Authors for correspondence:
Antoine Guiguet
e-mail: antoine.guiguet@protonmail.com
Heather M. Hines
e-mail: hmh19@psu.edu

THE ROYAL SOCIETY
PUBLISHING

Extreme acidity in a cynipid gall: a potential new defensive strategy against natural enemies

Antoine Guiguet[1], Nathaniel B. McCartney[2,3], Kadeem J. Gilbert[5,6], John F. Tooker[7], Andrew R. Deans[2], Jared G. Ali[2,3] and Heather M. Hines[2,4]

[1]Department of Biology, The Penn State University, University Park, PA 16802, USA
[2]Department of Entomology, [3]Center for Chemical Ecology, and [4]Department of Biology, The Pennsylvania State University, University Park, PA 16801, USA
[5]W.K. Kellogg Biological Station, Michigan State University, Hickory Corners, MI 49060, USA
[6]Department of Plant Biology, Program in Ecology and Evolutionary Biology, Michigan State University, East Lansing, MI 48824, USA
[7]Department of Entomology, The Penn State University, University Park, PA 16802, USA

AG, 0000-0002-3101-825X; KJG, 0000-0003-0105-8020; JFT, 0000-0002-9303-6699; ARD, 0000-0002-2119-4663; JGA, 0000-0001-9870-0299; HMH, 0000-0003-0299-5569

The morphology of insect-induced galls contributes to defences of the gall-inducing insect species against its natural enemies. In terms of gall chemistry, the only defensive compounds thus far identified in galls are tannins that accumulate in many galls, preventing damage by herbivores. Intrigued by the fruit-like appearance of the translucent oak gall (TOG; *Amphibolips nubilipennis*, Cynipidae, Hymenoptera) induced on red oak (*Quercus rubra*), we hypothesized that its chemical composition may deviate from other galls. We found that the pH of the gall is between 2 and 3, making it among the lowest pH levels found in plant tissues. We examined the organic acid content of TOG and compared it to fruits and other galls using high-performance liquid chromatography and gas chromatography–mass spectrometry. Malic acid, an acid with particularly high abundance in apples, represents 66% of the organic acid detected in TOGs. The concentration of malic acid was two times higher than in other galls and in apples. Gall histology showed that the acid-containing cells were enlarged and vacuolized just like fruits mesocarp cells. Accumulation of organic acid in gall tissues is convergent with fruit morphology and may constitute a new defensive strategy against predators and parasitoids.

1. Introduction

Many holometabolous insect species have developed defensive strategies to protect vulnerable larval and pupal stages. Plant galls are one such strategy: some herbivorous insects induce development of an abnormal organ on their host plant that houses and feeds the insects during their larval development. In addition to its nutritional function, these galls exhibit numerous morphologies that defend immature stages against enemies [1].

