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Adaptation to different host plant ages facilitates insect divergence without a host shift

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Host shifts and subsequent adaption to novel host plants are important drivers of speciation among phytophagous insects. However, there is considerably less evidence for host plant-mediated speciation in the absence of a host shift. Here, we investigated divergence of two sympatric sister elm leaf beetles, *Pyrrhalta maculicollis* and *P. aenescens*, which feed on different age classes of the elm *Ulmus pumila* L. (seedling versus adult trees). Using a field survey coupled with preference and performance trials, we show that these beetle species are highly divergent in both feeding and oviposition preference and specialize on either seedling or adult stages of their host plant. An experiment using artificial leaf discs painted with leaf surface wax extracts showed that host plant chemistry is a critical element that shapes preference. Specialization appears to be driven by adaptive divergence as there was also evidence of divergent selection; beetles had significantly higher survival and fecundity when reared on their natal host plant age class. Together, the results identify the first probable example of divergence induced by host plant age, thus extending how phytophagous insects might diversify in the absence of host shifts.

1. Introduction

Phytophagous insects are a highly diverse group and have long been recognized as outstanding models for studying the process of speciation [1–5]. One likely driver of diversification among phytophagous insects is their tendency to specialize on an array of host plant species [6–11]. Speciation commonly occurs via host shifts when an insect incorporates a new host into its diet. Subsequent host-associated adaptive divergence creates specialist host races or species that experience reduced gene flow [2,4,12–20]. Ecological speciation is commonly involved in the process of host specialization as ecologically divergent selection drives the evolution of barriers to gene flow between insects feeding on alternative hosts [3,21].

Although host-associated divergence is clearly an important mechanism in insect speciation, about half of phytophagous insect speciation events occur without a host shift [22]. For some phytophagous insect groups, allopatric divergence in the absence of host shifts may be a major factor causing insect radiations [23,24]. In addition, ecological processes other than host shifts also appear to have promoted divergence in the absence of a host shift. For example, divergence can be caused by within-plant species shifts in the feeding niche. Sympatric species of *Blepharoneura* flies have specialized to feed on a single sex of a host plant [25], and some gall-inducing insects have diverged by feeding on different plant organs [15,26] or by subdividing a single plant organ [27]. Thus far, however, we have few examples of within-host species divergence, probably because most work in phytophagous insects has appropriately focused on insect lineages where divergence is strongly associated with host

shifts [23]. Given the probable number of undescribed, cryptic insect species [28], a worthwhile goal is to assess the relative importance of within-host species divergence, between-host species sympatric divergence and allopatric speciation in phytophagous insects.

To this end, we examined divergence between the elm leaf beetles *Pyrrhalta aenescens* and *P. maculicollis* (Insecta: Coleoptera: Chrysomelidae). *Pyrrhalta aenescens* and *P. maculicollis* are serious pests of elm trees in China, including *Ulmus pumila*, *U. laevis* and *U. davidiana* [29,30]. Adults and larvae feed on elm foliage, and severe infestations can cause mortality of host trees. The beetles are distributed sympatrically over a large geographical area; *P. maculicollis* is found in China, Japan, Korea and Russia while *P. aenescens* is mainly restricted to northern China [29]. Phylogenetic analysis of mitochondrial and nuclear sequence data indicate that *P. aenescens* and *P. maculicollis* are reciprocally monophyletic and strongly support their placement as sister taxa [29]. Complete overlap in host species use coupled with a synchronized emergence phenology begs the question of how species boundaries formed between these closely related beetles. Casual field observations suggested that *P. aenescens* and *P. maculicollis* subdivide the host niche space within populations by feeding on different life-history stages of the plants (B.Z. 2013, personal observation). *Pyrrhalta aenescens* was observed to feed on adult elm trees, whereas *P. maculicollis* was found primarily on seedlings. Few beetles of either species feed on the intermediate age classes (B.Z. 2013, personal observation). Interestingly, strong sexual isolation between these species is driven by divergence in insect cuticular hydrocarbons (CHCs) [31]. Molecular phylogenetic analysis, and subtle differences in morphology further support this idea of divergence [29].

One possible explanation, then, is that evolutionary divergence between *P. aenescens* and *P. maculicollis* was driven by selection to use different age classes of *U. pumila*. In order for this to be the case, the beetles must be able to recognize host age via long-distance and/or contact signals. Host plant selection is to a large extent guided by phytochemistry [32,33], where volatile chemicals can strongly affect host plant recognition over long distances [34–36] and contact compounds such as plant surface waxes can play an important role in host choice once the insect has come into direct contact with the plant tissue surface [32,33,37–39]. Although other chemicals may also affect host plant choice [40,41], cuticular waxes are the primary chemicals encountered and have been shown to be critical in directing insect behaviour and development [33]. Since chemical composition of epicuticular waxes can vary with plant species [33], plant part [42], plant age [43] and differing environmental conditions [44], surface waxes may be especially important in determining host choice.

