Systematics, epidermal defense and bioprospecting of wild orchids

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Orchid-snail herbivory interactions

Chapter 4

The effect of orchid leaf ornamentation on snail adhesion

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Abstract. Protective structures in the epidermis are essential for land plants to defend themselves against herbivores. Intriguingly, field studies show that some orchid species are less prone to herbivory than other species. In this study, we investigated the effect of leaf ornamentation of the terrestrial orchids *Orchis mascula* (L.) L. and *Calanthe triplicata* (Willemet) Ames and epiphytic orchids *Dendrochilum pallidiflavens* Blume and *Trichotosia ferox* Blume on consumption and attachment of herbivorous land snails, using histochemistry, feeding and centrifuge experiments. Size and ornamentation of wax layers and density and histochemistry of epicuticular hairs and exudates on the orchid leaves were assessed with light microscopy, scanning electron microscopy and transmission electron microscopy. The possible contribution of epicuticular hairs to palatability was investigated by feeding snails untrimmed and trimmed orchid leaves. Total forces needed to detach two differently shaped snail species, *Subulina octona* and *Pleurodonte isabella*, were measured using a turntable equipped with a synchronized strobe. Snails were placed in two positions, either perpendicular or parallel to the main veins on the orchid leaves and on the adaxial (=upper) or abaxial (=lower) side. The results obtained provided three new insights. First of all, trimming of epicuticular hairs increased the palatability of young leaves of *Calanthe triplicata* (Willemet) Ames significantly. Secondly, a perpendicular or parallel position of the snails to the main veins did significantly affect the attachment performance of the smaller species tested, but only on the leaves of *Orchis mascula* (L.) L. that are protected by a wax layer. Thirdly, snails came off significantly faster on leaf sides covered with a high density of lignin filled epicuticular hairs. Our study highlights the importance of histology in combination with attachment force and feeding experiments for obtaining a better understanding of the defense mechanisms employed by different species of epiphytic and terrestrial orchids to deter herbivorous snails.

4.1 Introduction

The surface of sessile organisms like plants plays a crucial role in environmental interactions (Barthlott et al., 2017). Understanding the ecological and evolutionary interactions of plants and herbivores has been a subject of interest for many decades. Plants evolved different strategies to avoid consumption by herbivores. First of all, they have a physical barrier, either through development of a waxy cuticle, trichomes, spines, raphides or setae (Agrawal et al., 2009; Hanley et al., 2007; He et al., 2011; Konno et al., 2014; Sharma et al., 2009; Wagner et al., 2004). Secondly, they can defend themselves chemically by producing secondary metabolites (Barbehenn and Constabel, 2011; Rani and Jyothsna, 2010; Vandenborre et al., 2011). Thirdly, they often evolved a symbiosis with natural enemies of herbivores such as ants (Fiala and Maschwittz, 1992; Fischer et al., 2002; Gong and Zhang, 2014).

To feed on a plant, herbivores need to attach themselves to a plant's surface (Eigenbrode, 2004). Plant physical defensive structures are often embedded in the epidermal cells (Barthlott et al., 2017). Examples of such protective structures are epicuticular hairs and waxes. Trichomes are hair-like appendages extending from the epidermis that can be straight, spiral or hooked, branched, or unbranched, and glandular or non-glandular (Levin, 1973). Mechanically, trichomes can have both toxic and deterrent effects on herbivore attachment. (Hanley et al., 2007). Trichomes in high densities interfere with the movements of small herbivores on a plant, thus reducing access to the leaf surface (Agrawal et al., 2009). Secondary metabolites secreted by glandular trichomes can be poisonous, repellent or even trap insects, thus forming a combination of structural and chemical defense (Hanley et al., 2007; Sharma et al., 2009). Another structural feature produced by plants is a lipophilic material known as epicuticular wax (EW). The main function of EW is waterproofing the cuticle (Schönherr, 1976), but the complex chemical composition suggests additional functions. EW consists of minute crystals varying in size and shaped like filaments, rods, platelets, tubes, and complex dendritic structures (Barthlott et al., 2017; Jeffree, 1986). The precise mechanism of how EW reduces attachment of herbivores is not known, but four hypotheses were proposed by Gorb and Gorb (2002): (1) the aggregates of EW increase roughness of the plant surface, thus reducing the potential contact area between herbivore

foot pads and plant surface, (2) EW crystals contaminate foot pads and impair their function, (3) EW crystals draw lipophilic pad secretions away from their contact points by capillary adhesion, thus disrupting wet adhesion and lastly (4) the pad secretions (partially) dissolve EW crystals and form a thick layer of colloidal aggregate that disrupts the normal attachment process.

Orchids are among the largest families of flowering plants. Although mostly known for their spectacular floral diversity, orchids have a substantial anatomical diversity of the leaves as well (Dressler, 1981; Stern, 2014). Despite the large variation in epi- and subcuticular protective structures, orchids suffer from herbivore damage, both in the wild as well as in cultivation, which may be particularly detrimental to many endangered species in nature (Light and Macconaill, 2012) and causes huge annual capital losses to orchid nurseries worldwide (Hollingsworth and Armstrong, 2003). Common invertebrate orchid pests are slugs and snails (Hollingsworth and Sewake, 2002; Watson, 2002).

Compared to the many publications on orchid-insect herbivore biology and ecology (Light and Macconaill, 2014; Lucas-Barbosa, 2016; Subedi et al., 2011; Winkler et al., 2005), little has been published on understanding orchid-snail herbivory. Terrestrial snails and slugs adhere to and traverse many types of surfaces by using a thin layer (~10-70μm) of mucus secreted by the sole surface. To propel themselves, gastropods create a series of pulses by muscles in the foot that interact with the substrate through mucus secreted by the animal (Chan et al., 2005; Lai et al., 2010). Shirtcliffe et al. (2012) hypothesized that the amphiphilic (possessing both hydrophilic (water-loving) and lipophilic (fat-loving) properties) nature of the mucus plays an important role in adhesion of snails to many different types of surfaces. These authors based their hypothesis on the fact that they were able to reduce snail adhesion using a weak surfactant (a compound that lowers the interfacial tension between a liquid and a solid) that changed the wetting response of the surface of plant pots to the sole surface. The mucus layer helps the snail to create a stable attachment to any substrate at various inclinations (Lai et al., 2010). Adhesive locomotion on a smooth surface is less costly in terms of mucus production as compared to a rough surface (McKee et al., 2013). A recent study of Krings et al. (2019) showed that the radula is also involved in increasing mechanical interlocking with a substrate while feeding on a rough or wavy surface.

During fieldwork in tropical and temperate regions over the past twenty years, we observed that some orchid species are much more affected by herbivory than others (Gravendeel pers. comm.). We hypothesize that this phenomenon is caused by multiple factors, and that one of these factors involves leaf ornamentation acting as deterrent to herbivores. To test our hypothesis that orchid leaf ornamentation is involved in anti-herbivore defense, we investigated (i) the leaf anatomy and histology of four different orchid species, two epiphytic ones with leaves placed along the rhizome, and two terrestrial species with leaves in a basal rosette, with Scanning Electron Microscopy (SEM), Light Microscopy (LM), and Transmission Electron Microscopy (TEM); (ii) the palatability of young, juvenile and old leaves of one of the four orchid species, either left intact or with the main epicuticular properties trimmed, and (iii) adhesion of high spired-Subulinidae and low-spired Pleurodontidae snails in relation to the presence of three different epicuticular properties: trichomes, waxes and exudates.

