

RESEARCH PAPER

Nectar regulation in *Euphorbia tithymaloides* L., a hummingbird-pollinated Euphorbiaceae

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Keywords

Cyathium protogyny; homeostatic mechanism; hummingbird preference; nectar; sexual phases.

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Editor

A. Dafni

Received: 4 June 2012; Accepted: 25 September 2012

doi:10.1111/j.1438-8677.2012.00695.x

INTRODUCTION

Hermaphroditic flowers may benefit from separating male and female function since it decreases autogamy (Lloyd & Webb 1986). The sexual phases can be different in secondary sex characters such as nectar reward (Carlson & Harms 2006 and references therein). Gender-biased nectar production (GBNP) may allow plants to optimise each sexual function (pollen export for male function and pollen receipt affecting seed set for female function). In other words, floral phases would adaptively present dissimilar energetic rewards to pollinators (Brunet & Charlesworth 1995; Carlson & Harms 2006 and references therein; Carlson 2007). Another theory explaining GBNP assumes that differences between sexual phases in nectar availability decrease geitonogamy. The theory relies on the assumption that pollinators are risk-averse foragers. Under this theory, pollinators move from one plant to another when finding inadequate amount of nectar, which increases pollen export and outcrossing (Rathcke 1992; Boose 1997; Galetto & Bernardello 2004). Geitonogamy may be reduced if protogyny is associated with female-biased nectar production; presumably pollinators would first visit female-phase flowers (the more rewarding phase), depositing pollen from other individuals, and then visit male-phase flowers before departing for yet other individuals (Carlson & Harms 2006 and references therein).



Hummingbirds show risk-averse behaviour (Hurly & Oseen 1999) and are able to learn to detect differences in nectar rewards (Meléndez-Ackerman *et al.* 1997; Maloof & Inouye

ABSTRACT

Floral sexual phases can differ in nectar production and might be under selective pressure by pollinators. We studied *Euphorbia tithymaloides*, which has inflorescences that are initially female and then hermaphroditic. Volume and concentration of nectar were measured in both stages. Nectar production and the effect of extractions were determined using sets of bagged inflorescences; inflorescences in the hermaphroditic phase had higher values of nectar concentration, volume and sugar mass than inflorescences in the female phase. Nectar resorption was detected in senescent inflorescences. To test for homeostatic nectar regulation, artificial nectar was added and the response assessed after 24 h. The experiments showed that concentration and sugar mass are regulated within a narrow range, and the homeostatic points differ between the two sexual phases. These differences in nectar can be detected by hummingbirds, which prefer the female stage. Resorption and secretion seem to be part of a homeostatic mechanism by which nectar attributes are maintained to optimise sugar recovery.

2000 and references therein; Henderson *et al.* 2001). In addition, ornitophilous plants are parsimonious regarding nectar concentration (low), composition (sucrose dominant) and rates of secretion (slow) (Pyke & Waser 1981; Galetto & Bernardello 1993, 2004; Bernardello *et al.* 1994; Fenster *et al.* 2004; McDade & Weeks 2004; Chalcoff *et al.* 2006; Navarro *et al.* 2008). Given this past work, it is plausible to suppose that hummingbirds might exert selective pressures in favour of certain nectar attributes. It is likely that ornitophilous species may show a regulation mechanism on nectar attributes to reduce variability and to maintain nectar near an adaptive optimum (Castellanos *et al.* 2002). Flowers would accomplish this through homeostatic secretion and resorption (Búrquez & Corbet 1991).

In the current report, we consider nectar production patterns, the effect of nectar removal or addition, and the behaviour of hummingbirds – all in the context of protogyny. Other authors have indicated that the pattern of nectar secretion over a blossom's lifespan and the activity of pollinators may be coordinated. Nectar offerings may influence the frequency of visits (Mitchell 1991; Real & Rathcke 1991; Galetto & Bernardello 2004), and nectar removal may stimulate nectar secretion (Gutián *et al.* 1995; Navarro 1999). Nectar may also be resorbed. Resorption can have two functions: in senescent flowers it may recycle resources; in flowers that are in their prime, resorption combined with secretion can regulate nectar offerings, affecting the behaviour of pollinators (Nicolson 1995; Nepi *et al.* 2001; Nepi & Stpiczynska 2008). Homeostatic

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1 nectar secretion has been little studied (Galetto *et al.* 1994;
2 Nepi & Stpiczynska 2008). The molecular process of nectar
3 secretion is now under active investigation (Ren *et al.* 2007;
4 Kram & Carter 2009; Ruhlmann *et al.* 2010; Heil 2011). Our
5 report focuses on GBNP under natural conditions to comple-
6 ment the present findings.

7 We studied *Euphorbia tithymaloides*, formerly in the genus
8 *Pedilanthus* (Steinmann 2010). The lineage is thought to have
9 been derived from ordinary entomophilous *Euphorbia* and
10 then to have come under recent selective pressure by hum-
11 mingbird pollinators (Dressler 1957; Webster 1967; Cacho
12 *et al.* 2010). In this ornithophilous species, the sexual stages are
13 easily differentiated, the inflorescences are abundant and nectar
14 is easily manipulated. Ornithophilous plants generally have low
15 nectar concentrations, a generalisation that might be expected
16 to extend to *E. tithymaloides*. If, on the contrary, higher nectar
17 concentrations predominate, phylogenetic constraints can be
18 presumed because high nectar concentrations are the norm in
19 the genus *Euphorbia* (Traveset & Saez 1997; Narbona *et al.*
20 2005).

