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ORIGINAL ARTICLE

Metabolome and ionome analyses reveal the stoichiometric effects of contrasting geological phosphorus soils on seed-parasitic insects in subtropical oak forests

Huawei Ji[1,2] · Baoming Du[1,2] · Jiahao Wen[1,2] · Ningxiao Sun[1,2] · Mingjun Peng[3] · Hongmei Du[4] · Chunjiang Liu[1,2,5]

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Abstract

Phosphorus (P)-rich sites develop on phosphate-rock ores, while the soils are generally characterized with P deficiency in subtropical areas, resulting in contrasting nutrient environments for plants and herbivores. It remains unclear how in situ herbivorous insects cope with such two extreme nutrient habitats in terms of metabolome and ionome. Here, we investigated the metabolome and ionome of the weevil larvae (*Curculio davidi* Fairmaire), which were parasitizing in *Quercus variabilis* acorns at P-rich and P-deficient sites. Our results showed that there were significant differences in 34 identified metabolites (belonging to sugars, amino acids, lipids, vitamins, nucleosides, etc.) and four chemical elements (P, S, Mg, and Zn) in the two weevil larva populations of the two P-type sites. Moreover, the concentrations of P, Mg, Zn and the identified sugars were significantly higher; however, S, amino acids, and several other *N*-containing metabolites were lower in the weevil larvae at the P-rich site, in contrast to those at the P-deficient site. Arginine and proline metabolism and glutathione metabolism were the most relevant pathways differentially regulated between the two weevil larva populations at the two contrasting sites. In addition, some metabolites in the weevil larvae were indirectly associated with the P, Mg, Zn, and S concentrations of soils through bottom-up effects. Our results suggested that in situ herbivorous consumers altered their metabolites to a certain extent to adapt to nutrient-varying environments; and there were strong interactions between the nutrients of herbivorous insects and soil elements across variable nutrient sites.

Keywords Metabolomics · Ionomics · Phosphate-rock ores · Subtropics · Herbivore

Introduction

Under most natural conditions, the nutrition supply does not align with the nutritional requirements of organisms, and there are differential constraints on autotrophs and heterotrophs (Elser et al. 2000). When consumers are subject to elemental stoichiometric constraints in the primary producer across trophic levels, complex metabolic and biochemical adjustments are observed to occur (Jeyasingh et al. 2011). Wagner et al. (2014) employed metabolomics to determine the nutritional state of the freshwater invertebrate (*Daphnia magna*) and found significant changes in the metabolite composition of the animals in response to deficits in N or P. Based on Joern et al. (2012), in addition to N and P, multiple other plant nutrients were associated with insect herbivore populations. However, most studies have focused on the effects of availability of only one or two nutrient elements (e.g., N and P) only on plants, or consumers in control experiments. Fewer studies have endeavored to investigate both

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✉ Chunjiang Liu
chjliu@sjtu.edu.cn

1 School of Agriculture and Biology, Shanghai Jiao Tong University, Dongchuan Rd. 800, Shanghai 200240, China

2 Shanghai Urban Forest Research Station, State Forestry Administration, Dongchuan Rd. 800, Shanghai 200240, China

3 Yunnan Academy of Forestry, Lanan Rd. 2, Kunming 600000, Yunnan, China

4 Shanghai Jiao Tong University, Dongchuan Rd. 800, Shanghai 200240, China

5 Key Laboratory of Urban Agriculture (South), Ministry of Agriculture, Dongchuan Rd. 800, Shanghai 200240, China

the metabolic and ionic responses of herbivores to naturally occurring nutrient variations in soils (Sardans et al. 2011; Fester et al. 2014).

Ionomics, which involves the study of the ionome that represents the inorganic component of an organism or tissue, including mineral nutrients and trace elements, has recently emerged as a research focus for plants (Sanchez et al. 2011; Wu et al. 2013; Guo et al. 2016; Ribeiro et al. 2016). Moreover, both macro- and micro-elements acting as compounds and different elements have different physiological functions in animals, and availability of all these elements may be linked to variations of the metabolites (Amtmann and Armengaud 2009; Hänsch and Mendel 2009; Soetan et al. 2010). Thus, studies of consumers by ionomics, combined with metabolomics, may provide a significant elucidation of the nutritional state of consumers under multi-nutrient variations in natural soils.

As a main life element, P is involved in a variety of biochemical processes and is essential for the synthesis of many key compounds, such as nucleic acids, RNA, and membrane lipids (Amtmann and Armengaud 2009). Hence, P is of great importance for the growth and reproduction of animals (Sterner and Elser 2002). In ecosystems that possess P-rich soils, plants accumulate P in their cells, which can be further transferred to herbivores toward influencing their stoichiometric traits and population dynamics (Schade et al. 2003; Zhou et al. 2015; Ji et al. 2017). Weider et al. (1997) found that over the last 40 years, the P enrichment of lakes due to human activity has significantly altered the genetic makeup of *Daphnia galeata*. Moreover, genetic and phenotypic shifts in *Daphnia* populations were highly correlated with the availability of P (Jeyasingh and Weider 2007; Jeyasingh et al. 2009; Frisch et al. 2014). Since the metabolome can be considered as the organism's chemical phenotype (Rivas-Ubach et al. 2012) and metabolites are the end products of gene expression, natural soils with variable *P* concentrations may have an impact on the metabolomes and ionomes of consumers through the food chain.

Subtropical ecosystems are generally characterized as having P-deficient soils, resulting in the P-limited stoichiometry of plants (Reich and Oleksyn 2004; Han et al. 2005; Reich 2005; Sun et al. 2015). However, in some areas of subtropical China, P-deficient soils are often mixed with P-rich soils on P-rich ores, which results in significant variations in P and other elements (e.g., N, K, and Mg) between P-rich and P-deficient sites (Yan et al. 2011; Zhou et al. 2015; Ji et al. 2017). Moreover, it was revealed that there were significant differences in the multi-element compositions of leaves, acorns, and the parasitic insects within the acorns of *Q. variabilis* due to variable soil nutrients at the P-rich sites on phosphate rocks, and the P-deficient sites on non-phosphate rocks in the Central Yunnan Plateau, in subtropical China (Zhou et al. 2015; Ji et al. 2017, 2019). Thus, soils with such geologically derived P variations may also have a significant impact on the metabolomes of consumers.