4.2 Material and Methods

4.2.1 Snails and orchids

We observed snail species with long and short spired shells consuming orchids in the field and in cultivation (Table 4.1 and 4.2). To represent both groups, and for practical reasons, we chose to work with (sub)adults of *Subulina octona* (Subulinidae) and *Pleurodonte isabella* (Pleurodontidae). *Subulina octona* has a slender high spired shell; the crawling animal's foot surface, the sole, is elongate and narrow; *P. isabella*'s shell is much more globose and the foot of the crawling animal has a much wider sole surface (Figure 4.1). Live *Subulina octona* snails were collected from the greenhouses of plant breeder Elstgeest potplanten by placing traps, consisting of bricks with fresh cucumber slices underneath, below tables on which orchids were stowed. Live *Pleurodonte isabella* snails were purchased from a snail shop in Rotterdam (http://www.slakkenshop.nl). Live animals were placed in plastic tubes with a hole punched in the lid for fresh air access and fed with daily refreshed cucumber or lettuce ad libitum for the duration of the experiments. Both species of snails readily consumed leaves from any of the four orchid species investigated.

Table 4.1. Overview of different characteristics and epicuticular properties of four species of orchids investigated in this study.

Orchid species	Life strategy	Plant habit	Epicuticular properties of leaves								
			Leaf Position	Wax	Fine scale ridges	Exudates	Trichomes				
							Location	Length (mm)	Density (/mm2)	Diameter (mm)	Lignin in hairs
Orchis mascula	deciduous	tr.	basal	present on both sides	present	absent	absent on both sides	absent	absent	-	absent
Calanthe triplicata	evergreen	tr.	basal	absent	absent	absent	present on abaxial side	0.084±0.01	ca. 20±3 hairs	0.01± 0.001	absent
Dendrochilum pallidiflavens	evergreen	ep.	along rhizome	absent	absent	present	present on abaxial side	0.04±0.006	ca. 16±4 hairs	0.02± 0.001	absent
Trichotosia ferox	evergreen	ep.	along rhizome	absent	absent	absent	present on both sides	0.18± 0.04-1.0± 0.2	ca. 8±2-14±2 hairs	0.02± 0.004-0.03± 0.004	Present

*tr. terrestrial, ep. epiphyte

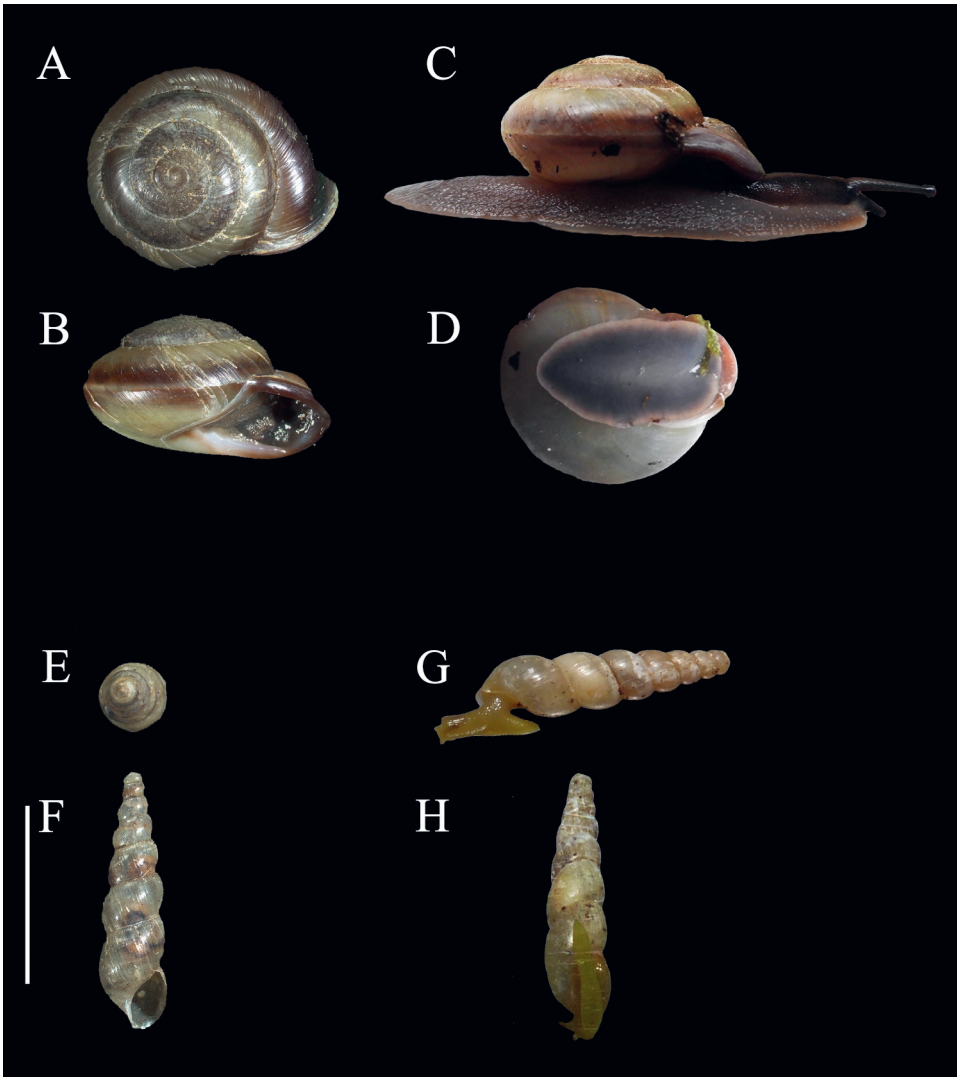


Figure 4.1. Different views of shells and live snails of the two species used in this study, *Pleurodonte isabella* (A-D) and *Subulina octona* (E-H) to illustrate the species' differences in size and shape. A, E, apical view of shells; B, F, apertural view of shells; C, G, extended (crawling) snails in lateral view; D, H, snail soles (crawling surface) attached to glass plate. Scale bar = 10 mm. Photographs by Anton J. de Winter.

We measured total mass, total surface in lateral view, and sole surface when viewed from below for both species of snails for a subset of 6-7 mature individuals per species from different size classes. Body mass was measured to the nearest 0.05 gram using a ProScale weighing scale. The lateral projected area was measured from lateral photographs, and sole surface area was measured from photographs of snails moving actively on a glass surface using ImageJ (Schneider et al., 2012). From these data, we used the correlation between body mass and lateral area to estimate the lateral area based on body mass for all specimens.

The herbivores investigated were a representative selection of snails eating from the leaves of four different locally common orchid species: the evergreen terrestrial *Calanthe triplicata* (Willemet) Ames (subfamily Epidendroideae - subtribe Collabiinae) and the epiphytic *Trichotosia ferox* Blume (subfamily Epidendroideae - subtribe Eriinae), both recorded in Manusela National Park in Seram, Indonesia in lower montane rainforest, between 1195 - 1272 m asl); the epiphytic *Dendrochilum pallidiflavens* Blume subfamily Epidendroideae - subtribe Coelogyninae) growing on Mount Salak in Java (800 – 1400 m asl); and the deciduous terrestrial *Orchis mascula* (L.) L. (subfamily Orchidoideae - subtribe Orchideae) growing in Grachterbos near Geulle, The Netherlands in mixed lowland Quercus and Carpinus coppice vegetation on a limestone slope, 61 m asl).

4.2.2 Light microscopy

Freshly harvested leaf samples were processed into microscopic slides to detect lignin, polysaccharides, carbohydrates, and calcium following protocols of Sheehan and Hrapchak (1980) and Dashek (2000). Leaf samples were first embedded in Paraffin Paraplast® Plus (Kendall Health Care Products, Japan) by rinsing the fixed samples in water and dehydrating them in a series of ethanol: xylene solutions. Then, they were stored in xylene for eight hours, infiltrated in Paraffin Paraplast® Plus (Kendall Health Care Products, Japan), and placed in an oven at 60°C for one day. Infiltrated samples were solidified and sectioned at 4–8 µm thickness with a Leica RM2265 rotary microtome (USA). Collected paraffin ribbons were laid in a 40-45ºC water bath, mounted on microscope slides, and dried on a hot plate set at 55ºC overnight. Deparaffination of samples was performed in

Table 4.2. Details of herbivorous snails and orchids that were found together, both in cultivation and in the field in The Netherlands or Indonesia.