21 We compared the nectar offerings in female-phase and
22 hermaphroditic-phase inflorescences of *E. tithymaloides*. If nec-
23 tar is an expensive resource for the plant, each sexual phase
24 may show a particular nectar secretion and resorption pattern
25 during a cyathium's life. Finally, nectaries may have a homeo-
26 static mechanism to regulate nectar volume and concentration
27 within a narrow range. Thus, we posed the following hypothe-
28 ses: (i) nectar dynamics differ between sexual phases, (ii)
29 cyathia of different sexual stages are differentially visited by
30 pollinators, and (iii) *E. tithymaloides* has a homeostatic mecha-
31 nism to regulate nectar features thereby optimising energy
32 investment.

35 MATERIAL AND METHODS

36 The species and study site

37 *Euphorbia* L. (Euphorbiaceae) comprises ca. 1600 species with
38 a cosmopolitan distribution in temperate and tropical regions
39 (Mabberley 1990; Benedí *et al.* 1997). Although dioecy is pres-
40 ent in the genus (Narbona *et al.* 2002; Zimmermann *et al.*
41 2010), the most common sexual system was formerly consid-
42 ered as monoecy with unisexual flowers. More commonly, it is
43 monoecious with unisexual flowers grouped in highly organ-
44 ised, tight inflorescences called cyathia, which acts as a 'blos-
45 som' functional unit for pollination (Webster 1994; Prenner &
46 Rudall 2007; Prenner *et al.* 2011). Each blossom is, in effect,
47 protogynously hermaphroditic: the pistillate flower develops
48 prior to the male flowers (Prenner & Rudall 2007). *Euphorbia*
49 is mostly a zoophilous genus with highly concentrated nectar
50 (Ehrenfeld 1976; Traveset & Sáez 1997; Narbona *et al.* 2005).

51 *Euphorbia tithymaloides* L. is a shrub 1.20–1.80-m high with
52 zigzag succulent branches. Its distribution includes Mexico,
53 Florida, northern South America, Central America and most
54 islands in the Caribbean (Cacho *et al.* 2010), and it has eight
55 recognised subspecies (Dressler 1957). It is native to Brazil,
56 occurring in Caatinga and Amazonia (Steinmann 2010). It has
57 pink cyathia that are zygomorphic and shoe-shaped (Burger &
58 Huft 1995). Flowers are hummingbird-pollinated (Cacho *et al.*
59 2010); the hummingbird inserts its beak into the spur lifting
60 the lid (Fig. 2c). *E. tithymaloides* cyathia start out in female
61 phase (Figs 1a and 2a) and then become hermaphroditic
(Figs 1b and 2b). The nectary is independent of male and
female flowers, thus the cyathium is the functional unit for
nectar-feeding pollinators (Fig. 2c). Cyathia have a basal spur
with four nectar glands (Fig. 1d). Inflorescences form dense
terminal cymose groups (Millspaugh 1913; Fig. 2c). The plants
employed in this study had on average 36.8 cyathia
(mean \pm 11.44 SE, range 6–125, $n = 10$).

Field observations were conducted in the Botanic Garden of
Recife, Recife, PE, Brazil (08°04'–08°05' S, 34°59'–34°57' W),
in an Atlantic Forest fragment of 8 ha, surrounded by two
other forest fragments of 100 ha each. Individuals were growing
within sight of one another. Field observations and experi-
ments were performed between December 2010 and January
2011. Voucher specimens are deposited in the Herbário UFP -
Geraldo Mariz in the Universidade Federal de Pernambuco,
Recife, Brazil (UFP n. 53.194).

Statistical analyses were done with Statistica v. 8 for Win-
dows (StatSoft, Inc. 2007).

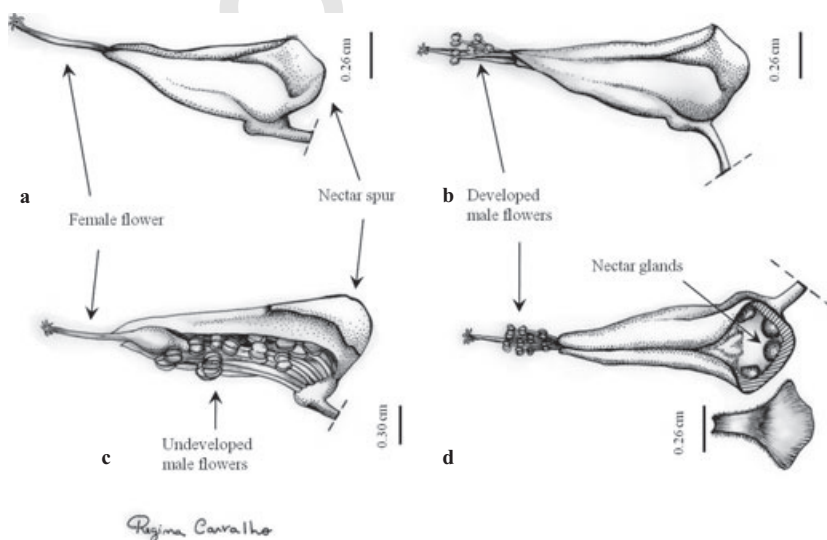


Fig. 1. *Euphorbia tithymaloides* (Euphorbiaceae) inflorescences in different sexual stages: (a) female, (b) hermaphrodite. In the female stage, male flowers are not yet fully developed and pollen is not exposed to pollinators (c). (d) Upper side of the inflorescence with the lid displaced. Nectar is secreted from four glands located inside the spur, which is physically separated from the flowers, thus each inflorescence works as a 'blossom' unit for pollination.



