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Water-repellent plant surface structure induced by gall-forming insects for waste management

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Abstract

Many animals and plants have evolved elaborate water-repellent microstructures on their surface, which often play important roles in their ecological adaptation. Here we report a unique type of water-repellent structure on plant surface, which develops as an insect-induced plant morphology in a social context. Some social aphids form galls on their host plant, in which they produce large amount of hydrophobic wax. Excreted honeydew is coated by the powdery wax to form "honeydew balls", which are actively disposed by soldier nymphs through an opening on their gall. These activities are enabled by a highly water-repellent inner gall surface, and we discovered that this surface is covered with dense trichomes that are not found on normal plant surface. The trichomes are coated by fine particles of the insect-produced wax, thereby realizing a high water repellency with a cooperative interaction between aphids and plants. The plant leaves on which the gall is formed often exhibit patchy areas with dense trichomes, representing an ectopic expression of the insect-induced plant morphology. In the pouch-shaped closed galls of a related social aphid species, by contrast, the inner surface was not covered with trichomes. Our findings provide a convincing example of how the extended phenotype of an animal, expressed in a plant, plays a pivotal role in maintaining sociality.

Keywords: gall, aphid, animal-plant interaction, water repellency, hierarchical structure

Introduction

The diversity of surface structures in plants and animals often reflects their adaptation to the environment. Water repellency is one of the well-understood adaptive features of biological surfaces. The water-repellent surfaces tend to exhibit microscopic and hierarchical roughness [1,2]. Such hierarchical structures are exemplified by self-cleaning lotus leaves covered by papillose epidermal cells with submicrometre-sized epicuticular waxes [3], floating legs of water striders covered by numerous needle-shaped setae with nanoscale groove structure [4], and others.

Liquid waste management is of critical importance for plant-sucking insects. The water problem is particularly serious for gall-inhabiting species, because they potentially suffer contamination or even drowning with their own liquid waste, which can destroy the colony if experimentally forced to accumulate inside their gall [5,6]. Probably for that reason, most gall-forming aphids produce a large amount of powdery hydrophobic wax from specialized epidermal glands, thereby forming wax-coated "honeydew balls" to protect colony members from wetting [6‑8]. In some social aphids, soldier nymphs actively dispose of the wax-coated honeydew balls and other wastes through openings on their gall to keep their habitat clean [6,9,10]. In several social aphids that form completely closed galls, the gall inner wall is specialized for absorption and removal of honeydew, which is regarded as a physiological manipulation of the plant tissue by the gall-forming aphids [5].

Here we report a previously unrecognized type of hierarchical microstructure, which confers hydrophobicity to a specific plant surface, the gall inner wall, induced by gall-forming aphids.

Materials and methods

Field observation and sampling

The woolly aphid *Colophina clematis* forms pouch-shaped galls with an opening on the tree *Zelkova serrata*, in which young nymphs exhibit defensive behaviours against intruders [9]. Galls of *C. clematis* were observed and collected at Okutama, Tokyo and Shomaru, Saitama, Japan. In the field, aphids around the gall opening were observed using a magnifying glass. Some twigs with a gall-harbouring leaf were brought to the laboratory and put into water, and aphids around the gall opening were video-recorded. Honeydew balls were collected from four galls on two trees using a fine brush and photographed, and from the photographs 100 balls were randomly chosen for size measurement. Nine gall-harbouring leaves collected from five trees were fixed in FAA (formaldehyde 3.7% and acetic acid 5% in 50% ethanol), dehydrated through an ethanol series and dried. Most of the aphid-derived wax on the gall inner surface was removed during this procedure. The dried samples were observed by a scanning electron microscope and photographed. Density and length of trichomes in a 0.5 x 0.5 mm square area of the sample surface were measured based on the photographs using ImageJ (https://imagej.nih.gov/ij/). Several unfixed galls were examined for distribution of wax particles on the trichomes. Other gall-forming aphids, *Colophina arma*, *Hemipodaphis persimilis* and *Paracolopha morrisoni,* are listed in table S1.

Hydrophobicity measurement

We compared the following three areas: (i) thin-sliced gall inner surface areas (n = 17, from 11 leaves on four trees); (ii) hairy leaf underside areas (n = 17, from 10 leaves on four trees); (iii) normal leaf underside areas (n = 13, from eight leaves on three trees). Each sample was affixed to an experimental table by double-sided adhesive tape to ensure an even surface. For contact angle measurement, 1.6 – 1.8 μl of distilled water was placed on the sample and photographed using a digital camera attached to a horizontally-mounted dissection microscope. The photographs were converted into grey scale and subjected to contact angle measurement using the Low-bond axisymmetric drop shape analysis plugin [11] implemented for ImageJ.

Gall surface manipulation

A total of 18 mature galls of *C. clematis* were collected from four trees and cut in half with a knife.

