Ecological Entomology

Ecological Entomology (2013), **38**, 11–22 DOI: 10.1111/j.1365-2311.2012.01397.x

Carnivores and carotenoids are associated with adaptive behavioural divergence in a radiation of gall midges

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Abstract. 1. Adaptive divergence in sympatry is supposed to be inhibited by the homogenizing role of gene flow. However, studies continue to uncover examples of sympatric divergence. In this study, two divergent phenotypes in a complex of four syntopic gall midge morphotypes [nominally *Asteromyia carbonifera* Osten Saken, Diptera: Cecidomyiidae: Alycaulini] are characterised. The first is a behavioural phenotype governing within-host tissue preference and the second is a trait governing accessory-gland carotenoid quality and quantity.

2. One gall morphotype (crescents) lay most of their eggs on mature tissue whereas the other three gall morphotypes oviposit only on young emerging leaves. Ecological maintenance of this divergent trait appears to be driven by enemy-reduced space. That is, nearly 40% of the crescent morphotype galls that develop high on the plant are attacked by the egg parasitoid *Platygaster solidaginis* Ashmed, whereas those low on the plant are relatively protected.

3. All morphotypes contain carotenoids in their accessory glands, but the quality and quantity of these pigments differs significantly between the morphotypes and is positively associated with the probability of parasitism by *P. solidaginis*.

4. Larval salivary glands also contain carotenoids and the plant hormone abscisic acid, which in plants is synthesized from carotenoid precursors and is involved in regulating plant defences. This hormone may facilitate gall development and influence gall morphology.

5. Ecological fitness trade-offs between carotenoids, parasitoid attack, and plant resistance may be fostering adaptive divergence in ovipositional phenotypes and sympatric speciation in this complex of gall midge morphotypes.

Key words. Adaptive divergence, adaptive radiation, *Asteromyia carbonifera*, carotenoids, Cecidomyiidae, Diptera, enemy-free space, parasitism.

Introduction

Adaptive divergence in sympatry is expected to proceed slowly (if at all) because of the homogenizing role of gene flow (reviewed briefly in Hendry *et al*., 2001; Servedio *et al*., 2011), but insect studies continue to reveal evidence of sympatric divergence (Wood, 1993; Dambroski & Feder, 2007; Joy & Crespi, 2007; Dorchin *et al*., 2009). In these systems, behavioural phenotypes and especially ovipositional phenotypes appear to be the forerunners to deeper physiological changes that may provide reproductive isolating mechanisms. It could be argued that the proximate reason for the evolution of the two host races of the apple maggot fly was host preference via associative learning (Prokopy *et al.*, 1982). This behavioural phenomenon may have led to the subsequent differentiation in diapause phenology that ultimately reduced gene flow between these sympatric host races (Dambroski & Feder, 2007). A similar argument could be made for the host races of *Eurosta solidaginis* (Fitch) on *Solidago altissima* L. and *S. gigantea* (Waring *et al.*, 1990; Craig *et al.*, 1993; Brown *et al.*, 1996).

Incipient species or host races are ideally suited for studying sympatric speciation because contemporary selective forces are likely to reflect those that drove the initial stages of

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divergence and continue to maintain discrete populations (Brown *et al.*, 1995; Schluter, 2000). Host shifts require simultaneous behavioural changes in host preference as well as physiological adaptation to the new host plant. These requirements are thought to hinder the likelihood of host shifts and adaptive divergence (Brown *et al*., 1995). However, lower predation risk on the new host or tissue is expected to accommodate maladaptation to host-plant resistance (Price *et al.*, 1980; Singer & Stireman, 2005). Gavrilets (2004, p. 396) concludes in his review of evolutionary models of sympatric speciation that models incorporating habitat preference or a 'magic trait' (Servedio *et al*., 2011) are the most likely to result in sympatric speciation. Similar constraints and facilitating factors may operate at even smaller scales; that is, within a host-plant (Joy & Crespi, 2007). In this study, we characterise two divergent phenotypes in a gall midge species complex occupying a single host. The first is a behavioural phenotype governing within-host tissue preference and the second is a trait governing accessory-gland carotenoid quality and quantity. These traits co-vary with each other and with the probability of parasitism.

System background

Our study system is comprised of a complex of gall midges, *Asteromyia carbonifera* Osten Saken on tall goldenrod, *S. altissima* (Crego *et al*., 1990; Stireman *et al*., 2008). The galls are the result of a mutualistic relationship between the gall midge and a symbiotic fungus, *Botryosphaeria dothidea* (Moug.) Ces. & De Not.; each partner requires the other for successful galls to form (Janson *et al*., 2009; Heath & Stireman, 2010). In this study, the focus is on a likely paraphyletic group of four gall morphotypes (hereafter, morphs) that occur in spatiotemporal syntopy on tall goldenrod. These morphs are referred to as crescents, cushions, flats, and irregulars according to their overall morphology, position on the leaf, position within the gall, and degree of loculation (Crego *et al.*, 1990). Current evidence indicates that the midge, not the fungus or the host plant, is responsible for variation in gall phenotypes (Heath & Stireman, 2010; Janson *et al*., 2010). The morphs are attacked by up to seven different parasitoids and gall structure and clutch size influences parasitism rates (Weis, 1982a,b; Weis, 1983; Weis *et al*., 1983; Stireman *et al*., 2008). Although some gall-making insects create sexually dimorphic galls (Cook & Gullan, 2008), have generation-specific morphotypes (Rey, 1992; Miller, 1998; Inbar *et al*., 2004) or appear to have diverged parapatrically (Mishima & Yukawa, 2007), *A. carbonifera* morphs are sympatric and genetically differentiated (Crego *et al*., 1990). The phylogenetic relationships of these four morphs depend heavily on the type of marker used, but amplified fragment length polymorphisms recover at least four major groups among these morphs with relatively little geographic genetic structure (Stireman *et al*., 2008, 2010), strongly suggesting that they have diverged and are maintained in sympatry.

Within-host preference and performance

Intuitively, one might predict that tissue preference and performance would be tightly correlated. However, this prediction depends on the number of trophic levels involved. In a bitrophic herbivore-plant system it is expected, but in a tritrophic system trade-offs may overshadow preference–performance correlations. That is, lower performance on a more defended tissue may be evolutionarily tolerated in order to realise a refuge from natural enemies (Price *et al*., 1980; Singer & Stireman, 2005). Such trade-offs may drive and maintain host shifts. Furthermore, physiological and behavioural optimisations for life in these new environments may positively feedback, further driving a wedge between diverging lineages (see Schluter, 2000 on ecological speciation).

