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TITLE:

Why do wasp-induced galls of *Acacia longifolia* photosynthesise?

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Abstract

While many stem and bud galls contain chlorophyll and have the potential to photosynthesise, these insect-induced growths are generally thought to act as strong carbon sinks, manipulating the normal phloem transport of the host plant in order to serve the demands of the galling herbivore. This study investigated the photosynthetic capacity of bud galls induced by the wasp, *Trichilogaster acaciaelongifoliae* (Pteromalidae) in the invasive *Acacia longifolia*. The role of this photosynthetic activity was examined in terms of its ability to subsidise carbon budgets, as well as to provide O_2 to the larvae and consume CO_2 in the dense gall tissue, thereby maintaining O_2 and CO_2 concentrations within the range of larval tolerance. Galls were found to contain an overall chlorophyll concentration that was less than half that of subtending phyllodes and a maximum stomatal conductance only 16% that of phyllodes. Gas exchange measurements indicated that while photosynthesis never fully compensated for the respiratory costs of the galls, light-induced carboxylation within galls contributed substantially to the maintenance and growth of galls, especially in the early stages of their development. Very low levels of O_2 were found within the larval chamber and internal tissues of galls, and these levels responded only marginally, if at all, to light, suggesting that the photosynthetic activity of galls does not play a critical role in providing O_2 to the larvae. The percentage mortality and metabolic response of larvae in reaction to various atmospheres of reduced O_2 and elevated CO_2 indicated that larvae were tolerant of hypoxia and capable of rapidly reducing their respiratory rates to cope with hypercarbia, at least over the short term. Sustained metabolic arrest may, however, have toxic consequences for insects, causing cell damage or even death. The photosynthetic activity of galls substantially reduced internal CO_2 concentrations, thus preventing CO_2 from accumulating within galls over prolonged periods. Hence, the capacity of galls to photosynthesise has significant implications for the survival of the developing larvae by reducing the risk of hypercarbic toxicity and supplying additional carbohydrates to the gall and its inhabitants, thereby creating a favourable microhabitat in which to live.

Introduction

Gall-inducing arthropods spend most of their lives inside the gall, and, as a result, have evolved highly specialised nutritional dependencies on their host plants (Florentine et al. 2005). While the mechanisms of gall formation are not fully understood, insects seem to control gall development by modifying the plant's developmental pathways so as to create a protected and favourable environment in which to live (Wool et al. 1999; Inbar et al. 2004). Acting concurrently, the chemical salivary secretions and feeding action of the inducing insect cause considerable stress in the attacked plant cells (Raman 2007). In response to this stress, the plant translocates metabolites (mostly photo-assimilates) and growth promoters to the site under stress, triggering an abnormal growth response and altering tissue differentiation, thus bringing about gall formation (Raman 2007). It has also been suggested that the larvae themselves contain large amounts of phytohormones (auxins and cytokinins), and are thus able to regulate the translocation of resources to the gall (Dorchin et al. 2009). Even the vascular system of the host plant can be modified so that it supplies nutrients and water to suit the demands of the inducing insect (Wool et al. 1999; Dorchin et al. 2009). Consequently, the gall becomes a sink for mineral nutrients and energy, manipulating phloem transport from sites of production or storage (e.g. mature leaves or phyllodes) and exploiting resources that would otherwise be used for plant growth and reproduction (Fay et al. 1993; Burstein et al. 1994; Florentine et al. 2005).

The demand for assimilates by galls and their ability to compete with other sinks (e.g. flowers, fruits or young shoots) varies, depending on their developmental stage and position on the plant (Larson and Whitham 1991; Dorchin et al. 2006). A common method used to determine the carbon costs of galls is to measure the photosynthetic rates of neighbouring leaves or adjacent leaf tissue. While some studies show that gall-formers have the capacity to stimulate the photosynthetic rates of nearby sources by increasing sink demand relative to source supply (Fay et al. 1993; Moseley et al. 2009), other studies reveal significantly reduced photosynthetic rates (Andersen and Mizell 1987; Florentine et al. 2005). The destructive feeding action of certain galling herbivores (e.g. *Epliblema strenuata* and *Phylloxera notablis*) has been found to disrupt the host plant's conductive tissue, and, hence, impair the uptake of CO_2 (Florentine et al. 2005). Species that feed on highly specialised nutritive tissues, however, do not cause direct damage to the host plant's vascular system, and may even promote greater differentiation within it (Wool et al. 1999), and are thus more likely to enhance assimilation rates in neighbouring source tissues (Dorchin et al. 2006).

Most studies investigating the photosynthetic capacities of galls focused on leaf galls, finding much lower photosynthetic rates in galls than in the surrounding leaf tissue (Andersen and Mizell 1987; Burstein et al. 1994; Larson 1998). To date, very few studies have measured the photosynthetic potential of chlorophyll-containing galls in other parts of the plant (e.g. stem and bud galls). Moseley et al. (2009) investigated the gas exchange characteristics of the galls induced by the cecidomyiid *Dasineura dielsi* in the reproductive tissues of *Acacia cyclops*, and found that, even in the light, the

net flux of CO_2 was outward from the gall, regardless of their developmental stage. In contrast, Dorchin et al. (2006) found that, when supplied with sufficient light, mature *Acacia pycnantha* galls induced by the wasp *Trichilogaster signiventris* maintained an inward flux of CO_2, although photosynthesis never fully balanced the respiratory costs of the immature galls. Radioactive ^{14}C labelling indicated that the influence of *T. signiventris* galls on the rest of the host plant was limited almost entirely to subtending phyllodes, suggesting that metabolites drawn from these, together with the photo-assimilates synthesised by the bud galls themselves, was sufficient for the growth and maintenance of the galls. Consequently, the authors suggest that galls of *A. pycnantha* (especially those in the final stages of development) have the potential to subsidise the carbon budget of the host plant substantially. This may explain why galling was not found to significantly impair the vegetative growth of afflicted stems or whole trees of *A. pycnantha* (Hoffmann et al. 2002).

Apart from factors such as the size and structure of galls and their capacity to photosynthesise, the metabolic activity of the insects within the gall tissue is thought to play a major role in explaining why some galls present higher carbon costs than others (Moseley et al. 2009). *Dasineura dielsi*-induced galls house up to 14 larvae per gall or more than 250 larvae per gall cluster (Moseley et al. 2009), while *T. signiventris* galls contain up to 40 larvae in individual chambers (Dorchin et al. 2006). While neither study measured the respiratory rates of the developing insects directly, the metabolic activity of the larvae, especially at such high densities, is believed to raise the overall respiratory rates of the gall considerably (Dorchin et al. 2006).

