



Effect of external stress on density and size of glandular trichomes in full-grown *Artemisia annua*, the source of anti-malarial artemisinin

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Abstract

Background and aims

Glandular trichomes (GT) of *Artemisia annua* produce valuable compounds for pharmaceutical and industrial uses, most notably the anti-malarial artemisinin. Our aim was to find out whether the density, number and size of GT can be manipulated to advantage by environmental stress. A range of external stress treatments, including stress response regulators, was therefore given to fully grown plants under field and greenhouse conditions.

Methodology

In a field experiment (Ex1), seed-grown plants were subjected to chemical or physical stress and plants analysed after 5 weeks. In a greenhouse experiment (Ex2), three groups of clonally derived plants were stressed at weekly intervals for 5 weeks. Stress treatments included sand-blasting, leaf cutting and spraying with jasmonic acid, salicylic acid, chitosan oligosaccharide (COS), H₂O₂ (HP) and NaCl (SC) at different concentrations. Leaves from an upper and a lower position on the plants were analysed by fluorescence microscopy to determine the density and size of GT.

Principal results

Densities of GT on upper leaves of full-grown *A. annua* plants generally showed no response to external stress and only plants from one clone of Ex2 supported the hypothesis that increased density of GT was inducible in upper leaves by stress (significant for SC, HP and COS). The density of GT on lower leaves was not affected by stress in any experiment. Glandular trichomes were significantly smaller on the lower leaves in response to stress in Ex2, and a similar non-significant trend was observed in Ex1.

Conclusions

The results indicate a dynamic system in which stress treatments of large *A. annua* plants had a minor promoting effect on the initiation of GT in developing leaves, and a maturing effect of GT later in the lifetime of the individual GT. The hypothesis that applying stress can induce larger GT or more numerous GT was rejected.

Introduction

Glandular trichomes (GT) on *Artemisia annua* produce and store the anti-malarial compound artemisinin (AN)

and other secondary metabolites (SM) that have several pharmaceutical and industrial uses. The present paper investigates the spatial and temporal distribution

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of GT on leaves and tests the hypotheses that environmental stress influences the size and density of GT.

Many vascular plants invest considerable resources in building, maintaining and filling GT on aerial surfaces (Levin 1973; Wagner 1991; Aagren and Schemske 1993). Glandular trichomes have several secondary functions and in *A. annua* GT are thought to contribute to plant defence (Duke and Paul 1993; Hu *et al.* 1993; Duke 1994). The biseriate capitate GT consist of 10 cells stacked in pairs (Duke and Paul 1993). The four lower cells function primarily as a stalk for the six topmost cells. These six cells all border the secretory cavity and contribute to filling the apical subcuticular space with SM. A laser dissection study of GT of *A. annua* (Olsson *et al.* 2009) showed that key enzymes of AN production were expressed exclusively in the two apical cells of GT. Studies on the initiation and development of GT in the genus *Artemisia* have established that differentiation of foliar cells into GT cells is completed in a very young primordial stage of the leaf (Duke and Paul 1993—in *A. annua*; Ascensão and Pais 1987—in *A. campestris*). As shown by Duke and Paul (1993) and discussed by Davies *et al.* (2009), the number of GT of the fully developed leaf in *A. annua* is predetermined at this early primordial stage.

Werker (2000) defined GT as mature when surrounding cells senesce and stop secreting SM to the central cavity. Glandular trichomes of some species continuously excreted SM, while in other species excretion of all the SM occurred during a single destructive rupture of the GT. In *A. annua*, Duke and Paul (1993) reported that the subcuticular sac was visibly broken in many mature glands, and they considered *A. annua* to be among the species that release SM by rupturing of the GT. Both Lommen *et al.* (2006—*A. annua*) and Shanker *et al.* (1999—*Mentha arvensis*) followed the development and found that GT densities were highest at the maximum size of leaves, after which time densities decreased rapidly, suggesting that some GT ruptured over time. Similarly, Arsenault *et al.* (2010—*A. annua*) found that the number of GT in relation to epidermal cells was optimal as leaves reached full size and decreased thereafter. Ferreira and Janick (1995) focused on the floral morphology of *A. annua* and found that physiological maturity of GT in the inflorescence coincided with full bloom.

If stress is defined as external conditions that are sub-optimal for growth, past work has demonstrated that stress can affect the formation of GT on leaves. For example, *Solanum lycopersicum* (Solanaceae) treated with methyl jasmonate developed nine times higher GT densities compared with untreated controls (Boughton *et al.* 2005). Similarly, jasmonic acid (JA), gibberellic

acid and benzylaminopurine (BAP, a synthetic cytokinin) applied to *Arabidopsis thaliana* (Brassicaceae) resulted in up to four times higher GT densities (Maes *et al.* 2008). In the same species, wounding and JA also significantly increased the number of GT, whereas salicylic acid (SA) decreased the number of GT (Traw and Bergelson 2003). In *Mimulus guttatus* (Scrophulariaceae), damage on leaves that appeared early in the season increased GT formation on leaves that developed later. It was also shown that this trait was maternally transmitted to progeny (Holeski 2007). *Madia sativa* (Asteraceae) subjected to leaf wounding and/or water shortage has been shown to produce increased GT density (Gonzales *et al.* 2008). Within *A. annua*, few published experiments are available linking external stress and the density and size of GT. Liersch *et al.* (1986) concluded that the growth regulators diaminozide and chlormequat may influence formation of GT, and Kapoor *et al.* (2007) demonstrated that arbuscular mycorrhizal fungi and added micronutrients increased GT densities, while Liu *et al.* (2009) showed that application of JA increased GT densities. Maes *et al.* (2011) demonstrated that the application of JA and gibberellic acid, but not cytokinin (BAP), increased the density of GT. They further determined that a cultivar with a low AN content exhibited a greater plasticity for change in the GT density than a cultivar with higher AN content. This finding suggested a mechanism whereby this species can regulate its capacity for GT on the leaves. Arsenault *et al.* (2010) determined that foliar GT distribution differed when plants were vegetative or reproductive and found a strong positive relationship between AN content and GT densities, regardless of leaf type. This strong relationship between GT densities, AN and key precursors was confirmed by Graham *et al.* (2010). Lommen *et al.* (2006) have given extensive insights into the differing densities of GT on individual leaves during development and maturation. Nguyen *et al.* (2011) reviewed comprehensively the influence of external factors on the production of GT and AN in *A. annua*.