We conducted a series of field surveys and laboratory experiments to assess divergence between *P. aenescens* and *P. maculicollis*. Specifically, we tested whether *P. aenescens* and *P. maculicollis* have non-random distributions among host plant ages in the field. These observations were followed with laboratory tests of preference for and performance on plant tissue from adult trees and seedlings. Finally, we compared leaf surface wax chemical profiles of adult trees and seedlings. We tested whether observed differences in leaf surface wax chemistry are critical for shaping beetle preference by testing preference for artificial leaves coated with leaf wax extracts.

2. Material and methods

To examine whether there is divergence associated with host plant age, we conducted field surveys in sympatric populations of *P. aenescens* and *P. maculicollis* and also feeding and oviposition preference trials in the laboratory. Because most individuals of both species tended to avoid trees of intermediate age class (3–5-year-old trees) in nature, we focused on seedling and adult trees. Here, we define adult trees as elms of 5 or more years in age and diameter at breast height greater than 10 cm, whereas seedlings are trees under 2 years of age that lack a distinct trunk.

(a) Field surveys

To assess whether the spatial distribution of *P. aenescens* and *P. maculicollis* on adult trees and seedlings was consistent across sites, we conducted field surveys at three locations in Beijing (Olympic Park, Chaoyang, 40.01° N, 116.39° E; Shanyangwa, Changping, 40.28° N, 116.05° E; Fadianzhan, Changping, 40.12° N, 115.98° E). These sites encompass an area where the two sister species are sympatric and occur on the same host species, *Ulmus pumila* L., which is the most abundant in nature [30]. Thus, there is ample opportunity for *P. aenescens* and *P. maculicollis* to use both adult trees and seedlings at these sites. Within each site, we haphazardly identified 10 sampling locations separated by at least 20 m that contained an adult tree and one to three seedlings growing less than 5 m from the adult tree. Per site and survey time, we counted the number of *P. aenescens* and *P. maculicollis* at each life-history stage (larva, pupa and adult) on adult trees and seedlings separately. Adult beetles were collected using sweep nets, and pupae and larvae were collected by hand. The surveys were conducted on a haphazardly selected subset of trees within each site from early June to mid-July, 2013 and 2014 (four to eight times per site), corresponding to the active season for these beetle species. We recorded the number of *P. aenescens* and *P. maculicollis* on adult trees and seedlings, and determined the relative proportion of life stages occupying adult trees versus seedlings. Larval identification followed Nie *et al.* [29]. Pupal identification methods were developed by allowing pupae to mature for at least 1 day, scoring them for colour, and then using species keys to identify the eclosed adults. By doing so, we were able to correlate pupal colour with species identification of adults. Mature pupae of *P. aenescens* are dark brown while pupae of *P. maculicollis* are bright yellow (electronic supplementary material, figure S1). We used a general linear model (GLM) to assess whether beetle species, site and beetle life stage affected the proportion of either beetle species found on adult trees or seedlings. All statistical tests were conducted using SPSS Statistics v. 19.0 (SPSS, Inc., Chicago, IL, USA).

(b) Preference

To experimentally confirm the observed divergence in beetle distribution between adult trees and seedlings, two laboratory experiments were designed to determine feeding and oviposition preference of *P. aenescens* or *P. maculicollis* for leaves of adult trees versus seedlings. The beetles used in these bioassays were collected from the northern section of Olympic Park. We collected third instar larvae or pupae that were then reared to adulthood in plastic cups (9.0 cm diameter, 9.0 cm deep) and placed in a climate box at 16 L : 8 D cycle at 25°C. Fresh leaves from actively growing branches were clipped from adult trees and seedlings, immediately placed into sealable plastic bags (450 × 340 mm) and refrigerated no longer than 2 days before the choice trials.

We first conducted feeding preference trials using newly emerged, naive adults. Two leaf discs (8 mm diameter) of each

elm age class were placed symmetrically around the edge of a 9 cm diameter Petri dish lined with moist filter paper. One male–female pair of adult beetles was placed at the centre of the Petri dish and then held at 25°C in a dark climate box. Ten replicates were conducted for each species [45]. The beetles were allowed to feed for 24 h before we determined the area consumed from each disc using calibrated graphing paper [46]. Preference for tree age class was evaluated separately for each beetle species by comparing the relative proportion of consumed leaf disc area per Petri dish trial.

