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Citation for published version:
Zhou, H-X, Milne, RI, Cui, P, Gu, WJ, Hu, MF, Liu, XY, Song, Y-Q, Cao, J & Zha, H-G 2022, 'Comparing the contents, functions and neonicotinoid take-up between floral and extrafloral nectar within a single species (Hemerocallis citrina Baroni)', *Annals of Botany*, vol. 129, no. 4, mcac002, pp. 429-441. https://doi.org/10.1093/aob/mcac002

Digital Object Identifier (DOI):
10.1093/aob/mcac002

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Annals of Botany

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Download date: 13. Jan. 2023

1 ORIGINAL ARTICLE

2 **1. TITLE:**

3 **Comparing the contents, functions and neonicotinoid take-up between floral**
4 **and extrafloral nectar within a single species (*Hemerocallis citrina* Baroni)**

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4 **5. Running title:**

5 **Floral and extrafloral nectar from *Hemerocallis citrina***

Abstract

Background and Aims Many angiosperms can secrete both floral (FN) and extrafloral (EFN) nectar. However, much remains unclear about how EFN and FN differ in secretion, composition and ecological function, especially when both FN and EFN are secreted on flowers of the same species.

Methods *Hemerocallis citrina* flowers secrete both FN and EFN. FN and EFN traits including volume, presentation pattern and temporal rhythms of secretion, were compared by field observation during 2019 and 2020. Sugar and amino acid contents, were analysed using regular biochemical methods, whereas proteomics were investigated by combined gel-based and gel-free approaches. FN and EFN animal feeders were investigated by field observation. *H. citrina* plants were exposed by soil drenching to two systemic insecticides, acetamiprid and imidacloprid, and the concentration of these in FN and EFN were measured by an ultra-high performance liquid chromatography coupled with electrospray ionization-quadrupole-time of flight-mass spectrometry.

Key Results *H. citrina* FN and EFN differed in timing of production and function. FN was concentrated and sucrose-dominant, secreted in the mature flower tube, and served as a reward for pollinators. Conversely, EFN was hexose-rich, more dilute and less sugar and amino acids-rich. EFN was secreted on the outside of developing floral buds, and was likely to attract predatory animals for defence. EFN had less phenolics, and lacked pathogenesis-related components, such as chitinase and glucanase.

Significantly different proteomic profile and enzymatic activities between FN and EFN suggest that they had different biosynthesis mechanisms. Both neonicotinoid insecticides examined became present in both nectar types soon after application, but in greater concentration within EFN. EFN also attracted a wider range of insect species than FN. These facts together mean that the EFN pathway could be a significant way for neonicotinoids to enter the wild food chain, and must be considered when evaluating the risks to the environment of any systemic insecticide.

Keywords: extrafloral nectar; floral nectar; *Hemerocallis citrina* Baroni; nectar proteome; systemic insecticides

1 **INTRODUCTION**

2 In angiosperms, nectar is a secreted sugar-rich liquid that mediates interactions
3 between plants and mutualists such as pollinators and non-pollination visitors. Nectar
4 can be secreted on almost any above-ground part of a plant, and usually fulfils
5 nutritive functions for diverse nectar feeders, either encouraging pollinators to visit, or
6 promoting the defense of plant tissues against herbivores by encouraging the presence
7 of mutualists such as ants (Heil, 2015). Usually, nectar is classified into two
8 categories according to the location where it is secreted, i.e. floral nectar (FN) and
9 extrafloral nectar (EFN) (Escalante-Pérez and Heil, 2012). In this paper, unless
10 otherwise specified, the term 'nectar' refers to both FN and EFN. FN is usually
11 secreted into the inner side of corolla and sited at the base of the ovary, during
12 blooming or a short time before. FN has long been discussed in the context of
13 pollination and is hence widely thought to promote beneficial plant-pollinator
14 interactions. However, EFN is secreted on the vegetative and less commonly on the
15 reproductive parts of a plant, and usually its secretion does not necessarily accompany
16 flowering (Heil, 2011). It is generally thought that EFN does not contribute to
17 pollination but acts as a reward for predators (Lundgren, 2009) that can deliver
18 top–down control of herbivore pests, possibly improving plant fitness (Cuautle et al.,
19 2005, Kost and Heil, 2008, Heil, 2015).

20 FN and EFN are similar in that both contain sugars, and most are colorless liquids
21 from a plant. In addition, both of them are deemed to hold protection functions, FN
22 for pollen by offering an alternative reward for visitors, and EFN for the tender

aboveground parts by attracting predatory animals which provide indirect defense (Willmer, 2011). However, it is still largely in doubt whether FN and EFN share a common evolutionary origin, and to what extent they share similar generation and biosynthesis mechanisms, especially given that some plants secrete EFN on their flowers. Some ferns can secrete sugary liquids on the leaves in response to herbivore damage (Koptur et al., 2013), and if this mechanism shares a common origin with angiosperm EFN, then it would follow that EFN evolution predates the origin of floral nectar, and even pollen (Lundgren, 2009). A comparison of metabolites in FN and EFN could be a good indicator to show differences in how they are generated, offering insights into whether they share a common origin, yet comparative studies between FN and EFN are still rare. However, rather few species secrete both FN and EFN, whereas comparison studies where the FN and EFN come from different plants face the problem that differences detected might be down to species differences, not functional ones. Nectar biosynthesis was shown to be conserved among floral and extrafloral nectaries in cotton (*Gossypium hirsutum*) using comparative nectary ultrastructure and transcriptomic analysis (Chatt et al., 2021), but no such work has been done among monocots.

In recent years, systemic insecticide contamination in floral nectar has caused global pollinator decline, arousing strong concerns (Raine, 2018). Systemic insecticides, mainly neonicotinoids, are water-soluble and can be taken into a plant by any of its living parts, following which they persist inside the plant and become distributed throughout its tissues via the vascular system. Compared with other types

1 of insecticide, e.g., organochlorine and pyrethroids, systemic insecticides have higher
2 mobility in a plant which can lead to their presence in some unwanted places, such as
3 pollen and nectar, thus resulting in the deaths of beneficial insects (Goulson, 2013).
4 For nectar, a lot of attention have been paid to FN in which systemic insecticide as
5 one the main drivers of worldwide pollinator decline (Pisa et al., 2015, Raine, 2018,
6 Sánchez-Bayo and Wyckhuys, 2019). However, to our knowledge, studies on the risk
7 of systemic insecticide through the EFN pathway have only looked at cotton and
8 sunflower (Stapel et al., 2000, Moscardini et al., 2014, Bredeson and Lundgren, 2018,
9 Jiang et al., 2018, Jones et al., 2020). Moreover, so far, there is no direct comparative
10 study on the environmental risks of systemic insecticide in both FN and EFN from the
11 same species.

