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Attachment of *Galerucella nymphaeae* (Coleoptera, Chrysomelidae) to surfaces with different surface energy

Constanze Grohmann*, Andreas Blankenstein, Sven Koops and Stanislav N. Gorb

ABSTRACT

Numerous studies deal with insect attachment onto surfaces with different roughness; however, little is known about insect attachment onto surfaces that have different chemistry. In the present study, we describe the attachment structures of the water-lily leaf beetle *Galerucella nymphaeae* and test the hypothesis that the larval and adult stages generate the strongest attachment on surfaces with contact angles that are similar to those of leaves of their host plants. The larvae bear a smooth attachment system with arolium-like structures at their legs and a pygopodium at the abdomen tip. Adults have pointed setae on the ventral side of the two proximal tarsomeres and densely arranged spatula-shaped ones on their third tarsomere. In a centrifugal force tester, larvae and adults attained the highest friction forces and safety factors on surfaces with a water contact angle of 83 deg compared to those of 6, 26 and 109 deg. This comes close to the contact angle of their host plant *Nuphar lutea* (86 deg). The similarity in larval and adult performances might be a result of the similar chemical composition of their attachment fluid. We compare our findings with previous studies on the forces that insects generate on surfaces with different surface energies.

KEY WORDS: Adhesion, Friction, Contact angle, Insect, Biomechanics, Locomotion

INTRODUCTION

The attachment of insects to surfaces is crucial for their survival. For example, they need to adhere to the plants they live and feed on, attach to their hosts or hold onto their copulation partners. The necessity to strongly attach to their host plant holds true especially for terrestrial beetles that are associated with water-plants. The terrestrial leaf beetle *Galerucella nymphaeae* (Linnaeus 1758), for example, lives and forages mainly on the upper surface of floating leaves of the water-lilies *Nuphar* and *Nymphaea* (Smirnov, 1960). Adult beetles overwinter in the riparian areas and colonise leaves in spring. After mating, females lay clutches of eggs on the upper surface of leaves. The larvae undergo three larval stages and pupate. All stages live on the upper surface of the leaves and larvae, as well as adults, feed on the leaves. At least three generations occur per year (Wesenberg-Lund, 1943). Along with other ecological impacts, *G. nymphaeae* strongly reduces *Nuphar* stands and thus increases light transmission into the water (Kouki, 1991a; Kouki, 1993; Stenberg and Stenberg, 2012; Wahl, 2008; Wallace and O'Hop, 1985).

For larvae, as well as for adults, it is essential to stay attached to the surface of host leaves during various environmental disturbances

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(wind, waves, rain, etc.), which can cause leaves to be covered by water. According to Hippa and Koponen (Hippa and Koponen, 1986), the larvae survive underwater temporarily, having an air film visible on their cuticle, but they sink immediately when dropped below the water surface (Kouki, 1991b). The body of adult beetles is almost completely covered with long setae. Between these setae, an air film renders the beetle water repellent for some time and persists for 2 days when submerging a freshly killed specimen (Balmert et al., 2011). Larvae, as well as adults, are able to move underwater (A.B. and S.K., personal observations).

A variety of attachment organs occur in insects (Beutel and Gorb, 2001; Gorb, 2001; Scherge and Gorb, 2001), which evolved many times independently based on two main principles – hairy and smooth. Hairy attachment organs can be found, for example, in adult beetles (Bullock and Federle, 2011; Gorb and Gorb, 2002) and flies (Gorb et al., 2001; Niederegger et al., 2002), whereas smooth adhesive pads are found – amongst other taxa – in representatives of Orthoptera (Gorb et al., 2000) and Hymenoptera (Federle et al., 2001). In both types of attachment systems, a fluid that is secreted into the contact zone with the substrate contributes to the attachment performance. In beetles, this fluid is a mixture of hydrocarbons, fatty acids and alcohols, similar to the composition of their cuticular lipids (Geiselhardt et al., 2009; Geiselhardt et al., 2010; Ishii, 1987). In this so-called wet adhesion, capillary forces account largely for the attachment ability (Langer et al., 2004). The capillary forces, in turn, strongly depend on the contact angle between the fluid and the surface of particular substrate (e.g. De Souza et al., 2008a).

There are a number of papers concerning the attachment of insects to surfaces of different roughness (e.g. Beutel and Gorb, 2001; Bullock and Federle, 2011; Dai et al., 2002; Gorb and Gorb, 2009; Voigt et al., 2008). However, the impact of the surface energy on the insects' attachment ability has been analysed in a few studies only. In most of them, instead of the free surface energy, the contact angle of water on the surface was measured. In general, one can say that the smaller the contact angle, the higher the free energy of a surface. Although in some studies (Gorb and Gorb, 2009; Gorb et al., 2010; Hosoda and Gorb, 2012; Lüken et al., 2009; Prüm et al., 2013) the focus was laid on hairy attachment systems of different beetle species, others were done on smooth attachment organs of moths (Al Bitar et al., 2009) and sawfly larvae (Voigt and Gorb, 2012). The results of these papers are not consistent. For example, in two studies (Al Bitar et al., 2009; Prüm et al., 2013), no differences in the ability of beetles to attach onto surfaces with different contact angles were detected, whereas in different studies (Lüken et al., 2009; Gorb et al., 2010), the insects performed, by far, better on surfaces with higher surface energies. Thus, the role of surface energy on the adhesion ability of the insects remains unclear. Because different insect species do not only have different attachment pad structures, but also have different degrees of specialisation to various natural substrates, it might be possible that

adaptation to the substrate chemistry is species specific and might be exhibited to a greater extent in 'specialist' species compared to 'generalist' ones.

