Oviposition site selection and attachment ability of *Propylea quatuordecimpunctata* and *Harmonia axyridis* from the egg to the adult stage

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Abstract. Surface features of plants can influence the searching efficiency and survival of predatory insects. Surfaces act as barriers preventing attachment of both phytophages and also their insect predators. In this regard, we focused on the oviposition site selection and the attachment ability of all life stages (eggs, larvae, imagines) of two common ladybird species, *Propylea quatuordecimpunctata* and *Harmonia axyridis* (Coleoptera: Coccinellidae), on artificial and natural substrates with different surface features and properties (roughness, wettability). Both species preferred a hydrophilic surface as the oviposition site and this can be correlated with the better performance of both larvae and adults on these substrates compared with hydrophobic ones. The egg glue of both ladybird species can wet hydrophobic surfaces such as those of many plant leaves and also with prominent 3D wax coverage. The surface roughness has an important role in the oviposition site selection in *P. quatuordecimpunctata*, but not in *H. axyridis*. The oviposition preference for smooth surfaces in *P. quatuordecimpunctata* could be due to better performance of larvae on smooth substrates compared with rough ones. The egg glue of both species can adapt to artificial and natural surfaces characterized by different asperity sizes faithfully replicating their shape, except for very high asperity sizes or big trichomes. The results of the present research can shed light on the mechanical ecology of the evolutionary successful Coccinellidae and may aid in the development of suitable substrates for coccinellid egg-laying, in order to improve the mass rearing technique of species used in biological control.

Key words. Adhesion, Coccinellidae, Coleoptera, egg glue, ladybird, larvae, ontogenesis, plant surface, roughness, wettability.

Introduction

The selection of a suitable oviposition site by gravid females is a fundamental factor for offspring growth, survival and reproduction. Good fixation of eggs to the substrate and the ability to attach firmly to plant surfaces during larval and adult stages are a fundamental prerequisite for phytophagous insects and can be important factors affecting the substrate choice for egg deposition (Al Bitar et al., 2012). Predatory and parasitoid insects specialized on phytophagous insects are associated with the same foraging habitat as their prey and, thus, focus predominantly on one, few or several host plant species. Consequently, they have to face all the mechanical barriers developed by plants against herbivore insects, such as epicuticular waxes, trichomes and other leaf surface features, typically reducing insect attachment ability (Gorb & Gorb, 2013). Such mechanical barriers, which can strongly reduce the performance of phytophagous insects (e.g. Gorb & Gorb, 2002; Salerno et al., 2018a, 2020a; Rebora et al., 2020a), can have a strong impact also on the...
effective of entomophagous insects (Eigenbrode, 2004; Lucas et al., 2004; Eigenbrode et al., 2009; Schmidt, 2014; Voigt, 2019; Rebora et al., 2020b).

Studies regarding the oviposition site selection in Coccinellidae considered mainly aphid density and chemical cues (e.g. Edward & Dixon, 1986; Scholz & Poehling, 2000; Griffin & Yeargan, 2002, Seagraves, 2009), whereas less is known about the role of plant surface features. Iperti & Quilici (1986), among the factors influencing the selection of oviposition site in Propylea quatuordecimpunctata (L.) (Coleoptera: Coccinellidae), tested plant surfaces with different hairiness, but they could not separate the chemical cues from the mechanical ones. The texture of plant surfaces can influence the searching efficiency and survival of Coccinellidae (Putman, 1955; Plaut, 1965; Belcher & Thurston, 1982; Shah, 1982; Carter et al., 1984), but only few studies focused on oviposition site selection in relation to the surface features of the potential substrate and coccinellid attachment ability. In this regard, Yao et al. (2021) highlighted positive correlation between oviposition preference and adult attachment force in the whitefly predator ladybird Serangium japonicum Chapin (Coleoptera: Coccinellidae).

In this regard, the aim of the present investigation is to deepen our knowledge of the oviposition site selection in relation with the attachment ability of eggs, larvae and adults of two common ladybird species, P. quatuordecimpunctata and Harmonia axyridis (Pallas) (Coleoptera: Coccinellidae), on substrates with different surface characteristics. We test the hypothesis that the choice of the oviposition substrate relates to peculiar surface features and properties of substrates and attachment ability of adults, eggs and larvae. In particular, our questions are as follows: (1) Which are the preferred substrates for oviposition in P. quatuordecimpunctata and H. axyridis females in regard to surface roughness and wettability? (2) Is there any difference in the attachment ability of the different life stages on surfaces with different roughness and wettability? Many studies are available regarding ladybird attachment ability, but they focused only on adults (Eigenbrode, 2004; Gorb et al., 2008, 2019; Eigenbrode et al., 2009; Heepe et al., 2017a, b; Gorb & Gorb, 2020) and only few data are available on larvae (Eigenbrode et al., 2009), whereas eggs were disregarded so far.

P. quatuordecimpunctata is typically mass reared and widely used as a biological control agent, whereas H. axyridis is considered an invasive species. Using centrifugal, traction and pull-off force experiments, we tested the adult, larval and egg attachment to artificial substrates having different roughness and wettability (hydrophilic, hydrophobic). We characterized surfaces using cryo scanning electron microscopy and measured their roughness and surface free energy. The interaction of the egg glue with some natural surfaces represented by plant leaves with different surface structures (trichomes, waxes, cuticular folds) was analysed.

Methods

Insects

Adults of P. quatuordecimpunctata (from Bioplanet, Cesena, Italy) and of H. axyridis (from laboratory culture, Dipartimento di Scienze Agrarie, Alimentari e Ambientali, University of Perugia, Italy) were reared from larvae kept inside net cages (300 mm x 300 mm x 300 mm) (Vermandel, Hulst, The Netherlands) and fed using aphids Aphis fabae Scopoli (Hemiptera: Aphididae) reared on young Vicia faba L. (Fabaceae) plants. The two insect species were kept in a controlled condition chamber (14 h photoperiod, temperature of 23 ± 1 °C, and a relative humidity of 60 ± 10%). Females and larvae of the fourth instar (Supporting information, Fig. S1) were used in the experiments.

Plants

Four common garden plant species with leaves having different surface features were used in this study: cabbage Brassica oleracea L. var. capitata (Brassicaceae), cherry Prunus avium L. (Rosaceae), rose Rosa hybrida L. ‘Schneesturm’ (Rosaceae) and eggplant Solanum melongena L. (Solanaceae).