The goals of this part of the study are to confirm experimentally the existence of tissue preference (i.e. ovipositional phenotype) among the gall morphs of *A. carbonifera* and evaluate trade-offs between performance and parasitism. These objectives are addressed with the following questions:

1 Do *A. carbonifera* morphs differ in their ovipositional niche?
2 Does this niche provide a refuge from parasitoid attack?
3 Do plant tissues vary in their suitability for different gall morphs?

Variation in carotenoids among gall morphs and its consequences

In insects and other organisms, carotenoids are important in vision, development, management of oxidative stress, diapause, photoperiod, mate signalling, resource location, and colouration (reviewed in Heath *et al*., unpublished). Carotenoids are bright orange or red, fat-soluble pigments and their presence in insect bodies is visually apparent. They have been found in insect glands (Eichenseer *et al.*, 2002; Sakudoh *et al.*, 2007), but it is generally assumed that animals cannot biosynthesise carotenoids and must obtain them from their diet. However, recent evidence of laterally transferred genes for carotenoid biosynthesis in some arthropods (Moran & Jarvik, 2010; Altincicek *et al*., 2011) challenges this dogma.

In our system, carotenoids are found in the adult female accessory glands (AG) and are deposited on eggs along with symbiotic fungal spores (Heath & Stireman, 2010). In addition to the AGs, a particularly dense localisation of carotenoids is also found in the filamentary region of the larval salivary glands (SG), which is the primary source of chemical agents that induce gall formation in cecidomyiids (Raman *et al.*, 2005). The accumulation of carotenoids in these glands, representing the biochemical interface between midge and host-plant, suggests that carotenoids play a functional role in gall development and performance, and may underlie divergence among gall morphs in *A. carbonifera*. We therefore hypothesised that carotenoid quantity and quality profiles represent a possible axis of cryptic phenotypic variation among gall morphs.

In addition to examining variation in carotenoids among morphs, we explored some of the possible roles of these compounds in midge–host and midge–enemy interactions. The basal region of the SGs appear to lack carotenoids, suggesting that they are broken down into unpigmented compounds

as they pass from the filament region to the basal region and eventually into the plant via the larval mouthparts. In plants, carotenoids are the precursors to abscisic acid (ABA), which is a hormone important in the regulation of plant defences (Mauch-Mani & Mauch, 2005) and stomatal aperture (Assmann, 2004) and can be found in the larvae of some herbivores (Tooker & De Moraes, 2011). This led us to hypothesise the presence of ABA in the SGs where it may play a role in gall development. Furthermore, because fungi and plants are known to degrade carotenoids to volatile apocarotenoids (Zorn *et al*., 2003; Lewinsohn *et al*., 2005) and hymenopterans are well known to use apocarotenoids as semiochemicals (reviewed in Heath *et al*., unpublished), we hypothesised that AG carotenoids would be positively associated with the risk of attack by the egg parasitoid *Platygaster solidaginis* Ashmed. To address these hypotheses we asked the following questions:

1 Do accessory gland carotenoids vary among the morphs?
2 Is the carotenoid break-down product and plant hormone, ABA, present in midge salivary glands?
3 Do accessory gland carotenoids covary with the probability of parasitoid attack?

Materials and methods

Testing for differences in ovipositional phenotype

We tested the hypothesis that morphs divide up the plant resource by leaf age by measuring gall diameter, gall height, and plant height of naturally occurring galls in a common garden of 100 *S. altissima* subplots (WSU site, Table 1). We established the common garden in June of 2007 with plants grown in the greenhouse that spring from rhizomes collected haphazardly from nearby wild populations. We transplanted the plants to the field in a 10-row by 10-column grid spaced 2 m on the centre in a randomised complete block design; each 2-row by 10-column block contained 2 replicates of each of the 10 clones (presumably different genotypes). We watered the plants after transplanting, but other than periodical weeding and mowing around the subplots, they were left to establish on their own. Starting in May 2009, we checked the subplots daily for new galls which were marked and measured at a rate of about one to two rows of the common garden per day. After about 1–2 weeks a complete census of the galls in the garden was achieved and the process began again, including additional measurements of the previously marked galls and measurements of any newly occurring galls. This was continued until August at which time several hundred galls had been tracked and measured (crescents, $n = 1579$; irregulars, $n = 210$; cushions, $n = 307$; and flats, $n = 188$). Morph assignment was confirmed after the marked galls were mature (i.e. when the rate of gall-diameter-increase was $<10\%$ per week). We could not determine gall age at the time of discovery and so the height of oviposition was uncertain. We inferred where the eggs were laid by plotting the relative gall height on the plant as a function of relative gall diameter on the day of discovery and took the intercept to indicate where oviposition occurred. We used a linear model to test if the intercepts were significantly different (R Development Core Team, 2010).

To determine whether the difference in gall heights was caused by delayed larval hatching or by actual oviposition on mature tissue, a common garden of 200 potted *S. altissima* plants representing 20 different clones was established. The plants were grown in 2.5-l pots of soilless media (Pro-mix BX/Mycorise® Pro, Premier Horticulture, Ltd., Quakertown, Pennsylvania, U.S.A.) from rhizome cuttings in the greenhouse and then placed in the field within a deer exclusion plot and watered regularly. We arranged pairs of clones in a randomized complete block design within a 10-row by 10-column grid with pairs spaced 0.5 m on centre; each 2-row by 10-column block contained 1 pair of each of 20 different clones (presumably different genotypes). All plants were free of galls at the start of the experiment in July 2009. One plant in each pair had the top 20-cm of young leaves including the terminal bud covered with screening ($n = 5$ pairs per clone). The screening was moved up as the plants grew. Galls were marked weekly as they appeared and were assigned to a morph when they were mature. The experiment lasted 2 months. If eggs were actually laid on mature tissue, then only galls of that morph should have appeared on plants with protected young tissue.

Table 1. List of study sites with abbreviations, names, and coordinates*.

Site abbreviation	Site name (all Ohio, U.S.A.)	Latitude (N)	Longitude (W)
BCWMA	Beavercreek Wildlife Management Area	39°45′59″	84°00′16″
GMP	Germantown Metropark	39°38′21″	84°24′50″
SSP	Sycamore State Park	39°48′08″	84°21′39″
WSU	Wright State University *Solidago altissima* Common Garden	39°47′15.40”	84°03′10.10″

*Coordinates obtained from Google Maps (retrieved 1 November 2011).

Assessing variation in parasitoid attack

To test whether parasitoids might be driving the divergence in ovipositional phenotype, parasitoid attack was measured at four prairie sites dominated by *S. altissima* in and around Dayton, Ohio (Table 1). We collected mature galls and either dissected them under a stereomicroscope or reared them in cotton-stopped vials in constant humidity chambers (~90% RH achieved with a saturated NaCl solution). For the WSU site, logistic regression over the height of the plant was performed on the binary variable of presence or absence of a specific parasitoid for the crescent morph. For all four sites, attack by *P. solidaginis* was analysed with a χ^2 test and Pearson's standardized residuals plotted by gall morph. Sample sizes for each site for crescents, cushions, flats, and irregulars were: BCW, $n = 100$, 113, 93, and 115; SSP, $n = 142$, 135, 78, and 151; GMP, $n = 88$, 106, 70, and 97; and WSU, $n = 1403$, 287,

173, and 204 galls; respectively. All analyses were conducted in R (R Development Core Team, 2010).

