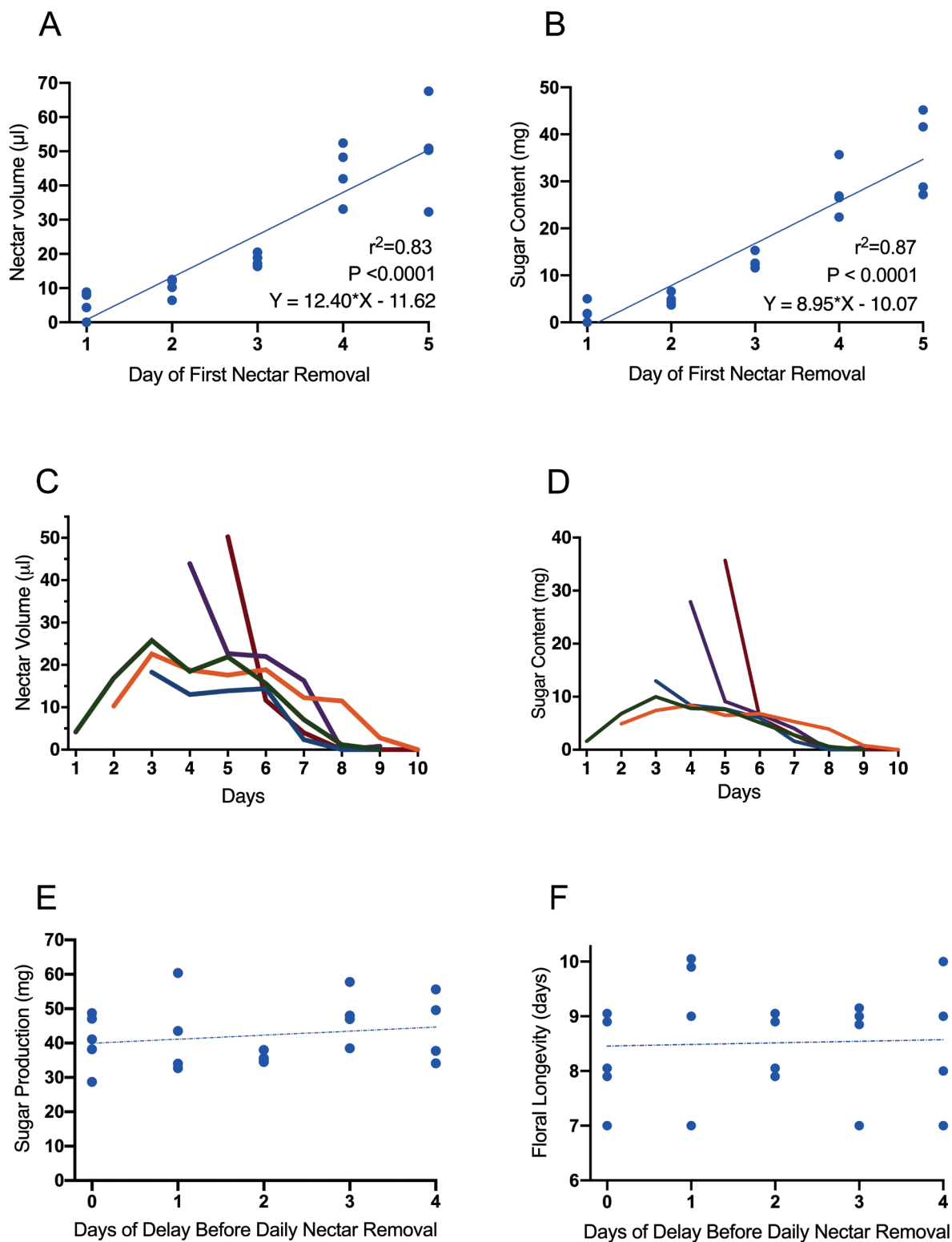
Figure 5 – Nectar volume (**A**) and sugar content (**B**) on day of first removal after no removal for 0, 1, 2, 3 or 4 days. 'Day 1' represents the first day the flower was open; 'Day 2' signifies that the flower had been open 2 days, and the nectar was removed on the second day of nectar accumulation, etc.; slopes are significantly non-zero. Daily nectar volume (**C**) and sugar content (**D**) during the life of the flower with no removal for 0, 1, 2, 3 or 4 days and then removal once every day. Cumulative nectar sugar (**E**), additive for the life of the flower, is not affected by delaying nectar removal. Floral longevity (**F**) is not affected by delaying nectar removal. For A, B, E and F each plotted point is a flower and the flowers of a set are aligned vertically; for C and D each line on the graph represents one flower set.

