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RESEARCH PAPER

Influence of light, dark, temperature and drought on metabolite and ion composition in nectar and nectaries of an epiphytic bromeliad species (*Aechmea fasciata*)

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Keywords
Amino acids; Bromeliaceae; drought; floral nectar; nectar composition; nectary composition; sugars.

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ABSTRACT

- Research into the influence of stress factors, such as drought, different temperatures and/or varied light conditions, on plants due to climate changes is becoming increasingly important. Epiphytes, like many species of the *Bromeliaceae*, are particularly affected by this, but little is known about impacts on nectar composition and nectary metabolism.
- We investigated the influence of drought, different temperatures and light–dark regimes on nectar and nectaries of the epiphytic bromeliad species, *Aechmea fasciata*, and also the influence of drought with the terrestrial bromeliad, *Billbergia nutans*. The content of sugars, amino acids and ions in nectar and nectaries was analysed using HPLC. In addition, the starch content and the activities of different invertases in nectaries were determined.
- Compositions of nectar and nectaries were hardly influenced, neither by light nor dark, nor by different temperatures. In contrast, drought revealed changes in nectar volumes and nectar sugar compositions in the epiphytic bromeliad as well as in the terrestrial bromeliad. In both species, the sucrose-to-hexose ratio in nectar decreased considerably during the drought period. These changes in nectar sugar composition do not correlate with changes in the nectaries. The total sugar, amino acid and ion concentrations remained constant in nectar as well as in nectaries during the drought period.
- Changes in nectar composition or in the production of floral pollinator rewards are likely to affect plant–pollinator interactions. It remains questionable how far the adaptations of the bromeliads to drought and diverse light or temperature conditions are still sufficient.

INTRODUCTION

In some tropical forests, vascular epiphytes represent about 50% of the flora, and they are responsible for the ecological complexity of these ecosystems (Gentry & Dodson, 1987a). Most species belong to only a few families, *e.g. Orchidaceae*, *Araceae* or *Bromeliaceae* (Gentry & Dodson, 1987b). Epiphytes are very sensitive to environmental changes such as frequent water shortage, as they absorb water through rain, where the duration of precipitation is more important than its quantity (Benzing, 1998; Nadkarni & Solano, 2002). Therefore, epiphytes show adaptations to absorb and store water. For example, many bromeliads have foliar trichomes, which function as moisture-absorptive appendages and also exhibit complex phyllotaxis to promote water retention (Benzing, 1980). In addition to morphological specializations, several epiphytic plants also show physiological adaptations to withstand drought, *e.g.* the Crassulacean acid metabolism (CAM) photosynthetic pathway (Pierce *et al.*, 2002; Silvera *et al.*, 2009). Epiphytes are also confronted with differing light or temperature conditions; on the one hand, they are exposed to almost full sun on tree branches, and on the other hand they live in deep shade at the stem base (Hietz & Briones, 2001).

The family *Bromeliaceae* is one of the species richest non-woody plant families in the Neotropics (Benzing, 2000). Approximately 60% of the species are epiphytes and the others have terrestrial live forms (Zotz, 2013). Furthermore, species with CAM and C_3 photosynthesis occur in approximately equal proportions, but the proportions within the genera differ greatly (Crayn *et al.*, 2015; Edwards, 2019). The flowers of bromeliads are unique within the taxon Poales in that they have septal nectaries for nectar production (Benzing, 2000; Sajo *et al.*, 2004).

Floral nectar is an aqueous solution rich in sugars, with the main sugars being the hexoses glucose and fructose and the disaccharide sucrose (Percival, 1961; Baker & Baker, 1983). Besides sugars, a wide range of amino acids, inorganic ions, organic acids and other secondary compounds can be found in the nectar, albeit at much lower concentrations (Baker & Baker, 1973; Calder & Hiebert, 1983; Adler *et al.*, 2006; Seo *et al.*, 2013). As the proportions of the three sugars in nectar are relatively consistent for a given species, they have often been

related to the plant's pollinator type (Nicolson & Thornburg, 2007; Krömer *et al.,* 2008; Tiedge & Lohaus, 2018; Göttlinger *et al.*, 2019).

There are several metabolic steps that are important for nectar production and secretion, including starch accumulation and degradation, sucrose synthesis and sucrose export from the nectaries (Ren *et al.,* 2007; Lin *et al.,* 2014). The proportion of hexoses in nectar depends on the activity of sucrose cleavage enzymes, *e.g.* invertases, or other metabolic processes during nectar secretion (Ruhlmann *et al.,* 2010; Tiedge & Lohaus, 2018). In general, invertases play an important role in plant reproductive development and are also involved in the adaptation to drought (Roitsch & González, 2004).

Several studies have investigated the general influence of drought on epiphytic plants such as bromeliads (Bader *et al.,* 2009; Freschi *et al.,* 2010). In addition, some studies have considered the influence of drought or other environmental conditions on nectar availability and nectar composition in non-bromeliad species (Kenoyer, 1917; Gardener & Gillman, 2001; Waser & Price, 2016; Clearwater *et al.,* 2018; Phillips *et al.,* 2018; Takkis *et al.,* 2018). However, so far there are no investigations of the influence of different light and temperature regimes or drought conditions on the nectar composition in bromeliads, as representatives of epiphytes.

The objective of this study, therefore, is to compare changes in the metabolite composition (sugars, amino acids, ions) in nectar and nectaries of an epiphytic bromeliad species in response to different light–dark regimes, temperatures or drought. The influence of flower age on nectar composition was first analysed to test whether flower age influences other factors related to nectar composition. All experiments were performed with *Aechmea fasciata*, one of the best-known species of the genus, that produces sufficient flowers from which to collect nectaries as well as abundant amounts of nectar for analysis. It grows as an epiphytic, forms a tank and uses CAM photosynthesis. To compare the effect of drought on epiphytic *versus* terrestrial bromeliads, the drought experiments were also performed on the terrestrial species *Billbergia nutans*, which has no water tank, but also uses CAM photosynthesis.

The nectaries of various plants have been studied several times, mostly with regard to cellular structure (Stahl *et al.,* 2012), gene expression (Kram *et al.,* 2009), activity of enzymes (Ruhlmann *et al.,* 2010; Lin *et al.,* 2014) and nectar secretion (Stpiczyńska *et al.,* 2012). A few studies also considered the metabolite content in nectaries, mainly with the amounts of sugar and starch (Tiedge & Lohaus, 2018; Solhaug *et al.,* 2019b). The present study is the first to consider the metabolite composition (sugar, starch, amino acids, ions) and the activity of different invertases in nectary tissue of bromeliad species. Furthermore, comparison of the metabolites in nectar and nectaries under different light or dark conditions, temperatures or induced drought will help to better understand regulation of nectar composition under these different conditions.

MATERIAL AND METHODS

Plant material

Aechmea fasciata (Lindl.) Baker and *Billbergia nutans* H. Wendl. ex Regel were provided by the Botanical Garden of the University of Göttingen (Germany) and cultivated in a glasshouse at the University of Wuppertal (Germany) with a 14-h light:10-h dark cycle, an irradiance of *ca.* 300 µmol photons $m^{-2} \cdot s^{-1}$ and a temperature regime of 25 °C day:18 °C night.

Experimental design

All experiments were performed in a closed glasshouse.

Influence of flower age in a normal light-dark cycle
Aechmea fasciata was exposed to a normal light–dark cycle. Six flowers of three plants were selected and nectar collected from each flower at six time points (08:00 h, noon, 16:00 h, 20:00 h, midnight, 04:00 h) At each time point, 5 µl nectar was taken from the same flower.

Influence of light and dark
Nectar and nectary samples of fresh flowers of *A. fasciata* were collected before imposition of different light and dark conditions. Since this bromeliad is day-pollinated and the flowers open in the morning, nectar samples were collected in the middle of the day (at 13:00 h; after normal light–dark cycles; control conditions). The plants were then exposed to either 24 h of light or 24 h of darkness. At 13:00 h of the following day, samples of nectar and nectaries were collected from freshly opened flowers (about 6 h after anthesis), *i.e.* flowers of the same age were used.