Oviposition preference was assessed in choice behaviour trials carried out in glass jars (11.5 cm tall by 12 cm diameter) containing two approximately equal-sized branches with leaves, one branch originating from an adult tree and the other from a seedling. Five male–female pairs of newly emerged, naive beetles were placed together into the test arena and allowed to mate and oviposit. In order to keep leaves fresh, the test branches were inserted into small floral tubes (5 cm tall by 1 cm diameter) containing water and covered with a ball of cotton. The branches were replaced every other day, and each time they were replaced, the eggs were counted. Ten and 12 replicates (each replicate containing five male–female pairs) were conducted for *P. aenescens* and *P. maculicollis*, respectively, and each trial lasted 20 days, as this time period allows the adult females to mature (approx. 6 days after emergence) and offers the opportunity to sample eggs over two weeks. We determined the cumulative number of eggs oviposited on each elm age class. Oviposition preference was evaluated by comparing the proportion of eggs placed on adult versus seedling branches.

We used paired *t*-tests to assess whether the proportion of leaf disc consumed and oviposition preference differed between tree age classes for each species. The test arenas represented the experimental unit, irrespective of the number of beetles tested in each trial. Percentage data were normalized with logit transformations prior to analysis [47].

(c) Performance

To determine whether there is a relationship between preference and performance on the different elm age classes and whether there is divergent selection, we conducted a series of no-choice feeding experiments. We first assessed performance in terms of adult fecundity, and then we examined larval survivorship and development for beetles reared on leaves from each elm age class. The fecundity experiments were carried out using the same methods and arenas used for the oviposition preference trials (see above), with the exception that only one age class of elm leaf was offered. Each replicate contained five male–female pairs of newly emerged adults (previously fed their natal host age class during the larval stages, i.e. *P. aenescens* on adult trees and *P. maculicollis* on seedlings) that were placed together in a glass jar arena containing a branch from either an adult tree or seedling. The branches were placed into floral tubes and replaced every other day. In total, there were four treatments (*P. aenescens* on adult trees, $n = 13$; *P. aenescens* on seedlings, $n = 11$; *P. maculicollis* on adult trees, $n = 11$ and *P. maculicollis* on seedlings, $n = 13$) where adults were allowed to feed, mature and lay eggs in no-choice trials. The experiment was conducted for 20 days. We compared the number of eggs laid by each beetle species when the adult stages were fed either adult trees or seedlings.

The eggs from the fecundity experiments (natal treatments only: *P. aenescens* on adult trees and *P. maculicollis* on seedlings) were used to test the effects of elm age class on development and survival of beetle larvae. Upon hatching, 20 larvae were inoculated onto fresh leaves of adult trees or seedlings and placed in a glass jar arena. Foliage was replaced every 2 days and insect development and mortality were checked daily. We conducted 10 replicates for each treatment (*P. aenescens* eggs on adult trees; *P. aenescens* eggs on seedlings; *P. maculicollis* eggs on adult trees; *P. maculicollis* eggs on seedlings), and recorded the number of days required to reach pupal stage. Dead beetles were not replaced during the experiment. We compared survival rates and development times across treatments, and each arena trial represented the experimental unit irrespective of the number of beetles tested in each trial. Two-way ANOVAs were performed to examine fecundity and larval development, logistic regression was performed to examine larval survivorship. Independent sample *t*-tests and Mann–Whitney *U*-tests were used to determine whether fecundity and survivorship differed when beetles were fed their natal versus non-natal elm age class, respectively.

(d) The role of leaf surface wax in feeding and oviposition preference

Because contact chemicals in elm surface wax may be important in determining beetle feeding and oviposition preference, we compared the chemical profiles of leaf waxes extracted from adult trees and seedlings and then tested whether these plant chemicals dictated beetle preference. For both of these experiments, we used fresh foliage to isolate leaf waxes. Leaf tissue for extraction was collected from Olympic Park and Shanyangwa, from mid-June to mid-July during the peak timing of beetle feeding and oviposition. To extract only the surface wax of fresh foliage, extractions were conducted at room temperature [33]. To make the analysis quantitative, we extracted from approximately equal leaf areas (about 50 cm²) across samples. For each sample, 5–10 leaves were individually dipped in 10 ml *n*-hexane (HPLC grade, Fisher, UK) in a 25 ml glass beaker for 20 s each. The extract was then filtered by anhydrous sodium sulfate, concentrated to a final volume of 0.5 ml by evaporating in a gentle stream of nitrogen, and transferred to a 1.5 ml glass vial (Agilent, USA). Samples were stored in a −30°C freezer until use.