Testing suitability of the oviposition site

To test whether cushions, flats, and irregulars could develop on mature leaves, eggs were collected from the terminal bud and adjacent young leaves of field populations of *S. altissima* and transplanted to the leaves of potted *S. altissima* clones. With the aid of a stereomicroscope, transplant eggs were placed in the middle of the underside of the leaf between the midvein and the margin of either young, newly emerged leaves or mature, fully expanded leaves. Gall morph, diameter, and midge stage were recorded after 5 weeks of development in the greenhouse. Final gall diameters were analysed with a one-way ANOVA followed by planned orthogonal decomposition. Logistic regression was used to test whether survival to adults or pupae was affected by tissue age, morph, or their interaction (R Development Core Team, 2010).

Crescent eggs are extremely difficult to find in the field, but very young crescent galls are relatively easy to locate and crescent morphs almost always develop on the very edge of the leaf. Several crescent eggs are often deposited along the edge of a single leaf (presumably by the same female) and all young *A. carbonifera* galls retain an orange spot of accessory fluid for about the first week after oviposition. Therefore, once a young crescent gall is located the entire edge of the leaf can be checked for unhatched eggs, neonates, or extremely young galls. By marking and tracking the development of these eggs, neonates, and young galls over time, the failure rate of crescents was estimated over the height of the plant. Galls rarely failed once they reached a diameter of about 2 mm; therefore, we are confident in this method of assessing plant resistance to crescent morphs. Logistic regression was used to test the effect of gall height on the failure rate of crescent galls (R Development Core Team, 2010).

Characterising carotenoid profiles in accessory glands

To examine variation in carotenoid quality and quantity among morphs, carotenoids were extracted in hexane from the AGs (Fig. 6c) of *A. carbonifera* morphs ($n = 34$) and one *A. modesta* female. The AGs were dissected from adult females in 0.9% NaCl and blotted dry on a piece of aluminium foil with a camel hair brush before being transferred to hexane and crushed with a flamed glass pipette. The UV/visible spectrum was obtained for a pooled *A. carbonifera* extract on an HP 8453 spectrophotometer (Agilent Technologies, Santa Clara, California, U.S.A.). Thin-layer chromatography was conducted on silica gel plates (2.5 × 7.5 cm, 200 um, F-254, 60A, Selecto Scientific Inc., Suwanee, Georgia, U.S.A.) and developed with a 3 : 1 : 1 ratio of petroleum ether, acetone, and chloroform. All the plates were scanned with the same flatbed scanner under identical conditions and the optical densities (ODs) of 17 spots determined using ImageJ (NIH) and the methods described in Valverde *et al*. (2007). Optical densities were converted to nanograms using a β,β-carotene (22040, Sigma-Aldrich, St. Louis, Missouri, U.S.A.) standard curve ($F_{1,5} = 236.4$, $r^2 = 0.98$, $P << 0.001$). The ODs measured were all within the linear range of the standard curve. Multivariate analysis of variance (MANOVA) and canonical discriminant analysis (CDA) was used to analyse the carotenoid profiles. Carotenoids c3, c5, c7, and c8 were strongly correlated; therefore, we used their mean (i.e. carotenoid, CA) in the MANOVA. Ninety-five per cent confidence ellipses of the mean CDA scores were calculated for each morph (crescents, $n = 6$; cushions, $n = 11$; flats, $n = 6$; and irregulars, $n = 11$) using the equations in Owen and Chmielewski (1985). The SGs of *A. carbonifera* larvae also contain orange pigments (Fig. 6b) for which a UV/visible spectrum was also obtained as above.

Associating carotenoid quantity and parasitoid attack

Midges attacked by *P. solidaginis* do not survive to the adult stage where the AG carotenoids can be measured. Therefore, to assess the association between *P. solidaginis* attack and AG carotenoid quantity of each morph, we regressed the mean total carotenoids by morph on the mean Pearson residual of *Platygaster* attack (i.e. the results from Fig. 4). To address concerns about the reduction in variation that taking the means might have on the analysis, we conducted both a conventional linear regression and a randomisation test. The randomisation was based on drawing four random samples from each normal distribution with the mean and SD estimated from our data and calculating an r^2 value 10 000 times. A histogram of these values was generated and the probability of obtaining an r^2 greater or equal to the one obtained by conventional analysis was calculated. A two-way ANOVA with interaction was also conducted to assess the association between total AG carotenoids by gall morph and the galls' relative height at maturity on the plant. Analyses were conducted in R (R Development Core Team, 2010).

Determining the presence of ABA in the SGs

In plants, ABA is formed from the oxidative cleavage of carotenoids and is a potent hormone controlling the size of the stomatal aperture. ABA is also up-regulated during periods of drought and is known to negatively interact with biotic stress signalling. In the absence of the midge larva, the mutualistic fungus cannot grow (Heath & Stireman, 2010); therefore, it is likely that the larva somehow manipulates plant chemistry to allow fungal growth. Based on the presence of carotenoids in the SGs, we tested the hypothesis that ABA is present in the SGs of these midges using two independent methods: a stomatal aperture bioassay and an enzyme-linked immunosorbent assay (ELISA).

The methods of Tucker and Mansfield (1971) and Ogunkanmi *et al.* (1973) were modified slightly for the stomatal aperture assay using *Tradescantia zebrina* Heynh. ex Bosse (Commelinaceae). This method is highly sensitive to ABA, unaffected by the presence of other plant hormones, and reproducible. Young galls of unknown morph were collected

from the field and the SGs dissected from the larvae under a stereomicroscope in 10-mM citrate buffer, pH = 5.5. Epidermal peels were taken in the morning from the bottom of dark-adapted leaves, divided into four equally sized pieces (*c*. 0.5 mm^2), and floated on 4 ml of SG extract in citrate buffer. Extracts were prepared by crushing an aliquot of SGs with a plastic pestle in an Eppendorf tube of citrate buffer. The tube was centrifuged and the supernatant drawn off and serially diluted. Four concentrations were tested: 0, 0.1, 1, and 10 midge equivalents per 4 ml of citrate buffer. Each midge larva contains a pair of SGs; therefore, one midge-equivalent (meq) is equal to two glands. After the peels were prepared the vials were incubated in a glass-pan water bath (24–26°C) over a bank of fluorescent lights (PAR, 80 µmols photons m^{-2} s^{-1}) for 2–3 h. Throughout the incubation time, each vial was bubbled with a stream of CO_2-free air created by filtering room air through two 1-l Erlenmeyer flasks each with a saturated aqueous solution of $Ca(OH)_2$ (i.e. 2 g l^{-1} pickling lime). Each replication of the concentration series ($n = 8$ per concentration) was tested on peels obtained from the same leaf at the same time. A mixed-effects model with replication treated as a random effect was used to analyse the stomatal response. The stomatal response was measured as the mean stomatal aperture of a random sample (*c*. $n = 30$) of stomata on the peels from each concentration. Analysis was conducted in R (R Development Core Team, 2010).