For this study, ionomic (N, P, S, K, Ca, Mg, Fe, Mn, Zn, Cu, and Na) and metabolomic investigations were conducted on parasitic weevil (*Curculio davidi* Fairmaire) larvae, which were distributed between P-rich and P-deficient sites. At the P-rich site, the soil was also substantially enriched with other mineral elements to form nutrient-rich soils in these subtropical areas, which are typically P, K, and Mg deficient (Zhou et al. 2015; Ji et al. 2017, 2019). For herbivores, the products of metabolic adjustments could be triggered by nutrient stress in primary producers (Jeyasingh et al. 2011). We hypothesized that the weevil larvae formed unique ion and metabolite compositions due to their adaption to different nutrient compositions of acorns as food resources at the P-rich and P-deficient sites.

Materials and methods

Study sites

The study areas are located in the Central Yunnan Plateau, Yunnan Province, in Southwestern China. The P-rich site is situated on rocks that are rich in phosphate in Wenquan Village, Anning County, which is in close proximity to Kunming City (102°26′47.58″E, 24°58′54.38″N, 1869 m a.s.l). The P-deficient site developed on non-phosphate rocks is situated in Fulong Village, Mouding County, Chuxiong City (101°32′35.62″E, 25°14′48.25″N, 1846 m a.s.l) (Zhou et al. 2015; Ji et al. 2017). The stands at both P-rich (Anning) and P-deficient (Mouding) sites are natural forests that have, historically, been left untouched (Wu et al. 1987).

Table S1 illustrates the compositions and relative chemical element content (%) of the phosphate rocks in Anning County (based on Tao (2005)), and non-phosphate rocks (the Matoushan Formation rocks) in Mouding County (based on Shi et al. (2011)). Within these areas, the primary soil groups are ferralsols and acrisols, according to WRB (FAO 1998). The physiochemical characteristics of the soils at the P-rich and P-deficient sites are shown in Table S2.

The study area has a plateau monsoon climate at low latitudes, and in 2015, the climate conditions were similar between the two sites, with a mean annual temperature of 15.5 °C and mean annual precipitation of 897.5 mm. The mean monthly temperatures and precipitation of Anning and Mouding Counties from 1981 to 2010 are shown in Fig. S1. The growing season is approximately 250 days.

In the study areas, the vegetation is characterized as diverse, consisting of plants from tropical, subtropical, and temperate areas (Wu et al. 1987). Historically, this area was heavily forested; however, over the last 300 years, the natural forests were destroyed due to cutting, fires, grazing, and

agricultural activities. The only natural forests remaining were either left as fragmented patches or located in remote mountainous areas. The vegetation at the two sampling sites is dominated by deciduous broadleaf trees. A stand with *Q. variabilis* as the dominant tree species was selected for experimentation at each site. The acorns (seeds of *Q. variabilis*), are generally infested by parasite *C. davidi* larvae.

Sampling

Within the mature *Q. variabilis* stands at each site, 15 trees were randomly selected (the distance from each tree was at least 20 m) (Ji et al. 2019). The height and diameter at breast height (DBH) were measured for all selected trees at each site. The selected *Q. variabilis* trees at the P-rich site (Anning), ranged from 8.5 m to 23 m in height and from 22.3 to 40 cm in DBH. At the P-deficient site (Mouding), the selected *Q. variabilis* trees ranged from 11 to 16 m in height, and from 18.9 to 38.5 cm in DBH.

Soil samples were collected in August, 2015. Surface soil cores (0–10 cm, 3 cm Ø) were systematically extracted from five spots arranged around each selected tree at 1.5 m from the trunk. The collected samples were subsequently mixed to form a single composite soil sample for each tree. The soil samples were air dried and sieved through a 60-mesh sieve (0.25 mm diameter) for chemical analysis.

Acorns that fell naturally from each tree were collected from the ground beneath the canopy of each tree (1 m from the trunk) during the peak acorn drop period, in October of 2015, and ~300 acorns were collected and transported to the laboratory. For each tree, a minimum of 50 acorns that were healthy, well developed and not infested by weevils were combined to make a composite acorn sample for chemical analysis. The composite acorn samples for chemical analysis were then scarified with a knife to remove the pericarp to obtain seeds, which were then dried at 65 °C, triturated with a blade mill to obtain a fine powder, and sieved through a 60-mesh sieve for chemical analysis.

The remaining acorn samples from each tree were then put into a basin and stored at room temperature for larva collection. When the larvae emerged from these acorns, they were immediately collected and frozen in liquid N_2 and then preserved in a freezer at − 80 °C for chemical and metabolite profiling analyses.

Thus, a total of 30 soil samples, 30 acorn samples, and 30 weevil larva samples were obtained for this study.

Chemical analysis

Soil, acorn, and weevil larva samples were then digested with trace metal-grade nitric acid and diluted with distilled water. Total N concentration was analyzed with an elemental analysis-stable isotope ratio mass spectrometer (EAI) (Vario ELIII; Elementar, Germany), while total P, S, K, Ca, Mg, Fe, Mn, Zn, Cu, and Na were determined with a plasma optical emission spectrometer (ICP–OES) (Iris Advantage 1000; Thermo Jarrell Ash, Franklin, MA). For EAI analysis, the larvae were dried at 50 °C to a constant weight, where after ≥ 7 weevil larvae were ground with a homogenizer at 5000 r min^{-1} to produce a composite weevil larva sample for each tree. For ICP–OES analysis, ~20 dried larvae were directly combined (without grinding) to produce a composite weevil larva for each tree. The element concentrations of all samples were milligrams per gram (mg g^{-1}) of dry weight. All analyses were conducted at the Instrumental Analysis Centre of Shanghai Jiao Tong University.

GC–MS analysis of metabolome

Prior to analysis, frozen weevil larvae were lyophilized and ground to a fine powder using a ball mill. The metabolites from the weevil sample powder were extracted according to Shi et al. (2015) with some modification. Samples of dry weevil larva powder (50 mg) were placed into 2-mL Eppendorf (EP) tubes to which 0.4 mL of extraction liquid (methanol-chloroform, 3/1, v/v) was added. In addition, 20 µL of L-2-chlorophenylalanine (1 mg mL^{-1} stock in dH_2O) was added as an internal standard and vortexed for 30 s. All samples were agitated via a ball mill for 4 min at 40 Hz, treated with ultrasound for 5 min (incubated in ice water), and centrifuged for 15 min at 12,000 rpm at 4 °C. A supernatant solution (0.34 mL) from each sample tube was placed in a clean 2-mL glass vial for GC/MS analysis. Moreover, 10 µL from each extract was removed and pooled into a clean 2-mL glass vial as a quality control (QC) sample.