In this paper, we study the morphology of the smooth and hairy attachment structures of larval and adult *G. nymphaeae* beetles. In an experiment, we tested the hypothesis that in *G. nymphaeae*, an insect species that has specialised to live on and to attach mainly to *Nuphar lutea* leaves, the highest attachment forces are reached on surfaces with surface energies similar to that of their host plant.

RESULTS
Morphology
Larvae

The third instar larvae were roughly 7 mm long (*N*=20). All thoracic legs bear an arolium-like structure and a claw at their distal end (Fig. 1A,B,G). The terminal abdominal segment is characterised by a fleshy pygopodium (Fig. 1C–F), which strongly contributes to the total adhesion force of the larvae (C.G., A.B. and S.K., personal observations). While the larvae attach to a surface, the thoracic legs, ventral parts of the abdominal segments and the pygopodium make contact with the surface (Fig. 1E–J).

Adults

Adult beetles were ~6 mm long and 3 mm wide (*N*=26). Each leg bears five tarsal segments and a claw. The third tarsal segment consists of two lobes and the reduced forth one is hardly visible (Fig. 2A–C,E,F). A claw is situated at the distal end of each tarsus. It is composed of two halves similar in shape, each with a longer strong tip at the outer side and a half as long at the inner side. All but the forth tarsal segment are covered with long and pointed setae; however, only the third tarsal segment has more densely packed spatula-shaped setae at its the ventral side (Fig. 2D). These spatula-shaped setae are cylindrical at their base with a diameter of 1 to 3 µm and a length of 40 to 62 µm. All but the most proximal setae widen towards their tips, reaching up to 6 µm in width at their end. The upper side of these spatula-shaped setae is covered with nubs that are up to 2 µm in length. There is a tendency that the width of the setae's ends and the number of nubs increase from the proximal to the distal end of the third tarsal segment.

Females outweighed males [12.3±1.7 (mean ± s.d., *N*=4) and 10.4±1.1 (*N*=5), respectively]. We did not find distinct differences between the shape of female and male adhesive setae (Fig. 2E,F). However, female beetles possessed more setae compared to males
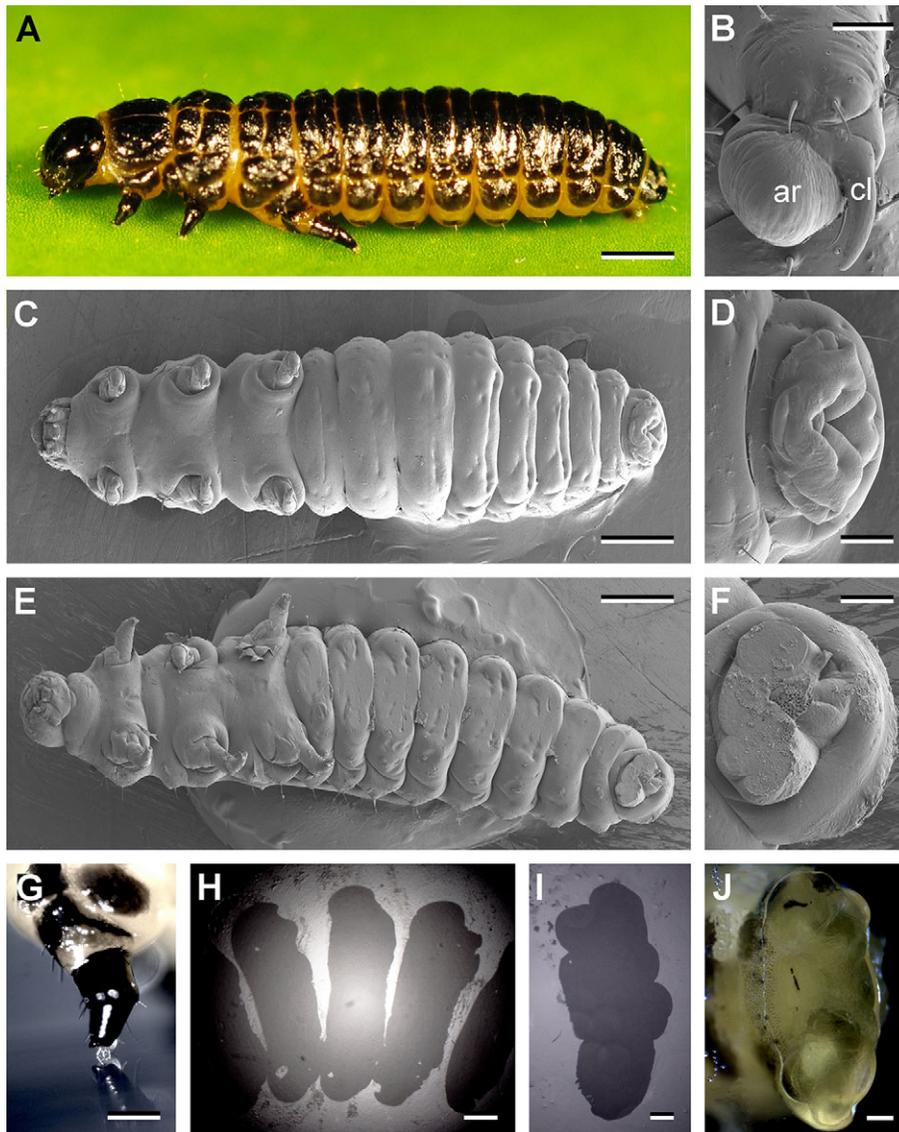