Fig. 2. *Euphorbia tithymaloides* (Euphorbiaceae) inflorescences in (a) female, (b) hermaphroditic phase. (c) *Amazilia fimbriata* (Gmelin 1788) (Trochilidae) visiting the inflorescence in the Botanical Garden of Recife, Brazil; the hummingbird inserts its beak into the spur, lifting the lid. Note the organisation of the inflorescences in cymose groups.

LOW RESOLUTION COLOR FIG

Differentiation of cyathium sexual phases and nectar measures

In order to define sexual phase duration, we followed ten inflorescences from unfurling to senescence. To test for stigmatic receptivity we applied hydrogen peroxide (Kearns & Inouye 1993) to five female flowers in female phase and in hermaphroditic phase. The inflorescences were isolated with bags made of bridal veil from the bud stage to prevent damage by pollinators.

To determine nectar volume and concentration (wt/wt of sucrose) we used a Hamilton (10 μ l) microsyringe and a hand refractometer (Atago Co. Ltd., Italy; Brix. 0–32%, 20 °C). If the concentration was higher than 32% we carefully diluted nectar with distilled water, adding an equal volume to that measured in the nectar sample. From volume and concentration data, we calculated sugar mass (μ g) using the tables of Bolten *et al.* (1979). These three variables were the response variables in the statistical analyses. We tagged equal numbers of cyathia from each plant within each treatment. We did not include ‘plant’ as a random factor in the statistical analyses, but all ‘treatments’ were performed on every plant, so plant variability is equally distributed within all the experimental treatments.

Nectar secretion measurements were done with bagged inflorescences from the bud stage to prevent any nectar removal by pollinators, with the exception of the standing crop, and hummingbird preference measurements, which were done using intact inflorescences. We selected the time of day from 09:00 to 14:00 h for the studies of nectar production and the effect of extraction to relate them to hummingbird activity, which is continuous throughout the day and maximal around 10:00 h (personal observations; Locatelli *et al.* 2004).

Nectar production

Volume and concentration of nectar were measured in female and hermaphrodite cyathia. Inflorescences (total $n = 65$) were assigned to different groups: group 1 inflorescences experienced one extraction of nectar (14:00 h); group 2 experienced two extractions (11:30 and 14:00 h); and group 3 experienced three extractions (09:00, 11:30 and 14:00 h). In order to assess if female and hermaphrodite phases vary in the pattern of nectar production, we used data from the first extraction (*i.e.* natural production without any manipulation) and the data were compared with a factorial ANOVA, with sexual phase and time of day as factors.

Effects of extraction on sugar mass production within a period of 5 h

To assess the effect of the number of extractions on nectar production within a 5-h period, we used the same data from the inflorescences ($n = 63$) and treatments of the previous section. We compared the accumulated sugar mass produced by each group of inflorescences for both sexual phases: group 1 (control) was the sugar mass measured from the extraction at 14:00 h; group 2 was the sum of the extractions made at 11:30 and 14:00 h (one nectar removal); and group 3 sugar was the sum of the extractions made at 09:00, 11:30 and 14:00 h (two nectar removals). To test for differences among groups, we used a factorial ANOVA with number of extractions and sexual phase as fixed factors, and accumulated sugar mass as the response variable.

Sugar mass production over 24 h

The same group of inflorescences ($n = 19$) of the control treatment (group 1 in the previous section) were measured again on the next day, after 24 h during which nectar could have accumulated. Thus, sugar mass measured in the first extraction (14:00 h) was compared with the accumulated sugar mass produced after 24 h. The accumulated sugar mass is the sum of the sugar mass obtained after the first extraction plus the sugar mass measured after 24 h. The test applied was a repeated measures ANOVA, with sexual phase and time (being the variable which compares the initial sugar mass with the accumulated sugar mass) as factors.

Resorption and homeostasis

Volume and concentration of nectar were measured in senescent inflorescences ($n = 66$ for volume; $n = 50$ for concentration) and were compared with nectar data obtained in the first extraction in the hermaphrodite phase using a non parametric Mann–Whitney *U*-test or the parametric *t*-test for independent samples (depending on normality and homoscedasticity; Zar 1999). If sugar mass of senescent inflorescences was significantly smaller than nectar values for hermaphrodite inflorescences, resorption was conjectured.

To test the homeostatic capacity of nectaries, we extracted the nectar from the inflorescences, discharged it, and added artificial nectars with high (75%) and low (25%) sucrose and high (10 μ l) and low (2 μ l) volume in hermaphrodite and female-stage inflorescences in the following combinations: (i) high volume and low concentration; (ii) low volume and high

1 concentration; (iii) high volume and high concentration. Nectar parameters were measured after 24 h ($n = 51$ for concentration; $n = 60$ for volume and sugar mass). Data were analysed using the Wilcoxon non-parametric test for paired samples; each pair of data points being the initial experimental nectar and the final nectar of each inflorescence.

