

INVITED REVIEW

# The why and how of sunken stomata: does the behaviour of encrypted stomata and the leaf cuticle matter?

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- **Background** Stomatal pores in many species are separated from the atmosphere by different anatomical obstacles produced by leaf epidermal cells, especially by sunken stomatal crypts, stomatal antechambers and/or hairs (trichomes). The evolutionary driving forces leading to sunken or ‘hidden’ stomata whose antechambers are filled with hairs or waxy plugs are not fully understood. The available hypothetical explanations are based mainly on mathematical modelling of water and CO<sub>2</sub> diffusion through superficial vs. sunken stomata, and studies of comparative autecology. A better understanding of this phenomenon may result from examining the interactions between the leaf cuticle and stomata and from functional comparisons of sunken vs. superficially positioned stomata, especially when transpiration is low, for example at night or during severe drought.
- **Scope** I review recent ideas as to why stomata are hidden and test experimentally whether hidden stomata may behave differently from those not covered by epidermal structures and so are coupled more closely to the atmosphere. I also quantify the contribution of stomatal vs. cuticular transpiration at night using four species with sunken stomata and three species with superficial stomata.
- **Conclusions** Partitioning of leaf conductance in darkness ( $g_{tw}$ ) into stomatal and cuticular contributions revealed that stomatal conductance dominated  $g_{tw}$  across all seven investigated species with antechambers with different degrees of prominence. Hidden stomata contributed, on average, less to  $g_{tw}$  (approx. 70 %) than superficial stomata (approx. 80 %) and reduced their contribution dramatically with increasing  $g_{tw}$ . In contrast, species with superficial stomata kept their proportion in  $g_{tw}$  invariant across a broad range of  $g_{tw}$ . Mechanisms behind the specific behaviour of hidden stomata and the multipurpose origin of sunken stomata are discussed.

**Key words:** Sunken stomata, leaf, epidermis, stomatal antechamber, stomatal encryption, nocturnal transpiration, cuticular transpiration, stomatal transpiration, trichomes, *Nerium oleander*, *Ficus elastica*, *Olea europaea*, *Clusia rosea*, *Arabidopsis thaliana*, *Capsicum annuum*, *Brassica oleracea*.

## INTRODUCTION

### *Sunken stomata*

Architectural characteristics of the stomatal apparatus are of profound importance for its function – prevention of fatal water loss while allowing sufficient carbon gain. During the light period of a day, stomata represent the most important plant–atmosphere interface, dynamically balancing the rates of transpiration and photosynthesis of a cuticle-sealed leaf. In an optimal mode, they are hypothesized to maximize CO<sub>2</sub> influx for a given physiologically tolerable water loss (Cowan and Farquhar, 1977; Vico *et al.*, 2013). At night, with stomata presumably closed, the cuticle and its cuticular wax are barriers that restrict water loss to a safe value that is not subjected to short-term control by the plant. Because of their key role in controlling CO<sub>2</sub> influx and water efflux, the stomata and cuticle

are assumed to be the primary targets of evolutionary adaptations to varying environmental conditions.

The position of stomata relative to the leaf surface has been a matter of interest to plant physiologists and anatomists for decades (Weyers and Meidner, 1990; Willmer, 1996; Larcher, 2001). In most species, a leaf cuticle that covers all epidermal cells forms external ledges arching over the stomatal pore, and the variable stomatal pore delimited by guard cells sits beneath the rigid ledges (Turner, 1994; Willmer, 1996). With this general arrangement, the central point of guard cells can protrude above, be in line with or be sunken below the surface of the epidermis. In the latter case, guard cells are located at the bottom of depressions, deep pits or crypts forming stomatal antechambers.

There is currently no consensus regarding the functional significance of sunken stomata. Traditionally, the stomatal antechamber has been considered as an adaptation to dry environments. However, the current distribution of species with

sunken stomata outside of dry habitats as well as fossil records indicate that the water deficit is not the sole and perhaps not even the primary factor promoting sunken stomata. It appears that more than one evolutionary factor may favour the location of stomata in recesses within leaves. What underlies the evolutionary success of stomata seated at the bottom of an epidermal recess?

In the first part of this study, an overview of stomatal antechamber anatomy and ecology is presented, followed by a short review of current ideas on the benefits conferred by stomatal antechambers. In the second part, original experimental results are presented, addressing the environmental response of various types of sunken or encrypted stomata that are relatively isolated from the external environment.

#### *Architecture of stomatal antechambers*

Hidden stomata are usually classified according to the ratio of the width of the aperture of the stomatal antechamber relative to its depth, by the antechamber volume or shape and/or by the number of stomata in the chamber. Two basic categories can be distinguished (Jordan *et al.*, 2008): (1) stomata encrypted in deep pits, crypts or longitudinal grooves and (2) stomata in shallow recesses protected by overarching external cuticular ledges. The term ‘sunken stomata’ in the following text, especially in the experimental part, includes both the above categories of stomatal antechamber, the deep crypts as well as the shallow pits covered with rigid ledges (papillae). In contrast, stomata with guard cells located at the same level as epidermal cells and without a discrete antechamber are called ‘superficial’ stomata.

Type (1) stomata in crypts can be formed by invaginations of the leaf epidermis protruding into abaxial mesophyll tissue or by entire leaf margins that curl tightly toward the middle vein. They can have different sizes and shapes, accommodating from one to many (8–35) stomata in one crypt as, for example, in six *Banksia* species investigated by Hassiotou *et al.* (2009a), or an average of 40 stomata per crypt in fossils of *Banksia paleocarpa* (Carpenter *et al.*, 2014). Here, the large crypts (approx. 300 µm in diameter) are often filled with a mesh of trichomes, which may obscure the aperture of the pit. Both the hairs and crypts have an uncertain role in hindering water loss (see below). Crypts evolved several times in the Australian Proteaceae from wet as well as presumably dry climates (Jordan *et al.*, 2008). However, several lines of evidence discussed below indicate that the location of stomata in deep grooves or crypts provides a selective advantage in xeric climates.

Type (2) stomata in shallow epidermal depressions covered partly by cuticular ledges (papilla) are common in most vascular plants. They can have different forms, for example pits where stomata appear sunken due to papillae surrounding the stomatal apparatus. In the Proteaceae, an abundance of species with papillose pits does not correspond to the level of aridity within their habitats. Jordan *et al.* (2008) argue that papillae and other epidermal features that increase the roughness of the leaf surface decrease its wettability, similar to trichomes in crypts and on the leaf surface. Lower wettability provides protection against waterlogging of stomatal pits, and hairs may prevent fungal growth into the stomatal antechamber and leaf interior via the stomatal pore. These evolutionary advantages may dominate in species with a paracytic stomatal complex, characterized by a lateral pair of subsidiary cells that

arch over the stomatal pore, creating a stomatal antechamber (Gray *et al.*, 2020).

Antechambers forming deep crypts have been of greatest interest to physiologists and plant anatomists. Crypts are the air-filled, roughly spherical or cylindrical spaces invaginating into mesophyll tissue with an opening to the atmosphere that is smaller than their diameter. Each usually accommodates a number of stomata located at the bottom and along the crypt walls (Fig. 1A–F). It is assumed that the walls are covered with a cuticle and do not represent any significant source of transpired water. Crypts are typical for xerophyllous or sclerophyllous species of dry habitats, commonly exemplified by *Nerium oleander* or many *Banksia* species. For example, the visible crypt openings (crypt pores) in *Banksia ilicifolia* occupy about 20 % of the total abaxial leaf surface area, and the internal surface area of the crypts exceeds the crypt pore area by five times (Roth-Nebelsick *et al.*, 2009). Thus, the crypt walls may enlarge the leaf area 100 % compared with the projected area. An interesting, extremely pronounced form of crypt was described recently in fossil needles of *Cryptokerpia sarlaccophora* (gymnosperm) from Permian deposits in Jordan (Blumenkemper *et al.*, 2019). The crypts are three cells deep and possess two additional papillae rings formed by neighbouring and subsidiary cells arranged in a circle (Fig. 2). The hook-shaped papillae restrict the diffusive path in two tiers, like a double bottleneck, and presumably protect a single stoma at the bottom of the crypt.

Crypts can be filled with trichomes, as is the case with *Banksia* or *N. oleander*, with stomata clustered along the walls of the crypt. The role of trichomes is enigmatic. Based on isoline estimates of water vapour concentration, they do not appear to hinder diffusion of water from the crypt (Roth-Nebelsick *et al.*, 2009). Haworth and McElwain (2008) proposed that they could protect stomata from mechanical damage, provide UV protection, shield against excessive irradiation and heat, or block the formation of water layers across the crypt pore. These and other commonly accepted ecophysiological functions of leaf hairs are likely to exist (Bickford, 2016). Interestingly, the hairs are hollow (Fig. 1C, F) and probably filled with air; unfortunately, data about the permeability of hair surfaces for water are lacking. The dramatic increase of the abaxial leaf surface by crypt walls and trichome surfaces raises a question about a possible role for these surfaces as water sources that moisten the air close to stomata, and thus change the crypt’s microclimate. Could stomata profit from such a crypt-specific microclimate? In what other ways might the presence of crypts benefit stomata, leaf and plant fitness?

#### *Benefits of sunken stomata*

*Adaptation to drought by reducing water loss more strongly than CO<sub>2</sub> uptake.* Antechambers are located in the diffusion pathways for both water vapour and CO<sub>2</sub>. Consequently, the increment in length of the diffusion pathway for both gases through the antechamber should be the same. However, because CO<sub>2</sub> diffusion does not end at the evaporation surfaces within the leaf but continues beyond, into mesophyll cells and chloroplasts, the diffusion path length for CO<sub>2</sub> is longer than that for water molecules. Antechambers *per se* thus increase the diffusive path length for water vapour proportionally more than for CO<sub>2</sub>. Following this principle, Jeffree *et al.* (1971) estimated

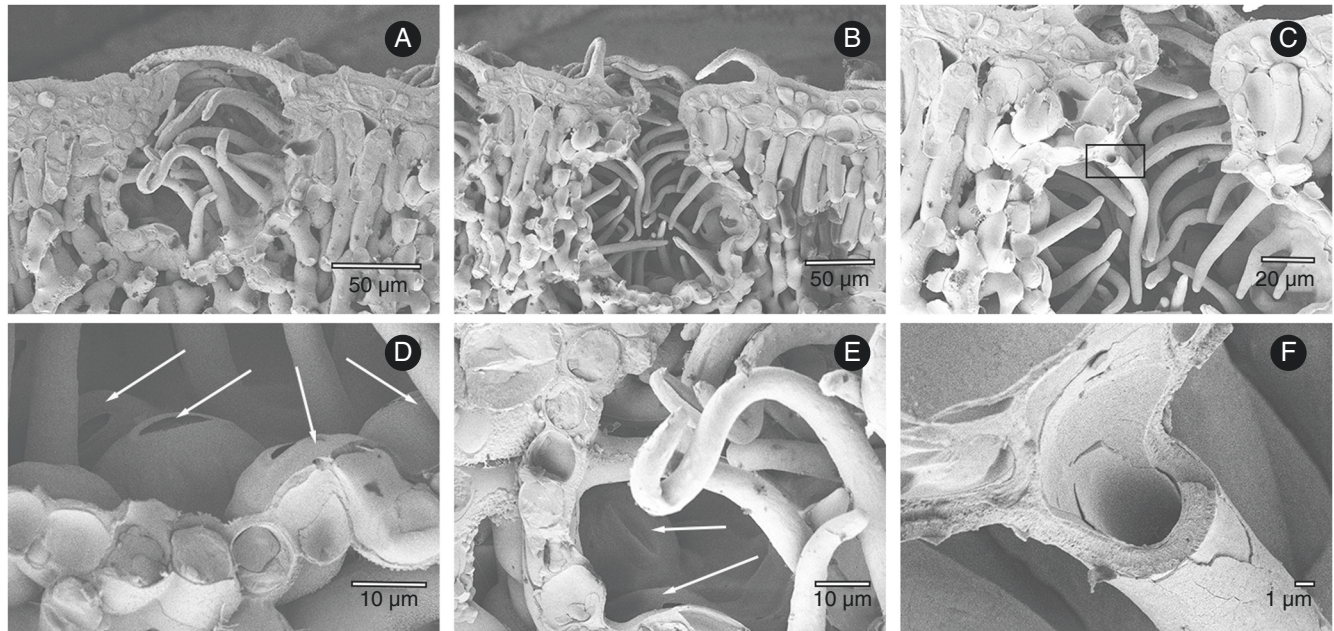


FIG. 1. Crypts with stomata and hairs in *Nerium oleander*. Cryo-scanning electron microscopy (SEM) images of transects through the whole crypts on the abaxial side of an *N. oleander* leaf, with crypt walls, internal trichomes and trichomes overarching the crypt aperture (A, B). Details of the crypt interior showing numerous stomata at the crypt bottom (arrows in D, E) and fractured crypt walls. Hollow trichomes are protruding from the individual cells forming the wall (C, F).