We first assessed whether adult trees and seedlings differed in leaf wax chemistry (adult trees from Olympic Park, $n = 10$; adult trees from Shanyangwa, $n = 12$; seedlings from Olympic Park, $n = 10$; seedlings from Shanyangwa, $n = 9$). Chemical identification was performed using a gas chromatograph (HP 6890 series, Agilent) fitted with a mass spectrometer (HP 5973, Agilent) with 70 eV electron impact mode, an HP5 column (30 m × 0.32 mm internal diameter × 0.25 μm film thickness, Agilent) and helium at 1.0 ml min⁻¹ as a carrier gas. Three microlitres of each extract was automatically injected at 280°C in the splitless mode. The oven temperature was controlled as follows: 50°C for 1 min, increased 50°C min⁻¹ to 200°C for 4 min, increased 5°C min⁻¹ to 300°C for 1 min and increased 2°C min⁻¹ to 320°C for 5 min. We used *n*-alkane (C6–C40) standards to calculate the retention indices (RIs) following Kováts [48]. Individual compounds were identified by their MS and RI [31]. We calculated peak area relative to the total peak area. To avoid limitations inherent to the analysis of compositional data, peak area was transformed as: $Zip = \ln[Aip/g(Ap)]$, where Aip is the peak area of i for sample p, $g(Ap)$ is the geometric mean of all peaks for sample p and Zip is the transformed area of peak i for sample p [49]. We used transformed data to conduct a canonical discriminant analysis with leaving-one-out cross-validation. A multivariate ANOVA was performed to test whether leaf chemistry differs between elm age classes, sites and the interaction between them [50,51].

We tested whether leaf wax chemicals play a role in beetle preference using choice experiments. Feeding and oviposition experiments were performed using artificial leaf discs painted with 0.1 ml extract from adult trees, seedlings, or *n*-hexane (control). One of each of these discs was placed symmetrically around

the outer edge of a 9 cm diameter Petri dish lined with moist filter paper. Once the solvent evaporated, a male–female pair of newly emerged, naive adults was placed in the centre of the arena. In the feeding trials (P. aenescens, $n = 10$; P. maculicollis, $n = 10$), we used discs of glass microfibre filters (25 mm diameter, Whatman, UK) and allowed the beetles to feed for 24 h before assessing the disc area removed. In the oviposition trials (P. aenescens, $n = 12$; P. maculicollis, $n = 11$), we moistened the filter paper every 12 h, used mated females (one female per replicate), and gave them tripartite fibre filter paper discs (90 mm diameter, Xinxing, China) as oviposition substrates. Oviposition experiments were conducted for 48 h, after which we counted the number of eggs deposited on each artificial leaf disc. All the assays were performed in the dark to remove the effect of potential visual cues. One-way ANOVA with sequential Bonferroni corrections for the three-choice experiments were used to test whether the proportion of leaf disc consumed and oviposition preference differed. Each arena trial was the experimental unit for the analyses, irrespective of the number of beetles tested per arena. Percentage data were normalized with logit transformations prior to analysis [47].

3. Results

(a) Field surveys

We observed a consistent, significant divergence in beetle population composition between adult trees and seedlings. More than 80% of P. maculicollis, irrespective of life-history stage, were collected from seedlings; conversely, most P. aenescens larvae (more than 81%), pupae (more than 97%) and adults (more than 77%) were found on adult trees (figure 1). Pupae exhibited the strongest fidelity, with over 94% of pupae located on (P. aenescens) or under (P. maculicollis) the preferred host plant stage across all three sites. Correspondingly, we also observed that pupae of P. aenescens usually aggregated at the base of the trunk or within cracks in the bark of adult trees, whereas P. maculicollis commonly hid in the leaf litter immediately beneath seedlings (figure 1a,b). The results of the GLM showed a significant effect due to beetle species identity (adult trees: $F_{1,94} = 1078.048$, $p < 0.001$; seedlings: $F_{1,103} = 2794.501$, $p < 0.001$), while neither site (adult trees: $F_{2,94} = 0.844$, $p = 0.434$; seedlings: $F_{2,103} = 0.169$, $p = 0.845$) nor beetle development stage (adult trees: $F_{2,94} = 0.051$, $p = 0.950$; seedlings: $F_{2,103} = 0.412$, $p = 0.664$) affected the proportion of beetles on adult trees versus seedlings.

(b) Preference

In the choice experiments where adult beetles were simultaneously offered leaves of adult trees and seedlings, P. aenescens and P. maculicollis exhibited divergent preferences. Pyrrhalta aenescens significantly preferred to feed and oviposit on adult tree leaves (feeding: $t_9 = 6.869$, $p < 0.001$; oviposition: $t_9 = 9.173$, $p < 0.001$, paired t-tests), whereas P. maculicollis showed a strong feeding and oviposition preference for seedlings (feeding: $t_9 = 6.483$, $p < 0.001$; oviposition: $t_{11} = 8.745$, $p < 0.001$, paired t-tests; figure 2).

