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ORIGINAL PAPER

Dynamics of soluble sugars and secondary metabolites in fruit of *Juglans australis* attacked by *Anastrepha fraterculus* and *Ceratitis capitata* (Diptera: Tephritidae)

Luis Oroño[1,2] · Martín Aluja[4] · Sergio Ovruski[3] · Juan Rull[3,4] · Roque Interdonato[6] · Fernando E. Prado[5] · Mirna Hilal[5,2]

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Abstract
The development and fitness of phytophagous insects are tightly linked to the nutritional quality of their host plants and many studies have examined the influence of primary and secondary metabolites of plants and their effects on the development of insects. Herbivore tactics to modify plant metabolic pathways to lower host toxicity need to be better understood as they are critical to a better understanding of herbivore–host plant relationships. To contribute to this end, in this study we analyzed temporal patterns of glucose, sucrose, fructose, and total soluble sugar contents, as well as tannins, phenols, and flavonoids in the mesocarp of fruit of native walnut (*Juglans australis*), uninfested and infested by *Anastrepha fraterculus* and *Ceratitis capitata* (sometimes simultaneously in a single fruit). Both fly species are polyphagous tephritids whose larvae feed on a wide variety of hosts. We observed a high correlation between infestation and adult emergence of these two insects which was positive in the case of sugar content and negative in the case of toxic secondary metabolites in fruit. At particular ripening stages, infested fruit contained higher levels of sugars and lower levels of phenols and tannins than non-infested fruit. We discuss the possibility that *A. fraterculus* and *C. capitata*, each with different egg-laying strategies, may modify metabolical pathways in the fruit for their own benefit through larval activity with the help of bacteria in their gut. Alternatively, the patterns observed may be simple side effects of larval feeding and associated growth of opportunistic microorganisms.

Keywords Herbivore offense · Herbivore manipulation · Plant defense · Nutritional content

Introduction

The suitability of most host plants for herbivorous insects is determined by the balance between the nutritional value of the host and its chemical and structural defenses (Bernays

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✉ Martín Aluja
martin.aluja@inecol.mx

[1] Centro de Investigaciones y Transferencia de Catamarca (CITCA), CONICET-UNCA, K4700AAP Prado 366, Argentina

[2] Facultad de Ciencias Exactas y Naturales UNCA, Av. Belgrano 300, San Fernando del Valle de Catamarca, Catamarca, Argentina

[3] Laboratorio de Investigaciones Ecoetológicas de Moscas de la Fruta y sus Enemigos Naturales (LIEMEN) - Planta Piloto de Procesos Industriales Microbiológicos y Biotecnología (PROIMI), CCT Tucumán – CONICET, Av. Belgrano y Pje. Caseros, T4001MVB San Miguel de Tucumán, Argentina

[4] Instituto de Ecología, A.C., Apartado Postal 63, C.P. 91000 Xalapa, Veracruz, Mexico

[5] Cátedra de Fisiología Vegetal, Facultad de Ciencias Naturales e IML, Universidad Nacional de Tucumán, Tucumán, Argentina

[6] Cátedra de Fisiología Vegetal. Fac de Agronomía y Zootecnia. UNT. El Manantial, Tucumán, Argentina

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and Chapman 1994, 2000; Awmack and Leather 2002; Aluja and Mangan 2008; Aluja et al. 2014; Papachristos et al. 2008; Chen 2008; Papachristos and Papadopoulos 2009). This balance is highly dynamic and can be influenced, or even manipulated, by herbivores, or associated microorganisms (Sampedro et al. 2011; Ben-Yosef et al. 2015). Herbivore activity frequently induces defensive responses in plants (Wu and Baldwin 2010; Machado et al. 2016a), but can also increase digestibility and nutritive value, and may also reduce the toxicity of the host environment (Feeny 1970; Bezemer and Mills 2001; Karban and Argawal 2002; Díaz-Fleischer and Aluja 2003; Alves et al. 2006). To gain further insight into this interesting phenomenon, we choose to work with wild walnuts (*Juglans australis* Grisebach, Juglandaceae) and two fruit fly species known to infest this fruit in Argentina: the native *Anastrepha fraterculus* and the exotic *Ceratitis capitata* (Ovruski et al. 2003, 2004).

Juglans australis occurs between 500 and 1500 m above sea level and is widespread in the Northwestern subtropical rainforest, locally known as "Yungas" (Brown et al. 2001). Its fruit is a subglobose drupe with a fleshy mesocarp and a tough shell (endocarp), containing the embryo (Digilio and Legname 1966; Wu et al. 2004, 2009). Of relevance to our objective is the fact that the fleshy mesocarp represents the most significant nutrient source for seed development (Wu et al. 2003, 2004, 2009). Based on the latter, feeding by fruit fly larvae could at best modify the chemical composition of the mesocarp and at worst disrupt the transport of nutrients to the embryo causing it to grow at a slower rate or even fail to accumulate enough nutrient reserves for the seed to be viable. Secondary metabolites, such as phenolic compounds, tannins, and flavonoids also present in the mesocarp, could act as defensive compounds against herbivores. Plant responses to herbivore attack are known to be regulated by phytohormonal networks (Machado et al. 2016b). For example, it has been found in the case of leaf-herbivores that auxin is strongly, specifically, and rapidly induced by herbivore feeding, leading to a jasmonate dependent accumulation of anthocyanins and phenylamides in plant parts under risk of attack (Machado et al. 2016b). Foliar herbivory can also disrupt nutrient dynamics by constraining sugar accumulation in the leaves (Machado et al. 2017). Nevertheless, plants appear to tailor responses according to the organ that is damaged (Tytgat et al. 2013). In the case of larvae feeding on ripening fruit, herbivory can lead to an ethylene burst, also triggering important metabolic changes in nutrient and secondary plant compounds content in the fruit pulp (Alagna et al. 2016). Of interest here, is the possibility that larvae or associated microorganisms (Ben-Yosef et al. 2015) could influence or even manipulate metabolic pathways to gain more nutrients (e.g., glucose) and reduce levels of toxic secondary metabolites, such as polyphenols, which in apples and mango have been shown to be deleterious to fruit fly larvae (Verghese et al. 2012; Aluja et al. 2014).