Influence of temperature
Aechmea fasciata was exposed to different temperature regimes (20 °C day:18 °C night and 35 °C day:22 °C night), starting 6 days before the first flower opened. Nectar and nectary tissue were collected at 13:00 h from both plant sets (about 6 h after anthesis), *i.e.* flowers of the same age were used.

Influence of drought
The influence of drought was studied in two bromeliad species (*A. fasciata*, *B. nutans*). To produce drought conditions, the pots were not watered and the stored water in the tank of *A. fasciata* was removed at the beginning of the experiment. This was not necessary for *B. nutans* as this species does not forms tanks. At the same time, control plants of both bromeliad species received sufficient water. In *A. fasciata*, the tanks were always half-filled with water and the soil was watered to 10% pot volume every third day. In *B. nutans* the soil was watered to about 10% of pot volume and the leaves were sprayed with water every second day. Drought conditions and control conditions were continued for 14 days, corresponding to initiation of the flowering period for both species. Nectar and nectary tissue were collected at 13:00 h (about 6 h after anthesis) from all available flowers throughout the entire flowering period of the two bromeliads (14 days).

Collection of nectar and sample size

All nectar samples were collected from single flowers and there was no pooling of samples from different flowers. Nectar (5–10 µl) was collected in a micropipette on the first day of anthesis (Göttlinger *et al.,* 2019) and stored at −80 °C until analysis.

At least six samples were taken at each point of measurement and for each of the different growth conditions (normal light:-dark conditions, 24 h light or 24 h dark; different temperatures; well-watered or droughted).

Collection of nectaries and sample size

The septal nectary tissue was dissected from the flower according to Sajo *et al.* (2004), using a scalpel under a binocular microscope. For each nectary sample (~25 mg), nectary tissue from about 15 flowers was pooled. At least three pooled samples were taken at every testing time and for each of the different growing conditions (normal light:dark, 24 h light or 24 h dark; different temperatures; well-watered or droughted). The extracted tissue was rinsed with ultrapure water to remove external sugars. All nectary samples were immediately frozen in liquid nitrogen and stored at −80 °C until further analysis.

Extraction of soluble metabolites from nectaries

In order to extract soluble metabolites from nectary tissue, a chloroform–methanol–water extraction was performed (Nadwodnik & Lohaus, 2008) using 25 mg of finely milled powder.

Analyses of sugars in nectar and nectaries

The sugars were analysed *via* HPLC (Thermo-Scientific Dionex ICS-5000 + HPIC System, Dreieich, Germany) according to Lohaus & Schwerdtfeger (2014). Sugars were detected with a pulse amperometric detector. The sugar concentrations in samples were determined from calibration curves for the different sugars. The concentrations of different sugars in nectar are given as millimoles (mM) and in nectaries as $\mu mol \cdot g^{-1}$ fresh weight (FW).

Testing changes in secreted nectar sugar composition

Nectars of plants under all experimental conditions (normal light:dark, 24 h light or 24 h dark; different temperatures; well-watered and droughted) were measured immediately after sampling, as well as 24 and 48 h later. During this time, the samples were stored at room temperature to allow any metabolic processes to continue after the nectar secretion.

Analyses of free amino acids in nectar and nectaries

The analysis of free amino acids was performed *via* HPLC according to Göttlinger *et al.* (2019). Amino acids with a primary amino group were processed by precolumn derivatization with *o*-phthaldialdehyde; amino acids with a secondary amino group (*e.g.* proline) were processed by pre-column derivatization with fluorenylmethyloxycarbonyl. The amino acids were detected with a fluorescence detector. The concentration of amino acids (alanine, arginine, aspartate, asparagine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine) in the nectaries and in nectar was determined from calibration curves for the different amino acids. All concentrations of amino acids in nectar are given as millimoles (mM) and in nectaries as $\mu mol \cdot g^{-1}$ FW.

Analyses of inorganic anions in nectar and nectaries

Inorganic anions (chloride, phosphate, sulphate) and cations (potassium, sodium, magnesium, calcium) were analysed separately *via* HPLC according to Lohaus *et al.* (2001). The ions were detected as their electrical conductivity. The concentration of the inorganic ions in the nectaries and nectar was determined from calibration curves for the different inorganic ions. All concentrations of inorganic ions in nectar are given as millimoles (mM) and in nectaries in $\mu mol \cdot g^{-1}$ FW.

Analyses of starch in nectaries

According to a modified protocol from Riens *et al.* (1994), the starch content of nectaries was determined by measuring the glucose released after treatment with KOH, α-amylase and amyloglucosidase.

Enzyme assay for cell wall invertase (CW-INV), vacuolar invertase (V-INV) and neutral invertase (N-INV)

To analyse enzyme activity of the different invertases, proteins from 25 mg nectary tissue were extracted as described in Tiedge & Lohaus (2018). For cell wall invertase, an aliquot of insoluble protein extract was added to 0.6 M sucrose and 0.125 M sodium acetate, pH 5.0. The soluble acids (vacuolar) and neutral invertases were measured in the soluble protein fraction. Both enzymes require different pH values for the substrate solution. An aliquot of the protein extract was added to 0.6 M sucrose and 0.125 M sodium acetate, pH 5.0 (soluble acid invertase) or pH 7.5 (soluble neutral invertase). The enzyme reaction was stopped by boiling the solution after 10 min. The amount of glucose released during each reaction was quantified using coupled optical enzyme assays.

Statistical analysis

Light–dark, temperature and drought effects on metabolite and ion concentrations were analysed with *t*-tests or one-way ANOVA, followed by multiple comparisons with Tukey's HSD test. All analyses were performed using R (version 3.6.1, www.r-project.org).

RESULTS

Flower phenology

Flowers of *A. fasciata* had a lifespan of about 24 h (Fig. 1; Figure S1). Anthesis started at about 07:00 h with the separation of petals from each other at the tip of the flower. In addition, petals showed a remarkable colour change throughout their phenological stages. In the morning, flowers changed from blue (before anthesis) to purple, and after about 10–12 h, they became pink, the petals were softer, contracted and underwent constant shrinking. After 24 h, the petals had dried up completely.

In the case of *B. nutans*, flowers also opened in the morning at about 07:00 h and had a life span of about 24 h, similar to *A. fasciata*.