Herbivore and orchid species	Localities in cultivation and natural habitat
Calanthe triplicata	Manusela National Park, Moluccas, Indonesia
Curvella sp. (Subulinidae)	Elstgeest potplanten, Nieuwe Wetering, The Netherlands
Subulina octona (Subulinidae)	Cibodas Botanic Gardens, Java, Indonesia
Ariophanta sp. (Ariophantidae)	Kampung Loa Loa, Moluccas, Indonesia
Orchis mascula	Grachterbos, Geule, The Netherlands
Aegopinella nitidula (Oxychilidae)	Hortus botanicus, Leiden, The Netherlands
Arion sp. (Arionidae)	
Cepaea hortensis (Helicidae)	
Cepaea nemoralis (Helicidae)	
Cochlodina laminate (Clausiliidae)	
Discus rotundatus (Discidae)	
Helix pomatia (Helicidae)	
Merdigera obscura (Enidae)	
Monachroides incarnatus (Hygromiidae)	
Trochulus hispidus (Hygromiidae)	
Trichotosia ferox	Manusela National Park, Moluccas, Indonesia
Leptopoma sp. (Cyclophoridae)	
Dendrochilum pallidiflavens	Cibodas Botanic Gardens, Java, Indonesia
Curvella sp. (Subulinidae)	

a series of xylene: ethanol solutions and the following stains were applied to the paraffin sections: an aqueous solution of Toluidine Blue O (TBO) 1% (w/v) in 1 % (w/v) sodium borate for 30 seconds to detect mucins, Etzold's staining (Basic Fuchsin 10 mg, Safranin 40 mg, Astra Blue 150 mg, Acetic acid 2 ml, and distilled water to complete 100 ml) for 3 min to detect lignin, Periodic Acid-Schiff (PAS) staining for 5 min the detection of insoluble polysaccharides and starch (Ruzin, 1999) and van Kossa (Sigma-Aldrich) for 30 min to visualizing calcium crystals. All sections were mounted in Entellan® (Merck) after dehydration and examined under a Axiolab 5 (Zeiss, Cambridge) directly after staining.

4.2.3 Scanning Electron Microscopy (SEM)

Fixed leaves were dehydrated for 20 minutes in a series of ethanol solutions (70%-96%-≥99.9%) and twice in fresh acetone ≥99.8%. Critical-point drying using ≥99.8% acetone and liquid CO2 as exchange fluids was performed in an Automated Critical Point Dryer Leica EM CPD300 (Leica Microsystems, Wetzlar, Germany). The drying protocol included a cooling step at 15°C, 50% stirrer speed with auto version, slow CO2 influx in the pressure chamber, with a delay of 120 seconds after influx of CO2 and before starting the exchange process, 18 exchange cycles (CO2: 99.8% acetone), with a fast (10 s) heating speed and medium (1 min) gas out speed. Dried samples were mounted on stubs with adhesive carbon conductive tabs and sputter-coated with 20 nm of Pt/Pd in a Quorum Q150TS (Quorum Technologies Ltd, East Sussex, United Kingdom) sputter-coater. The resulting samples were observed with a JEOL JSM-7600F Field Emission Scanning Electron Microscope (JEOL Ltd, Tokyo, Japan), at an accelerating voltage of 10 kV.

4.2.4 Transmission Electron Microscopy (TEM)

Fresh leaves were fixed in modified Karnovsky fixative (2.5% glutaraldehyde, 2% formaldehyde, in 0.1M sodium cacodylate buffer, pH 7.2) for 3 hours in a turntable and rinsed three times in 0.1M sodium cacodylate buffer (pH 7.4). Staining was performed in the dark for at least 2 hours in 2% osmium tetroxide in 0.1M sodium cacodylate buffer, and rinsing three times with 0.1M sodium cacodylate buffer (pH 7.4). Fixed samples were dehydrated in a proportion of ethanol (30%,50%,70%, 96% with 1% UAR-EMS uranyl acetate replacement,

and twice in ≥99.9% ethanol) for 15 minutes, each step in a turntable. The ≥99.9% ethanol was later replaced by propylene oxide in two steps of 15 minutes each. The sample were infiltrated in Epon (21.1% DDSA, 47.5% Embed 812, 29% NMA and 2% BDMA, all from Electron Microscopy Sciences) by submerging them in a mixture of propylene oxide and Epon (2:1, 1:1, 1:2) for 20 minutes for each step. After overnight evaporation of the remaining propylene oxide, the samples were placed in fresh Epon for 3 hours in a turntable at room temperature. Later, moderate vacuum pressure was applied for 20 minutes. The samples were embedded in fresh Epon in plastic molds and polymerized at 60°C for 48 hours. Resulting Epon blocks were trimmed in a rotary microtome with glass knives. Then, ultrathin sections of 95 nm were cut with a Leica EM UC7 ultratome (Leica Microsystems, Wetzlar, Germany), with a diamond knife and mounted on film-coated copper slot grids and post stained with uranyl acetate and lead citrate. Resulting samples were observed and photographed with a JEM-1400 PlusTEM (JEOL Ltd, Tokyo, Japan).

4.2.5 Feeding experiment

We performed feeding experiments for *S. octona* with *C. triplicata* as we had access to sufficient live animals and plants of these two species. Sterilized plastic containers were each supplied with one individual snail that had been starved for 4 days. This individual was subsequently fed with a freshly cut (2x2 cm sized) leaf piece of *C. triplicata*. Remaining leaf fragments were removed after 10 days and measured from digital photographs using ImageJ (Schneider et al., 2012). The experimental treatments were: (1) leaves either young (maximum of 1 week old), juvenile (1-3 weeks) or old (older than 3 weeks); (2) leaves with or without trimmed trichomes. Trichomes were trimmed using adhesive tape. Each treatment was replicated 8 times with different animals of comparable size and weight.

4.2.6 Attachment forces

To measure the attachment forces of the herbivorous snails investigated, we used a centrifuge technique similar to the method described by Federle et al. (2000). A part of a freshly cut leaf was clipped under two strips of acrylic (80 x 18 mm), held in place by two small magnets, which was placed on a horizontally orientated

turntable (radius r=80·mm) mounted on a rotor (Figure 4.2). We used a strobe light synchronized to the revolutions of the centrifuge through a photoelectric barrier so that a standing image of the snail on the rotating surface could be observed. The centrifuge was filmed from above (distance 50 cm) with a Nikon D5 camera. Cycle duration (in ms) was recorded with an optical tachometer, and the output was displayed on a display on the upper side of the centrifuge housing so that the speed of rotation was visible in the video image.

The snails were placed on a piece of freshly cut orchid leaf, either with the main veins perpendicular to the herbivore or in parallel and on the adaxial (=facing towards stem) or abaxial side (=facing away from stem). Individual herbivorous snails were placed on the turntable facing its centre, and the centrifuge was slowly accelerated at 0.231cycles*s-2 once it could be visually assessed that the snail had attached itself to the piece of orchid leaf. As soon as the snail flew off the leaf, the acceleration was stopped. Between every experiment, the orchid leaf fragment was refreshed. The force due to centripetal acceleration of the disk (Fc) was calculated based on the individual body mass in kilograms (Mb), the distance of the centre of the snail from the centre of rotation in meters (r) and the cycle duration in seconds (cycle) following the following formula:

$$F_c = M_b\, r \left(\frac{2\pi}{cycle}\right)^2$$

The air resistance force due to the drag (Fd) was calculated from air density (ρ), the lateral projected area (A_l) of the snail in m2 estimated based on its individual body mass, and the cycle duration in seconds (cycle) following the formula below (assuming a drag coefficient of 1):

$$F_d = 0.5\ \rho\ A_l \left[\left(\frac{2\,\pi\, r}{cycle}\right)\right]^2$$

The magnitude of the total force (Ft) parallel to the surface of the centrifuge disk needed for removal of the snail was calculated from the centrifugal force and the air drag acting perpendicular to it using the formula below:

$$F_t = \sqrt{\left({F_c}^2 + {F_d}^2\right)}$$

For each snail species, we conducted experiments with ten different animals per orchid species. For each animal, we did three consecutive measurements to not mix naive with experienced animals. We took the maximum value for analysis. The animals were allowed to recover for at least 15 min between these three measurements. Temperature and humidity were measured during every experiment to correct for their influence on air density (Federle et al., 2004).