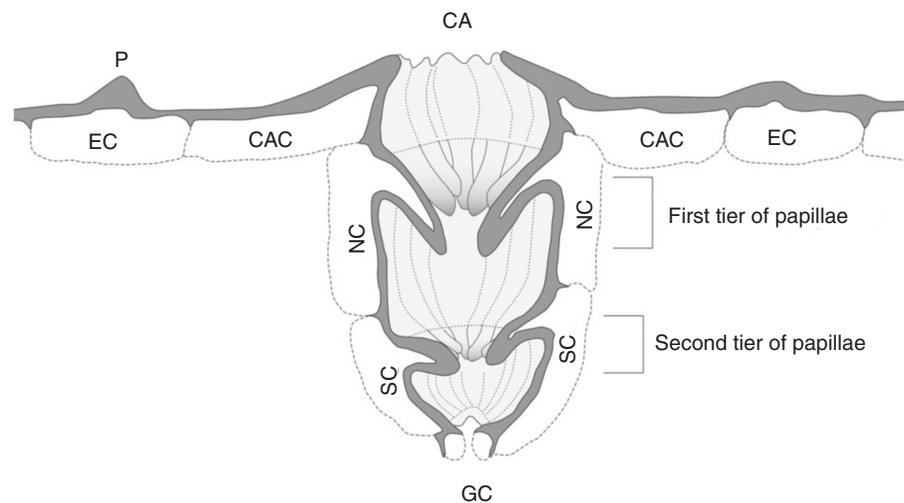


FIG. 2. Reconstruction of the stomatal crypt of *Cryptokerpia sarlaccophora*. CA, crypt aperture; CAC, crypt aperture cell; EC, epidermal cell; NC, neighbouring cell; SC, subsidiary cell; GC, presumed position of guard cells. According to [Blomenkemper et al. \(2019\)](#).

that a modelled stomatal antechamber in Sitka spruce increased diffusion resistance for water by about the same magnitude as open stomatal pores. Diffusion is hindered further by wax occlusions – amorphous or crystalline structures occurring inside and around the antechambers ([Kim et al., 2010](#)). They reduce the effective cross-section for free diffusion in air; moreover, if the distance between wax particles is close to or less than the mean free path of the diffusing molecules (approx. 100 nm), the free diffusion regime changes to much slower Knudsen diffusion. With Knudsen diffusion, the gas molecules collide more frequently with wax particles and hairs than with each other, and the diffusion coefficients decrease substantially ([Cussler, 1987](#)). With the lengthening of the common water and CO<sub>2</sub>

diffusion path lengths and the introduction of wax occlusions, the placement of stomata in antechambers selectively promotes CO<sub>2</sub> uptake over water loss and, therefore, increases the instantaneous water use efficiency of photosynthesis. Using a simple mathematical model, [Jeffree et al. \(1971\)](#) showed that the antechambers of spruce, plugged with wax, formed two-thirds of the total needle resistance to water loss while they accounted for only one-third of the resistance to CO<sub>2</sub> uptake. An even greater effect of wax plugs on stomatal conductance was estimated by [Brodrribb and Hill \(1997\)](#) for a group of Southern Hemisphere conifers. Partitioning of the total leaf diffusion conductance to crypts and stomata in *Banksia* revealed a dominant role for stomata (77 %) over crypts (23 %) and a 7–11 % improvement in

water use efficiency compared with leaves with superficial stomata (Hassiotou *et al.*, 2009a).

If this principle works on an evolutionary time scale, the number of species with sunken stomata may be expected to increase with greater aridity in their environment. Jordan *et al.* (2008) observed this phenomenon in the fossil record of Proteaceae species exhibiting deep stomatal crypts. Deep crypts appeared relatively recently after the aridification of Australia had started. Other epidermal features considered as antitranspirants are papillae and hairs overhanging stomatal crypts. However, the paleorecord of their occurrence in an extinct Cretaceous conifer (*Pseudofrenelopsis*) was not consistent with a change in paleoclimate aridity (Haworth and McElwain, 2008). Also, numerical modelling of water diffusion through the crypts of Proteaceae render the significance of their transpiration-reducing role doubtful (Roth-Nebelsick *et al.*, 2009). In addition, stomata sunken in pits also occur in species native to habitats well supplied with water or even occasionally flooded, mainly in conifers (Waldhoff *et al.*, 2002).

*Facilitation of CO<sub>2</sub> access in thick leaves.* Groups of stomata buried in deep grooves or crypts are also common in sclerophytes that cope with a deficiency of nutrients rather than with water shortage (Salleo *et al.*, 1997). These observations indicate that selective pressures other than drought probably contributed to the evolution of hidden stomata. Mohammadian (2005) and Hassiotou *et al.* (2009a) showed for a set of ten *Banksia* species that crypt depth correlated significantly with leaf thickness and dry mass per area (LMA). The volume of crypts and density of stomata inside the crypts, especially at their bottom, also increased with LMA and leaf thickness. This indicates that deep, voluminous crypts with walls covered with numerous stomata may function as porous ‘pipelines’ facilitating diffusion of CO<sub>2</sub> by shortening the distance to densely packed mesophyll and especially palisade tissue. Mathematical simulation of diffusion through the stomatal complex (antestomatal chamber, stomatal pore and sub-stomatal cavity) showed that the volume and shape of the antestomatal cavity are important parameters. An increase in both the area of the bottom of a crypt and density of stomata at the bottom should facilitate CO<sub>2</sub> uptake relative to water loss (Roth-Nebelsick, 2007) because the bottom part represents the internal air–mesophyll interface with the highest CO<sub>2</sub> demand.

*Protection of stomata from formation of water films in fog-rich environments.* Leaf surfaces have water-repellent properties that prevent formation of water films over stomatal pores, which would hinder CO<sub>2</sub> influx into the leaf and slow photosynthetic CO<sub>2</sub> assimilation. Diffusion of CO<sub>2</sub> through water is almost 10 000 times slower than through air (Nobel, 1991), so waterlogging of stomatal pores or even stomatal antechambers would effectively uncouple the leaf from the ambient atmosphere. Hydrophobicity of waxes deposited inside and on the top of the cuticular membrane extending through antestomatal chambers and stomatal pores reduces the risk of water-plugged stomata. Stomatal papillae, elevating the aperture of the stomatal antechamber above the general surface of the epidermis, are also considered as one of the adaptive traits avoiding stomatal waterlogging (Hill, 1998). Without wax and papillae, this phenomenon could frequently occur, especially in cloud-rich, foggy or rainy environments.

In some species, typically conifers, antestomatal chambers are completely filled with porous plugs of wax (Jeffree *et al.*, 1971; Brodribb and Hill, 1997; Kim *et al.*, 2010). This leaf trait was traditionally assumed to be an adaptation reducing transpiration and increasing water use efficiency (Jeffree *et al.*, 1971); however, fossil records show that the plugs appeared first in wet environments (Hill, 1998), and work with living species indicates that stomatal plugs inefficiently protect trees from water loss. Removal of stomatal plugs in *Drimys winteri*, an angiosperm tree from the wet tropics, did not increase the transpiration rate (Feild *et al.*, 1998). Wax plugs in *Sequoia* seem inefficient in preventing transpiration when stomata remain partially open. Burgess and Dawson (2004) found that night-time transpiration could often amount to 20 % of maximum daytime summer transpiration in *S. sempervirens*, a tree of coastal vegetation subjected to frequent occurrence of fog. These findings indicate that the plugs played only a minor role in saving water, especially at high air humidity that is common in the tree’s habitat. Instead of saving water, the main advantage of the wax plugs is likely to be prevention of continuous water films forming on the leaf surface. Therefore, the antechambers with waxy plugs obscuring the stomata could be most adaptive in wet habitats and cloud forest vegetation.

*Protection against environmental stress factors beyond drought.* Investigations of the epidermis morphology of *Erica arborea* leaves fumigated with toxic volcanic gases revealed that stomata occurred deeper in the leaf mesophyll than in control leaves from non-polluted locations (Bartirolo *et al.*, 2013). These results demonstrate another potential function of stomatal encryption – protection against harmful effects of polluted air. A specific action of small aerosols (<2 µm) such as soot, ash and fine dust is that the particles can settle on leaves and pass through stomatal openings, and, when moistened, can form a continuous film of water extending from the leaf surface through the stomata and into the leaf interior. This can create a liquid water connection between the leaf apoplast and the external atmosphere, forming in essence a wick that can transport liquid water directly from the internal epidermis or mesophyll cell walls. In doing so, stomatal control can be bypassed by what has been termed hydraulic activation of stomata (HAS) (Burkhardt, 2010). Extended boundary layer and micromorphological obstacles provided by papillae or trichomes arching over the antestomatal aperture can prevent deposition of fine airborne particles, reduce HAS and, consequently, save water. Consistent with this possibility, species with sunken stomata are common in fire-prone environments where smoke can regularly deposit fine aerosols.

Elevated leaf surface temperature is another factor which probably contributes to sunken stomata in leaves. For example, the depth of stomata in leaves from 23 *Banksia* species was correlated with the maximum annual temperatures in their respective habitats but not with minimum annual rainfall (Ratnawati, 2001). Higher leaf temperature increases leaf to air water vapour pressure difference (VPD), which as a driving force of transpiration could increase water loss in warm environments. A dual adaptation to increased leaf temperature and VPD can also explain the high abundance of deeply encrypted stomata in fossil leaves of vegetation from open habitats (Jordan *et al.*, 2014).