The weevil larva extract samples were dried in a vacuum concentrator without heating, to which 80 µL methoxyamine hydrochloride (20 mg mL^{-1} in pyridine) was added and incubated for 20 min at 80 °C. Subsequently, 100 µL of *N,O-bis* (trimethylsilyl)-trifluoroacetamide (BSTFA) reagent with 1% trimethylchlorosilane (TMCS, v/v) was added to the sample aliquots, incubated for 1 h at 70 °C, and cooled to room temperature. Moreover, 6 µL FAMEs (standard mixture of fatty acid methyl esters, C8–C16: 1 mg mL^{-1}; C18–C24: 0.5 mg mL^{-1} in chloroform) was added to the QC sample to facilitate the further calculation of the retention time index (RI) of the metabolites.

GC/TOFMS analysis was performed using an Agilent 7890 gas chromatography system coupled with a Pegasus HT time-of-flight mass spectrometer. This system utilized a DB-5MS capillary column coated with 5% diphenyl cross-linked with 95% dimethylpolysiloxane (30 m × 250 µm inner diameter, 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA). A 1 µL aliquot of the analyte was injected in splitless mode. Helium was used as the carrier gas, the front inlet purge flow was 3 mL min^{-1}, and the gas flow rate

through the column was 1 mL min^{-1}. The initial temperature was maintained at 80 °C for 1 min, which was then raised to 290 °C at a rate of 10 °C min^{-1}, and maintained at 290 °C for 12 min. The injector, transfer line, and ion source temperatures were 280, 270, and 220 °C, respectively. The energy was − 70 eV in electron impact mode. Mass spectrometry data were acquired in full-scan mode with the *m/z* range of 50–600 at a rate of 20 spectra per second after a solvent delay of 8.1 min.

LC–MS analysis of metabolome

For UHPLC–QTOF–MS analysis, metabolites from the samples of dry larva powder were extracted according to Want et al. (2013) with some modification. 50 mg samples in 2 mL EP tubes were homogenized by employing a ball mill (40 Hz, 5 min.) after adding two steel balls, 200 µL H_2O and 800 µL MeOH:ACN (1:1, v/v). The samples were subsequently incubated for 1 h at − 20 °C, followed by 15 min centrifugation at 12,000 rpm at 4 °C. A supernatant solution (800 µL) from each resulting extract was obtained and evaporated to dryness in a vacuum concentrator. The dry extracts were subsequently re-dissolved in 100 µL of ACN:water (1:1, v/v), vortexed for 30 s, and then sonicated for 10 min in a water bath at 4 °C. The extracts were then centrifuged 15 min at 12,000 rpm at 4 °C, whereafter 10 µL of supernatant solution from each tube was pooled together as a QC sample. Extract-derived supernatant solutions of 70 µL were used for UHPLC–QTOF–MS analysis.

UHPLC–QTOF–MS analysis was performed using an Agilent 1290 UHPLC system (Agilent Technologies) with a UPLC BEH Amide column (1.7 µm, 2.1 × 100 mm, Waters) coupled to a TripleTOF 6600 (Q-TOF, AB Sciex). The mobile phase consisted of water (including 25 mM NH_4OAc and 25 mM NH_4OH) (pH = 9.75) (A) and acetonitrile (B). The elution profile was as follows: 0–1 min, 85% B (15% A) (isocratic); 1–12 min, 85–65% B (15–35% A) (linear gradient); 12–12.1 min, 65–40% B (35–60% A) (linear gradient); 12.1–15 min, 40% B (60% A) (isocratic); 15–15.1 min, 40-85% B (60–15% A) (linear gradient); 15.1–20 min, 85% B (15% A) (isocratic). The flow rate was 0.3 mL min^{-1} and the sample injection volume was 2 µL. The analysis duration was 20 min.

A Triple TOF mass spectrometer was employed for its ability to acquire MS/MS spectra on an information-dependent basis (IDA) during the LC/MS experiments. In this mode, the acquisition software (Analyst TF 1.7, AB Sciex) continuously evaluated the full-scan survey MS data as it collected and triggered the acquisition of MS/MS spectra, depending on preselected criteria. For each cycle, six precursor ions whose intensity was greater than 100 were selected for fragmentation at the collision energy (CE) of 35 V (15 MS/MS events with the product ion accumulation time of 50 ms each). The ESI source conditions were set as follows: ion source gas 1, 60 psi; ion source gas 2, 60 psi; curtain gas, 30 psi; source temperature, 550 °C; and ion spray voltage floating (ISVF), 5500 V or − 4500 V in positive or negative modes, respectively. To assess the column condition and data quality, the QC sample was analyzed six times during the all sequence of UHPLC–MS analysis.

Data processing for GC–MS analysis

For GC–MS analysis, the Chroma TOF4.3X software from LECO Corporation and the LECO-Fiehn Rtx5 database were applied for raw data extraction, baseline filtering and calibration, peak alignment, deconvolution of mass spectra, peak area integration, and metabolite identification (Kind et al. 2009). The LECO/Fiehn Metabolomics Library was utilized to identify the compounds and it provided a similarity value for the compound identification accuracy. If the similarity value was > 600, the metabolite identification could be considered as reliable. The RI method also was used in the peak identification, and the RI tolerance was 5000. A total of 573 peaks were detected in the weevil larva samples. The missing values of the raw data were filled up by half of the minimum value. The internal standard normalization method was then employed in this data analysis. The resulting three-dimensional data, which included the sample name, peak number, and normalized peak area, were used for statistical analyses.

Data processing for LC–MS analysis

Raw LC/MS data were processed using XCMS software for peak detection, chromatogram alignment, and peak identification (Smith et al. 2006). The identifications were completed by matching the acquired MS/MS data, which were assembled in an in-house developed database. A total of 30,191 and 29,326 mass peaks in the weevil samples were detected in positive and negative modes, respectively, where 2624 and 3145 metabolites could be left, respectively, through an RSD < 30% denoising method, whereafter the missing values of raw data were filled up by half of the minimum value. Further, an overall normalization method was employed in this data analysis. Subsequently, the resulting three-dimensional data in positive or negative modes, which involved the peak number, sample name, and normalized peak area, were utilized for statistical analyses.