8 Standing crop and hummingbird preference

10 Volume and concentration of nectar were determined between 11 09:00 and 14:00 h from non-bagged female and hermaphroditic inflorescences available to pollinators (standing crop hereafter). Data were analysed with t -tests for independent samples (total $n = 76$ for volume; $n = 71$ for concentration).

15 To assess hummingbird preference on cyathia sexual phase, 16 we made observations from 09:00 to 14:00 h, counting for the 17 number of inflorescences visited, their sexual stage and the 18 plant to which they belonged. The only animal that visited the 19 cyathia of *E. tithymaloides* was *Amazilia fimbriata* (Gmelin 20 1788) (Trochilidae; Fig. 2c), which has been described as being 21 continuously active from 08:00 until 15:00 h, with maximum 22 activity around 10:00 h in a tropical wet evergreen forest (Locatelli *et al.* 2004). We recorded a total of 108 inflorescences 24 visited in a 3-h period of observations accumulated over 3 days 25 between 09:00 and 14:00 h; on the first day from 09:00 to 26 10:00 h, on the second day from 11:00 to 12:00 h and on the 27 third day from 13:00 to 14:00 h; giving a total of three cen-

28 suses, each of 1 h. For each plant we counted the number of inflorescences in each stage to understand plant display in order to calculate the proportion of inflorescences in female and hermaphroditic phases available to pollinators. We considered this proportion as the null hypothesis of no pollinator preference. To determine if the frequency of visits observed to each phase was equal to that expected on the per plant display, we applied a chi-square test of independence.

RESULTS

Differentiation of sexual phases and nectar production

A cyathium's female phase (Figs 1a and 2a) lasted for 2 days, and the stigma of the female flower is receptive from the start of this period. Afterwards, male flowers developed almost synchronously with one another. The cyathium remained for 3 days in the hermaphroditic stage because the stigma remained receptive throughout this phase (Figs 1b and 2b). There were no obvious morphological differences between female and hermaphroditic phases other than exertion of stamens. Senescence was recognised when stamens had no visible pollen and the stigma began to curve downwards.

There were no differences in nectar attributes due to time of day, but cyathia in hermaphroditic phase had higher values for concentration, volume and sugar mass than female cyathia (Table 1, Fig. 3).

Table 1. ANOVA for nectar production and removal effects on total nectar production within 5 h or after 24 h in *E. tithymaloides* inflorescences at female and hermaphrodite sexual stages.

study	statistical test	response variable	total n	factor	df	SS	F	P
nectar production	factorial ANOVA	concentration (wt/wt)	65	sexual stage	1	9533.2	184.99	<0.001
				time of day	2	17.9	0.17	0.841
				S × T	2	253.4	2.46	0.094
				error	59	3040.5		
		volume (μl)	65	sexual stage	1	516.254	116.85	<0.001
				time of day	2	6.053	0.69	0.508
				S × T	2	5.866	0.66	0.519
				error	59	260.667		
		sugar mass (μg)	65	sexual Stage	1	32337.1	183.91	<0.001
				time of day	2	224.8	0.64	0.531
				S × T	2	321	0.91	0.407
				error	59	10373.9		
effect of extractions	factorial ANOVA	accumulated sugar mass (μg)	63	sexual stage	1	4.49*10 ⁸	168.52	<0.001
				no extractions	2	1.61*10 ⁵	0.03	0.970
				S × N°	2	2.34*10 ⁶	0.44	0.647
				error	57	1.52*10 ⁸		
				production over 24 h	repeated measures ANOVA	accumulated sugar mass (μg)	19	sexual stage
				time	1	731.8	38.97	<0.001
				S × T	1	157.5	8.39	0.01
				error	17	319.2		

59 Sugar mass of nectar production and accumulated sugar mass production over 24 h were square root-transformed ($Y + 0.5$). Statistically significant results are highlighted in bold ($P < 0.05$). The levels of the factors considered in the different analyses were: Sexual stage (female or hermaphrodite), Time of day (09:00, 11:30 or 14:00 h), N° of extractions (three, two or control), Time (initial sugar mass at 14:00 h *versus* accumulated sugar mass over 24 h later).

Effects of extraction on sugar mass within a period of 5 h and over 24 h

We found no differences related to the number of nectar extractions, but again cyathia in the hermaphroditic stage produced more sugar mass (mean \pm SE: $6992 \pm 349 \mu\text{g}$) than in the female stage ($1646 \pm 215 \mu\text{g}$; Table 1). Accumulated sugar mass after 24 h was higher than initial sugar mass values in both sexual stages. Cyathia in the female stage secreted sugars at a higher rate (from 1542 ± 404 to $2658 \pm 562 \mu\text{g}$; a mean secretion rate of $46.5 \mu\text{g h}^{-1}$) than during the hermaphrodite stage (from 7085 ± 643 to $7885 \pm 644 \mu\text{g}$; a mean secretion rate of $33.3 \mu\text{g h}^{-1}$). The difference between initial sugar mass production and that accumulated over 24 h was statistically significant for cyathia in the female stage, but not for cyathia in the hermaphroditic stage (Table 1, Fig. 4).

Resorption and homeostasis

The three nectar variables – concentration, volume and sugar mass – had smaller values for senescent cyathia than for those in the hermaphroditic sexual stage (Fig. 5).