Our treatments were selected to represent a broad range of stress-inducing agents. Mechanical damage was applied by sandblasting (SB; Ex1 and Ex2) or cutting the leaves (Ex1). Indirect damage resulting from the osmotic stress was induced by spraying with NaCl (SC; Ex2). Mimicking stress conditions by triggering the recognition of molecules associated with insect and fungus attacks (Zheng *et al.* 2010; Lei *et al.* 2011) was achieved by applying chitosan oligosaccharide (COS; Ex1 and Ex2). Treatments with H₂O₂ (HP; Ex2) mimicked the bursts of reactive oxygen species triggered by the plants under stress (Neill *et al.* 2002; Mittler *et al.* 2011). The hormones JA (Ex1) and SA (Ex1 and Ex2)

were applied since they are directly involved in the internal stress management of the plants (Parthier 1990; Wasternack 2007; Pu *et al.* 2009).

These treatments helped test two primary hypotheses: firstly, that stress treatments on pre-primordial leaves can increase the density and size of GT; secondly, that stress treatments applied to mature leaves are capable of altering GT density and size. These hypotheses were tested on both seed-grown and clonally propagated *A. annua* in the field and greenhouse.

Materials and methods

Plant material Ex1

Seeds of *A. annua* (cv. Artemis, F₂ seeds; Mediplant, Switzerland) were sown in a greenhouse, and 6 weeks later (June 2009) planted in the field at Research Station Aarslev, Denmark (55°18'N, 10°27'E). The soil was a sandy loam, fertilized with 100 kg N ha⁻¹ prior to planting. No artificial irrigation was provided and weeding was manual. Plants were 18 weeks old and 90–110 cm in height at the onset of treatments, and 150–180 cm tall at sampling.

Plant material Ex2

From a field population of seed-propagated *A. annua* (cv. Artemis, F₂ seeds; Mediplant), three plants were randomly selected as mother plants for clonal propagation by cuttings and sufficient plants were obtained during two rounds of multiplication carried out in a greenhouse. Tip cuttings were ~10 cm long, comprising 4–5 internodes longer than 1 cm. Cuttings were rooted after 2–3 weeks, and potted in 3.5-L containers with Pindstrup no. 2 peat moss (Pindstrup Mosebrug, Denmark). Plants were transferred from the nursery to the experimental greenhouse, and allowed to acclimatize for 7 days before the onset of treatments. Plants were 9 weeks from propagation and 80–110 cm tall at the onset of the treatments, and 150–190 cm tall at sampling. During the experiment, plants were drip irrigated twice diurnally with a liquid fertilizer adapted for Asteraceae.

Experimental design Ex1

The experiment was carried out during September–October 2009. A randomized complete block design with subsampling with three blocks was set up in a field. The 12 different treatments were randomly represented within each block. Beds with three rows of plants were subdivided into plots containing 18 plants, which all received the same treatment. Three random plants from each plot were sampled for the present experiment. Plants were spaced 50 cm apart and individual beds were separated by 150 cm of bare soil. With a

manual garden vapourizer (Gardena), JA (J2500; Sigma Aldrich) solutions (95 % water:5 % ethanol) and SA (S7401; Sigma Aldrich) solutions (100 % water) were sprayed in aliquots of 10 mL per plant at concentrations of 0.05, 0.5 and 5.0 mM. Chitosan oligosaccharide (provided by Dalian Glycobio Ltd, China) was sprayed in aliquots of 10 mL per plant in a concentration of 200 mg L⁻¹. Sandblasting was carried out using a small hobby sandblaster (Badger, model 260-3, aluminium oxide particles), and leaf cutting was carried out with hand-held scissors (see Table 1 for an overview of treatments). Chemical treatments and SB were focused exclusively on the top section of the main shoot including the lower leaf (Fig. 1) by placing a plastic container around the shoot while spraying/SB. Leaf cutting consisted of systematic removal of parts of the leaves on all side branches above the lower leaf marking. Treatments were carried out in September 2009 and repeated on half of the sandblasted and leaf-cut plots once each week for a 5-week time period. Treatments caused no physical changes to the plants, except for the scissor cuts and tiny brown spots caused by SB. The upper leaf was defined as the first leaf below the apex on the main stem with internodes longer than 2 cm (Fig. 1). To recognize leaves that were at a comparable developmental stage at the onset of the experiment, the main stem below the 10th internode from the upper leaf was marked on all the plants at the onset of treatments. The leaf above this mark was the lower leaf. On 29 September 2009, the lower leaves and the upper leaves were sampled along with the two leaves above and below the respective leaves. Leaves were immediately placed in plastic bags between filter papers, soaked in ice-cold water and stored at 3 °C. To investigate differences in morphology of adaxial and abaxial leaf sides and to follow the GT development in time, leaves from nine untreated plants were sampled once a week during the experiment from leaf positions as described above.

Experimental design Ex2

The experiment was carried out during April–May 2010. A randomized complete block experimental design with subsampling was set up in a greenhouse divided into two self-contained compartments. In each compartment, three beds of plants were established, each bed consisting of 3 × 23 plants of one of the three clones. Plants were 50 cm apart and individual beds were separated by 100 cm of gravel. Every second row of three plants was left as a guard row, allowing for 11 treatable subplots of three plants in each bed (2 blocks × 3 clones × 3 plants = 18 subplots per treatment). Treatments were performed weekly for 5 weeks, and included NaCl (Salina) at 1 and 10 g L⁻¹ aqueous solutions, SA

Table 1 All treatments of the field experiment (Ex1) and greenhouse experiment (Ex2) with the abbreviations used in the text and figures.