Statistical analyses

The ionomics and metabolomics data obtained through GC–MS or LC–MS analyses in positive mode or negative mode were log transformed to improve normality and then used separately for multivariate analysis using the

SIMCA13.0 software package (Umetrics, Umea, Sweden). For the initial steps, principal component analysis (PCA) was applied to reveal the homogeneity of the data, any groupings, trends, and outliers. Moreover, in the case of using unsupervised PCA, the obtained results could be influenced by many factors, such as biological variability, pathological variations, instrumental drift, artifacts, and other experimental conditions, which might divert the focus of a PCA model to systematic variation unrelated to the scientific question of interest (Wiklund et al. 2008). Therefore, to specify the metabolic and ionomic differences between weevil larva samples from P-rich and P-deficient sites, data were analyzed using a supervised classification method—orthogonal projections to latent structures discriminant analysis (OPLS-DA) that separates predictive variations (between groups) from non-predictive variations (within groups).

Sevenfold cross validation was employed to estimate the robustness and the predictive ability of the supervised OPLS-DA, whereas for the significance testing of the OPLS-DA, ANOVA of the cross-validated residuals (CV-ANOVA), or a permutation test was applied. In addition, an R^2 value of >0.7 and a Q^2 value of >0.4 denoted a highly significant model (Lundstedt et al. 1998). Based on OPLS-DA, a loading plot was constructed, and the first principal component of variable importance projection (VIP) was obtained. To select metabolites or chemical elements that had the strongest and most significant effects on the discrimination of weevil larva populations at the P-rich and P-deficient sites, VIP values that exceeded 1.2 and Student's t test ($p<0.05$) between two comparison groups were applied.

Weight analysis of weevil larvae between P-rich site and P-deficient site was performed with the application of MetaboAnalyst 3.0(MetPA) (http://metaboanalyst.ca/) based on all identified and discriminating metabolites obtained from the OPLS-DA analysis. The pathway impact value (pathway impact, PI) calculated from pathway topology analysis and enrichment analysis was used to evaluate the weight of the metabolic pathway in the whole metabolic regulatory network, and the pathway impact value threshold was set at 0.1 (Xia and Wishart 2010; Xia et al. 2015).

Results

Among soil elements (N, P, S, K, Ca, Mg, Fe, Mn, Zn, Cu, and Na) measured in the study, all elements except for Ca and Na had higher concentrations at P-rich sites than at P-deficient sites (Table 1). Accordingly, the concentrations of P, S, K, Ca, Mg, Mn, and Zn were higher in both acorns and weevil larvae at the P-rich sites compared with those at P-deficient sites (Table 1). However, acorn Cu was higher at P-deficient sites than at P-rich sites. Principal component analysis (PCA) with the ionomics

Table 1 Content of chemical elements in soils, acorns, and weevil larvae at P-rich and P-deficient sites (mg g^{-1})

Element	Soils						Acorns						Weevil larvae					
	P-rich site		P-deficient site		p value	df	P-rich site		P-deficient site		p value	df	P-rich site		P-deficient site		p value	df
	Mean	CV, %	Mean	CV, %			Mean	CV, %	Mean	CV, %			Mean	CV, %	Mean	CV, %		
N	1.31	22.5	0.99	26.5	**	28	6.77	18.5	6.10	17.1	n.s.	28	59.78	11.9	55.73	6.5	n.s.	28
P	6.53	134.4	0.25	22.4	*	14	1.76	18.8	1.11	12.5	***	19	4.12	6.0	3.83	4.6	**	28
S	0.37	47.6	0.16	20.7	***	15	1.14	18.1	0.67	16.6	***	22	1.46	28.2	1.88	11.8	**	22
K	11.41	29.0	7.16	56.7	**	28	9.43	14.8	7.54	9.9	***	28	7.83	18.0	6.77	14.5	*	28
Ca	7.76	205.0	0.54	62.7	n.s.	14	0.80	16.9	0.52	15.5	***	28	0.61	26.0	0.49	19.0	*	28
Mg	3.31	16.7	2.25	48.1	**	28	0.80	17.0	0.66	12.3	**	28	2.06	11.7	1.66	12.7	***	28
Fe	43.753	32.7	26.457	42.2	**	28	0.030	35.1	0.033	75.0	n.s.	28	0.162	219.5	0.032	21.6	n.s.	14
Mn	0.642	46.3	0.281	56.4	***	21	0.230	35.7	0.146	35.0	**	28	0.046	14.8	0.039	13.3	**	28
Zn	0.197	26.5	0.105	148.5	*	28	0.012	21.8	0.009	17.0	***	28	0.050	13.9	0.043	7.8	**	28
Cu	0.062	30.0	0.026	42.6	***	23	0.014	36.8	0.010	46.0	*	28	0.019	13.1	0.018	16.5	n.s.	28
Na	1.218	81.2	0.671	35.2	n.s.	16	0.386	100.7	0.587	49.8	n.s.	28	0.174	78.5	0.095	55.5	n.s.	28

Significance of differences in the element content between two sites: *$p<0.05$, **$p<0.01$ and ***$p<0.001$

or metabolomics data, respectively, revealed no noticeable separation between the two weevil larva populations among the P-rich site and the P-deficient site (Fig. 1a, b, c, d).

Based on the OPLS-DA analysis of ionomic data or metabolomic data obtained from LC–MS or GC–MS analysis (excluding the outlier data), respectively, the populations of weevil larvae at the P-rich site could be discriminated from those at the P-deficient site (Fig. 2a, b, c, d). CV-ANOVA data and the permutation test both revealed that the differences between the two weevil larva populations in these OPLS-DA models all were statistically significant and reliable (Fig. S2). As listed in Tables 2 and Tables S3 and S4, 34 metabolites and four chemical elements (P, S, Mg, and Zn) had the strongest power in discriminating the two weevil larva populations.