4.2.7 Statistical analyses

To compare attachment performance across individuals that differ in mass and size, we used relative centrifugal force (RCF) rather than absolute force in our statistical analyses, as absolute force depends on the individual's mass. We did not include the forces induced by air drag or disk acceleration in the statistical analysis, as we found these to be negligible (<1% of the total force). Mean RCF was calculated from the three replicates per specimen, resulting in a single value for each snail. To identify effects of the variables that we tested (perpendicular, parallel, abaxial and adaxial position), we then performed a crossed ANOVA using the package LME4 (Bates et al., 2015) in R version 3.5.0 (R Core Team, 2018) with log-transformed mean RCF as the response variable, snail species and individual as nested independent variables, and orchid species, leaf side, leaf direction and individual as separately nested independent variables. The individual was coded as a random variable. We also tested between a more elaborate model that included the log-transformed mass of each specimen (LogMeanRCF ~ (Snail/(1 | Specimen)) *logMass + Orchid/Side/Direction). The model comparison found the more elaborate model to be only a marginally better fit than the simpler model excluding mass (p=0.0508).

To test if surface properties had a significant effect on the attachment RCF, we also tested a model containing the only variables that varied across and within orchid species: trichome length and density. Unfortunately, our dataset did not include enough differences in the presence of wax and exudates to test the effect of these two variables on attachment. The absence of trichomes was coded as very short trichomes (10-6mm), to allow log transformation. The variables

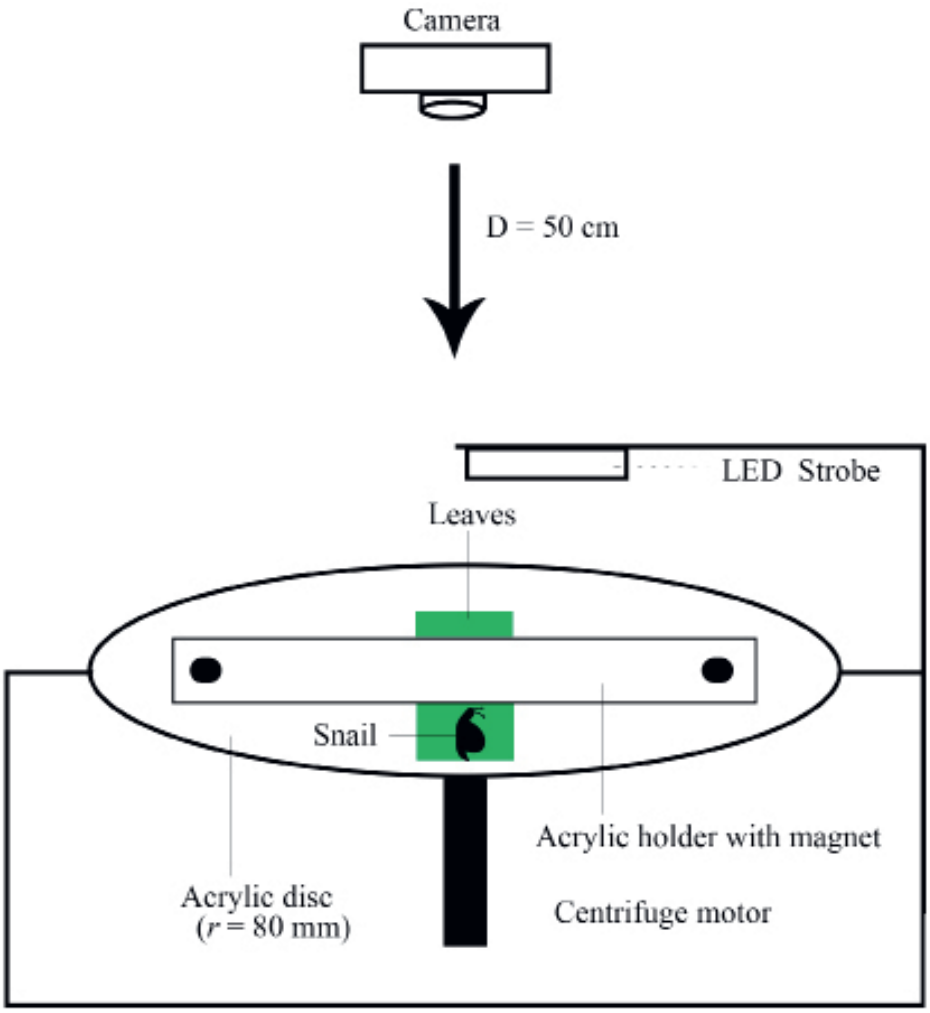


Figure 4.2. Schematic illustration of the centrifuge method used for measuring surface attachment forces of two different species of herbivorous snails. The snail was placed on a freshly cut piece of orchid leaf that was clipped to an acrylic strip on a horizontally rotating platform, which was accelerated until the snail became detached. A high-speed video camera recorded the run from above and was used to determine the maximum force of detachment. D, distance from the camera to the centre of the rotating platform; r, radius of platform.

snail species, snail specimen, and leaf side and direction were coded as nested random variables. Because we expected the effect of trichome length and density to have an interaction, we initially designed the model with that interaction. As this interaction term had no effect, it was removed. We here present the results of the following mixed model (variations of this model did not significantly alter the outcome) regarding the trichome variables: LogMeanRCF ~ (Snail / (1|Specimen)) + Orchid / (1|Side) / (1|Direction) + LogTrichomeLength + LogTrichomeDensity.

4.4 Results

4.4.1 Epicuticular properties

Detailed SEM, TEM, and LM images of the four different orchid species investigated revealed various epicuticular structures of orchid leaves surface (Figure 4.3). Both the abaxial and adaxial side of the leaves of *O. mascula* were found to be covered by an epicuticular wax layer. In contrast, *C. triplicata* has short non-glandular trichomes (ca. 0.1 mm long) in a relatively high density (ca. 20/mm2) on the abaxial side of the leaves. The leaves of *D. pallidiflavens* are covered by short glandular trichomes (ca. 0.5 mm long) on the abaxial side in a relatively low density (ca. 16/mm2) that secrete exudates. Leaves of *T. ferox* are covered by relatively large (ca. 0.2-1.6 mm long) paired trichomes on both sides. The density of these trichomes is higher on the abaxial (ca. 14/mm2) than the adaxial side (ca. 8/mm2).

The TEM images revealed a relatively thick wax layer along the cell wall of the parenchyma of the leaves of *O. mascula* on both the adaxial and abaxial side. This wax layer was thicker and more finely wrinkled on the adaxial side (ca 160-210 nm thick) as compared to the abaxial side (ca 30-40 nm thick). The non-glandular trichomes of *C. triplicata* and *T. ferox* have a thicker cell wall as compared to the glandular trichomes of *D. pallidiflavens*. We detected lipid droplets inside the glandular trichomes of *D. pallidiflavens*. The different stainings applied revealed that the paired trichomes on the leaves of *T. ferox* contain lignin. Proteins and polysaccharides were detected in the trichomes on the leaves of *C. triplicata*, *D. pallidiflavens*, and *T. ferox*. Exudates on the leaves of *D. pallidiflavens* were identified as polysaccharide substances by periodic acid staining.