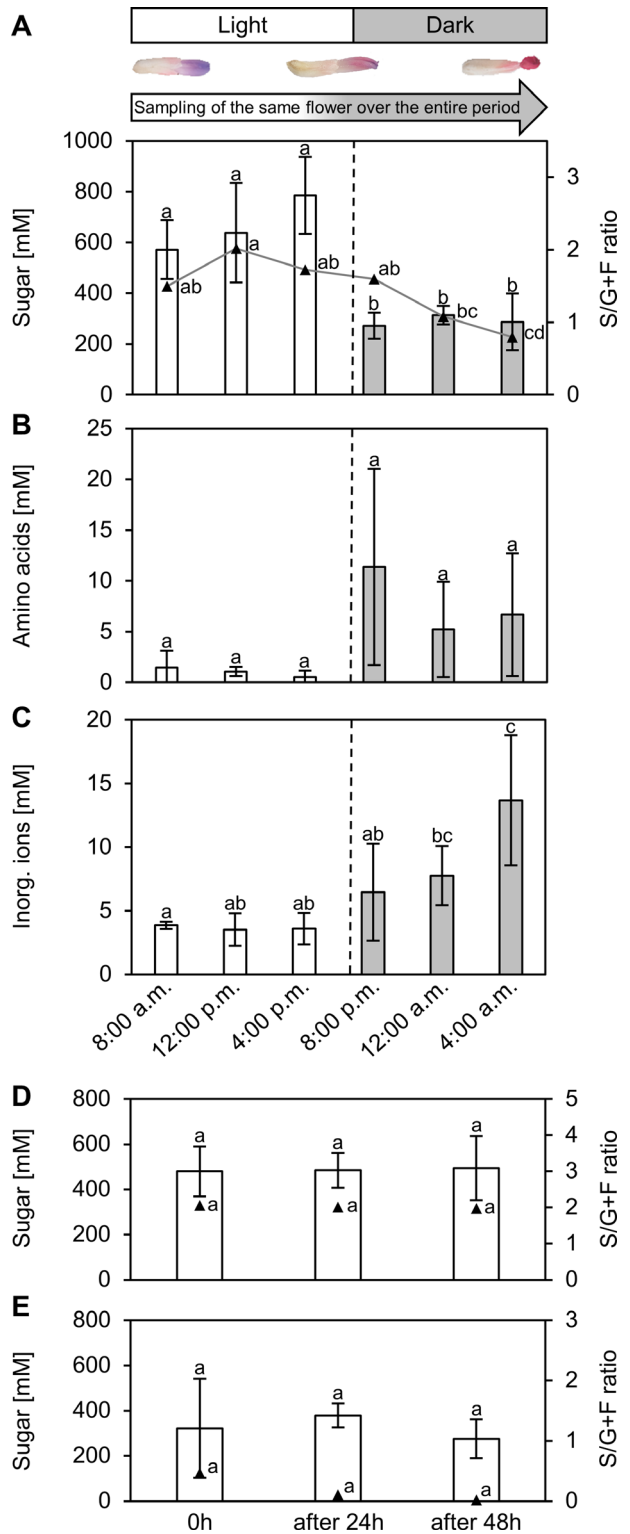

Fig. 1. Concentration of different compounds in nectar of *Aechmea fasciata* under a normal light/dark regime and post-secretory changes in sugar concentrations in the nectar. Bar charts illustrate (A) total sugar concentration and sucrose:hexose ratio, (B) total amino acid concentration and (C) the total inorganic ion concentration. Post-secretory changes in sugar concentrations in the nectar. Samples were analysed immediately after collection, as well as 24 and 48 h later. Bar charts illustrate nectar of (D) fresh flowers and (E) senescent flowers. Mean ± SD of n = 6 independent samples. Different letters represent significant differences in sugars, sucrose:hexoses ratio, amino acids and ions (Tukey's HSD; $P < 0.05$).

Sugar, amino acid and ion concentrations in nectar at different flower ages

Nectar accumulated from the start of anthesis in the morning. The highest amount was around noon and in the early afternoon (about 30 µl flower^{-1}), whereas older or senescent flowers only contained small amounts of nectar.

In nectar of *A. fasciata*, sampled under ambient day–night conditions at six time points, there were large differences in sugars, amino acids and ion concentrations and compositions (Fig. 1A-C; Tables S1–S3). The total sugar concentration (sum of glucose, fructose and sucrose) in nectar taken from flowers between 08:00 h and 16:00 h (referred to as *fresh flowers*) was about three-fold higher than that sampled from flowers between 20:00 h and 04:00 h (referred to as *senescent flowers*). The ratio of sucrose:hexoses decreased from 1.5 to 2.0 in fresh flowers to 0.7 in senescent flowers (Fig. 1A). The amino acid concentration in nectar of fresh flowers was low and increased about five-fold in senescent flowers (Fig. 1B). The main amino acids in all nectar samples were asparagine, glutamine, aspartate, glutamate, serine and alanine (Table S2). The concentration of inorganic ions (cations and anions) was also higher in senescent flowers compared to fresh flowers (Fig. 1C). The main cation was potassium, the main anion was chloride, and these ions increased in senescent flowers (Table S3).

Neither the sugar composition (sucrose:hexoses ratio) nor sugar concentrations in nectar of fresh flowers changed significantly over 48 h at room temperature. In senescent flowers, however, sucrose:hexoses ratios decreased during this time period (Fig. 1D,E).

Sugar, amino acids and ion concentrations in nectar and nectaries after 24 h of light or darkness

Light or dark periods of 24 h caused no visible changes to flower morphology or colour. Also, the nectar volume was also unchanged.

After 24 h of light, the total sugar concentration and sucrose:hexoses ratio in sampled nectar were similar to those of plants under ambient day–night conditions (Fig. 2A). Furthermore, amino acid (Fig. 2B) and ion concentrations (Fig. 2C) in the nectar did not differ significantly.

After 24 h in darkness, however, the total sugar concentration in nectar decreased from 750 to 600 mM; but the difference was not significant (Fig. 2A). The sucrose:hexoses ratio, again, was similar in nectar of plants under ambient day–night conditions and 24 h of darkness (Fig. 2A). Amino acid concentrations in nectar increased from 2.4 to 4.1 mM; however, this difference was also not significant because of the high SD (Fig. 2B). After 24 h of darkness, the proportion of asparagine and glutamine increased, whereas that of other essential amino acids decreased (Table S2). Total ion concentration also increased from 2.2 to 6.5 mM, but this difference was also not significant (Fig. 2C).

In nectaries there was no change in total sugar content after 24 h of light, whereas after 24 h in darkness, total sugars decreased, albeit not significantly, from 140 µmol·g^{-1} FW to