(c) Performance

The no-choice experiments examining the effect of elm age class on fecundity, larval development and mortality showed differential performance indicating divergent selection on

beetles occupying adult trees versus seedlings (figure 3a,b). The beetles that were fed their preferred plants performed better in terms of fecundity (two-way ANOVA, $F_{3,47} = 9.341$, $p < 0.001$) and survival (logistic regression, $\chi_3^2 = 25.313$, $p < 0.001$). For example, P. aenescens reared on adult trees had significant higher fecundity and larval survival rate than P. aenescens reared on seedlings. Similarly, P. maculicollis exhibited improved performance in both fecundity and larval survival rate when reared on seedlings (figure 3a,b). For both beetle species, however, larval development rate did not differ between the two elm age classes (two-way ANOVA: $F_{3,39} = 1.272$, $p = 0.302$).

(d) The role of leaf surface wax in feeding and oviposition preference

The leaf surface wax profiles contained n-alkanes, esters, acetates, triterpenoids and a number of other compounds (electronic supplementary material, table S1 and figure S2). Discriminant analysis clearly separated the epicuticular wax profiles between the elm age classes (figure 4), and the first canonical root was associated strongly with elm age class, accounting for 99.4% of the total variance. The second canonical root was associated with differences among sites, but only explained 0.5% of the variance (figure 4). MANOVA analysis of these data revealed a significant effect of elm age class on the epicuticular wax profiles (Wilks' $\lambda = 0.0002$, $F_{3,38} = 1664.039$, $p = 0.009$), whereas site (Wilks' $\lambda = 0.001$, $F_{3,38} = 24.562$, $p = 0.159$) and the interaction term (Wilks' $\lambda = 0.001$, $F_{3,38} = 50.466$, $p = 0.111$) contributed little to these differences.

The choice tests using leaf wax extracts on artificial leaf discs showed a consistent pattern of preference to the feeding trials described above. Beetles exhibited significant preference for feeding and oviposition on the discs painted with extracts from their natal elm age class than discs painted with extracts from the non-natal age class or hexane (figure 5). Pyrrhalta aenescens consumed a larger disc area and laid more eggs on the artificial leaf discs painted with adult tree extract (ANOVA: feeding: $F_{2,9} = 59.125$, $p < 0.001$; oviposition: $F_{2,11} = 29.718$, $p < 0.001$). Similarly, the extract from seedlings was more attractive to P. maculicollis than adult tree extracts or hexane (ANOVA: feeding: $F_{2,9} = 47.679$, $p < 0.001$; oviposition: $F_{2,10} = 20.048$, $p < 0.001$). Finally, as compared to controls, the artificial leaf discs painted with leaf surface wax attracted more feeding and ovipositing beetles (ANOVA: $p < 0.05$ for all pairwise comparisons; figure 5).