The experiments in which artificial nectar was added to female and hermaphroditic cyathia showed that concentration fell from high values (75% in the treatments 2, low volume and high concentration, and 3, high volume and high concentration) to lower values (medians 26 and 29%, respectively; Fig. 6). Related to volume, there were differences between female and hermaphroditic responses: cyathia in the female stage decreased the volume from 7.6 to 4.3 μL in treatment 1 (high volume and low concentration) and increased it from 2.0 to 7.75 μL in treatment 2 (low volume and high concentration). Hermaphrodites reduced the initial volume in treatment 3 (high volume and high concentration) from 10.0 to 1.85 μL (Fig. 6). Regarding sugar mass, cyathia in both sexual stages reduced the initial quantity in treatment 3 (high volume and high concentration): females from 8290 to 1879 μg , and hermaphrodites from 10363 to 510 μg (Fig. 6). Although marginally significant, there was a decrease of sugar mass in treatment 2 (low volume and high concentration) for the hermaphrodite stage, which helps to explain the concentration decrease without a volume changes (Table 2, Fig. 6). In summary, cyathia showed complex responses for nectar traits according to sexual phase and nectar parameters of the experiments, returning volume and concentration to within natural ranges (standing crop).

Standing crop and hummingbird preference

A smaller number of female cyathium were found by pollinators than hermaphroditic cyathium (female:hermaphrodite ratio = 1:2.7). Concentration of nectar in hermaphroditic cyathia ($30 \pm 1.71\%$, wt/wt) was higher than in the female stage ($26 \pm 0.68\%$, wt/wt; $n = 71$, $t = 2.28$, $df = 69$, $P = 0.026$). There were no differences between sexual phases for volume or sugar mass ($n = 76$, $t = 0.56$, $df = 74$, $P = 0.58$; $n = 76$, $t = 0.002$, $df = 74$, $P = 0.99$; respectively).

The frequency of visits was very high; values observed (60 visits for females and 48 visits for hermaphrodites) were significantly different from expected (30 visits for females and 78 visits for hermaphrodites); hummingbirds visited inflorescences

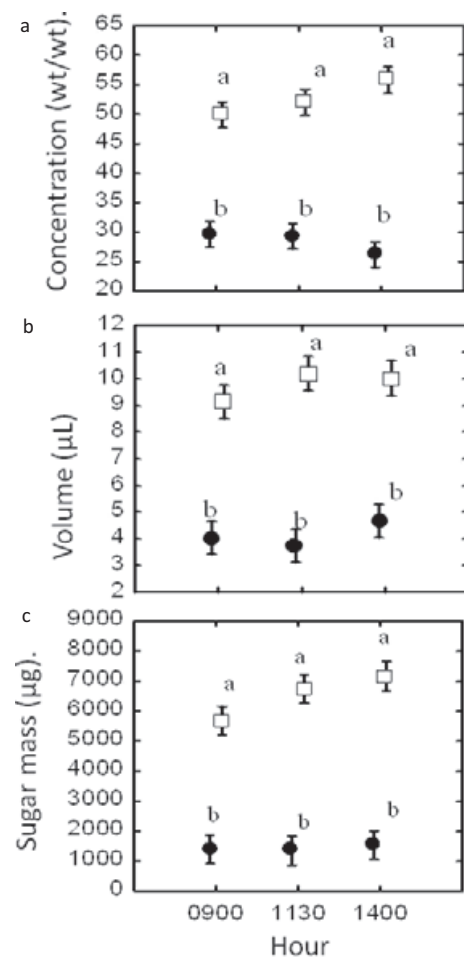


Fig. 3. Nectar production throughout the day from *E. tithymaloides* inflorescences in female and hermaphroditic stages. Data are mean \pm SE for (a) concentration (w/w), (b) volume (μL) and (c) sugar mass (μg) measured at 09:00, 11:30 or 14:00 h. Significant differences are marked with different letters (see statistical analyses in Table 1). Circles denote female anthesis phase, squares denote hermaphrodite anthesis phase.

in the female stage more frequently than expected (test of independence, $n = 108$, $df = 1$, $\chi^2 = 43.07$, $P < 0.001$).

DISCUSSION

Differentiation of sexual phases and nectar production

Sexual phase differentiation of cyathia in *E. tithymaloides* is accompanied by changes in nectar attributes. Concentration, volume and sugar mass in the hermaphroditic phase were much higher than in the initial female phase for bagged inflorescences. Such gender-biased nectar production (GBNP) is not unusual; Carlson & Harms (2006) reviewed the GBNP of 41 species and found that about half the species have female-biased nectar production and the other half have male-biased nectar production. Nevertheless, the large difference in nectar concentration between phases was a surprise because the concentration for the hermaphroditic phase (52% wt/wt) was far above the usual range (20–30% wt/wt) for hummingbird-adapted flowers (Pyke & Waser 1981; Perret *et al.* 2001; Nicolson & Fleming 2003; McDade & Weeks 2004; Chalcoff