The ELISA kit (PDK 09347/0096, Agdia, Inc., Elkhart, Indiana, U.S.A.) was also used to directly assay the quantity of ABA in SG extracts. Salivary glands were dissected in 0.9% aqueous NaCl (with 0.2% Tween 20) and extracts were prepared as above in 0.5 ml of an 8 : 2 ratio of methanol/water (v/v). The manufacturer's instructions were subsequently followed. A linear model generated in R (R Development Core Team, 2010) was used to examine the relationship between the number of SGs in the extracts and the amount of (+)ABA measured by the ELISA. Only the flat morph was analysed because only this morph had levels of (+)ABA significantly above the detection limit.

Results

Ovipositional phenotype

The intercept estimates (Fig. 1; crescent, 0.58 ± 0.01; cushion, 0.93 ± 0.02; flat, 0.88 ± 0.03; and irregular, 0.93 ± 0.03; intercept ± SE) indicated that crescents develop lower on the plant than the other three morphs: the main effect of morph was highly significant in a two-way ANOVA ($F_{3,2276} = 618$, $P << 0.001$) as was the covariate, relative gall diameter ($F_{1,2276} = 263$, $P << 0.001$). Because there was a significant interaction between morph and gall diameter ($F_{3,2276} = 4.63$, $P = 0.003$), we were suspicious about whether the differences were the result of delayed larval hatching or an ovipositional phenotype. However, the manipulative experiment indicated that only crescent morphs oviposited on plants with screened young leaves, whereas all gall morphs oviposited on unprotected plants (Fig. 2).

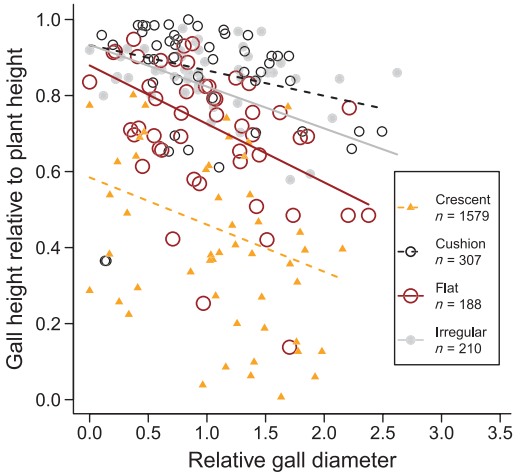

Fig. 1. Relative gall height on the plant at gall discovery as a function of relative gall diameter. Only 50 randomly selected points are plotted per morph to avoid cluttering the plot, but the lines are fit to all the data (i.e. flat $n = 188$, cushion $n = 307$, irregular $n = 210$, and crescent $n = 1579$). The intercept of each of the lines represents an estimate of the height of oviposition. See online colour version.

Parasitoid attack

Among the mortality factors for crescents, *P. solidaginis* was the most significant in logistic regressions of these variables over the height of the plant (Fig. 3; deviance = 38.8; d.f. = 1, 1370; $P << 0.001$). For crescent galls located high on the plant the probability of *Platygaster* attack was nearly 40% (Fig. 3a). Importantly, across the morphs and sites, *P. solidaginis* consistently attacked the crescent morph more than expected by chance (Fig. 4) and the χ^2 analyses were significant for every site (BCW, $\chi^2 = 16.0$, d.f. = 3, $P = 0.001$; SSP, $\chi^2 = 11.5$, d.f. = 3, $P = 0.009$; GMP, $\chi^2 = 26.5$, d.f. = 3, $P << 0.001$; WSU, $\chi^2 = 19.0$, d.f. = 3, $P < 0.001$).

Egg transplants and crescent failure rates

Crescent morphs failed slightly more often when they developed on mature leaves (Fig. 3f), but this result only approached significance (deviance = 3.4; d.f. = 1, 1525; $P = 0.06$). However, when other gall morphs were forced to develop on mature leaves they failed to produce full size galls as a whole (Fig. 5, $F_{5,179} = 15.6$; d.f. = $P << 0.001$) and for each gall morph individually (cushion-young vs. cushion-mature, $P << 0.001$; flat-young vs. flat-mature, $P << 0.001$; and irregular-young vs. irregular-mature, $P = 0.035$). Likewise, these three morphs exhibited low survival to pupae or adults on mature tissue (≤8%, deviance = 28.2, d.f. = 1, 179; $P << 0.001$), but there was no effect of morph (deviance = 3.47, d.f. = 2, 179, $P = 0.178$) or the interaction of morph and tissue age on their survival probability (deviance = 0.06, d.f. = 2, 179, $P = 0.972$).

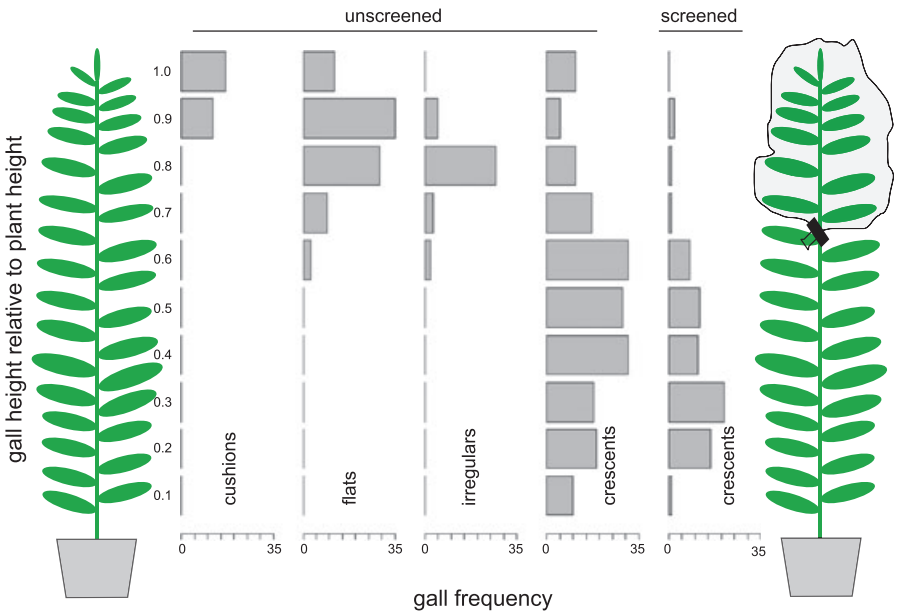

Fig. 2. Histograms of gall morphs on *Solidago altissima* plants with either the top 20 cm of foliage protected with fine-mesh screening ('screened') or not ('unscreened'). Under protected conditions the histograms for the flat, cushion, and irregular morphs are not shown because there were absolutely no galls of these morphs after 2 months in the field.