Fig. 1. Habitus and attachment organs of *Galerucella nymphaeae* at the 3rd larval stage. (A) Lateral view. (B–F) Cryo-scanning electron microscopy images. (B) Thoracic leg, right forefoot. (C) Ventral view. (D) Detail of the abdominal appendage (pygopodium). (E,F) Ventral view of a specimen frozen while it attached to a smooth surface. (E) Whole specimen. (F) Detail of the pygopodium. (G) Arolium-like structure of a thoracic leg attaching to a surface. (H,I) Contact area of abdominal segments (H) and the pygopodium (I) of a larva attaching upside down to a smooth surface. Areas in contact with glass appear dark at coaxial illumination. (J) Pygopodium attaching to a smooth glass surface, ventral view. ar, arolium-like structure; cl, claw. Scale bars: 1 mm (A,C,E); 50 µm (B); 250 µm (D,F–H); 100 µm (I,J).
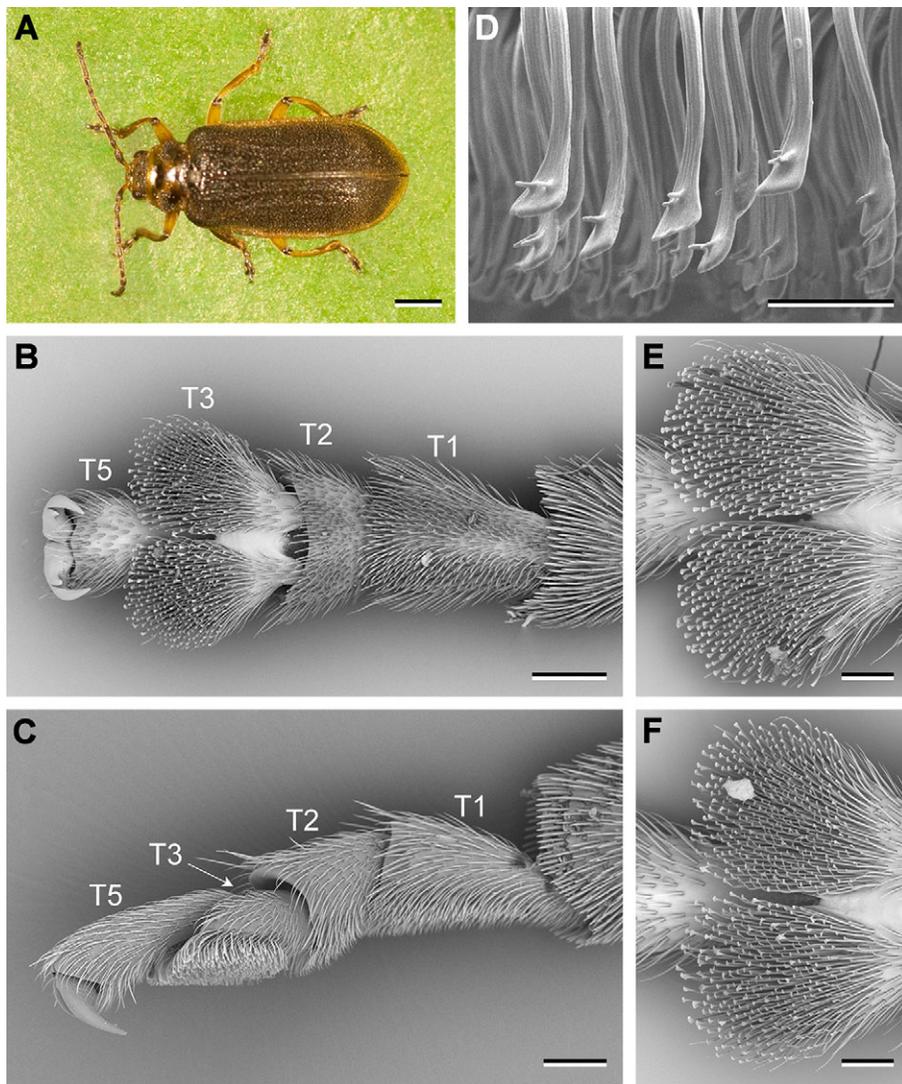
Fig. 2. Habitus and attachment organs of adult *G. nymphaeae*. (A) Beetle on its typical substrate, a floating leaf of *N. lutea*. (B) Tarsus, ventral view. (C) Tarsus, lateral view. (D) Setae of the third tarsal segment in detail, right hind leg of a male, disto–lateral view. (E,F) Third tarsal segment of a right middle leg of a female (E) and male (F) beetle, ventral view. T1–T5, tarsal segments; T4 is reduced. Scale bars: 1 mm (A); 100 µm (B,C); 10 µm (D); 50 µm (E,F).