Cryo scanning electron microscopy

Samples of (1) adult and larval tarsi of the two coccinellid species, (2) egg glue (eggs detached from the different tested surfaces with a micromanipulator, see below), (3) polishing paper, (4) abaxial sides of the tested plant leaves, and (5) eggs glue/plant surface interaction were studied in a scanning electron microscope (SEM) Hitachi S-4800 (Hitachi High-Technologies Corp., Japan), equipped with a Gatan ALTO 2500 cryo-preparation system (Gatan Inc., U.K.). For details of sample preparation and mounting for cryo-SEM, see Gorb & Gorb (2009a). Whole mounts of insect tarsi, eggs and plant leaves were sputter-coated in frozen conditions with gold–palladium (thickness 10 nm) and examined at 3 kV acceleration voltage and temperature of −120 °C at the cryo-stage within the microscope.

Substrate preparation and characterization

Artificial (epoxy resin) substrates with different roughness have been prepared to minimize the possible influence of chemical properties or colour of different substrates on experimental data. Epoxy resin casts of a clean glass surface and of polishing papers with defined asperity sizes were made using a two-step moulding method (Gorb, 2007) to get artificial substrates with different roughness parameters – 0 (smooth), 0.3, 1, 3, 9, 12, 35 (P400) and 269 (P600) μm asperity sizes. A two-component dental silicone polymer polyvinylsiloxane (President light body, Coltene, Switzerland) was used to obtain silicone negative casts of glass and of the polishing papers of different roughness. The negative casts were applied as templates to make positive resin moulds using Spurr’s low-viscosity resin (Gorb, 2007). The roughness (0, 0.3, 1, 3, 9, 12 μm) of the different surfaces and of their negative silicone casts and positive resin moulds has been characterized (as reported in Salerno et al., 2017) using the white light interferometer NewView 6000 ( Zygo, Middlefield, Connecticut) with the objectives x5 and x50 (N.A. 0.4, window 0.1, numerical aperture 0.1)
size 1400-μm × 1050-μm and 140-μm × 105-μm, respectively). Ten individual measurements (n = 10) were performed for each substrate. Roughness data for above surfaces are given in Supporting information, Table S1.

Hydrophobic glass has been prepared by treatment of a glass plate by vapour phase deposition silanization method using 4 μL of dichlorodimethyl-silane for 12 h. The wettability of surfaces used in the experiments (smooth hydrophilic glass, smooth hydrophobic glass, smooth plastic of Petri dishes, abaxial side of plant leaves) was characterized by determining the contact angles of water (aqua millipore, droplet size = 1 μL, sessile drop method) using a high-speed optical contact angle measuring instrument OCAH 200 (Dataphysics Instruments GmbH, Germany). Ten measurements (n = 10) were performed for each substrate.

Oviposition choice experiments

The experiments were performed in a controlled climate chamber (14 h photoperiod, temperature of 23 ± 1 °C, and a relative humidity of 60 ± 10%). Choice tests were performed using plastic Petri dishes (Polystyrene). Insect couples of both species were isolated until the end of mating and mated females were used for the oviposition site selection experiments. A female was placed inside the Petri dish and, in case of oviposition, the position of the egg clutch (or of the different egg clutches, if more than one were deposited by the same female), was recorded after 24 h. After 24 h, the female was replaced and each ladybird was used only once.

Preliminary behavioural tests were performed to evaluate the positive or negative geotropism in the oviposition behaviour. A female was placed inside the Petri dish having 6 cm in diameter and in case of oviposition, the position of the egg clutch (top, bottom, and border) was recorded. In total, 36 females of P. quatuordecimpunctata and 32 females of H. axyridis were tested.

Once confirmed the negative geotropism in the oviposition site selection of both species, experiments were performed in Petri dishes with the internal side of the lid in different conditions.

1. To evaluate the oviposition preference of both species towards a rough or a smooth surface, dual choice experiments were conducted in a Petri dish (6 cm in diameter) with the lid (deprived of the border) manually scratched with polishing paper on half of its internal surface to realize a smooth or a rough surface. A filter paper sheet was placed over the Petri dish to uniform the light conditions. A female was placed inside the Petri dish and in case of oviposition, the position of the egg clutch on the lid (smooth, rough) was recorded. In total, 31 females of P. quatuordecimpunctata and 27 females of H. axyridis were tested.

2. To evaluate the substrate roughness effect on oviposition behaviour of P. quatuordecimpunctata, a Petri dish (9 cm in diameter) with its lid internally covered with eight different sectors characterized by substrates with different asperity sizes was prepared. The eight substrates were obtained by cutting eight sectors of the same area from the artificial epoxy resin replicas with different roughness, 0, 0.3, 1, 3, 9, 12, 35 and 269 μm. Each substrate was attached with Patafix (UHU Bostik, Milano, Italy) to the internal side of the lid of the Petri dish. A female was placed inside the Petri dish and in case of oviposition, the position of the egg clutch on the lid was recorded. In total, 42 females of P. quatuordecimpunctata were tested.

3. To evaluate the oviposition preference of both species towards a hydrophilic or hydrophobic surface, dual choice experiments were performed in a Petri dish (6 cm in diameter) with the lid constituted of two microscope glass slides of the same surface, characterized by different wettability (hydrophobic glass, showing a water contact angle of 104.5 ± 0.88° (mean ± SD) and hydrophilic glass, showing a water contact angle of 44.38 ± 3.25° (mean ± SD). A female was placed inside the Petri dish and in case of oviposition, the position of the egg clutch on the lid (hydrophobic glass, hydrophilic glass) was recorded. In total, 13 females of P. quatuordecimpunctata and 22 females of H. axyridis were tested.

Force measurements

The attachment ability of adults, larvae and eggs of P. quatuordecimpunctata and H. axyridis to the different surfaces was studied using two different instruments: a centrifugal force tester and a Biopac force tester. The centrifugal force tester was applied to measure the attachment ability of adults and larva to the artificial substrates with different roughness (polishing paper 0, 0.3, 1, 3, 9, 12, 35 and 269 μm for adults and 0, 0.3, 1, 3, 9, 12, 35 μm for larvae) and wettability [hydrophobic glass with a water contact angle of 112.62 ± 3.49° (mean ± SD) and hydrophilic glass with a water contact angle of 32.49 ± 4.17° (mean ± SD)]. The Biopac force tester was used to measure (1) the traction force (parallel to the substrate) of adults walking upright and upside down on a Petri dish surface and (2) the egg pull-off (adhesion) force (perpendicular to the substrate) on the artificial substrates of different roughness (0, 0.3, 1, 3, 9, 12, 35 and 269 μm) and on the abaxial (lower) side of different plant leaves (B. oleracea, S. melongena, P. avium and R. hybrida).