but these flowers had secreted nectar by day two. Open flowers having no nectar were also seen during the last day or two of anthesis; 19 of 21 flowers had no nectar during the last one to two days of anthesis.

DISCUSSION

Nectar production may cost a plant up to 37% of its available energy and this may reduce vegetative growth and reproductive ability (Pike 1991). For flowers of *Blandfordia nobilis* Sm., nectar removal resulted in increased production of nectar but also decreased a plant's ability to produce seeds (Pyke 1991). For two bromeliad species, the cost of replenishing nectar was high for one species (seed set was reduced approximately by half) and negligible for the other (Ordano & Ornelas 2005). In our replenishment study, sugar concentrations were significantly higher in control flowers but the concentration differences (table 1) were likely due only to more evaporation of water from nectar that remained in place for the 8 hours of the experiment. Because the mean sugar content was higher in the nectar of the experimental flowers, but the sugar concentrations were higher in the control, we conclude that the higher sugar content in the experimental flowers was due to the greater volume of nectar produced if sugar is not being reabsorbed in control flowers.

Luo et al. (2014) noted that replenishment of nectar may be overestimated in experimental studies relative to field studies. Our study took place in the greenhouse where water was never limiting. Conditions in Arequipa, Peru, where this species grows are generally dry: on average there is only 10 cm of rain per year mostly in the months December through March (Holmgren et al. 2001) when the plants flower. Plants grown in dry soil generally produce less nectar (Boose 1997; Carroll et al. 2001; Gallagher & Campbell 2017), and had this study been done with wild plants, nectar volumes almost certainly would have been lower.

Flowers remained open seven to ten days in our nectar dynamics study. During earlier observations in the same greenhouse, minimum floral longevity was four days (Mione et al. 2019). Although the temperature was not measured during the earlier observations, we have observed during several years of growing *Jaltomata quipuscoae* in the greenhouse that floral longevity is inversely correlated with seasonally varying (unmanipulated) temperatures. The temperature was quite cool in the greenhouse during this nectar dynamics study, at times as low as 15.6°C when nectar was measured and cooler yet at night. In this study, the lack of a relationship between number of days of daily nectar removal and floral longevity (fig. 5F) suggests that the method of nectar removal, gently inserting microcapillary tubes into nectar troughs, does not damage flowers.

Starting on day one or two, with once daily removal of nectar, flowers produced nectar every day except for the last day or two when flowers were usually open but produced no nectar. Comparison of sets of flowers that were unmanipulated for zero, one, two, three or four days (depending on the flower set) showed that nectar volume and sugar content rose linearly. This linear increase suggests that floral nectar is not reabsorbed or reabsorption is slow relative to the rate of secretion. Daily nectar removal began the day after flowers were unmanipulated for a given number of days; once daily nectar removal began the volume of nectar and sugar content were similar for all sets of flowers (fig. 5C, D). Total sugar production over the life of the flower was similar for flowers experiencing one nectar removal per day and flowers experiencing one nectar removal per day that was delayed one or more days (fig. 5E). In contrast, in our nectar replenishment study, removal every two hours increased the nectar volume and sugar production relative to controls. This underscores the assertion made by Luo et al. (2014) that nectar production is not a fixed trait and varies depending on removal, and is concordant with their statement that replenishment of nectar after removal is 'more the rule than the exception.' Our results demonstrate that the nectaries of *Jaltomata quipuscoae* respond to nectar removal, repeatedly providing a reward to pollinators.

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