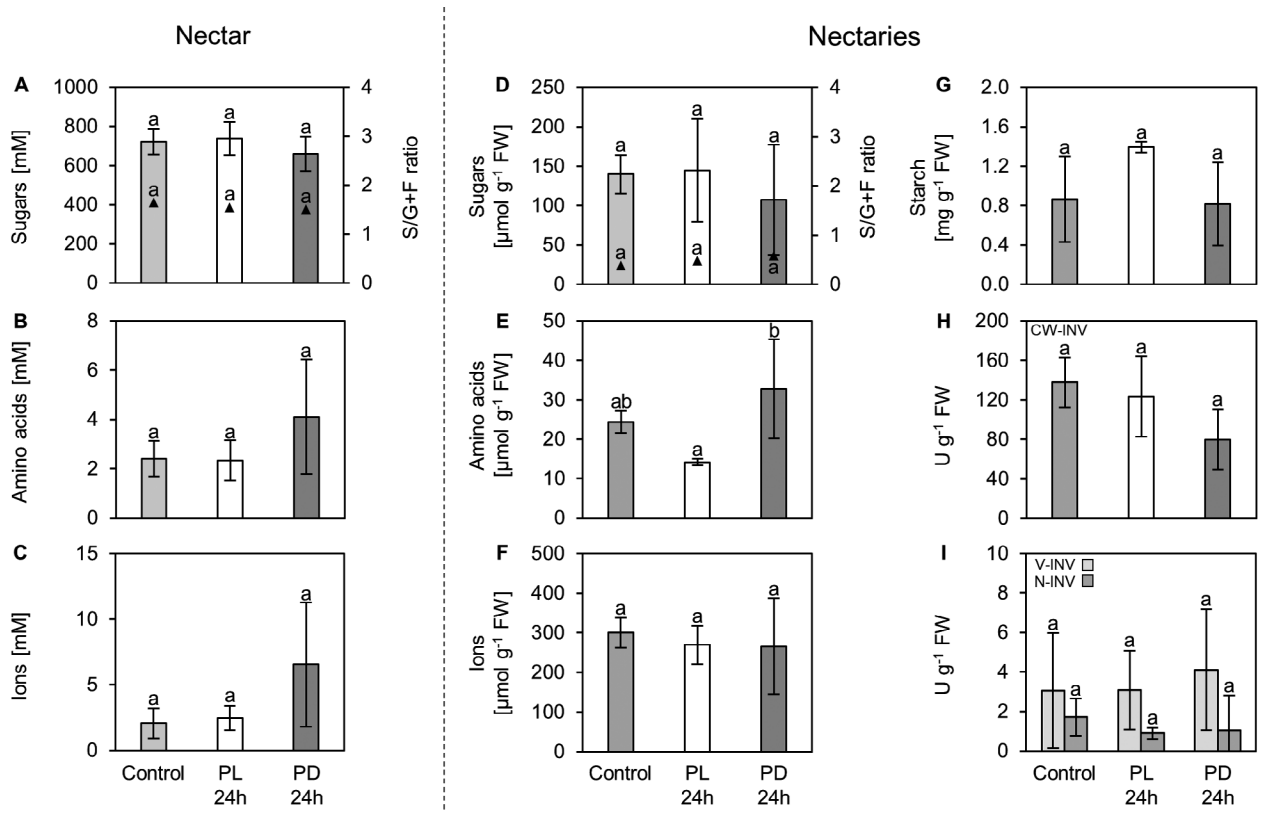

Fig. 2. Concentration of different compounds in nectar and nectary tissue of *Aechmea fasciata* after 24-h light (PL) or 24-h dark (PD) in comparison to normal light–dark exposure. Plants exposed to normal light–dark regimes are presented as control. Bar charts illustrate: (A) total sugar concentration and sucrose:hexose ratio, (B) total amino acid concentration and (C) total inorganic ion concentration in nectar; (D) total sugar content and sucrose:hexoses ratio, (E) total amino acid content, (F) total ion content, (G) starch content, (H) CW-INV activity and (I) V-INV and N-INV activity in nectaries. Mean ± SD of n = 6 (nectar) and n = 3 (nectaries) independent samples. Different letters represent significant differences in sugars, sucrose:hexoses ratio, amino acids, ions, starch and invertase assay between normal exposure, permanent light and darkness (Tukey's HSD; $P < 0.05$).

110 µmol·g^{-1} FW (Fig. 2D). The sucrose:hexoses ratios were comparable for both 24 h light and dark periods (Fig. 2D). Neither light nor darkness had a significant effect on the total amino acids or total ion content (Fig. 2E,F). The amino acid profile of nectaries was similar in the 24 h light and 24 h dark treatments (Table S2). In both cases, the main amino acids were asparagine, glutamine, aspartate, glutamate, serine and alanine (Table S2). Starch content of control plants and plants after 24 h of darkness was similar, whereas in plants after 24 h of light, levels of starch were slightly higher (Fig. 2G).

Activity of cell wall invertases (CW-INV) in nectaries after 24 h of darkness was 40% lower compared to control plants, but the difference was not significant (Fig. 2H). Soluble acid invertases (V-INV) and soluble neutral invertases (N-INV) were also active in the nectaries, but mean activity was about 20-fold lower compared to that of CW-INV activity (Fig. 2I) and did not differ significantly between light and dark conditions.

To test for changes in nectar sugar composition after secretion, nectar of flowers after 24 h of light or 24 h of darkness was measured immediately after sampling, as well as 24 and 48 h later. The sugar concentration and sucrose:hexoses ratio in nectar did not change significantly during this period (data not shown).

Sugars, amino acids and ion concentrations in nectar and nectaries at different growth temperatures

The different temperature treatments (20 °C or 35 °C) did not lead to any visible changes in flower morphology or colour, nor in nectar volume.

In nectar, the total sugar concentration and sucrose:hexoses ratio of plants at the two temperatures were similar (Fig. 3A). Total amino acids increased significantly from 4.7 to 11.0 mM (Fig. 3B; $F_{(11)} = 18.63$; $P < 0.05$), but the amino acid profile was comparable at both temperatures (Table S2). Furthermore, the ion concentration (Fig. 3C) in nectar did not change significantly.

As for nectaries, the two temperatures did not significantly influence the total sugar content or sucrose:hexoses ratio (Fig. 3D), as also found for content of total amino acids, total ions and starch (Fig. 3E,F,G).

The activity of CW-IN, V-INV and N-INV in nectaries did not differ in response to the two temperatures (Fig. 3H,I).

To test for changes in nectar sugar composition after secretion, nectar of flowers in both temperature treatments was measured immediately after sampling, as well as at 24 and 48 h later. Neither sugar concentration nor sucrose:hexoses ratio in nectar changed significantly during this period (data not shown).

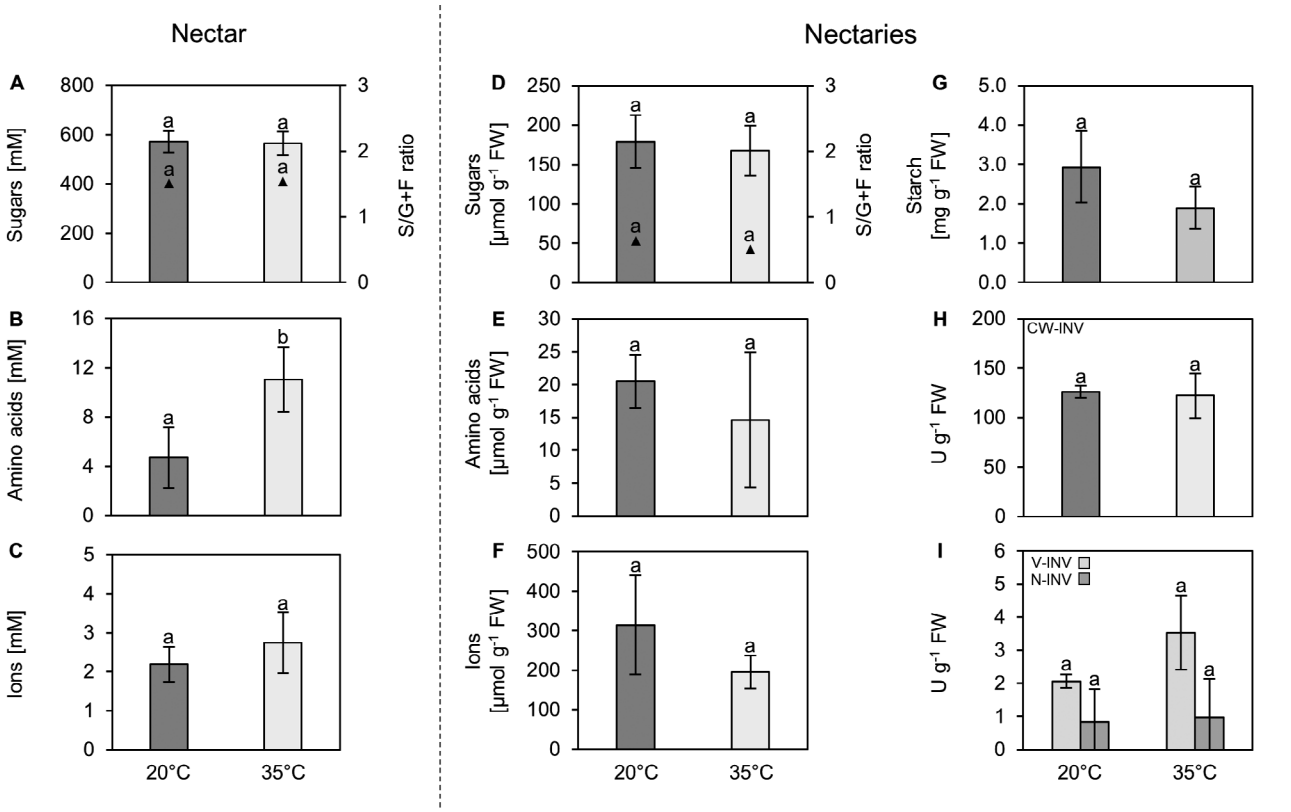

Fig. 3. Concentration of different compounds in nectar and nectary tissue of *Aechmea fasciata* at 20 °C and 35 °C. Bar charts illustrate: (A) total sugar concentration and sucrose:hexoses ratio, (B) total amino acid concentration and (C) total inorganic ions concentration in nectar; (D) total sugar content and sucrose:hexoses ratio, (E) total amino acid content, (F) total ion content, (G) starch content, (H) CW-INV activity and (I) V-INV and N-INV activity in nectaries. Mean ± SD of n = 6 (nectar) and n = 3 (nectaries) independent samples. Different letters represent significant differences in sugars, sucrose:hexoses ratio, amino acids, ions, starch and invertase assay between different temperatures (*t*-test; $P < 0.05$).