12 *Hemerocallis citrina* Baroni (Asphodelaceae) is an ideal study system for
13 contrasting mechanisms of FN or EFN biosynthesis and ecophysiological function
14 (Rodriguez-Enriquez and Grant-Downton, 2013), because it secretes both FN and
15 EFN in considerable amounts, EFN on the outside of young floral bud and FN in the
16 deep inside of opened flowers. Also called edible daylily or nightlily, *Hemerocallis*
17 *citrina* is endemic to the mountain areas of South China but widely cultivated as a
18 vegetable for its edible young flower bud (Rodriguez-Enriquez and Grant-Downton,
19 2013). It is a strictly outcrossing entomophilous species, which uses two kinds of
20 rewards to attract pollinators, pollen for honeybee and bumble bees, and FN for
21 lepidopterans (Rodriguez-Enriquez and Grant-Downton, 2013).

22 Nectar is notoriously plastic mainly due to postsecretory hydrolysis of its

1 components, and the activities of microorganisms inhabiting it (Parachnowitsch et al.,
2 2019). However, such postsecretory changes can be largely ignored in both FN and
3 EFN because *H. citrina* flowers are open for only one night. Furthermore,
4 neonicotinoid insecticides such as imidacloprid are widely used to deal with aphids
5 and spider mites that attack *H. citrina* plants cultivated in China (Jin et al., 2019).
6 However, the risk of these systemic insecticides for non-target insects via the *H.*
7 *citrina* FN and EFN pathways has not been investigated; indeed there has been very
8 little investigation of insecticides entering the food chain via EFN from any plant. In
9 this study, we used two systemic insecticides, imidacloprid (IMI) and acetamiprid
10 (ACE). Systemic insecticides currently used in China include imidacloprid (IMI),
11 which is banned in many western countries (PPDB, 2021b), and acetamiprid (ACE)
12 which is highly toxic to birds and earthworms but supposedly only moderately so to
13 bees (PPDB, 2021a).

14 The current study therefore has two goals. First, the differences between FN and
15 EFN from a monocot will be examined for the first time based on the chemical
16 composition, proteome, secretion pattern and ecological function of nectar in *H.*
17 *citrina*. And second, the presence of two systemic insecticides in *H. citrina* FN and
18 EFN was quantified and compared as preliminary evidence for further environmental
19 risk assessment.

1 **MATERIALS AND METHODS**

2

3 ***Hemerocallis citrina cultivation and floral nectar (FN) and extrafloral nectar (EFN)***

4 ***secretion observation***

5 Forty *H. citrina* plants grown since 2017 in the open experimental field at Huangshan

6 University (29°41′N, 118°17′E; Anhui province, China) were used in this study. To

7 determine when *H. citrina* starts to secret FN, floral tubes from flowers at different

8 developmental stages were opened using blades, and the existence of FN was then

9 checked by eye (Fig. 1). Because secreted EFN is presented on the outside of the *H.*

10 *citrina* flower bud, its secretion can be directly observed without disturbing the plants.

11 Ten individual flowers from each developmental stage, each from a separate

12 individual, (Fig. 1A) were examined for the presence of FN and EFN in this way

13 during its peak flowering season, June to July in 2019. To determine whether *H.*

14 *citrina* FN and EFN was reabsorbed after secretion, we selected 20 inflorescences,

15 each containing 5-8 individual flowers at different developmental stages. Each was

16 bagged at 8:00 in the morning using a 40-mesh nylon net to block animal visitors, but

17 left attached to grow normally. After bagging, flower buds within were checked by

18 eye at noon the next day to see if any EFN remained on the outside of the flower buds.

19 Any flowers that had wilted after opening were taken out from the bag, and opened by

20 hand to check whether any FN remained in the tube. If no visible nectar remained, it

21 would be deemed that reabsorption occurred.

22

***H. citrina* FN and EFN collection and Physicochemical character measurements**

Raw FN and EFN samples were collected using a pipette and autoclaved tips between 7:00 and 8:00 am during June to July 2019. Because *H. citrina* FN sits deep inside the *H. citrina* flower tubes, the flower tube was cut using a blade just above the ovary then FN was pipetted out from the cut end (Fig. 1C). FN from an individual *H. citrina* plant in the same day was pooled and formed a single sample. The same was done with EFN, pooling samples from different development stages from the same plant and day. All nectar samples were centrifuged at 12000 g for 5 min to remove any dirt and pollen granules and stored at -20 °C prior to use.

The pH of fresh FN and EFN was tested using narrow range pH test strips (Supelco MQuant® pH 4.0-7.0, Merck). The total dissolved solids in nectar was measured using a handheld refractometer (MASTER-500, Atago, Tokyo, Japan) as its refraction index (°Brix). The sugar composition of FN and EFN was determined using an EClassical 3100 high-performance liquid chromatograph (HPLC) (Elite, Dalian, China) equipped with a refractive index detector (RI-201H, Shodex, Shoko Science, Tokyo, Japan) as described in Zhou et al. (2018). The concentration of total free amino acids in the nectar samples was measured using leucine as a standard according to Rosen (1957). The protein content in the *H. citrina* FN and EFN samples was determined according to Bradford (1976). The Folin–Ciocalteu method (Meda et al., 2005) was used to measure total phenolic content in the nectar samples. Gallic acid was taken as a standard, and the content was expressed in μg of Gallic acid equivalents (GAE) of fresh *H. citrina* FN or EFN mL^{-1}. The level of hydrogen

peroxide in *H. citrina* FN or EFN was analysed using a commercially available kit (Sangon Biotech Co, Shanghai, China), according to the manufacturer's instructions.

Ultraviolet-visible (UV-VIS) absorbance spectra for wavelengths from 200 to 400 nm of FN and EFN were measured in triplicate using a spectrophotometer (Model TU1901; Pgeneral, Beijing, China) within a quartz cuvette.

Nectar chitinase and glucanase activity assay

The most common enzymes detected so far in EFN, FN, and pollination drops from Gymnosperms are chitinase and glucanase, both typical pathogenesis-related (PR) proteins (Gonzalez-Teuber et al., 2009, Roy et al., 2017, von Aderkas et al., 2018). Profiling of *H. citrina* FN and EFN nectar chitinolytic activity in gel after SDS-PAGE was performed according to Song et al. (2019). Fresh *H. citrina* FN or EFN (20 μL per well) were loaded in gels without boiling beforehand. The clear lytic zones of chitinase isoforms were visualized as dark bands against a fluorescent background under the UV transilluminator, and then photographed. Chitinase activity in *H. citrina* FN and EFN was also determined using a fluorimetric chitinase assay kit (CS1030, Sigma-Aldrich) following the manufacturer’s instructions with minor modification according to Song et al. (2019) using a SpectraMax i3x microplate reader (Molecular Devices, CA, USA). One unit of chitinase activity was defined as the amount of enzyme that liberated 1 nmol 4-MU from the substrate per min at pH 5.0 and 30 °C.