*Protection against pathogen attack*. Spores and bacteria accumulate around stomata and can invade the leaf interior via stomatal pores (Kroupitski *et al.*, 2009). Siting stomata deep below the epidermal leaf surface, and especially obstructions in the the aperture of antechambers such as waxy plugs, can prevent hyphae of fungi and other pathogens from entering the leaf (Mohammadian *et al.*, 2009). Fungal invasion is common in the humid environment of rain forest trees and, consistently, waxy plugs are frequently found in stomatal antechambers in leaves from these habitats (Mohammadian, 2005). Such features are postulated to increase leaf longevity (England and Attiwill, 2006). Stomatal wax repels water from the vicinity of the antestomatal cavity and prevents germination of fungal spores. However, the antestomatal cavity and wax plug can also provide a niche that enhances survival of phyllosphere fungi. Deckert *et al.* (2001) examined this plant–fungus relationship on needles of *Pinus strobus* but found no clear evidence of any mutual benefit.

*Creation of a humid micro-environment and improved hydraulic ‘safety’*. The stomatal antechamber represents an additional, invariable and, especially in the case of CO<sub>2</sub>, a moderating element in the chain of diffusion resistances into or out of a leaf. Numerical modelling has shown that the zone of steep vertical humidity depression above a superficially positioned stomatal pore (cup-shaped concentration isolines) is significantly reduced when the stoma is buried in the antechamber (Roth-Nebelsick, 2007). As a result, relative humidity near the sunken guard cells increased to 75 %, compared with 65 % for a non-sunken stoma. Hassiotou *et al.* (2009a) speculated that the additional fixed resistance imposed by the antechamber creates a safety buffer, which attenuates negative impacts of rapid variation in the ambient environment, such as from wind effects that reduce boundary layer thickness or sunflecks that suddenly warm leaves. A more humid and stable microclimate in stomatal crypts filled with hairs and/or waxy plugs may thus be possible, thereby enabling greater stomatal conductance at higher leaf temperatures and leaf to air VPDs. In experiments with the rain forest conifer *Agathis robusta*, Mohammadian (2005) observed maximum photosynthesis at a higher temperature (30 °C) in leaves with intact waxy plugs than in leaves where the plugs had been artificially removed (25 °C). He suggested that stomata in the empty crypts were exposed to less humid air and had partially closed, relative to the situation where plugs were intact. An additional benefit of the buffering effect of sunken stomata is that mesophyll resistance to CO<sub>2</sub> entry could be reduced with respect to LMA, given the disproportional shift of the overall resistance in the diffusion path to the water flux pathway that occurs with sunken stomata (Hassiotou *et al.*, 2009b).

Numerical 3-D simulation of water vapour diffusion across crypts of *Banksia ilicifolia* showed that the crypts cannot be considered as an invariable (fixed) conductance element (Roth-Nebelsick *et al.*, 2009). Their conductance depends not only on their shape and dimensions but also on the level of stomatal opening and relative humidity inside the crypt: the crypt conductance decreases with increasing stomatal conductance and with rising relative humidity. For example, the authors showed that crypt conductance decreased by 30, 21 and 10 % for narrow, standard and large stomatal pore size, respectively, when relative humidity inside the crypt increased from 50 % to 80 %. In

spite of the generally small effect of crypts on transpiration rate (10–15 % reduction) for narrow and intermediate stomatal openings, which the authors predicted, a much higher reduction (48 %) was calculated for an extremely large stomatal conductance. However, the ‘large’ values of conductance used in the simulation model were about ten times higher than those measured by gas exchange in *B. ilicifolia*, which makes a reduction of almost 50 % unrealistic. The lack of information about water sources inside the crypts, i.e. stomata, hairs and cuticle, and about the actual relative humidity inside crypts leaves the role of crypts in controlling transpiration rate unresolved.

Do stomata in deep pits or crypts respond differently from those located superficially? The solution to this problem is currently restricted by the lack of methods allowing separation of stomatal and cuticular contributions to leaf surface transpiration and conductance *in planta*. Without accounting for cuticular conductance, it is hardly possible to make any judgements about stomata, especially when fluxes through both the cuticle and stomata are of the same order of magnitude, as is the case in darkness or in drought-stressed plants.

Here, I hypothesize that the increased level of humidity within the stomatal crypt, or any other specific trait of the antechamber climate, could modify the behaviour of encrypted stomata. For example, stomata could remain open for an extended period of time during the day, contributing to increased carbon gain. Also a larger opening at night could bring benefits, especially for plants that suffer from nutrient deficiency and depend for their supply on continuous nocturnal xylem flux (Scholz *et al.*, 2007; Rohula *et al.*, 2014). Shortage of nutrients on nutrient-poor soils leads to the evolution of sclerophyllous species with thick and hard leaves and well-developed papillae around the stomata. In order to test the hypothesis of a specific behaviour of sunken stomata at night, a technique allowing partitioning of the leaf surface conductance in the dark into its components was developed and employed here. The technique is described in the Appendix. Stomatal and cuticular conductance at the dark–light and light–dark transitions was measured in seven species with antestomatal chambers with different degrees of prominence.

## MATERIALS AND METHODS

### *Plant species and growth conditions*

To test the above hypothesis, the following set of species with well-pronounced antechambers of different types was investigated: *Nerium oleander* L. (oleander), a Mediterranean sclerophyte shrub with typically voluminous crypts, each accommodating numerous stomata and filled with a dense mesh of trichomes (Fig. 1A–F); *Ficus elastica* Roxb. ex Hornem. (fig tree), a hemi-epiphyte native to tropical Asia, having large antechambers with one stoma at the bottom and no trichomes inside the crypt (Fig. 3A–C); *Clusia rosea* Jacq. (autograph tree), a tropical tree native to the Caribbean, with smooth leaves covered by a deep layer of epicuticular wax and medium-sized stomatal antechambers (Fig. 3D, F); and *Olea europaea* L. (olive

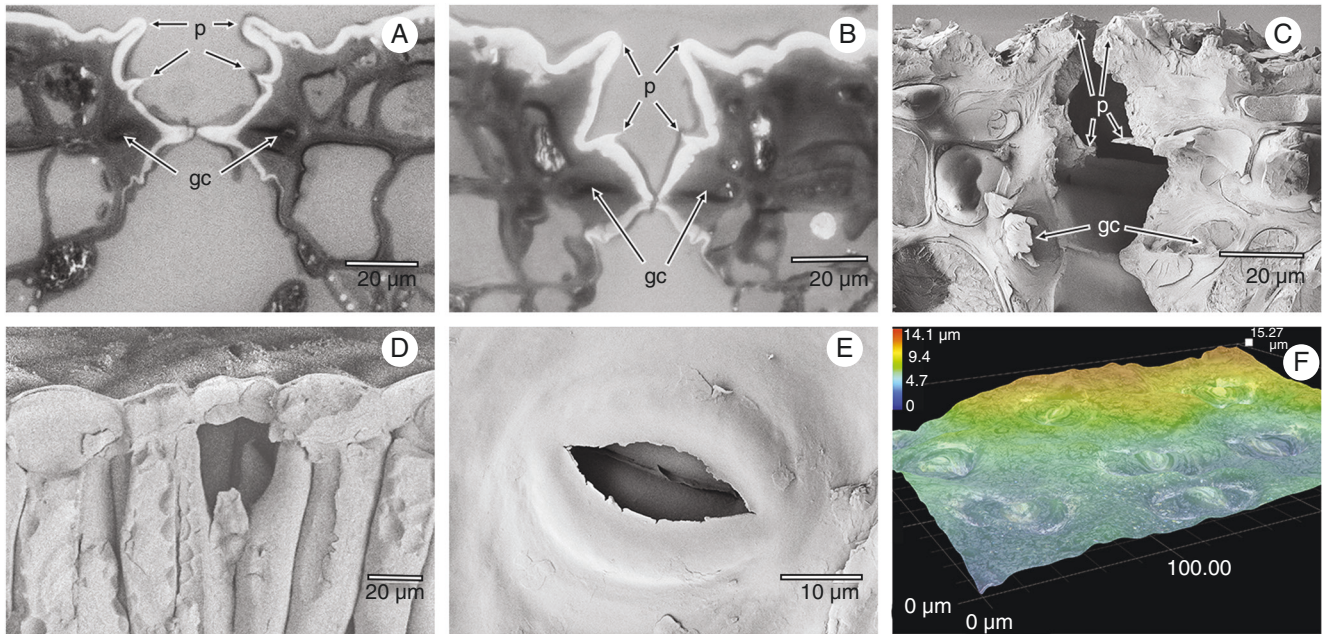


FIG. 3. Antestomatal chambers of *Ficus elastica* (A–C) and *Clusia rosea* (D–F) leaves. Optical microscope images of semi-thin leaf transversal sections (A, B) and cryo-SEM image (C) of transects through stomatal antechamber pits of *F. elastica*, guard cells and through the underlying substomatal cavity. The pits are arched with external papillae (p). Guard cells (gc), located about 20–40 µm deep at the bottom of pits, produce internal papillae (p). The whitish layer in (A) and (B) covering the leaf surface and antechamber walls and protruding into the sub-stomatal cavity consists of epicuticular wax, indicating the existence and extent of an internal cuticle. Cryo-SEM images (D, E) and the digital microscope (Keyence WHX-7000) relief map (F) of *C. rosea* show the sub-stomatal cavity (D), upper view into the antestomatal chamber (E) and shallow elliptical furrows around the external stomatal papillae (F).

tree), a sub-tropical dry-forest tree native to Africa, Asia Minor and the Mediterranean region, whose abaxial leaf side is fully covered with disc-shaped trichomes hiding the stomata beneath (Fig. 4A–F). All four species are hypostomatous, with thick leaves and relatively slow growth. For comparison, I included a set of three fast-growing annual species with thin amphistomatous leaves bearing relatively small-volume stomatal antechambers formed only by the papillae and the wall of thin epidermal cells: *Arabidopsis thaliana* L. (arabidopsis, Fig. 5A, D), *Capsicum annuum* L. (pepper, Fig. 5B, E) and *Brassica oleracea* (broccoli, Fig. 5C, F).

Plants from the ‘large antechamber’ group were obtained from a local garden shop as 3- to 4-year-old saplings and grown in a glasshouse for 3–7 months (*N. oleander* and *O. europaea*) or for 3–5 years (*C. rosea* and *F. elastica*) before the gas exchange measurements. The *F. elastica* plants were acclimated to deep shade conditions [photosynthetic photon flux density (PPFD) 0–40 µmol m<sup>-2</sup> s<sup>-1</sup>]. Plants from the ‘small antechamber’ group were grown from seeds in 1 L pots filled with garden substrate (*C. annuum* and *B. oleracea*) or in approx. 100 mL pots (*A. thaliana*) in a growth chamber (Percival Scientific, Inc, USA). The PPFD was 450 ± 50 µmol m<sup>-2</sup> s<sup>-1</sup> (*C. annuum* and *B. oleracea*) or 250 ± 50 µmol m<sup>-2</sup> s<sup>-1</sup> (*A. thaliana*), day/night temperatures 25/17 °C at a 16 h photoperiod, and the free atmospheric CO<sub>2</sub> concentration was 360–420 µmol mol<sup>-1</sup>. Four to six different plants from *C. rosea*, *A. thaliana*, *C. annuum* and *B. oleracea* were used, and one plant each from the other species. Four to twelve attached leaves per species were tested for nocturnal stomatal opening. All plants were regularly watered, keeping the soil surface moist.