Gall wasps (Hymenoptera: Cynipidae), the second largest group of gall-inducing insects (approx. 1400 spp.) [2], have a large diversity of predictable gall architectures that have demonstrated defensive properties [1]. Parasitoid wasps, the main enemies of gall wasp larvae, are efficiently deterred by dense coatings of trichomes [3] and large gall size [4], and their oviposition is made more difficult by the presence of internal air spaces, thick walls or multiple larval chambers [5]. Other morphological traits, like the presence of spines on gall surfaces, secretion of sticky substances on the gall epidermis or a mobile
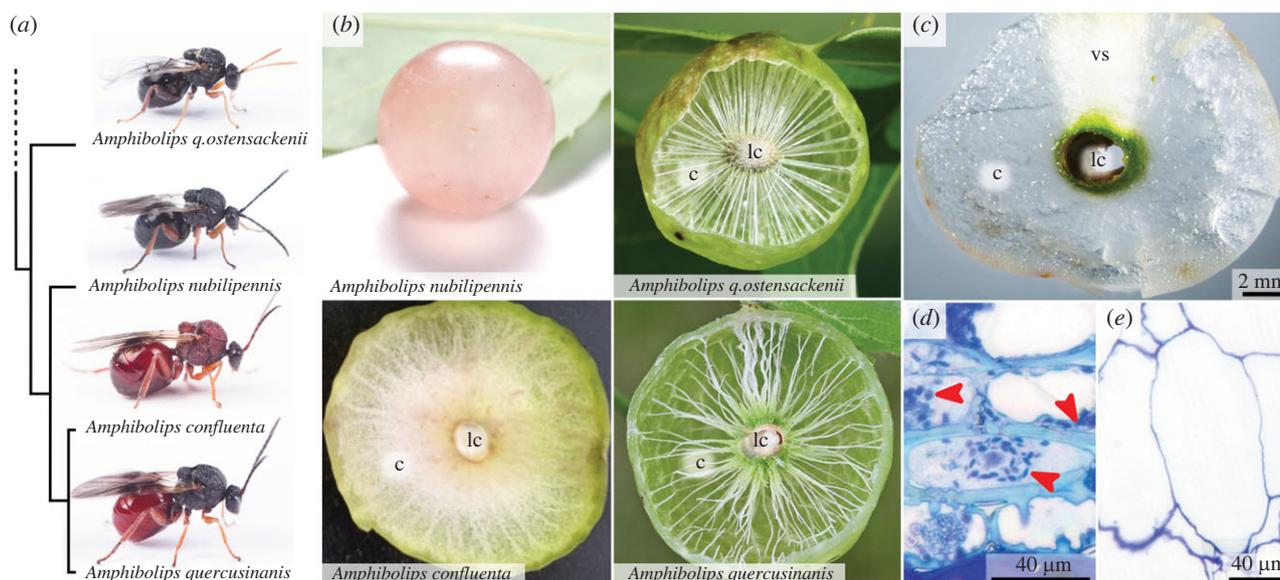
Figure 1. Morphology of the TOG (*Amphibolips nubilipennis*, sexual generation) induced on *Quercus rubra* compared to other *Amphibolips* galls. (*a*) Phylogeny of the genus *Amphibolips* adapted from [15], with photos of adult females of the sexual generation. (*b*) Morphology of the gall of *A. nubilipennis* compared to the galls of three other related *Amphibolips*. Photo of *A. confluenta* gall by Rebecca Gerken, photos of *A. quercusostensackenii* and *A. quercusinanis* galls by Joe Boggs. (*c*) Cross-section of the TOG. (*d,e*) Histology of the wall of the larval chamber and of the gall cortex, respectively. Legend: lc: larval chamber; c: cortex; vs: vascular system; red arrow: chloroplasts.

larval chamber may also have defensive functions. Some galls have indirect defences by attracting ants that deter parasitic wasps. Ants are attracted either through production of nectar secretions on which ants feed [6–8] or nutrient-rich elaiosome-like tissues that promote dispersal of galls into ant colonies [9].

Chemical properties of cynipid galls have also been suspected of contributing to larval defence against insects that may consume galls. Phenolic compounds, such as flavonoids and tannins, have toxic and anti-feedant effects on insect herbivores by altering digestive activity [10]. Likely for this purpose, most cynipid galls have high concentrations of tannins [11]. These compounds accumulate in exterior tissues of galls [12–14]. Gall tannins also decrease larval mortality due to fungal infestation [14], a major cause of death in cynipids.

In this study, we focus on the translucent oak gall (TOG), the gall of the sexual generation of cynipid wasp *Amphibolips nubilipennis*, that is induced on leaves of red oak, *Quercus rubra*. This spherical gall is translucent (figure 1*b*), which [15] suggests it lacks a high concentration of tannins, which are brown/black. As a result, its protective chemistry must differ from most oak galls. In addition, its appearance and succulent texture shares similarity to some stone fruits, rather than more typical lignified galls [1,16]. Thus, its physical defence may be reduced compared to other species. In this study, we examine the structure and chemistry of this gall to explore possible adaptive values of its morphology.

2. Material and methods

(a) Field observation and sampling
Galls of the sexual generation of *Amphibolips* reach their maturity in June in central Pennsylvannia. Galls of the sexual generation of *Amphibolips nubilipennis*, the TOG, and closely related species *Amphibolips confluenta*, the spongy oak apple gall (SOAG), were each collected from several different trees of *Quercus rubra* between 15 and 23 June 2021 in Pennsylvania, Ohio and Virginia and analysed within 3 days of processing.