Prior to the force measurements, insects were weighed on a micro-balance (Mettler Toledo AG 204 Delta Range, Greifensee, Switzerland). Experimental adult insects were anaesthetized with carbon dioxide for 60 s and made incapable of flying by gluing their forewings together with a small droplet of melted wax. Before starting the experiments, adults were left to recover for 30 min. All the experiments were performed during the daytime at 25 ± 2 °C temperature and 50 ± 5% RH.

Centrifugal force tester experiments (larvae, adults)

The centrifugal force tester (Gorb et al., 2001) is constituted of a metal drum covered by a substrate disc to be tested. The metal drum is driven by a computer-controlled motor. Just above the disc, the fibre-optic sensor monitored by the computer is placed.
After the positioning of the insect on the horizontal disc, the centrifuge drum was allowed to begin the rotation at a speed of 50 rev min$^{-1}$ (0.883 rev s$^{-1}$). The position of the insect on the drum was monitored by using a combination of a focused light beam and a fibre-optical sensor. The drum speed was continuously increased until the insect lost its hold on the surface under centrifugal force. The rotational speed at contact loss, the last position of the insect on the drum (radius of rotation) and the insect mass were used to calculate the maximum frictional component of the attachment force. In total, 10 females of 	extit{P. quatuordecimpunctata} and 10 females of 	extit{H. axyridis} as well as 11 larvae (fourth instar) of 	extit{P. quatuordecimpunctata} and 11 larvae (fourth instar) of 	extit{H. axyridis} were tested.

**Biopac force tester experiments (adults, eggs)**

The Biopac force tester consisted of a force sensor FORT-10 (10 g capacity; World Precision Instruments Inc., Sarasota, Florida) connected to a data acquisition unit MP 100 (Biopac Systems Ltd., Goleta, California) (Gorb et al., 2010). Data were recorded using AcqKnowledge 3.7.0 software (Biopac Systems Ltd.).

1. **Adults.** Experimental insects were anaesthetized with carbon dioxide for 60 s and one end of a human hair (about 15 cm long) was fixed with a droplet of molten wax to their thorax. Before starting the experiments, insects were left to recover for 30 min. The insect was attached to the force sensor by means of the human hair glued to its thorax (Supporting information, Fig. S1a,b) and was allowed to move on the test substrate in a direction perpendicular to the force sensor (and parallel to the substrate). The force generated by the insect walking upright and upside down (in consideration of the negative geotropism shown by the tested species during oviposition) on plastic [the plastic from Petri dishes used in the oviposition choice experiments, water contact angle of 91.94$\pm$1.76$^\circ$ (mean$\pm$SD)] was measured. Force–time curves were used to estimate the maximal pulling force produced by tethered running insects (traction). In total, seven females of 	extit{P. quatuordecimpunctata} and nine females of 	extit{H. axyridis} were tested.

2. **Eggs.** To test egg adhesion to the different artificial surfaces (represented by silicone negative casts obtained from polishing paper with different roughness of 0, 0.3, 1, 3, 9, 12, 35 and 269 $\mu$m) and natural surfaces (abaxial side of the leaves of 	extit{S. melongena}, 	extit{B. oleracea}, 	extit{P. avium} and 	extit{R. hybrida}), the substrate with the egg clutch (about 1 h after deposition) was firmly attached to a glass microscope slide, which was fixed to a motorized micromanipulator DC3314R and a controller MS314ZU (World Precision Instruments Inc.). At the beginning of each experiment, the apical portion of a single egg was attached by means of a small drop of glue (Super Attack, Locite Henkel Adhesives, Düsseldorf) to a pin (with the sharp point removed) connected to the force sensor (Supporting information, Fig. S1e,f). The micromanipulator was applied to pull the microscope slide with the egg clutch in the direction perpendicular to the microscopic slide away from the force sensor until the egg detached from the surface. The pull-off force during the detachment event was measured and related to the mean contact area of the egg glue of each species. To measure the egg glue contact area, observations under stereomicroscope of the ladybird eggs laid on a microscope slide, stained with methylene blue, were performed. Twenty-one eggs of 	extit{H. axyridis} and 13 of 	extit{P. quatuordecimpunctata} were measured. The number of replicates for each species and substrate is reported in Table 1.

**Statistical analysis**

In the oviposition choice experiments, the percentage of tests out of the total number of replicates in which the ladybird females laid the eggs on each area or surface was calculated. The dichotomous paired data obtained in the oviposition choice experiments were analysed using the Cochran’s Q test to evaluate the positive or negative geotropism and the substrate roughness effect on oviposition behaviour of the two ladybird species, and using the McNemar test to evaluate the oviposition preference of both species towards a rough or smooth surface and towards a hydrophilic or hydrophobic surface.

In the force experiments, the force produced by insects was divided by the body mass to obtain the safety factor. In the experiment on plastic surface with ladybird females walking upright and upside down, data were analysed with two-way repeated measures ANOVA (Statistica 6.0, Statsoft Inc. 2001) considering the two species and the walking positions as factors. In the force experiments performed with adult females and with larvae of the two ladybird species on substrates covered with polishing papers with different roughness, data were analysed with two-way repeated measures ANOVA considering the two ladybird species and the different roughness as factors. For significant factors, the unequal N HSD Tukey test was used as post-hoc test (Statistica 6.0, Statsoft Inc. 2001). In the force experiments with females to hydrophilic and hydrophobic glass, data were analysed with two-way repeated measures ANOVA.