### Theory

The principle for estimating nocturnal stomatal conductance with gas exchange measurements is explained in the Appendix. Briefly, the light response of net CO<sub>2</sub> exchange rate,  $A$ , [photosynthesis minus (photo-)respiration rate] was measured. The data were used to plot  $A$  vs. the leaf internal CO<sub>2</sub> concentration,  $c_i$ , showing the effects of both  $c_i$  and light on the rate of net CO<sub>2</sub> exchange. Typically, when the  $A(c_i)$  relationship is analysed,  $c_i$  is manipulated by varying the external CO<sub>2</sub>,  $c_a$ . Here,  $c_i$  varied in response to the light-dependent  $A$ , and both were recorded when the leaf total conductance for water vapour,  $g_{tw}$ , reached a steady state. Thus, each point of the  $A$  vs.  $c_i$  plot as shown in the Appendix (Fig. A1) was recorded at a different incident light intensity (PPFD). Special attention was given to the  $A$  and  $c_i$  values at zero or very low light levels (0–5 µmol photons m<sup>-2</sup> s<sup>-1</sup>), i.e. to the dark–light transitions in the morning (at artificial dawn) or the light–dark transitions after the daily light period (at artificial dusk). Typically, the  $A$  and  $c_i$  values fall on a straight line, which indicates that the flux ( $A$ ) is linearly proportional to its driving force ( $c_a - c_i$ ) and the conductance invariant during the dark–light–dark transitions. The slope of the line corresponds to the stomatal (gaseous) conductance for CO<sub>2</sub>,  $g_{sc}$ , in the dark, and can be converted to water vapour conductance of stomata,  $g_{sw}$ , using the ratio of water and CO<sub>2</sub> diffusivities ( $D_{H_2O}/D_{CO_2} = 1.6$ ). The calculations of  $c_i$  used as a standard in gas exchange analysis are erroneous due to the incorrect assumption of zero water flux through the cuticle. However, the  $g_{sc}$  slope is insensitive to the erroneous calculations of  $c_i$  provided that the stomatal and cuticular conductances to water

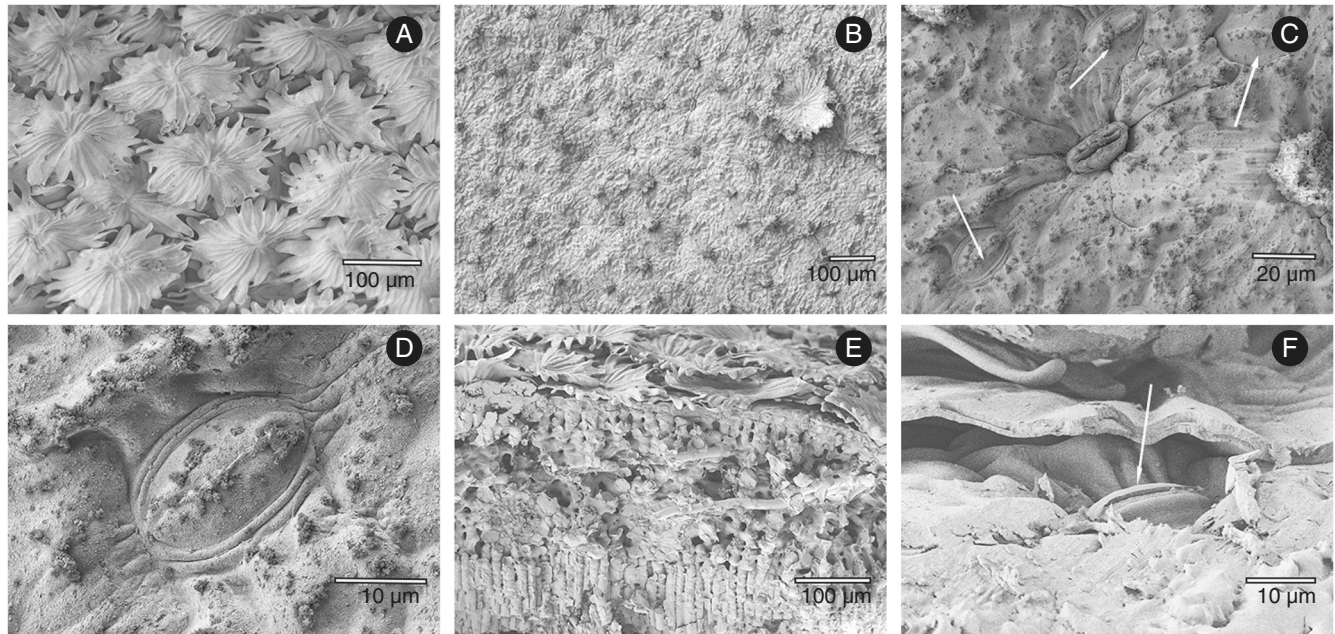


FIG. 4. Abaxial surface and section through an *Olea europaea* leaf. The leaf is covered with flat, disc-shaped trichomes (A), which fully cover the stomata underneath (arrows in C, F and D) and form a special type of crypt that can be uncovered when the trichome is broken (F). The disc trichomes can be removed (torn off) with adhesives (here: collodion) and the stomata exposed (B–D). The abaxial surface and a transect through mesophyll tissue is shown in (E).

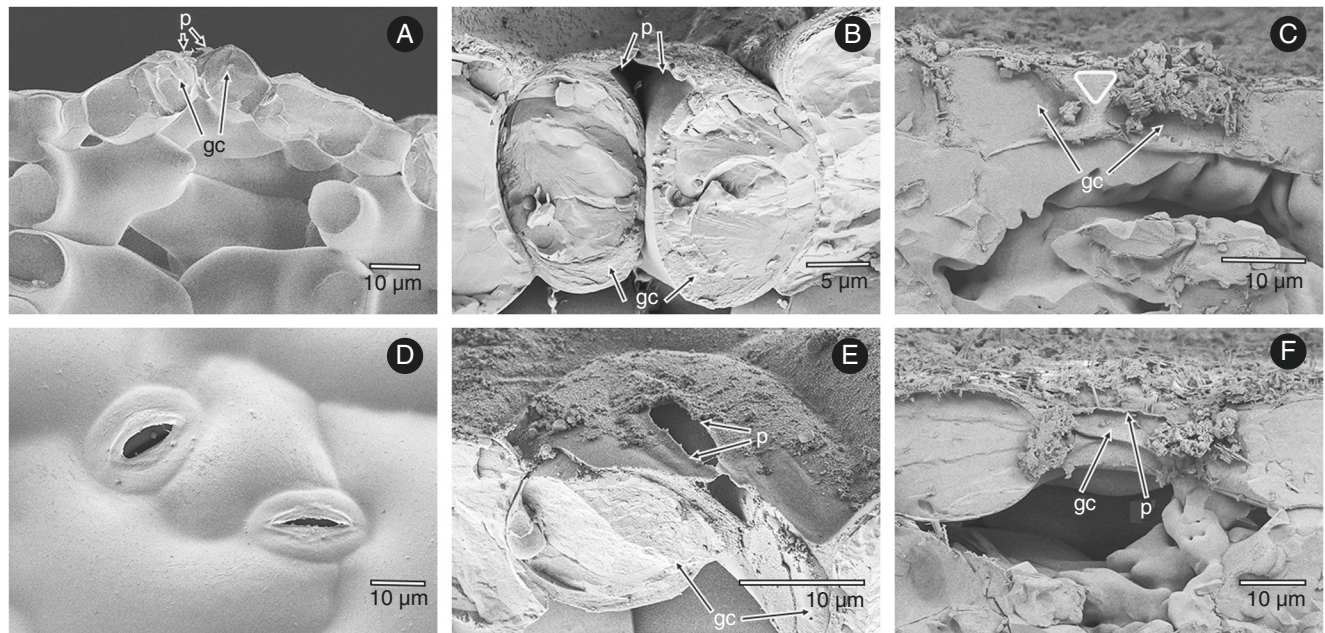


FIG. 5. Surface and transect cryo-SEM views of leaves of *Arabidopsis thaliana* (A, D), *Capsicum annuum* (B, E) and *Brassica oleracea* (C, F). Abaxial surface of *A. thaliana* with transect of guard cells (gc) and papillae (p) (A) and a surface view of stomata (D) with the aperture formed by cuticular ledges (papillae) arching over a shallow antestomatal cavity. Freeze fracture through guard cells of *C. annuum* with papillae arching over a shallow antestomatal chamber (B, E). Freeze fracture of *B. oleracea* leaf showing a trans-sectional (C) and longitudinal (F) imprint of guard cells and papilla, delimiting a small antestomatal cavity (triangle in C) filled with cuticular wax. Epicuticular wax crystals are visible on the leaf surface.

( $g_{sw}$  and  $g_{cw}$ ) do not change during the dawn or dusk measurements. The knowledge of stomatal conductance to water, together with that of leaf total conductance  $g_{tw}$  as estimated with a standard gas exchange device, allows the calculation of cuticular conductance for water and partitioning of  $g_{tw}$ .

#### Gas exchange measurements

The portable photosynthetic system LI-6800 (LI-COR, Lincoln, NE, USA) has been used to record the light-driven  $A(c_i)$  relationship. Measurements started in the morning with the following leaf chamber settings: air flow through the chamber 250  $\mu\text{mol}$

(air)  $s^{-1}$ , chamber - ambient pressure difference ( $\Delta P$ ) 0.1 Pa, leaf-air water VPD 1.2 kPa,  $CO_2$  concentration in the leaf cuvette  $410 \mu mol mol^{-1}$ , leaf temperature 20 or 25 °C (specific for each species and selected for being closest to the ambient temperature) and fan speed 10 000 rpm. The  $6 \times 6 cm^2$  leaf chamber (6800-13) equipped with the large source (6800-03) of white light was used for all species. Typically, the following light levels were applied with 30–50 min intervals between the measurements: 0, 1, 3, 5, 10, 20, 50, 100, 300, 600, 1000, 1200, 7, 4, 2, 1 and 0  $\mu mol m^{-2} s^{-1}$ . Usually, recording the full light response took between 8 and 14 h so the light-dark transition was applied close to actual dusk. The leaf area exposed in the leaf chamber was estimated after the measurements by weighing a paper replica of the leaf, and data were recalculated accordingly. The high irradiance level (PPFD  $\geq 1000 \mu mol m^{-2} s^{-1}$ ) was omitted in the low-light-acclimated species (*F. elastica*). To investigate the effect of the disc-shaped trichomes covering stomata on *O. europaea* leaves, the trichomes were removed (torn off, see Fig. 4B–D) using collodion (a solution of nitrocellulose in diethyl ether and ethanol, Merck, Germany). The collodion treatment and light response measurements were carried out with the same leaf, which was measured intact on the previous day.

#### Cryo-scanning electron microscopy

A fresh leaf sample was transferred onto a holder with a slit and clamping mechanism, glued on with Tissue-Tek and frozen by plunging it into liquid nitrogen slush. After freezing, the sample was transferred to a high vacuum cryo-preparation chamber (ALTO 2500, Gatan), where the sample was fractured at  $-160$  °C, sublimated at  $-95$  °C for 2 min and coated with 5 nm of gold at  $-135$  °C. The coated sample was inserted into a Field Emission Electron Microscope (JSM-7401-F, JEOL) pre-cooled at  $-135$  °C. Images were obtained using an Everhart-Thornley detector at an accelerating voltage of 1.5 kV in Gentle Beam (GB) low mode.