Raman (2007, p. 750) described the use of O_2 in gall tissues as being "so intense" that it is capable of depriving certain regulatory enzymes (e.g. indoleacetic acid (IAA) oxidase) of O_2, thereby inhibiting the enzymes' normal activity and allowing growth hormones, like IAA, to accumulate, unchecked, at insect-feeding sites. The dense mass of large-celled parenchyma tissue surrounding the larval chamber (Dorchin et al. 2009) is unlikely to allow O_2 to readily diffuse in from outside the gall to meet the respiratory demands of either the insect or the gall tissue. Thus, the internal space of galls may represent a severely hypoxic habitat, with consequences for the developing larvae within. While several insects possess adaptations which enable their survival in environments of low O_2 (Hoback and Stanley 2001), increases in mortality and/or developmental abnormalities have been observed in insects when reduced O_2 concentrations and elevated levels of CO_2 cross critical thresholds of tolerance (Loudon 1988; Greenberg and Amos 1996; Zhou et al. 2000). Depleted levels of O_2 and high internal CO_2 concentrations are to be expected in galls demonstrating high respiratory rates, but the mechanisms facilitating gas exchange to maintain O_2 and CO_2 concentrations within the range of larval tolerance have not been studied. In addition, little is known about the physiological adaptations of galling insects living in these hypercarbic and hypoxic habitats (Zhou et. 2000).

Several studies have recognised the photosynthetic potential of chlorophyll-containing galls, yet galls are generally found to rely on the host plant for carbon (Andersen and Mizell 1987; Larson and

Whitham 1991; Moseley et al. 2009). This begs the question, why are galls green? It is hypothesised that the photosynthetic activity of galls plays a key role in providing O_2 to the larvae and consuming CO_2 in the dense gall tissue, thereby ensuring the survival of the larvae in an otherwise hypoxic environment. An alternative hypothesis is that gall photosynthesis may make a substantial contribution toward their own carbon budget, thus reducing the influence of galls and their inhabitants as a significant carbon-sink on the host plant. This might serve to minimise the impact of galls on the host plant, allowing the plant to carry a large gall biomass. These hypothesises were tested by studying galls in the reproductive buds (referred to here as 'reproductive galls') of the Australian long-leaved wattle, *Acacia longifolia* (Andr.) Willd. induced by the wasp, *Trichilogaster acaciaelongifoliae* Froggatt. The mortality and respiration rates of larvae were studied under various atmospheres of reduced O_2 and elevated CO_2, respectively, in order to gauge insect tolerances. The effect of light on gall gas exchange with the environment (CO_2 and water) and O_2 production within the gall tissue was examined, and the consequences for gall growth and larval survival assessed.

The study system

Trichilogaster acaciaelongifoliae became the first biocontrol agent to be used against an invasive acacia in South Africa when it was introduced in 1982 to control the spread of its host, *Acacia longifolia* (Dennill and Donnelly 1991). As an agent of biological control, this bud-galling wasp has had tremendous success. Galls are characteristic of almost all *A. longifolia* trees in South Africa and studies demonstrate a >95% reduction in seeding (Hoffmann et al. 2002). Adults emerge from the galls in mid-summer and live for three to four days, during which time, the parthenogenic females lay eggs in the reproductive and vegetative buds of the host plant (Hoffmann 2001). Eggs remain dormant until the following spring, and hatch as the bud begins to enlarge (Dennill and Donnelly 1991). The developing larvae then induce the formation of roughly spherical galls, completely prohibiting the inflorescence from developing normally (Dorchin et al. 2009). When several eggs are laid in the same bud, individual galls may coalesce, creating convoluted clusters with up to 24 chambers (Dennill 1987). Each chamber is surrounded by a mass of parenchyma tissue and by numerous vascular bundles which extend in a centripetal pattern from the chamber toward the gall periphery, connecting the larval chamber and its surrounding tissue with the vascular system of the host plant (Dorchin et al. 2009). Dennill (1988) reports that the production of galls expends up to 23 times more energy than the production of seeds, thus proving very costly carbon sinks indeed. Despite this, the plants manage to support very dense clusters of galls (Fig. 1) without noticeable consequences for vegetative growth (Hoffmann et al. 2002).

Figure 1. Vegetative and reproductive galls frequently occur together on the same branch and at varying stages of development. Galls that develop from inflorescences bear small, shrivelled flower-buds that protrude from the surface of the gall, while vegetative galls have mostly smooth surfaces.

Methods

Shading experiment

A shading experiment was performed in the field on naturally growing invasive *Acacia longifolia* trees on the Spier Wine Estate near Stellenbosch, in the Western Cape, South Africa (35^0 58'S, 18^0 47' E), during the month of July 2011. Galls, induced by the wasp, *Trichilogaster acaciaelongifoliae*, were chosen on predominantly north-east-facing exposed sides of trees to control for sun exposure. Forty-five pairs of approximately spherical reproductive galls were selected such that individuals within a pair were of a similar size (or developmental stage) and occupied adjacent branches on the same tree. Across the sample, gall diameters ranged from 8 to 16 mm (i.e. immature to maturing). Galls larger than this were excluded from selection as the majority were multilocular and irregular in shape and their larvae had already pupated.

To test whether the exclusion of light from galls influenced gall development and the survival of larvae, diameters were measured, and one gall from each pair was covered with aluminium foil. The foil was loosely applied to exclude light without hindering gas flux. Five days after initial measurements, all gall diameters were re-measured and the aluminium coverings replaced. At this time, 15 gall-pairs were excised from the tree and dissected to determine levels of larval mortality (established from the motility of larvae) in covered versus uncovered galls. The same procedure was repeated 10 and 20 days after initiation of shading. Only the 15 pairs that had remained on the tree for the whole 20 day period were used in growth rate analyses, and larvae that had pupated or galls that had fallen off the tree were excluded from both growth rate and mortality analyses. Growth rates of covered and uncovered galls were calculated separately over the periods 0-5 d, 5-10 d and 5-20 d, and compared using a Student's t-test (Microsoft Excel 2007). A Chi square test was used to determine whether the percentage mortality of larvae in covered galls differed significantly from the percentage mortality in uncovered galls after 5, 10 and 20 d.

Measurements of gas exchange in galls

Galls for gas exchange experiments (and all other laboratory work) were obtained from six *Acacia longifolia* trees (serving as six independent biological replicates) on the campus of the University of Cape Town (33^{0}58'S, 18^{0}27'E) during August and September 2011. An infrared gas analyser (Li-6400 portable photosynthesis system, LI-COR, Lincoln Nebraska, USA) was used to measure the rates of photosynthesis (A), conductance (g_s) and transpiration (E), as well as internal carbon dioxide concentrations (Ci) of both mature (>20mm diameter) and immature (<10mm diameter) reproductive galls. Similar amounts of material in each size class were removed from each tree and carefully arranged in the LI-COR 6400-05 "conifer" cuvette to eliminate shading.