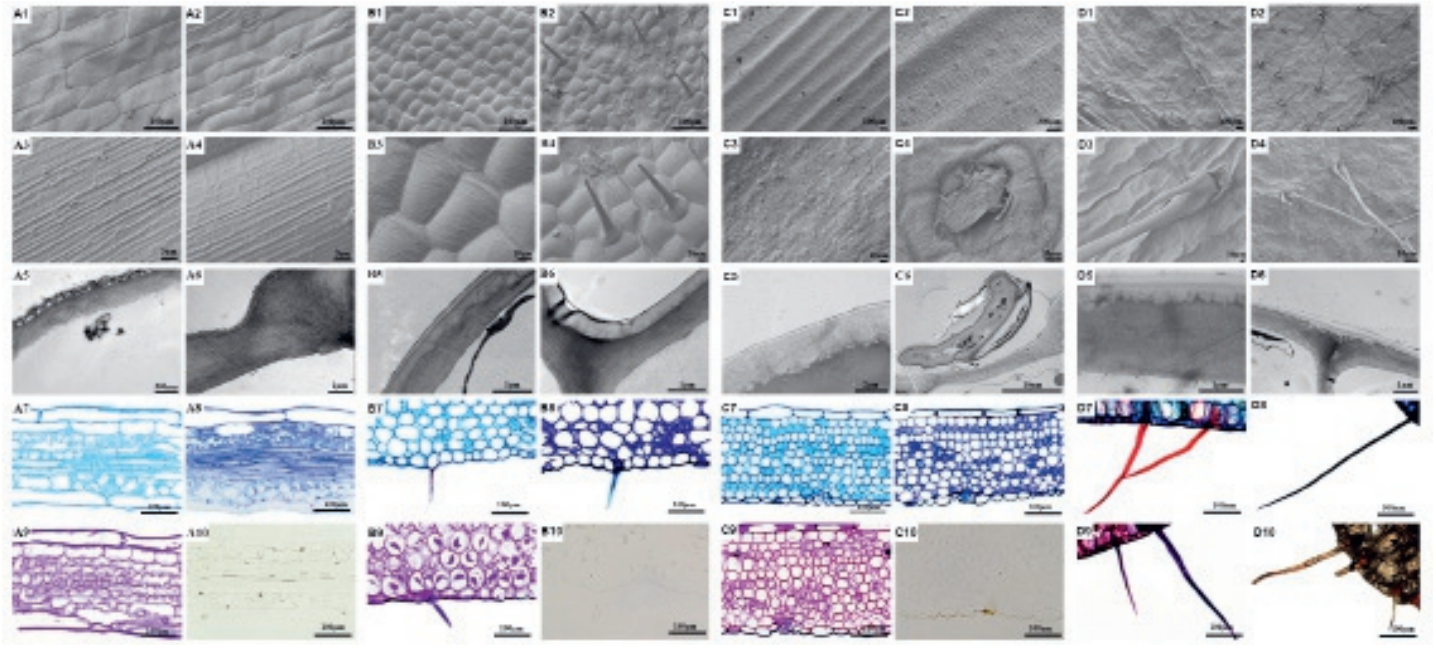


Figure 4.3. Leaf anatomy and histochemistry of orchid species used in the experiments: A1. Parenchyma cells on adaxial side of leaf of *O. mascula*. A2. Parenchyma cells on abaxial side of leaf of *O. mascula*. A3. Ornamentation of wax layer on adaxial side of leaf of *O. mascula*. A4. Ornamentation of wax layer on abaxial side of leaf of *O. mascula*. A5. Roughly waved thick wax layer on adaxial side of leaf of *O. mascula*. A6. Finely waved thinner wax layer on abaxial side of leaf of *O. mascula*. B1. Parenchyma cells on adaxial side of leaf of *C. triplicata*. B2. Parenchyma cells on abaxial side of leaf of *C. triplicata*. B3. Parenchyma cell surface on adaxial side of leaf of *C. triplicata*. B4. Trichomes on abaxial side of leaf of *C. triplicata*. B5. Section through epidermal surface on adaxial side of leaf of *C. triplicata*. B6. Section through epidermal surface on abaxial side of leaf of *C. triplicata*. C1. Ornamentation of adaxial side of leaf of *D. pallidiflavens*. C2. Ornamentation of abaxial side of leaf of *D. pallidiflavens*. C3. Parenchymal cell surface on adaxial side of leaf of *D. pallidiflavens*. C4. Trichome on abaxial side of leaf of *D. pallidiflavens*. C5. Section through epidermal surface on adaxial side of leaf of *D. pallidiflavens*. C6. Trichome basal cell on abaxial side of leaf of *D. pallidiflavens*. D1. Surface of parenchymal cells on adaxial side of leaf of *T. ferox*. D2. Surface of parenchymal cells on abaxial side of leaf of *T. ferox*. D3. Paired trichomes on leaf of *T. ferox*. D4. Paired trichomes on leaf of *T. ferox*. D5. Section through epidermal surface on adaxial side of leaf of *T. ferox*. D6. Section through epidermal surface on abaxial side of leaf of T. ferox. 7-10: Histochemistry of parenchymal cells of the epidermis of leaves of *O. mascula* (A), *C. triplicata* (B), *D. pallidiflavens* (C), *T. ferox* (D). A-D7. Staining with Etzold (lignin), A-D8. Staining with TBO (proteins), A-D9. Staining with PAS (polysaccharides), A-D10. Staining with van Kossa (calcium).

4.4.2 Feeding experiments

We found that *S. octona* preferred young leaves of *C. triplicata* over juvenile and old leaves (see Supplementary Figure 1). The mean proportion of remaining leaf fragments of untrimmed leaves was significantly larger (2.53 cm2) as compared to trimmed fragments (1.15 cm2) (Figure 4.4).

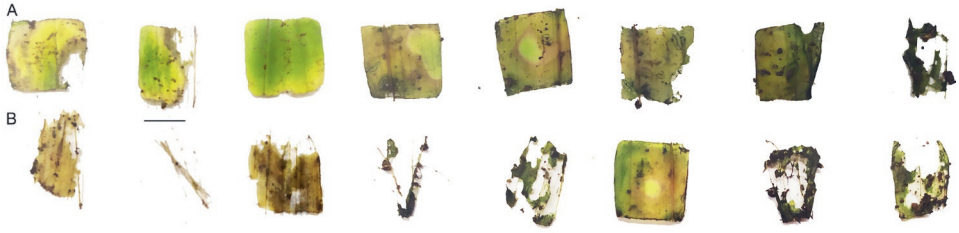


Figure 4.4. Feeding experiments result of *C. triplicata* grazed by *S. octona* after 10 days. A. untrimmed leaves; B. trimmed leaves. Scale bar: 1 cm.

4.4.3 Attachment performance

The snails that were used in the centrifuge experiments differed in size, shape, and sole surface area. We compared the weight and sole surface area for both species for 6-7 individuals per species from different size classes (Figure 4.5). The mass scaled with sole surface area with an exponent of 1.35 (95% confidence interval: 1.30-1.41) across the two species, a little less than the theoretical isometric value of 1.5. Despite this non-isometric scaling, *P. isabella* had 2.5 times more mass (0.0106 g/mm2) per unit of sole surface area than *S. octona* (0.00424 g/mm2). Both the position of the snail to the main veins on the orchid leaves, either perpendicular or in parallel ($p=0.0087$, see Table 4.3), and the side of the orchid leaf, either adaxial or abaxial ($p=0.030$), was found to influence RCF needed for snail removal. These findings are discussed in more detail below.

We ran the mixed models of mean RCF against predictor variables. Since the difference in fit between the model that included mass, and that which excluded mass was marginal, with the results being very similar between the two models, we here will mostly present the results of the simpler mixed model, excluding mass. Where

Table 4.3. Analysis of Deviance Table (Type III Wald F tests with Kenward-Roger df) with mean. Dependent variable is the log of the mean of three observations of detachment RCF per snail specimen. Formula: LogMeanRCF ~ (Snail/(1|Specimen)) + Orchid/Side/Direction. P-values that are significant at α=0.05 are shown in bold face.