In the field, these three gall morphs tend to be multilocular, but in this experiment only a single egg was used to initiate galls. As predicted, the morphs still differed in their mature sizes; cushions and flats were larger than irregulars (Fig. 5, $P < 0.001$) and flats were larger than cushions (Fig. 5, $P = 0.009$).

Carotenoid profiles

The orange pigments found in the AGs and SGs (Fig. 6) of adult female and larval midges were non-polar pigments readily extractable in hexane. The UV/visible spectra of a sample of each in hexane (Fig. 6d, AGs: I = 430 nm, II = 450 nm, III = 478 nm, %III/II = 27; SGs: I = 435 nm, II = 458 nm, III = 485 nm, %III/II = 17) bears a strong resemblance to typical carotenoids, including β, β-carotene in hexane (I = 425 nm, II = 450 nm, III = 477 nm, %III/II = 25). Multivariate analysis of 17 scored carotenes and xanthophylls indicate that the quality and quantity of the AG carotenoids differs consistently and significantly by gall morph (Fig. 7 and Table 2; approximate $F = 48.7$; d.f. = 3, 30; Roy = 35.9; num. d.f. = 14; den. d.f. = 19; $P << 0.001$) regardless of the test employed (i.e. Roy, Pillai, Wilks or Hotelling–Lawley).

Relationship between carotenoids and Platygaster attack

In a linear model, the mean total amount of carotenoids in the AGs of each of the morphs significantly predicted their mean susceptibility to *P. solidaginis* (Fig. 8, $F_{1,2} = 26.6$, $r^2 = 0.93$, $P = 0.036$). Furthermore, the interaction term in a linear regression of total AG carotenoids indicated that only the crescent morph carotenoids increase with their relative height on the plant (Fig. 9, $F_{3,15} = 4.41$, $P = 0.021$). Because the slopes of the other morphs were not significantly different from zero, only a single linear model for the crescent morph is presented ($F_{1,2} = 29.1$, $r^2 = 0.94$, $P = 0.033$). Therefore, a positive association of AG carotenoid quantity and *Platygaster* attack exist both within (compare Figs 3a and 9) and across morphs (Fig. 8).

ABA in the SGs

Two independent experiments confirmed the presence of ABA in the SGs of these midges. As expected, increasing the quantity of glands extracted caused a concomitant effect in the response of both the stomatal bioassay and the ELISA assay. In the ELISA assay, only the flat morphs were analysed because only they showed values above the detection threshold (Fig. 10, $F_{1,8} = 14.0$, $r^2 = 0.64$, $P = 0.006$). In the stomatal bioassay (data not shown), stomatal aperture decreased with increasingly concentrated SG extracts ($F_{1,23} = 9.71$, $P = 0.005$).

Discussion

Two independent experiments indicate that crescent morphs of the gall midge, *A. carbonifera* oviposit and develop on mature leaves of *S. altissima*, whereas three other sympatric and syntopic morphs do not. Furthermore, when the others are forced to develop on mature tissue they perform poorly. The ability of the crescents to initiate and develop galls on mature tissue is unusual among gall midges and gall-forming

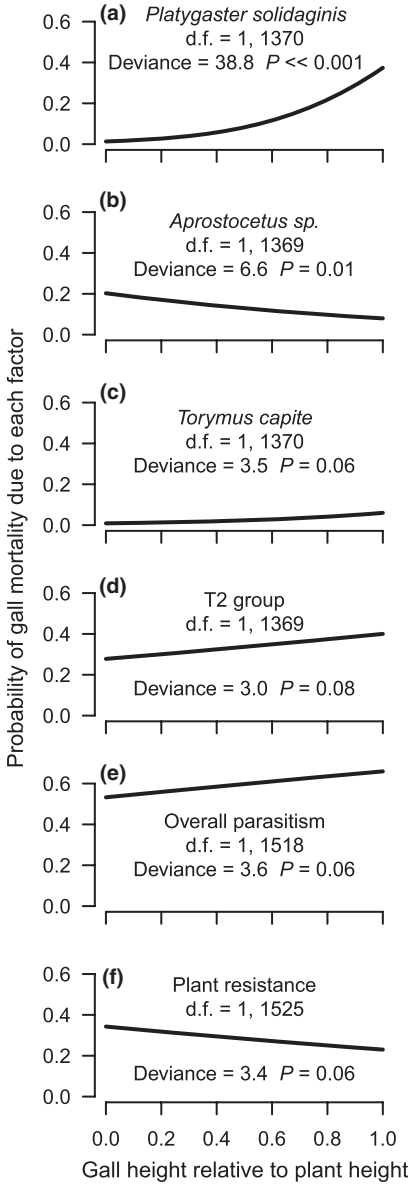

Fig. 3. Crescent-gall death probabilities over the height of the plant for different mortality factors (a) parasitism by P. solidaginis, (b) Aprostocetus sp., (c) Torymus capite, or the (d) T2 group, (e) overall parasitism, (f) plant resistance. The 'T2' parasitoids are now known to represent four different parasitoid species: *Aprostocetus tesserus*, *Aprostocetus homeri*, *Baryscapus fumipennis*, and *Closterocerus solidaginis*. Plant resistance was determined by the obvious presence of the hypersensitive response by the plant, which resulted in dead galls soon after they were discovered (see Heath & Stireman, 2010).