	Treatment	Abbreviation	Cumulative treatment per plant
Field (Ex1)	Control	Control	–
	Salicylic acid 0.05 mM	SA1	0.07 mg
	Salicylic acid 0.50 mM	SA2	0.69 mg
	Salicylic acid 5.00 mM	SA3	6.91 mg
	Jasmonic acid 0.05 mM	JA1	0.11 mg
	Jasmonic acid 0.50 mM	JA2	1.05 mg
	Jasmonic acid 5.00 mM	JA3	10.51 mg
	Chitosan oligosaccharide ^a 0.2 g L ⁻¹	COS	20.00 mg
	Sandblasting × 1	SB × 1	× 1
	Sandblasting × 4	SB × 4	× 4
	Cutting × 1	Cut × 1	× 1
	Cutting × 4	Cut × 4	× 4
	Greenhouse (Ex2)	Control	Control
NaCl 1.0 g L ⁻¹ ~17.11 mM		SC 1	140.00 mg
NaCl 10.0 g L ⁻¹ ~171.12 mM		SC 2	1400.00 mg
H ₂ O ₂ 0.1 %~29.37 mM		HP 1	0.14 mL ^b
H ₂ O ₂ 1.0 %~293.71 mM		HP 2	1.40 mL ^b
Salicylic acid 0.1 g L ⁻¹ ~0.72 mM		SA 1	14.00 mg
Salicylic acid 1.0 g L ⁻¹ ~7.24 mM		SA 2	140.00 mg
Chitosan oligosaccharide ^a 0.1 g L ⁻¹		COS 1	14.00 mg
Chitosan oligosaccharide ^a 1.0 g L ⁻¹		COS 2	140.00 mg
Sandblasting		SB	× 5

The cumulative treatment was calculated as the amount of compound applied per plant during one or over several treatments.

^aThe molecular weight of mixed product was unknown.

^bMillilitres of 100 % H₂O₂.

(S7401; Sigma Aldrich) at 0.1 and 1 g L⁻¹ aqueous solutions, HP (Matas, 10 %) at 0.1 and 1.0 %, COS (provided by Dalian Glycobio Ltd) in the aqueous solutions 0.1 and 1.0 g L⁻¹, and SB (Badger, Model 260-3, aluminium oxide particles). A manual garden vapourizer (Gardena) delivered 1 mL of liquid per spray, and 24 sprays covered the majority of a plant in a water film. To compensate for the growth during the 5-week treatment period, two additional sprays were added each week (32 sprays at last treatment). Sandblasting was carried out for 2 × 15 s at the onset of the experiment and for 2 × 20 s at the last treatment (see Table 1 for an overview of treatments). Treatments were carried out during the late afternoon to minimize any sun scalding effect from water droplets on the leaf surface. No physical changes were observed after the treatments, apart from the highest dose of SC, which caused reddened midribs in leaves of some plants. Lower leaves and upper leaves were marked and defined as in Ex1 (Fig. 1). At sampling, the lower leaves and the upper leaves were collected and immediately placed into

plastic bags between filter papers, soaked with ice-cold water and stored at 3 °C.

Fluorescence microscopy and picture analyses Ex1

Leaf samples were analysed under an Olympus BH2 microscope equipped with a 100-W mercury fluorescence lamp at ×40 magnification using a standard broad BG filter and a Canon EOS 10D camera. From each sample, the adaxial side of three leaf tips was exposed and two predefined areas from each of these were photographed, giving a total of six photographs per sample. Photographs were analysed using NIS-elements BR 3.1 software (Nikon Instruments). On each photograph, an area of 0.2–0.9 mm² was measured exactly and the number of GT within the area was counted. Four GT from each area were randomly selected and the lengths were measured. One entire leaf from each sample was photographed and the area was quantified using WinRHIZO 5.1 (Regent Instruments).

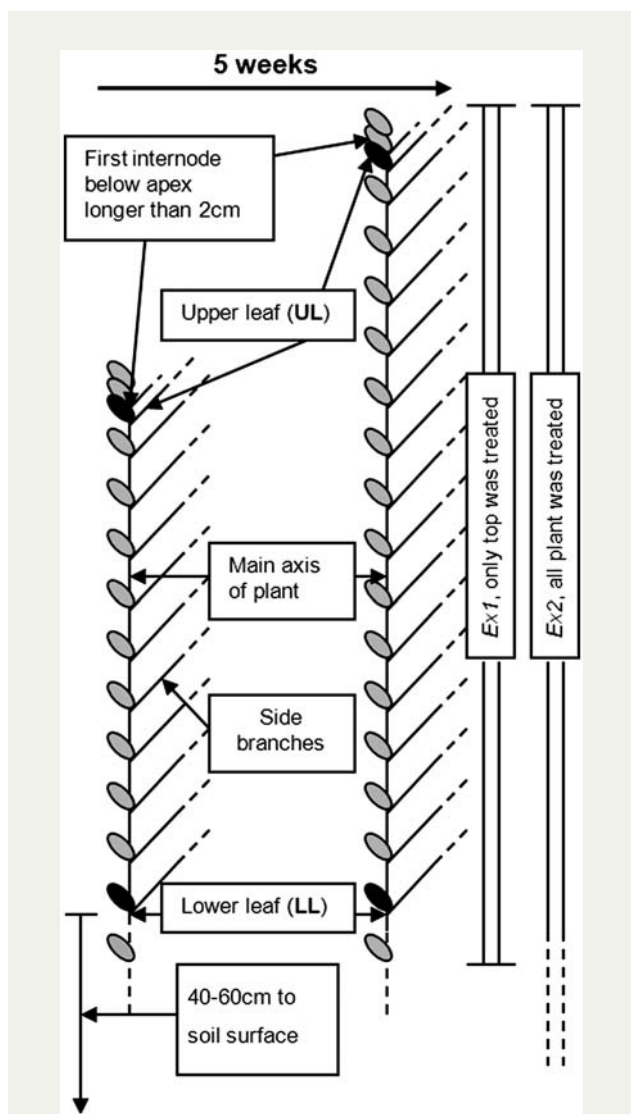


Fig. 1 Treatment and sampling methods. To sample leaves from the same position throughout the experiment, the lower leaves, positioned 10 internodes below the upper leaf on the main axis, were marked on all plants at the start of the experiment. Lower leaves were assumed to be fully expanded at the beginning of the experiment. Upper leaves were positioned at progressively higher positions as time progressed, thus enabling leaves that were initiated after the application of stress treatments to be examined.