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**Table 1.** Number of replicates in Biopac force tester experiments to evaluate the egg adhesion to artificial surfaces and natural surfaces.

| Asperity size ($\mu$m) | Tested plants  | Solanum melongena | Brassica oleracea | Prunus avium | Rosa hybrida |
|-----------------------|----------------|--------------------|-------------------|--------------|-------------|
| 0                     |                |                    |                   |              |             |
| 0.3                   |                |                    |                   |              |             |
| 1                     |                |                    |                   |              |             |
| 3                     |                |                    |                   |              |             |
| 9                     |                |                    |                   |              |             |
| 12                    |                |                    |                   |              |             |
| 35                    |                |                    |                   |              |             |
| 269                   |                |                    |                   |              |             |

- **Propylea quatuordecimpunctata**
  - 32
  - 20
  - 23
  - 25
  - 20
  - 18
  - 22
  - 21
  - 18
  - 9
  - 8
  - 18
- **Harmonia axyridis**
  - 41
  - 26
  - 20
  - 32
  - 33
  - 20
  - 22
  - 33
  - 14
  - 10
  - 34
  - 8
considering the two ladybird species and the two different substrates as factors.

In the egg adhesion experiments on artificial surfaces with different roughness and on the abaxial side of the different plant leaves, data were analysed with two-way ANOVA considering the two ladybird species and the different substrates as factors. For significant factors, the unequal N HSD Tukey test was used as post-hoc test. Before the analysis, all the data were subjected to Box–Cox transformations to reduce data heteroscedasticity and nonparametricity (Sokal & Rohlf, 1998).

Results

Oviposition site selection

In the behavioural assays examining geotropism in the oviposition behaviour of the two ladybird species, the percentage of the tests with eggs laid on the top of the Petri dish was significantly higher than that of tests with eggs laid on the bottom or on the border in both species. No significant difference was observed between tests with eggs laid on the border vs. on the bottom of the Petri dish (P. quatuordecimpunctata: Cochran’s Q, \( X^2 = 28.47, df = 2, P < 0.001 \); H. axyridis: Cochran’s Q, \( X^2 = 28.58, df = 2, P < 0.001 \)) (Fig. 1a,b).

In the dual choice experiments testing the oviposition preference of both species towards a rough or smooth surface, the percentage of tests with eggs laid on the rough surface was higher than that of tests with eggs laid on the smooth surface in P. quatuordecimpunctata (McNemar’s, \( X^2 = 12.9, df = 1, P < 0.001 \)), whereas there was no significant difference in the percentage of tests with eggs laid on the two kinds of surfaces in H. axyridis (McNemar’s, \( X^2 = 0, df = 1, P = 1.00 \)) (Fig. 1c,d).

In the experiments aimed to evaluate the substrate roughness effect on oviposition behaviour of P. quatuordecimpunctata (on the different sectors with substrates having different asperity sizes), the percentage of tests with eggs laid on the different sectors characterized by substrates with different asperity sizes was significantly higher on the surfaces with a small asperity size, whereas it tends to reduce on the surfaces with higher asperity size (Cochran’s Q, \( X^2 = 48.05, df = 7, P < 0.001 \)) (Fig. 1e).

The dual choice experiments with hydrophilic vs. hydrophobic surfaces revealed a higher percentage of tests with eggs laid on the hydrophilic surface than on the hydrophobic surface in both species (P. quatuordecimpunctata: McNemar’s, \( X^2 = 7.69, df = 1, P = 0.003 \); H. axyridis: McNemar’s, \( X^2 = 13.14, df = 1, P < 0.001 \)) (Fig. 1f,g).

Adult attachment

The tarsi of P. quatuordecimpunctata and H. axyridis are composed of four tarsal segments. The tarsal attachment organs in both species are similar and consist of a pair of pretarsal claws with a basal tooth and two hairy pads located on the ventral side of the first and second tarsal segments (Fig. 2a,b). Each hairy pad is covered with numerous ‘tenent setae’ with a
Fig. 2. Attachment devices of the different life stages of *Harmonia axyridis* (a,g,h) and of *Propylea quatuordecimpunctata* (b–f,i) visualized with cryo-SEM. (a,b) Ventral view of the tarsus of the female. Note the tarsal attachment organs represented by a pair of pretarsal claws (CL) with a basal tooth and two hairy pads (HP) located on the ventral side of the first (I) and second (II) tarsal segments; (c,d) Details of hairy pads covered by numerous ‘tenent setae’ composed of a shaft and terminal plate (arrows), whose shape changes from a sharp tip in the proximal tarsus (c) to a widened tip in the distal tarsus (d); (e,g) Lateral view of the tarsi of larvae showing a single pretarsal claw (CL) and tenent setae (arrows); (f,h) Larval fleshy pygopodium located at the end of the abdomen; (i) Eggs attached to a microscope slide showing a thin layer of glue typically forming a disc (arrow) on the surface.
Salerno et al. setal shaft and a terminal plate (endplate), whose shape changes from the proximal to the distal tarsomere. Type 1 setae with a pointed, sharp tip (Fig. 2c) are localized mainly on the first tarsal segment, whereas type 2 setae with a flattened and widened end plate called spatula (Fig. 2d) are localized mainly on the second tarsomere (Fig. 2c,d).

In the traction force experiments on plastic surface with ladybird females walking upright and upside down, we observed no significant difference in the attachment ability during upright vs. upside down walking in either species (F = 0.07, df = 1,15, P = 0.789). Moreover, no significant difference was recorded either between species (F = 112.21, df = 1,15, P = 0.004) and also no significant interaction between species and surfaces was found (F = 0.811, df = 1,15, P = 0.382) (Fig. 3a).

The centrifugal experiments on substrates with different roughness (0, 0.3, 1, 3, 9, 12, 35 and 269 μm) revealed no significant difference in the overall attachment ability between the two species (F = 2.46, df = 1,18, P = 0.251). The attachment ability of P. quatuordecimpunctata was significantly higher on smooth surface and on surfaces with an asperity size of 9 and 12 μm, intermediate on surfaces with an asperity size of 3, 35 and 269 μm and significantly lower on surfaces with an asperity size of 0.3 and 1 μm (Fig. 3b). The attachment ability of H. axyridis was significantly higher on smooth surface and on surfaces with an asperity size of 3 and 12 μm, intermediate on surfaces with an asperity size of 9, 35 and 269 μm and significantly lower on surfaces with an asperity size of 0.3 and 1 μm (Fig. 3b) (F = 46.74, df = 7,126, P < 0.001). No significant interaction between species and surfaces was recorded (F = 0.74, df = 7,126, P = 0.642).