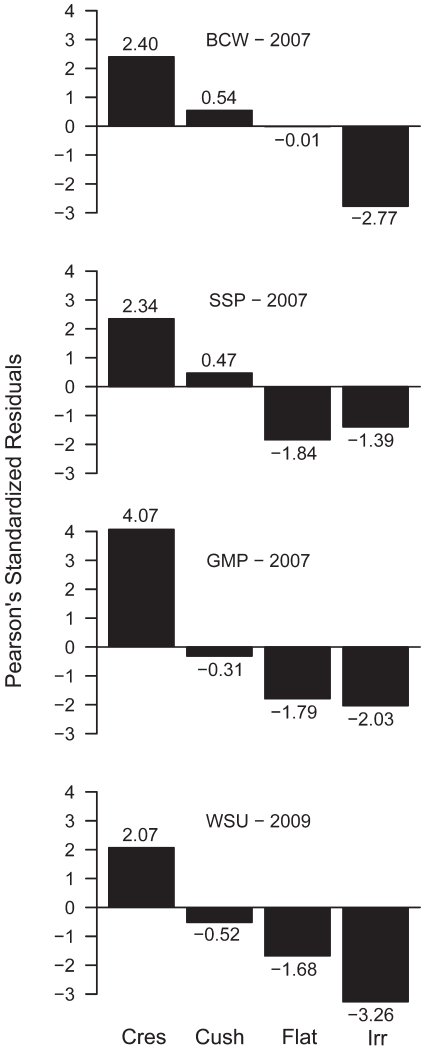

Fig. 4. Pearson's standardised residuals from a χ^2 analysis showing deviations from the expected value under a random *Platygaster solidaginis* attack scenario for four different sites around Ohio, U.S.A. See Table 1 for site information.

taxa in general. It is well known that galling insects generally prefer younger, less differentiated, actively growing tissue (Espirito-Santo *et al*., 2007). Presumably, young tissues are more amenable to physiological manipulation by the galler. As stated previously, *A. carbonifera* galls are not formed by deformations of plant tissue, but are rather owing to a controlled growth of their fungal symbiont (Heath & Stireman, 2010). This strategy of building the gall structure from fungus may release these midges from the physiological constraints associated with forming typical plant galls. Although the other three morphs performed poorly on mature tissue, some individuals did successfully pupate and emerge from galls initiated there. This suggests that the evolutionary lability of ovipositional behaviour may differ across lineages. Field observations have suggested that other members of the genus *Asteromyia* may also have the capacity to develop on mature tissue; that is, this ability may have evolved several times or been shared by a common ancestor.

Among the selective forces that might maintain this ovipositional phenotype, *P. solidaginis* attack was the most significant. That *P. solidaginis* parasitism is a potent selective force maintaining this phenotype is evident in the fact that crescents continue to oviposit low on the plant even though the plant is more resistant to them there. Singer and Stireman

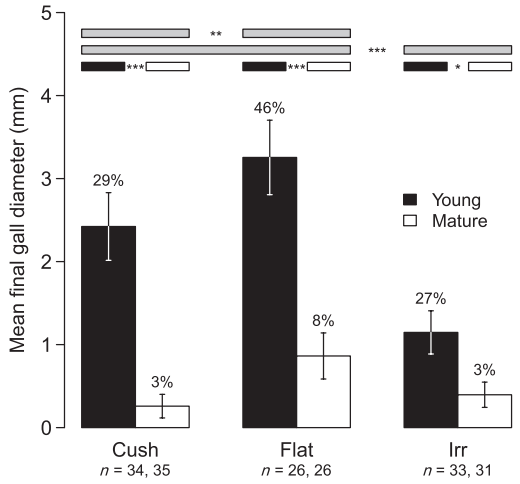

Fig. 5. Results of *Asteromyia carbonifera* egg transplants from field *Solidago altissima* plants to greenhouse grown *S. altissima*. The numbers above the bars are the per cent survival to either healthy pupae or adults. Crescent eggs were not included in this experiment because they are extremely difficult to find, but estimates of their survival on mature and young tissue can be found in Fig. 3f (i.e. 65–75%, respectively). Asterisks correspond to the level of significance in planned orthogonal decomposition of the final gall diameters (***$P < 0.001$; **$P < 0.01$, *$P < 0.05$). See the text for results of logistic regression of the per cent survival to healthy pupae or adults.

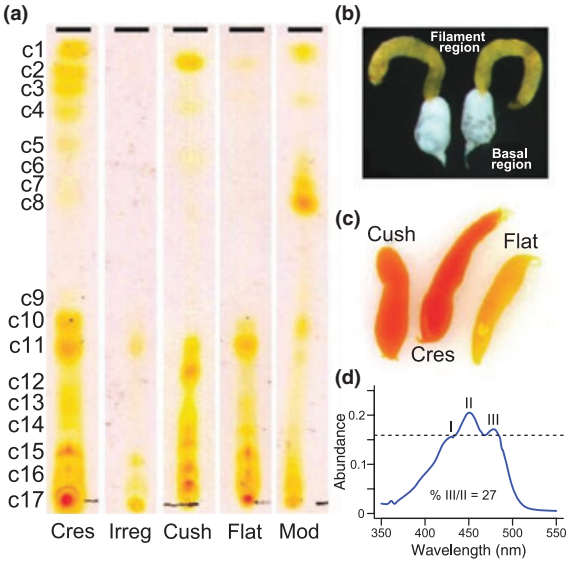

Fig. 6. (a) Representative thin-layer chromatography plates of the carotenoids in the accessory glands of each of the *Asteromyia carbonifera* morphs and one *A. modesta* (Mod). Carotenoid numbers indicate and correspond to the labels in Fig. 7. The red colour is a result of equal contrast image enhancement: all spots were uniformly orange in the original scans. (b) A pair of *A. carbonifera* larval salivary glands showing carotenoids in the filament region only. (c) Three adult female accessory glands from three different morphotypes. (d) UV/Vis spectrum of a pooled sample of *A. carbonifera* accessory glands in hexane. See online colour version.

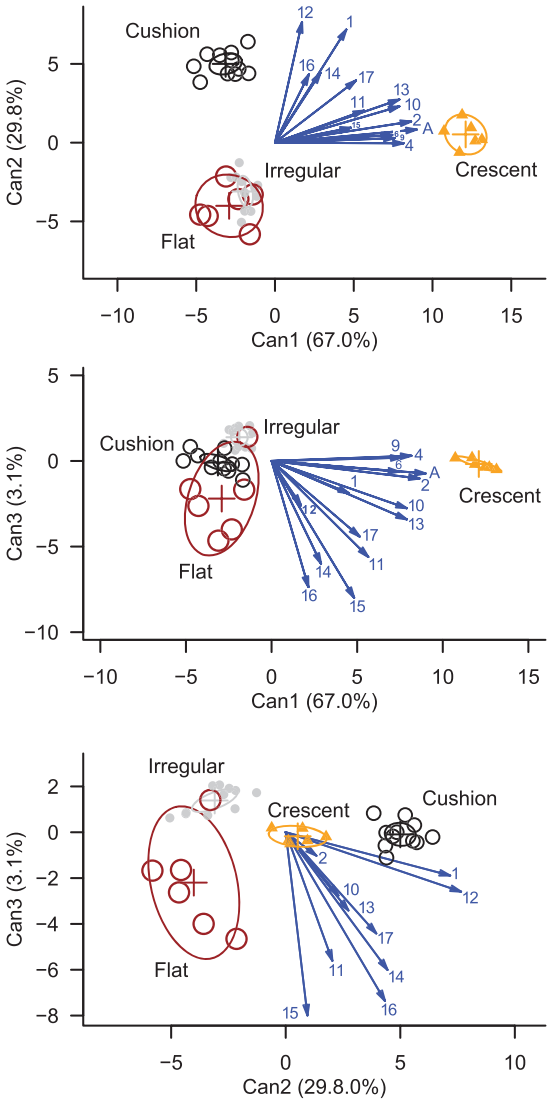

Fig. 7. Canonical discriminant ordination of the carotenoids found in the accessory glands of adult female *Asteromyia carbonifera* morphs. The 95% confidence ellipses on the means indicate significant differences in carotenoid quality and quantity. The vectors indicate the direction of increase with respect to each carotenoid (c1–c17) or strongly correlated group of carotenoids (cA = mean of c3, c5, c7, and c8). See online colour version.