Independent variables	F	Df	Df.res	Pr(>F)
(Intercept)	903	1	162	**< 2.2e-16**
Snail Species	83.9	1	159	**2.53e-16**
Orchid Species	52.2	3	144	**< 2.2e-16**
Orchid: Side	2.36	4	144	0.0561
Orchid: Side: Direction	3.09	8	144	**0.00301**

the differences between the models are of interest to understand the role of mass and snail species in attachment, we will refer to the more elaborate model, including mass.

Snail and orchid species and direction had a significant correlation (see Table 4.4). Leaf side was only marginally significant in the overall analysis. However, the orchids *T. ferox* and *D. pallidiflavens* showed significant differences in attachment RCF between leaf sides (Table 4.5). Of all the orchid species, *T. ferox* stood out as different from the other species, as RCF necessary for detachment was much lower on this species. There was a significant effect of direction on detachment RCF for *O. mascula*, with snails aligned in parallel with the axis of the leaf of this species requiring higher RCF than those oriented perpendicular to the axis of the leaf. In the more elaborate model (see Table 4.6), including snail specimen mass, snail species was non-significant (p=0.12), and mass was marginally non-significant (p=0.067), while their interaction term was also non-significant (p=0.83).

We found that trichome density and length both had a significant effect on detachment RCF, even taking variables like orchid species and leaf side into account. Higher trichome density had a negative effect on detachment RCF, while trichome length had a positive effect on detachment RCF.

We also calculated the force necessary to remove a snail from a leaf surface (Figure 4.6). The component of force due to centripetal acceleration (CF) was always much larger than that of air resistance (AD; ADmean/CFmean= 0.0043. The safety factor calculation (the snail weight divided by total force) showed that both of the snail species had poor attachment on *T. ferox* (Supplementary Figure. 2).

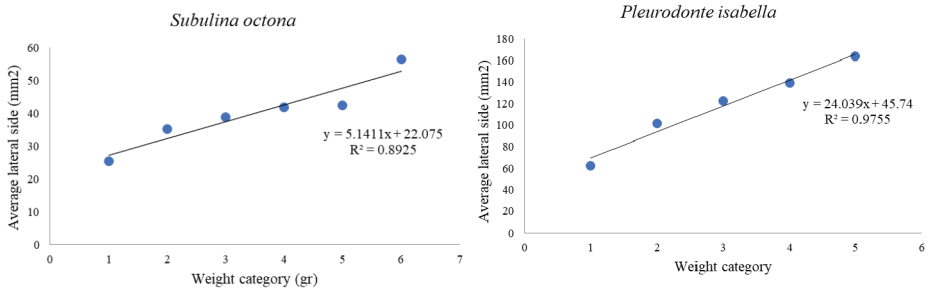


Figure 4.5. Relationship between body mass and footpad area (mm2) of *S. octona* and *P. isabella*. Weight category for *S. octona*: 1 (0.05 g), 2 (0.07 g), 3 (0.08 g), 4 (0.09 g), 5 (0.1 g); 6 (0.11 g). Weight category for *P. isabella*: 1 (2.3 g); 2 (2.4-2.6 g); 3 (2.7 g); 4 (3-3.1 g); 5 (3.2-3.3 g).

4.5 Discussion

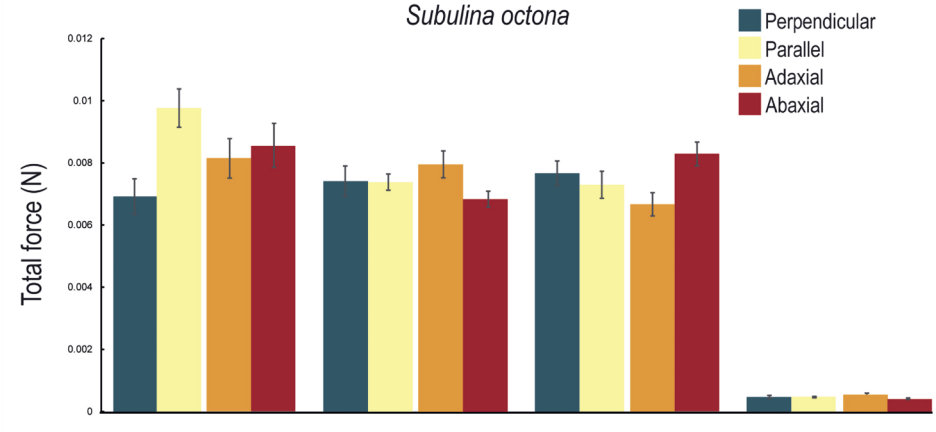
For *O. mascula*, wax layers were detected on both the adaxial and abaxial sides of the leaves. Wax layers on the epidermis of many plants species function as barrier against herbivores, to reduce water loss, and as a selective medium for spectral light penetration and reflection (Barthlott et al., 2017). The adaxial part of plant leaves, oriented parallel to the soil such as the basal leaves of *O. mascula*, is more exposed to sunlight and, therefore possibly covered with a thicker wax layer, as found in this study. The lipids detected in the wax layer have hydrophobic properties and could act as anti-adhesive for herbivorous snails (Shirtcliffe et al., 2012). The presence of epicuticular wax on the leaves of *O. mascula* might reduce the adhesion of the mucus of snails to the surface. The fact that a larger force was needed to remove the *P. isabella* snails from the abaxial as compared with the adaxial side of the leaves of *O. mascula* could be explained by our TEM observations of a slightly higher surface roughness created by fine folds of the epicuticular wax layer on the adaxial side. Such a fine pattern might significantly disrupt the attachment area below the sole surface of the larger snails. This result is in line with a study by Prüm et al. (2012) that showed that traction forces of Colorado potato beetles on plant surfaces covered by cuticular folds were reduced with 88 percent in comparison to smooth plant surfaces. According to our TEM

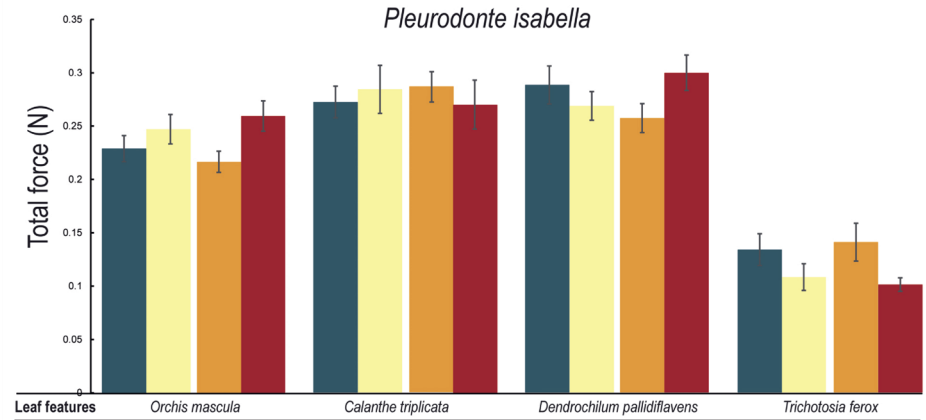
Table 4.4. Linear mixed model fit by REML. t-tests used the Satterthwaite's method ['lmerModLmerTest']. The dependent variable is the log of the mean of three observations of detachment RCF per snail specimen. P-values that are significant at α=0.05 are shown in bold.