Fluorescence microscopy and picture analyses Ex2

Leaf samples were analysed under a Nikon AZ100 microscope equipped with a 200-W fluorescent light source and a short-pass fluorescein isothiocyanate filter (Ex 465–495/DM 505/Ba 515–555) at $\times 40$ magnification. The abaxial side was exposed, and within the first major secondary lobe from the leaf tip, three areas

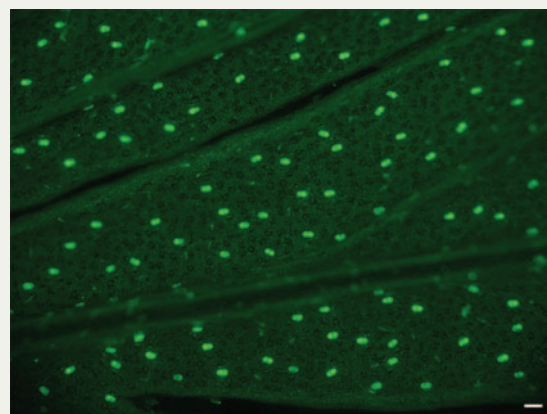


Fig. 2 Glandular trichomes of *A. annua* showing content of autofluorescing aromatic oils. Bar: 100 μm .

were photographed using the ‘binning’ option, exposure times of 2 s and Gain 1 (Fig. 2). Pictures were analysed with NIS-elements BR 3.1 (Nikon Instruments). Bright light intensity was used to discriminate the GT from the background, and false positives were excluded by size and shape recognition. From each of the three pictures derived from the same leaf, two areas (0.3–2.4 mm^2) were marked, and GT counts and size parameters based on pixel counts were recorded.

Data analyses

Statistical analyses were performed in R ver. 2.11.1 (R Development Core Team 2010). Graphs were prepared in Sigmaplot (2000 for Windows, ver. 6.00). Stress effects were analysed in a linear mixed model approach (lmer, lme4 package). Statistics for Figs 3–5 were performed in linear mixed models by relating the desired variables (GT density, GT area, GT length, GT width, leaf area and height of plant) to the fixed variable ‘Treatment’ and adding the relevant random effects. Probabilities of significance were given as $P > |t|$ in an MCMC algorithm. Statistical analyses of data from the adaxial and abaxial leaf sides (Table 2) were performed as paired *t*-tests, and differences between sampling times (Fig. 6) were performed as honestly significant difference (HSD) Tukey’s tests.

Results

In Ex1, the densities of GT on upper leaves treated with SA (all concentrations) and COS were significantly lower than those in control leaves (Fig. 3A). Lengths of GT on treated upper leaves were not significantly different from the control, but it is noteworthy that almost

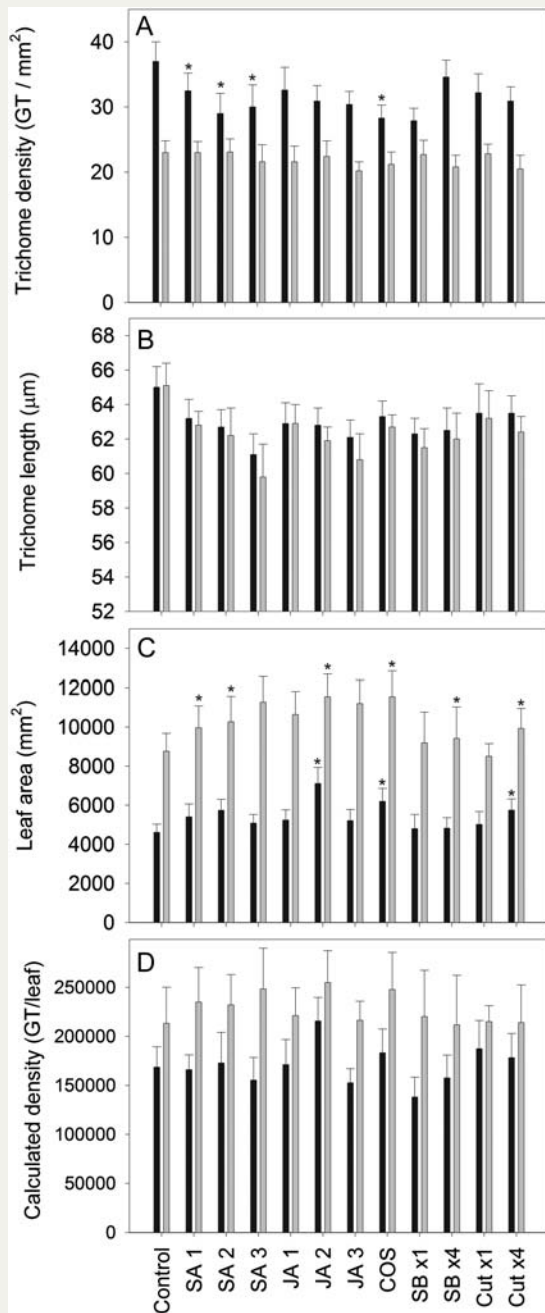


Fig. 3 Effects of stress treatments in the field experiment (Ex1) on trichome density (A), trichome length (B), leaf area (C) and calculated trichome density of entire leaves (D). Black columns are results from upper leaves (not initiated at onset of treatments) and grey columns are results from lower leaves (fully developed at onset of treatments). Error bars represent the SEM ($n = 9$) and an asterisk indicates significant difference from control ($P < 0.05$) in a linear mixed model. Abbreviations of treatments are given in Table 1.