Although the carbohydrate deposits in the fleshy pericarp are the main requirement for seed development, at a late stage of growth, carbohydrates are mainly transformed into lipids and proteins (Wu et al. 2009). However, at earlier stages of seed development, the fleshy pericarp becomes a significant sink of carbohydrates and thus an ideal medium for the development of fruit fly larvae. During development and ripening of walnut fruit both protein composition and carbohydrate reserves undergo energy-supporting changes, but carbohydrates of the mesocarp also act as a substrate to sustain the synthesis of lipids and other primary and secondary metabolites (Cosmulescu et al. 2010).

Walnuts are rich in secondary metabolites, mainly phenolic compounds. Thirty-seven compounds, including four novel hydrolysable tannins and two derivates of dicarboxylic acid have been isolated from the *J. regia* walnut extract (Colaric et al. 2005). Similarly, Fukuda et al. (2006) and Cosmulescu et al. (2010) identified thirteen phenolic compounds in walnut mesocarp. Since the carbohydrate supply to developing fruit depends upon leaf photosynthesis it is reasonable to assume that the carbohydrate pool of walnut mesocarp must also be affected by environmental factors. Therefore, the chemical composition of walnut fruit is dynamic (Corelli-Grappadelli and Lakso 2004; López and Dejong 2007). Thus, a complex network involving carbohydrate, lipid, protein, secondary metabolism, and environmental influences must be occurring in the mesocarp of ripening walnut fruit.

During the life cycle of many insects, egg-laying preference is a form of maternal investment. Females spend time and energy in such activity, which may result or not in offspring development. They therefore have to choose an adequate host to provide the best possible nutritional resources and habitat conditions to ensure both egg-laying and larval growth (Janz 2005; Fontellas-Brandalha and Zucoloto 2004). Walnuts of many cultivated and wild *Juglans* species are hosts for oviposition of different fruit fly species worldwide (Smith and Bush 2000; Guillen et al. 2011; Rull et al. 2013). Wild walnut fruits from the Argentinean Northwestern forest are hosts to two native tephritids *Anastrepha schultzi* (Blanchard) and *A. fraterculus* (Wiedemann) (South American Fruit Fly) (Schliserman et al. 2004), as well as the exotic *Ceratitis capitata* (Wiedemann) (Mediterranean Fruit Fly) (Ovruski et al. 2003). While *Anastrepha* schultzi and *A. fraterculus* tend to lay a single egg per clutch (JR personal observation), *C. capitata* lays egg clutches that vary in number depending on a range of host characteristics (McDonald and McInnis 1985). All three species can frequently be found simultaneously exploiting individual walnuts (Ovruski et al. 2003; Schliserman et al. 2004), with *A. fraterculus* tending prevail in greater numbers depending on the degree of habitat disturbance (Schliserman et al. 2014).

Chemical composition can affect host plant selection of many herbivorous insects, which display selective attractiveness to flowers and fruit structures that frequently lack characteristic secondary compounds present in other parts of the plant (Ikonen 2002). As fruit ripen, defensive compounds (secondary metabolites) disappear (Fitt 1990). Some fruit fly species feed on seed tissue that may be more toxic than the pulp. For example, in case of *Anastrepha*, primitive species such as *A. cordata, A. hamata, A. crebra*, and their closely related relatives (flies in the sister group *Toxotrypana*) specialize on seeds or associated tissue and attack latex-producing plants (e.g., *Apocynaceae, Asclepiadaceae*, and *Sapotaceae*). In contrast, most derived species (e.g., *fraterculus* group) feed almost exclusively on fruit pulp and are highly polyphagous (Aluja and Norrbom 2000; Aluja et al. 2000). Additionally, species such as *A. ludens* (also within the highly derived *fraterculus* group) have retained the ability to feed on both types of substrates (Aluja et al. 2000). In addition to maternal host choice, offensive tactics that may foster benefits to herbivores using highly defended host plants, include enzymatic metabolization of plant compounds, sequestration, morphological adaptations, symbionts, induction of plant galls, and induced plant susceptibility, trenching, and gregarious feeding (see review by Karban and Argawal 2002, and more recently; Ben-Yosef et al. 2015).

The aim of this study was to examine time-dependent variations associated with fruit ripening related to soluble phenols, flavonoid-like compounds, tannins, and soluble carbohydrates (sucrose, glucose, and fructose), and to determine whether such variations are related to fruit infestation by *A. fraterculus* and *C. capitata* in wild *J. australis*. By doing so, we hoped to start gaining insight into possibility that larval feeding could possibly induce metabolic pathways in the fruit leading to an increase of beneficial compounds and a decrease of toxic ones.

Methods and materials

Study site

The study was conducted in a disturbed natural vegetation area of the nature reserve "Parque Sierra de San Javier" at 26°44′S latitude and 65°16′W longitude, at elevations ranging from 500 to 600 masl. This site encompasses nearly 4000 ha within a protected area of native vegetation belonging to the "Yungas" forest, classified as low mountain forest (LMF) (Brown et al. 2001). The climate in the study site is defined as humid subtropical (*Cwa*, Köppen climatic classification) with a summer rainy season and winter dry season (Papetti-Villada 1978). Daily rainfall and maximal and minimal temperatures during the census period were obtained from the Estación Experimental Agro Industrial Obispo Colombres (EEAOC) (Tucumán, Argentina). The study was repeated over two growing seasons. There were no significant differences in minimal, maximal temperatures or rainfall between the 2003/2004 and 2004/2005 growing seasons with mean maximal temperatures and precipitation recorded in January of 32.1 °C and 163.1 mm month^{-1}, respectively.