Fixed effects	**Estimate**	**Std. Error**	**df**	**t value**	**Pr(>|t|)**
(Intercept)	0.817	0.0971	303	30.1	**< 2e-16**
Snail	0.121	0.269	303	9.16	**< 2e-16**
Dendrochilum pallidiflavens	0.0739	0.209	303	1.98	0.0485
Orchis mascula	0.0792	0.311	303	2.12	0.0345
Trichotosia ferox	-0.323	0.0375	303	-8.66	**2.88E-16**
Calanthe triplicata: Side	0.0278	0.0378	303	0.745	0.457
Dendrochilum pallidiflavens: Side	-0.0819	0.0373	303	-2.20	0.0288
Orchis mascula: Side	0.0334	0.0378	303	0.895	0.376
Trichotosia ferox: Side	0.0674	0.0371	303	1.81	0.0718
Calanthe triplicata: Abaxial: Direction	-0.0316	0.0371	303	-0.846	0.398
Dendrochilum pallidiflavens: Abaxial: Direction	0.00509	0.0372	303	0.136	0.892
Orchis mascula: Abaxial: Direction	0.0166	0.0380	303	0.446	0.656
Trichotosia ferox: Abaxial: Direction	-0.0345	0.0372	303	-0.925	0.356
Calanthe triplicata: Adaxial: Direction	0.0323	0.0371	303	0.866	0.387
Dendrochilum ferox: Adaxial: Direction	0.0366	0.0373	303	0.983	0.327
Orchis mascula: Adaxial: Direction	-0.170	0.0374	303	-4.57	**7.24E-06**
Trichotosia ferox: Adaxial: Direction	0.0231	0.0371	303	0.619	0.536

Table 4.5. Mean detachment RCF and force for each combination of factors.

Snail Species	Orchid Species	Side	Direction	RCF(G)	Force(N)
S. octona	*O. mascula*	Adaxial	Perpendicular	7.47	0.00618
S. octona	*O. mascula*	Adaxial	Parallel	15.7	0.00765
S. octona	*O. mascula*	Abaxial	Perpendicular	13.9	0.0101
S. octona	*O. mascula*	Abaxial	Parallel	11.4	0.00942
S. octona	*C. triplicata*	Adaxial	Perpendicular	9.48	0.00818
S. octona	*C. triplicata*	Adaxial	Parallel	8.54	0.00665
S. octona	*C. triplicata*	Abaxial	Perpendicular	7.95	0.00774
S. octona	*C. triplicata*	Abaxial	Parallel	8.43	0.00703
S. octona	*D. pallidiflavens*	Adaxial	Perpendicular	8.30	0.00699
S. octona	*D. pallidiflavens*	Adaxial	Parallel	7.28	0.00833
S. octona	*D. pallidiflavens*	Abaxial	Perpendicular	9.52	0.00634
S. octona	*D. pallidiflavens*	Abaxial	Parallel	9.41	0.00824
S. octona	*T. ferox*	Adaxial	Perpendicular	5.81	0.00567
S. octona	*T. ferox*	Adaxial	Parallel	5.30	0.00373
S. octona	*T. ferox*	Abaxial	Perpendicular	3.82	0.00520
S. octona	*T. ferox*	Abaxial	Parallel	4.76	0.00427
P. isabella	*O. mascula*	Adaxial	Perpendicular	6.09	0.205
P. isabella	*O. mascula*	Adaxial	Parallel	6.45	0.253
P. isabella	*O. mascula*	Abaxial	Perpendicular	6.73	0.228
P. isabella	*O. mascula*	Abaxial	Parallel	7.41	0.266
P. isabella	*C. triplicata*	Adaxial	Perpendicular	8.38	0.283
P. isabella	*C. triplicata*	Adaxial	Parallel	7.79	0.260
P. isabella	*C. triplicata*	Abaxial	Perpendicular	6.61	0.289
P. isabella	*C. triplicata*	Abaxial	Parallel	7.19	0.278
P. isabella	*D. pallidiflavens*	Adaxial	Perpendicular	8.17	0.275
P. isabella	*D. pallidiflavens*	Adaxial	Parallel	7.85	0.301
P. isabella	*D. pallidiflavens*	Abaxial	Perpendicular	9.09	0.238
P. isabella	*D. pallidiflavens*	Abaxial	Parallel	8.85	0.298
P. isabella	*T. ferox*	Adaxial	Perpendicular	3.58	0.158
P. isabella	*T. ferox*	Adaxial	Parallel	3.64	0.110
P. isabella	*T. ferox*	Abaxial	Perpendicular	3.00	0.124
P. isabella	*T. ferox*	Abaxial	Parallel	2.78	0.0926





Leaf features	*Orchis mascula*		*Calanthe triplicata*		*Dendrochilum pallidiflavens*		*Trichotosia ferox*	
	adaxial	abaxial	adaxial	abaxial	adaxial	abaxial	adaxial	abaxial
Non-glandular trichome								
Single trichome	-	-	-	+	-	-	-	-
Paired trichome	-	-	-	-	-	-	+	++
Lignified	-	-	-	-	-	-	+	+
Length	-	-	-	+	-	-	++	++
Density	-	-	-	+++	-	-	++	+
Glandular trichome								
Density	-	-	-	-	-	+	-	-
Wax layer								
Thickness	++	+	-	-	-	-	-	-

Figure 4.6. Attachment forces measured (in newton N) of *Subulina octona* and *Pleurodonte isabella* snails on four orchid leaf surfaces. Two positions were tested: perpendicular and parallel to the main veins and adaxial and abaxial side of the leaves. A: *Orchis mascula*, B: *Calanthe triplicata*, C: *Dendrochilum pallidiflavens*, D: *Trichotosia ferox*. Error bars mark the upper and lower 5%.

Table 4.6. Analysis of Deviance Table (Type III Wald F tests with Kenward-Roger df) with means. Dependent variable is the log of the mean of three observations of detachment RCF per snail specimen. Formula: LogMeanRCF ~ (Snail / (1|Specimen)) + Orchid / (1|Side) / (1|Direction) + LogTrichomeLength + LogTrichomeDensity. P-values that are significant at α=0.05 are shown in bold face.

Independent variables	F	Df	Df.res	Pr(>F)
(Intercept)	161	1	151	**< 2.2e-16**
Snail Species	75.9	1	159	**3.73e-15**
Orchid Species	41.8	3	33.6	**1.97e-11**
Trichome Length	10.3	1	152	**0.00163**
Trichome Density	10.0	1	153	**0.00187**

photographs, the amplitude of the wax folds is higher on the adaxial side of the leaves than on the abaxial side. This could explain the higher force needed to remove a snail from the abaxial side of a leaf of *O. mascula*.

During our field observations, we found many snails attached to the adaxial sides of leaves of shrubs in the forest surrounding the orchids during day time. The snails had retracted their soft parts into their shell and became active after sunset (Gravendeel pers. comm.). Terrestrial snails are known to do this to protect themselves against dehydration and predatory birds, mammals, insects and snakes (Heller and Ittiel, 1990). A slightly higher surface roughness of the epicuticular wax layer on the adaxial side of the leaves of O. mascula might have evolved in response to the above described behavior.

Several field studies showed that herbivore grazing is higher on young versus mature leaves (Coley, 1980; Williams-Linera and Baltazar, 2001; Zvereva and Kozlov, 2014) as found in our feeding experiment with *C. triplicata* leaves. This difference might be caused by the increasing concentration of chemical defense compounds built up during the lifespan of a leaf and needs further investigation. Lack of any lignin staining suggests that the trichomes on the leaves of *C. triplicata* are involved in evaporation only (Rosinski, 1992). The results of our feeding experiment, however, show that these trichomes do have a role in antiherbivore defense after all, indicating that trichomes lacking any lignin can still function as physical barriers against snails.

A higher force needed to be applied to remove both snail species from the adaxial side of the leaves of *T. ferox*. Leaves of *T. ferox* were found to be covered on both sides by trichomes containing lignin, with a slightly higher density of hairs on the abaxial than the adaxial side. The lower density of trichomes on the adaxial side could explain why the snails were able to hold on longer in the centrifuge experiments. This may be due to the fewer trichomes providing more uncovered leaf surface, and thus a larger area for the footpad of the snails to attach to than on the abaxial leaf side.

The glandular trichomes on the leaves of *D. pallidiflavens* did not affect the attachment of any of the snails. This result suggests that these trichomes on their own do not function as anti-herbivore defence, a hypothesis further supported by a study of Podroužková et al. (2015) that showed that faeces of *Succinea putris* and *Urticicola umbrosus* snails contained glandular trichomes of *Helianthus tuberosus*, indicating that snails readily consume leaves covered by glandular trichomes. The lipid droplets detected in the trichomes of *D. pallidiflavens* might, similar to the elaiosomes on the seeds of this orchid species, attract ants foraging for food, that keep snails away by their aggressive behavior, in this way protecting the orchid from herbivores in a mutualistic relationship (van Leeuwen, 1929; Van der Wall et al., 2005).