Endo-β-1,3-glucanase activity of *H. citrina* FN and EFN was measured using

1 Azurine-crosslinked-curdlan (AZCL-curdlan, Megazyme, Ireland) as the substrate
2 according to the method of Morohashi and Matsushima (2000) with minor
3 modifications. In brief, the assay mixture contained in a total volume of 200 μL
4 containing 25 μL FN or EFN, 1 mg AZCL- curdlan, and 175 μL water, and was
5 incubated at 30 °C for 2 h. The amount of soluble dyed fragments released from
6 AZCL-curdlan was determined colorimetrically at 590 nm using a SpectraMax i3x
7 microplate reader. One unit of enzyme activity represents an increase in 0.1
8 absorbance units under the conditions used.
9
10 ***H. citrina FN and EFN proteomic analysis***
11 To profile the proteome of *H. citrina* FN and EFN, 10 ml of each type of nectar
12 was examined. Protein content was first concentrated by 30 times via ultracentrifuge
13 using Amicon Ultra centrifugal filters (10-kD cutoff; EMD Millipore).
14 Tricine-SDS-PAGE (Schägger, 2006) was conducted to separate the nectarins from
15 FN or EFN. Each well contained 5 μg total FN or EFN nectarins, and samples were
16 run in triplicate. After electrophoresis, nectarins were visualized by Coomassie
17 Brilliant Blue (CBB) G250 staining. Visible protein bands were manually excised
18 from gels and subjected to in-gel digestion using trypsin as the protease. The samples
19 were analysed in a MALDI-TOF/TOF mass spectrometer (Model 5800, Applied
20 Biosystems-Sciex). The combined mass spectrometry (MS) and tandem MS (MS/MS)
21 peak lists were analysed using Global Proteome Server (GPS) Explorer Software 3.6
22 (Applied Biosystems) with a Mascot search engine (MASCOT version 2.3; Matrix

Science, London, UK). The following settings were selected for searching: cysteine carbamidomethylation as fixed modifications; methionine oxidation as variable modifications; peptide mass tolerance of 300 ppm at the most and a general fragment mass tolerance of 0.5 Da. Protein identifications were accepted if they contained at least two identified peptides. According to the search engine, a score of 56 represents a significant identification ($P < 0.05$) when the database is restricted to the Viridiplantae taxonomy (NCBInr 20191120).

Because only one nectarin from EFN and none from FN had been successfully identified by the above approach, we next used a gel-free based analysis with liquid chromatography coupled to high-definition mass spectrometry (LC-MS/MS). For this, 20μg each of *H. citrina* FN and EFN nectarin isolate was sent to the proteomic facility of Institute of Microbiology (Chinese Academy of Sciences) for identification. Trypsin-digested protein samples were analysed using an EASY-nLC 1000 liquid chromatograph that was connected in-line with an Orbitrap Fusion Tribrid mass spectrometer equipped with a nanoelectrospray ionization (nanoESI) source (Thermo Fisher Scientific, Waltham, MA). The mass spectrometric data were analysed using the Mascot database search engine. Peptide sequences were interpreted from the MS/MS spectra by searching across all plants in NCBI protein database. Carbamidomethylation of cysteines was set as a fixed modification and methionine oxidation was set as a variable modification. The peptide mass tolerance was set at 15 ppm and the fragment mass tolerance at 0.6 Da. Trypsin was specified as the proteolytic enzyme, and two missed cleavage events were allowed. The mass

1 spectrometry proteomics data including experimental details have been deposited to
2 the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via
3 the PRIDE partner repository (Perez-Riverol et al., 2019) with the dataset identifier
4 PXD025892.
5

6 ***H. citrina FN and EFN feeder observation***

7 All observations of nectar feeding animals were done during peak *H. citrina*
8 flowering season (mid to late June), on a 2 m^2 land with 12 *H. citrina* plants, within
9 the open experimental field in Huangshan University. Because *H. citrina* secretes
10 both FN and EFN between dawn and dusk (Hong-Guang Zha, personal observation),
11 observations were conducted during three time periods during the day: morning
12 (6:00-8:00, 20 hours in 2019 and 10 hours in 2020), evening (18:00-20:00, 20 hours
13 in 2019 and 10 hours in 2020), and night (21:00-22:00, 10 hours in 2020). A red
14 headlamp was used in the night observations to avoid deterring nocturnal feeding
15 arthropods. A "visit" is defined here as an insect making some form of contact with an
16 inflorescence, hence if it contacts >1 flowers it is still classed as a single visit. For
17 flying visitors, the number of visits were counted, whereas for non-flying insects
18 (mostly EFN feeders) it was possible to count the number of individuals of each
19 species that visited a plant with the assumption that only one plant was visited per
20 individual. Predatory arthropods that were present on the *H. citrina* inflorescence and
21 preyed on visiting FN or EFN feeders, but were not observed to feed on nectar
22 themselves, were recorded as indirect feeders. Arthropods that landed on *H. citrina*

1 flowers or plants without feeding on nectar, such as sap-suckers, were not counted in
2 this study. Because the sole focus here is on nectar feeders, visitors were also
3 excluded if they collected pollen but not nectar; these were bees such as *Apis*
4 *mellifera*, *A. cerana* and *Bombus spp*.

5 Only those visitors that appeared to have successfully fed on FN were deemed as
6 FN feeders. These were either those with long (≥ 3 cm) mouthparts that were
7 observed reaching into the *H. citrina* floral tube, or those with tough mouthparts that
8 were observed biting through the end of floral tube where FN is stored inside, or very
9 small insects like thrips that can enter *H. citrina* floral tubes to feed on nectar. These
10 last were checked for by opening by hand 30 fully open *H. citrina* flowers between 7
11 and 8 am in the morning, in 2019 and 2020, respectively. Visitors were deemed EFN
12 feeders if their mouthparts were observed contacting extrafloral nectars. Where
13 possible, visiting species were identified by eye, otherwise photos or collected
14 specimens were used.

15

16 ***Systemic insecticides (acetamiprid and imidacloprid) in H. citrina FN and EFN***

17 Acetamiprid (ACE) and imidacloprid (IMI) were purchased from Sichuan
18 Guoguang Agrochemical Co., Ltd., JianYan, China. Two grams each of ACE and IMI
19 were dissolved in 10 L water and at 8.00 am on July 7th 2019 this was applied as a
20 soil drench to an 8 m^2 open field plot, where grew thirty *H. citrina* plants, avoiding
21 getting any insecticide directly onto the shoots. Ten *H. citrina* plants on a separate
22 plot without any insecticide treatment, 2 m away from the treated plot, were used as a

control.

Nectar was sampled from each *H. citrina* plant at one, 6 and 11 days after insecticide application, always between 7 and 8 am. FN was collected only from fully opened flowers, and EFN from the outside of young buds using a pipette and autoclaved tips. Where an individual had >1 flowers open on a given day, all FN was pooled, and all EFN was pooled from that plant. When a sample of FN or EFN from a single plant was < 150 μL, then two or more samples were combined to form a sample containing ≥ 150 μL for all subsequent tests. Six samples of each nectar type, from each of treated and control plots, and from each time period, were examined. Each nectar sample was filtered through 0.22 μm syringe filters (Millipore) to remove any dirt or pollen grains therein and stored at -20 °C prior to use, then directly analysed without any extraction or clean-up steps. The contents of the nectar were separated on a UPLC-MS/MS (ACQUITY ultra performance liquid chromatograph; Waters, Milford, MA) with a BEH Shield RP C18 column (100 mm × 2.1 mm internal diameter, particle size 1.7 μm) following Jiang et al. (2018). Raw chromatographs and mass spectrogram data were processed with MassLynx 4.1 Software (Waters). The peak area ratio of ACE and IMI to external standards was used for quantification.