Readings were taken shortly after excision of galls, between 10h00 and 15h00, in a well-ventilated climate-controlled growth chamber with the air temperature set to 25^{0}C. The CO_2 concentration in the cuvette was set to 425 ppm and a gall temperature was maintained at 29^{0}C. Readings were taken at various light intensities, starting at *c.* 1800 µmol m^{-2} s^{-1} PAR (i.e. within the range of natural, direct sunlight at midday) and decreasing by increments of *c.* 250 µmol m^{-2} s^{-1} PAR until dark, by adjusting the distance between the light source and the cuvette and using a black shade cloth to achieve lower intensities. The diameters of all galls were measured before being dried at 60 ^{0}C for 72 h, and weighed.

Light response curves for mature and immature galls were constructed for each variable measured. Because the galls had negative photosynthetic rates (i.e. net respiration) the usual curve fitting for leaf light response (e.g. Sharkey et al. 2007) was not implemented. Instead, the curve-fitting programme, CurveExpert Professional v.1.2.3 (Hyams 2011), was used to select the model that fitted the data without exhibiting overfitting (Morgan-Mercer-Flogin). The fitted curves were then used to calculate the gas exchange parameters at each selected light intensity.

Gall and phyllode characteristics

To investigate reasons for the vastly different photosynthetic capacities of galls versus phyllodes, tissue chlorophyll concentrations and stomatal size and density were analysed. To examine the distribution of chlorophyll through the gall, spherical reproductive galls (n = 5) were sectioned and subdivided into three concentric layers; - the outer layer, including the epidermis; middle layer; and inner layer, including the nutritive tissue around the locule. Tissue from five fully expanded phyllodes, subtending those selected galls, was also sampled. A modified version of the extraction protocol described by Hiscox and Israelstam (1978) was followed. One hundred milligrams of gall or phyllode tissue was placed into a 2 ml eppendorf tube and ground using a ball mill (MM 400, Retsch, Haan, Germany). Dimethyl sulphoxide (DMSO, 1.5 ml) was added to this, and the chlorophyll extracted by incubating for 4 h at 65 ^{0}C. The extract was made up to 5 ml with DMSO and a sample of chlorophyll was transferred to a 96-well plate (Greiner Bio-One, Solingen, Germany) and the

absorbance measured at 645 and 633 nm was read using a Multiskan Spectrum microplate reader (Thermo Fisher Scientific, Finland), against a DMSO blank. Chlorophyll content was calculated according to the equation used by Arnon (1949).

Epidermal impressions were made by coating phyllodes and galls (n = 5) with nail varnish, and peeled off with sellotape when dry. Impressions were examined under 400x magnification using a transmission light microscope, and average measures of stomatal density and guard cell size (length x width of the guard cell pair) were calculated (after Franks and Beerling 2009). Maximum diffusive conductance to water vapour and CO_2 in galls and phyllodes was calculated (Franks and Farquhar 2007):

$$g_{\text{turmax}} = \frac{d}{v} \cdot D \cdot a_{\max} \Big/ \left(l + \frac{\pi}{2} \sqrt{a_{\max}/\pi} \right)$$

where d is the diffusivity of water vapour in air (2.42 x 10^{-5} m^2 s^{-1}) at 20°C; *v* is the molar volume of air (2.40 x 10^{-2} m^3 mol^{-1}) at 20°C; *D* is stomatal density; a_{max} is approximated as $\pi(p/2)2$, where *p* is stomatal pore depth, approximated as L/2; and *l* is stomatal pore depth for fully open stomata, taken as W/2.

Gall tissue oxygen concentrations

Branches with galls were cut from trees and immediately stood in water. These were transferred to the laboratory where they were maintained in light. Reproductive galls were excised from the branches and transferred to a Perspex water bath (ca. 75 ml volume) through which distilled water at 25 ^{0}C was flowed at a rate of *c.* 100 ml min^{-1}. Galls were illuminated from opposite sides by two 35 mm slide projectors (Elmo Omnigraphic 253AF, Nagoya, Japan) placed 40 cm from the gall. The slide projectors provided 950 µmol m^{-2} s^{-1} PAR from both sides. An Ocean Optics (Dunedin, FL, USA) FOXY micro-probe (0.5 mm diameter; serial number HTP-1-PNA) was positioned in a micro-manipulator (World Precision Instruments, Sarasota, FL, USA). The probe was attached to an Ocean Optics Neofox spectrophotometer and calibrated using the supplied multi-point calibration files and a single point calibration check. The single point calibration check was conducted by recording the tau value when the electrode was in distilled water at 25 ^{0}C.

The manipulator was used to advance the micro-probe into the gall tissue. Several readings were taken at a depth of *c.* 10 mm inside the gall. Once a stable oxygen tension was recorded in the gall, the projectors were switched on or off to determine the effect of photosynthetic activity on gall oxygen concentration. In a separate analysis, the probe was advanced from the gall surface toward the centre at 2 mm increments and the effect of illumination was determined at each depth. Galls were also sectioned to expose the tissue just beyond the locule. The exposed surface was then immediately sealed with Parafilm (Pechiney Packaging Company, Chicago, Illinois, USA) and left to equilibrate for 30 minutes at 25 ^{0}C. The sectioned gall was positioned in the water bath and the

probe advanced through the Parafilm into the locule. Projector lamps were switched off and on again and oxygen concentrations inside the locule were recorded.

Mortality responses to reduced O_2

The mortality of larvae was tested under 21, 5, 2, 1 and 0% O_2 to determine the tolerance of larvae to different levels of O_2, and hence, assess their minimum oxygen requirements. Three 50 ml plastic vials with suba-seal caps were used per treatment. Vials were positioned on their sides and damp tissue paper was placed at one end to create an environment of high relative humidity, at an ambient temperature of 20 ^{0}C. Ten larvae (at varying stages of development) were placed into each vial and their caps tightly fitted and sealed with silicon sealant. Vials (except those intended for 21% O_2 treatment) were flushed with nitrogen at a rate of c. 100 ml min^{-1} and immediately injected with the appropriate volume of air to achieve the desired oxygen concentration within each vial. After 24 hours, vials were opened and the percent mortality per treatment was calculated.

Measurement of larval respiration

The Licor infrared gas analyser was used to determine the specific respiration rate of larvae (nmol CO_2 g^{-1} fresh wt s^{-1}). Several larvae (n = 5) at varying stages of development) were placed into a LI-COR 6400-15 "arabidopsis" cuvette and respiration was recorded at varying CO_2 concentrations (0, 250, 425, 500, 1000 and 1500 ppm) at 20 ^{0}C. The total fresh weight of the sample was subsequently determined and the same process was repeated 5 times, using a different set of larvae on each occasion. The rate of O_2 uptake was estimated using a respiratory quotient (RQ) of either 1.0 (assumed) or 0.6 (as described by Zhou et al. 2000, for *Platynota stultana* larvae).