4. Discussion

Shifts to novel host plants have been identified as a critical factor in speciation among phytophagous insects [2,4,8, 10–12,15–20], yet about half of speciation events occur without a host shift [22,23]. Non-ecological processes such as genetic drift in allopatry could easily explain diversification with host conservatism, but examples particularly from galling insects suggest that ecological mechanisms could also be acting [15,25–27]. Ecological speciation is possible if, for example, the host niche is subdivided such that the insects specialize and adapt to different organs of the host (e.g. leaves, flowers). Here we provide evidence that insect divergence is correlated with differential preference for host plant
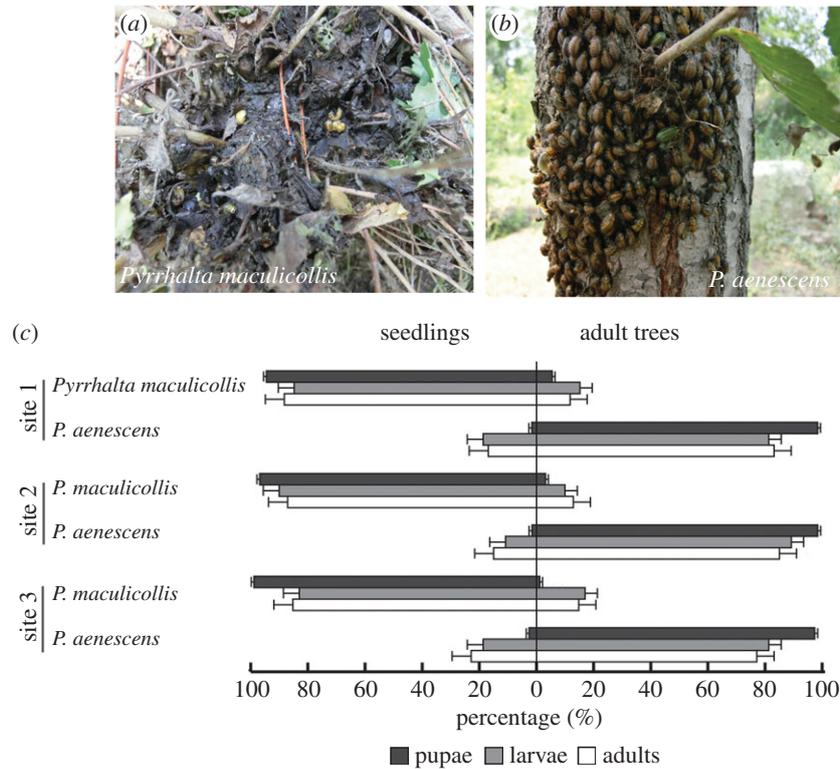
Figure 1. Pupation sites (*a,b*) and distribution of *Pyrrhalta maculicollis* and *P. aenescens* on seedlings versus adult trees (*c*). (Online version in colour.)
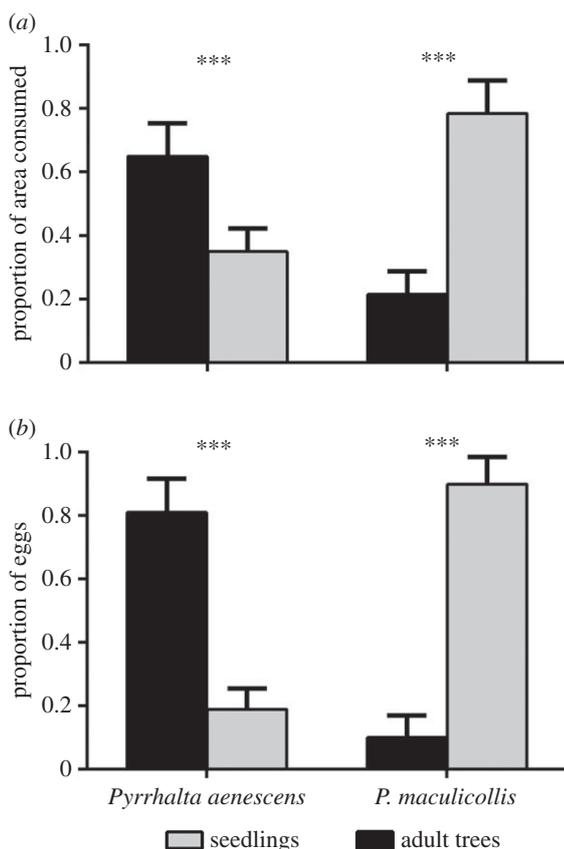
Figure 2. Feeding (*a*) and oviposition (*b*) preference of *Pyrrhalta maculicollis* and *P. aenescens* on adult trees and seedlings in choice experiments. Significance level is indicated with asterisks (***$p < 0.001$).

age, thus extending how the feeding niche might be further subdivided between sister species.

The results demonstrated a clear pattern of divergent selection caused by feeding on plants differing in age. This

is one component of ecological speciation, where divergent adaption and barriers to gene flow ultimately evolve via ecologically based divergent selection [3]. Divergent selection imposed by adult trees and seedlings was detected such that both *P. aenescens* and *P. maculicollis* had highest fecundity and survivorship on their preferred age class. Together, these differences in fecundity and survival would promote fidelity in host age selection as using the non-preferred plant was highly maladaptive. Divergent selection was also supported by the chemical analysis that demonstrated a quantitative difference in leaf surface wax between adult trees and seedlings. These quantitative differences strongly impacted beetle preference for feeding and oviposition as demonstrated by the assays using artificial leaf discs painted with surface wax extracts. Although we only examined differences in leaf surface wax, it is likely that other plant chemicals differ between the host plant ages. Whether these other chemicals affected beetle performance is unknown, but the experiments using artificial leaf discs suggest that preference is primarily governed by leaf surface wax.