(2005) have proposed the tritrophic niche concept, which predicts that insects diverging with respect to enemy-free space will illustrate exactly such a pattern. That is, natural selection will tolerate lower performance with respect to plant resistance in order to realise a net fitness benefit associated with enemy-free space. Given this theory, we would predict that the ancestor of crescents oviposited exclusively on newly emerging leaves and that the abilities to oviposit and develop on mature tissue are derived traits.

We found that AG carotenoids varied qualitatively and quantitatively across gall morphs. Given that flat, irregular, and cushion morphs culture their symbiotic fungus on the same host

Table 2. Results of individual ANOVAS on the effect of morph on accessory-gland carotenoid quantity and quality.

	Mean (SE) carotenoid quantity by morph (ng)				ANOVA statistics	
ID	Crescent	Cushion	Flat	Irregular	F	*P*
c1	163 (31)	132 (16)	21 (12)	6 (2)	24.1	<<0.001
c2	193 (43)	16 (3)	4 (2)	0 (0)	30.1	<<0.001
c3	175 (40)	5 (2)	0 (0)	1 (1)	30.9	<<0.001
c4	157 (34)	0 (0)	0 (0)	16 (16)	18.6	<<0.001
c5	58 (10)	3 (2)	1 (1)	0 (0)	44.2	<<0.001
c6	15 (6)	0 (0)	0 (0)	0 (0)	12.0	<0.001
c7	22 (7)	0 (0)	0 (0)	0 (0)	16.6	<<0.001
c8	53 (7)	1 (1)	0 (0)	2 (2)	62.5	<<0.001
c9	29 (8)	1 (1)	0 (0)	3 (3)	12.8	<0.001
c10	352 (69)	92 (10)	74 (37)	11 (4)	23.9	<<0.001
c11	723 (144)	349 (24)	440 (124)	108 (33)	12.1	<0.001
c12	123 (21)	153 (13)	49 (22)	20 (14)	16.5	<<0.001
c13	236 (24)	81 (7)	69 (32)	19 (9)	30.1	<<0.001
c14	125 (20)	107 (14)	94 (30)	17 (7)	9.7	<0.001
c15	564 (75)	282 (20)	488 (114)	84 (23)	17.5	<<0.001
c16	468 (77)	443 (31)	444 (118)	87 (25)	12.8	<0.001
c17*	905 (218)	558 (68)	448 (81)	137 (25)	11.1	<0.001

∗Carotenoid c17 is the thin layer chromatography origin.
All degrees of freedom are the same (d.f. = 3, 30).

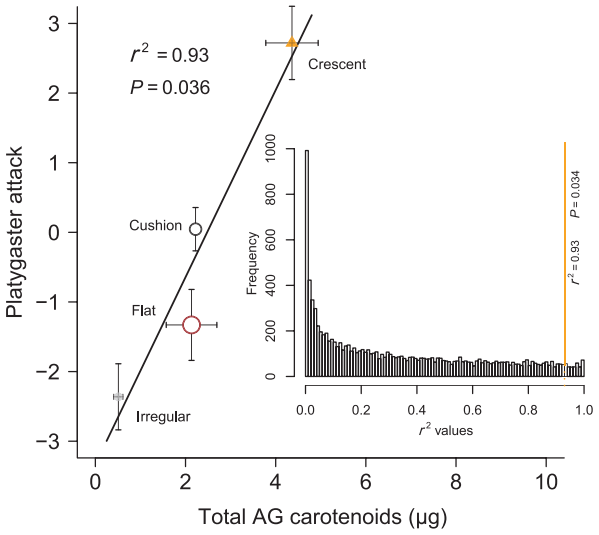

Fig. 8. The association between mean total carotenoid content of the accessory glands of each morph and the preference of *Platygaster solidaginis* for that morph (preference taken from Fig. 4). Error bars around the points represent ± 1 SEM. See online colour version.

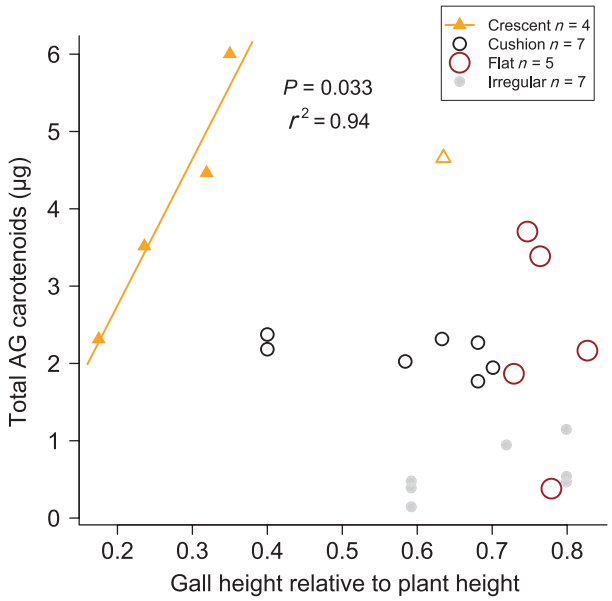

Fig. 9. The relationship between total accessory-gland carotenoids and the relative height of the gall on the plant at maturity. Note that gall heights will appear lower on average compared with those in Fig. 1 because these were measured when the gall was mature, whereas Fig. 1 galls were measured at the time of discovery. One crescent (open triangle) was not included in the regression because it was identified as a strong outlier according to Cook's distance and leverage estimates. See online colour version.

species and tissue (i.e. young leaves), the observed variation in carotenoid composition cannot be explained simply by host carotenoids. We suspect that this variation is functionally related to gall morphology, but we currently lack a complete mechanistic hypothesis for how this might occur. Carotenoids in the AGs of flats and irregulars were qualitatively similar. Indeed, natural variability in the morphology of these two morphs can also make them sometimes difficult to distinguish. However, only unambiguous morphs were used in this analysis making it unlikely that morph was incorrectly assigned. If AG carotenoid quality and quantity is genetically determined, this similarity may suggest a close relationship or some level of gene flow between these morphs. Indeed, amplified fragment length polymorphism analysis of these four morphs suggests