1 **RESULTS**

2

3 ***Hemerocallis citrina* FN and EFN showed different secretion patterns**

4 In the area where our study was conducted, *H. citrina* flowered in summer from June
5 to July. Its flowers started to open at dusk (~19:00), fully opened by ~21:00, remained
6 open overnight and then wilted in the following morning whether pollinated or not.
7 From an early stage where buds are ~1 cm long to full opening takes nearly ten days
8 (Fig. 1A). *H. citrina* EFN was exclusively secreted on the middle area of the outer
9 surface of the young flower bud, lasting for 8 days from when flower buds reached 1
10 cm long until it reached half its final length and turned yellowish (one day before
11 opening) without any externally visible structure associated with EFN exudate (Fig.
12 1A, B). *H. citrina* EFN secretion also showed a very clear circadian rhythm, starting
13 from dusk (~19:00) and ending at dawn (~7:00) each day during our investigation
14 (Fig. 1B). An individual flower daily produced up to about 50 µl EFN, but the amount
15 varied dramatically depending on the age of the bud and the weather conditions, such
16 as humidity. Usually, younger and smaller flower buds (<3 cm long) secreted more
17 EFN than the bigger ones (Hong-Xia Zhou; personal observation). Because no EFN
18 residue could be seen left on bagged *H. citrina* flower buds in the middle of the day,
19 reabsorption apparently did happen even though evaporation might also play a role in
20 the disappearance of EFN.

21 *H. citrina* has deep-tubed flowers with a gynoecial nectary which is situated at
22 the base of the ovary (Fig. 1C). Individual *H. citrina* flowers produce 10~40 µl (22.5

± 9.5 µl, mean ± SD, n=30) of FN which sits in the deep end of floral tube. The *H. citrina* floral tube was 3-4 cm long and its inside diameter was <3 mm. *H. citrina* FN was not visible from outside and only accessible to feeders with a long proboscis, or to very small insects such as thrips that can enter the floral tube (Fig. 1C). *H. citrina* flowers begin to secrete FN about one day before they fully open (Fig. 1A). FN was never present in wilted flowers by ~10:00 am in the morning after they opened, including in bagged flowers that received no insect visits, indicating that FN was completely reabsorbed. Therefore, FN is present in each *H. citrina* flower for less than two days, from when secretion starts to full reabsorption (Fig. 1C).

Therefore, even though *H. citrina* plants were rich in both FN and EFN every night during its flowering season, the timing of EFN and FN secretion on an individual flower never overlapped (Fig. 1A) which also indicated a shift from EFN production to FN production during flower development.

H. citrina FN and EFN have different physiochemical characters

Both *H. citrina* FN and EFN were colourless liquids and slightly acidic, with the pH value of 5.4 ± 0.2 and 5.4 ± 0.1, respectively (mean ± SD, n=16) (Table 1). FN contained >5 times more solutes than EFN, with brix values of 17.0 ± 0.33 and 3.3 ± 0.35 respectively (mean ± SD, n=8) (Table 1). Both FN and EFN contained glucose, fructose and sucrose, but FN was sucrose dominant whereas EFN was hexose rich (Table 1, Fig 2A). This supports the hypothesis that sucrose is usually dominant in deep hidden nectars which are preferred by longer-tongued bees, hummingbirds, and

1 moths (Willmer, 2011). *H. citrina* FN contained significantly higher amount of free
2 amino acids and total phenolics than did EFN (Table 1). The average concentration of
3 hydrogen peroxide in *H. citrina* FN was 2.5 μM, which was much lower than it
4 detected in tobacco FN (~1 mM) in which hydrogen peroxide was reported to limit
5 microbial growth (Carter and Thornburg, 2004). No hydrogen peroxide was detected
6 in EFN samples. Chitinase and glucanase were deemed important enzymes for
7 protecting FN or EFN from infection by microorganisms (Gonzalez-Teuber et al.,
8 2009, Roy et al., 2017). In our study, *H. citrina* FN contained lower chitinase activity
9 than EFN (FN: 0.04±0.03, EFN: 1.46 ± 0.31, mean ± SD, n=8) and no glucanase
10 activity unlike EFN (45.7±15.1 U mL^{-1}, mean ± SD, n=8) (Table 1). *H. citrina* FN
11 and EFN were very different in absorbance spectra in the wavelength range 200-400
12 nm (Fig. 2B). EFN only had one absorbance peak at 215 nm, whereas FN had two
13 absorbance peaks at 220 nm and 263 nm; furthermore FN had higher absorbance in
14 the wavelength range 200-300 nm (UV region) than did EFN. Distinct UV absorption
15 spectra between FN and EFN indicated they contained different UV-absorbing
16 substances. Both *H. citrina* FN and EFN had no absorbance at wavelengths >400 nm
17 in the visible region (data not shown).
18
19 ***Nectarins of H. citrina FN and EFN***
20 Most reported nectarins are enzymes, and these are the only components in nectar
21 with catalytic activity, playing important roles in nectar biosynthesis and
22 post-secretory modification (Heil, 2011, Roy et al., 2017, Ma et al., 2019). In this

study, both *H. citrina* FN and EFN contained low but almost equal concentration of nectarins, 15.1 ± 8.5 and 15.8 ± 2.6 μg mL^{-1}, respectively (Table 1) which were lower than the mean value of nectarin concentration in some other floral nectars (ca. 100 μg mL^{-1}) (Nicolson and Thornburg, 2007). CBB G-250 staining in a Tricine-SDS-PAGE gel visualized completely different proteome profiles between FN and EFN (Fig. 3A). Unlike many reported nectar proteome profiles (Park and Thornburg, 2009, Roy et al., 2017), *H. citrina* FN presented weakly, forming a smear area across the lane (Fig. 3A) indicating that it comprised diverse proteins at low concentration with different molecular weights, among which none predominated. We excised ten bands from the top to the bottom across the lane which were slightly CBB stained, but none could be identified by MALDI-TOF/TOF (data not shown). Different from FN, EFN yielded 4 distinct bands visualized by CBB G-250 staining, ranging in size from 30 kDa to 35 kDa with a predominant band separated at approximately 33kDa (Fig. 3A), from which two peptides were identified: "AIETYLFAMFDENQK" and "QPEVEK". Both of these matched the identity of a beta-1,3-glucanase from *Hevea brasiliensis* (ACZ74626) or *Sesamum indicum* (XP_011083775) with a score of 134. No other nectarins from EFN were successfully identified by this approach. Chitinase zymograms (Fig 3B) revealed that both *H. citrina* FN and EFN contained chitinolytic activity but in different profiles. FN showed only one faint band whereas EFN showed four bands with different mobility across the lane. This indicated that EFN had multiple nectarins with chitinolytic activity but FN only had one.