Estimation of vascular transport of O_2 into the gall

Transpiration rates recorded from gas exchange analysis were used to calculate the amount of O_2 that could potentially be delivered to the gall in the transpiration stream, assuming an that the xylem sap was at equilibrium with atmospheric O_2 and using the fact that O_2 solubility is 7.69 mg O_2 l^{-1} water at 29 ^{0}C (the temperature at which gas flux experiments were conducted). The vasculature of the gall was stained by standing cut shoots (with galls) in 1% safranin for 24 h. This was sufficient for phyllodes to be stained dark red. Galls were cut open and photographed using both a Nikon Stereoscopic Zoom Microscope (Model SMZ1500; Tokyo, Japan) and a handheld camera (Nikon Coolpix P500, Melville, NY, USA).

Results

Shading experiment

Galls that were covered to exclude light for 20 d grew at significantly lower rates than galls exposed to light for an equivalent time (Student's t test; P = 0.021) (Fig. 2). Galls covered for 10 d also

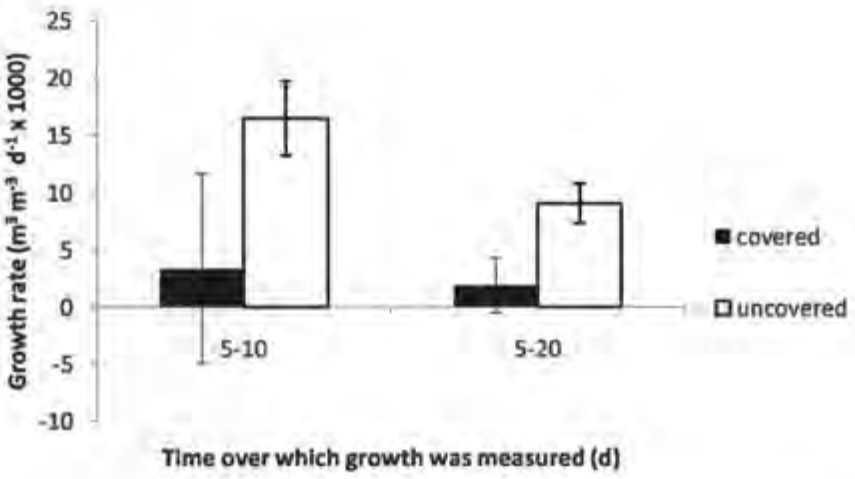

Figure 2. The effect of excluding light on the growth rates ($m^3 m^{-3} d^{-1}$ x 1000) of *Acacia longifolia* galls, over the periods 5-10 d and 5-20 d (mean ± SE, n = 12).

presented lower growth rates than their uncovered counterparts, although rates did not differ significantly ($P > 0.05$). The measurements over the first 5 d were not included in the analysis as galls displayed very low growth rates and an overall reduction in size. Significantly lower mean ($P < 0.001$) and maximum ($P < 0.05$) daily temperatures were experienced over that period in comparison to the periods 5-10 d and 5-20 d (Appendix, Table 1) and may have contributed to the low growth rates observed over this period. There was no significant difference in the levels of larval mortality in covered or uncovered galls after 5, 10 or 20 d of shading (Table 1).

Table 1. Percentage mortality of larvae in covered and uncovered galls at 5, 10 and 20 d after initiation of shading. Chi-squared values (X^2) comparing numbers of living versus dead larvae in covered and uncovered galls are shown for each time interval (df = 1) were not significant.

Time from covering	5 d		10 d		20 d	
	covered	uncovered	covered	uncovered	covered	uncovered
Mortality (%)	0.0	7.7	16.7	7.1	9.1	8.3
Sample size (n)	12	13	12	14	12	12
X^2	0.96, P > 0.05		0.57, P > 0.05		0.00, P > 0.05	

Gall gas exchange

Both mature (large) and immature (small) galls exhibited negative rates of photosynthesis, i.e. net respiration (nmol $CO_2 g^{-1} s^{-1}$), even in the light (Fig. 3a). However, the rates of CO_2 efflux decreased when galls were exposed to light intensities sufficient to drive photosynthesis. Small galls respired less vigorously than mature galls at light intensities greater than 500 µmol $m^{-2} s^{-1}$ PAR, but displayed significantly higher respiration rates than mature galls in the dark ($P < 0.01$). The mean surface area to volume ratio of immature galls was significantly greater than that of large, mature galls (632 m^{-1} and 295 m^{-1}, respectively, $P < 0.001$), and this may have contributed to the observed difference in CO_2 flux between size classes.