(b) Histology
Histological sections of *A. nubilipennis* were obtained to determine the cellular structure of the fruit-like exterior. Cross-sections were fixed overnight with 2.5% paraformaldehyde and 0.4% glutaraldehyde in 0.1 M PBS at low pressure. After dehydration in a graded series of ethanol, samples were embedded in medium grade LR White resin (London Resin Company Ltd, UK). Semi-thin sections were made with an ultramicrotome (EM UC6, Leica) and stained with Toluidine Blue O. Tissues were visualized with a microscope Olympus BX43 and its attached camera (CellSens Entry 1.18, Olympus DP73).

(c) pH measurement
We compared the pH of galls of the sexual generations of *A. nubilipennis* ($n = 8$) and *A. confluenta* ($n = 7$) by measuring the pH of the surface of the leaf bearing the gall, the gall surface and the inside of the gall cortex (the fleshy exterior outside the larval chamber) using a flat-tipped pH probe (HI981037, Hanna Instruments Ltd, Befordshire) [17].

(d) Organic acid composition analysis
We compared the composition of the primary acids known for fruits and galls in *A. nubilipennis* ($n = 4$), *A. confluenta* ($n = 5$), apples (variety Granny Smith) ($n = 4$) and lemons ($n = 5$). Gall and apple tissues were ground by mortar and pestle cooled with liquid nitrogen. Lemons were hand-extracted. To prepare extracts, 100 mg of tissue or juice was added to 900 µl of distilled water containing 5 mg of the internal standard tricarballylic acid and sonicated at room temperature for 30 min. Extracts were then centrifuged at 14 000 rpm for 5 min and the supernatant was filtered using 0.2 µm syringe filters. The pH of samples was checked with pH test strips to ensure the pH of analysed samples is consistent with previous measures and the literature (electronic supplementary material, figure S1). Samples were stored at −80°C prior to analysis.
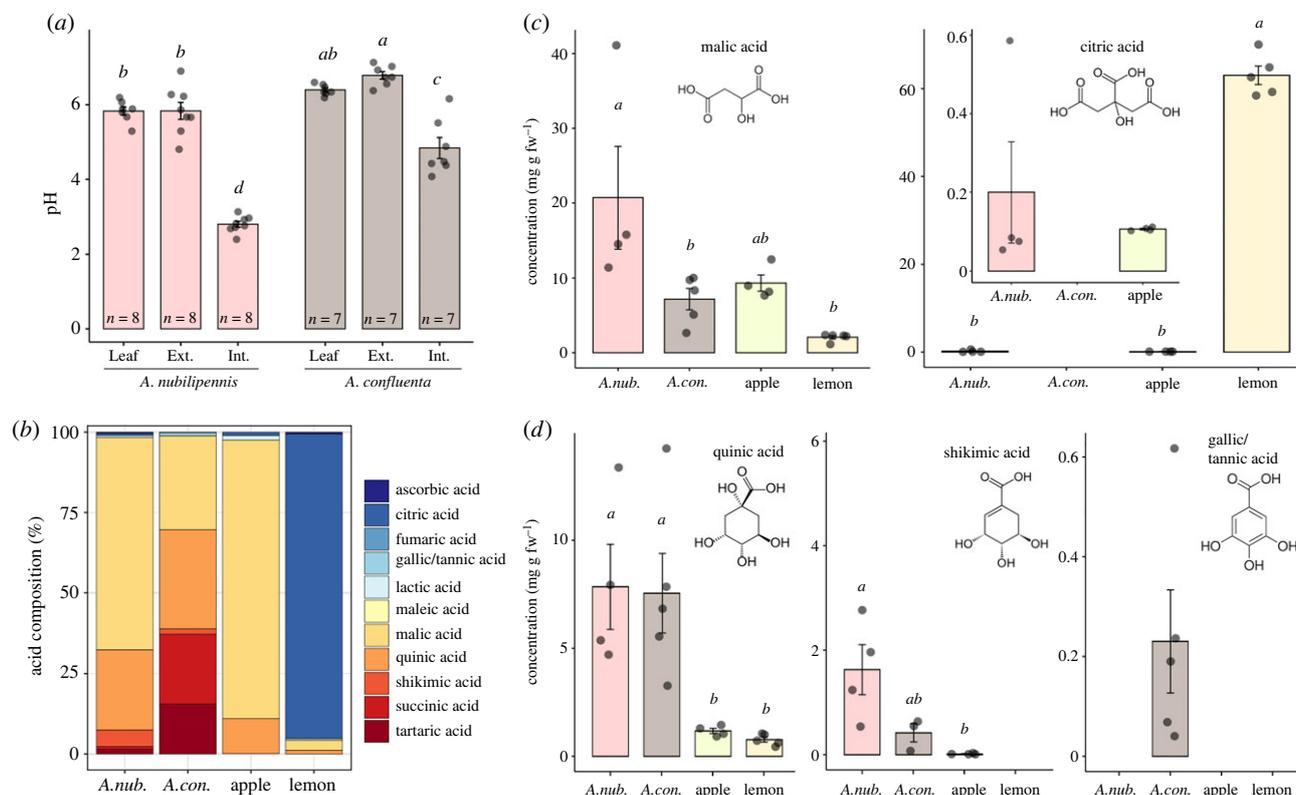
Figure 2. Chemical properties and composition of the TOG (*Amphibolips nubilipennis*, sexual generation) induced on *Quercus rubra* compared to other galls and to acidic fruits. (*a*) pH of gall tissues of *A. nubilipennis* and *A. confluenta*. Leaf: leaf surface; Ext.: gall surface; Int.: gall cortex. (*b*) Acid composition of the galls tissues of *A. nubilipennis* and *A. confluenta*, apple and lemon. (*c*) Concentration of malic and citric acids. (*d*) Concentration of acids involved in tannin biosynthesis (quinic, shikimic and gallic/tannic acids).