In the centrifugal force measurements on hydrophilic and hydrophobic glass, the attachment ability of both species was significantly higher on a hydrophilic than on a hydrophobic surface. No difference between the species and no significant interaction between species and surfaces were found (species: F = 1.41, df = 1,18, P = 0.251; substrates: F = 251.43, df = 1,18, P < 0.001; species x substrates: F = 0.147, df = 1,18, P = 0.706) (Fig. 3c).

Larva attachment

The attachment devices of larvae of all four instars of both species are similar (Fig. 2e–h) and are constituted of a single pretarsal claw, tarsal tenent setae (Fig. 2e,g) and a fleshy pygopodium or ‘anal organ’ at the end of the abdomen (Fig. 2f,h). Tenent setae are located both on the dorsal and ventral sides of the tarsus. The distal-most have a widened end plate (spatula) (Fig. 2e,g).

In the centrifugal experiments on substrates covered with polishing paper having different roughness (0, 0.3, 1, 3, 9, 12, 35 and 269 μm), significant differences between the species, among the substrates and in the interaction between species and surfaces were recorded (species: F = 211.61, df = 1,19, P < 0.001; substrates: F = 1106.38, df = 4,76, P < 0.001; species x substrates: F = 34.86, df = 4,76, P = 0.011). In particular, the attachment ability of both species was significantly higher on the smooth surface than on all the other surfaces; in both species
The eggs of ladybird species can be attached to the surface owing to a thin layer of glue typically forming a disc on the surface (Fig. 2i). In the pull-off experiments with silicone negative casts obtained from polishing paper having different roughness, significant differences between the species and among the substrates were recorded, whereas no significant interaction was present between the species and the surfaces (species: $F = 13$, $df = 1,386$, $P < 0.001$; substrates: $F = 1,386$, $P < 0.001$; species $\times$ substrates: $F = 1, df = 7,386$, $P = 0.174$) (Fig. 5a). In particular, the eggs of *P. quatuordecimpunctata* revealed a similar adhesive strength to the surfaces with an asperity size of 0, 0.3, 1, 3, 9 and 35 $\mu$m and a significantly lower adhesive strength to the surface with an asperity size of 269 $\mu$m. The eggs of *H. axyridis* showed a higher adhesive strength to surfaces characterized by an asperity size of 0, 0.3, 1, 3 and 35 $\mu$m, an intermediate to surfaces with an asperity size of 12 $\mu$m and a lower to the surface with an asperity size of 269 $\mu$m (Fig. 5a). The comparison of the morphology of the glue disc surface of the eggs detached from the different tested silicone surfaces with the original polishing paper having different roughness (Fig. 6) revealed a great ability of the egg glue in both species to adapt properly to the different surfaces faithfully reproducing the shape and size of the asperities. This was especially true for smooth substrates (Fig. 6a–c) and for substrates with a small or medium asperity size (Fig. 6d–l), whereas for substrates with a very high roughness, such as 269 $\mu$m (Fig. 6n,o), the glue of most of the laid eggs could not form a complete basal disc on the surface (Fig. 6m) and could not reach the deep cavities on the surface (Fig. 6n), thus not allowing a firm attachment of the egg.

In the experiments with the abaxial side of different plant leaves (*S. melongena*, *B. oleracea*, *P. avium* and *R. hybrida*), significant differences between the ladybird species and among the plant species as well as their interaction were recorded (ladybird species: $F = 41$, $df = 1133$, $P < 0.001$; plant species: $F = 50$, $df = 3133$, $P < 0.001$; ladybird species $\times$ plant species: $F = 11$, $df = 3133$, $P < 0.001$) (Fig. 5b). In particular, the eggs of *P. quatuordecimpunctata* revealed a similar adhesive strength to the abaxial side of the leaves of *P. avium* and *R. hybrida*, which was significantly higher than that recorded on the leaves of *S. melongena* and *B. oleracea*, showing any significant difference between the latter. The eggs of *H. axyridis* demonstrated a higher adhesive strength to *R. hybrida* leaf, which was significantly higher than that on *S. melongena* leaf, whereas the adhesive strength to *B. oleracea* and *P. avium* was intermediate.

The surface features of the abaxial side of the leaf of the different tested plant species were characterized under cryo-SEM (Figs 7 and 8). In particular, the abaxial side of the *B. oleracea* leaf (water contact angle 152.5 $\pm$ 45.05$^\circ$, mean $\pm$ s.d.) is entirely and uniformly covered by prominent three-dimensional epicuticular wax projections (Fig. 7a). The dense wax coverage (ca. 20 projections per 100 $\mu$m$^2$) consists of round or angular tubules with characteristic dendrite-like branches on their tops (Fig. 7b). The tubules stay approximately perpendicular to the leaf surface and are nearly completely hidden under the branches situated almost parallel to the surface. The branches are composed of thin

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filaments having various length (length: $1.98 \pm 0.85\, \mu m$, $N = 13$; diameter: $0.06 \pm 0.02\, \mu m$, $N = 10$). These filaments are often partly fused to different degrees. Stomata occur very densely (ca. 250 mm$^{-2}$) on the leaf surface (Fig. 7a).

In *P. avium*, the abaxial leaf surface (water contact angle $102.64 \pm 4.70^\circ$, mean ± s.d.) looks rather uneven because of the slightly convex epidermal cell shapes and convex leaf veins. There are several types of surface structures here. Nonglandular, uniseriate, very long thread-shaped (length: $902.08 \pm 211.80\, \mu m$, $N = 10$; diameter: $17.76 \pm 2.27\, \mu m$, $N = 14$) trichomes having a microsculptured surface and bearing a multicellular socket at the base are scattered in a rather small number (4–7 mm$^{-1}$) mostly along the main vein (Fig. 7c,e). Numerous stomata (abundance: ca. 200 mm$^{-2}$) are spread all over the leaf area (Fig. 7d). Cuticular folds building groups around the stomata are rather short (length: $14.68 \pm 7.00\, \mu m$, $N = 20$) and shallow, and their running directions often differ between neighbouring domains (Fig. 7f). The folds associated with veins are much longer (length: $14.68 \pm 7.00\, \mu m$, $N = 20$) and always run clearly parallel to the