Among the 34 metabolites with the most strong discriminating power, the sugars (sn-glycerol 3-phosphate, stachyose, D-fructose, D-mannose, D-ribose and maltotriose), and L-proline, S-methyl-5′-thioadenosine, 2′-*O*-methyladenosine, adenosine, riboflavin (vitamin B2), L-ascorbic acid, L-pipecolic acid, L-pipecolic acid, and homoveratric acid in the weevil larvae were all higher in those at the P-rich site than at the P-deficient site; however, most amino acids and lipids and other *N*-containing molecules exhibited the opposite pattern (Table 2).

To find metabolic pathways related to differences between the two P-type sites, we used weight analysis to analyze the metabolic pathways of weevil larvae related to 34 identified and discriminating metabolites (Table 2). The *p* value was calculated from the enrichment analysis and the PI value was calculated from the pathway topology analysis. With the application of MetPA, we identified three pathways with PI ≥ 0.1; however, there were only two pathways with both PI ≥ 0.1 and $p < 0.05$: arginine and proline metabolism, and glutathione metabolism (Fig. 3 and Table S5), which were the most relevant pathways differentially regulated between the two weevil larva populations at the P-rich and P-deficient sites (Fig. 3).

Discussion

Characteristic metabolites of weevil larvae at P-rich and P-deficient sites

Our results showed, for the first time, the characteristics of metabolomics of in situ parasitic weevil larvae at

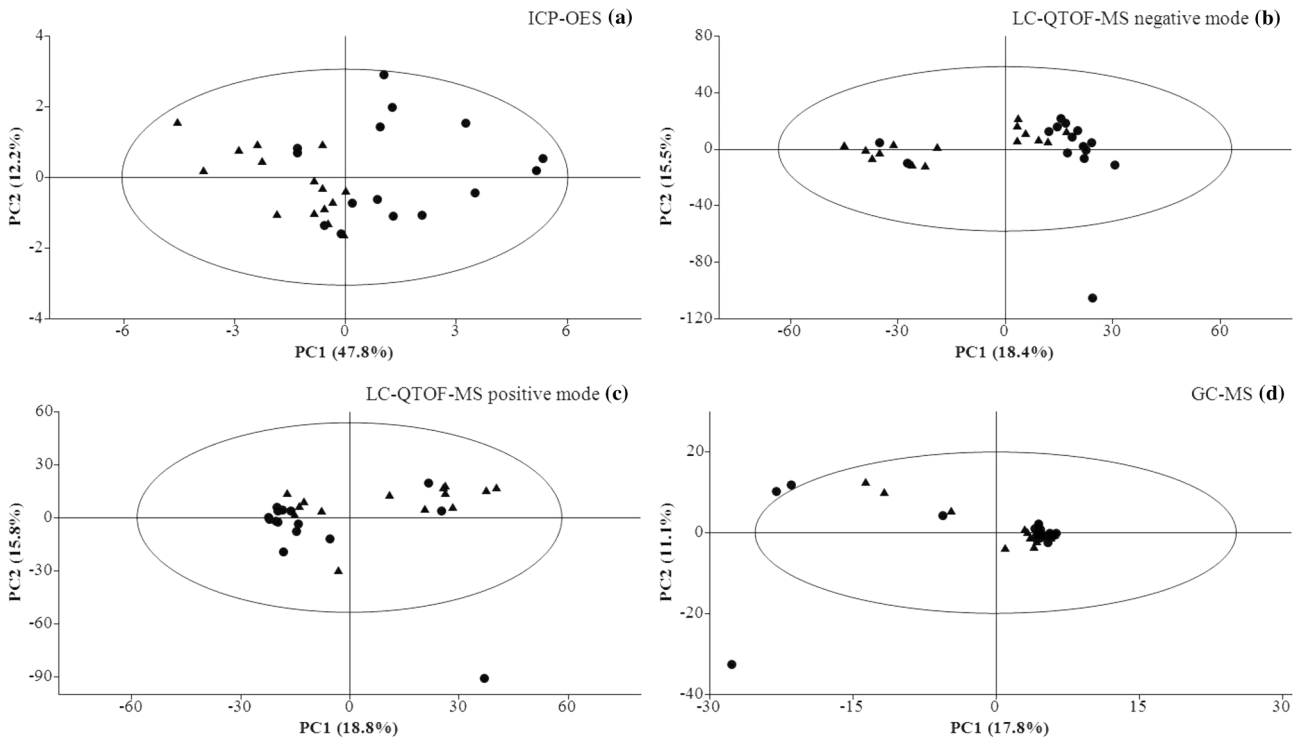

Fig. 1 Score plots of principal component analysis (PCA) of ionomic (**a**) and metabolomic data obtained from LC–QTOF–MS in negative mode (3145 variables) (**b**) and LC–QTOF–MS in positive mode (2624 variables) (**c**) and GC–MS (517 variables) (**d**) of the weevil larvae distributing at two experimental sites with P-rich (circles) and P-deficient (triangles) content in soils. PC1, the first principal component; PC2, the second principal component. The ellipse indicates Hotelling's T^2 (95%)