Acid chemical analysis was performed using a Shimadzu Prominence LC-20 series liquid chromatograph system fitted with a 250 mm × 4.6 mm Phenomenex Gemini 5u C18 column and a Shimadzu SPD-M20A photo diode array detector with detection at 250 nm (ascorbic acid) and 210 nm (all other analytes). Mobile phase consisted of 0.02 M KH_2PO_4 in water at pH 2.45 pumped at 0.8 ml min^{-1} for 20 min with column temperature maintained at 40°C and injection volumes of 10 µl. Authentic standards for tartaric acid, malic acid, ascorbic acid, citric acid, succinic acid, tannic acid, gallic acid, quinic acid, lactic acid, maleic acid, shikimic acid, fumaric acid and tricarballylic acid were used to prepare a 13-level dilution series covering the range of 3.1 ng µl^{-1} to 12.5 µg µl^{-1}. Instrument calibration was performed using duplicate injections of the standard dilutions and yielded linear peak area responses with R^2 values ≥ 0.987. Ascorbic acid and shikimic acid co-eluted under these conditions, but differences in absorbance spectra allowed for satisfactory quantification using 250 nm and 210 nm, respectively.

Components of the gall and fruit samples were preliminarily identified by comparing HPLC retention times and UV absorbance spectra to authentic standards. Identifications were then confirmed using gas chromatography–mass spectrometry analysis of derivatized sample aliquots. Briefly, 10 µl aliquots were dried before being derivatized by trimethylsilylation using 100 µl of 10% MSTFA in acetonitrile incubated at 60°C for 30 min. Samples were promptly analysed using 1 µl injections onto an Agilent Technologies 7890 gas chromatograph fitted with a 30 m × 0.25 mm × 0.25 µm HP-5MS UI column interfaced to an Agilent 5977 mass spectrometer operated in EI mode using standard tune parameters. Identification was performed by comparing retention times and mass spectra to those of trimethylsilyl-derivatized authentic standards as well as by matching to reference spectra from the NIST 2017 mass spectral library. All analytes could be detected by this method with the exception of ascorbic acid, which is unstable at elevated temperatures [17].

(e) Statistics

Statistical analyses were performed using R [18]. pH and acid concentrations were analysed using one-way ANOVA. The comparisons were performed with Tukey's honest significant differences test for *post hoc* multiple comparisons.