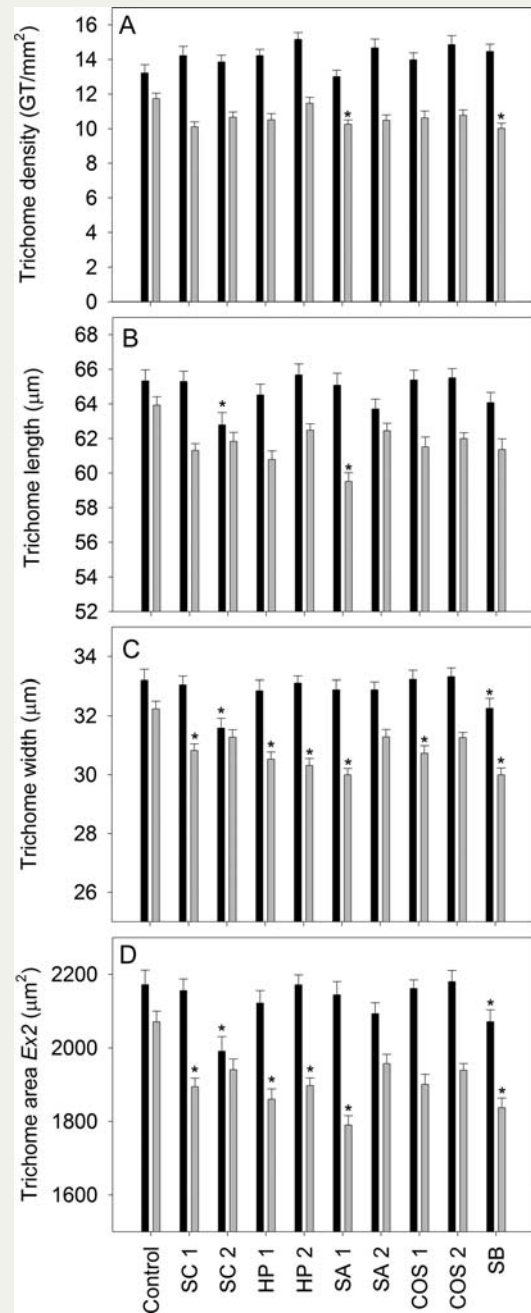


Fig. 4 Effects of stress treatments in greenhouse experiment (Ex2) on trichome density (A), trichome length (B), trichome width (C) and trichome area (D). Black columns are results from upper leaves (not initiated at onset of treatments) and grey columns are results from lower leaves (fully developed at onset of treatments). Error bars represent the SEM ($n = 18$) and an asterisk indicates significant difference from control ($P < 0.05$) in a linear mixed model. Abbreviations of treatments are given in Table 1.

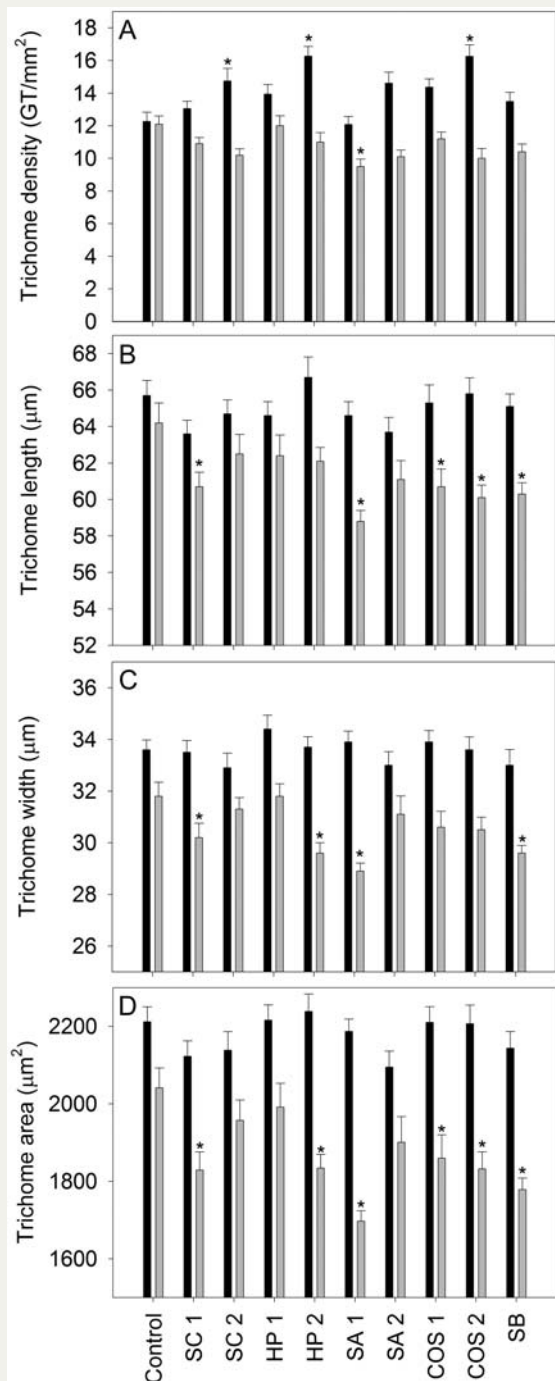


Fig. 5 Effects of stress treatments on Clone 2 (unaffected by mildew) in the greenhouse experiment (Ex2) on trichome density (A), trichome length (B), trichome width (C) and trichome area (D). Black columns are results from upper leaves (not initiated at onset of treatments) and grey columns are results from lower leaves (fully developed at onset of treatments). Error bars represent the SEM ($n = 6$) and an asterisk indicates significant difference from control ($P < 0.05$) in a linear mixed model. Abbreviations of treatments are given in Table 1.

all mean values of stress-treated leaves were lower than the control (Fig. 3B). In the lower leaves, no significant effects of the treatments on either the densities or the length of GT were observed, though most treated plants had a lower mean length of GT as compared with controls (Fig. 3A and B). Treatment with SA1, SA2, JA2, COS, SB \times 4 and Cut \times 4 produced significantly larger leaf areas than the control (Fig. 3C). Multiplying the values of GT densities with the leaf areas produced a data set of calculated GT per leaf (Fig. 3D). None of the calculated GT per leaf values showed a significant relationship to the treatments. Neither the final height nor the increment in height during the experiment was significantly affected by the treatments (data not shown).

In Ex2, GT densities on upper leaves of treated and control plants were not significantly different, though it is noteworthy that the mean densities of the control plants were lower than those of most of the treated groups (Fig. 4A). Two treatments (SC1 and SB) showed significantly smaller areas and widths of GT in upper leaves compared with the control (Fig. 4C and D). Densities of GT in the lower leaves showed significantly lower densities in treated leaves in SA1 and SB, and mean values of all the remaining treated groups were non-significantly lower than controls (Fig. 4A). Compared with control plants, the area, length and width of GT of the lower leaves were significantly lower in several of the treated plants, and non-significantly lower in the remaining groups (Fig. 4B–D). Neither the final plant height nor the increment in height during the experiment was significantly affected by the treatments (data not shown). Plants of two of the three clones in Ex2 (Clones 1 and 3) were infected by a powdery mildew (*Golovinomyces* sp.). The growth of the infected plants between propagation and the onset of treatments was affected, and infected plants were significantly ($P < 0.0001$) shorter than uninfected plants within the same clone (data not shown). At the end of the experiment, this difference in height was non-significant. The level of fungus infection was scored (levels: 0, 1 or 2) and the random effect caused by the infection was included in the linear mixed models.