Sugars, amino acids and ion concentrations in nectar and nectaries during drought treatment

The influence of drought was studied in the *A. fasciata* (epiphytic bromeliad; Fig. 4) and *B. nutans* (terrestrial bromeliad; Fig. 5). As control plants showed no differences in metabolite concentrations in nectar or nectaries during the course of the experiment, they are presented as 'control day 0' samples for each parameter and species.

Aechmea fasciata produced a large number of flowers per plant, of which three to six open each day during the flowering season. Under drought conditions, flower formation and number of flowers per day decreased until no further flowers opened after 14 days. The nectar volume decreased by up to 50%.

Total sugar concentration in nectar was almost constant (about 600 mM), whereas the sucrose:hexoses ratio decreased from 1.6 to 0.5 during the drought period of 14 days (Fig. 4A; $F_{(41)} = 19{,}94$; $P < 0{,}001$). Total amino acid concentration (Fig. 4B) and concentration of inorganic ions (Fig. 4C) increased slightly under drought, but these changes were not significant. The amino acid profile in nectar of droughted plants and under control plants was similar (Table S2).

Nectary tissue of *A. fasciata* was sampled from day 7 to day 14 of drought. The sugar, amino acid and ion content in nectaries did not differ significantly between control plants and droughted plants (Fig. 4D–F). The sucrose:hexoses ratio in nectaries was lower than in nectar.

The starch content in nectaries was low, <0.5 mg·g^{-1} FW (measured as glucose equivalents; Fig. 4G), and drought had no significant impact on starch content.

The activity of CW-INV, V-INV and N-INV in nectaries did not change under drought conditions (Fig. 4H,I).

Billbergia nutans produces fewer flowers per plant, with only one or two opening per day during the flowering season. Under drought conditions (14 days), there was no reduction in number of flowers opening per day, but the nectar volume per flower decreased by up to 20%.

The total sugar concentration (glucose, fructose and sucrose) in nectar was similar under control and drought conditions (about 900 mM; Fig. 5A); sugar composition, however, changed during the drought period. The sucrose:hexoses ratio decreased significantly from 4.6 on the first day to 0.7 on the last days of the drought period (Fig. 2A; $F_{(20)} = 24{,}41$; $P < 0.001$). Like sugar concentrations, amino acid concentrations in nectar were also constant during the drought period (about 0.15 mM; Fig. 5B). During the first 11 days of the drought, there were no differences in the ion content; after day 11, however, it increased about twofold, but this difference was not significant (Fig. 5C).

Nectary tissue of *B. nutans* was sampled on day 7 to day 14 of the drought period. In contrast to the sugar and ion content of nectary tissue (Fig. 5D,F), drought treatment resulted in a

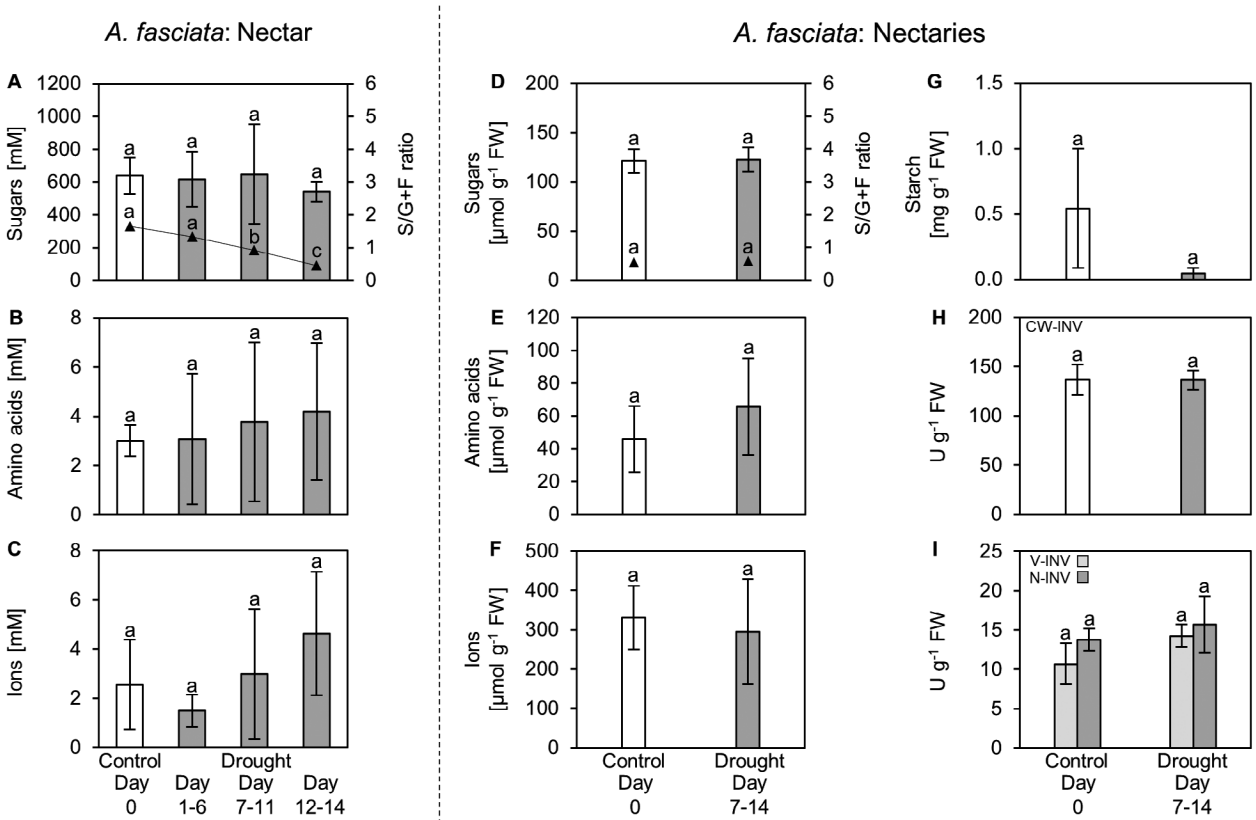

Fig. 4. Concentration of different compounds in nectar and nectary tissue of *Aechmea fasciata* under drought conditions. Plants with normal irrigation are presented as control on day 0. For nectar, each graph shows the time period of both conditions, from day 1 to day 14, at shorter intervals (A–C), and for nectary glands, drought stress for each compound between days 7–14 are provided in one graph (D–I). Bar charts illustrate: (A) total sugar concentration and sucrose:hexoses ratio, (B) total amino acid concentration and (C) total inorganic ions concentration in nectar; (D) total sugar content and sucrose:hexoses ratio, (E) total amino acid content, (F) total ion content, (G) starch content, (H) CW-INV activity and (I) V-INV and N-INV activity in nectaries. Mean ± SD of n = 6 (nectar) and n = 3 (nectaries) independent samples. Different letters represent significant differences in sugars, sucrose:hexoses ratio, amino acids, ions, starch and invertase assay between normal irrigation and drought conditions (Tukey's HSD; $P < 0.05$; *t*-test; $P < 0.05$).

significant increase in amino acid content (Fig. 5E; $F_{(4)} = 31{,}78$; $P < 0{,}05$).

No starch was detected in nectaries of *B. nutans* under control conditions (Fig. 5G), whereas starch content increased significantly during the drought period, but the content was still low (0.1 mg·g^{-1} FW; Fig. 5G; $F_{(4)} = 12{,}00$; $P < 0.05$).

Activity was similar for CW-INV in nectaries of *B. nutans* under control and drought conditions (Fig. 5H), whereas activity of both soluble invertases increased significantly under drought conditions (Fig. 5I; $F_{(4)} = 241{,}85$; $P < 0.001$).

To test for changes in nectar sugar composition after secretion, nectar of flowers under control or drought conditions was measured immediately after sampling as well as 24 and 48 h later. Sugar concentration and sucrose:hexoses ratio in nectar of flowers under control and drought conditions did not change significantly (Fig. 6A–D). This was similar in both *A. fasciata* and *B. nutans*.