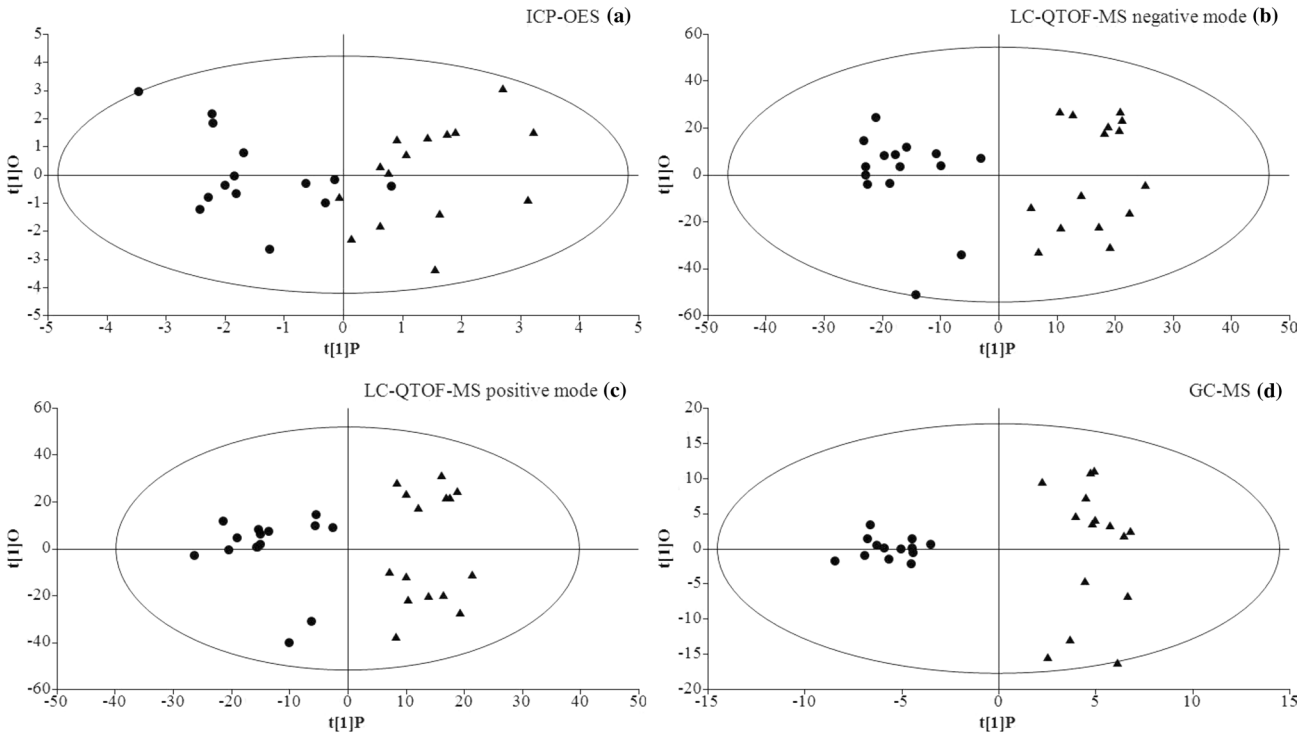

Fig. 2 Score plots of orthogonal projections to latent structures discriminant analysis (OPLS-DA) of ionomic data (**a**, $R^2Y[1]=0.68$, $Q^2[1]=0.41$, CV-ANOVA $p<0.01$) and metabolomic data obtained from LC–QTOF–MS in negative mode (**b**, $R^2Y[1]=0.88$, $Q^2[1]=0.52$, CV-ANOVA $p<0.001$) and LC–QTOF–MS in positive mode (**c**, $R^2Y[1]=0.86$, $Q^2[1]=0.36$, CV-ANOVA $p<0.05$) and GC–MS (**d**, $R^2Y[1]=0.94$, $Q^2[1]=0.42$, CV-ANOVA $p<0.05$) from the weevil larvae distributed at two experimental sites with P-rich (circles) and P-deficient (triangles) content in soils

geologically derived P-rich and P-deficient sites in the subtropics, which are typically characterized by P-limiting soils. As shown, 34 metabolites were identified, with the strongest power in discriminating weevil larva populations between the two P-type sites (Table 2). In particular, of the 34 metabolites, most were sugars, amino acids, lipids, vitamins, and nucleosides and analogs, etc., which characterized the metabolites under such nutrient-contrasted environments. These data greatly advanced our understanding of how in situ insects adapt to contrasting nutrient environments at the metabolic level.

Such characteristics of metabolites in weevil larvae may be the result of biochemical interactions between the requirements and the supply of nutrients in acorns that are associated with soils during their growth. Meanwhile, for the growth and development of organisms, several essential elements play key roles (Marschner 2012). Although the amount of demand for P in weevil larvae at the P-rich or P-deficient site was unknown, based on the growth rate hypothesis, the weevil larvae at the P-rich site were thought to have a higher growth rate than those at the P-deficient site, due to a fact that more dietary P, sugars and protein are required to sustain their higher growth rate (Wagner and Frost 2012; Ibanez et al. 2017). Moreover, our results indicated that the weevil larvae at the P-rich site contained a higher sugar content, but lower levels of most *N*-containing metabolites (including the amino acids) (Table 2), which seemed to be consistent with the growth rate hypothesis.

Boer et al. (2010) found that N limitation decreased the intracellular amino acids in yeast; however, limitations in P resulted in the converse. Wagner et al. (2017) found that algal with high C:P ratios increased the body content of free amino acids in *Daphnia*, and suggested that algal food quality may have potent impacts on the biochemistry of planktonic consumers. In this study, both the N:P and C:P molar ratios of acorns were significantly higher at the P-deficient site, in contrast to the P-rich site, which indicated poor food quality for weevil larvae at the P-deficient site (Table 1). Hence, consistent with the above research, higher amino acid content in the weevil larvae at the P-deficient site was most likely due to low-quality acorns. Moreover, in contrast to the P-rich site, the higher content of N-containing metabolites at the P-deficient site might be due to that low P food decreased the growth rate of the weevil larvae and created a surplus of non-P metabolites (such as AA) needed for biomass production (Wagner et al. 2014; Ibanez et al. 2017; Wagner et al. 2017).

Table 2 Chemical elements and metabolites responsible for discrimination of weevil larvae from two experimental sites with P-rich and P-deficient soils that were selected from the OPLS-DA models on the basis of VIP > 1 and *t* test ($p < 0.05$) values