Analysing values from the fungus-free Clone 2 alone (Fig. 5) eliminated the variation caused by the fungal infection. Glandular trichomes densities in the upper leaves were significantly higher after treatment with SC2, HP2 and COS2 as compared with control plants, and the remaining treatments produced non-significantly higher mean values than control plants. Treatments tended to result in non-significantly lower mean values of GT densities in lower leaves compared with control plants (Fig. 5A). Area, width and length of the GT on the upper leaves were not significantly

Table 2 Trichome densities and trichome length on adaxial and abaxial leaf sides of control plants of the field experiment (Ex1) and greenhouse experiment (Ex2).

		Trichome density		Trichome length	
		Adaxial	Abaxial	Adaxial	Abaxial
Ex1	Upper leaves	32.4 ± 1.8**	39.1 ± 2.5	66.1 ± 0.9 NS	65.2 ± 0.8
Ex1	Lower leaves	23.7 ± 3.0**	31.7 ± 3.1	65.2 ± 0.7 NS	66.3 ± 0.8
Ex2	Upper leaves	8.1 ± 0.9**	13.0 ± 1.7	64.0 ± 1.5 NS	62.2 ± 1.1
Ex2	Lower leaves	5.9 ± 1.2*	9.3 ± 1.1	60.2 ± 2.2 NS	59.3 ± 1.5

Values are means of mean values from six photographs per sample ± SEM ($n = 5$ (Ex1) and $n = 9$ (Ex2)).

Asterisks indicate significant difference between adaxial and abaxial values (NS, not significant; * $P < 0.05$, and ** $P < 0.01$) in paired t -tests.

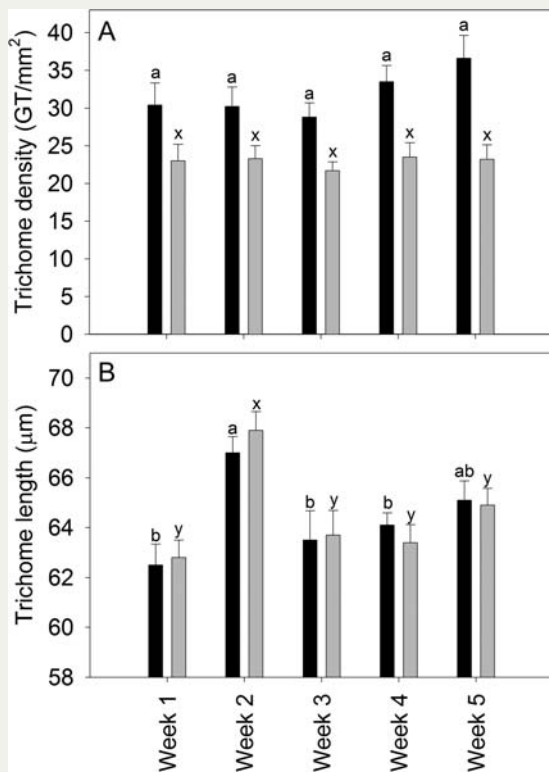


Fig. 6 Trichome density (A) and trichome length (B) of untreated plants from the field experiment (Ex1) sampled at weekly intervals over 5 weeks. Black columns show upper leaves (sampled at progressively higher positions of the main stem) and grey columns show the lower leaves (sampled at the same position throughout the experiment). Lower-case letters show significant differences ($P < 0.05$) in a HSD Tukey's test. Error bars represent the SEM ($n = 9$).

affected by the treatments, but on the lower leaves there were several significantly lower size values as compared with controls (Fig. 5B–D).

Observation of untreated control plants from Ex1 over 5 weeks (Fig. 6A) revealed constant GT densities in the lower leaves (sampled at the same position on the plant), whereas the upper leaves (sampled at progressively later initiation positions) had a non-significant tendency to produce higher GT densities. The lengths of GT (Fig. 6B) of both lower leaves and upper leaves were significantly larger at Week 2 compared with other weeks. These measurements coincided with the precipitation pattern of the period, as heavy rains saturated the soil before Week 2, and Weeks 4 and 5 had light rainfalls. Prior to Weeks 1 and 3, conditions were dry and windy. Investigation of the adaxial and abaxial sides of leaves of control plants (Table 2) revealed that densities of GT on the abaxial side were significantly higher than those on the adaxial side. Length of GT did not differ significantly between the two sides.

Discussion

Hypotheses

It was hypothesized that the applied stress on full-grown *A. annua* plants would result in higher GT densities and larger GT in developing leaves (upper leaves), and that GT on mature leaves (lower leaves) would remain unchanged. The stress treatments induced higher densities of GT only in some treatments on newly developed leaves (upper leaves) of Clone 2 from Ex2 (Fig. 5A). However, the stress treatments did not increase the size of any GT relative to the control. On the contrary, several treatments in Ex2 resulted in significantly smaller GT in the lower leaves in response to stress, and similar non-significant trends were observed in the remaining lower leaves of both Ex1 and Ex2. Thus, in this study, the hypothesis that stress is able to initiate higher GT densities in upper leaves of large plants could only be confirmed in a limited subset of the

plants, and the hypothesis that stress will induce larger GT was rejected. Reports in the literature concluding that GT morphology in young *A. annua* plants is relatively plastic in response to stress treatments were not confirmed in mature plants grown under exposed or protected conditions.