From one half, aphid-derived wax was collected into a plastic tube using a fine brush. The other half was further cut into an approximately 5 mm x 5 mm square. To remove aphid wax, the gall slice was soaked in 1 ml hexane for 1 min, taken out and left until residual hexane completely evaporated. Then, 1.6 – 1.8 μl of distilled water was placed on the sample and photographed. After removing the distilled water, the aphid wax collected in the plastic tube was spread onto the sample surface. Again, the same amount of distilled water was placed on the same location of the sample and photographed. The photographs were subjected to contact angle measurement as described above.

Water absorption by galls of P. morrisoni

In the field, on each of six galls of *P. morrisoni* formed on leaves of *Z. serrata*, a 1 x 1 mm square hole was bored using a fine edge of chisel. Then, 3 μl of food dye water (0.2% Food Red No. 102, Kyoritsu Foods) was injected into each gall using a micropipette. The hole was immediately filled with an adhesive [5]. After 15 h, the galls were brought to the laboratory and inspected for the injected solution.

Results and Discussion

Housekeeping behaviour of young nymphs in C. clematis *galls*

In 5 of 8 galls (63%) of *C. clematis* examined in the field (figure 1*a*), honeydew balls came out through a slit-like opening during 30 min observation (figure 1*b*), where first and second instar nymphs actively pushed honeydew balls out of the galls (figure 1*c* and movie S1). These observations indicate that young nymphs of *C. clematis* perform not only defense against enemies but also housekeeping by disposing of colony wastes, as previously reported in other social aphids [6,9].

Inner surface structure of C. clematis *galls*

Microscopic observations revealed that the inner surface of the galls of *C. clematis* was covered with minute trichomes (figure 1*d*). The trichome density was 221.7±61.3 / mm^2 (n = 36), which was 30 times higher than the trichome density on the opposite underside of the same leaf (7.2±5.9 / mm^2) (n = 36, table 1). The average pairwise distance between two neighbouring trichomes was 42.1±14.3 μm (n = 149, table 1), which was far smaller than the diameter of honeydew balls (405.3±176.8 μm, n = 100). Hence, a honeydew ball is expected to sit on several tens or hundreds of trichomes in the gall of *C. clematis*. In mature galls, the trichomes were coated with fine wax particles, which were obviously aphid-derived, thereby forming a unique hierarchical microstructure (figure 1*e*, *f*). Notably, we found that 14 of 31 galled leaves (45%) exhibited a patchy hairy area outside the gall, where the trichome density was as high as 203.1±35.9 / mm^2 (n = 36, table 1 and figure S2*a*), whereas none of the ungalled leaves we observed contained such hairy area. The hairy region may represent a remote effect of the galling activity by *C. clematis*, as observed in some insects whose galls are induced at a plant part distant from their infesting site [12].

Comparison of inner gall structure between galls formed by different aphid species on the same plant

Not only *C. clematis* but also closely related aphids, including *C. arma*, *H. persimilis* and *P. morrisoni*, form galls on leaves of the same plant *Z. serrata* (figure S3*a*, *d* and *g*). In the pouch-shaped open galls of *C. arma* and also in the leaf-roll open galls of *H. persimilis*, the inner surface was covered with dense trichomes (table 2 and figure S3*b* and *e*). The trichomes were significantly denser and longer in *C. clematis* and *C. arma* than in *H. persimilis* (table 2). In the pouch-shaped closed galls of *P. morrisoni*, by contrast, the inner surface was not covered with trichomes (table 2 and figure S3*h*). The different surface structures of the galls on the same plant strongly suggest that these morphological traits of the plant are controlled by the insects and regarded as their extended phenotypes, consistent with the previous phylogenetic study that demonstrates that aphids determine the gall morphology [13].

Functional difference between hairy inner wall of open galls and smooth inner wall of closed galls

When food dye solution was introduced into open galls of *C. clematis* and *H. persimilis*, the solution was repelled by the waxy and trichome-covered inner surface, thereby forming round droplets (figure S3*c* and *f*). By contrast, the dye solution introduced into closed galls of *P. morrisoni* was not repelled by the inner surface (figure S3*i*). Notably, when 3 μl of the dye solution was injected into 6 galls of *P. morrisoni*, the solution was completely absorbed in 5 galls within 15 h, whereas the solution was covered with aphid-derived wax and remained as a honeydew ball in 1 gall after 15 h. These observations suggest that gall openness, surface trichomes, and waste managing strategies are ecologically interconnected to each other in these gall-forming aphids: namely, the aphids forming open galls induce water-repelling inner surface covered with dense trichomes and facilitate disposal of honeydew droplets from the opening [8], whereas the aphids forming closed galls induce water-absorbing inner surface with few trichomes and remove honeydew through the plant vascular system [5].