#### Data evaluation and statistics

Determination of the regression coefficients relating  $A$  to  $c_i$  at the dark-light and light-dark transitions was the critical step in separation of cuticular and stomatal conductance. Usually, either three or four  $[A;c_i]$  points were used for each artificial dawn and dusk transition. The slope of the best fit, with the coefficient of determination being nearest to one, was used for further evaluation. The slopes and coefficients of determination were determined using the MS Excel software package, which was also used for corrections of the slopes for  $CO_2$  permeability of the cuticle and the partitioning of leaf total conductance into its stomatal and cuticular fractions. Means and standard deviations were calculated using the MS Excel or SigmaStat packages.

## RESULTS

#### Leaf water conductance in the dark (at dawn and dusk)

Leaf surface conductance,  $g_{tw}$ , was higher at dawn than at dusk in all species (Fig. 6A, B, horizontal axis). The former

ranged from 0.4 to 99.3  $mmol m^{-2} s^{-1}$  and the latter from 0.2 to 86.6  $mmol m^{-2} s^{-1}$  across all species except for *B. oleracea*. Dark conductance in *B. oleracea* ranged up to 362  $mmol m^{-2} s^{-1}$  at dawn (Fig. 6A). The dawn conductance exceeded that at dusk by a factor of almost two in the sunken stomata group ( $g_{tw}^{dawn} = 100 \pm 83 mmol m^{-2} s^{-1}$  and  $g_{tw}^{dusk} = 57 \pm 71 mmol m^{-2} s^{-1}$ , mean  $\pm$  s.d.) while their ratio in arabidopsis was only 1.2 ( $g_{tw}^{dawn} = 286 \pm 124$  and  $g_{tw}^{dusk} = 222 \pm 131 mmol m^{-2} s^{-1}$ ).

#### Stomatal conductance at dawn and dusk

In all plant species and in the majority of measurements, the coefficients of determination of the regressions relating  $A$  to  $c_i$  were close to 1 at dawn ( $R^2 = 0.984 \pm 0.024$ ,  $n = 76$ ) and slightly lower at dusk ( $0.946 \pm 0.076$ ,  $n = 53$ ) (means  $\pm$  s.d.). Slopes of the regression lines, which provide the stomatal conductance for  $CO_2$ ,  $g_{sc}$ , were converted to stomatal conductance for water,  $g_{sw}$ , using eqn (A7). In the vast majority of measurements,  $g_{sw}$  was lower than the leaf conductance for water,  $g_{tw}$ , obtained directly from standard gas exchange measurements, so almost all data points lie below the 1:1 line in Fig. 6A and B.

Stomatal conductance in the dark increased with increasing total leaf conductance in an approximately linear pattern for both the species with sunken stomata (red line in Fig. 6) and species with superficial stomata (blue line). The vertical distance between the coloured lines and the  $x$ -axis indicates the stomatal fraction of total leaf conductance, while the vertical distance between the coloured and 1:1 lines shows the cuticular fraction. The slopes of the regression lines indicate that stomata accounted for 85 and 57 % of leaf conductance at dawn in the superficial and sunken stomata groups, respectively (Fig. 6A), while at dusk the respective stomatal contributions decreased to 81 and 26 % (means across all species in the respective group).

#### Response of sunken stomata

The linear approximations in Fig. 6 fail to uncover the group-specific stomatal behaviour, especially at dawn (Fig. 6A). A closer look at the stomatal contribution to leaf conductance in Fig. 7 reveals that the prevalence of stomatal over cuticular conductance in darkness persists in the species with superficial stomata over the whole range of leaf conductances (see blue lines in Fig. 7). In contrast, the fraction of stomatal over total leaf conductance decreased with increasing leaf conductance in species with sunken stomata (red lines). At both dawn and dusk, superficial stomata were responsible for about 80–87 % of leaf conductance. In contrast, the relative contribution of sunken stomata decreased with increasing total leaf conductance, and the sensitivity of stomata to  $g_{tw}$  (slope of the red line) was even higher at dusk than at dawn.

#### Cuticular and stomatal conductance in the dark: interspecies comparison

Stomatal conductances in darkness span almost three orders of magnitude across all seven species (0.24–143  $mmol m^{-2} s^{-1}$ , see Fig. 8). Cuticular conductance was proportional to stomatal



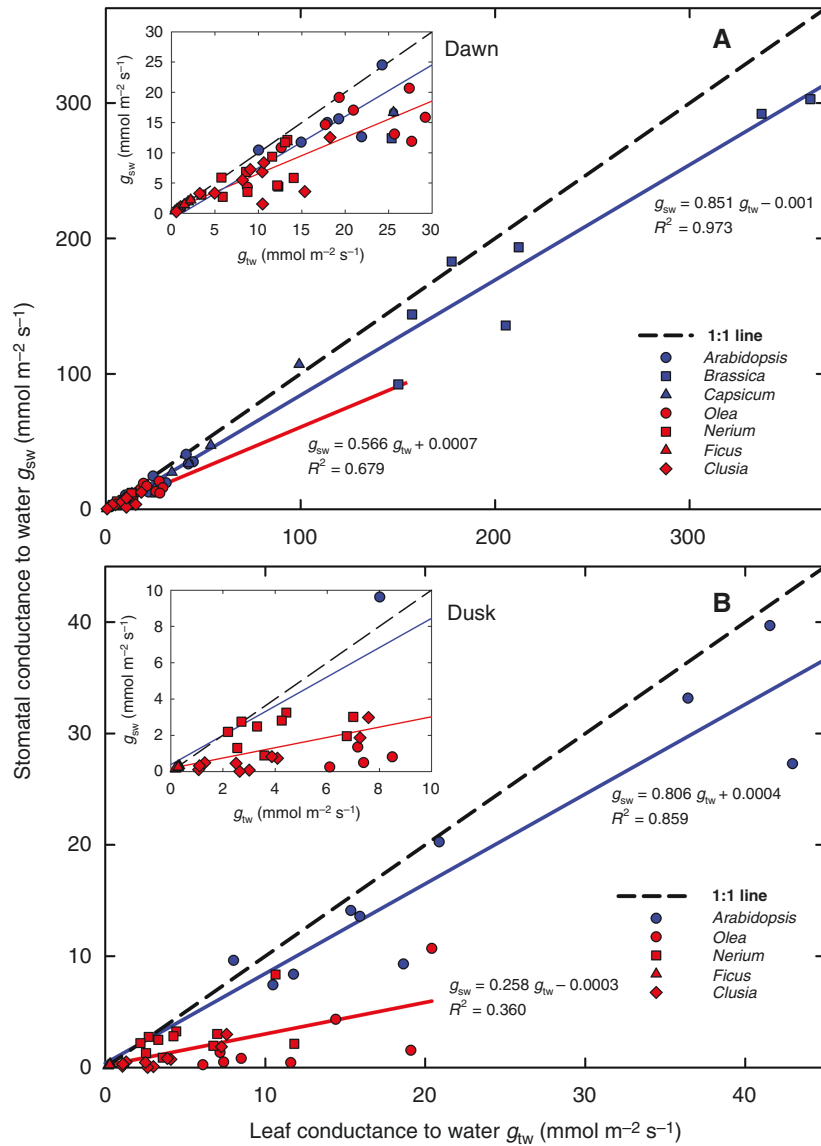


FIG. 6. Contributions of stomatal and cuticular conductance to total leaf water conductance at the dark–light (dawn) and light–dark (dusk) transitions. Stomatal conductance  $g_{sw}$  was estimated from the regression of  $A$  vs.  $c_i$  as described in the Appendix [eqn (A7)] while the total conductance  $g_{tw}$  was measured with a gas exchange device (LI-6800). Values found at artificial dawn (A) and dusk (B) are shown. Each point indicates a measurement on an individual leaf in one of four species characterized by well-pronounced stomatal antechambers (*N. oleander*, *F. elastica*, *O. europaea* and *C. rosea*, red symbols and red regression line) and in a group of species with superficial location of stomata (*A. thaliana*, *C. annuum* and *B. oleracea*, solid blue symbols and lines). The dusk data for superficial stomata include only *A. thaliana*. Insets depict low  $g_{tw}$  ranges at higher resolution. The 1:1 lines (black dashed) indicate the theoretical situation where stomata fully account for total conductance  $g_{tw}$ . Slopes of the regression lines indicate the estimated relative contributions of stomata.

conductance, but its range of absolute values was somewhat less ( $0.05\text{--}48.5 \text{ mmol m}^{-2} \text{ s}^{-1}$ ). In both cases, the extremes belong to *F. elastica* and *B. oleracea*. The means of cuticular conductance in the other five species ranged within a relatively narrow interval from 1 to  $10 \text{ mmol m}^{-2} \text{ s}^{-1}$ , while stomatal conductance was between 5 and  $40 \text{ mmol m}^{-2} \text{ s}^{-1}$ . After the leaf trichomes on *O. europaea* have been removed,  $g_{sw}$  at dawn increased by a factor of 4 and returned to the value of the non-treated leaves at dusk. In contrast, cuticular conductance decreased to one half in the leaves without trichomes at dawn and increased 3-fold at dusk. Interestingly, the  $g_{sw}$  values at dawn were higher than those at dusk in each species and in the vast majority of leaves that were measured, but the pattern was ambiguous in the case

of cuticular conductance without any trend of  $g_{cw}$  between or within species (Supplementary data Fig. S1).

## DISCUSSION

Sunken stomata have traditionally been examined by describing that what is readily visible – the antechamber cavity, its shape, volume or content (Jeffree *et al.*, 1971; Weyers and Meidner, 1990; Willmer and Fricker, 1996; Brodrribb and Hill, 1997; Larcher, 2001; Roth-Nebelsick, 2007; Haworth and McElwain, 2008; Hassiotou *et al.*, 2009a; Roth-Nebelsick *et al.*, 2009). The functional implications of the structure of the guard and subsidiary cells, and the mechanical and physiological consequences of their being sunken have been less investigated