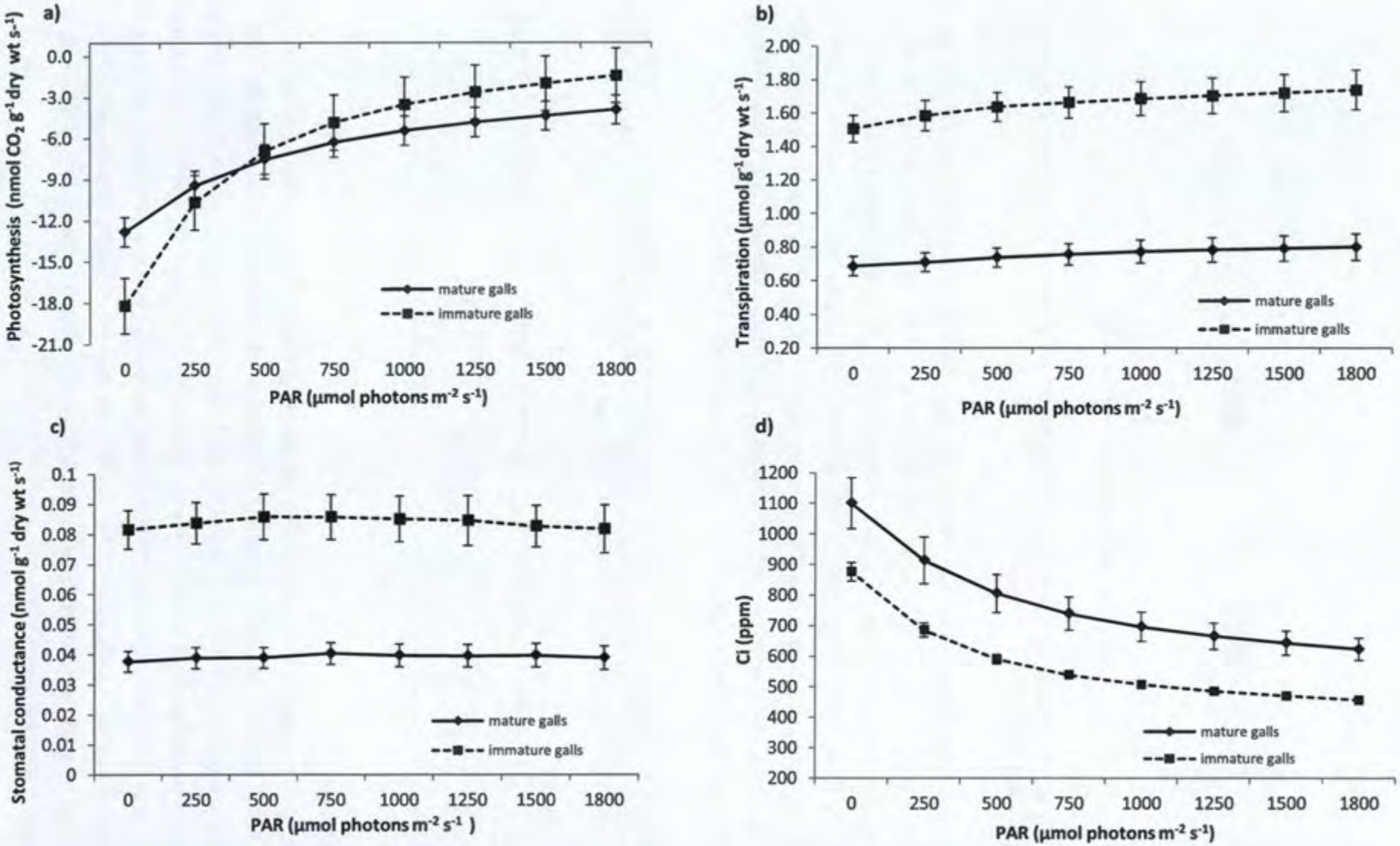

Figure 3. Light response curves for mature and immature galls (n = 6 replicates), showing the influence of various light intensities on mean (bars indicate ± SE) a) photosynthetic rates, b) transpiration rates, c) stomatal conductance, and d) intercellular CO_2 concentrations (Ci).

The transpiration rates (μmol g^{-1} s^{-1}) of immature galls were significantly higher than those of mature galls, in darkness and in the light ($P < 0.001$) (Fig. 3b). Both types of galls displayed a gradual increase in transpiration with light intensity due to a combination of lowered stomatal resistance (through increased stomatal opening) and an elevated energy balance (through increased photon supply). Stomatal conductance to water vapour (mmol g^{-1} s^{-1}) showed no increase with light intensity (Fig. 3c). Immature galls did, however, present significantly higher rates of stomatal conductance relative to those of mature galls at all light intensities ($P < 0.001$). Internal CO_2 concentrations (Ci) were consistently higher in mature galls than immature galls (Fig. 3d) and both types of galls exhibited a similar decline in Ci with increased light intensity.

Gall and phyllode characteristics

The outer layer of the gall, which included the epidermis, contained most (63%) of the total gall chlorophyll, while the middle and inner layers contained much lower levels at 17 and 20%, respectively (Fig. 4). Total gall tissue chlorophyll concentration (i.e. 1.07 mg chlorophyll g^{-1}) was *c.* 2.2 times less than that of phyllodes, suggesting that the photosynthetic potential of galls is far lower than that of phyllodes.

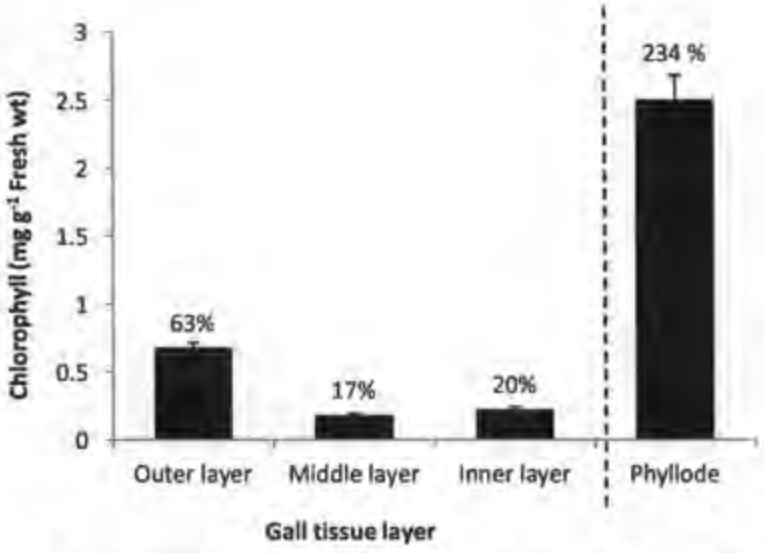

Figure 4. Chlorophyll concentrations in different sections of the gall (outer; middle and inner layers), contrasted with phyllode chlorophyll concentrations, expressed per gram fresh weight (mean ± SE; n = 5). The percentage of the average total gall chlorophyll concentration (i.e. 1.07 ± 0.11 mg chlorophyll g^{-1} fresh weight) is indicated per tissue section. Bars indicate SE of mean.

The mean stomatal density (D) of galls was *c.* 5 times lower than that of phyllodes, and the mean gall guard cell size (S) was half that of phyllodes (Table 2). Consequently, maximum diffusive conductance to water vapour (g_{wmax}), calculated from stomatal parameters, *D* and *S*, indicated that the g_{wmax} of galls was only *c.* 16% that of phyllodes.

Table 2. Mean ± SE stomatal density (D) and guard cell size (S) of galls versus phyllodes (n = 5). Maximum diffusive conductance, g_{wmax} was calculated from *D* and *S* parameters, following Franks and Farquhar (2007).

	Gall	Phyllode	(Gall as % Phyllode)
Stomatal density ($m^2 \times 10^{-6}$)	28 (±4.9)	122 (±3.6)	23
Guard cell size (μ^2)	413 (±13.1)	832 (±53.2)	50
g_{wmax} (mol H_2O m^{-2} s^{-1})	0.6 (±0.1)	3.8 (±0.1)	16

Gall tissue O_2 concentrations

Very low O_2 levels were recorded within galls at a depth of *c.* 10 mm (Table 3). These levels decreased further, albeit by a small margin, in the absence of light. This suggests that, to some extent, the photosynthetic activity of galls influences the O_2 tension within the gall tissue. The effect of illumination, however, was far greater at the gall's surface and at a depth of 2 mm within the gall tissue (Fig. 5), where chlorophyll concentrations are at their highest (Fig. 4), and light absorbance would be greatest. Net photosynthesis was observed close the gall surface, where O_2 levels reached 24% (v/v) in the light. When the light was turned off, however, a large, instantaneous decrease in the O_2 concentration occurred. Below the gall surface (2 mm), O_2 levels declined sharply to <5%; yet a further decrease was again observed when the light was turned off. At a depth of 4 mm below the gall surface, the O_2 concentration was *c.* 3%, where it remained relatively stable, changing very little under the influence of illumination or with increased depth. Within the larval chamber (Fig. 5b), O_2 levels were at their lowest (*c.* 2.4%), and no discernible difference in O_2 tension was observed under the presence or absence of light.