Plant material and fruit sampling

Five *J. australis* trees with easily accessible fruit were chosen at random. Unripe, uninfested control fruit on branches of previously selected trees were covered with an organdy mesh bag to prevent natural fruit infestation by either wild *C. capitata* or *A. fraterculus* individuals during late spring (November). Fruit with evident signs of oviposition, identified by the presence of brown patches that appear on the surface caused by larval feeding, was considered as infested. Both control and infested fruits were collected weekly during a 50-days period from early summer (December) during the 2003/2004 and 2004/2005 growing seasons, when signs of infestation were clearly evident on fruits. The ripening period for *J. australis* is 120–140 days after full bloom. Day 1 of the collection corresponded to fruit with ~70 days of development. Fruit were collected from the basal section of the tree canopy (up to 2 m height from the ground), in the morning between 10.00 and 12.00 h. For infestation and fly emergence analyses, a set of 20 infested and 20 uninfested fruits per tree (a total of 200 fruit per replicate) were placed into cloth bags and transported to the Planta de Procesos Industriales Microbiológicos (PROIMI) (Tucumán, Argentina). For chemical determinations, collected fruits (a separate set of five infested and five uninfested fruits from each tree) were placed in liquid nitrogen and then transported to the Plant Physiology Laboratory of the Faculty of Natural Sciences The University (Tucumán, Argentina). A single (infested and uninfested) fruit per tree was analyzed for chemical content while the remaining four were stored frozen in case of need.

Infestation and emergence analyses

Fruits from each sampled tree were separated, weighed and rinsed with 20% sodium benzoate solution. After that, fruits were individually placed in a 220-ml plastic cup containing sterilized damp sand as pupation substrate and covered with an organdy cloth lid. Cups were placed in a growth chamber at 26 ± 2 °C and 70 ± 5% relative humidity (RH) for 5 weeks. Sand was sifted weekly to collect pupae. Afterwards, fruits were dissected to recover the remaining pupae in the walnut mesocarp. All pupae from each sample were counted and then placed in a plastic cup for emergence of adult flies. Adults were identified and the number of individuals per fruit sample was recorded. Fruit infestation level was expressed as the number of *A. fraterculus* or *C. capitata* puparia per kg of

fruit. Fly emergence values correspond to adult flies per kg of fruit and adult flies recovered from the total puparia obtained from fruit (percentage of adult emergence).

Fly identification

Pupae of *C. capitata* and *Anastrepha* spp. were distinguished and separated based on characters described in White and Elson-Harris (1992). Fruit fly adults were identified using the taxonomic key of Zucchi (2000). Voucher specimens were placed in the entomological collection of the Fundación Miguel Lillo (FML), Museo de Ciencias Naturales, Tucumán-Argentina.

Equatorial diameter and fruit weight

Fruit diameter was measured by assuming walnuts were spherical in shape, using a Mitutoyo micrometric caliper. Fruit weight was determined using an analytical balance (Ohaus PA214, USA). Twenty randomly selected fruits were measured on each sampling date.

Soluble sugar content

Soluble sugars were extracted from 500 mg fresh weight (FW) of frozen walnut mesocarp by homogenization in 4 ml of 80% ethanol with a mortar and pestle. The homogenate was heated in a water bath at 75 °C for 10 min and the insoluble fraction removed by centrifugation at 5000 × *g* for 10 min. After a second extraction with 4 ml of 80% ethanol, supernatants from the first and second extraction were pooled and dried under a stream of hot air. The dry residue was resuspended in 1 ml of distilled water and desalted by filtration through an ion-exchange column (Amberlite MB3, BDH, UK). Soluble sugars were determined colorimetrically. Total soluble sugars were determined according to Dubois et al. (1956), sucrose following the protocol of Cardini et al. (1955), and fructose by the method of Roe and Papadopoulos (1954). Glucose was determined using a glucose oxidase–peroxidase coupled assay according to Jorgensen and Andersen (1973).

Soluble phenols and tannins

Total soluble phenols and tannins were extracted from 100 mg FW of frozen walnut mesocarp with 90% methanol at 5 °C for 12 h. After centrifugation at 5000 × *g* for 5 min, the supernatant was collected and used for soluble phenol and tannin measurements. Total soluble phenols were determined using the Folin–Ciocalteau reagent as described by Swain and Hills (1959). Briefly, phenolic extract was mixed with the Folin–Ciocalteau reagent and kept for 2 min at room temperature before addition of 7.5% (w/v) sodium carbonate. After 5 min at room temperature, the absorbance was measured at 760 nm in a UV–Vis spectrophotometer (Hitachi U-2800A, Japan). Soluble phenol content was expressed as µmol phenol equivalent g^{-1} FW. Tannins were determined according to Hagerman and Butler (1978). Briefly, a sample of supernatant (2 ml) was evaporated until dryness and resuspended in distilled water. An aliquot of aqueous extract (0.2 ml) was added with 0.1% BSA in 0.2 M acetate buffer, pH 5.0 containing 0.17 M NaCl in a final volume of 1.5 ml, mixed thoroughly, left to stand for 15 min at room temperature, and then centrifuged at 10,000 × *g* for 10 min. The resulting precipitate was resuspended in 1.6 ml of SDS/TEA reagent (1 g sodium dodecyl sulfate and 5 ml triethanolamine in a final volume of 100 ml). Immediately, after that, 0.5 ml of acidic $FeCl_3$ reagent (10 mM anhydrous $FeCl_3$ in 0.1 M HCl) was added and mixed and left to stand for 30 min at room temperature. Absorbance was read at 510 nm. Tannin content was expressed as mg tannic acid equivalent g^{-1} FW.