DISCUSSION

The nectar composition is relatively consistent for a given plant species but varies between species. This might be related to a plant's pollinator type (Nicolson & Thornburg, 2007; Krömer *et al.*, 2008; Tiedge & Lohaus, 2018; Göttlinger *et al.*, 2019). However, it is also possible that features of the flowers or changes in environmental factors might influence nectar composition (Petanidou *et al.*, 1996; Gardener & Gillman, 2001; Waser & Price, 2016; Clearwater *et al.*, 2018).

Flower age is known to have a strong influence on nectar composition and volume (Petanidou *et al.*, 1996; Quintana-Rodríguez *et al.*, 2018), as confirmed here for the epiphytic bromeliad *A. fasciata* (Fig. 1A–C). It is possible that the decreased sugar concentration and nectar volume of older flowers is caused by nectar resorption by nectaries or other flower cells (Nepi & Stpiczyńska, 2007). Furthermore, nectar sugar composition changed with flower aging, where the proportion of sucrose decreased and that of hexoses increased with flower age (Fig. 1A). This is similar to *Psittacanthus calyculatus* (Mexican mistletoe), where the change in sugar composition of nectar was related to activity of CW-INV in nectaries (Quintana-Rodríguez *et al.*, 2018). Activity of CW-INV is generally important for production of hexose-rich nectars during nectar secretion (Ruhlmann *et al.*, 2010). In addition, the decreased in the sucrose:hexoses ratio in senescent flowers is probably related to sucrose cleavage enzymes in nectar of these flowers

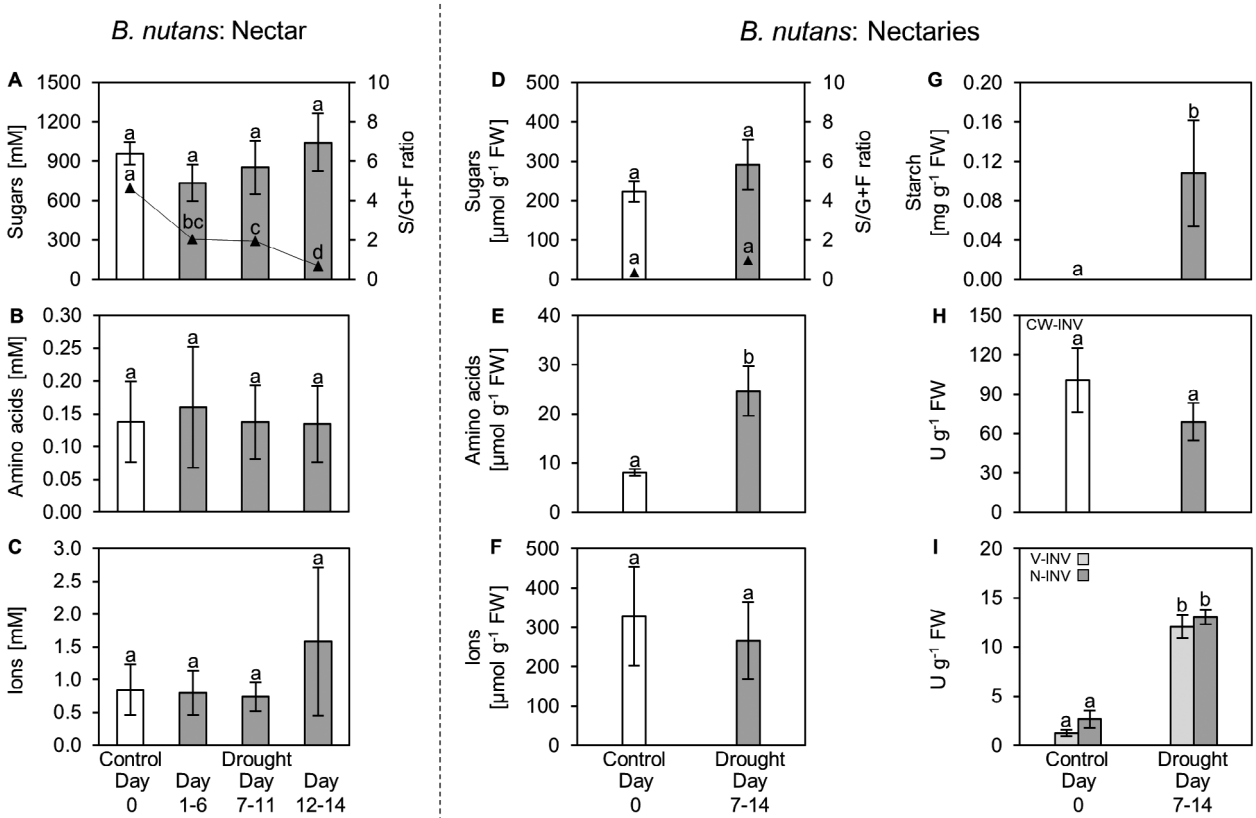

Fig. 5. Concentration of different compounds in nectar and nectary tissue of *Billbergia nutans* under drought conditions. Plants with normal irrigation are presented as control on day 0. For nectar, each graph shows the duration of both conditions from day 1 to day 14 at intervals (A–C) and for nectary glands, drought stress for each compound between days 7–14 are displayed in one graph (D–I). Bar charts illustrate: (A) total sugar concentration and sucrose:hexoses ratio, (B) total amino acid concentration and (C) total inorganic ion concentration in nectar; (D) total sugar content and sucrose:hexoses ratio, (E) total amino acid content, (F) total ion content, (G) starch content, (H) CW-INV activity and (I) V-INV and N-INV activity in nectaries. Mean ± SD of n = 6 (nectar) and n = 3 (nectaries) independent samples. Different letters represent significant differences in total sugars, sucrose:hexoses ratio, total amino acids, total ions, starch and invertase assay between normal irrigation and drought conditions (Tukey's HSD; $P < 0.05$; *t*-test; $P < 0.05$).

(Heil *et al.*, 2005). This is supported by the post-secretory changes in the sucrose:hexoses ratio in nectar of senescent flowers, but not in nectar from fresh flowers (Fig. 1D,E). The increased concentrations of amino acids and inorganic ions in nectar of senescent flowers may be related to proteolytic activity in older flowers, as reported by Petanidou *et al.* (1996). This is similar to observations in extrafloral nectaries of *Acacia cornigera*, where proteolytic activity was highest after peak nectar secretion (Orona-Tamayo *et al.*, 2013).

Based on these results, further experiments on the influence of light or darkness, different temperatures and drought on nectar composition were performed where the influence of flower age was eliminated.

Light and dark periods have little impact on nectar composition

Exposure of the epiphytic bromeliad *A. fasciata* to 24 h of light did not significantly alter the nectar volume or composition of either nectar or nectaries (Fig. 2). However, light stress can influence growth and development of epiphytic plants (Stancato *et al.*, 2002; Díez *et al.*, 2017). Furthermore, in *Inga* species (tropical, non-epiphytic plant) nectar production in extrafloral nectaries increased in response to light (Bixenmann *et al.*, 2011). In the light experiment with *A. fasciata*, a light intensity of 300 µmol photons·m^{-2}·s^{-1} was used; it is possible that this light intensity did not have a major impact on photosynthesis and other physiological processes, including sugar composition of nectar or nectaries.