Gel-free mass spectrometry (LC-ESI-MS/MS) analysis revealed that *H. citrina*

1 FN and EFN had significantly different protein compositions (supplementary Table
2 S1 S2). *H. citrina* EFN contained 11 unique proteins (Table 2), 6 of which belonged
3 to the glycoside hydrolase (GH) family and probably played roles in carbohydrate
4 metabolic processes. The major nectarin in EFN, a beta-1,3-glucanase that was
5 recognized by gel-based mass spectrometry, was also identified in this approach, and
6 is a member of pathogenesis-related (PR) family 2. Three other PR proteins were also
7 identified in EFN, endochitinase (PR-3), Peptidase S8 (PR-7), and an uncharacterized
8 protein with cysteine-type endopeptidase inhibitor activity (PR-6). This indicates that
9 these PR proteins might function in antimicrobial processes in EFN, consistent with
10 previous findings from other species (Gonzalez-Teuber et al., 2009, Park and
11 Thornburg, 2009, Roy et al., 2017).
12 In FN, 1333 proteins were identified by LC-ESI-MS/MS but only three of them
13 were the same as identified in EFN (Table 2 and supplementary Table S1). These
14 were beta-D-xylosidase (ONK73945), Cobalamin-independent methionine synthase
15 (KJB09184), and 14-3-3 h-1 protein (EPS73301). Even though beta-1,3-glucanase
16 was the major nectarin in *H. citrina* EFN and was frequently identified in FN or EFN
17 from different plant species (Roy et al., 2017), it was not detected in *H. citrina* FN.
18 This was consistent with no glucanase activity having been detected in *H. citrina* FN
19 by our enzymatic analysis in this study.
20 No chitinase was identified in FN by this sequence-based proteomic analysis,
21 however weak chitinolytic activity was detected in FN by enzymatic methods with
22 chitin substrates. This suggests that *H. citrina* FN might contain a chitinase whose

sequence was different from known ones. One tenth of proteins identified by proteomics analysis were heat shock proteins (HSPs), especially HSP70, also including HSP81, HSP82, HSP83, HSP90 (Supplementary Table S1). It is known that HSP70 is required for flower opening under normal temperatures or mild heat stress (Chen et al., 2019), and in *Nicotiana* FN it is thought to protect other nectarins from oxidative damage caused by high levels of H_2O_2 (Silva et al., 2020). However, in this study, neither HSPs nor H_2O_2 were detected in EFN. We did detect some non-secretory proteins presented in *H. citrina* FN, e.g. Proteasome subunit alpha type-5 protein (PHU25788; Nucleus or Cytoplasm located), 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (NP_001311960; Cytoplasm located). Because the FN is sited deeply in the end of long *H. citrina* floral tube, we had to cut the tube to collect it and could not make it totally free of contaminants from floral tissue. These non-secretory proteins in FN proteome could be collection-related contaminations but they were present in very small amounts and would thus have little effect on the comparative proteomic analysis much between EFN and FN.

***H. citrina* FN and EFN attracted different types of arthropods to visit and feed**

Even though both FN and EFN presented simultaneously during the night, they attracted different types of animal nectar feeders. Three hawkmoths, *Agrius convolvuli*, *Theretra suffuse*, and *Ampelophaga rubiginosa* (Sphingidae), were the most frequently observed FN feeders, probing into the narrow floral tube and sucking

FN using their long mouthparts during the evening and night (Supplementary Data Video S1), during which their bodies touched the stigma and anther. Therefore, hawkmoths were observed to be the dominant *H. citrina* FN feeders and pollinators (Table 2). Thrips (*Frankliniella intonsa*) also fed on FN feeder, by entering the floral tube (they are less than 2 mm long). We found thrips in 20 out of 60 opened *H. citrina* flowers examined. Thrips are well known to feed on both FN and pollen (Ananthakrishnan, 1993). However, in this study, no contact was observed between thrips and *H. citrina* anthers or stigma, and furthermore their small size makes them poor pollen vectors, hence thrips appear to be FN feeders but nor pollinators for *H. citrina*. *Xylocopa nasalis* and *X. appendiculata* (Apidae) had been observed piercing through the end of *H. citrina* floral tube with their tough mouthparts and sucking out FN, but these were not observed collecting *H. citrina* pollen (Supplementary Fig. S1).

Comparing with FN, EFN was completely exposed and accessible to more diverse animals. *H. citrina* EFN's feeders were mainly flies (*Rivellia nigroapicalis*, *Sarcophaga peregrine*, *Lucilia sericata*, *Drosophila melanogaster*, *Musca domestica*, and *Episyrphus balteatus*), ants (*Camponotus japonicas* and *Monomorium pharaonis*), and wasps (*Vespa affinis*, *Polistes jokahamae, Polistes snelleni,* and *Parapolybia varia*) (Table 2). Notably, wasps are well known predatory insects, but we frequently watched these wasps feeding on EFN on *H. citrina* flower buds in the early morning instead of hunting their prey, such as flies or aphids on *H. citrina* (Supplementary Data Video S2). Ants could be seen on *H. citrina* flower buds almost throughout the day, especially in the morning before sunrise because of ample EFN on *H. citrina*

1 floral buds at that time. However, we seldom observed the ants entering the opened
2 flower for FN (Supplementary Data Video S3).

3 EFN consumption by some regular predatory animals has been reported, e.g.,
4 crab spiders (Taylor and Foster, 1996). On *H. citrina* flowers, spiders (*Ebrechtella*
5 *pseudovatia*, *Tetragnatha praedonia, Argiope bruennichi*), katydid (*Ducetia japonica*),
6 and mantis (*Hierodula patellifera*), were observed preying on flies or other insects on
7 *H. citrina* flowers (Table 2; Supplementary Fig. S1), but none of these were observed
8 directly feeding on FN or EFN. Hence they may only be regarded as indirect feeders
9 of *H. citrina* FN and especially EFN.

10 No animals other than arthropods were observed feeding on *H. citrina* FN or EFN
11 during our investigation. Furthermore, no animal species was observed to feed on
12 both *H. citrina* FN and EFN during our investigation. Honeybee and bumble bees
13 were observed collecting pollen on *H. citrina* flowers, but none of these were
14 observed to feed on *H. citrina* FN or EFN.