The adhesive footpad area is the main factor that affects the adhesive force of climbing animals such as herbivorous snails (Chan and Carlson, 2019; Labonte et al., 2016). The production of mucus as adhesive substance enables snails to traverse various surfaces (Chan et al., 2005; Pawlicki, 2004). *Pleurodonte isabella* did detach at a lower relative centripetal acceleration (RCF) than *Subulina octona*. This may be attributed to the scaling of footpad area to mass, which results in more mass per unit of footpad area as a snail gets larger. Perhaps to partially correct for this problem, the larger species of snails had a slightly larger footpad to mass ratio compared to what would be expected under isometric scaling. Isometric scaling describes the condition where objects of different size share the same shape, whereas the relationship between two size measures fits a power function with a particular scaling exponent (Vogel, 2013). Nonetheless, the larger species of snails (*P. isabella*) still had about 2.5 times more mass per unit foot area. If the snails would all detach at the same foot stress, we would expect the smaller species *S. octona* to detach at about 2.5 times the RCF of *P. isabella*. This

was not the case, as *S. octona* detached at 1.37 times the RCF of *P. isabella*. This difference in foot stress may be due to size-related effects of the surfaces of the orchid leaves, a stronger adhesive mucus, or shell shape (ovate versus elongate) and size-related and different sole surface effects of the snails (e.g. elongate versus more oblong shape). The scale-related factors of leaf irregularity, snail sole surface area, mucus layer thickness and physical properties of the mucus could also interact to explain the observed results. This may be an interesting avenue to explore in future research.

The differences between the models with and without mass as an independent variable seem to indicate that much of the difference in attachment RCF between the two snail species may be mediated by their difference in mass. However, when including mass in the model, the variance seemed to be divided between species and mass, leading to a marginal non-significant effect for both these variables. Even when including mass, some variance in attachment RCF may be explained by snail species. The non-isometric scaling of the snails and the potential scaling effects suggested above may contribute to this effect.

We found significant differences in detachment RCF across orchid species. The lowest values were seen on *T. ferox*, both trichome density and length are significant factors affecting detachment RCF. The safety factor results showed that both snails attached poorly on the lignified trichomes of *T. ferox*. Interestingly, increasing trichome density decreased attachment, but increasing trichome length increased attachment. Trichome density was earlier shown to have a negative effect on ovipositional behavior, feeding and larval nutrition of insect pests (Handley et al., 2005). Increasing herbivore attachment with increasing trichome length was also recorded by Voigt et al. (2007) for *Dicyphus errans* bugs on the surface of *Brassica oleracea* leaves, with a significant positive correlation between force and both trichome length and diameter. According to these authors, the trichomes provide extra grasp for the claws, thus enabling a stronger attachment of the bug. Expanding this study to more orchid species with relatively long trichomes might provide more insights in the overall effect of trichome length on snail detachment. Trichomes may reduce wet adhesion by creating large asperities on a surface. A wet adhesion mechanism is created by producing a thin fluid layer that creates capillary and viscosity forces between a pad and the surface (Hanna and Barnes, 1991). The texture of the attachment surface is an important factor for this

mechanism. A study by Crawford et al. (2016) revealed that the wet adhesion of tree frogs was strong on a smooth surface or a surface with small asperities (< 10 μm) but not on a surface with large asperities. This might be explained by insufficient production of fluid to fill the space between the large asperities and creating air bubbles that reduce the attachment to the surface. Alternatively, the large asperities may increase the drainage of fluid from the pad. Both trichomes and epicuticular wax ornamentation could thus disrupt wet adhesion of snails and other herbivores to leaf surfaces.

A waxy layer did not result in a lower detachment RCF. In *O. mascula*, the only species with a waxy outer layer, the smaller snail species *Subulina* was able to attach to an RCF of over 10g. However, *O. mascula* was the only species that showed differences in detachment RCF by direction. Our observation that a higher force and a higher RCF was needed to remove the *S. octona* snails from leaves of *O. mascula* when placed parallel to the main veins as compared with a perpendicular position might be caused by the surface roughness created by fine folds of the epicuticular wax on the leaves of this orchid species. The orientation of this fine ornamentation, as revealed by our SEM photographs is generally perpendicularly oriented to the main parallel longitudinal venation. It is yet unclear why orchid leaves evolved small-scale epicuticular wax in this orientation. The wax morphology is affected by the wax content, composition and cuticle permeability between glaucous and non-glaucous near-isogenic lines (Zhang et al., 2013). The waxes contain fatty acids, primary alcohols, esters, aldehydes, alkanes, hentriacontane-14, 16-dione (β-diketone), and OH- β-diketone that vary depending on the organs by which they are produced (Wang et al., 2017). A possible adaptive explanation might be that the main parallel veins running towards the tip of the leaf ensure quick removal of debris and small insects during heavy rainfall. The main veins, predominantly the one on the midrib, channel down raindrops towards the tip. Under the weight of accumulating raindrops, such leaves start to droop until all the water falls off the pointed apex, commonly called a ‘drip tip’. If a drip tip is cut off, rainwater starts to pool (Burd, 2007), and the leaf will become more vulnerable to infection by pathogens and less effective in photosynthesis. Small-scale roughness of orchid leaves possibly evolved in a direction perpendicular to the main veins to improve water drainage towards the main veins. In addition, such a pattern might disrupt the attachment area below the

sole surface area of small herbivorous snails. This in turn could change the area/edge ratio of the attachment or may cause stress concentrations. These hypotheses need further experimental validation.

Whether leaf surface ornamentation indeed helps plants to rid themselves of snail herbivores, depends of course on the forces that may be induced by wind or rain to a leaf. Plant appendages seem to act as damped oscillators (Peltola et al., 1993; Spatz and Bruechert, 2000) with a random input by wind or rain. The two tested snail species detached at accelerations between 2.0 g and 24.2 g (19.6-237 m/s2). Although it seems that at least the lower end of this range can be reached in plants swaying in the wind (Finnigan, 2000), measurements of g-forces on leaves exposed to natural conditions will be necessary to confirm if this is a viable mechanism for the plants included in this study to eject snails. In terrestrial species, low wind, and a small travel distance needed for the snail to regain its position on the plant may make this mechanism less viable. In epiphytic species, however, loss of footing by the snail may result in it tumbling several meters to the ground, with a lower likelihood of the snail returning to the same plant.

Our study is the first to quantify adhesion forces of herbivores on orchids. The accelerations applied were in the natural range of those induced by wind or rain to plant leaves. Our measurements provide a first estimate of total forces needed to detach herbivorous snails from orchid leaves. A high density of calcium and lignin containing hairs, and a thick wax layer were found to be effective in inducing loss of footing of the snails, possibly due to reduction of the contact area of the sole. Of the leaf epicuticular compounds detected with staining in our study, cutin, lignin, lipids, and polysaccharides such as cellulose are hydrophobic (de Candolle, 1813; Schönherr, 1982) whereas one particular group of carbohydrates, e.g., sugars, are lipophobic. Further studies on the hydrophilic and hydrophobic capacities of the epicuticular protective structures of the leaves investigated here, next to their chemistry and nanostructures as detected for several other plant species (Barthlott et al., 2017) might reveal additional details by which orchids protect themselves against snail herbivores. This might ultimately contribute to the design of a bio-coating with sufficient anti-adhesive properties. Alternatively, the attachment ability of snail pests can be decreased by the selection of orchid individuals with a relatively high amount of leaf trichomes and waxes to develop new cultivars and improve conservation of species in botanic gardens.

Supplementary material

Supplementary information is available online at Figshare, https://doi.org/10.6084/m9.figshare.13060046.v1