Table 3. Gall tissue oxygen tensions (n = 4, mean ± SE) in the light (PAR = 2 x 950 µmol photons m^{-2} s^{-1}) and in the dark. There was no significant difference between light and dark measurements ($P > 0.05$).

	Light	Dark	Ratio (light: dark)
Oxygen % (Saturated = 21%)	2.68 (±0.8)	2.48 (±0.8)	1.1
Oxygen (mg O_2 l^{-1} at 25 °C)	1.06 (±0.3)	0.98 (±0.3)	1.1

Influence of hypoxia and hypercarbia on larvae

There was less than 10% larval mortality under 21 and 5% O_2 (Fig. 6), but mortality increased with decreasing O_2 concentration. Under 0% O_2, larval mortality was substantially higher at 63%, indicating an intolerance of anoxic environments. Gall tissue O_2 measurements (Fig. 5) indicated that concentrations between 2 and 5% O_2 were sufficient to supply respiratory demand. Thus, mortality observed at these concentrations (and at 21% O_2) may have been caused by stress experienced during translocation from the gall to the vial, rather than hypoxia, per se.

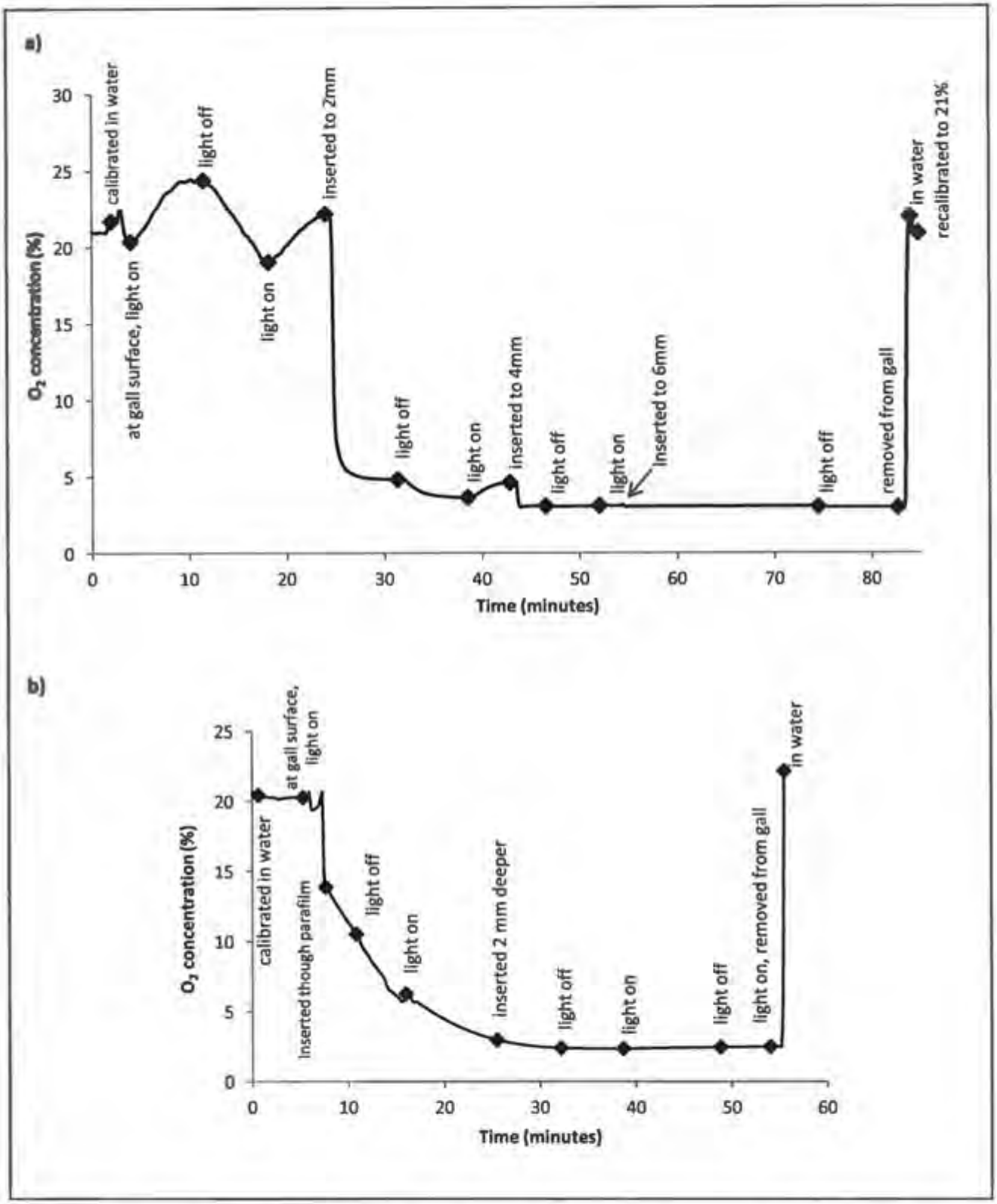

Figure 5. The effect of illumination on gall-tissue and locule O_2 concentrations (%): a) with increasing depth through the gall, and b) in the larval chamber. Labelled data points indicate specific actions. In a), the microprobe was advanced into the gall at 2 mm increments to a total depth of 6 mm. Total gall diameter was 17.3 mm. Light was 950 µmol photons m^{-2} s^{-1} PAR from two sides, and temperature 25 °C.

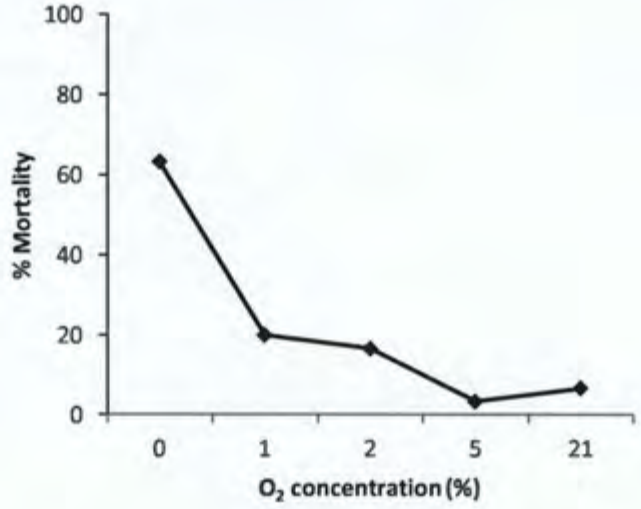

Figure 6. Percentage mortality of *Trichilogaster acaciaelongifoliae* larvae, under 0, 1, 2, 5 and 21% O_2, over a 24 h treatment period (n = 30 larvae per O_2 treatment).