(Student's t-test, t_7=2.521, P=0.040). When the number of setae were normalised to the body mass of the beetles, significant differences between sexes were no longer detected (Student's t-test, t_7=−0.171, P=0.869). We found different numbers of attachment hairs on the fore-, middle and hind leg (one-way repeated measures ANOVA, F_2=11.517, P<0.001) with most hairs on the middle leg (1320±134, mean ± s.d., N=12), followed by the hind leg (1285±150) and least hairs on the foreleg (1242±139; pairwise comparisons with Holm–Sidak method, P<0.001 for comparison of fore- and middle legs, P=0.031 for fore- versus hind legs, P=0.041 for middle versus hind legs).

Surface roughness of *N. lutea* leaves

The surface roughness of *N. lutea* leaves yielded roughness average (R_a) values of 5.143±1.413 µm and root mean square (rms, R_q) values of 6.729±1.493 µm (mean ± s.d., n=7).

Friction force

The median friction force calculated for all data pooled together for all surfaces was 1.50 mN (0.78–2.63 mN) for larvae and 6.97 mN (3.67–14.13 mN) for adults (median values, the interquartile range is given in brackets; N=18 larval individuals, N=30 adult individuals, n=5 repetitions for each individual on each surface).

The dimensionless safety factor (friction force divided by body weight) was calculated to account for weight differences between individuals. Its values revealed that larvae were able to withstand forces, on average, 15 (9–27) times higher than their body weight and that adults resisted forces 63 (34–109) times higher than their weight.

Both friction forces and safety factors of larval as well as adult individuals differed significantly between surfaces of different contact angles [P<0.001, linear mixed effects model with individual (N=30 adults, N=18 larvae, n=5) as the random factor and contact angle (N=4) as the fixed factor]. The friction forces and safety factors were highest on surfaces with a contact angle of 83 deg for both larval and adult individuals (Fig. 3). For Tukey's multiplicity-adjusted pairwise comparisons of the safety factors please refer to Fig. 4.

DISCUSSION
Morphology
Larvae

The general habitus of third larval instars of *G. nymphaeae* resembles those of other representatives of the family Chrysomelidae (e.g. Baselga, 2007; Hua et al., 2013). Arolium-like structures between the claws (e.g. Gannon et al., 1994; Nie et al.,
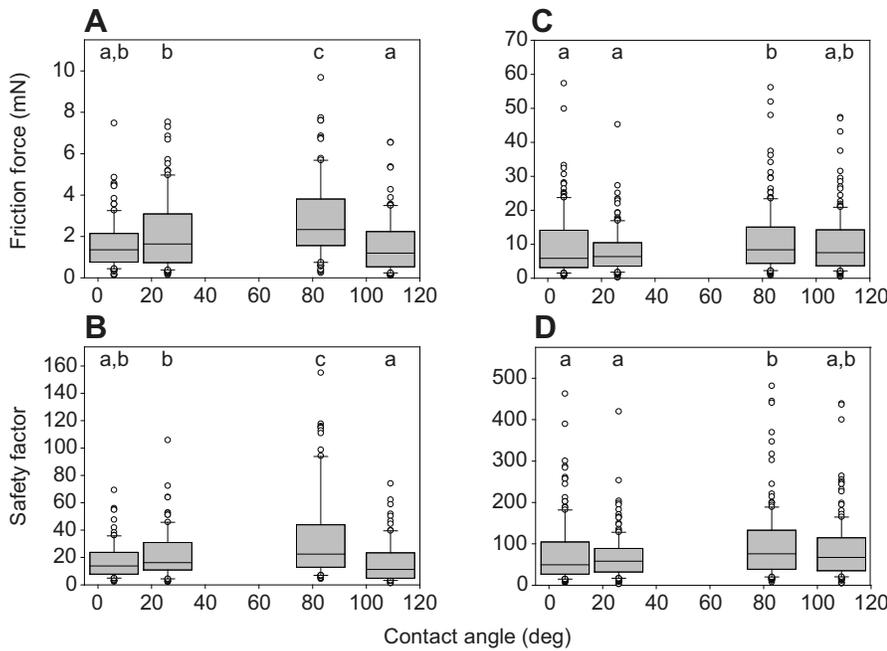
Fig. 3. Friction forces and safety factors of *G. nymphaeae* on glass surfaces with different water contact angles. (A,C) Friction forces, (B,D) safety factors, (A,B) larvae, (C,D) adults. The plots show the medians (lines within the boxes), 25th and 75th percentiles (ends of boxes), 10th and 90th percentiles (error bars) and outlying values (circles). Pairwise comparisons within a single plot differ significantly if they do not share the same letter.

2012) and the pygopodium (Nie et al., 2012) have been previously described for other chrysomelid species, but not yet for *G. nymphaeae*. The contribution of the ventral surface of abdominal segments to the attachment performance of leaf beetle larvae was demonstrated here for the first time. Based on our own observations, we assume that the pygopodium contributes more to the attachment forces than the attachment devices associated with legs. Abdominal segments seem to contribute even less to overall adhesion than the pads of thoracic legs.