Trichomes and wax jointly contribute to water-repellent inner surface of C. clematis *galls*

By using contact angle measurements, we quantitatively evaluated the water-repelling properties of the inner surface of the galls of *C. clematis* in comparison with other plant parts of *Z. serrata*. The gall inner surface (with both trichomes and wax) was highly water-repellent with contact angles of 149.5±3.5° (n = 17); the hairy underside area of the leaf (with trichomes but no wax) was also water-repellent with slightly lower contact angles of 127.6±10.6° (n = 17), and the normal underside area of the leaf (with neither trichomes nor wax) showed remarkably smaller contact angles of 81.5±11.1° (n =13) (figure 2*a*). The differences between these three areas were all statistically significant (Tukey's HSD test, $P < 0.001$), indicating that both factors, mainly trichomes and additionally wax, contribute to the water repellency. The hierarchically rough surface consisting of trichomes and wax reduces contact area of a liquid drop with the surface, thereby attaining higher contact angle and increased water repellency than smoothed surface [2,8]. Wax removal and re-addition experiments reproduced the significant shift of contact angles between 131.1±9.8° and 145.3±8.6° (n = 18, paired t-test, $t_{17} = -6.28$, $P < 0.001$), confirming the cooperative contribution of trichomes and wax to the water repellency of the gall inner surface (figure 2*b*).

Conclusion

In conclusion, *C. clematis* and closely related aphids induce dense trichomes on the inner surface of their galls, and by adding the aphid-derived fine wax particles, the trichome-wax complex constitutes a highly water-repellent surface, thereby facilitating the waste management in combination with the behavioral honeydew disposal by soldier nymphs. Our finding highlights the ecological relevance of gall openness, the inner surface structure, and the waste management strategies, in which the intricate manipulation of plant morphology plays a pivotal role in the aphid social system. A larger comparative study across aphids and host plants will clarify the general applicability of this unrecognized animal-plant interaction.

Ethics

We followed the Association for the Study of Animal Behaviour Guidelines for the Use of Animals in Research.

Data accessibility

Additional data, details of statistical analyses and a movie are available as electronic supplementary material and in the Dryad Digital Repository (https://doi.org/10.5061/dryad.q9p2q59) [14].

Authors' contributions

All authors designed the study. K.U. and M. K. collected data and performed analysis. K. U. and T. F. wrote the manuscript. All authors revised the manuscript, gave their final approval and agree to be held accountable for the content therein.

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Competing interests

We have no competing interests.

Acknowledgements

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Repository. (doi:10.5061/dryad.q9p2q59)

Figure legends

Figure 1. Gall of *C. clematis*. (*a*) A mature gall on a leaf of *Z. serrata*. (*b*) A slit-like gall opening (arrow). (*c*) Young nymphs and honeydew balls in a mature gall. The gall inner cavity is full of aphid-derived powdery wax. (*d*) A scanning electron micrograph of trichomes on the gall inner surface. Note that aphid-derived wax is removed during fixation. (*e*) A fresh cross section image of the gall inner surface. Note that trichomes are coated with aphid-derived white wax. (*f*) A scanning electron micrograph of the wax-coated trichomes.

Figure 2. Hydrophobic effects of trichomes wax on the galls and gall-harbouring leaves of *C. clematis*. (*a*) Contact angles of water droplets measured on normal underside areas (left, n = 13), hairy underside areas (middle, n = 17), and gall inner surface areas (right, n = 17). (*b*) Contact angles of water droplets measured on normal underside areas (left, n = 18), gall inner surface areas from which aphid-derived wax was removed by hexane (middle, n = 18), and gall inner surface areas to which the wax was removed and re-added (right, n = 18). Lines indicate the changes of contact angle values measured on the same gall inner surface. The box plots depict median, quartiles, and minimum and maximum values. Corresponding water droplet images are shown below (bars 1 mm).

Table 1. Trichomes on the different areas of *Z. serrata* leaves harbouring a *C. clematis* gall. Stastistical significance was analyzed using linear mixed model (*lmer* function in the *lme4* package) with gall identity treated as a random factor followed by Tukey's post-hoc test using *glht* function in the *multcomp* package. Values indicate mean±SD.

Area	Trichome density (No. of trichomes / mm^2)	Trichome length (µm)	Distance between trichomes (µm)
Gall inner surface	221.7^{a}±61.3 (N = 36)	104.9^{a}±35.5 (N = 160)	42.1^{a}±14.3 (N = 149)
Trichome-dense area on the underside	203.1^{a}±35.9 (N = 36)	197.4^{b}±70.0 (N = 180)	37.9^{a}±11.8 (N = 180)
On the underside	7.2^{b}±5.9 (N = 36)	111.1^{a}±66.1 (N = 178)	244.2^{b}±176.9 (N = 175)
On the upperside	8.7^{b}±5.3 (N = 36)	115.1^{a}±68.4 (N = 126)	286.7^{c}±119.2 (N = 115)

^{abc}Values within a column with different superscripts are significantly different ($P < 0.01$). Details of the staistical analyses are shown in electronic supplementary material, table S2.