Effect of stress on GT density

The two stress-based experiments showed very limited effects on GT densities of newly developed leaves (upper leaves) of adult plants. Only the upper leaves of Clone 2 in Ex2 showed a significant increase in GT densities following repeated application of the strongest doses of SC, HP and COS. This difference in response may be partly related to differences in the impact of uncontrolled stress between and within the two experiments. The relatively heterogeneous appearance of plants of Ex1 was probably related to the harsh and variable outdoor environmental conditions, whereas the plants of Ex2 were protected in a greenhouse. Clones 1 and 3 of Ex2 included many individuals infected by powdery mildew, which significantly affected their height and growth pattern. The mildew-free individuals of Clone 2 were the least affected by factors other than the stress treatments, and these plants were visibly more uniform in growth. The results indicated that stress treatments could induce small changes in GT densities, but that the effect could easily be overwhelmed by other factors. These factors included stress by environmental conditions, interplant variation in density of GT, physiological age, proximity to flowering, genetic predispositions and how 'saturated' the leaf was with GT at the time of stress treatment. This 'saturation' effect was reported by [Maes *et al.* \(2011\)](#), who found a lower ability to increase GT densities in plants which initially had higher GT densities. There is limited information available on the possible change in this plasticity in response to stress when plants grow larger or approach the onset of flowering. [Holeski \(2007\)](#) demonstrated that the GT density on the fifth leaf of *M. guttatus* was more responsive to damage than the seventh leaf. The initial density of GT is shown to be a heritable trait ([Graham *et al.* 2010](#)), and possibly, though not documented in the literature, the plasticity in the GT density response to stress is similarly heritable.

The differences in results of GT densities in Ex1 and Ex2 illustrated the variability and complexity in GT morphology of adult *A. annua*. Interestingly, the GT densities of upper leaves in Ex1 in several stress treatments (Fig. 3A) were decreased, as opposed to the increased densities of upper leaves observed in several treated groups of Ex2. This might be explained by the significant effect that several treatments had on leaf

size of upper leaves of Ex1 (Fig. 3C). When leaf size increases, there is an implied 'dilution' of GT due to the increased expansion of treated leaves as compared with controls. When applying a method from [Lommen *et al.* \(2006\)](#) to calculate the total GT number per leaf (Fig. 3D), we determined a non-significant effect of stress on the density of GT in Ex1.

Salicylic acid This was applied in both Ex1 and Ex2. The significant decrease in GT density on upper leaves of Ex1 (Fig. 3A) was possibly due to the dilution effect mentioned above. The upper leaves of the overall Ex2 (Fig. 4A) showed no significant response in GT density to SA, and Clone 2 of Ex2 showed a limited, but non-significant, increase in GT density in the SA2 treatment. The effect of SA on GT densities of *A. annua* is undocumented, but [Traw and Bergelson \(2003\)](#) showed no effect of SA on the density of GT of *A. thaliana*.

Jasmonic acid This was applied only in Ex1, and no significant differences were found in GT densities on the upper leaves as compared with the controls. [Maes *et al.* \(2011\)](#) reported a several-fold increase in GT density in young *A. annua* plants after applying JA and [Liu *et al.* \(2009\)](#) reported similar significant increases in GT densities following treatment with JA. In the present study treatments were applied to older plants, which may already have been saturated by relatively high GT densities or had lost phenotypic plasticity in the ability to respond to stress.

Chitosan oligosaccharide Plants were treated with COS in both Ex1 and Ex2. The significant decreases in GT density in upper leaves of Ex1 (Fig. 3A) were possibly due to the dilution effect of leaf expansion mentioned above. The upper leaves of the overall Ex2 (Fig. 5A) showed no response to COS, but the upper leaves of Clone 2 in Ex2 (Fig. 6A) showed a significant increase in the density of GT in response to the COS2 treatment, and a non-significant increase in the COS1 treatment. The effect of COS on GT densities in *A. annua* is undocumented in the literature, but [Lei *et al.* \(2011\)](#) reported a significant increase in both AN and HP levels in leaves after foliar application of COS.

H₂O₂ The upper leaves of the overall Ex2 (Fig. 5A) showed no response to HP, but the isolated Clone 2 (Fig. 6A) showed a significant increase in the HP2 treatment, and a non-significant increase in the HP1 treatment. The effect of HP on GT densities in *A. annua* is undocumented in the literature.

NaCl The upper leaves in Ex2 overall (Fig. 5A) showed no response to SC, but the upper leaves of Clone 2 in Ex2 (Fig. 6A) showed a significant increase in the

density of GT in response to the SC2 treatment, and a non-significant increase in the SC1 treatment. The effect of foliar application of SC on GT densities in *A. annua* is previously undocumented in the literature.

Artificial wounding Wounding from SB in both Ex1 and Ex2 and from scissor cutting (Cut) in Ex1 failed to raise GT densities. The effect of wounding on GT densities in *A. annua* is previously undocumented in the literature, but [Traw and Bergelson \(2003\)](#) and [Travers-Martin and Muller \(2008\)](#) showed a significant increase in GT density in response to leaf wounding in *A. thaliana* and *Sinapis alba* (both Brassicaceae), respectively.

Effect of stress on the size of GT

[Maes *et al.* \(2011\)](#) observed a significant increase in the area of GT following treatment with JA and gibberellic acid, and a significant decrease in the GT area in cytokinin-treated leaves of young individuals of a low-AN-producing *A. annua* cultivar. They interpreted the increase in GT area to be linked with a more advanced maturation stage of the GT and/or increased biosynthetic activity, and that the decreased areas were linked with cytokinin maintaining cells in division rather than proceeding towards maturity. In the present study, decreased sizes (area, length or width) of GT in stress-treated plants were observed for both upper leaves and lower leaves in Ex1 (Fig. 3B), and the decreased GT sizes in the lower leaves were significant for most treated plants in Ex2 (Fig. 4B–D) and Clone 2 (Fig. 5B–D). In contrast to [Maes *et al.* \(2011\)](#), we interpreted the smaller GT as an indication that the GT of stressed plants were more mature than the GT of the control plants. How stress affects the maturation of GT thus remains unresolved, though it is clear that stress had an effect on the size of GT. In our observations, no collapsed GT or any remnants of GT were observed. The present study leads to the theory that not only proximity to flowering, but also the influence of stress, may promote the maturation of GT. Clearly, more knowledge is needed on the maturation and rupture processes of GT in *A. annua*.