UV-B absorbing compounds

UV-B absorbing compounds (flavonoid-like compounds) were extracted from 10 mg FW mesocarp tissue in darkness with 2 ml of acidified methanol (methanol/water/HCl, 79:20:1) according to Mirecki and Teramura (1984). Absorbance was measured at 305 nm using a UV–Vis spectrophotometer (Hitachi U-2800A, Japan).

Protein content

Soluble protein was extracted from 1 g FW of frozen walnut mesocarp tissue using 2.5 ml of 100 mM Tris–Cl buffer, pH 7.6 containing 1 mM β-mercaptoethanol, 10 mM $MgCl_2$ and 5 mM EDTA. Protein was determined according to Bradford (1976) using bovine serum albumin (BSA) as standard.

Statistical analyses

Results are means of two sampling seasons represented as means ± SD (standard deviation). Because in some cases, it was not possible to obtain enough fruit for the entire sampling period (some trees during dry years yielded few fruit), chemical composition of infested and uninfested fruit was compared with Student's *t* tests. Data were analyzed using Statistica v 7.1 (StatSoft, Inc). Differences among treatments were analyzed by means of a nested MANOVA followed by Univariate ANOVAs. *P*-values of less than 0.05 were considered as statistically significant. The interactions between chemical content (for every tested compound) and sampling season were not significant (data not shown). Correlation of infestation and emergence of *C. capitata* and *A. fraterculus* and content of soluble sugars, tannins, soluble phenolics and UV-B absorbing compounds were established using correlation matrices (Table 1).

Table 1 Correlations of infestation or emergence of *A. fraterculus* and *C. capitata* with soluble sugars, tannins, or soluble phenolics

Metabolites	Species			
	Emergence of *A. fraterculus*	Infestation of *A. fraterculus*	Emergence of *C. capitata*	Infestation of *C. capitata*
Total sugars	$r=0.85$	$r=0.82$	ns	$r=0.88$
	$P<0.05$	$P<0.05$		$P<0.05$
Sucrose	$r=0.89$	ns	ns	$r=0.92$
	$P<0.005$			$P<0.005$
Glucose	$r=-0.97$	$r=0.76$	$r=0.89$	$r=0.94$
	$P<0.05$	$P<0.05$	$P<0.05$	$P<0.005$
Tannins	ns	ns	$r=-0.96$	$r=-0.94$
			$P<0.05$	$P<0.005$
Soluble phenolics	$r=-0.94$	ns	$r=-0.98$	$r=-0.96$
	$P<0.05$		$P<0.001$	$P<0.005$

Results

Fruit weight and diameter

Patterns of fresh weight and diameter evolution of *J. australis* fruit throughout the walnut ripening period are shown in Fig. 1a, b, respectively. Both parameters exhibited a similar linear increase over time.

Level of infestation and adult tephritid emergence during fruit ripening

To assess seasonal susceptibility of *J. australis* fruit to *A. fraterculus* and *C. capitata* during walnut fruit development, an infestation index consisting of pupae recovered per kg of fresh fruit and adult emergence was used (Fig. 2). Infestation indexes for the two fly species increased during fruit ripening, but the increase was significantly higher for *A. fraterculus* than for *C. capitata*. Maximum values were 150 pupae kg^{-1} and 42 pupae kg^{-1} for *A. fraterculus* and *C. capitata*, respectively, and they were reached at the 50-days census time point (Fig. 2a). Infestation indexes slowly increased until 21-days after initiation of the census, and then exhibited a pronounced increase until the end of the census period. The emergence index (adult flies per kg of fresh fruit) also showed a higher increase for *A. fraterculus* than for *C. capitata*. Maximum values for emergence indexes were 97 adult flies kg^{-1} and 38 adult flies kg^{-1} for *A. fraterculus* and *C. capitata*, respectively (Fig. 2b). Emergence index patterns showed similar trends as infestation indexes. Simultaneous infestation by both fly species was observed for 3.97% of the sampled fruit during the early season and tended to increase as the season progressed, reaching in one case a maximum of 29.6% of doubly infested fruit per tree. There was a positive correlation between fruit size and infestation by *A. fraterculus* ($N=9345$ $r=0.67$ $P<0.0001$), but not for *C. capitata*.