ID	Name	Classification	Relative content		Fold change	*p* value	VIP
			P-rich site	P-deficient site	Log (P-rich site/ P-deficient site)		
	P	Chemical elements	4.102	3.834	0.029	0.002	1.203
	S	Chemical elements	1.452	1.885	−0.113	0.002	1.219
	Mg	Chemical elements	2.039	1.660	0.089	0.000	1.473
	Zn	Chemical elements	0.050	0.043	0.069	0.001	1.252
4391	sn-Glycerol 3-phosphate	Sugars	1.551	0.799	0.288	0.018	1.502
4407	sn-Glycerol 3-phosphate	Sugars	0.417	0.211	0.296	0.019	1.667
6446	Stachyose	Sugars	2.269	0.680	0.524	0.001	2.445
16,078	D-Fructose	Sugars	0.014	0.007	0.315	0.000	2.235
21,175	D-Mannose	Sugars	26.393	19.687	0.127	0.003	2.011
24,364	D-Ribose	Sugars	0.196	0.076	0.409	0.030	1.548
28,282	Maltotriose	Sugars	5.160	2.486	0.317	0.002	2.028
270	Aspartic acid	Amino acids	0.017	0.033	−0.296	0.008	1.237
1988	L-Pyroglutamic acid	Amino acids	0.162	0.212	−0.119	0.042	1.342
5152	L-Glutamine	Amino acids	3.182	5.023	−0.198	0.006	1.727
9553	5-L-Glutamyl-L-alanine	Amino acids	0.221	0.366	−0.220	0.037	1.260
24,539	L-Proline	Amino acids	0.658	0.514	0.107	0.045	1.503
393	Putrescine	Polyamine	2.048	5.001	−0.388	0.012	2.225
586	Spermidine	Polyamine	1.014	1.464	−0.159	0.007	2.299
514	Palmitic acid	Lipids	0.009	0.234	−1.395	0.005	1.450
4609	Capric acid	Lipids	0.695	1.851	−0.425	0.035	1.786
16,061	*cis*-9-Palmitoleic acid	Lipids	40.277	68.793	−0.232	0.001	1.927
22,958	S-Methyl-5′-thioadenosine	Nucleosides, nucleotides, and analogs	0.016	0.011	0.153	0.023	1.390
3069	2′-*O*-methyladenosine	Nucleosides, nucleotides,and analogs	0.040	0.029	0.147	0.015	1.925
3133	*N*2,*N*2-Dimethylguanosine	Nucleosides, nucleotides,and analogs	0.340	0.620	−0.261	0.009	1.784
5138	*N*2,*N*2-Dimethylguanosine	Nucleosides, nucleotides,and analogs	0.047	0.093	−0.292	0.017	1.771
5835	Inosine	Nucleosides, nucleotides,and analogs	5.437	8.093	−0.173	0.014	1.374
5932	Inosine	Nucleosides, nucleotides,and analogs	27.443	40.896	−0.173	0.018	1.246
6010	2-Methylguanosine	Nucleosides, nucleotides,and analogs	0.030	0.050	−0.224	0.015	1.771
13,739	Adenosine	Nucleosides, nucleotides,and analogs	0.023	0.014	0.194	0.014	1.668
5501	Hypoxanthine	Purines	23.789	34.446	−0.161	0.011	1.362
5413	Nicotinate	Vitamins and cofactors	0.019	0.066	−0.548	0.010	1.770
6434	Thiamine	Vitamins and Cofactors	2.279	4.898	−0.332	0.014	1.628
6619	Thiamine	Vitamins and Cofactors	0.023	0.043	−0.272	0.020	1.572
8113	Riboflavin (Vitamin B2)	Vitamins and Cofactors	0.020	0.010	0.305	0.005	1.778
26,219	L-Ascorbic acid	Vitamins and Cofactors	0.018	0.011	0.233	0.005	1.677
25,778	L-Pipecolic acid	Alkaloids	2.338	0.897	0.416	0.016	1.728
26,186	L-Pipecolic acid	Alkaloids	0.315	0.119	0.425	0.019	1.436
6279	Homoveratric acid	Others	0.014	0.005	0.443	0.046	1.823

Furthermore, at the P-deficient site, the higher content of amino acids might be converted into lipids; thus, leading to a higher content of lipids in the weevil larvae. Wagner et al. (2014) found that dietary N or P stress both resulted in slower growth and increased total lipid content in Daphnia, and Yang et al. (2006) also showed that inadequate dietary phosphorus resulted in poor growth and increased lipid content in the body of juvenile silver perch. This appeared to be consistent with the increased content of palmitic acid, capric acid, and *cis*-9-palmitoleic acid in the weevil larvae from the P-deficient site, which may be an indicator of energy storage. Moreover, aspartic acid, L-glutamine, and L-proline were glycogenic amino acids, and may be subsequently used for the generation of glucose (Morcuende et al.

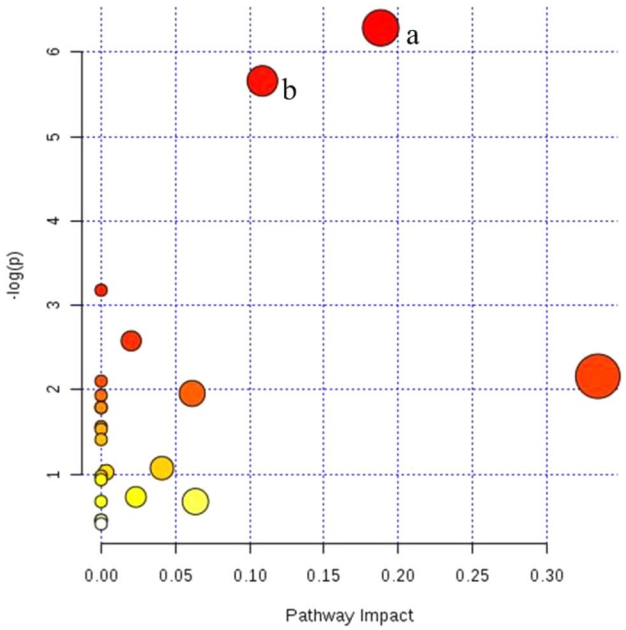

Fig. 3 Weight analysis of the metabolic pathways of weevil larvae between P-rich site and P-deficient site. Each dot represents a metabolic pathway; there is positive correlation between the size/color of the dots and metabolic pathways. **a** Arginine and proline metabolism; **b**, glutathione metabolism

2007; Wu 2009; Bender 2012). Aspartic acid and L-glutamine may also be used for the synthesis of nucleotides and thus likely to increase the content of several nucleotides in the weevil larvae from the P-deficient site (Wu 2009). Polyamines (including putrescine and spermidine) are essential for cell proliferation and differentiation and have been discovered to be important for the growth of insects (Heby 1986; Shim and Fairlamb 1988; Mitsuhashi 1998).

For this study, except for L-proline, all of the metabolites (aspartic acid, L-pyroglutamic acid, L-glutamine, 5-L-glutamyl-L-alanine, putrescine, spermidine) involved in the arginine and proline metabolism and glutathione metabolism were higher in the weevil larvae at the P-deficient site than those at the P-rich site, which likely indicated that the two metabolic pathways (arginine and proline metabolism and glutathione metabolism) both were differentially regulated in the two weevil larva populations. Moreover, there were significant relationships between the P concentrations and C:P ratios of acorns and four metabolites (aspartic acid, L-pyroglutamic acid, L-glutamine, and 5-L-glutamyl-L-alanine) in weevil larvae at the P-deficient site; however, no such relationships were found for the P-rich site (Table S6). These results suggested that, at the P-deficient site, the nutritional quality of the acorns may have strong association with amino acid metabolism in the weevil larvae, particularly the arginine and proline metabolism and the glutathione metabolism. Therefore, the two amino acid metabolic pathways might be related to the growth of the weevil larvae and have great significance in adapting them to the P-deficient soil site and the low-quality food. These results suggested that geologically derived P variations strongly influenced the N metabolism of weevil larvae.