Adults

The ventral sides of the two proximal tarsal segments of the species studied are covered with pointed setae, the third one is densely covered with spatula-shaped setae. In many chrysomelids, all three

visible tarsal segments have setae that can be pointed, spatula shaped or discoidal (Bullock and Federle, 2009; Gorb and Gorb, 2002; Stork, 1980a; Voigt et al., 2008). We could not find any differences in the shape of the adhesive hairs of male and female *G. nymphaeae*. This is different to the pad morphology of many other beetle species. Most male individuals in the Chrysomelidae, Cerambycidae and Coccinellidae bear discoid setae that cannot be found in females (Stork, 1980a). However, they occur only in species with rather glaborous elytra in the females. Such setae are probably adapted for attachment to the smooth elytra of females during copulation (Stork, 1980a; Stork, 1980b; Stork, 1983). We assume that the lack of discoid setae in *G. nymphaeae* is owing to the surface morphology of the females' elytra, as the elytra of *G. nymphaeae* are densely covered with water-repellent setae. Owing
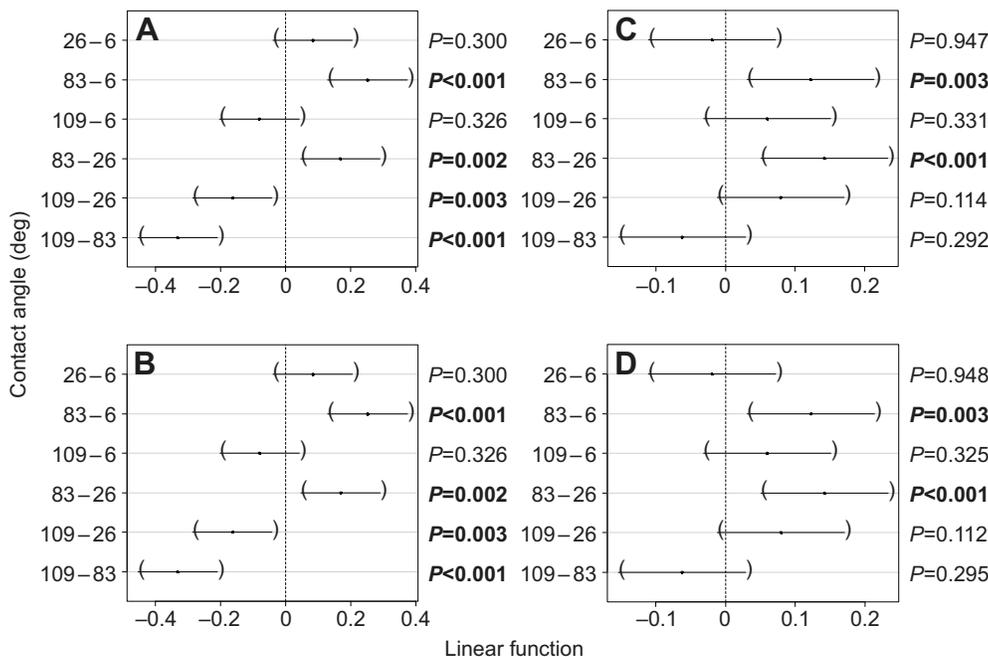

Fig. 4. *Post hoc* performance of the linear mixed model of friction forces and safety factors of *G. nymphaeae* on glass surfaces with different contact angles. (A,C) Friction forces, (B,D) safety factors, (A,B) larvae, (C,D) adults. Values of the safety factor were log_{10}-transformed before analysis. The estimators and the confidence intervals of Tukey's all-pair comparisons are given. The corresponding *P*-values are displayed on the right-hand side of each plot. *P*-values in bold indicate significant differences. If the confidence interval lies completely to the left (right) side of the zero line, the safety factor of the contact angles given first are significantly smaller (larger) compared to the contact angle given second.

to these hairy structures, it might be impossible for a male to make the proper adhesive contact between its adhesive hairs and the surface of the elytra.

The presence of small nubs or corrugations on the upper side of spatula-shaped insect setae has been described previously (Federle, 2006; Haas and Gorb, 2004). It was assumed that such surface structures prevent conglutination of neighbouring setae.

Friction force

The comparison of the friction force measured in this study with the data from other studies reveals great diversity, as the force depends not only on the insect species, but also on the weight of an individual and the method used to measure forces (centrifugal force device, traction force measurements in tethered walking animals).

We computed the safety factor to account for differences in the weight of animals and yielded values of 15 (9–27) (median value, the interquartile range is given in brackets) for larvae and 63 (34–109) for adults. Safety factor data on larval coleopterans are completely lacking in the literature. However, the safety factor values calculated from measured friction forces of adult *G. nymphaeae* lie within the same range as previously shown in similar studies using various insect taxa. Previously published safety factors are 60–71 (*Leptinotarsa decemlineata*, Chrysomelidae, female and male on glass) (Voigt et al., 2008), 45–268 (*Cylas puncticollis*, Brentidae, male and female on glass and silanised glass) (Lüken et al., 2009) and 39–70 (*Chrysolina polita*, Chrysomelidae, male and female on glass) (Stork, 1980b). In the first two studies, friction forces were measured using a centrifugal force tester, whereas the latter study dealt with traction forces generated by walking tethered beetles. For safety factors of other taxa, please, see reviews (Federle et al., 2000; Voigt and Gorb, 2012).