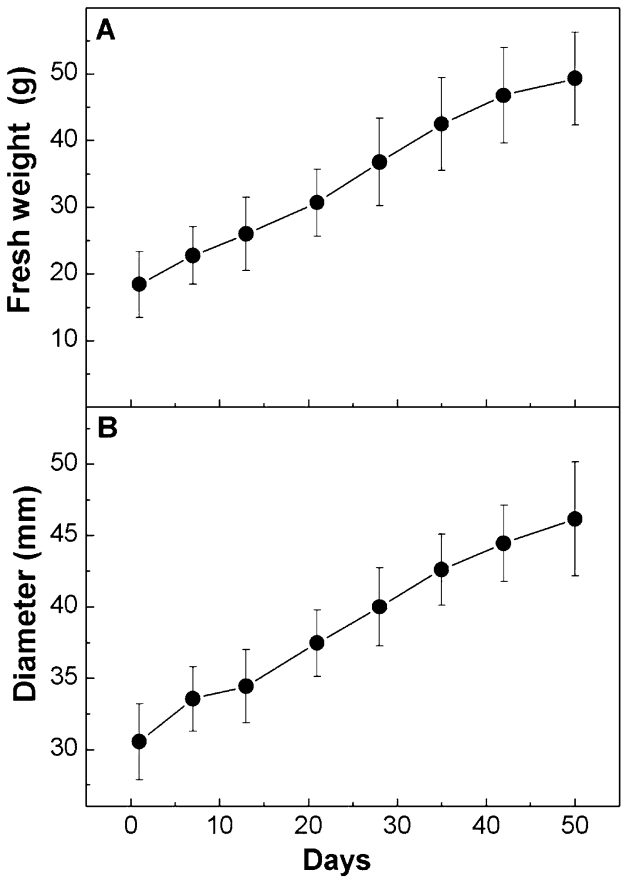

Fig. 1 Temporal patterns of fresh weight (**a**) and diameter (**b**) of developing walnut fruit. Each value is the mean ± SD of samples recorded during the 2003/2004 and 2004/2005 periods ($n=20$)

By contrast, adult emergence expressed on the basis of infestation level was significantly higher for *C. capitata*

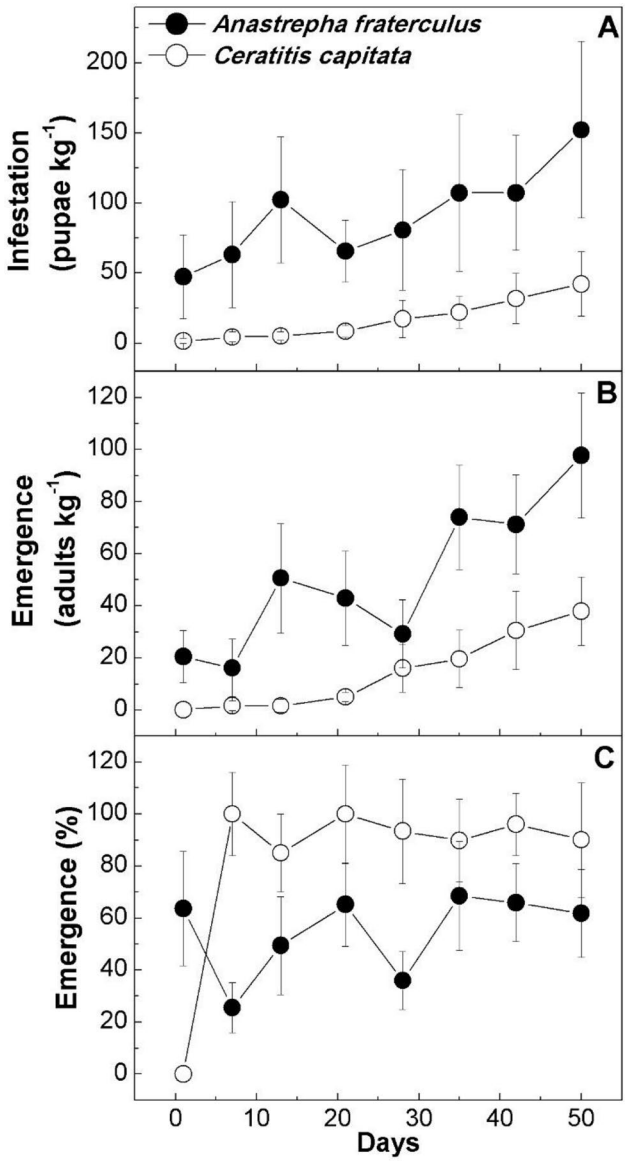

Fig. 2 Temporal evolution of infestation (**a**), emergence in adult kg^{-1} (**b**) and emergence in percentage (**c**) of *A. fraterculus* and *C. capitata* in walnut during ripening fruit ($n = 20$)

than for *A. fraterculus* (Fig. 2c). Maximum percentages of emergence were nearly 100% for the former and 60% for the latter. Interestingly, percentage values remained practically unchanged across the census period.

Soluble sugars

All soluble sugars started to increase at about 5 days after the start of census period, with increases in total sugars, sucrose and glucose being most pronounced (Fig. 3a–c). Fructose content showed a less pronounced initial increase until 23 days after the start of the census period, and then progressively decreased. There were no significant differences in