The respiration rates of larvae (nmol g^{-1} s^{-1}) declined with increasing CO_2 concentration, especially at concentrations above 500ppm (Fig. 7), suggesting that larvae are capable of quickly reducing their metabolic rates under hypercarbic conditions. The specific respiration rate of larvae at 425 ppm (the ambient CO_2 concentration at which gall gas exchange measurements were conducted) was *c.* 0.14 nmol g^{-1} s^{-1}, representing only a fraction of the overall respiratory rate (nmol g^{-1} s^{-1}) of the gall (Fig. 3a), i.e. 6.7 and 0.9% in the light (1800 µmol photons m^{-2} s^{-1}, PAR) and dark, respectively. However, since larvae experienced much higher levels of CO_2 inside the gall (Fig. 3d), their respiration rates would have decreased accordingly and their percentage contribution to the overall respiratory rate of the gall would have been even smaller, especially in the dark.

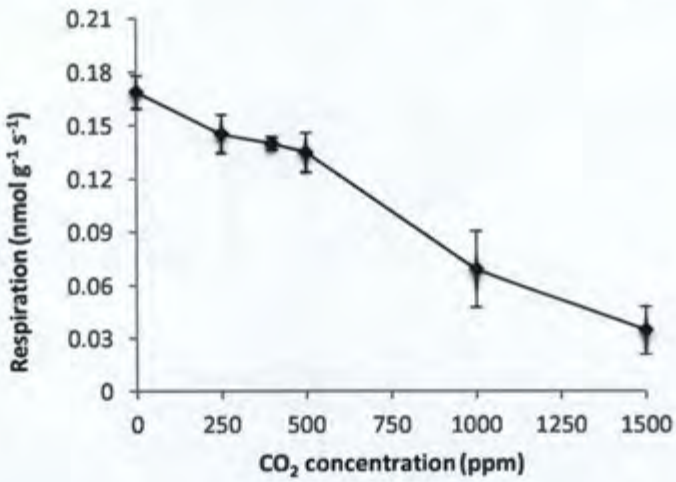

Figure 7. Larval respiration rates, expressed per gram larval fresh weight, under increasing CO_2 concentration (n = 5 replicates). Bars indicate SE of mean.

Vascular transport of O_2 into the gall

No significant difference was observed in the transpiration rates of excised galls and those still on branches stood in water (data not shown, $P > 0.05$). Hence, the amount of O_2 that could potentially be delivered to the gall tissue in the transpiration stream was calculated from gas exchange measurements using excised galls (Fig. 3b). In the light (1800 µmol photons m^{-2} s^{-1}), xylem-flow may supply the tissue of mature and immature galls with 3.5 and 7.5 nmol O_2 g^{-1} s^{-1}, respectively. This more than compensates for the O_2 used by larvae, calculated as 0.14 nmol O_2 g^{-1} s^{-1} (assuming an RQ of 1) or 0.23 nmol O_2 g^{-1} s^{-1} (using an RQ of 0.6, following Zhou et al. 2000) at 425 ppm. The stained vasculature of the gall revealed that the xylem sap was first delivered to the vascular bundles at the periphery of the gall and proceeded to diffuse toward the larval chamber (Fig. 8). Small amounts of safranin were observed in the nutritive tissue at the locule.

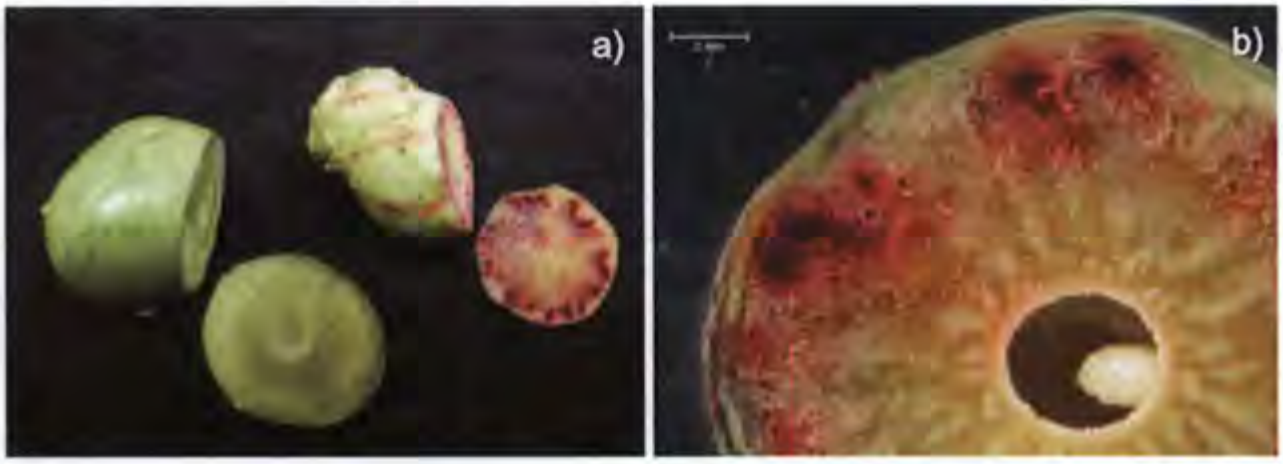

Figure 8. a) Sectioned galls of *Trichilogaster acaciaelongifoliae*: left, unstained; right, stained. Note red staining of the veins just beneath the gall surface. b) The larval chamber is surrounded by numerous centripetal vascular bundles, but those at the gall periphery have been stained the darkest, such that xylem sap appears to diffuse inwards from the outer margin. Note light staining of the nutritive layer surrounding the locule.

Discussion

Galls are generally regarded as major carbon sinks, diverting resources away from the normal metabolic pathways of the host-plant in order to meet the demands of the inducing insect (Larson and Whitham 1991; Fay et al. 1993; Florentine et al. 2005). While many galls are green, their ability to photosynthesise and subsidise carbon budgets has largely been overlooked (Dorchin et al. 2006). Although the present study found that bud galls of *Acacia longifolia* contained an overall chlorophyll concentration that was less than half that of subtending phyllodes, shading experiments revealed that the photosynthetic capacity of galls contributed significantly toward gall growth, and may have offset their carbon costs considerably, particularly in the early stages of development.

While covered galls maintained positive growth rates by continuing to acquire carbon from adjacent phyllodes, their growth rates were substantially lower than galls left exposed to sunlight, especially after 20 days (Fig. 2). This suggests that the photosynthate generated by the gall itself contributes to the maintainence of the gall and the synthesis and construction of its cellular components.

Relative to phyllodes, *A. longifolia* galls have a limited ability to take up CO_2 from the atmosphere, as their estimated maximum diffusive conductance to water vapour (g_{wmax}) is constrained by the small size and low density of their stomata. This may, in part, explain why gall photosynthesis never fully compensated for respiratory costs. Gas exchange measurements indicated that the net flux of CO_2 was always outward from the gall, despite significant light-induced carboxylation (Fig. 3a). When supplied with sufficient light, the flux of CO_2 from small, immature galls was much less than that from large, mature galls. Small galls have a higher surface area to volume ratio than large galls and are thus able to supply a greater proportion of their tissue with CO_2, thereby achieving higher photosynthetic rates than mature galls in the light. Large galls possess longer pathways for gas diffusion compared to small galls (Dorchin et al. 2006), and, as a result, are not as capable of rapidly assimilating carbon to offset their respiratory costs. Small galls also displayed higher rates of water-loss compared to larger galls (Fig. 3b). Rapid transpiration may allow for increased CO_2 acquisition and could also facilitate nutrient acquisition through mass-flow (Cramer et al. 2008), whereby minerals are transported from the soil to the photosynthesising tissues of the gall and the developing larvae within.