Table 2. Differences among the gall inner surfaces of Eriosomatini aphids on *Zelkova serrata.* Values indicate mean±SD.

Species	No. of galls	Gall morphology	Trichome density (trichomes / mm^2)	Trichome length (µm)
Colophina clematis	9	Open pouch	$221.7^{a}\pm61.3$ (N = 36)	$104.9^{a}\pm35.5$ (N = 160)
Colophina arma	3		$254.3^{a}\pm66.2$ (N = 12)	$87.6^{a}\pm18.4$ (N = 30)
Hemipodaphis persimilis	5	Open leaf-roll	$114.1^{b}\pm44.4$ (N =19)	$37.8^{b}\pm15.0$ (N = 48)
Paracolopha morrisoni	4	Closed pouch	0 (N = 16)	N/A

^{abc}Values within a column with different superscripts are significantly different ($P < 0.001$). *P. morrisoni* was excluded from the statistical analyses. Details of the staistical analyses are shown in electronic supplementary material, table S2.

Figure 1.

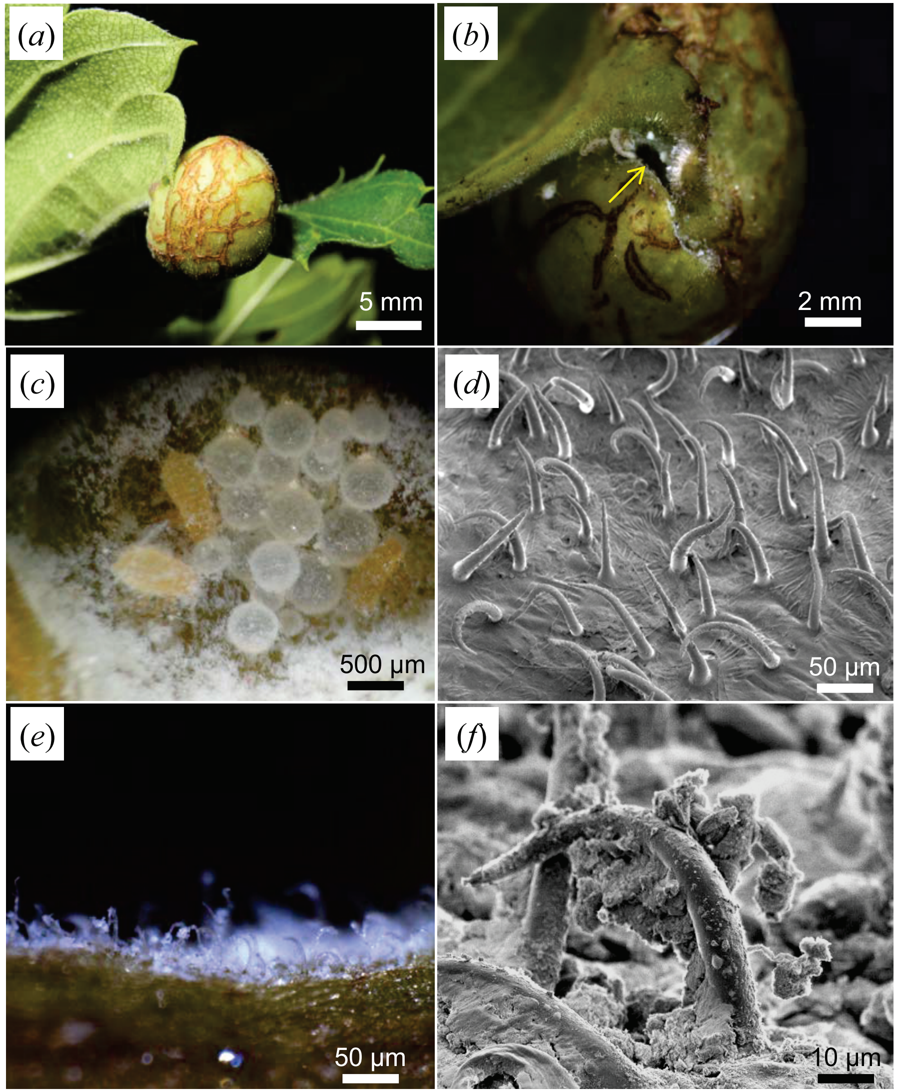
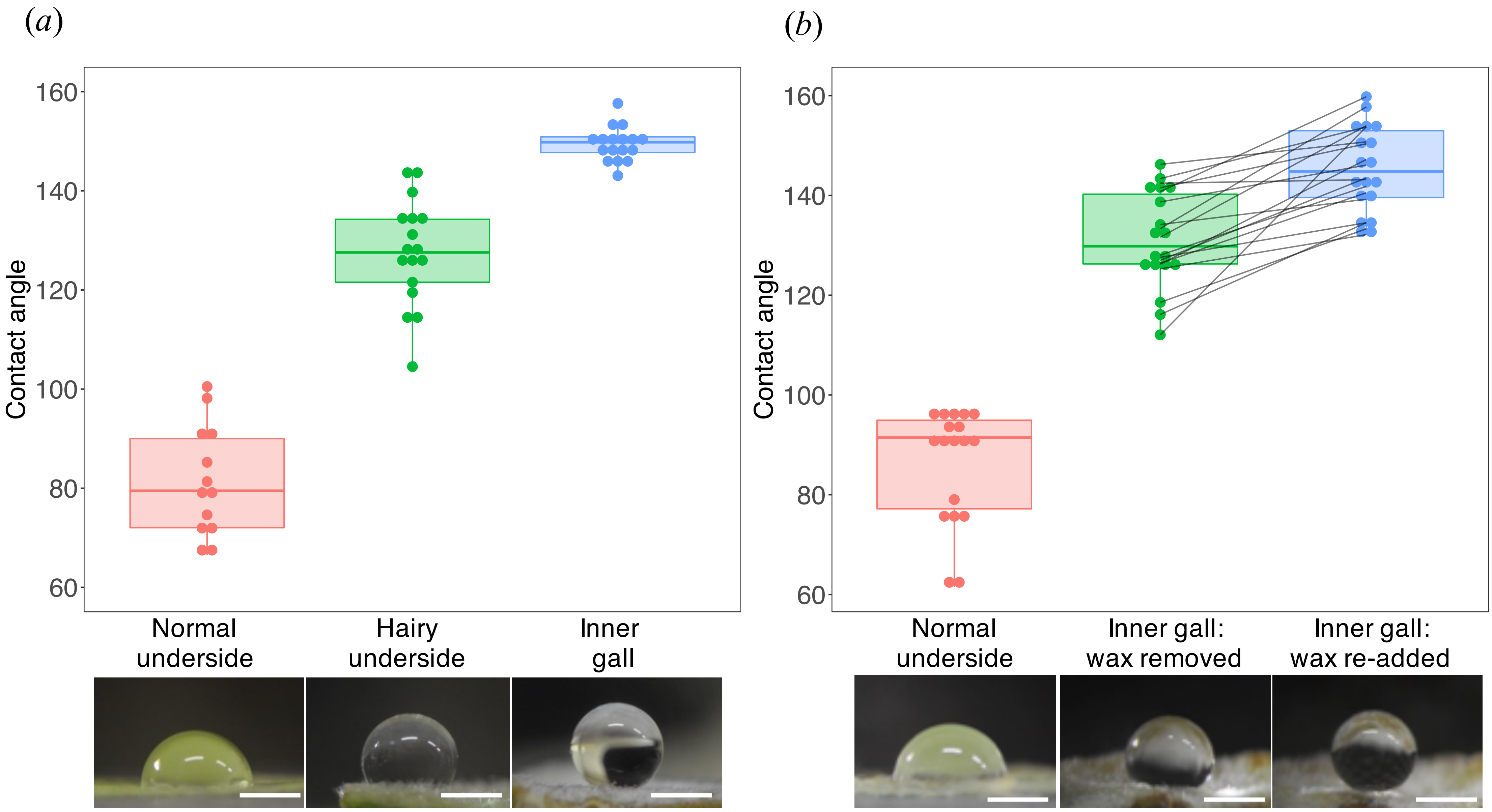