In *Fagopyrum esculentum*, the number of open flowers decreased, but secretion of nectar in the remaining opening flowers was not completely prevented in darkness for 1 day (Cawoy et al., 2008). These authors suggest that lack of light stops photosynthesis in leaves and hence energy supply – in the form of sugars – necessary for flower opening. In the present study with *A. fasciata*, however, flowers still opened, and nectar was produced after 24 h of darkness. This suggests that storage compounds in leaves and other organs may be involved in supplying the flowers with sugars. Moreover, in darkness, phloem transport is reduced but not completely stopped (Riens *et al.*, 1994). In the epiphytic bromeliad, total sugar concentration in nectar and nectaries decreased only slightly after 24 h of darkness, whereas in squashes, a decrease in total sugar content could already be observed if the normal dark period was extended by 5 h (Solhaug *et al.*, 2019a). As epiphytes can also grow in different light conditions, *i.e.* also in shade, they

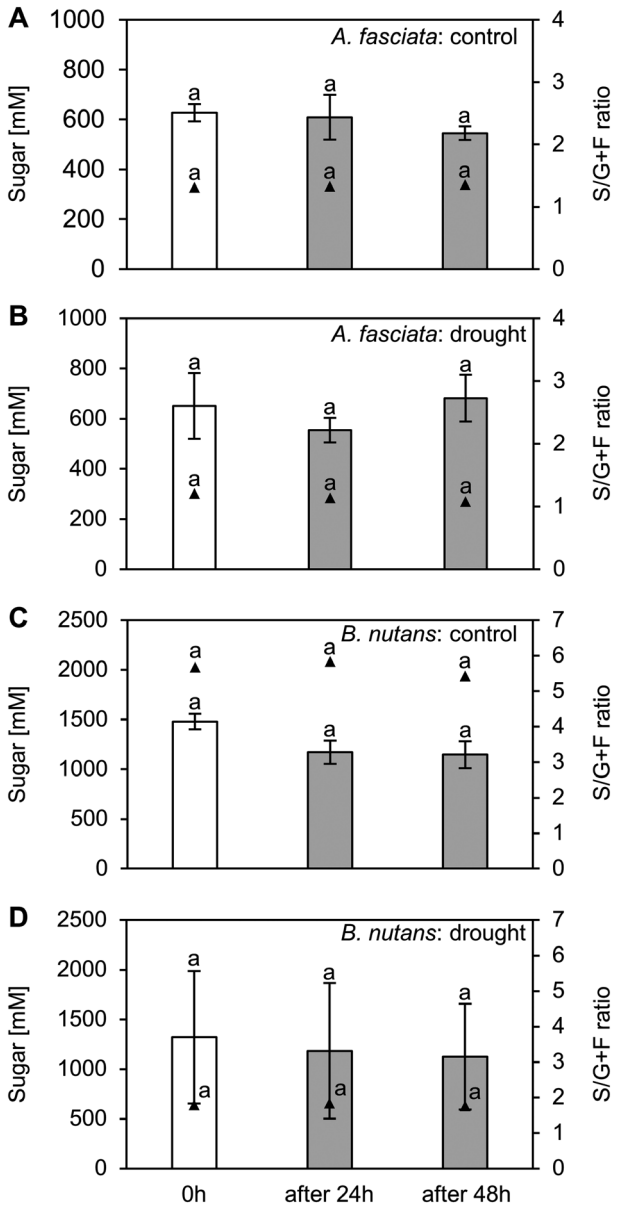

Fig. 6. Post-secretory changes in sugar concentrations in the nectar. Nectar samples were analysed immediately after collection, as well as 24 and 48 h later. Bar charts: nectar of *Aechmea fasciata*, (A) control conditions, (B) drought conditions; nectar of *Billbergia nutans*, (C) control conditions, (D) drought conditions. Mean ± SD of each n = 3 independent measurements. Different letters represent significant differences in sugars (Tukey's HSD; $P < 0.05$).

probably react more slowly to permanent darkness than other plant species, such as squash, which generally grows in full sun.

The starch content in the nectaries of different *Nicotiana* species was found to be higher in night-flowering species compared to day-flowering species (Tiedge & Lohaus, 2018). Due to reduced phloem transport at night, night-flowering species may use a larger amount of starch for production of nectar sugar. In *A. fasciata*, the starch content in nectaries of plants after 24 h of light or 24 h of darkness was very similar and generally comparably low (0–1 $mg \cdot g^{-1}$ FW; Fig. 2G). One reason for this could be the time when the nectaries were collected. This was about 6 h after the beginning of anthesis. Solhaug *et al.* (2019a) found that in squash, rapid starch degradation starts about 3 h before anthesis, and 3 h after anthesis nectary starch is nearly completely degraded. In leaves of *A. fasciata*, starch content increased during the light period and decreased during the dark period (data not shown), which is similar to that of other plant species. Therefore, starch metabolism in nectaries appears to function independently of starch metabolism of light-dependent, photosynthetically active parts of the plant (Tiedge & Lohaus, 2018). Furthermore, activity of enzymes crucial for starch degradation or sucrose cleavage in nectaries are also mostly independent of light (Millán-Cañongo *et al.,* 2014; Solhaug *et al.,* 2019a). In general, it seems that the carbon metabolism in floral nectaries and in the other parts of the plant are regulated independently, as already found for extrafloral nectaries (Orona-Tamayo *et al.,* 2013).

After 24 h of darkness, amino acid and ion concentrations in nectar increased; however, these differences were not significant (Fig. 2B,C). The origin of these components in nectar has not yet been conclusively clarified (Nicolson & Thornburg, 2007). It can be assumed that under dark conditions, fewer sugars are transported from the nectaries into the nectar, while at the same time more amino acids and ions are transported or leak out of the nectaries (Fig. 2D–F).

Different temperatures have little impact on nectar composition

A rise in temperature to 35 °C has little effect on nectar sugar concentrations of *A. fasciata* (Fig. 3A). This is similar to the results of a 6-year warming experiment with a perennial herb (*Saussurea nigrescens*), in which the concentrations remained unchanged. The nectar volume per flower and the flower number per capitulum, however, decreased (Mu *et al.,* 2015). Moreover, a 5-week temperature experiment (between 21 and 27 °C) with *Borago officinalis* showed that nectar volume is more influenced than nectar sugar concentration (Descamps *et al.,* 2018). With *A. fasciata,* we did not observe any influence of temperature on nectar volume or number of flowers. This is probably due to the shorter duration of the experiment. In addition, *A. fasciata* is a species native to subtropical regions and therefore adapted to higher temperatures. Also, the nectar sugar compositions of *A. fasciata* were similar at the different temperatures (Fig. 3A). Whether this applies to *A. fasciata* only or also for other plant species cannot be answered as of yet because no additional studies on the subject are available.

Nectar volume and sugar composition in nectar are mainly influenced by drought

Under drought conditions, *A. fasciata* had a decreased number of open flowers and lower nectar volumes. In contrast, in *B. nutans* the number of open flowers was not reduced, but there was a reduction in nectar volume per flower. Similar results have been described for other plant species (Phillips *et al.,* 2018). In *Borago officinalis,* for example, drought increased flower abortion, which resulted in a lower number of open flowers (Descamps *et al.,* 2018). Also, drought usually induces a reduction in the volume of secreted nectar (Carroll *et al.,* 2001; Mu *et al.,* 2015; Waser & Price, 2016; Gallagher & Campbell, 2017).

The total sugar concentrations of *A. fasciata* and *B. nutans* remained constant under drought conditions, the sucrose:hexoses ratios in nectar, however, decrease in both species (Figs 4A,5A). The fact that nectar sugar concentration is not affected by drought had already been demonstrated for other plant species (Carroll *et al.,* 2001; Mu *et al.,* 2015); however, there are no uniform results on the effect of drought on nectar composition in the current literature (Borghi *et al.,* 2019). It is therefore likely that different plant species react differently to strong and rapid drought. A possible reason for the reduced sucrose and increased hexose content in nectar is that hexose-rich nectar may reduce evaporation (Corbet, 1978). For the same sugar concentration, hexose-rich nectars have a higher osmolality than sucrose-rich nectars, and this leads to slower evaporation (Nicolson, 1994). As a result, there is reduced loss of water through evaporation of the nectar, and that is advantageous during dry conditions.