Friction forces and safety factors of both larval and adult *G. nymphaeae* were the highest on the silanised glass surface with a contact angle of 83 deg compared with those on plasma-treated glass (6 deg), untreated glass (26 deg) and silanised glass (109 deg). The similarity of the data obtained for larvae and adults might be explained by a possible similarity in the chemical composition of the liquid delivered into the contact zone. Because *N. lutea* leaves exhibited water contact angles of around 86 deg, the beetles' attachment abilities might be the result of a long-term evolutionary adaptation. For other insect taxa, such as ants, flies and locusts, it has been previously discussed that the oil-water mixture of the fluid allows them to attach to surfaces with different surface energies (Federle et al., 2002; Gorb, 2001; Vötsch et al., 2002). For instance, the production of a non-lipoid fraction in the tarsal secretion of *Stenus* beetle species shows that the composition of the tarsal liquid might be adapted rapidly to the contact surface because these components are produced by different glands (Betz, 2003). Further studies should hence consider (i) whether insects can adapt their attachment fluid to the surface chemistry in a short-term range, which might be the case in generalist species; (ii) whether the properties of the fluid are constant, which might be the case in specialist species; or (iii) whether modifications of the fluid are not possible in any species.

The similarity of the trend in larval and adult *G. nymphaeae* regardless of the number of contacts might be evoked by a physical principle (De Souza et al., 2008b). De Souza et al. calculated the strength of capillary forces between two surfaces that have different contact angles and state that if the sum of the two contact angles is constant, the forces decrease with increasing asymmetry in the two contact angles. Transferred to our results, we would expect the highest forces on the surface of 83 deg if the contact angle of water

on the setae tips also lies in the magnitude of 83 deg, but less forces on the surface with a 109 deg contact angle if the contact angle on the setae tips is 57 deg. In both cases, the two contact angles sum up to 166 deg. Unfortunately, to the best of our knowledge, there is no data on the contact angles of the attachment setae of insects.

However, it cannot be excluded that other factors are responsible for the generation of strong friction forces on surfaces with contact angles of around 80 deg. The dependence of capillary forces on the contact angle of the surface has also been the subject of models (De Souza et al., 2008a). The authors analysed the separation force between two homogeneous surfaces with a constant liquid volume between them and used the number of liquid bridges and the contact angle as variable factors. Their results showed that, at numerous liquid bridges, contact angles of approximately 70 deg are favourable if a high total capillary force is to be achieved. Angles of 90 deg optimise the capillary force per contact area. We conclude from this outcome that, if there is no evolutionary pressure on the number or area of contact elements, insects in general should achieve the highest attachment forces on surfaces with contact angles of 90 deg and on those of 70 deg if the number or area of contact elements underlie evolutionary pressure. However, it remains unclear to what extent these models fit to the contact angle between the beetles' fluid and *Nuphar* or *Nymphaea* leaves. Further experimental studies on the properties of the attachment fluid of insects on their host plants and various further substrata are needed in order to draw conclusions on a possible adaptation of the fluid on the insects' host plant. While walking, insects must renew their attachment fluid because at least part of it remains attached to the surface after foot detachment. It is not solved yet whether the attachment abilities of insects are limited by the production of the fluid. If the production is limited, a high surface energy of the attachment hairs would prevent insects from a high rate of fluid loss (Kovalev et al., 2012). Hence, the surface energy of insect attachment hairs might either be adapted to achieve similar contact angles between the setae–liquid and liquid–surface interface or it might be relatively high to prevent extensive fluid loss from the setae. The latter case might have evolved in species walking on surfaces with fine roughness (of much smaller dimensions compared with the terminal contact elements): these species might need a higher amount of the fluid owing to its strong absorption by the fine roughness (Gorb et al., 2010; Kovalev et al., 2012).

In experiments, beetles showed strongly reduced friction forces on surfaces with low roughness values (R_a) between 74 and 198 nm compared with those higher than 953 nm roughness and smooth surfaces (R_a=30 nm) (Voigt et al., 2008). Because *N. lutea* does not possess such a fine roughness (R_a=5143 nm), the need for a high rate of fluid production to fill the fine surface irregularities should not apply to *G. nymphaeae*.

Our hypothesis is that surface energy does play an important role in insect attachment. However, the results of previous studies on this matter are rather heterogeneous. We analysed literature data dealing with force measurements of insects on surfaces with different surface energies (Table 1; Fig. 5). In five experiments from four different studies, no significant dependencies of friction forces on contact angles were detected. However, six experimental set-ups from five different studies (excluding the present one) revealed lower friction forces on surfaces with contact angles above 100 deg than those with contact angles below 40 deg. Interestingly, all studies that detected significant differences showed decreasing friction forces with increasing contact angle, although different species, developmental stages, sexes and experimental designs were taken into account.

Table 1. Literature in insect adhesion on surfaces with different but defined surface energies