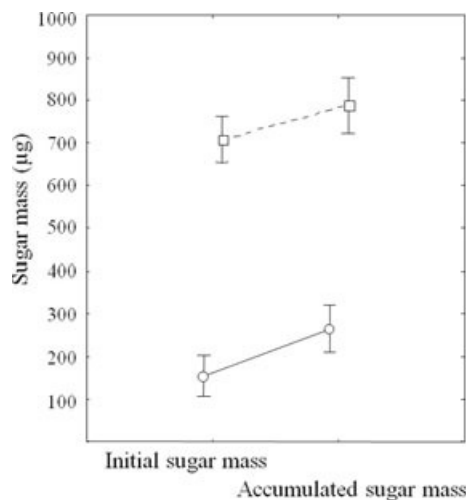


Fig. 4. Accumulated sugar mass secreted from *E. tithymaloides* inflorescences in female and hermaphroditic stages of anthesis. Data are mean \pm SE for the initial sugar mass (μg at 14:00 h) and the accumulated sugar mass (μg of initial sugar mass plus μg secreted after 24 h of nectar accumulation). Accumulated sugar mass was higher than initial sugar mass in both flower stages (see statistical analyses in Table 1). Circles denote female anthesis phase, squares denote hermaphrodite anthesis phase.

et al. 2006). However, this percentage coincides with the tendency described for *Euphorbia* of having highly concentrated nectar (Ehrenfeld 1976; Traveset & Sáez 1997), which may indicate phylogenetic conservatism of nectar attributes. On the other hand, concentration values found from nectar standing crop data for both phases (*i.e.* 26–30%) were in the range expected for flowers visited by hummingbirds. Thus, high nectar concentrations in unvisited inflorescences could be due to prolonged evaporation, which might be unusual, considering the observed high rates of hummingbird visitation in *E. tithymaloides*. Alternatively, the decrease in nectar concentration from female to hermaphrodite phase could be caused by nectar yeast through sugar fermentation. When a hummingbird visits a cyathium in the female phase it would transmit yeasts (Herrera *et al.* 2008; de Vega *et al.* 2009; de Vega & Herrera 2012).

Nectar traits did not vary with time of day, and removals did not affect the rate of nectar secretion. This pattern of nectar secretion assures rewarding inflorescences for pollinators for the period of major pollinator activity (personal observations, Locatelli *et al.* 2004). In the literature, there is no single explanation regarding removal effects for the various ornitophilous species that have been studied; some of them increase secretion following removal, which may be seen as a strategy to increase flower visitation rates, but others, such as *E. tithymaloides*, lack this response (Gill 1988; Guitián *et al.* 1995; Navarro 1999; McDade & Weeks 2004; Ornelas *et al.* 2007).

Resorption and homeostasis

Resorption occurred in senescing, bagged inflorescences, which indicates that this process follows a fixed schedule, independent of pollinator activities (Bernardello *et al.* 1994; Rivera *et al.* 1996). Sugar mass accumulation over 24 h was higher in female-stage cyathia than hermaphroditic cyathia, the latter being closer to senescence. Given these findings and the rapid

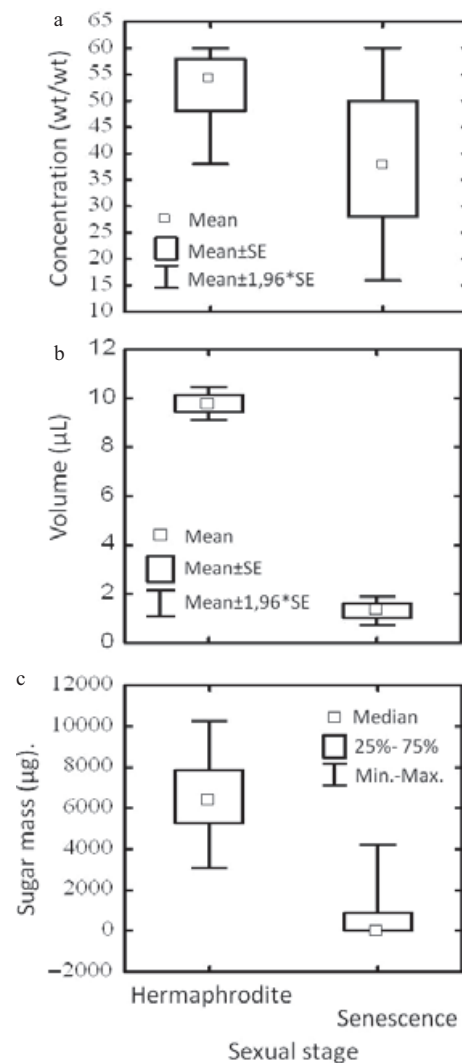


Fig. 5. Hypothesised nectar resorption through comparisons of *E. tithymaloides* inflorescences in the hermaphroditic and senescence stages. (a) Concentration (wt/wt), (b) volume (μL) and (c) sugar mass (μg). Differences are statistically significant (concentration: $n = 50$, $Z = 3.39$, $df = 50$, $P < 0.001$; volume: $n = 66$, $t = 18.51$, $df = 64$, $P < 0.001$; sugar mass: $n = 66$, $Z = 6.85$, $df = 66$, $P < 0.001$).

responses recorded after nectar manipulations, we infer a homeostatic mechanism. It seems that solute concentration is key for the nectar regulation process. Inflorescences of both stages decreased sugar mass when it was high, demonstrating active resorption during both sexual phases, even though final values of sugar mass were higher for female than for hermaphroditic phase cyathia.