3. Results

Contrary to many other *Amphibolips* that have radiating fibres connecting the exterior to the internal chamber housing the larva, the cortex of the gall of *A. nubilipennis* has evolved a condition of having a cortex that is instead succulent and translucent (figure 1*a,b*). The wall of the interior larval chamber is leaflike in its thickness, texture and green colour (figure 1*c*). Histology reveals that the cortex is made of enlarged vacuolized cells whereas the larval chamber wall comprises four layers of sclerified cells with chloroplasts (figure 1*d*).

The pH of the fleshy cortex of the gall of *A. nubilipennis* was 2.8 ± 0.22 (mean ± s.d.), which was considerably more acidic than the cortex of the *A. confluenta* gall (4.61 ± 0.5) (figure 2*a*). Both of these values were lower than the pH of the external surface of the respective galls (5.83 ± 0.64 and 6.75 ± 0.26) and the leaves bearing the galls (respectively, 5.83 ± 0.29 and 6.38 ± 0.15) (ANOVA $F_{6,85} = 78.97$, $p = 2 \times 10^{-16}$).

Malic acid was the most abundant acid in the *A. nubilipennis* gall and apple (respectively, 66% and 86%), whereas citric acid represented 95% of the acid composition in the lemon (figure 2*b*). The *A. confluenta* gall had considerably different acid composition from *A. nubilipennis* as it exhibited proportionally more tannin-related acids, with the two most abundant

4

royalsocietypublishing.org/journal/rsbl   *Biol. Lett.* **19**: 20220513

acids being quinic acid (33%) and malic acid (31%). The concentration of malic acid was two times higher in TOG than in SOAG and the apple (figure 2*c*). Citric acid was detected in TOG and apple at similar but low concentrations, and this acid was not detected in SOAG. The concentration of quinic acid and shikimic acid, two precursors of tannins, was similar in both galls, whereas gallic acids and tannins are detected only in the apple gall (figure 2*d*).

4. Discussion

With a pH level below 3, the translucent gall of *A. nubilipennis* is among the most acidic plant tissues measured to date; mostly only *Citrus* fruit tissues are known to be capable of this extreme [19,20]. The low pH in TOG is likely to be caused in part by the accumulation of malic acid inside the gall. Malic and citric acids contribute to acidity in fruits [21] and malic acid is common in most fruits where it imparts a tart flavour [22]. The acidic juice present in the cortex of TOG is stored within large vacuolized cells that are similar to mesocarp cells [23]. This suggests that malic acid is accumulated in large vacuoles, as in mature fruit cells [21]. These traits represent a new case of convergence between galls and fruits [24].

The pH of the gall cortex is much lower than typical leaf tissues and more similar to the most acidic digestive fluid excreted by carnivorous plants, and is more acidic than the stomachs of insect-eating animals [25,26]. Therefore, the gall content could harm insects trying to feed on it and may represent a novel defence against gall enemies. This could be an alternative strategy to the tannin accumulation observed in most other oak galls. Like tannins, low pH decreases the efficiency of protein digestion in insects because caterpillar hindguts are highly alkaline [27]. In contrast with tannins, this strategy could also be efficient against parasitoid wasps, as it may degrade tissue of the thin ovipositors of these wasps. Nevertheless, at least one parasitoid wasp species has been reported from TOG [28]. Acidity of the gall may also limit the success of inquilines—gall wasps that feed on tissue induced by other gall wasps—as it may limit the ability for inquilines to oviposit and survive in the cortex. Notably, no inquilines have been reported from TOG [29]. While the acidic exterior could be generated from a plant defence response, the gall wasp itself is protected by the inner chamber.

An alternative and non-mutually exclusive hypothesis for acidity of the gall is that the translucence of the gall cortex, which appears to be a consequence of high concentrations of acids, has nutritional benefits for the gall-inducing insect. Indeed, light conductance by the cortex appears to allow for photosynthetic activity that is not typically found in the centre of galls (figure 1*c*). Gall photosynthesis can enhance the probability that galls will reach maturity by reducing dependence of the insect-induced gall on the host plant for photosynthates [30]. In addition, the proximity of the photosynthetic tissue to the central chamber suggests it may provide oxygen to the larva [31]. Thus, by allowing growth of a large translucent tissue, gall acidity may provide a form of defence for the gall-inducing larvae with concomitant nutritional benefits of photosynthetic tissues near the larval chamber.