The correlation between ecological differences in preference and performance and strong sexual isolation between the species is suggestive of the role of host plant specialization in speciation. As has been documented for many other plant-feeding insect taxa, speciation is accompanied by changes in host use (e.g. [9,13,14,16]). One of the challenges in linking speciation as a direct result of ecological selection is to understand the mechanism by which selection drives reproductive isolation [5]. For *P. aenescens* and *P. maculicollis*, it is unclear whether the differences in insect CHCs that generate sexual isolation [31] caused divergence in preference to host plant chemistry, are the result of feeding on different host plant ages, or are a secondary trait that may act as reinforcement in sympatry. At the very least, the strong ecological differences suggest that hybrids would perform poorly on
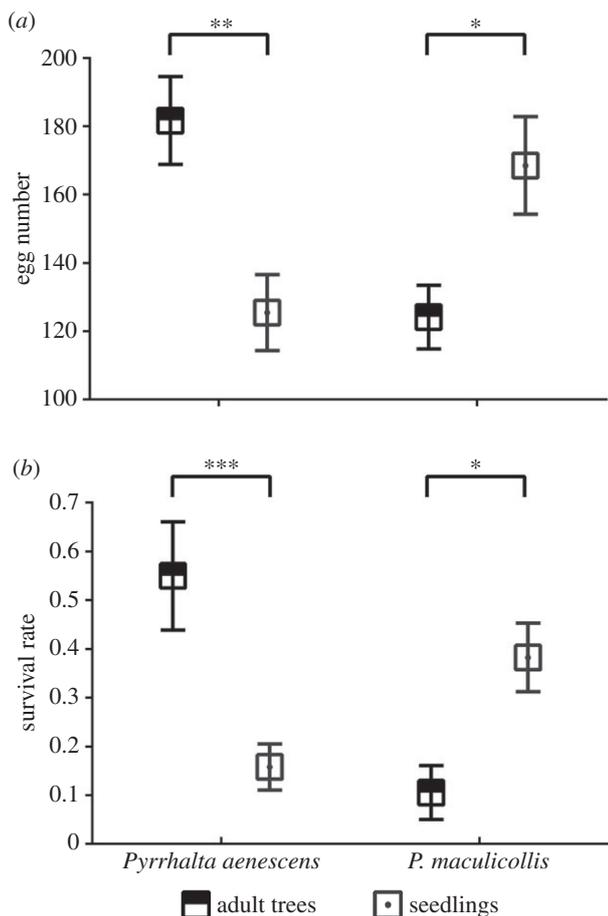
Figure 3. Performance of *Pyrrhalta maculicollis* and *P. aenescens* in a no choice feeding experiment. (*a*) Fecundity and (*b*) survival rate of larvae (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Figure 4. Canonical discriminant analysis of the leaf surface wax profiles between adult trees and seedlings from Olympic Park and Shanyangwa. (1) Adult trees from Olympic Park, (2) adult trees from Shanyangwa, (3) seedlings from Olympic Park and (4) seedlings from Shangyangwa. Each symbol represents a single sample. (Online version in colour.)

either host plant age, and hence lead to the evolution of strong sexual isolation. Additional studies on the historical geographical ranges and the role of host plant chemistry in generating differences in insect CHCs are needed to further clarify the role of changes in host use with speciation.

Here we focused on seedling versus adult age classes, yet the intermediate age classes (3–5 years of age) would seem to present an opportunity for the beetles to feed and hybridize. However, field surveys of elm trees show that heavy feeding damage occurs only on young and old trees, and the intermediate age classes have little damage. If the intermediate age classes were attractive to either beetle species, we would have predicted much higher levels of damage than those observed in the field. Beetle surveys also showed that *Pyrrhalta* are only rarely found on intermediate-aged trees, suggesting that they seldom overlap on these potentially intermediate hosts. While we cannot rule out the possibility of rare hybridization events, there are several lines of evidence suggesting that this is highly unlikely. First, molecular analyses of our species show reciprocal monophyly in both nuclear and mtDNA, indicative of no gene flow between them [29]. Second, strong pre-mating isolation is based on preference for CHC profiles [31]. Third, we have shown complete post-mating reproductive isolation in laboratory trials (B.Z., unpublished data). Together, this evidence suggests that the beetle species rarely interact and when they do come together on a host plant, that hybrids are unlikely to form. While it would be interesting to know how the beetles perform on the intermediate age class, this information would
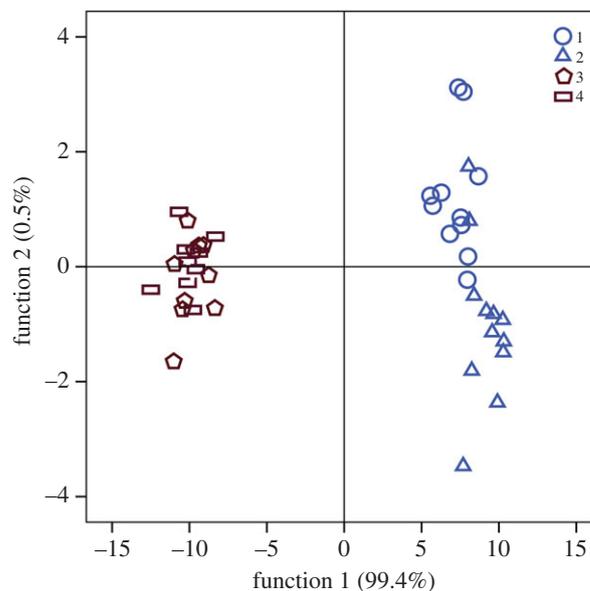
not change the overall pattern observed in this study: that host age is correlated with divergence. Indeed, the evidence seems to point towards rather strong selection to use the natal host age.