Characteristic elements of weevil larvae at contrasting sites

Our results clearly revealed that there were significant differences in the concentrations of most individual elements (P, S, K, Ca, Mg, Mn, and Zn) in weevil larvae at the P-rich and P-deficient sites; however, this did not significantly alter the basic ionomic features in organisms based on principal component analysis (PCA). This might suggest that in spite of the variations in the concentrations of individual elements in weevil larvae between the two contrasting sites, they maintained a stricter ionic homeostasis.

Interestingly, of the elements that were measured, the concentrations of P, K, Mg, Mn, and Zn were not only higher in weevil larvae, but also in the acorns and soils, respectively, at the P-rich site compared with those at the P-deficient site (Table 1). It has been shown that the nutritional state of herbivorous insects is closely related to the status of soil nutrients (such as P and N) across P-variable habitats (Schade et al. 2003; Zhang et al. 2014; Ji et al. 2017). Our results suggested that there are bottom-up effects of multiple elements, including not only P and N, but also other relevant elements, along the plant–insect parasitism food chains in subtropical ecosystems, where P, K, and Mg are typically the limiting elements in subtropical areas.

Such bottom-up transfer patterns of multiple elements along the parasitism food chains might be associated with the biological functions of these elements, or their status in soils. In previous studies, the primary focus was on the effects of N and P on the performance of insect herbivores (Perkins et al. 2004; Cease et al. 2012; Zhang et al. 2014; Cease et al. 2016) through nutrient addition experiments. Ibanez et al. (2017) showed that for grasshoppers, the existence of P was linked to dietary P, and P-rich grasshoppers consumed plants that were high in P. Moreover, Joern et al. (2012) suggested that not just N and P, but plant Mg, Na, and K had potential effects on herbivorous insect populations. Numerous enzymes require Mg to facilitate phosphate transfer, whereas zinc is a critical constituent of many enzymes for protein synthesis and energy production, and plays important roles in cell replication, gene expression, and in nucleic acid and amino acid metabolism. Further, sulfur is present in two amino acids, and thus in proteins (Soetan et al. 2010). In addition, the effects of correlations between elements also influence the transfer of multiple elements from low to high trophic levels. For instance, Yang et al. (2006) found that whole body P and Mg concentrations

in juvenile silver perch increased with higher dietary phosphorus levels. Therefore, in the present study, the consistent variations in P, S, Mg, and Zn, from soils, to acorns, and to weevil larvae might be a result of their functions in physiological metabolism.

Bottom-up effects of elements in soils on herbivorous metabolites

An elucidation of how metabolites in consumers are differentially associated with multiple elements is essential toward understanding the mechanisms underlying the transfer of nutrition in ecosystems (Jeyasingh et al. 2011; Wagner et al. 2014). Here, our results revealed the possible relationships of variable elements in soils and metabolites in parasitic weevil larvae, with bottom-up effects along food chains. Firstly, across contrasting P sites, Mg, P, S, and Zn had strong correlations with several metabolites (e.g., nucleosides and analogs, vitamins and cofactors, sugars, lipids, etc.) in weevil larvae (Table S7). Secondly, it was shown that Mg, P, and Zn were significantly higher in weevil larvae, while each of these three elements was higher in acorns and soils, at the P-rich site, in contrast to the P-deficient site (Table 1). Logistically, this might suggest that the differences in Mg, P, S, and Zn in soil leads to variations in the counterpart elements of acorns and weevil larvae, and subsequently relevant metabolites in weevil larvae at contrasting P sites. These results seem to confirm the hypothesis that there are soil nutrient-associated bottom-up effects on the metabolites of herbivorous insects along host–parasite food chains.

In fact, it has been shown in controlled experiments that various elements have different physiological functions, and the availability of essential elements in the environment might influence biochemical reactions and metabolism products in organisms (Amtmann and Armengaud 2009; Hänsch and Mendel 2009; Soetan et al. 2010). Furthermore, metabolites participated in cellular reactions, and metabolomics provided phenotypical responses at the metabolic level under particular environmental conditions (Villas-Bôas et al. 2005; Mapelli et al. 2008; Sardans et al. 2011). In this sense, the differences in some metabolites in the weevil larvae between the two P-type sites might be an adaptation to local soils at the metabolic level.

Interestingly, as an essential element, *N* did not demonstrate significant impacts on the metabolites of the weevil larvae, although a substantial difference occurred in the concentration of NO_3^--N in soils across the two P-type sites (Table S2). In fact, there was no difference in the concentrations of N in both the acorns and weevil larvae between the two P-type sites in this study. This means that to a certain extent, soil resident N appeared not to be a limiting element for the development and growth of the weevil larvae at these sites. There is abundant evidence that N is not a limiting element for the growth of plants in subtropical areas (Han et al. 2005).

Conclusion

Our results clearly showed that there were significant differences in 34 metabolites and four chemical elements (P, S, Mg, and Zn) between two weevil populations distributed among a P-rich and a P-deficient site. Moreover, the concentrations of P, Mg, Zn, and the identified sugars were significantly higher, but S, amino acids, and several additional N-containing metabolites were lower in weevil larvae at the P-rich site, in contrast to the P-deficient site. Metabolic pathway analysis revealed that the arginine and proline metabolism and the glutathione metabolism were most likely to be differentially regulated in the two weevil larva populations. The metabolites of herbivorous insects were indirectly associated with the nutrient elements in soils due to bottom-up effects along the host–parasite food chain. These results suggested that in situ herbivorous consumers altered their metabolites to a certain extent to adapt to nutrient-varying environments; and there were strong interactions between the nutrients of herbivorous insects and soil elements across variable nutrient sites.

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Declaration of authorship C. Liu conceived and designed the experiments and wrote the manuscript. J. Wen, B. Du, N. Sun, and M. Peng performed the experiments. H. Ji and H. Du performed the experiments, analyzed the data, and wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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