Future investigation should assess the parasitism rate of the TOG and examine anti-caterpillar properties of these acids. This study highlights a potentially novel manipulation of host-plant chemistry by a gall-inducing species to lower pH to defend the gall while simultaneously allowing for gall tissues to have some photosynthetic activity. Additional research is necessary to determine if other gall-inducing species similarly influence the pH of their galls.

References

1. Stone GN, Schönrogge K. 2003 The adaptive significance of insect gall morphology. *Trends Ecol. Evol.* **18**, 512–522. (doi:10.1016/S0169-5347(03)00247-7)

2. Ronquist F, Nieves-Aldrey JL, Buffington ML, Liu Z, Liljeblad J, Nylander JAA. 2015 Phylogeny, evolution and classification of gall wasps: the plot thickens. *PLoS ONE* **10**, e0123301. (doi:10.1371/journal.pone.0123301)

3. Bailey R, Schönrogge K, Cook JM, Melika G, Csóka G, Thuróczy C, Stone GN. 2009 Host niches and defensive extended phenotypes structure parasitoid wasp communities. *PLoS Biol.* **7**, e1000179. (doi:10.1371/journal.pbio.1000179)

4. Price PW, Clancy KM. 1986 Interactions among three trophic levels: gall size and parasitoid attack. *Ecology* **67**, 1593–1600. (doi:10.2307/1939090)

5. Zwölfer H, Arnold-Rinehart J. 1994 The evolution of interactions and diversity in plant–insect systems: the *Urophora–Eurytoma* food web in galls on Palearctic Cardueae. In *Biodiversity and ecosystem function* (eds E-D Schulze, HA Mooney), pp. 211–233. Berlin, Germany: Springer.

6. Washburn JO. 1984 Mutualism between a cynipid gall wasp and ants. *Ecology* **65**, 654–656. (doi:10.2307/1941429)

7. Seibert TF. 1993 A nectar-secreting gall wasp and ant mutualism: selection and counter-selection shaping gall wasp phenology, fecundity and persistence. *Ecol. Entomol.* **18**, 247–253. (doi:10.1111/j.1365-2311.1993.tb01097.x)

8. Inouye BD, Agrawal AA. 2004 Ant mutualists alter the composition and attack rate of the parasitoid community for the gall wasp *Disholcaspis eldoradensis* (Cynipidae). *Ecol. Entomol.* **29**, 692–696. (doi:10.1111/j.0307-6946.2004.00652.x)

9. Warren RJ, Guiguet A, Mokadam C, Tooker JF, Deans AR. 2022 Oak galls exhibit ant dispersal convergent with myrmecochorous seeds. *Am. Nat.* **200**, 292–301. (doi:10.1086/720283)

10. Barbehenn RV, Peter Constabel C. 2011 Tannins in plant–herbivore interactions. *Phytochemistry* **72**, 1551–1565. (doi:10.1016/j.phytochem.2011.01.040)

11. Hartley SE. 1998 The chemical composition of plant galls: are levels of nutrients and secondary compounds controlled by the gall-former? *Oecologia* **113**, 492–501. (doi:10.1007/s004420050401)

12. Ikai N, Hijii N. 2007 Manipulation of tannins in oaks by galling cynipids. *J. For. Res.* **12**, 316–319. (doi:10.1007/s10310-007-0016-x)

13. Allison SD, Schultz JC. 2005 Biochemical responses of chestnut oak to a galling cynipid. *J. Chem. Ecol.* **31**, 151–166. (doi:10.1007/s10886-005-0981-5)

14. Taper ML, Zimmerman EM, Case TJ. 1986 Sources of mortality for a cynipid gall-wasp (*Dryocosmus dubiosus* (Hymenoptera: Cynipidae)): the importance of the tannin/fungus interaction. *Oecologia* **68**, 437–445. (doi:10.1007/BF01036752)

15. Ward et al. 2022 Speciation in Nearctic oak gall wasps is frequently correlated with changes in host plant, host organ, or both. *Evolution* **76**, 1849–1867. (doi:10.1111/evo.14562)

16. Dixon KA, Lerma RR, Craig TP, Hughes KA. 1998 Gall morphology and community composition in *Asphondylia flocossa* (Cecidomyiidae) galls on *Atriplex polycarpa* (Chenopodiaceae). *Environ. Entomol.* **27**, 592–599. (doi:10.1093/ee/27.3.592)