Figure 2.



(a)
(b)
Contact angle
160
140
120
100
80
60
Normal underside
Hairy underside
Inner gall
Inner gall: wax removed
Inner gall: wax re-added

Supplementary figures

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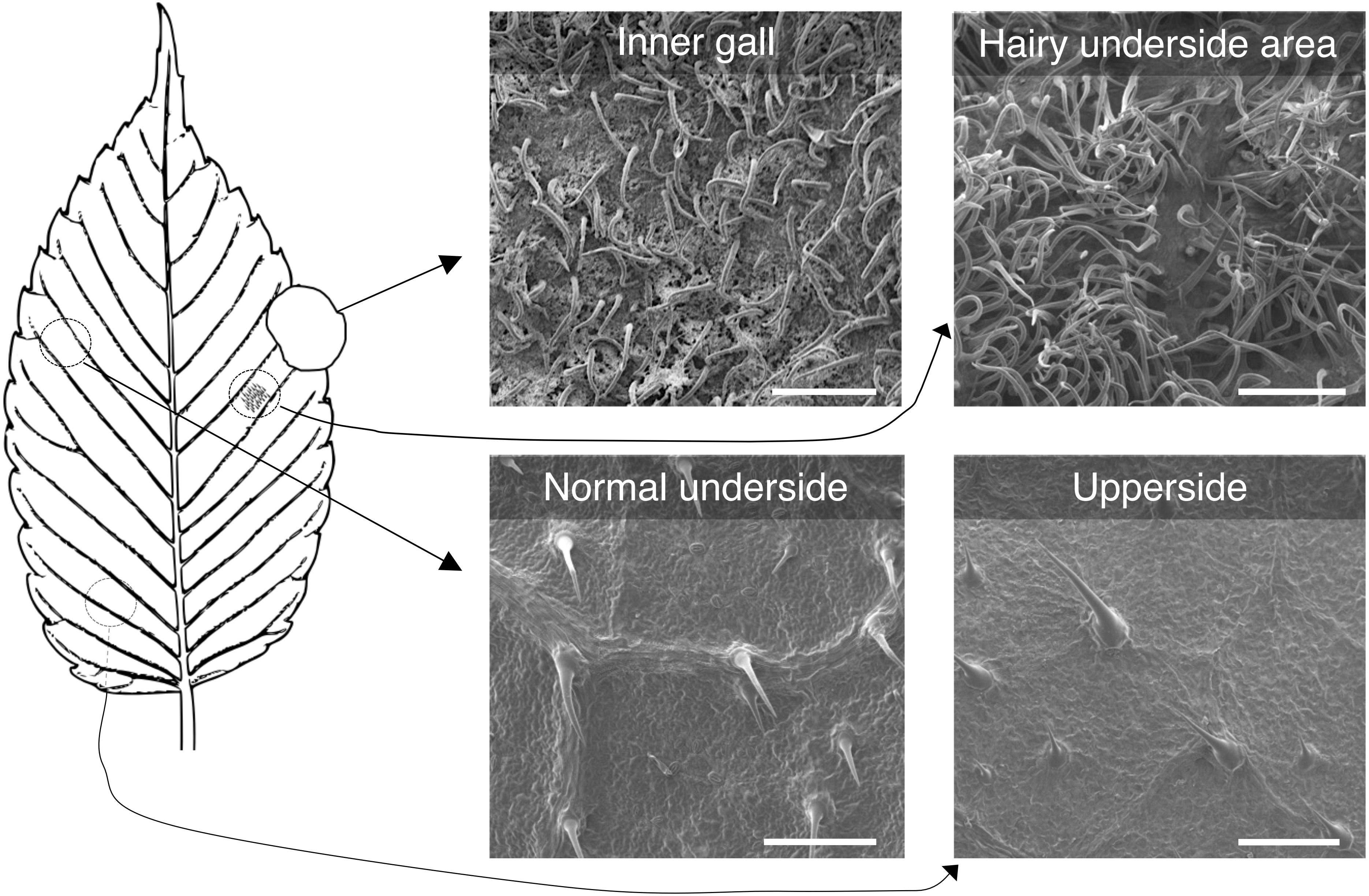

Figure S1.
Leaf areas and corresponding SEM figures on the underside of a *Z. serrata* leaf harbouring a *C. clematis* gall. Scale bars indicate 200 µm.