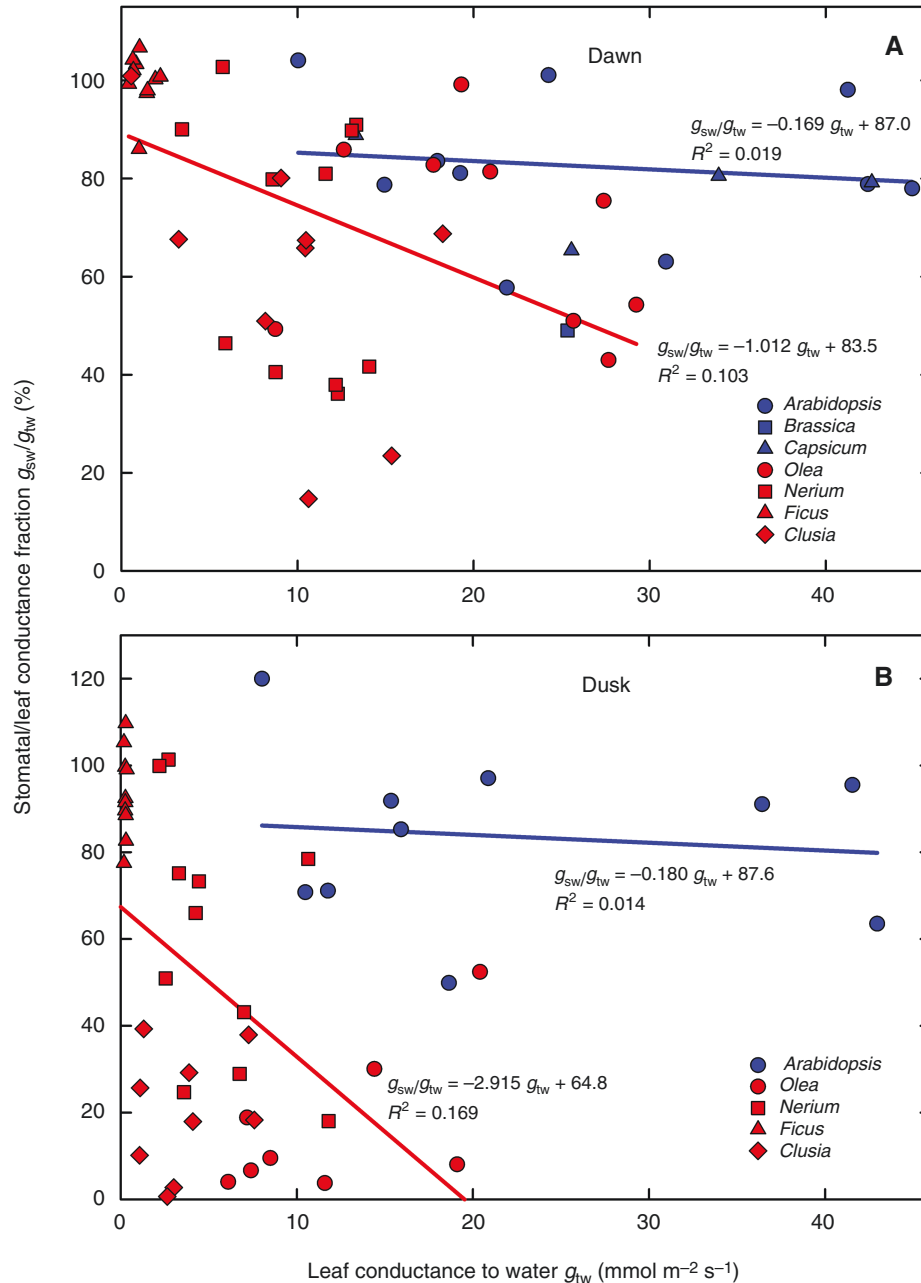


FIG. 7. Fraction of stomatal to total leaf conductance at dawn (A) and dusk (B). Points indicate individual measurements from all investigated species with well-pronounced stomatal antechambers (*N. oleander*, *F. elastica*, *O. europaea* and *C. rosea*, red symbols and red regression lines) and ten measurements on different *A. thaliana* leaves with a superficial location of stomata (solid blue symbols and lines). The ratio  $g_{sw}/g_{tw}$  is shown for the morning period – at dawn (A) – and for the evening when the measurements of light response of photosynthesis with the same leaf were concluding – at dusk (B).

(Feild *et al.*, 1998; Eamus *et al.*, 2008; Mott and Peak, 2013). Here I address this deficiency by examining the behaviour of sunken and superficial stomatal complexes in response to identical environmental drivers. As a prerequisite, it was necessary to design a technique allowing measurements of stomatal conductance without the interference of cuticular water loss. It was more feasible to do this for darkened leaves. Therefore, the discussion starts with a phenomenon that is generally acknowledged and experimentally more accessible – nocturnal transpiration – and specific effects of the formation of crypts on stomatal and cuticular function on that process.

#### Stomatal conductance dominates nocturnal leaf conductance

Nocturnal transpiration is a phenomenon that has been explored frequently during the last decades but its significance and ecological diversity are still unclear (de Dios *et al.*, 2019; Fricke, 2019). The leaf surface conductance ( $g_{tw}$ ) at dawn and dusk ranged, in the measurements presented here, from some tens to several hundred  $\text{mmol (H}_2\text{O) m}^{-2} \text{s}^{-1}$  across seven selected species and was higher at dawn than at dusk in all but two of 49 measured intact leaves (Supplementary data Fig. S1). The dawn vs. dusk difference in  $g_{tw}$  seems to be a ubiquitous feature, probably controlled by the endogenous circadian clock

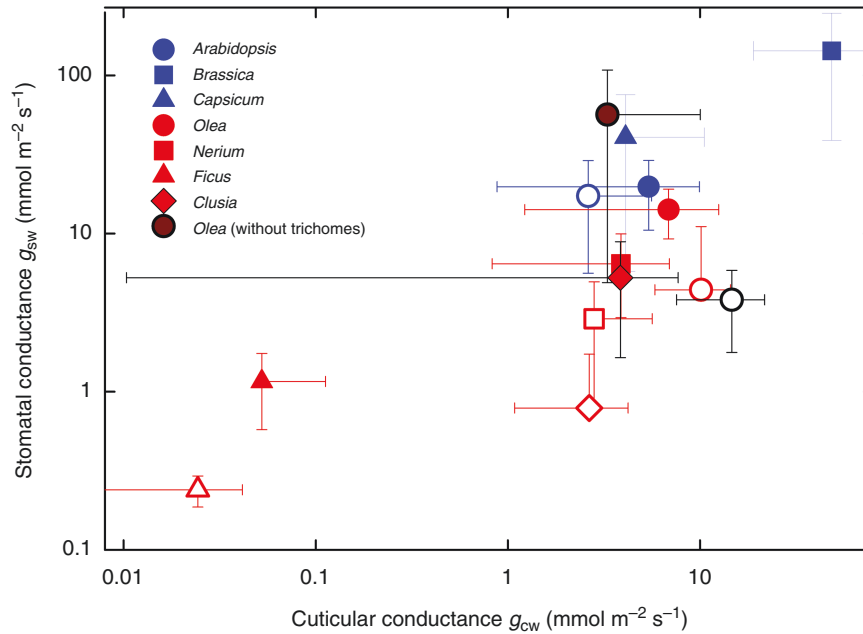


FIG. 8. The relationship between stomatal and cuticular conductance in seven species with stomatal antechambers of different prominence. Means and standard deviations of  $g_{sw}$  and  $g_{cw}$  at dawn (solid symbols) and dusk (open symbols) of four species with well-pronounced stomatal antechambers (*N. oleander*, *F. elastica*, *O. europaea* and *C. rosea*) and three species with superficially located stomata with less pronounced low-volume antechambers (*A. thaliana*, *C. annuum* and *B. oleracea*) are presented. Only the dawn values for *B. oleracea* and *C. annuum* are shown. All means and standard deviations were calculated from 6–10 measurements on different leaves. Note that the scales on both axes are logarithmic. The black-rimmed circles indicate *O. europaea* leaves with trichomes removed by collodion (see also Fig. 4B–D).

(Caird *et al.*, 2007; Costa *et al.*, 2015). Interestingly, the amplitude of the dawn–dusk oscillations was higher in species with sunken stomata. I can only speculate about the reasons, which might arise from the greater diurnal fluctuation of turgor in sclerophyllous vs. mesophytic leaves, a different type of osmoregulation in guard cells (Lasceve *et al.*, 1997) and/or specific cuticular traits in species with sunken stomata. Dawn and dusk conductances correlated in the data here with maximum stomatal conductance (Supplementary data Fig. S2) and photosynthetic rate (not shown) during the light response measurements. The correlation supports the hypothesis that nocturnal transpiration promotes leaf metabolism and plant growth, possibly by flushing respiratory  $\text{CO}_2$  out of the leaf and avoiding toxic  $\text{CO}_2$  concentrations in mesophyll cells (Fricke, 2019).

The partitioning of conductance in (near)darkness was a prerequisite of the experimental part of this study. The method, which was designed and applied here (see Appendix), is in principle similar to that published recently by Marquez *et al.* (2022). Both methods use a standard commercial gas exchange device for *in planta* measurements, but the technique of Marquez *et al.* was developed for red-light-illuminated leaves while the technique presented here was for measurements in the dark or in light–dark transitions. Nevertheless, both methods yielded comparable results. For example, cuticular conductance for water in *C. annuum* leaves was about  $8 \text{ mmol m}^{-2} \text{ s}^{-1}$  and  $6.5 \text{ mmol m}^{-2} \text{ s}^{-1}$  in the measurements of Marquez *et al.* and in this study, respectively. Cuticular conductance at dawn and dusk averaged over all seven species measured here was  $7.8$  and  $3.7 \text{ mmol m}^{-2} \text{ s}^{-1}$ , respectively, which is in the range of values reported for 160 species in the recent review of Schuster *et al.* (2017).

I hypothesized that increased stomatal opening and transpiration in darkness could provide a selective advantage to plant species with sunken stomata. They often are epiphytes, or sclerophytes inhabiting nutrient-poor soils, and nocturnal transpiration could increase the efficiency of water used for nutrient uptake and/or increase nutrient acquisition *per se* (Scholz *et al.*, 2007; Rohula *et al.*, 2014). Leaf surface conductance in the dark,  $g_{tw}$ , was high in three thin-leaved amphistomatous species with superficial stomata (arabidopsis, pepper and broccoli,  $23$ – $232 \text{ mmol m}^{-2} \text{ s}^{-1}$ ) and low in the group of thick-leaved hypostomatous species with encrypted stomata (fig tree, oleander, autograph tree and olive tree,  $1$ – $20 \text{ mmol m}^{-2} \text{ s}^{-1}$ ). Stomata were usually responsible for more than half of the leaf conductance in darkness but, contrary to my hypothesis, they dominated more when located on the leaf surface (about 80 % of  $g_{tw}$  at both dawn and dusk) and less when embedded in antechambers (73 % of  $g_{tw}$  on average across four species). The dominance of stomata in nocturnal transpiration has been assumed in the literature but, to date, experimental evidence had been lacking. The assumption was based on the comparison of relatively high nocturnal leaf conductance (median of  $40 \text{ mmol m}^{-2} \text{ s}^{-1}$  in a global dataset, de Dios *et al.*, 2019) with the substantially lower cuticular conductance assessed by various techniques [median of  $0.74 \text{ mmol m}^{-2} \text{ s}^{-1}$  obtained from measurements of isolated cuticles or astomatous leaf sides of 57 species, Kerstiens (1996); median of  $1.47 \text{ mmol m}^{-2} \text{ s}^{-1}$  across a set of 160 species, Schuster *et al.* (2017); mean of  $4.89 \text{ mmol m}^{-2} \text{ s}^{-1}$  across 221 species, measured as mass loss from detached leaves, Duursma *et al.* (2019)]. Cuticular conductance in most of the species examined here ranged from 1 to  $10 \text{ mmol m}^{-2} \text{ s}^{-1}$ .

except for *F. elastica* with a  $g_{cw}$  of 0.05 and 0.02 mmol m<sup>-2</sup> s<sup>-1</sup> at dawn and dusk, respectively (Fig. 8).

#### *Are stomata in crypts controlled by water loss across the cuticle?*

As mentioned above, the sunken stomata were less open in darkness and, across four species, they accounted for a smaller fraction of total leaf conductance than the superficial stomata (Fig. 6). It seems that they are more conservative compared with the non-sunken stomata. However, when looking at the dynamics of their contribution to the total leaf conductance, the opposite picture emerges: stomata in deep voluminous pits control their fraction of dark transpiration more sensitively while superficial stomata keep their proportion invariable (Fig. 7). This seems to be a unique feature of sunken stomata across the four investigated species, though this feature is not well preserved intraspecifically, i.e. when each species is observed individually (Supplementary data Fig. S3). The explanation for the greater stomatal response among the four species with sunken stomata is not clear. It could possibly be based on stomata–cuticle interactions. Eamus *et al.* (2008) have shown that the stomatal aperture response to leaf-to-air VPD is mediated by cuticular permeability in *Eucalyptus*, *Commelina* and *Vicia*, and a similar response may explain the observations of Feild *et al.* (1998) in *Drimys*, a tropical wet-forest tree with encrypted stomata. The markedly enlarged area of the leaf cuticle in species with stomata sunken in crypts may enhance this effect (Roth-Nebelsick *et al.*, 2009).