Because of the relatively deep divergence between the sister taxa, inferences about the mechanism of speciation should be taken with caution. Current observations of reproductive isolation may not reflect the processes that initially caused speciation; yet, lessons from classic studies of divergent taxa show that we can still learn about the process of reproductive isolation [52,53]. Our results demonstrating strong preference and performance relationships with respect to host plant age show that there are multiple mechanisms separating these two ecologically equivalent species in present-day populations. Although we cannot directly test whether divergent selection caused speciation between *P. aenescens* and *P. maculicollis*, this strong plant component to current reproductive isolation in *Pyrrhalta* is characteristic of species undergoing incipient speciation in other phytophagous insects. Thus, divergence due to host plant age seems a feasible process leading to speciation, if not in *Pyrrhalta*, then possibly in other insect species.

Although the current data cannot identify the mechanism of speciation, this study does embellish upon previously identified mechanisms of sympatric speciation. Studies have shown how differences in plant organ type or tissue age (within plant) can favour subdivision of the niche [15,26,27], and here we extend those processes to also include host plant age. To the best of our knowledge, this is the first study to document divergent selection associated with host plant age, a process that has the potential to be quite common especially among insects that feed on long-lived plants. Particularly for sessile, gall-inducing insects, the age of host plant tissue can significantly impact insect fitness [54–56]. Ontogenetic changes in plant defence have been well characterized [57], and age-specific differences in defensive chemistry, physical defence traits or nutrient content [58–60] could restrict the feeding patterns of phytophagous
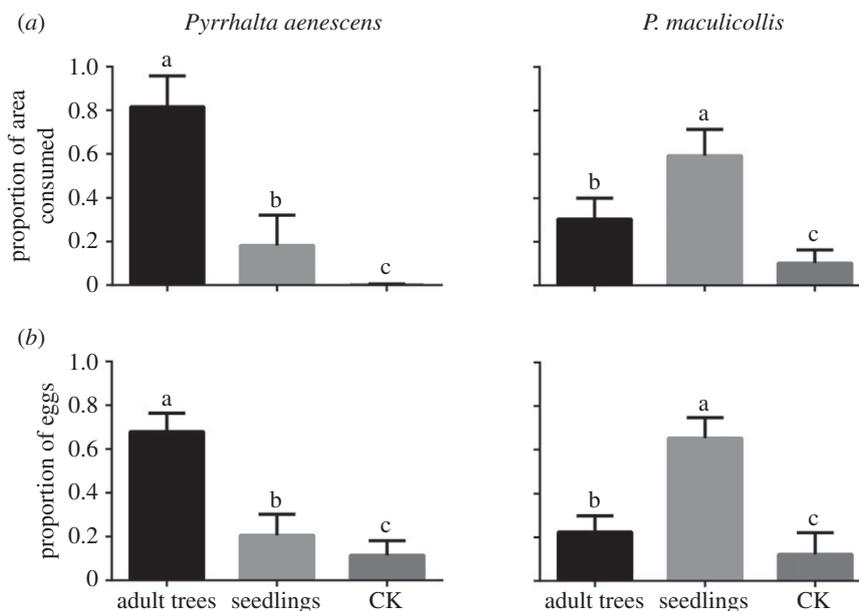
Figure 5. Response of beetles to leaf surface wax extracts from adult trees and seedlings. Effect on feeding (*a*) and oviposition choice (*b*) using artificial leaf discs painted with leaf extracts. Different letters on bars indicate significant differences. 'CK' indicates control.

insects and/or require specialized adaptations. These host age-specific effects can also translate into differences in the ability of insects to resist predation [59]. Together, these lines of evidence suggest that differences between host plant age classes may be nearly as effective in driving divergence within insect populations as shifts to new host plant species.

Studies of insect speciation have predominantly focused on host-associated divergence, where diversification is caused by a shift to a novel host species [23]. While this work has been incredibly important in identifying mechanisms of speciation, this bias may miss alternative mechanisms that occur without host shifts. For instance, studies on the fig–fig wasp mutualism had previously assumed a one-to-one species ratio until a genetic analysis repeatedly showed the presence of cryptic wasp species within single fig species [61,62]. Such unrecognized diversity is common in phytophagous insects, and searching for new species within host plants may show that host age-associated divergence is common.

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