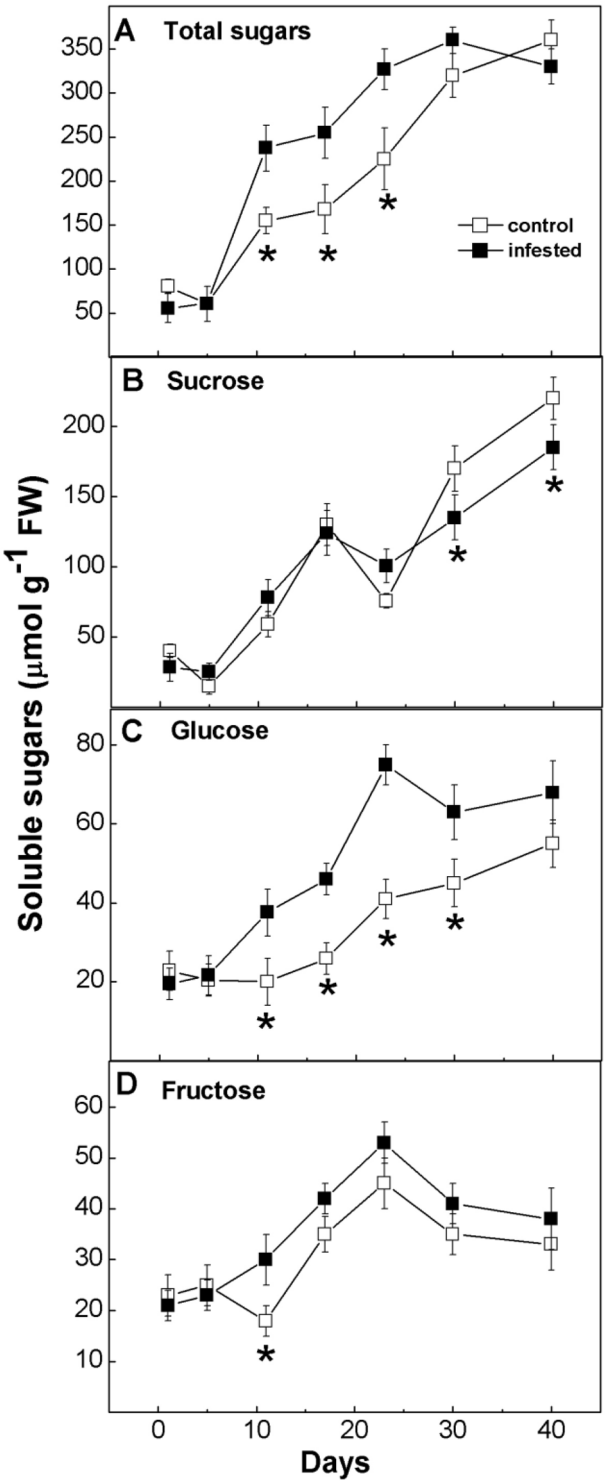

Fig. 3 Temporal evolution of soluble sugar content in mesocarp developing walnut fruit; infested (closed symbols) or uninfested (open symbols) with fruit fly larvae. **a** Total soluble sugars, **b** sucrose, **c** glucose, and **d** fructose. Values are means ± SD of two different experiments ($n = 10$). *Significant differences between infested and uninfested ($P < 0.05$) determined by Student's *t* tests

fructose content between infested and uninfested fruits; except at the 11th day ($P < 0.0001$, $t = 49.41$, df = 8) (Fig. 3d). In general, soluble sugars were higher in infested fruits. There were significant differences in glucose content at the 11th ($P < 0.0001$, $t = -43.60$, df = 8), 15th ($P < 0.0001$, $t = -45.37$, df = 8), 23rd ($P < 0.0001$, $t = -189.5$, df = 8), and 30th ($P < 0.0001$, $t = -40.62$, df = 8) days of sampling. In case of total soluble sugars, there were significant differences between infested and uninfested fruit at the 11th ($P < 0.0001$, $t = -33.74$, df = 8), 15th ($P < 0.0001$, $t = -45.83$, df = 8) and 23rd ($P < 0.0001$, $t = -14.27$, df = 8) days. The glucose and total soluble sugar contents were similar at the beginning and end of the period, in both uninfested and infested fruits. There were no significant differences in sucrose content until the 23rd day of the census period; however, at the 30th and 40th days sucrose content was significantly higher in uninfested than infested fruits ($P < 0.0001$, $t = 14.07$, df = 8 and $P < 0.0001$, $t = 18.08$, df = 8, respectively) (Fig. 3b). Interestingly, sucrose content showed a pronounced decrease beginning at 23 days that was coincident with significant increases in both glucose and fructose contents. Towards the end of the ripening period (40-days) the increases for total sugars, sucrose, glucose, and fructose were nearly 7-, 6-, 3-, and 2.5-fold, respectively. Notably, the highest fructose increase was observed at 23 days (Fig. 3d).

Tannins and soluble phenolics

As opposed to soluble sugars both tannins and soluble phenolics decreased across the ripening period, but they were over all significantly higher in uninfested than in infested fruits (Fig. 4a, c). It is noteworthy that in the early stages of the census and at the ending, both tannins and soluble phenolics contents were not different between infested and uninfested fruit. Differences between infested and uninfested fruit in total soluble phenolics contents were significant in samples taken at days 15 ($P < 0.005$, $t = 5.85$, df = 8), 23 ($P < 0.0001$, $t = 12.86$, df = 8) and 30 ($P < 0.0001$, $t = 19.34$, df = 8). Tannins contents were significantly lower in infested fruit than in uninfested fruit in samples taken on days 11 ($P < 0.0001$, $t = 32.40$, df = 8), 15 ($P < 0.0001$, $t = 38.25$, df = 8), 23 ($P < 0.0001$, $t = 22.01$, df = 8), and 30 ($P < 0.0001$, $t = 4.12$, df = 8).

By contrast, there were no significant differences in UV-B absorbing compounds between infested and uninfested fruits (Fig. 4b). In addition, initial and final concentrations of UV-B absorbing compounds did not differ significantly.

Proteins

Protein content did not vary significantly across the census period in control or infested fruits. Mean values ranged between 500 and 650 μg g^{-1} FW (data not shown).