Both [Pu *et al.* \(2009\)](#) and [Aftab *et al.* \(2010\)](#) applied SA as a 1 mM spray on young *A. annua* plants, JA was applied by [Liu *et al.* \(2009\)](#) as a 1 mM spray and by [Maes *et al.* \(2011\)](#) as a combination of a 0.1 mM foliar spray and substrate application. [Lei *et al.* \(2011\)](#) applied COS as a 100 mg L⁻¹ foliar spray. All these stress treatments caused significant changes in GT morphology or SM content. For SC, HP and wounding, no comparable studies of *A. annua* were found in the literature. In the present study, the chosen doses and frequencies of treatments (Table 1) reflected what had previously been shown to be effect in the literature but

doses were increased in proportion to the physically larger plants. The frequency and degree of application of stress differed between Ex1 and Ex2, as only the upper part of the plants of Ex1 were treated once, whereas the entire plants were treated five times in Ex2.

General observations

Mean GT densities within treatment groups were 20–37 GT mm⁻² for the field experiment (Ex1) (adaxial side) and 10–15 GT mm⁻² in the greenhouse experiment (Ex2) (abaxial side). These ranges corresponded well to the 5–37 GT mm⁻² reported by [Arsenault *et al.* \(2010\)](#), the 12–75 GT mm⁻² by [Maes *et al.* \(2011\)](#), the 20–75 GT mm⁻² by [Lommen *et al.* \(2006\)](#) and the 2–80 GT mm⁻² by [Graham *et al.* \(2010\)](#). Other studies have reported different GT densities. For example, [Kapoor *et al.* \(2007\)](#) reported 80–140 GT cm⁻² (corresponding to only 0.8–1.4 GT mm⁻²) and [Hu *et al.* \(1993\)](#) reported 190–225 GT mm⁻². Mean values of the GT length and width for the present study were in the ranges of 60–65 and 30–33 μm, respectively. In the literature, only [Hu *et al.* \(1993\)](#) presented GT dimensions of *A. annua* and reported results of 45–48 and 27–30 μm, respectively. Mean values of projected GT areas were in the range of 1700–2300 μm², as measured by bright pixels in fluorescence microscope images. By estimating GT area from graphs presented by [Maes *et al.* \(2011\)](#), a range of 400–1100 μm² was determined. Differences between these ranges could be attributed to the fact that [Maes *et al.* \(2011\)](#) studied very young plants and areas were calculated by using the formula $LW\pi/4$. When we applied the same formula to our data, estimated values showed a 20–30 % lower GT area than the projected values.

None of the leaves that were stress treated at an already developed stage (lower leaves) showed increases in GT density as compared with controls with similar leaf sizes (Figs 3A, 4A and 5A). The present study thus supported the general understanding that GT of *Artemisia* are only initiated in leaves at an early developmental stage ([Ascensão and Pais 1987—*A. campestris*; \[Duke and Paul 1993—*A. annua*\\). Investigation of control plants during a 5-week period \\(Fig. 6A\\) showed constant densities of GT on lower leaves, whereas GT densities of upper leaves increased slightly, but non-significantly. Similar trends were observed in *A. annua* by \\[Lommen *et al.* \\\(2006\\\)\\]\\(#\\) and \\[Liu *et al.* \\\(2009\\\)\\]\\(#\\), and in *M. arvensis* by \\[Shanker *et al.* \\\(1999\\\)\\]\\(#\\). The significant differences in length of GT observed in both upper leaves and lower leaves during the 5-week study \\(Fig. 6B\\) followed the precipitation pattern closely, and it was proposed that the length of GT was a flexible parameter linked to the water status of the leaf.\]\(#\)](#)

The comparison of GT on the adaxial and abaxial sides of the leaf in untreated plants of Ex1 and Ex2 (Table 2)

showed significantly higher densities of GT on the abaxial than the adaxial side. Ascensão and Pais (1987) found a similar trend on leaves of *A. campestris*, whereas Hu et al. (1993) found the opposite trend in *A. annua*. Kelsey and Shafizadeh (1980) reported no difference between the adaxial and abaxial GT density in *A. nova* and, based on estimations of graphs of GT densities on vegetative leaves of *A. annua*, neither did Arsenault et al. (2010). In the present study, the length of the GT did not differ significantly from the abaxial to the adaxial side of the leaf irrespective of leaf age.

Several researchers have shown that various types of stress can increase the content of AN and other SM in *A. annua* (Liersch et al. 1986—chlormequat; Qureshi et al. 2005—oxidative stress; Ferreira 2007—potassium deficiency; Qian et al. 2007—salinity; Kapoor et al. 2007—mycorrhiza; Özgüven et al. 2008—nitrogen; Pu et al. 2009—SA; Liu et al. 2009—JA; Mannan et al. 2010—dimethyl sulphide; Liu et al. 2010—wounding; Lei et al. 2011—chitosan; Maes et al. 2011—JA; and Nguyen et al. 2011—review). Although differences in the developmental age and size of the investigated plants have to be taken into account, the present study suggested that stress may increase the AN production or conversion of AN precursors within existing GT, rather than directly influencing the number or size of GT.

Conclusions and forward look

The results showed very little impact of applied stress on the GT densities of leaves initiated after or under the influence of stress (i.e. the upper leaves). Only a subset of plants in a greenhouse experiment (Ex2) produced significantly higher GT densities on upper leaves in response to multiple treatments of SC, HP or COS at high concentrations. The density of GT on older (i.e. lower) leaves that had already developed at the time of treatment was largely unaffected by stress. The limited effect on upper leaves, especially in Ex1, compared with previous reports is attributed to the larger size of the plants, interference by uncontrolled environmental stresses in the field, and a possible 'saturation' effect of GT in this study. The size of GT was largely unaffected on the upper leaves of Ex2, but several stress treatments decreased GT size on the lower leaves, probably as an outcome of accelerated maturation. The results of this study showed that it was not possible to induce similar stress responses on GT in full-grown plants grown under less protected conditions as found in previous research on young plants. Future biochemical analyses of plant samples from the present experiments are needed to examine this relationship further.

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Contributions by the authors

A.K. executed the experiments and analyses, and wrote the paper in collaboration with K.G. and M.J.

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Conflict of interest statement

None declared.

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