Total leaf conductance in the dark,  $g_{tw}$ , and dark transpiration rate were proportional to each other under the experimental conditions here because the driving force for evaporation (VPD) was kept constant. Low  $g_{tw}$  implies a low rate of water loss, which should increase in parallel with rising  $g_{tw}$ . Results show that in species with sunken stomata, the contribution of stomatal conductance to  $g_{tw}$  decreased relative to the contribution of cuticular conductance,  $g_{cw}$ , at higher transpiration rates at night. In short, each increment in dark transpiration occurs more through the cuticle than through the stomata. This stomata–cuticle complementarity is much more pronounced among species with antechamber-embedded stomata and might result from the phenomenon traditionally called peristomatal transpiration (Maier-Maercker, 1983). As the term indicates, water evaporation directly from the exposed surface of guard cells is assumed to be higher than that from other epidermal cells. The sunken guard and/or subsidiary cells may be covered with a more permeable cuticle that induces closing of the stomatal pore when water flux through the cuticle is rising. Higher water permeability of the stomata-bearing cuticle compared with the astomatous one was reported for several species (Santrucek *et al.*, 2004; Karbulkova *et al.*, 2008; Marquez *et al.*, 2022) and could close stomata by reducing guard cell turgor. Nevertheless, an unambiguous and truly reliable dataset that quantifies permeability of the guard cell and pavement cell cuticles is lacking. Cuticular permeability *per se* can also be affected by the humidity of the surrounding air. It has repeatedly been shown that the permeability increases at elevated water vapour pressure or relative humidity (Hoad *et al.*, 1997; Schönherr and Merida, 1981; Schreiber *et al.*, 2001). Cuticle shrinkage or

swelling induced by changes in leaf turgor are accompanied by changes in cuticular permeability (Boyer, 2015b) and might also contribute to the lower stomatal and variable cuticular conductance at dusk compared with dawn (Fig. 8; Supplementary data Fig. S1).

#### *Other mechanisms affecting sunken stomata*

It has been shown several times that the response of stomata to humidity is affected predominantly by the water potential of neighbouring cells rather than directly by the humidity of the surrounding air (Nonami *et al.*, 1991; Buckley, 2005). Guard cells located at the bottom of an antestomatal cavity may experience smaller fluctuations of water potential in response to variations in leaf energy budgets (resulting from changes in ambient temperature or irradiance). Their location deeper in the leaf interior results in a higher resistance to transfer of heat from the leaf surface to evaporation sites and, conversely, to a lower resistance for water vapour diffusing from evaporating sites to guard cells (Mott and Peak, 2013). These authors verified that the ratio of these two resistances is much higher in *N. oleander* with stomata in crypts than in *Xanthium strumarium* and *Pastinaca sativa*, with more superficially positioned stomata, and speculated that it could help the plants with sunken stomata to maintain an isohydric water regime in harsh conditions. Isohydry is characterized by a relatively stable mid-day leaf water potential independent of seasonal or daily fluctuations in pre-dawn water potential of the plant shoot, so stomata of isohydric species are more responsive to fluctuating transpiration or VPD (Franks *et al.*, 2007). Isohydry typically occurs in trees of xeric environments, for example in savannah trees, and occurs in environments with high potential risk of embolism and high costs of its repair (Bucci *et al.*, 2005). In contrast, stomata of anisohydric plant species, usually fast-growing crops or annuals such as sunflower, respond less sensitively to changes in transpiration rate or VPD. The stomatal responses of the two groups of species investigated here, one with sunken and the other with superficial stomata, resemble the stomatal response of iso- and anisohydric species, respectively. I can only speculate as to whether the apparent isohydry of the species with markedly sunken stomata is just a coincidence or whether there is a causal link between guard cells ‘hidden’ in deep antestomatal chambers and their response to evaporative demand.

## CONCLUSION

Sunken stomata have multiple functions. Their advantages in xeric and/or infertile conditions with fluctuating soil water availability and VPD are readily apparent, linked to water saving and avoidance of fatal, unrepairable depressions in leaf water potential and embolism (Fig. 9). Guard cells devoted to this purpose follow the isohydric mode of operation, i.e. they sensitively control transpiration, keeping the water potential stable and above the critical threshold for embolism (Tardieu and Simonneau, 1998). The results presented here indicate that, indeed, the species with sunken stomata control even the mild

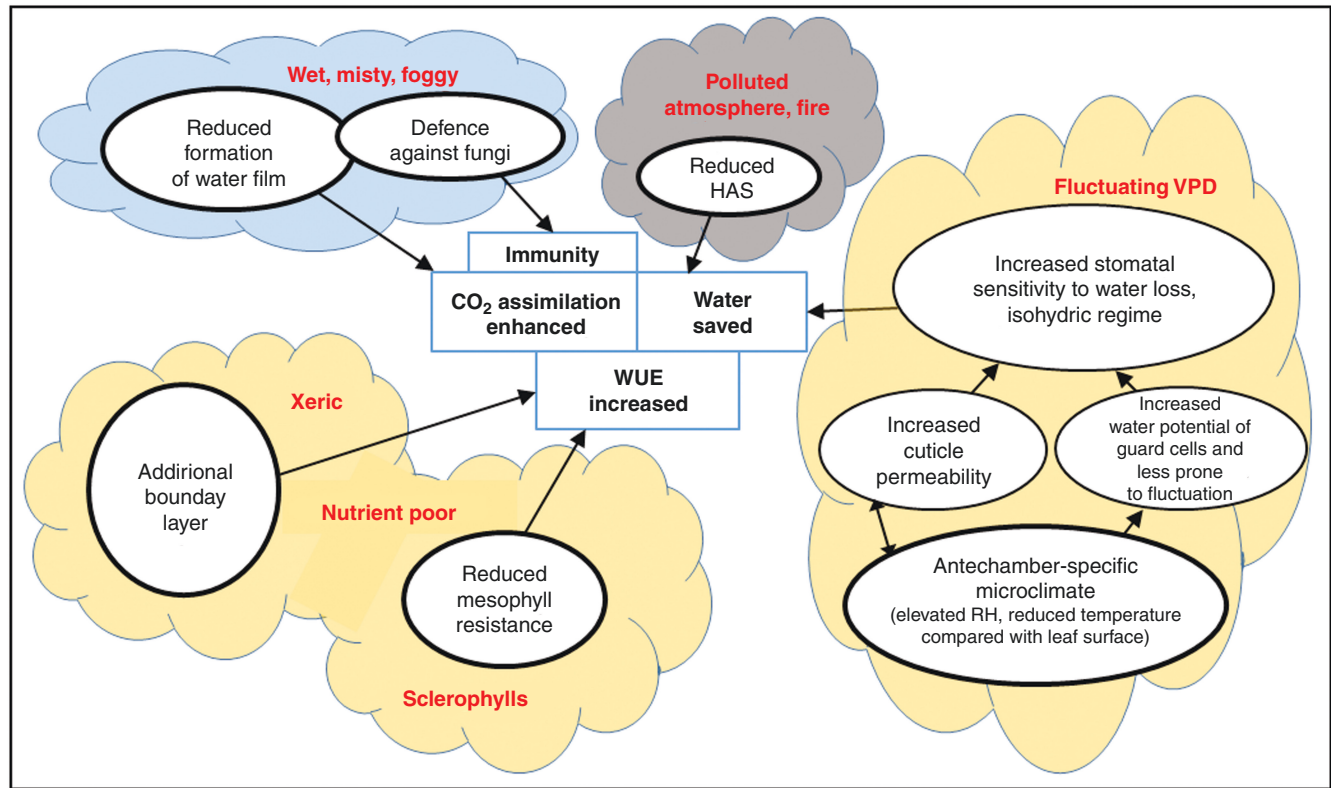


Fig. 9. The multipurpose character of antestomatal chambers. At least four different and/or subsequently acting selective pressures contributed to evolution of antestomatal chambers: (i) xeric and/or nutrient-deficient soil environment; (ii) VPD fluctuating atmosphere; (iii) polluted atmosphere and fire-prone environment; and (iv) frequent occurrence of a water film on the leaf surface (see the four ‘clouds’ indicating relevant environmental conditions or leaf morphotypes, e.g. hard thick sclerophyllous foliage). Plant species adapted to the particular environment may profit from their antestomatal chambers via physical mechanisms affecting water, CO<sub>2</sub> transport, stomatal behaviour or defending against leaf surface pathogens (ovals with bold lines) and leading to at least one of four benefits shown in the rectangular boxes. The underlying physical mechanisms including hydraulic activation of stomata (HAS) are described in detail in the text. The three main benefits to gas exchange provided by stomatal antechambers and sunken stomata can be coupled proportionally, e.g. water savings usually increase water use efficiency (WUE), but may act independently, optimize carbon gain or water loss depending on the environment and affect the plant physiology status. The arrows indicate the line and direction of causality in the short term; the long-term evolutionary feedbacks are not considered here. However, a mutual effect of cuticle permeability and antechamber climate can also be expected in the short-term range. Wax plugs, hairs or internal leaf cuticle often accompany stomatal antechambers and contribute to all physical mechanisms shown here. The antechamber-specific microclimate could increase stomatal sensitivity to the transpiration rate as indicated by experiments in this study. Two possible links between the stomatal sensitivity and antechamber climate, i.e. cuticle permeability increasing with relative humidity (RH) and guard cell water potential stabilized by relatively steady antechamber humidity and temperature, are suggested.

night-time transpiration more sensitively than the species with superficially located stomata.

Stomatal antechambers evolved many times over and may have been shaped perhaps by selection for functions other than to resist xeric conditions, although this ‘pre-adaptation’ might have been valuable in later evolution (Mast and Givnish, 2002). It is possible that the dominant primary function was protection against the formation of continuous water films that could prevent assimilatory CO<sub>2</sub> flux into the leaf (Fig. 9). Although this function is based on very different physical mechanisms, it results in a benefit almost identical to the previous one – keeping carbon flux for photosynthesis running. Both the functioning of encrypted guard cells and reduced leaf wettability share a common link in (epi) cuticular waxes, their structure and chemical composition, which brings another challenge for future research. Finally, the ‘sequestration’ of stomata in various leaf depressions, crypts or leaf grooves confers a broad range of protection from abiotic (mechanical, airborne particle, radiation or temperature) stresses and biotic attacks to stomata.

#### SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. Figure S1: the dawn–dusk effect on stomatal and cuticular conductance. Figure S2: the relationship between maximum leaf conductance at light saturation and leaf conductance in darkness. Figure S3: the percentage contribution of stomatal conductance to leaf surface conductance in darkness in seven plant species with stomatal antechambers differing in prominence.

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## APPENDIX

Partitioning of leaf water loss into its stomatal and cuticular components has been a matter of some interest for several decades, and various attempts to do so experimentally have been made, most recently by [Marquez \*et al.\* \(2022\)](#). Here, a gas exchange-based technique is suggested which enables partitioning total leaf transpiration into its cuticular and stomatal fractions and detecting incomplete stomatal closure at dark–light or light–dark transitions, i.e. at dawn and dusk.