	Source	Species	Systematic position	Force measured (method)	Surfaces (contact angles, deg)	Comments
1	Al Bitar et al., 2009	Cydia pomonella	Lepidoptera, Tortricidae	Friction force (centrifuge)	Glass (39), Plexiglas (74, females only), silanised glass (109)	Adults (females and males)
2	Gorb and Gorb, 2009	Gastrophysa viridula	Coleoptera, Chrysomelidae	Friction force (centrifuge)	Glass (30), silanised glass (102)	Adults (not differentiated between sexes)
3	Lüken et al., 2009	Cylas puncticollis	Coleoptera, Brentidae	Friction force (centrifuge)	Glass (39), silanised glass (110)	Adults (females and males)
4	Gorb et al., 2010	Coccinella septempunctata	Coleoptera, Chrysomelidae	Traction force (force transductor)	Glass (30), sapphire samples (85)	Adults (females and males)
5	Hosoda and Gorb, 2012	Gastrophysa viridula	Coleoptera, Chrysomelidae	Traction force (force transductor)	Soda-lime glass (43), smooth polycarbonate (59), modified spurr (104), smooth polycarbonate (108)	Adults (females)
6	Voigt and Gorb, 2012	Rhadinoceraea micans	Hymenoptera, Tenthredinidae	Friction force (centrifuge)	Glass (39), silanised glass (109)	Larvae (in longitudinal and transverse orientation)
7	Prüm et al., 2013	Leptinotarsa decemlineata	Coleoptera, Chrysomelidae	Traction force (force transductor)	Glass (20), epoxy resin replica untreated (83) and hydrophobised with antispread (105)	Adults (males)
8	Present paper	Galerucella nymphaeae	Coleoptera, Chrysomelidae	Friction force (centrifuge)	Plasma treated glass (6), glass (26), 2× silanised glass (83, 109)	Larvae, adults (no differences between sexes)

References are numbered according to Fig. 5.

Most studies compared performances on surfaces with contact angles of roughly 40 deg with those on surfaces of 105 deg. However, on surfaces with contact angles above 100 deg, changes in the contact angle might influence friction forces in a totally different manner than on surfaces with contact angles between 40 and 100 deg. A single study (Hosoda and Gorb, 2012) differentiated between two surfaces that had contact angles higher than 100 deg. Although friction forces were lower on the surface with a 104 deg contact angle compared to that with a 59 deg contact angle, higher friction forces were detected on surfaces of 108 deg compared with those of 104 deg, although these two contact angles differed by 4 deg only.

In all the studies we analysed, the friction force either decreased with an increasing contact angle (but see angles above 100 deg) or remained the same, whereas in our study, friction force increased with an increasing contact angle. The reason for these differences might be owing to particular surfaces used in the experiments. Surfaces with contact angles of approximately 83 deg were rarely included in other studies. If we omit this surface from the analysis, we likewise would detect decreasing (or the same) friction forces with an increasing contact angle for larvae (or adults; see Fig. 4). Similarly, the contact angles of host plants were seldom given in other studies. We assume that, if surfaces with contact angles, similar to the host plants, were included, an increase in the contact angle up to the contact angle of the host plant would be accompanied by an increase in attachment forces. For example, study number 5 (Hosoda and Gorb, 2012) worked on *Gastrophysa viridula*, a species that almost entirely lives on *Rumex obtusifolius* that in turn has a contact angle of 63 deg (Gorb and Gorb, 2009). Hosoda and Gorb tested a surface with a contact angle similar to that of *R. obtusifolius* (59 deg) and indeed detected a slight, although not significant, increase in the friction forces from the surface with a contact angle of 43 deg to that with a contact angle of 59 deg. We hence propose that further studies should (i) cover a larger variety of surfaces with different contact angles and different individual chemistry even at the same contact angles, (ii) concentrate on representatives of a single insect order and (iii) provide the contact angle(s) of their host plant(s) to reveal adaptations, which might be the underlying factors ruling the attachment behaviour of species on different surfaces.

MATERIALS AND METHODS
Eggs, larvae and adult individuals of *G. nymphaeae* were collected from floating leaves of *Nuphar lutea* in the Botanical Garden, Kiel, Germany. Animals were kept individually under laboratory conditions (22–24°C, 45–55% relative humidity) in Petri dishes of 35 mm in diameter. Petri dishes were supplemented with moist tissue underneath a disc of *N. lutea* leaves, which was replaced every third up to fifth day. For handling reasons, we studied larvae of the third larval stage. Adults used in the experiments were partly bred from these larvae and partly collected from the field.

Scanning electron microscopy
The samples were sputtered with a layer of ~20 nm thick gold–palladium. Images of the larvae were taken with a Hitachi S4800 (Hitachi High-Technologies, Tokyo, Japan) scanning electron microscope (SEM) equipped with a Gatan ALTO 2500 cryo-preparation system (Gatan, Abingdon, UK) at 3 kV accelerating voltage and a temperature of −140°C in the preparation chamber. The attachment system of adult beetles was studied with a Hitachi TM-3000 (Hitachi High-Technologies, Tokyo, Japan) at 5 kV. Detailed images of the setae were taken with the above mentioned Hitachi S4800 device at 3 kV.