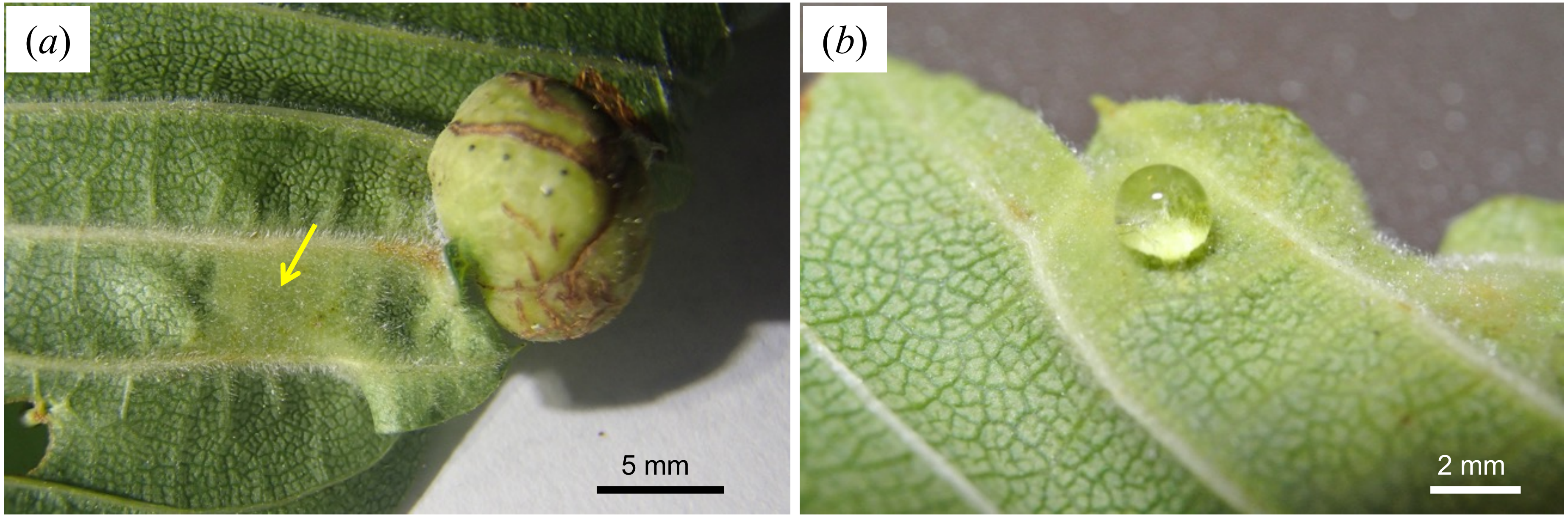

Figure S2.
Hairy areas on the *Z. serrata* leaf harbouring a *C. clematis* gall. (*a*) An underside area covered with dense trichomes (arrow). (*b*) A water droplet placed on the hairy area.

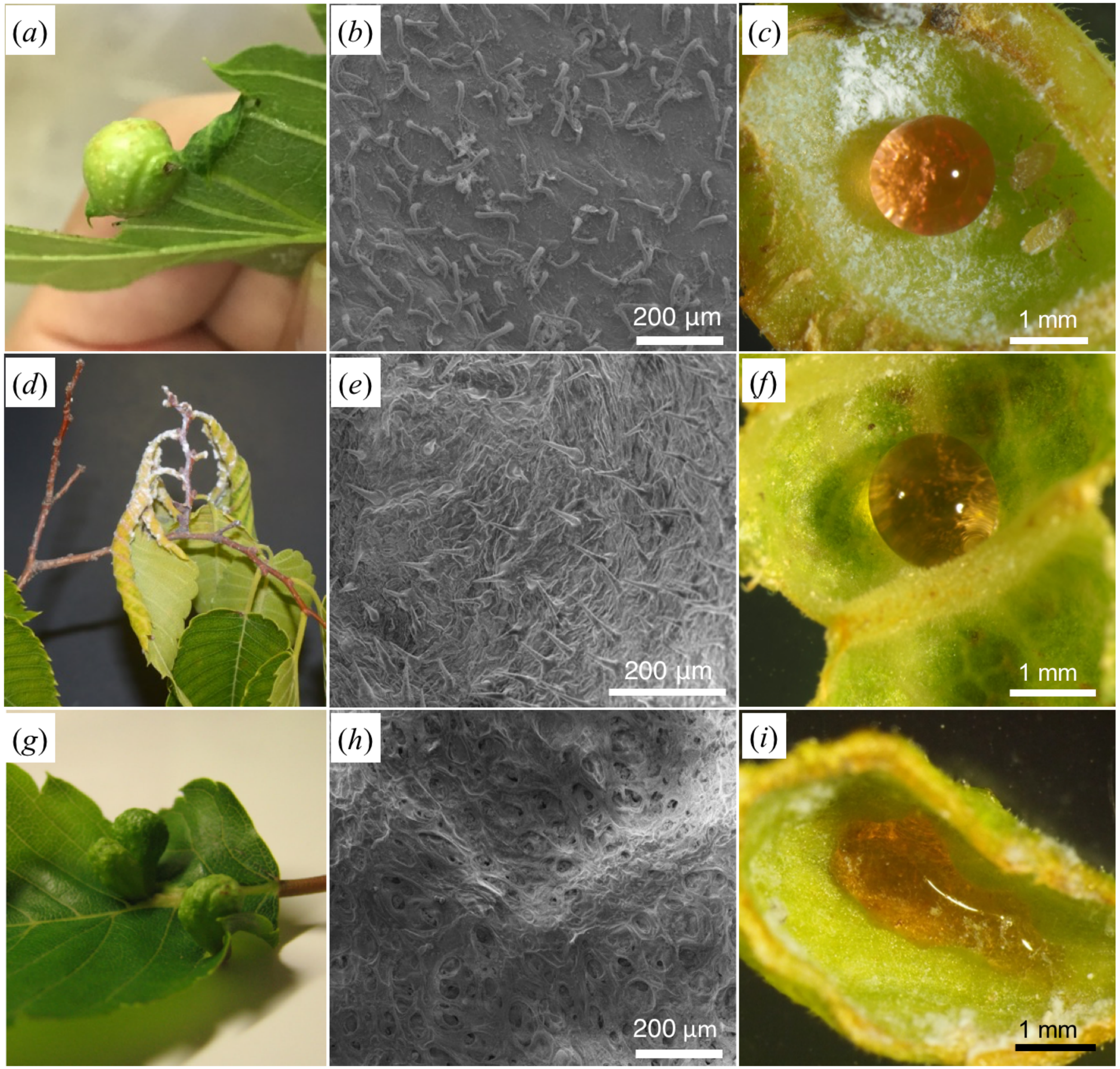

Figure S3.
Galls of Eriosomatini aphids on *Zelkova serrata*: *Colophina arma* (*a,b*), *Colophina clematis* (*c*), *Hemipodaphis persimilis* (*d-f*), and *Paracolopha morrisoni* (*g-i*). (*a,d,g*) Mature galls; (*b,e,h*) inner gall surfaces; (*c,f,i*) food-dye solution on gall inner surfaces.