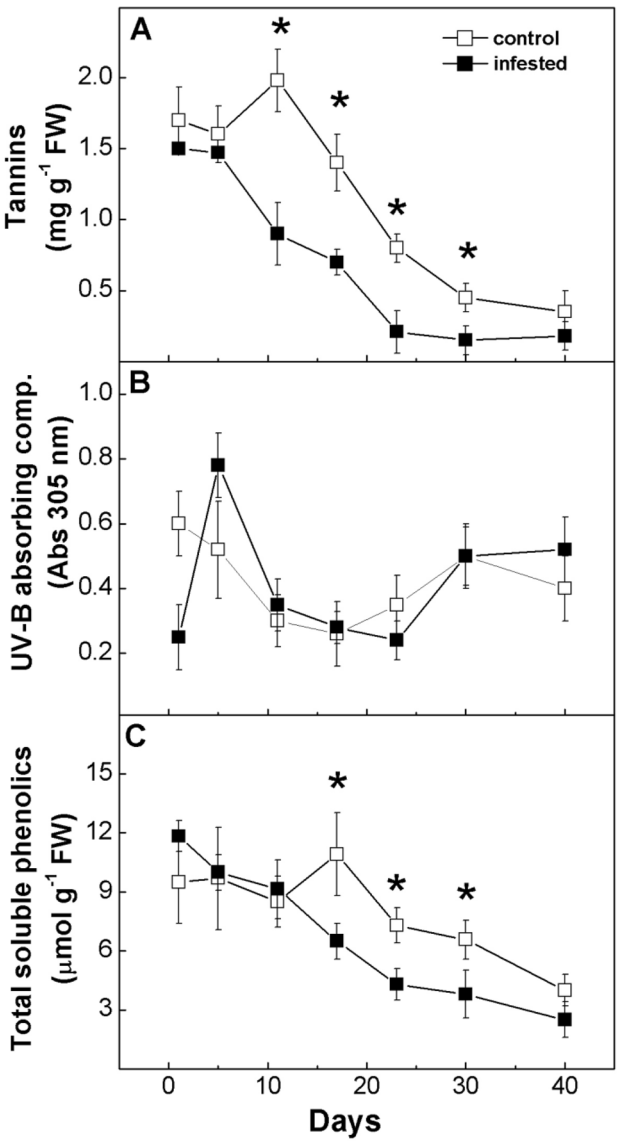

Fig. 4 Temporal pattern of phenolic content in mesocarp developing walnut fruit; infested (closed symbols) or uninfested (open symbols) with fruit fly larvae. **a** Tannins, **b** UV-B absorbing compound and **c** total soluble phenolics. Values are means ± SD of two different experiments ($n = 10$). *Significant differences between infested and uninfested ($P < 0.05$) determined by Student's *t* tests

Statistical analyses

A MANOVA revealed significant differences in content between infested and uninfested walnut fruit $F = 5.794$, df = 8, $P < 0.005$; Wilk's lamda = 2.45. But univariate analyses showed that some metabolites were different when comparing the two fruit types. Only glucose and tannin content differed significantly between infested and uninfested fruit $F = 5.1151$, df = 1, $P < 0.05$; Wilk's lamda = 4.64 and $F = 4.4443$, df = 1, $P < 0.05$; Wilk's lamda = 6.52, respectively.

Discussion

Size, weight, nutrient, and secondary compound content during fruit development for *Juglans australis* in our study followed similar patterns to those reported for other species of *Juglans*, with a linear increase in size, a peak in sugar content, and a sharp drop of phenolic compounds at the end of the ripening period (Wu et al. 2003; Cosmulescu et al. 2010; Li et al. 2012). Fruit fly larval development was positively correlated with sugar content and negatively correlated with tannin and phenolic compounds which peaked at the end of the ripening period. Interestingly, fruit fly infestation early in the ripening process produced apparent metabolic changes in fruit that altered temporal variation in sugar and phenolic compounds, resulting in an early peak in glucose and a sharp drop in tannins that likely favored fruit fly larval development. *Anastrepha fraterculus* was better able than *C. capitata* to infest fruit during the early season, yet adults of *C. capitata* emerged in consistently high numbers despite the fact that females infested fruit much later than *A. fraterculus*.

Developmental studies of walnut fruit are mostly based on work on *Juglans regia* L. (Wu et al. 2004, 2009; Li et al. 2012). Such studies have revealed that during the general developmental pattern of fruit (fresh weight and diameter) follows a sigmoidal pattern that can be divided into four stages, established from florescence. The third stage (60–100 days) is the period of more metabolic activity during which there are important changes in content of both soluble sugars and phenolic compounds. This maturation stage is characterized by an increase in soluble sugar content (total soluble sugars, sucrose, glucose, and fructose) and a drop in phenolic compounds (total phenols, tannins, and flavonoids), total soluble phenolics and tannins in particular. Towards the end of the maturation period fruit tissues enter senescence (Wu et al. 2004, 2009; Li et al. 2012). Mesocarp chemical composition varies across fruit development in association with seed development requirements. The mesocarp, which represents a nutrient reserve, becomes a suitable host for egg-laying and larval development for several herbivore species, including fruit flies.

In this study, using *J. australis*, a wild walnut species, we observed (over a 5-year period) that the entire period of walnut ripening lasted 120–140 days, depending on environmental conditions (LO, unpublished data). At the beginning of collections, fruit had approximately 70 days of development; corresponding to fruit in active growth and high metabolic activity (Wu et al. 2004; Li et al. 2012). Consistent with patterns in *J. regia*, an inverse relationship between sucrose and hexose content was observed at the 23 days census period, with significant increases in both glucose and fructose contents and a drop in sucrose, which would be associated with an increase in invertasic activity of the cell wall (Wu et al. 2004). Interestingly, at this stage (21-days) infestation of walnuts by *C. capitata* was first observed, whereas *A. fraterculus* larvae were present in fruit earlier.

The central finding of this work was a strong negative relationship between temporal variation in glucose and tannin content in infested fruit (−0.97). We found higher glucose values than those detected in uninfested fruit, while tannins (anti-nutritional, toxic compounds) were lower (Figs. 3, 4). The same pattern was observed for total sugars and total phenols. Egg-laying and larval feeding appeared to affect fruit development, while fruit chemical content influenced survival and development of larvae and adults. This relationship has ecological consequences, both for reproduction and plant seed dispersal and for herbivore population growth. The mechanism underlying the correlation of fruit fly infestation and accelerated fruit ripening awaits further investigation and merits examination in the future.