Effect of drought on epiphytic or terrestrial bromeliad species

The effect of the drought period on nectar or nectaries of *A. fasciata* and *B. nutans* was similar, although *A. fasciata* is epiphytic while *B. nutans* is terrestrial. During the drought period, the content of sugars, amino acids and ions in nectaries was largely unchanged, compared to plants under control conditions, for both plant species. Probably these two species were not particularly affected by drought in the analysed period because they have thick and waxy leaves that reduce water loss (Bernhardt, 2003; Moore, 2008). In addition, *A. fasciata* and *B. nutans* perform CAM photosynthesis, so that plant stomata open during the night to take up carbon dioxide, and close during the day to avoid water loss (Winter & Smith, 1996; Dodd *et al.,* 2002). This type of photosynthesis allows bromeliads to endure extended periods without water loss, so that there are no immediate drastic effects on the composition of nectaries in either the epiphytic or terrestrial plant. This also generally applies to the nectar composition, with the exception that the ratio of sucrose:hexoses was affected here. However, in both CAM species, the nectar volume and number of flowers containing nectar were reduced during the drought period, which corresponds to results for other non-bromeliad C_3 species (Descamps *et al.,* 2018; Phillips *et al.,* 2018).

Comparison of nectar and nectary metabolism

Drought has an obvious effect on the sucrose:hexoses ratio in nectar, which decreased considerably under this condition. The corresponding ratio in nectaries, however, remained constant, and towards the end of the drought period, the ratios of both nectar and nectaries became similar (Figs. 4A,D and 5A,D). In addition, post-secretory modifications of the sugar composition in nectar is unlikely because there were no changes after secretion (Fig. 6A–D). Therefore, it can be assumed that the changed sugar ratio in nectar is a result of a regulated sugar secretion from the nectaries or metabolic processes during secretion. Activities of different enzymes, *e.g.* invertases, or different sugar transport proteins, *e.g.* SWEET, can lead to differences in nectar composition (Ruhlmann *et al.,* 2010; Lin *et al.,* 2014). As in *A. fasciata,* the activity of the different invertases was similar in nectaries of control plants and in those under drought conditions (Fig. 4H,I), nectaries are unlikely to be the cause for the decreased sucrose:hexoses ratios. Therefore, it cannot be excluded that additional sucrose cleavage enzymes are involved in this process. It is also conceivable that, under drought conditions, the export of sucrose from the nectaries decreased and that of hexoses increased. In *B. nutans*, the decreased sucrose:hexoses ratio in nectar correlates with increased activity of V-INV and N-INV in nectaries (Fig. 5A,I). The V-INV may be important in liberating hexoses from sucrose stored in the vacuole during secretion (Solhaug *et al.,* 2019b). However, the extent to which these enzymes are actually the cause for differences in the sugar ratio needs to be further investigated.

To compare metabolite and ion concentrations in nectar and nectaries, the concentration units must be the same for both. Metabolite and ion concentrations in nectar were measured in millimoles (Figs. 4D–F and 5D–F), whereas the content in nectaries was measured in micromoles per gram fresh weight (Figs. 4D–F and 5D–F). When taking the water content of nectary cells (75%; Tiedge & Lohaus, 2018) into account, metabolite and ion concentrations (in mM) in the nectaries can also be calculated. The sugar, amino acid and ion concentrations in the nectaries of *A. fasciata* are about 150, 70 and 400 mM respectively, and the corresponding values for *B. nutans* are about 300, 20 and 400 mM, respectively.

Based on these calculations, the ratios between metabolite concentrations in nectar and in nectaries can be estimated. In general, the total sugar concentrations were three- to four-fold higher in nectar than in the nectary cells of both analysed bromeliad species under control or drought conditions (Figs. 4A, D and 5A,D). This suggests that sugar is actively transported when secreting nectar, perhaps *via* monosaccharide transporters (MST) and/or sucrose transporters (SUT), which were known in flowers of *Arabidopsis* and *Nicotiana* (Lemoine *et al.,* 1999; Sherson *et al.,* 2003). However, so far, the occurrence and function of such transporters in flowers, and particularly in nectaries, is not fully elucidated. Another class of transporters, so called SWEET, are clearly involved in nectar secretion in *Arabidopsis* and *Nicotiana* (Lin *et al.,* 2014); SWEET9 functions as a facilitated diffusion transporter for sucrose and is probably responsible for sucrose efflux from nectary cells. Several SWEET genes were also identified in the bromeliad *Ananas comosus* (Guo *et al.,* 2018), but functional analysis of these genes has yet to be done. As the sugar concentration in nectar is higher than in nectary cells of the analysed bromeliad species, the role of SWEET in sugar export from nectaries should be investigated. It is also possible, however, that sucrose concentrations in sub-domains of the nectaries or in nectar-secreting cells (*e.g.* epithelial cells) are much higher than the calculated sucrose concentration for whole nectary tissue, and mass-flow transport of sucrose *via* SWEET still plays a role in sucrose transport into nectar (Roy *et al.,* 2017). Whether this is also the case for bromeliad species, is currently unknown and further experiments are required to understand the process.

In contrast to the sugars, the concentration of amino acids and inorganic ions is much higher in nectaries than in nectar under control and under drought conditions (Figs 4B,C,E,F and 5B,C,E,F). For *A. fasciata*, amino acid concentration was about 20-fold higher in nectaries than in nectar, and for *B. nutans,* the difference was even 100-fold higher. The concentration of inorganic ions was more than 100-fold higher in nectaries than in nectar in both bromeliad species. In addition,

the amino acid concentration in nectar varies much more than the sugar concentration. Therefore, it is possible that facilitated diffusion transporters or channels for amino acids or ions mediate efflux of these substances from nectary cells.

Further ecological impacts

Future climate changes are predicted to result in increased occurrence and intensity of droughts in many regions of the world (IPCC, 2014). Beside their influence on whole plant metabolism, such changes could also influence nectar composition and secretion, as well as plant–pollinator interactions (Brown *et al.,* 2016; Borghi *et al.,* 2019). Under drought conditions, *A. fasciata* and *B. nutans* had a modified nectar composition, changing from sucrose-rich to hexose-rich nectar, with no differences between the epiphytic and the terrestrial bromeliad species. This means that drought produces nectar that is no longer attractive to typical pollinators of the plants (hummingbirds), as they prefer a sucrose-rich nectar (Benzing, 2000). Moreover, due to the decreasing number of flowers and nectar volume, it is questionable to what extent attraction of pollinators, and thus reproductive success of the plant, will be successful (Scaven & Rafferty, 2013; Descamps *et al.,* 2018; Phillips *et al.,* 2018). In particular as the reduced number of open flowers and nectar volume will provide fewer food resources for the pollinators. In this study, two bromeliad species were investigated under glasshouse conditions; therefore, the results might not fully reflect the influence of drought under natural conditions. In nature, drought is usually accompanied by other factors, such as elevated temperature and irradiation, which are additional influencing factors and will probably accelerate and intensify the changes in nectar composition (Schweiger *et al.,* 2010).

In summary, the results suggest that drought significantly influences sugar composition in nectar of both epiphytic and terrestrial bromeliad species, whereas the influence of different light and temperature conditions is less pronounced. Although these bromeliads have developed adaptations to local environmental conditions, the future climatic changes constitute a challenge for these plants, and it is not known if these adaptations will prove sufficient. Moreover, not only the plants are affected, but also the pollinators, because the quantity and quality of floral resources, like nectar, might be reduced. This is one of the first studies to investigate the effects of different environmental factors on bromeliads, with special emphasis on the composition of nectar and nectaries. In order to improve understanding of such changes and processes, further investigations of additional bromeliad species are necessary.

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AUTHOR CONTRIBUTIONS

GL planned and designed the research. TG designed and performed the experiments. TG and GL carried out the data analysis. TG and GL wrote the manuscript.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Inflorescence of *Aechmea fasciata* with flowers of different ages.

Table S1. Effect of light or dark, temperature and drought on sugar composition in nectar and nectaries of *Aechmea fasciata* and *Billbergia nutans*.

Table S2. Effect of light or dark, temperature and drought on amino acid composition in nectar and nectaries of *Aechmea fasciata* and *Billbergia nutans*.

Table S3. Effect of light or dark, temperature and drought on inorganic ion composition in nectar and nectaries of *Aechmea fasciata* and *Billbergia nutans*.

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