15

16 ***Both acetamiprid and imidacloprid accumulated more in EFN than FN***

17 Both acetamiprid (ACE) and imidacloprid (IMI) compounds were detected in *H.*
18 *citrina* FN and EFN at a time 24 hours after ACE and IMI were applied into the soil,
19 and the concentration of both in both nectars continuously increased over 11 days
20 from application (Fig. 4). Both ACE and IMI concentrations in EFN were
21 significantly higher than in FN at the three checked time points (Fig. 4). After one day,
22 ACE concentration was 30.2 ± 2.0 ppb in EFN and 3.0 ± 0.3 ppb in FN; after 11 days

1 it was 97.0 ± 4.7 and 12.2 ± 0.9 ppb respectively (Fig. 4A). The IMI concentration

2 was 3.4 ± 0.7 in EFN ppb and 0.8 ± 0.1 ppb in FN; after 11 days it was 131.8 ± 10.7

3 and 19.9 ± 2.5 ppb respectively (Fig. 4B). Metabolites, e.g. 6-chloronicotinic acid

4 from the breakdown of IMI and 6-chloro-pyridilmethyl alcohol from ACE (Malev et

5 al., 2012), could not be detected in the EFN or FN samples using mass spectrometer

6 (data not shown) which indicates that *H. citrina* couldn't degrade or detoxify either

7 compound during any stage of nectar production. Hence there are different generation

8 processes or pathways for EFN versus FN, which ACE and IMI become involved in.

9 Comparing with IMI, ACE reached significant higher level concentrations in FN or

10 EFN which might due to ACE having higher water solubility (2950 mg l^{-1} at 20 °C,

11 compared to 610 mg l^{-1} at 20 °C for IMI, PPDB: Pesticide Properties DataBase,

12 https://sitem.herts.ac.uk/aeru/ppdb/en/index.htm, accessed 1st May, 2021). No

13 morphological changes between treated and untreated groups or phytotoxicity caused

14 by IMI and ACE application were observed.

15

1 **DISCUSSION**

2

3 ***Similarities and differences between Hemerocallis citrina FN and EFN biosynthesis,***

4 ***composition, and secretion***

5 The similarities between *H. citrina* FN and EFN were as expected, in that both

6 are acidic and have sugars as their major components, with smaller amounts of amino

7 acids, proteins, phenolics, etc. Both FN and EFN were secreted on flowers between

8 dusk and dawn, and attracted a lot of animal visitors. However, many differences were

9 detected between *H. citrina* FN and EFN, for example FN had sucrose as the

10 dominant sugar, while EFN had hexose. Hexose in nectar is believed to result from

11 sucrose hydrolysis by a cell wall-bound invertase in the nectary (Nicolson and

12 Thornburg, 2007, Zhou et al., 2018), but if so this process was important in *H. citrina*

13 EFN generation but not FN generation. Sugar type in nectar is considered to be a

14 determinant for which visitors are attracted to a flower (Baker and Baker, 1983,

15 Nicolson and Thornburg, 2007). For example, sucrose dominant nectar can be less

16 viscous than a hexose dominant nectar of equivalent caloric value, making it more

17 suitable for lepidopterans sucking nectar from deep-tubed flowers with a long

18 proboscis (Willmer, 2011). Given that hawkmoths were the most commonly observed

19 pollinators for *H. citrina*, sucrose dominant FN appears to be an ecological adaptation

20 to its native pollinators and flower shape. Conversely, because EFNs are always

21 presented on plant surface, viscosity is not a serious limitation for visitors to feed on,

1 and furthermore higher viscosity makes it less likely that externally presented nectar
2 will drip from the flower.
3 The comparative proteomic analysis showed further differences between FN and
4 EFN. *H. citrina* EFN's proteome was quite simple, with only 11 detected proteins, of
5 which four belonged to four different pathogenesis-related (PR) proteins families,
6 PR-2, PR-3, PR-6, and PR-7. This strongly supports the idea that nectarins in EFN, as
7 well as FN, mainly serve to protect the nectar, and surrounding tissues, from
8 microbial infections (Park and Thornburg, 2009, Heil, 2011). A chitinase was the
9 predominant protein in *H. citrina* EFN, and both chitinase and glucanase activity were
10 detected in EFN, both of which enzymes also have antimicrobial properties
11 (Gonzalez-Teuber et al., 2009, Ma et al., 2017). However, neither enzyme could be
12 detected in FN, even using high-resolution mass spectrometry based proteomic
13 analysis. Furthermore, the FN proteome was significantly lacking in PR proteins,
14 despite being far more complex than that of EFN, comprising mainly HSPs with
15 hundreds of other proteinaceous components. These differences likely reflect the
16 different roles and situations of the two nectars. Microbial defences are not obligate in
17 nectars (Ma et al., 2017), and there may be little selection pressure on *H. citrina* FN to
18 develop such defences, because the FN is deeply concealed, accessible to only a
19 selection of visitors, and only present for half a day from when it first becomes
20 accessible, all of which limits opportunities for pathogen establishment. Conversely,
21 *H. citrina* EFN is continuously secreted on the outside of flower buds for at least one
22 week and completely exposed to any visitors or wind, giving ample time for microbes

to arrive and initiate infection, making an antimicrobial mechanism essential. In this context, it is surprising that hydrogen peroxide was detected in *H. citrina* FN but not EFN, given that this compound has an antimicrobial role in *Nicotiana* sp. (Carter and Thornburg, 2004). However, the hydrogen peroxide concentration in *H. citrina* FN was very low, only around 2.5 μM, over 1000 times less than 4 mM detected in *Nicotiana* sp. (Carter and Thornburg, 2004), making it quite possible that hydrogen peroxide doesn't play any antimicrobial roles in *H. citrina* FN.

In addition, we observed that there was almost no EFN present on the flowers when it was about to rain, whereas on such days, no difference in FN production was detected. Higher sensitivity to weather conditions in EFN secretion, relative to that of FN, indicated that they have different secretion regulation mechanisms.

EFN is secreted on flowers but does not function directly in encouraging pollination

H. citrina is a strict outcrosser, and provides pollen and FN as a reward for its animal pollinators, which are mainly hawkmoths, although honey and bumble bees also pollinate the flowers. However, during our two years' observation, bees of all kinds were only observed to collect pollen, but never nectar (FN or EFN). The likely reason is that the relatively short mouthparts of bees cannot reach the FN deep within the corolla tube. The EFN of *H. citrina* is very dilute (<4% sugars), making it an unsuitable energy source for flying pollinators, especially bees (Parachnowitsch et al., 2019). Consistent with this, across two years of observation, we never once saw any

1 of the insects that pollinate *H. citrina* (e.g. honeybee, or bumble bees), feeding on the
2 EFN. Furthermore, we found that *H. citrina* EFN secretion stops on each flower at
3 least two days before it opens, so it cannot help the flower it is on attract pollinators.
4 EFN secretion on *Bixa orellana* similarly reaches a peak on the mature floral buds,
5 and ceases entirely by the time the flowers open (Bentley, 1977, de Miranda et al.,
6 2017). Conversely, EFN is attractive to various insects that do not pollinate *H. citrina*,
7 such as wasps, ants, mantises and flies. To these it offers water and nutrients as well
8 as limited amounts of sugar. For example, it has been shown that on summer days,
9 solitary wasps are more likely to choose flowers with diluted nectar which can give
10 them safe water as well as sugar (Willmer, 1985). The question therefore becomes,
11 how are visits from these insects, that are attracted by EFN, beneficial to *H. citrina*?
12 Because many are predatory, they might serve a defensive role for the flower,
13 discouraging herbivores and sap-suckers, although this has yet to be tested.
14 That EFN secretion always stopped before FN secretion began suggests a
15 trade-off between herbivore defence and pollination attraction (Fig. 1A). This could
16 be due to the cost of nectar production: it is estimated that 4 to 37% of daily
17 photosynthate assimilated during blossoming is secreted as nectar sugar (Southwick,
18 1984), and *H. citrina* might not have the resources to produce both FN and EFN on
19 the same flower at once. However, both are produced on the same inflorescence when
20 flowers are in different development stages. An alternate explanation is that EFN
21 ceases to be advantageous, and even becomes disadvantageous, as the flower opens. If
22 the EFN function is to attract predatory arthropods, then these might discourage

pollinators from visiting, making it necessary to cease EFN production before the flower opens.