17. Guiguet A, McCartney N, Gilbert KJ, Tooker JF, Deans AR, Ali J, Hines H. 2023 Data from: Extreme acidity in a cynipid gall: a potential new defensive strategy against natural enemies. Dryad Digital Repository. (doi:10.5061/dryad.v41ns1s0x)

18. R Core Team. 2020 *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. See http://www.R-project.org/.

19. Müller ML, Irkens-Kiesecker U, Rubinstein B, Taiz L. 1996 On the mechanism of hyperacidification in lemon: comparison of the vacuolar H^+–ATPase activities of fruits and epicotyls. *J. Biol. Chem.* **271**, 1916–1924. (doi:10.1074/jbc.271.4.1916)

20. Strazzer P, Spelt CE, Li S, Bliek M, Federici CT, Roose ML, Koes R, Quattrocchio FM. 2019 Hyperacidification of citrus fruits by a vacuolar proton-pumping P–ATPase complex. *Nat. Commun.* **10**, 744. (doi:10.1038/s41467-019-08516-3)

21. Etienne A, Génard M, Lobit P, Mbeguié-A-Mbéguié D, Bugaud C. 2013 What controls fleshy fruit acidity? A review of malate and citrate accumulation in fruit cells. *J. Exp. Bot.* **64**, 1451–1469. (doi:10.1093/jxb/ert035)

22. Batista-Silva W, Nascimento VL, Medeiros DB, Nunes-Nesi A, Ribeiro DM, Zsögön A, Araújo WL. 2018 Modifications in organic acid profiles during fruit development and ripening: correlation or causation? *Front. Plant Sci.* **9**, 1689. (doi:10.3389/fpls.2018.01689)

23. Cerri M, Reale L. 2020 Anatomical traits of the principal fruits: an overview. *Sci. Hortic.* **270**, 109390. (doi:10.1016/j.scienta.2020.109390)

24. Schultz JC, Edger PP, Body MJA, Appel HM. 2019 A galling insect activates plant reproductive programs during gall development.

Sci. Rep. **9**, 1833. (doi:10.1038/s41598-018-38475-6)

25. Freund M, Freund M, Graus D, Fleischmann A, Gilbert KJ, Lin Q, Renner T, Stigloher C, Albert VA, Hedrich R, Fukushima K. 2022 The digestive systems of carnivorous plants. *Plant Physiol.* **190**, 44–59. (doi:10.1093/plphys/kiac232)

26. Gilbert KJ, Renner T. 2021 Acid or base? How do plants regulate the ecology of their phylloplane? *AoB PLANTS* **13**, plab032. (doi:10.1093/aobpla/plab032)

27. Felton GW. 2005 Indigestion is a plant's best defense. *Proc. Natl Acad. Sci. USA* **102**, 18 771–18 772. (doi:10.1073/pnas.0509895102)

28. Hanson P. 1992 The Nearctic species of *Ormyrus* Westwood (Hymenoptera: Chalcidoidea: Ormyridae). *J. Natural Hist.* **26**, 1333–1365. (doi:10.1080/00222939200770761)

29. Nastasi L, Deans A. 2021 Catalogue of rose gall, herb gall, and inquiline gall wasps (Hymenoptera: Cynipidae) of the United States, Canada and Mexico. *Biodivers. Data J.* **9**, e68558. (doi:10.3897/BDJ.9.e68558)

30. Haiden SA, Hoffmann JH, Cramer MD. 2012 Benefits of photosynthesis for insects in galls. *Oecologia* **170**, 987–997. (doi:10.1007/s00442-012-2365-1)

31. Pincebourde S, Casas J. 2016 Hypoxia and hypercarbia in endophagous insects: larval position in the plant gas exchange network is key. *J. Insect. Physiol.* **84**, 137–153. (doi:10.1016/j.jinsphys.2015.07.006)

32. Guiguet A, McCartney NB, Gilbert KJ, Tooker JF, Deans AR, Ali JG, Hines HM. 2023 Extreme acidity in a cynipid gall: a potential new defensive strategy against natural enemies. Figshare. (doi:10.6084/m9.figshare.c.6431706)