Visualisation of contact areas
We used two different techniques to study the contact areas of the larvae. First, we let larvae attach themselves to the reverse side of a Petri dish and
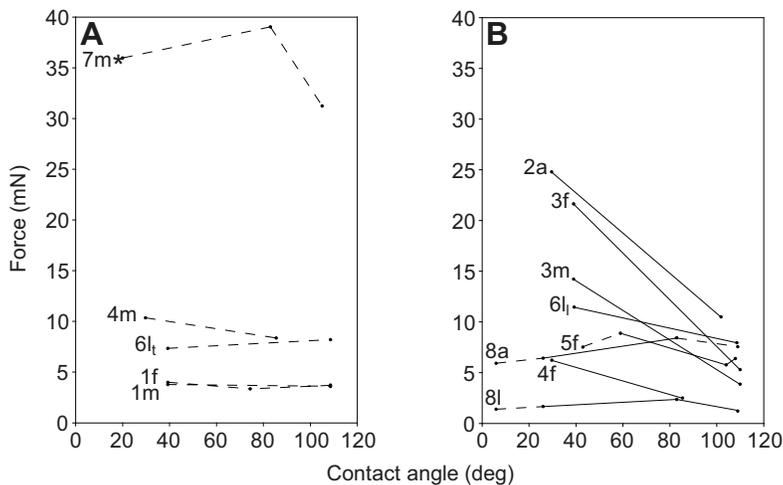
Fig. 5. Adhesion forces yielded on smooth surfaces with different contact angles of water. The means (Gorb et al., 2010) and medians (all other papers) were measured from the plots given in the previous studies (numbered 1–8, for more information see Table 1). (A) Experiments that did not detect significant differences between contact angles. (B) Significant differences between two proximate contact angles are indicated by solid lines. *The contact angle was not measureable for most droplets as they were spreading on the surface. 1f and 1m, adult females and males, respectively (Al Bitar et al., 2009); 2a, adult females (Gorb and Gorb, 2009); 3f and 3m, adult females and males, respectively (Lüken et al., 2009); 4f and 4m, adult females and males, respectively (Gorb et al., 2010); 5f, adult females (Hosoda and Gorb, 2012); $6l_l$ and $6l_t$, larvae in longitudinal and transversal orientation, respectively (Voigt and Gorb, 2012); 7m, adult males (Prüm et al., 2013); 8l and 8a, larvae and adults, respectively (present paper).

filled it with liquid nitrogen. The contact areas were frozen immediately. The deep-frozen larvae were removed from the Petri dish and glued with their dorsal side onto a metal holder. Second, we visualised the contact areas with a Leica MZ 205A stereomicroscope equipped with an integrated coaxial incident illuminator (Leica GmbH, Wetzlar, Germany) and a video camera. For this purpose, larvae were placed in Petri dishes that were subsequently inverted so that the attaching organs faced upwards. Surfaces in contact with glass appeared dark in coaxial illumination, whereas others appeared bright.

Surfaces used for experiments

The experiments were run on glass discs of 10 cm diameter and different surface energies. To obtain discs with high surface energies, they were treated in the plasma device ZEPTO B (Diener electronic GmbH + Co. KG, Ebhausen, Germany) with oxygen as the carrier gas for ~2 min at 0.2 mbar. Discs treated in this way obtained a contact angle of 6±5 deg (mean ± s.d.; N=6 discs, n=10 measurements on each disc). The non-treated disc had a contact angle of 26±2 deg (N=1, n=10). To prepare discs with higher contact angles, two glass plates were silanised to contact angles of 83±3 deg (N=1, n=10) and 109±3 deg (N=1, n=10).

We measured the contact angles of bi-distilled water on the surfaces as a representative parameter of the surface energy: the lower the contact angle, the higher the free energy of a surface. Contact angles of the surfaces were measured at 10 randomly chosen spots on each disc using the contact angle measurement device OCA 20 (Dataphysics, Filderstadt, Germany).

Surfaces of *N. lutea* leaves

Leaves of *N. lutea* bore a contact angle of 86±17 deg (mean ± s.d., N=7 leaves measured, n=10 times each). Their surface roughness was measured with the white light interferometer Zygo NewView 5000 (Zygo Corporation, Middlefield, USA) using the ×5 lens.

Friction force

We used the computer-controlled centrifugal force tester Tetra Zentri-01-P (Tetra GmbH, Ilmenau, Germany) to assess attachment forces of larval and adult beetles on different surfaces (Gorb et al., 2001). A drum of 10 cm in diameter was rotated with a speed of 50 rounds min^{-1} and accelerated up to a maximum of 3000 rounds min^{-1} within 20 s. The position of the individual on the drum was located using a light barrier. The maximum friction force in each single run was calculated from the distance of the insect to the centre of rotation, the rotational speed of the drum at the moment the individual was dropped and the mass of the individual (Gorb et al., 2001).

We used friction forces to allow for comparisons of the observed trend with other studies that did not compute the safety factors. However, to account for differences in weights, we obtained the dimensionless safety factors by dividing the friction force by the body weight of each individual (Gorb and Gorb, 2004). Owing to the high mobility of the individuals, it was not possible to consider the orientation of the beetles in the drum (e.g. individuals facing inwards versus outwards) at the moment of detachment.

In total, 18 larvae and 30 adult individuals were tested on each surface, each of them five times.

Statistical analyses

Prior to the analyses, the obtained friction forces and safety factors were log$_{10}$-transformed to achieve a homogeneous distribution of the variances of the residuals. We compared the values the individuals yielded on different surfaces using a linear mixed effects model. Surface (N=4) was included as a fixed factor and individual (N_{larva}=18, N_{adult}=30, n=5 repetitions per individual on each surface) as a random factor. Differences in the friction forces and safety factors between surfaces were analysed using Tukey's multiplicity-adjusted pairwise comparisons.

We ran the linear mixed effects models with the software R (R Development Core Team, 2012) using the *lme* function implemented in the package *nlme* (Pinheiro et al., 2012). *Post hoc* comparisons were performed with the *glht* function of the package *multcomp* (Hothorn et al., 2008).

Author contributions

S.G. conceived, S.G. and C.G. designed the research. A.B. and S.K. performed the experiments. C.G., A.B. and S.K. took the images and analysed the results. C.G., A.B. and S.K. wrote the manuscript. S.G. interpreted the results and commented on the manuscript.

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