Neonicotinoid insecticides in EFN is a potential threat to nontarget animals

The use of systemic insecticides, especially neonicotinoids, is well documented as causing global pollinator decline (Goulson, 2013). Acetamiprid (ACE) and imidacloprid (IMI), are the two most frequently detected neonicotinoids in agricultural products and honey in China (Wang et al., 2020). Both are currently legal in China, despite being highly toxic to a broad range of insects and other animals, especially to birds and earthworms (Pisa et al., 2015).

Thus far most of the attention to the problems caused by insecticides has focussed on poisoning pollinators through FN and pollen (Goulson, 2013, Raine, 2018). Here, we demonstrate that both ACE and IMI can become components of EFN, as well as FN, at least in *H. citrina*. The presence of insecticides in EFN indicates they will hence kill a further range of nontarget animals more diverse than those that feed on FN, because EFN is more accessible and because being hexose-rich it is more digestible (Heil et al., 2005). In this case, both ACE and IMI became more concentrated in *H. citrina* EFN than in FN. It is predictable that the insecticides will also be passed on to the predators of all these EFN feeders. Moreover, because EFN appears to have evolved to attract those arthropods that prey on herbivorous insects, the effect of killing these insects will be to reduce natural predation on the very pest

species that the insecticides are supposed to control, hence worsening the exact problem the chemicals are supposed to be dealing with.

EFN is always ignored in environmental risk evaluation because of usually small amount, not always adjacent to flowers and easily cleaned by feeders. Our results show that it is important and must be included. Thus far, 4017 angiosperm species across 110 families are known to secrete EFN, and the numbers keep growing (http://www.extrafloralnectaries.org/, accessed 1st June, 2021). Though the number is small compared to with FN-bearing species, it includes several major crops, such as castor oil (*Ricinus communis*), beans (*Phaseolus vulgaris* and *P. lunatus*), sunflower (*Helianthus annuus*) and cotton (*Gossypium hirsutum*). Moreover cotton, like *H. citrina*, secretes more EFN than it does FN (Jones et al., 2020, Chatt et al., 2021), and the same is true of the widespread and common genus *Acacia* (Heil, 2015). *H. citrina* itself is a common vegetable in China with an annual yield of fresh weight 1.25 million tons in 2020 (Zhi-Xin Qin, Hunan Agriculture University; personal communication). Furthermore, it generally flowers in June and July, a time when relatively few species are flowering in China relative to spring or late summer, making it a significant food source for pollinating insects. Systemic pesticides, including both IMI and ACE are commonly used on farmed *H. citrina* (Jin et al., 2019), and because flowers are always harvested before they have opened, EFN but not FN provides a route for these chemicals to enter the wild food chain, and might have a major ecological impact. Our work demonstrates that EFN is a potential

pathway for these chemicals to enter the wild food chain, and must be considered when evaluating the ecological risk of any such chemical.

Conclusions

This study demonstrates that FN and EFN differ in content, timing of production and function, within *H. citrina*, with FN being concentrated and sucrose-dominant, secreted in the mature flower tube, and serving as reward for pollinators. Conversely, EFN is dilute and hexose-rich, secreted on the outside of developing floral buds, and is likely to attract predatory animals for defence. There were also significant physiochemical differences between FN and EFN, especially concerning the proteins they contained, with microbial defence proteins only evident in EFN. The two neonicotinoid insecticides examined, IMI and ACE, became present in both nectar types soon after application, but in greater concentration within EFN. This, plus the fact that a wider range of insect species were seen to feed on EFN, means that EFN is a more significant pathway for these chemicals to enter the wild food chain. Therefore, the EFN pathway must be considered when evaluating the risks to the environment of any systemic insecticide.

Acknowledgements

This study was supported by Collaborative Innovation Centre of Plant Physiology and Active Ingredient Research of Huangshan University (to HG Zha); National Natural Science Foundation of China Science (32070258 to HG Zha); Key Research Projects of Natural Science in Universities in Anhui Province (KJ2016A680 to YQ Song and KJ2020A0689 to HX Zhou); National College Students' Innovation and Entrepreneurship Training Program (S202010375056 to HG Zha, and S202010375048 to HX Zhou); and Open Fund of Laboratory of Ecology & Evolutionary Biology, Yunnan University (to HG Zha); Science and Technology program of Yunnan province (2019FY003026 to Jun Cao). We thank Mr Hong-Cao Wang (Nanjing Forestry University) for arthropod identification and Qiu-Yuan Zha for his help in field work.

Compliance with ethical standards

Conflict of interest

Nothing declared.

Ethical statement

Our work complies with the ethical rules applicable for this journal.

Author contributions

Conceived and designed the experiments: HGZ, HXZ, YQS. Performed the experiments: PC, MFH, XYL, WJG. Analyzed the data: HGZ, RIM, JC. Wrote the paper: HGZ, RIM.

1 **Figure legends:**

2

3 **Fig. 1** *Hemerocallis citrina* flower and presentation of FN and EFN. (A) The
4 development of *H. citrina* flower is divided into different stages based on day. (B)
5 EFN on *H. citrina* flower bub, indicated by an arrow. (C) FN in *H. citrina* floral tube,
6 indicated by an arrow.

7

8 **Fig. 2** HPLC chromatogram (A) and UV-VIS absorption spectrogram (B) of *H.*
9 *citrina* FN and EFN.

10

11 **Fig. 3** SDS-PAGE of *H. citrina* FN and EFN nectarins. From left to right: FN, floral
12 nectar; M, molecular weight marker; EFN, extrafloral nectar. Predominant nectarin
13 (beta 1-3 glucanase) in EFN was indicated by an arrow.

14

15 **Fig. 4** Imidacloprid (IMI) and acetamiprid (ACE) residue in *H. citrina* FN and EFN.
16 A, ACE; B, IMI. Error bars represent standard error.