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**Fig. 5.** Egg adhesion of *Propylea quatuordecimpunctata* and *Harmonia axyridis* to artificial and natural surfaces. (a) Adhesive strength (pull-off force/attachment area) to artificial substrates of different roughness (0, 0.3, 1, 3, 9, 12, 35 and 269 μm); (b) Adhesive strength to the abaxial side of plant species having different surface features. Boxplots show the interquartile range and the median ( ), whiskers indicate 1.5× interquartile range, × shows the arithmetic mean and 'o' shows outliers. Boxplots with different upper case letters and lower case letters, respectively, are significantly different at $P < 0.05$ (Two-way ANOVA, Tukey unequal N HSD post-hoc test).
Fig. 6. Glue disc of the eggs of *Propylea quatuordecimpunctata* detached from silicone negative casts obtained from polishing paper with different roughness, 0 μm (a,b), 1 μm (d,e), 12 μm (g,h), 35 μm (j,k) and 269 μm (m) and the corresponding polishing paper, 0 μm (c), 1 μm (f), 12 μm (i), 35 μm (l) and 269 μm (n,o), visualized with cryo-SEM. Note that the egg glue adapted perfectly to the different surfaces faithfully reproducing the shape and size of the asperities in all tested substrates (a–l) except on the highest tested roughness (n,o), where the glue could not form a complete basal disc on the surface (arrow).

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Fig. 7. Abaxial leaf surfaces in *Brassica oleracea* (a,b) and *Prunus avium* (c–g), cryo-SEM. (a) General view of the surface; (b) Details of the epicuticular wax coverage; (c) Leaf area with the main vein bearing nonglandular trichomes; (d,f) Leaf area with stomata and cuticular folds; (e) Surface of the nonglandular trichome; (g) Surface microsculpturing. CF, cuticular fold; FL, wax filament; NT, nonglandular trichome; ST, stoma; TU, wax tubule; VN, vein; WP, wax projection.

veins. The surface additionally bears microscopic epicuticular wax irregularities having various irregular shapes (Fig. 7g).

The *R. hybrida* abaxial leaf surface (water contact angle 101.75 ± 10.64°, mean ± s.d.) is not flat, but noticeably sculptured with a dense network of prominent veins, slightly convex epidermal cells and slightly sunk stomata occurring in a high number (ca. 150 mm⁻²) (Fig. 8a). The surface microtopography is clearly rough because of the presence of epicuticular wax irregularities of extremely varying shapes – from irregular to scale-like projections (Fig. 8b).

The abaxial side of *S. melongena* (water contact angle 147.14 ± 10.30°, mean ± s.d.) leaf bears a prominent pubescence due to densely situated (ca. 17 mm⁻²) nonglandular stellate trichomes (Fig. 8c). These multicellular surface features are composed of one vertical arm and several (usually >5; 7.39 ± 0.82 trichome⁻¹, N = 18) spreading side arms, which are incumbent to the leaf surface (Fig. 8d). The arm length (269.13 ± 107.36 μm, N = 25) varies greatly both between different trichomes and within the same trichome. Due to a high number and relatively high length of the side arms, as well as high trichome abundance, a multi-layer structure of the indumentum is formed (Fig. 8c). The trichome surface, although bearing indistinct flat microscopic irregularities, looks rather smooth (Fig. 8e). In addition to nonglandular stellate
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Fig. 8. Abaxial leaf surfaces in *Rosa hybrida* (a,b) and *Solanum melongena* (c–f), cryo-SEM. (a) General view of the surface; (b) Surface microsculpturing; (c) General view showing nonglandular trichomes; (d) Nonglandular trichome in detail; (e) Surface of the nonglandular trichome; (f) Glandular trichome and stoma. NT, nonglandular trichome; SA, side arm; ST, stoma; TH, glandular trichome head; TS, trichome stalk; VA, vertical arm; VN, vein; WP, wax projection.

trichomes, solitary (<1 mm⁻²) glandular capitate trichomes, being rather small in size (length: 61.21 ± 19.51 μm, N = 4) and having relatively short stalks and ellipsoid multicellular heads (stalk to head lengths ratio ca. 1:1), are present (Fig. 8f). The epidermal cells surface underneath trichomes is smooth, but somewhat uneven, and bears numerous (ca. 150 mm⁻²) stomata (Fig. 8f).

In the cryo-SEM, we could observe that the big stellate trichomes of *S. melongena* did not allow the egg glue to reach the leaf surface (Fig. 9a,b), while the glue could adhere to the wax projections of *B. oleracea* (Fig. 9c–e); however, the wax projections detached very easily from the leaf surface, thus causing the detachment of the eggs (Fig. 9f). On the surfaces of *P. avium* and of *R. hybrida* leaves, the egg glue could adhere to the leaf surface, thus replicating the leaf decorations (Fig. 9g,h).

Discussion

Few insect species have been studied regarding their attachment ability along their life cycle, among them the beetles *Gastrophysa viridula* (De Geer) and *Galerucella nymphaea* (L.) (Coleoptera: Chrysomelidae) (Grohmann et al., 2014; Zurek et al., 2017), the giant stick insect *Eurycantha calcarata*
Fig. 9. Egg glue interaction of *Harmonia axyridis* eggs with the abaxial side of the leaves having different surface features, visualized with cryo-SEM. (a,b) Egg interaction with the big stellate trichomes (ST) of *Solanum melongena*, which did not allow the egg glue (G) to reach the leaf surface (arrow); (c–f) Egg interaction with the wax projections (WC) of *Brassica oleracea*. Note that the glue adhered to the wax projections (arrows), but these last detach easily from the leaf surface (arrow heads) leaving egg glue prints (asterisk) on the leaf; g,h, Egg interaction with the leaf of *Prunus avium* (g) and *Rosa hybrida* (h). The egg glue could adhere well to the leaf surface, thus replicating the leaf decorations.
Oviposition and attachment in ladybirds

Adult stage: oviposition site selection and attachment ability

Our oviposition choice experiments revealed similarities and differences between the two tested species. In particular, coccinellids express different degrees of geotropism in egg-laying behaviour (Iperti & Quilici, 1986), and our experiments confirmed the negative geotropism of *P. quatuordecimpunctata* (Iperti & Quilici, 1986) and extended this preference also to *H. axyridis*. Such a negative geotropism is in agreement with the good attachment ability of both species at the adult stage when walking upside down on plastic surfaces, which was not different from their attachment ability when walking upright, as observed in our experiments. Such ability allows ladybirds to keep a firm attachment when ovipositing on the abaxial leaf side, allowing eggs to be protected from predators, solar radiation and rain.