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Table S1. Galls of aphids of the tribe Eriosomatini (subfamily: Eriosomatinae) on *Zelkova serrata* examined in this study.

Species	Collection locality[1]	Collection date
Colophina clematis	Okutama, Tokyo Shomaru, Saitama	Jun 2016 - Jun 2018
Colophina arma	Aki-Ota, Hiroshima	22 Jul, 2016
Hemipodaphis persimilis	Sapporo, Hokkaido Matsumoto, Nagano	Jul 2016 - Jun 2018
Paracolopha morrisoni	Tsukuba, Ibaraki Shomaru, Saitama	2 Jun, 2016

[1]All localities are in Japan.

Table S2. Statistical information reported in this study.Stastistical significance was analyzed using linear mixed model (lmer function in the lme4 package) with gall identity treated as a random factor followed by Tukey's post-hoc test using *glht* function in the *multcomp* package in R version 3.4.3.

(a) Trichome density among different leaf areas (no. of trichomes / mm^2)

	Mean difference (left - right)	95 % CI of the difference (lower limit, upper limit)	z score	p-value
Inner gall vs. Hairy underside	18.6	-1.8, 38.9	2.34	0.089
Inner gall vs. Normal underside	214.4	194.1, 234.8	27.06	< 0.0001
Inner gall vs. Normal upperside	213	192.6, 233.4	26.88	< 0.0001
Hairy underside vs. Normal underside	195.9	175.5, 216.3	24.72	< 0.0001
Hairy underside vs. Normal upperside	194.4	174.1, 214.8	24.54	< 0.0001
Normal upperside vs. Normal underside	1.4	-18.9, 21.8	0.18	0.998

(b) Trichome length among different leaf areas (μm)

	Mean difference (left - right)	95 % CI of the difference (lower limit, upper limit)	z score	p-value
Inner gall vs. Hairy underside	-94.0	-110.4, -77.6	-14.69	< 0.0001
Inner gall vs. Normal underside	-2.8	-19.3, 13.7	-0.43	0.973
Inner gall vs. Normal upperside	-11.4	-29.5, 6.7	-1.61	0.370
Hairy underside vs. Normal underside	91.2	75.0, 107.4	14.46	< 0.0001
Hairy underside vs. Normal upperside	82.6	64.9, 100.4	11.96	< 0.0001
Normal upperside vs. Normal underside	8.6	-9.1, 26.2	1.25	0.597

(c) Distance between trichomes (μm)

	Mean difference (left - right)	95 % CI of the difference (lower limit, upper limit)	z score	p-value
Inner gall vs. Hairy underside	4.2	-26.5, 34.9	-14.69	0.985
Inner gall vs. Normal underside	-201.8	-232.7, -171.0	-0.43	< 0.0001
Inner gall vs. Normal upperside	-244.6	-279.0, -210.1	-1.61	< 0.0001
Hairy underside vs. Normal underside	-206.0	-235.4, -176.6	14.46	< 0.0001
Hairy underside vs. Normal upperside	-248.7	-281.8, -215.7	11.96	< 0.0001
Normal upperside vs. Normal underside	42.7	9.5, 76.0	1.25	0.006

(d) Trichome length among the species (μm)

	Mean difference (left - right)	95 % CI of the difference (lower limit, upper limit)	z score	p-value
C. clematis vs. *C. arma*	16.9	-7.2, 41.0	1.637	0.227
H. persimilis vs. *C. arma*	-50.1	-77.0, -23.2	-4.35	< 0.0001
H. persimilis vs. *C. clematis*	-67.0	-87.1, -46.9	-7.778	< 0.0001

(e) Trichome density among the species (no. of trichomes / mm2)

	Mean difference (left - right)	95 % CI of the difference (lower limit, upper limit)	z score	p-value
C. clematis vs. *C. arma*	-31.4	-97.1, 34.4	-1.12	0.502
H. persimilis vs. *C. arma*	-138.7	-208.8, -68.6	-4.62	< 0.0001
H. persimilis vs. *C. clematis*	-107.3	-161.6, -53.0	-4.62	< 0.0001

(f) Contact angle among different plant surfaces

	Mean difference (left - right)	95 % CI of the difference (lower limit, upper limit)	z score	p-value
Normal underside vs. Inner gall	-68.6	-76.1, -61.2	-21.58	< 0.0001
Hairy underside vs. Inner gall	-22.1	-29.0, -15.3	-7.577	< 0.0001
Hairy underside vs. Normal underside	46.5	39.1, 53.9	14.701	< 0.0001