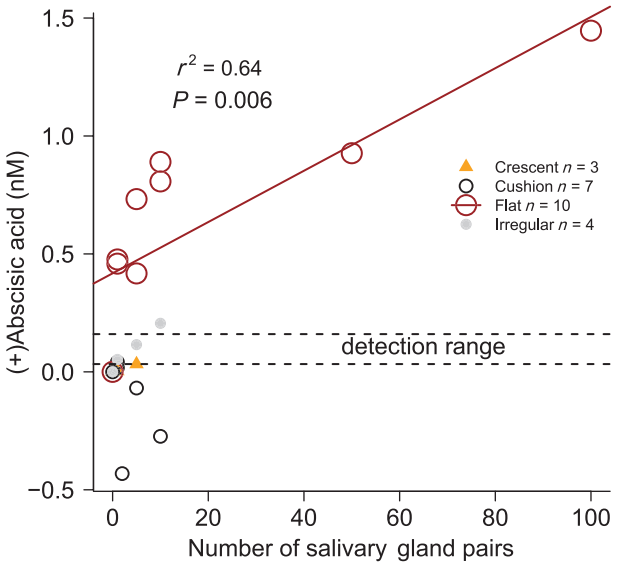

Fig. 10. The concentration (nM) of abscissic acid (ABA) in the salivary-gland extracts of the four morphs of *Asteromyia carbonifera*. Only the flat morph was analysed because only it showed levels significantly above the detection threshold. See online colour version.

possible gene flow between the irregular and flat morphs (Stireman *et al*., 2010).

Across gall morphs, the total quantity of carotenoids was positively associated with *Platygaster* attack. This association was also apparent within the crescent morph as crescents developing higher on the plant had more AG carotenoids and a higher probability of *Platygaster* attack. The source of carotenoids in these midges is not known, but carotenoids do increase in *S. altissima* plant tissue with plant height (J. J. Heath, unpublished), suggesting that crescents sequester carotenoids in proportion to the concentration in plant tissue. While the within-morph association may be explained by factors that affect both carotenoid profiles and risk of parasitism (e.g. younger plant tissues), the relationship between parasitism and carotenoids across gall morphs suggests a possible causal link. Carotenoids could directly influence parasitoid behaviour as precursors to attractive volatile apocarotenoids (Zorn *et al*., 2003; Lewinsohn *et al*., 2005), illustrating the potential complexity of tritrophic interactions in this system.

The presence of ABA in the SGs of the flat morphs was confirmed in this study and its localization in the SGs suggests that it is secreted into plant cells during the early stages of gall development, possibly inhibiting induced plant defences. Furthermore, the growth of some pathogenic fungi is enhanced by the topical application of ABA (Kettner & Dorffling, 1995). While ABA is involved in the induction of some defences, ABA can also negatively affect the induction of other defences through inhibitory pathway interactions (Anderson *et al*., 2004). This suggests that ABA in the SGs of *A. carbonifera* may be used to manipulate plant physiology to enhance the growth of its symbiotic fungus.

Increased carotenoid production or sequestration may provide crescent morphs a key innovation that allows them to attack unutilised mature tissue and gain enemy-free space, but it also has the potential to increase their apparency to *P. solidaginis* when they develop on younger tissues. The mechanism underlying this innovation is still under investigation, but the presence of ABA and possibly other carotenoid-derived hormones in the larval SGs is worthy of further investigation. Regardless of whether carotenoids provide a key innovation, enemy-free space is probably maintaining the crescent ovipositional phenotype.

Although gall morphology itself may be adaptive (Stone & Schonrogge, 2003) and we are currently preparing work on its significance, behavioural phenotypes also appear to be important in this system as in other adaptive radiations such as orb-weaving spiders, *Anolis* lizards, and possibly Hawaiian *Drosophila* (West-Eberhard, 2003, p. 573; Blackledge & Gillespie, 2004; Johnson *et al*., 2010). We have examined a coarse ovipositional phenotype here, but crescents also oviposit on the edge of the leaf whereas the other morphs are laid on the interior and Weis *et al*. (1983) have shown that the degree of loculation, determined proximately by oviposition behaviour, affects parasitism rates in *A. carbonifera*. Furthermore, larval feeding behaviour within the gall may influence gall morphology and parasitism risk. It is not only midge behaviour, but also parasitoid behaviour that may be important in maintenance of these cryptic species (Weis, 1982a,b). However, forthcoming analyses of the adaptive value of gall morphology are required before the relative importance of behavioural versus morphological traits can be adequately assessed.

Conclusions

In this study, we have assessed the fitness value of a behavioural (i.e. ovipositional) phenotype as well as its association with gall morphology and glandular carotenoid quality and quantity in an incipient radiation of gall midges. This ovipositional phenotype may represent an early diverging trait in the radiation of the *A. carbonifera* clade. Documenting this ovipositional phenotype within the radiation of *Asteromyia* and subsequently illustrating its utility in terms of a fitness advantage satisfies two of Schluter's (2000) requirements for an adaptive radiation, whereas Stireman *et al*. (2010) have provided evidence of common ancestry and the rapidity of the radiation. Understanding and describing these phenotypes provides the experimental framework for testing for similar ovipositional and carotenoid phenotypes in other clades of *Asteromyia* on different host plants where anecdotal evidence suggests they also occur (see Stireman *et al*., 2010). In addition, this study provides the preliminary evidence for future manipulative experiments that will directly test putative causal links between carotenoids and parasitism, carotenoids and gall development, and carotenoids and gall morphology. It may be that many of the major interactions in this system are mediated, at least in part, by carotenoids and their derivatives.

We illustrate that behavioural divergence can be favoured by enemy-free space even when an organism may be physiologically maladapted to the new environment. The

net benefit of escaping natural enemies may allow natural selection to mould a physiological adapted phenotype in the new environment, which may have pleotropic effects that further drive a wedge between diverging lineages. Conversely, behavioural inflexibility may constrain natural selection and hinder the colonisation of new niches.

Acknowledgements

Seth Jenkins, Diego Javier Inclan Luna, Hilary Devlin, and Patrick McAfee helped transplant the goldenrod accessions to the field. Andrew J. Kroft assisted with carotenoid analysis and field work. Tom Rooney and Thad Tarpey provided helpful advice with respect to randomization testing and canonical discriminant analysis, respectively. Jennifer L. Heath facilitated the field and laboratory studies and provided helpful comments on the manuscript. The comments and suggestions of two anonymous reviewers were also helpful. This research was supported by an NSF grant DEB-0614433 to J.O.S. and the Wright State University Environmental Science PhD program.

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Accepted 14 September 2012
First published online 21 November 2012