Experimental changes in nectar showed different responses during each sexual phase. For the female stage, volume changes seemed to be mainly linked with water movements to regulate solute concentration (26–30% wt/wt): if the concentration was high and volume low, volume increased and consequently solutes were diluted and nectar concentration reduced (*i.e.* active secretion mainly of water); if the initial volume was high and concentration low, nectar volume decreased, but solutes were maintained and concentration increased (*i.e.* active resorption mainly of water). Furthermore, if the experimental volume and

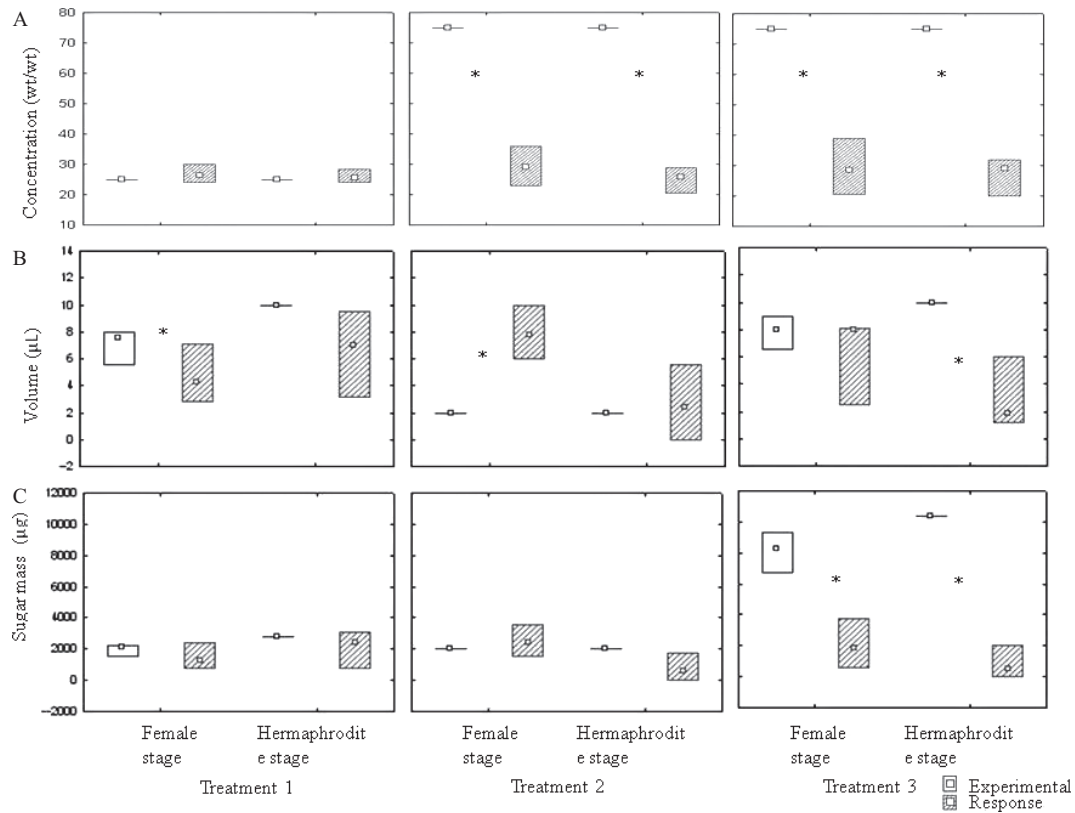


Fig. 6. Extreme nectar manipulations applied to female and hermaphroditic stages. Treatments were: (i) initial high volume (10 μL) and low concentration (25%), (ii) initial low volume (2 μL) and high concentration (75%), and (iii) initial high volume (10 μL) and high concentration (75%). Box-plots with median and 25–75% percentiles for (a) concentration, (b) volume and (c) sugar mass. The comparisons were performed between the initial experimental values (Exp) of nectar traits and the plant response (Resp) after 24 h. Significant differences between experimental and response values are marked with * ($P < 0.05$).

concentration were high, then solutes seem to be differentially resorbed to maintain nectar concentration within a naturally lower range (*i.e.* active resorption mainly of solutes). For the hermaphroditic stage, the priority seems to be the recovery of resources: if experimental concentration was high and volume low, there was little change in volume, but sugar mass decreased with a concomitant concentration decrease (*i.e.* active nectar resorption mainly of solutes). If the experimental concentration and volume were high, there was active uptake of sugar and water (*i.e.* active resorption of water and solutes). This trend could be related to the active nectar resorption of cyathia that are becoming senescent.

These results indicate complex responses involving secretion and resorption mechanisms in the nectary. The dissimilar responses of the two sexual phases to experimental nectar manipulations can be explained considering the different rates of nectar secretion of each phase and the apparently continuous functioning of both homeostatic and resorption mechanisms throughout the entire secretion process. The next issue would be to try to understand homeostatic GBNP in terms of optimal patterns of pollen flow; altogether nectar secretion patterns and pollinator behaviour seem to explain the higher resorption rate of solutes during flower senescence.

Standing crop and hummingbird preference

Narbona *et al.* (2008) studied nectar production in a protogynous and entomophilous *Euphorbia* and found male-biased

nectar production. Hummingbirds visited female-stage inflorescences of *E. tithymaloides* differentially, in the phase with less concentrated nectar. This is surprising since previous reports suggest that if hummingbirds have a choice, they prefer more concentrated nectar (Stiles 1976; Tamm & Gass 1986; Mitchell & Waser 1992; Roberts 1996; Blem *et al.* 1997; Mitchell *et al.* 1998). In addition, hummingbirds can distinguish flowers without nectar and quickly learn which flowers have rewards (Meléndez-Ackerman *et al.* 1997; Maloof & Inouye 2000 and references therein; Henderson *et al.* 2001). Other research on ornitophilous flowers have also found a positive relation between number of pollinator visits and nectar reward, explaining it as a plant strategy to decrease geitonogamy or increase pollen export (Devlin & Stephenson 1985; Carlson 2008).