Both ladybird species at the adult stage showed oviposition preference towards a hydrophilic surface compared with a hydrophobic one. Such a preference is in line with higher attachment ability of the females of both species to hydrophilic glass in comparison with hydrophobic glass. A decrease in the attachment force on artificial surfaces with an increasing water contact angle has been reported in many other insect species belonging to Diptera such as *Ceratitis capitata* Wiedemann and *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) (Rebora et al., 2020a; Salerno et al., 2020a), Heteroptera such as *N. viridula* (Salerno et al., 2017) and in different other species belonging to Coleoptera such as *G. viridula* (Gorb & Gorb, 2009b), *Cylas puncticollis* (Boheman) (Coleoptera: Brenidae) (Läken et al., 2009), *Cryptoplaenus montrouzieri* Mulsant (Coleoptera: Coccinellidae) (Gorb & Gorb, 2020) and *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) females (Gorb et al., 2010; Hosoda & Gorb, 2012). The reduction of the attachment force on hydrophobic substrates could be due to the reduced role of the adhesive fluid in generation of capillary forces (England et al., 2016). In any case, as the aphids, which are considered a factor for oviposition, show a preference for hydrophilic substrates (Friedemann et al., 2015), it is coherent to find the same preference in Coccinellidae as well.

Regarding the role of surface roughness in the oviposition choice behaviour, differences were observed between the two tested species. For *P. quatuordecimpunctata* females, the surface roughness is an important factor in the egg-laying behaviour: they preferentially chose smooth surfaces and surfaces characterized by low asperity sizes compared with surfaces with high asperity sizes. On the contrary, for *H. axyridis*, the substrate roughness had no effect when choosing the oviposition substrate. Such a behavioural difference and the ability of *H. axyridis* to use substrates with different surface features as oviposition site could be linked to the high ecological valence of this species, contributing to increase its role as invasive species threatening the diversity of native aphidophagous species, such as *P. quatuordecimpunctata*, through direct competition (Roy & Wajnberg, 2008).

When considering the attachment ability of the adult *P. quatuordecimpunctata* to artificial surfaces with different asperity sizes, we can observe the typical trend described in most of the insects tested so far on rough surfaces, such as the flies *Musca domestica* L. (Diptera: Muscidae) (Peressadko & Gorb, 2004) and *C. capitata* (Salerno et al., 2020a), the beetles *G. viridula* (Bullock & Federle, 2011; Zurek et al., 2017) and *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) (Voigt et al., 2008), the bug *N. viridula* (Salerno et al., 2017, 2018b), the much larger stick insects *Sungava inexpetcta* Zompro (Phasmatodea: Heteropterygidae) and *Medauroidea extradentata* (Wat-tenwyl) (Phasmatoidea: Phasmatidae) (Büscher & Gorb, 2019) and the spider *Philodromus dispar* Walckenaer (Araneae: Philodromidae) (Wolff & Gorb, 2012). Indeed, the higher attachment ability of ladybird females was recorded on smooth surfaces and on surfaces with larger asperities, whereas microrough substrates with asperity sizes 0.3 and 1 μm strongly decreased attachment ability owing to the reduction of the contact area of hairy pads. The higher attachment ability recorded on surfaces with larger asperities (3–12 μm) can be due to the adhesion of tenent setae to smooth islands within rough substrates, but also due to the role of claws, which typically can be used, when the substrate roughness is larger than the claw tip diameter (Dai et al., 2002; Song et al., 2016).

Larval attachment ability

The higher attachment ability on hydrophilic glass compared with hydrophobic glass has been recorded also in the larvae of both species. A greater affinity to hydrophilic glass compared with hydrophobic one was reported also in other insect nymphs and larvae such as the green stinkbug *N. viridula* (Salerno et al., 2020b), the leaf beetle *G. viridula* with thoracic legs and pygopodium (Zurek et al., 2015) and *Rhadinoceraea micans* (Schrank) (Hymenoptera: Tenthredinidae) provided with thoracic legs, pygopodium and abdominal legs (Voigt & Gorb, 2012), whereas the larvae of *G. nymphaea* with thoracic legs and pygopodium showed a higher attachment ability to surfaces with water contact angle similar to that of their host plant (80°) and lower attachment ability on other surfaces with higher or lower water contact angles (Grohmann et al., 2014). We can hypothesize that the chemical composition of the tarsal fluid of the tested ladybird species at the larval stage is similar to that of the adult stage and shows a higher affinity to hydrophilic surfaces.

When looking at the attachment of larvae on surfaces with different roughness, we can observe high attachment ability, especially on smooth surfaces, and a reduction in attachment on all other tested surfaces. The larval safety factors of the tested ladybird species on smooth surfaces were about 64 for *P. quatuordecimpunctata* and 142 for *H. axyridis* and were quite high if compared with those of other Coleoptera larvae equipped.

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with smooth tarsal adhesive pads and pygopodium, which were tested so far: 15 in *G. nymphea* (Grohmann et al., 2014) and 35 in *G. viridula* (Zurek et al., 2017). As in adults, the larval attachment ability of *P. quatuordecimpunctata* and *H. axyridis* was strongly reduced on microrough surfaces and intermediate on surfaces with larger irregularities, but differently from the adult, larvae could not attach to rough surfaces with a force similar to that exerted on the smooth surface. This behaviour is similar to that observed in the larva of *G. viridula* (Zurek et al., 2017), the only coleopteran larva tested so far on rough surfaces, where even on the coarsest surface, friction forces remained lower than those on a smooth substrate. This could be probably due to the different attachment devices of adults and larvae. Larvae have a single pretarsal claw, which probably reduces their ability to interlock with rough surfaces compared with adults equipped with a pair of claws. Additionally, we hypothesize that the pygopodium is more effective in the attachment to smooth than to rough surfaces.