In the dark, mature galls exhibited lower specific respiratory costs than immature galls (Fig. 3a). As the larvae mature, their feeding activity decreases, and gall growth, thought to be triggered by this feeding action, slows down (Raman 2007). Thus, the reduced metabolic activity of mature galls and their inhabitants may account for their lower respiratory costs in the dark. This is in accordance with conclusions of Dorchin et al. (2006) for bud galls of *Acacia pycnatha*. However, contrary to the results presented here, mature *A. pynantha* galls were found to respire less than immature galls in the light as well, maintaining an inward flux of CO_2 substantial enough to off-set all respiratory costs, at least during daylight hours. Such galls may house *c.* 40 larvae (Dorchin et al. 2006), thus the resultant respiratory costs of small, immature galls are so large that the benefit derived from having a larger surface area to volume ratio is nullified. The carbon costs of galls are, therefore, a consequence of the metabolic activity of the larvae, and the size (or developmental stage) and photosynthetic capacity of the galls.

Many hypoxic habitats and the adaptations of insects living therein have been identified (reviewed in Hoback and Stanley 2001), yet this is the first study to recognise galls as an hypoxic habitat. Very low levels of O_2 are to be expected in galls demonstrating high respiratory rates (Raman 2007), and such conditions could prove insecticidal (Zhou et al. 2000) should levels fall below a

critical point or tolerance threshold (Fig. 6). However, this study found little evidence to support the hypothesis that the photosynthetic activity of galls is, in fact, the mechanism maintaining O_2 concentrations within the range of larval tolerance. Shading galls to prevent their photosynthetic activity appeared to have no effect on larval mortality (Table 1), and gall tissue O_2 measurements revealed that the O_2 tension within the galls increased only marginally in response to light (Table 3), and not at all inside the central chamber (Fig. 5). Since light penetration decreases exponentially through the dense parenchyma tissue of the gall, there is little need for photosynthetic pigments beyond the gall's outer margin (Fig. 4). While a substantial amount of O_2 was produced by photosynthesis at the gall periphery, it remains unclear whether the O_2 generated in that region might diffuse inward toward the larval chamber. Nonetheless, anoxia was never observed within the gall, although low O_2 levels (< 5%) seemed a persistent feature.

Since it appears unlikely that the photosynthetic activity of galls plays a key role in providing this O_2 to the larvae in the dense gall tissue, an alternative source of O_2 was sought. The O_2 that could potentially be transported to the gall in the transpiration stream was found to be more than sufficient for the respiratory demands of the larva. However, staining showed that the xylem sap was first delivered to the gall periphery (Fig. 8). This was surprising as it was previously reported that the vascular strands of the gall connected the larval chamber with the inflorescence axis and stem directly (Dorchin et al. 2009), thus first supplying the demands of the larva and then those of the gall tissue. The passage of stain first to the gall periphery and then inward to the nutritive layer of the gall may indicate that the xylem delivered the safranin to the outer photosynthetic tissue by transpiration pull, after which it passed into the phloem and was delivered to the nutritive layer around the locule. This raises the possibility that both the O_2 and the photosynthate produced within the gall are conveyed to the developing larva in the central chamber.

While hypoxia limits the respiratory metabolism of insects, elevated CO_2 has been found to prevent insects from using O_2 by inhibiting respiratory enzymes such as succinic dehydrogenase, resulting in reduced oxidative phosphorylation and a decline in ATP generation (Edwards 1968, Zhou et al. 2000). In the dark, CO_2 accumulates inside the gall and may reach very high concentrations due to elevated rates of gall tissue respiration and increased stomatal resistance. However, *Trichilogaster acaciaelongifoliae* larvae seemed capable of tolerating severely hypercarbic environments, at least over the short term, by rapidly reducing their respiratory rates (Fig. 7), thereby proving successful metabolic conformers. Prolonged periods of metabolic arrest and decreased ATP production may, however, have lethal consequences for insects (Hochachka 1986). As a result of energy deficiencies, membrane ion pumps may fail to regulate the influx of ions such as Ca^{2+}, leading to cell damage or even death (Hochachka 1986). Gas exchange measurements indicated that the Ci of galls ranged between 400 and 600 ppm in the light (depending on the size of the gall and the intensity of the light), but reached levels as high as 1100 ppm in the dark (Fig. 3d). Thus, it appears

that gall photosynthesis is capable of substantially reducing the internal CO_2 concentration of galls and may, therefore, have significant implications for the survival of the larvae.

Conclusion

Understanding the role of photosynthesis in galls is fundamental to gall-herbivore ecology. While it appears that the photosynthetic activity of galls does not play a critical part in providing O_2 to the larvae, it may at least contribute some O_2 and help to prevent CO_2 from accumulating in the gall over prolonged periods, thereby reducing the risk of hypoxic and hypercarbic toxicity. Furthermore, although galls may still rely heavily on the host plant for photosynthate and other resources, the capacity for CO_2 fixation in galls contributes substantially to carbon budgets. This is important not only for the growth of galls and the supply of carbohydrates to the larvae, but could also help to minimise the impact of galls and their inhabitants on the host plant, ultimately enabling the plant to support a greater gall biomass.

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Appendix

Table 1. Daily mean, maximum and minimum temperatures (°C) over the period 05-Jul-2011 to 25-Jul-2011. Measurements of gall diameters were performed between 11h00 and 14h00 on day 0, 5, 10 and 20. Temperatures were recorded at the Cape Town International Airport (33°58' S, 18°35' E).

Date	Day	Mean Temp (°C)	Max Temp (°C)	Min temp (°C)
05-Jul	0	10	16	5
06-Jul	1	12	17	6
07-Jul	2	10	19	1
08-Jul	3	12	19	4
09-Jul	4	12	21	4
10-Jul	5	16	25	7
11-Jul	6	16	24	9
12-Jul	7	14	22	7
13-Jul	8	12	17	7
14-Jul	9	14	22	5
15-Jul	10	16	25	7
16-Jul	11	16	26	7
17-Jul	12	14	21	7
18-Jul	13	12	19	6
19-Jul	14	12	18	6
20-Jul	15	16	20	11
21-Jul	16	13	19	7
22-Jul	17	14	17	12
23-Jul	18	14	19	9
24-Jul	19	14	17	12
25-Jul	20	13	16	10

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