17

18 **Supplementary Fig. S1** *H. citrina* Flower in the daytime 14:00 (A) and night 20:00
19 (B).
20 **Supplementary Fig. S2** Some *H. citrina* flower visitors. *Bombus trifasciatus* Smith
21 (A); *Apis mellifera* Linnaeus (B); *Xylocopa nasalis* Westwood (C); *Camponotus*
22 *japonicus* Mayr (D); *Vespa affinis* (Linnaeus) (E); *Ebrechtella pseudovatia* (Schenkel)
23 (captured a fly) (F); *Ducetia japonica* (Thunberg) (G); *Frankliniella intonsa* (Trybom)
24 (H); *Hierodula patellifera* Serville (I); *Musca domestica* Linnaeus (J).
25 **Supplementary Table S1** Summary of proteins identified in *H. citrina* FN.
26 **Supplementary Table S2** Summary of proteins identified in *H. citrina* EFN
27 **Supplementary Video S1** Hawkmoth fed on *H. citrina* FN-1.
28 **Supplementary Video S2** Hawkmoth fed on *H. citrina* FN-2.
29 **Supplementary Video S3** A wasp patrolling on an *H. citrina* flower bud and
30 searching for EFN.

1 **Supplementary Video S4** Ants patrolling on an *H. citrina* flower buds.

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1 **Table 1 The chemical traits of *H. citrina* FN and EFN (means ± SD, n = 8).**

Test	**FN**	**EFN**
pH (n=16)	5.4±0.2	5.4±0.1
Total soluble solids (Brix)	17.0±0.33	3.3±0.35**
Sucrose (%)	12.9±1.6	0.8±0.5**
Glucose (%)	3.0±0.3	1.7±0.3**
Fructose (%)	2.4±0.4	1.8±0.3**
Class of nectar[a]	Sucrose dominant	Hexose rich
Total free amino acids (μg mL^{-1})	0.37±0.12	0.08±0.01**
Total protein (μg mL^{-1})	15.1±8.5	15.8±2.6
Total phenolics (μg GAE mL^{-1})	114.0±44.4	14.5±4.5**
Hydrogen peroxide (μM)	2.5±1.9	nd
Chitinolytic activity (U mL^{-1})	0.04±0.03	1.46±0.31**
Endo-β-1,3-glucanase activity (U mL^{-1})	nd	45.7±15.1

2 Values are means ± s.d. [a] Nectar categorization was based on sugar type ratios, defined as the ratio
3 by weight of sucrose to the combined hexose sugars, S/(G + F). Nectar was defined as “hexose
4 rich” if the sugar ratio was between 0.1 and 0.5, or “sucrose dominant” if it was >1.0 (Nicolson
5 and Thornburg 2007). [b] nd = not detected. **, $P < 0.01$, significant differences (using the Student's
6 test)

Table 2 Proteins identified in EFN from *Hemerocallis citrina*

N°	Protein identified	Access number (Genbank)	Organism	Protein Mascot score/ Count of distinct sequences	Biological process[1]	PR protein family[2]	GH family[1]	others
1	Beta-D-xylosidase	ONK73945	*Asparagus officinalis*	243/6	carbohydrate metabolic		3	
		ACL53913	*Zea mays*	79/5			3	Also detected in FN
		PKA56762	*Apostasia shenzhenica*	61/5			3	
		SPT20554	*Triticum aestivum*	133/4			3	
		OIV96210	*Lupinus angustifolius*	147/3			3	
		NP_196535	*Arabidopsis thaliana*	146/2			3	
2	Beta-galactosidase	KVH90786	*Cynara cardunculus var. scolymus*	74/4	carbohydrate metabolic		35	
3	Alpha-amylase	CDP01359	*Coffea canephora*	62/2	carbohydrate metabolic		13	
4	Polygalacturonase	RCV19800	*Setaria italica*	65/2	carbohydrate metabolic		28	
5	Endochitinase	NP_181890	*Arabidopsis thaliana*	68/2	carbohydrate metabolic	3	19	
6	Endo-1,3-beta-glucanase	XP_020700743	*Dendrobium catenatum*	57/2	carbohydrate metabolic	2	17	
7	Peptidase S8	OVA17070	Macleaya cordata	64/2	Proteolysis	7	-	
8	Cobalamin-independent	KJB09184	*Gossypium raimondii*	50/2	cellular amino	-	-	Also detected in FN

	methionine synthase				acid biosynthetic			
9	Uncharacterized protein (with cysteine-type endopeptidase inhibitor activity)	RRT32118	*Ensete ventricosum*	122/2	unknown	6	-	
10	Fasciclin-like arabinogalactan protein	KNA25811	*Spinacia oleracea*	63/2	unknown	-	-	
11	14-3-3 h-1 protein	EPS73301	*Genlisea aurea*	51/2	unknown	-	-	Also detected in FN

: 1. Source: InterPro- https://www.ebi.ac.uk/interpro.

2. Pathogenesis-related (PR) proteins classification according to Sels et al. (2008).

1 **Table 3 *Hemerocallis citrina* FN and EFN's feeders in the two years of study.**

Nectar feeder type	Order /Species	Visits or number of FN and EFN feeder[1]	
		2019	2020
FN	Thysanoptera		
	Frankliniella intonsa	12[4]	8[4]
	Hymenoptera		
	Xylocopa nasalis	5	2
	Xylocopa appendiculata	8	3
	Lepidoptera		
	Agrius convolvuli	5	11
	Theretra suffusa	12	26
	Ampelophaga rubiginosa	4	13
EFN[2]	Scutigeromorpha		
	Scutigera coleoptrata	45	13
	Blattodea		
	Blattella bisignata	25	9
	Coleoptera		
	Chauliognathus sp.	9	7
	Hymenoptera		
	Camponotus japonicus	135	74
	Monomorium pharaonis	51	16
	Vespa affinis	32	14
	Polistes jokahamae	9	5
	Polistes snelleni	5	2
	Parapolybia varia	114	62
	Diptera		
	Rivellia nigroapicalis	15	6
	Sarcophaga peregrina	10	7
	Lucilia sericata	17	23
	Drosophila melanogaster	74	33
	Musca domestica	15	18
	Episyrphus balteatus	19	11
Indirect feeding on FN or EFN[3]			
	Araneae		
	Ebrechtella pseudovatia	44	27
	Tetragnatha praedonia	18	13
	Argiope bruennichi	17	4
	Orthoptera		
	Ducetia japonica	12	4
	Mantodea		
	Hierodula patellifera	6	2
	Coleoptera		
	Harmonia axyridis	33	18

2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41

42 : 1. Arthropods that visited *H. citrina* flowers but were not seen feeding on FN or EFN, e.g., honeybees and
43 aphids, were not counted in this investigation.
44 2. For flightless or weakly mobile arthropods, numbers of individuals observed was treated as number of
45 visits, for each species were counted during our observation.
46 3. Predatory arthropods which had not been observed directly feeding on FN or EFN but preying on FN or
47 EFN feeders.
48 4. Numbers of flowers out of 30 adult flowers checked which had Frankliniella intonsa found within them.
49

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