**Egg attachment ability**

Coccinellidae egg glue is a hydrogel with a high protein content (glycine and serine) (Li et al., 2008) forming both a very thin layer on the egg chorion and a basal disc able to keep the egg anchored to the surface. When observing the egg attachment ability to different surfaces, it is clear that the egg glue of both ladybird species can adapt to artificial surfaces characterized by different asperity sizes without significant differences. Detachment experiments revealed the lowest pull-off force on substrates with a very high roughness (269 μm), because the glue of most of the laid eggs was not able to form a complete basal disc on the surface and to reach the deep cavities on the surface, as was clearly evident in our observations in cryo-SEM. In any case, such a reduction was not significantly different from less rough surfaces. These results seem to contradict the basic rule that the rougher the surface, the larger the contact area and the stronger the adhesion of a glue (Habenicht, 2002). However, if the viscosity of the adhesive on the eggs’ surface is rather high and the attraction to the chorion is rather strong, the actual contact area does actually not sufficiently increase, as the glue does not reach into the depths of the surface corrugations. This could be the case in coccinellid eggs covered by a rather tight envelope of glue (Fig. 2i), which was also described for the egg adhesive of the leaf insect *Phyllium philippinicum* Hennemann et al. (Phasmatodea: Phylliidae) (Büscher et al., 2020a, b). In other studies, testing insect egg attachment such as of the codling moth *Cydia pomonella* L. (Lepidoptera: Tortricidae) to fruit and leaf surfaces of different apple cultivars (Al Bitar et al., 2012, 2014), stronger forces were recorded to detach the eggs from the lower leaf side compared with those from the upper leaf side. This result was explained by the presence of trichomes, dispersed wax projections, corrugations and cuticle foldings on lower leaf surfaces, leading to an increase in the contact area with the egg glue. In our experiments on plant leaves with different surface features, we could observe that the presence of big trichomes or waxes reduced ladybird egg adhesion in comparison with other leaf surface structures, such as cuticular folds, prominent veins, convex epidermal cells and stomata. Indeed, in the presence of big trichomes, such as those of *S. melongena*, the glue could not reach the leaf surface as evident in our cryo-SEM observations. In the presence of a thick layer of structural waxes, such as in *B. oleracea*, the glue could adhere to the wax coverage, but the wax projections detached very easily from the leaf surface, thus causing the detachment of the eggs. In this regard, the egg glue of *P. quatuordecimpunctata* and *H. axyridis* reveal properties similar to those observed in the egg glue of the asparagus beetle *Crioceris asparagi* (L.) (Coleoptera: Chrysomelidae), when adhering to the waxy leaves of its host plant (Voigt & Gorb, 2010): the egg glue can wet the wax projections and incorporate them into the adhesive matrix. The same properties were described in the egg glue of *C. pomonella*, able to wet the hydrophobic apple fruit surface (Al Bitar et al., 2014). Our similar results on the eggs of predaceous insects such as ladybirds could indicate that the surfactant-like nature of the egg glue is not limited to specialized species but could be widespread in insects owing to the proteinaceous nature of the egg adhesive helping to overcome the hydrophobicity of plant surfaces. In any case, the egg detachment happens easily due to fracture of wax projections or their separation from the plant epidermis, as evident from our cryo-SEM observations and force experiments, thus making the wax an effective protective structure against adhesion of phytophagous insects (e.g. Salerno et al., 2018a) and predatory insects, such as ladybirds, not only at the adult and larval stage (Eigenbrode, 2004; Eigenbrode et al., 2009), but also at the egg stage.

Further studies testing ladybird egg adhesion to artificial surfaces could better clarify the egg glue properties because the egg glue of some insect species such as the stick insect *P. philippinicum* adhere better to hydrophilic than to hydrophobic substrates (Büscher et al., 2020a).

**Conclusions**

In the present study, using the experiments on the oviposition preference in females and attachment ability along all the life cycle in *P. quatuordecimpunctata* and *H. axyridis*, we tried to answer to the following questions. Is there any difference in the attachment ability of the different life stages to surfaces with different roughness and wettability? Is there any relationship between the choice of a substrate with peculiar surface features and attachment ability of adults, eggs and larvae? We found both differences and similarities between the two species. In particular, both species preferred a hydrophilic surface as oviposition site and this can be correlated with the better performance of both larvae and adults on these surfaces compared with hydrophobic ones owing to the specific chemical composition of their adhesion fluid. In our experiments, we could not compare the egg attachment force to hydrophilic and hydrophobic glass because the eggs of both species adhered too strongly to hydrophilic glass and during the pulling experiments the chorion always went broken. This suggests a very strong adhesion of the egg glue to hydrophilic surfaces in agreement with adhesion of adults and larvae. In any case, the ladybird egg glue of both ladybird species, similar to other insect species tested so far, can wet

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hydrophobic surfaces, such as those of many plant leaves. The egg glue can partially wet 3D wax covered plant surfaces, but the egg adhesion is prevented here owing to the wax projections detachment in the plant species studied.

The surface roughness has an important role for the oviposition site selection in *P. quatuordecimpunctata*, but not in *H. axyridis*. The preference for smooth surfaces in *P. quatuordecimpunctata* could be due to the better performance of larvae on smooth surfaces compared rough ones. The egg glue of both species can well adapt to artificial surfaces having different asperity sizes faithfully replicating their shape. This is true also for natural surfaces characterized by cuticular folds, prominent veins, convex epidermal cells and stomata. However, substrates with large surface asperities or big trichomes hinder egg adhesion, because the egg glue cannot form proper basal anchoring disc.

The results of the present research can help to better understand the interactions between predators and plants as well as shed light on the mechanical ecology of evolutionary successful group of coccinellids. Finally, it may aid in development of suitable substrates for coccinellid egg-laying, in order to improve the mass rearing technique of species used in biological control and also potentially contributes to the pest control of the invasive *H. axyridis*.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Developmental stages of *Propylea quatuordecimpunctata* and *Harmonia axyridis* used in the experiments.

Table S1. Roughness average Ra of the tested substrates.

Acknowledgements

We are very grateful to Lorenzo Austeri for his help in measuring the egg attachment forces and to Alexander Kovaliev for his help in surface roughness measurements. Bioplanet (Cesena, Italy) is greatly acknowledged for providing *P. quatuordecimpunctata* for the experiments. All the authors declare that they have no conflict of interest.

Author contributions

The study was designed by all the authors. S.N.G. and E.V.G. performed the cryo-SEM investigations. G.S., M.R. and T.H.B. performed the force experiments. G.S., M.R. and S.P. performed the behavioural experiments. E.V.G. and M.R. characterized the tested surfaces. The manuscript was written by G.S., M.R. and E.V.G. All authors discussed the analysis and interpretation of the results and participated in the final editing of the manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Accepted 6 July 2021
First published online 21 July 2021