In our study, nectar standing crops differed between hermaphrodites (30% wt/wt) and females (26% wt/wt), although both were within the range described as preferred by hummingbirds (Perret *et al.* 2001; Nicolson & Fleming 2003; McDade & Weeks 2004). The hummingbird *Selasphorus rufus* has been shown to distinguish differences in sugar content as small as 3% (Blem *et al.* 1997). Thus, it is likely that *Amazilia fimbriata* can distinguish the differences in nectar traits between sexual stages in *E. tithymaloides*.

Why then do hummingbirds prefer the sexual phase with less concentrated nectar? Could there be another explanation for this preference? Hurly & Oseen (1999) offered *Selasphorus rufus* choices of different degrees of nectar variability, and

Table 2. Extreme nectar manipulations performed in female (F) and hermaphrodite (H) cyathium stages of *E. tithymaloides*.

variable	treatment	anthesis	median	n	Z	P	
concentration (wt/wt)	1	F	26.5	10	1.27	0.203	
		H	25.6	8	0.91	0.363	
	2	F	29	10	2.80	0.005	
		H	25.8	8	2.52	0.012	
	3	F	28.5	8	2.52	0.012	
		H	29	7	2.37	0.018	
volume (µl)	1	F	4.3	10	2.09	0.037	
		H	7	10	1.83	0.067	
	2	F	7.75	10	2.70	0.007	
		H	2.4	11	0.62	0.534	
	3	F	8	9	1.68	0.093	
		H	1.85	10	2.67	0.008	
	sugar mass (µg)	1	F	1255	10	1.38	0.169
			H	2369	10	1.17	0.241
		2	F	2409	10	0.97	0.332
H			639	11	1.87	0.062	
3		F	1879	9	2.67	0.008	
		H	510	10	2.80	0.005	

Treatments were: (1) initial high volume (10 µl) and low concentration (25%), (2) initial low volume (2 µl) and high concentration (75%) and (3) initial high volume (10 µl) and high concentration (75%). The nectar trait (volume, concentration and sugar mass) comparisons were performed between the initial experimental values (Exp) and the plant response (Resp) after 24 h. Wilcoxon non-parametric test was applied for dependent samples. Statistically significant results are highlighted in bold ($P < 0.05$).

found that if the choice was binary, birds preferred constant reward over low- or high-variance rewards. In other words, hummingbirds might be risk-averse. In *E. tithymaloides*, nectar concentration in the standing crop was more variable for hermaphroditic than for female-phase cyathia (SD = 10.12 and 4.10, respectively); hence, the female phase could be preferred for that reason by *Amazilia fimbriata*. Moreover, Carlson (2008) also suggested that hummingbirds may remain faithful to less-rewarding flowers if they have faster refill rates. This may be the case for female-phase cyathia of *E. tithymaloides*, considering the higher nectar secretion rate over 24 h for the

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female stage ($46.5 \mu\text{g h}^{-1}$ for females compared to $33.3 \mu\text{g h}^{-1}$ for hermaphrodites).

Hummingbirds match predictions of foraging theory: they move from more rewarding flowers to less rewarding flowers (Carlson 2008). Protogynous species such as *E. tithymaloides* may benefit from preferences for the female phase because pollen deposition can be assured before male flowers develop, which reduces self-pollination. In addition, if pollinators move to other plants more frequently after visiting a hermaphroditic blossom, the chance of geitonogamy is decreased (geitonogamy avoidance hypothesis; Carlson 2008). Nevertheless, it is necessary to obtain further data (*i.e.* the relation between the number of visits and the reproductive success of each phase) to elucidated whether the sexual selection hypothesis explains this case of GBNP (Carlson & Harms 2006; Carlson 2008).

Concluding remarks

Our main conclusions are that (i) sexual phase differentiation of the cyathium in *E. tithymaloides* is accompanied by differences in nectar production, (ii) consequently, hummingbirds preferred the female stage, and (iii) resorption and secretion may form a homeostatic mechanism by which the cyathium maintains nectar traits within a narrow range and recovers energy if nectar is not removed by pollinators. Homeostasis seems to be gender-specific, evidencing complex physiological responses to biotic and abiotic changes. Considering that nectar secretion rates can be affected by endogenous as well as exogenous factors, more research is needed to understand how flowers produce nectar, the most important mediator of their interactions with pollinators (Heil 2011). Other techniques, *e.g.* radiolabelled sucrose (Nepi & Stpiczynska 2007), can provide a more accurate method to improve our interpretation.

ACKNOWLEDGEMENTS

We thank P. Wilson, E. Narbona and an anonymous reviewer for useful suggestions on an early version of this paper. This project was financially supported by the Erasmus-Mundus interchange programme. We thank the Botanic Garden of Recife for providing the study sites, the laboratory Polinizar of the CCB, UFPE for providing material resources and the CNPq for a research grant to ICM, and Universidad Nacional de Córdoba (SECyT-UNC) and CONICET for financial support to LG as the Carrera del Investigador of CONICET.

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