ESTIMATION OF STOMATAL CONDUCTANCE FOR CO<sub>2</sub>

Net CO<sub>2</sub> exchange between a leaf and its environment at steady state,  $A$ , can be expressed in its simplest form as

$$A = g_{tc} \cdot (c_a - c_i) \quad (\text{A1})$$

where  $g$  and  $c$  are conductance and CO<sub>2</sub> concentration, respectively, and the indexes  $t$ ,  $c$ ,  $a$  and  $i$  stand for ‘total’, ‘CO<sub>2</sub>’, ‘atmospheric’ and ‘leaf internal’, respectively. Total leaf conductance for CO<sub>2</sub>,  $g_{tc}$ , is made up of the conductances of stomata,  $g_{sc}$ , cuticle,  $g_{cc}$ , and boundary layer,  $g_{bc}$ ;  $c_i$  denotes CO<sub>2</sub> concentration at surfaces evaporating water inside the leaf. The conductances  $g_{sc}$  and  $g_{cc}$  are arranged in parallel (CO<sub>2</sub> can diffuse via either the stomata or the cuticle) and both the stomata and cuticle pathways are linked in series with the boundary layer (before diffusing through the stomata or cuticle, each CO<sub>2</sub> molecule must pass the boundary layer). This arrangement requires for the total conductance for CO<sub>2</sub>:

$$g_{tc} = \frac{1}{\frac{1}{g_{bc}} + \frac{1}{g_{sc}}} + \frac{1}{\frac{1}{g_{bc}} + \frac{1}{g_{cc}}} = \frac{g_{bc} \cdot g_{sc}}{g_{bc} + g_{sc}} + \frac{g_{bc} \cdot g_{cc}}{g_{bc} + g_{cc}} \quad (\text{A2})$$

As a first approximation, we may ignore  $g_{bc}$  because it is 2–3 orders of magnitude higher in a ventilated leaf chamber than stomatal conductance in darkness. Further, the cuticle is almost impermeable for CO<sub>2</sub> diffusion into or out of the leaf, so  $g_{cc}$  is small relative to  $g_{sc}$  ([Boyer, 2015b](#)) and can be neglected, too, in the first approximation (see below for a revision of this). Then, the total leaf conductance to CO<sub>2</sub> can be approximated and substituted in eqn (A1) with stomatal conductance:  $g_{tc} \approx g_{sc}$ .

Provided stomata do not change their aperture during measurement, i.e.  $g_{sc}$  is invariant, it is possible to determine  $g_{sc}$  as the slope of the regression line relating  $A$  to the difference  $c_a - c_i$  in eqn (A1), or for constant  $c_a$ , as the slope of the line relating  $A$  to  $c_i$ . Presumably, stomata are not completely closed in the dark and they start to open further at low but non-zero irradiance. A typical example of such a dark–light transition in terms of the  $A(c_i)$  response is shown in [Fig. A1](#).

The calculation of  $c_i$  via photosynthesis measurements is biased, especially at low degrees of stomatal opening, because the unknown contribution of cuticular conductance is ignored and cuticular transpiration is treated in calculations as stomatal water loss ([Boyer, 2015a](#)). The error in  $c_i$  is usually small when stomata are open and their conductance for water vapour is much higher than the cuticular conductance ( $g_{sw} \gg g_{cw}$ , where the index  $w$  indicates water). However in the dark, when stomata are almost closed, the error can be significant ([Stinziano \*et al.\*, 2020](#)).

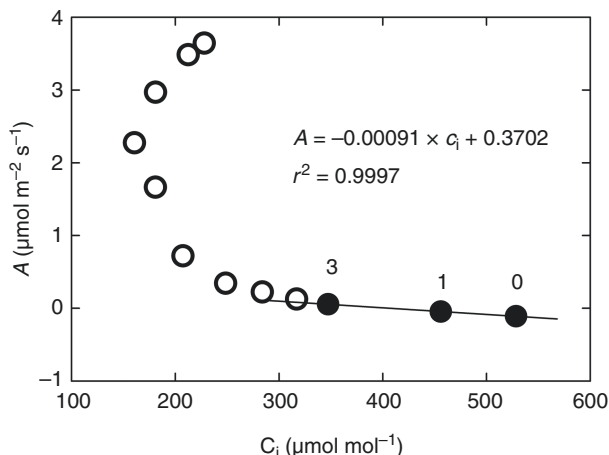


FIG. A1. CO<sub>2</sub> response of the photosynthesis rate at variable irradiance, following stomatal conductance reaching a steady state at each level. A *Ficus elastica* leaf was exposed to darkness for 40 min in the leaf chamber of a gas exchange system (LI-6800, USA) and, at that point, the photosynthesis rate ( $A$ ) and leaf internal CO<sub>2</sub> concentration ( $c_i$ ) were recorded (the data point marked as 0). Then, the light was increased in steps to 1, 3, 5, 7, 10, 20, 50, 100, 300, 600 and 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  with time intervals of 40 min between steps, and the  $A$  and  $c_i$  values were recorded when the new steady state of leaf water conductance was reached. Other leaf environmental parameters (leaf temperature, leaf–air water vapour pressure difference and CO<sub>2</sub> concentration in ambient atmosphere) were kept constant. The values recorded at the dark–light transition (solid circles with PPFD level indicated above them) yielded the parameters of the regression line shown in the graph. The slope of the regression line ( $-0.00091 \text{ mol m}^{-2} \text{ s}^{-1}$ ) gives the leaf conductance for CO<sub>2</sub>,  $g_{\text{sc}}$ , as defined in eqn (A1), which is identical to stomatal conductance for CO<sub>2</sub>,  $g_{\text{sc}}$ , if cuticular conductance for CO<sub>2</sub> is neglected. The coefficient of determination ( $r^2$ ) being close to 1 indicates that stomata were not responding to low levels of irradiance ( $0\text{--}3 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ).

Is the  $g_{\text{sc}}$  value, estimated as the slope of the  $A(c_i)$  regression line shown in Fig. A1, affected by the erroneous calculation of  $c_i$  yielded by a conventional gas exchange analysis? When the dilution effect of water vapour is ignored for simplicity's sake,  $c_i$  is calculated as

$$c_i = c_a - 1.6 \cdot \frac{A}{g_{\text{tw}} - g_{\text{cw}}} \quad (\text{A3})$$

The constant 1.6 represents the ratio of diffusion coefficients,  $D$ , of water and CO<sub>2</sub> in air ( $D_w/D_c = g_{\text{sw}}/g_{\text{sc}} = 1.6$ ). Total leaf conductance for water,  $g_{\text{tw}}$ , is assessed from the measured leaf transpiration rate and leaf–air water vapour pressure difference (VPD). Cuticular conductance to water ( $g_{\text{cw}}$ ) is assumed to be zero, so  $g_{\text{sw}}$  is (incorrectly) considered identical to  $g_{\text{tw}}$ . This concept was first introduced by Moss and Rawlins (1963) and became the common algorithm used by contemporary gas exchange systems for calculating  $c_i$  values. However, these values overestimate the real ones, which are lower at positive  $A$  (and higher at negative  $A$ ) because  $g_{\text{sw}}$  also includes  $g_{\text{cw}}$ . Nevertheless, the  $g_{\text{sc}}$  value, estimated as the slope of the  $A(c_i)$  regression line shown in Fig. A1, is not affected by the erroneous calculation of  $c_i$  in the case where all conductances to water ( $g_{\text{sw}}$ ,  $g_{\text{cw}}$  and  $g_{\text{tw}}$ ) as well as  $c_a$  do not change during the measurements for constructing the  $A(c_i)$  plot. Differentiation of  $A$  in eqn (A3), with respect to  $c_i$ ,  $dA/dc_i$ , yields for the slope  $(g_{\text{cw}} - g_{\text{tw}})/1.6$ , which can be expressed as  $-g_{\text{sw}}/1.6$ , or  $-g_{\text{sc}}$ . Provided that  $g_{\text{tw}}$ ,  $g_{\text{sw}}$  and  $g_{\text{cw}}$  do not change during the dark–light transition, the  $[A; c_i]$  values should fall on a line having, in an ideal case, a coefficient of determination equal to 1 and a slope equal to  $-g_{\text{sc}}$ .

## CORRECTION FOR CO<sub>2</sub> FLUX ACROSS CUTICLE

CO<sub>2</sub> diffuses across the lipidic cuticle at a much lower rate than water. Boyer (2015b) estimated that the cuticular conductance for CO<sub>2</sub> across the astomatous upper side of grape leaves was about 40 times lower than for water. Total leaf conductance for CO<sub>2</sub>,  $g_{\text{tc}}$ , estimated from the slope of the regression line in Fig. A1, should therefore be reduced to account for the cuticular CO<sub>2</sub> leakage. Due to the parallel arrangement of stomatal and cuticular pathways, we can write for CO<sub>2</sub>:

$$g_{\text{tc}} = g_{\text{sc}} + g_{\text{cc}}, \text{ and for water } g_{\text{tw}} = g_{\text{sw}} + g_{\text{cw}}, (\text{A4a,b})$$

In air-filled stomatal pores, the ratio of conductances to water and CO<sub>2</sub> is equal to the ratio of their diffusion coefficients in air (Moss and Rawlins, 1963):

$$\frac{g_{\text{sw}}}{g_{\text{sc}}} = 1.6 \quad (\text{A5a})$$

The ratio of the two conductances in the lipidic cuticle is (Boyer, 2015b):

$$\frac{g_{\text{cw}}}{g_{\text{cc}}} \approx 40 \quad (\text{A5b})$$

Rearrangement of eqns (A4a, b) and (A5a, b) yields for  $g_{\text{sc}}$ :

$$g_{\text{sc}} = g_{\text{tc}} \cdot \left(1 - \frac{1.6}{1.6 - 40}\right) + g_{\text{tw}} \cdot \frac{1}{1.6 - 40}$$

where  $g_{\text{tc}}$  is the slope of the regression line in Fig. A1 and  $g_{\text{tw}}$  is obtained from gas exchange measurements at the dark–light transition.

## Partitioning of total leaf conductance for water vapour into stomatal and cuticular parts

The knowledge of stomatal conductance for CO<sub>2</sub> ( $g_{\text{sc}}$ ) allows us to calculate stomatal conductance for water ( $g_{\text{sw}}$ ):

$$g_{\text{sw}} = 1.6 \cdot g_{\text{sc}} \quad (\text{A6a})$$

The  $g_{\text{sw}}$  value represents stomatal conductance and, if additional water flows through the cuticle,  $g_{\text{sw}}$  should always be lower than the leaf surface conductance  $g_{\text{tw}}$  measured directly with a gas exchange device. Then, the residual conductance can be attributed to the cuticle ( $g_{\text{cw}}$ ):

$$g_{\text{cw}} = g_{\text{tw}} - g_{\text{sw}} \quad (\text{A6b})$$

The boundary layer of laminar air at the leaf surface affects water vapour diffusion and, thus, water conductance of the boundary layer,  $g_{\text{bw}}$ , should be taken into account. Then, eqn (A6a) modifies to:

$$g_{\text{sw}} = 1.6 \cdot g_{\text{sc}} \cdot \frac{g_{\text{bw}}}{g_{\text{bw}} - 1.37 \cdot g_{\text{sc}}} \quad (\text{A7})$$

where the term 1.37 relates the rates of water and CO<sub>2</sub> diffusing across the laminar boundary layer. The value of  $g_{\text{bw}}$  is regularly estimated by gas exchange instruments and depends mainly on the speed of the air mixing fan in the leaf chamber.