Many induced responses in plants are systemic (van Dam and Oomen 2008). In the case of leaf-herbivores, it has been found that jasmonic acid, a plant produced signal induced by larval feeding, can trigger rapid changes in carbohydrate transport and partitioning to storage organs (Babst et al. 2005). In the case of frugivory, response to attack may result in transport from the pulp to the seed, possibly triggered by an ethylene burst. Larval feeding or associated bacteria (see Ben-Yosef et al. 2015) could have induced a redistribution of metabolites (less flow of carbon from primary to secondary metabolism), by altering activity of associated enzymes, resulting in a more suitable environment for larval feeding and growth. Only in infested fruit, did we observe a sharp drop in the content of phenolic compounds (tannins and phenols) concurrent with a temporary increase in levels of glucose and other sugars which may be indicative of an increase in glucose content in infested walnut mesocarp. Nevertheless, Machado et al. (2017) recently discovered that herbivore-induced hormonal cross-talk can suppress growth and carbohydrate accumulation independently of the production of defensive metabolites. Notwithstanding the above, this alteration in the dynamics of fruit metabolism would contribute to the energy requirement of fruit fly larvae resulting in more benign and suitable conditions for their development. Similar relationships between insect pests and their hosts have been observed among gall forming insects (Waring and Priice 1988), sap feeders (Awmack and Leather 2002), and the specialized tephritid *Bactrocera olea*e (Rossi), where fruit infestation induces a strong ethylene burst (a phytohormone linked to fruit ripening) (Alagna et al. 2016). Machado et al. (2015) found that jasmonates can reduce constitutive and herbivore-induced concentration of glucose and fructose in leaves, leading to an unexpected increase in *Manduca sexta* growth. Changes induced by insects in plants can therefore be beneficial, both for the

offspring, to the insect itself that causes the change, and even to their congeners.

Walnuts have a high concentration of chemical defenses (tannins) but these fruit are also highly nutritious. In the case of *A. fraterculus*, which also infests peaches and guava, walnuts contain up to 8 times more protein than the latter (Oroño et al. 2013). The high protein content in the walnut mesocarp and the lack of variation in protein content in the presence of fruit fly infestation mean that this nutrient is more abundant in this particular fruit, perhaps facilitating the simultaneous development of several fruit fly larvae. This suggests that if chemical defenses can be overcome, intraspecific competition could be reduced in comparison to that which occurs in other less nutritious fruit. This would also favor females that lay large clutches of eggs or engage in repeated egg-laying bouts. Additionally, deposition of several eggs, whether by a single female, several conspecific females, or several species of Tephritidae, appears to trigger chemical changes that favor fruit fly offspring development (Díaz-Fleischer and Aluja 2003).

Whereas *A. fraterculus* infested the mesocarp during the whole fruit maturation process, infestation by *C. capitata* only began when fruit attained an advanced maturity stage from 21 days on. The *C. capitata* females, which have a short ovipositor, could be using egg-laying wounds on the fruit skin left by *Anastrepha* females to overcome skin hardness of unripe fruit. Alternatively, delayed egg-laying activity could indicate that this species is more selective and perhaps requires that a certain threshold in secondary compound content has been reached for egg hatch and larval development to proceed. Tannin content and an adequate sugar/tannin–phenol ratio may be particularly important factors in limiting development. It is noteworthy, however, that adult emergence from puparia for this species was remarkably high (near 100%) suggesting that selectiveness in the timing of host utilization by females has important consequences in offspring fitness.

We observed that *A. fraterculus* can survive in fruits with a high concentration of tannins, especially early in the census period, and moreover, that this species may be tolerant to the possible production of reactive oxygen species induced in the plant by the insect. In contrast, *C. capitata* may be more sensitive to oxidation. This would allow *A. fraterculus* to infest walnuts with high levels of tannins and phenols early in the fruiting season with adequate adult fly emergence that can reach between 40 and 60% (i.e., relatively low fitness costs). As noted above, this species uses a different egg-laying strategy, and is not as dependent on fruit quality as *C. capitata*. Other walnut-infesting species of insects have also been shown the effects of secondary compounds, as is the case for the codling moth *Cydia pomonella*, which can survive in walnut in the presence of the secondary metabolite juglone, since the intestine of the larvae can metabolize it (Piskorski and Dorn 2011). Some species in the genus *Anastrepha* (e.g., *Anastrepha ludens* Loew) lay larger egg clutches in unripe than in ripe mangos. Although immature mortality in unripe, chemically defended fruit is greater than in ripe fruit, the surviving adults exhibit similar fitness parameters as those stemming from ripe fruit (Díaz-Fleischer and Aluja 2003). In the case of *A. fraterculus* exploiting walnuts early in the season, it would be interesting to examine if females (single egg-layers) lay more often, and therefore place more eggs, in unripe than in ripe walnuts and if they adjust the number of eggs laid per fruit according to chemical and physical properties.

As shown in this study, individual fruit of *J. australis* can simultaneously sustain the development of several *A. fraterculus* and/or *C. capitata* larvae. Such is the case for several other native and introduced species of walnuts exploited by fruit flies in the genus *Rhagoletis* in North America and Europe (Prokopy and Papaj 2000; Guillén et al. 2011; Rull et al. 2013). Multiple species infestations in single fruit have also been reported in the case of *Anastrepha* (Sivinski et al. 2004; Birke and Aluja 2011).

In sum, we possibly indirectly detected an association between fruit infestation (larval feeding and/or activity of associated bacteria), with the induction of metabolic changes in the fruit. These changes involved changes in the temporal pattern of variation in certain plant compounds during ripening, which favored larval survival and feeding.

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