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Authors for correspondence:
Linyi Zhang
e-mail: lz41@rice.edu
Scott P. Egan
e-mail: scott.p.egan@rice.edu

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Temporal isolation between sympatric host plants cascades across multiple trophic levels of host-associated insects

Linyi Zhang[1], Glen R. Hood[1,2], James R. Ott[3] and Scott P. Egan[1]

[1]Department of Biosciences, Rice University, Houston, TX 77005, USA
[2]Department of Biological Sciences, Wayne State University, Detroit, MI 48202, USA
[3]Population and Conservation Biology Program, Department of Biology, Texas State University, San Marcos, TX 78666, USA

Phenological differences between host plants can promote temporal isolation among host-associated populations of insects with life cycles tightly coupled to plant phenology. Divergence in the timing of spring budbreak between two sympatric sister oak species has been shown to promote temporal isolation between host plants and their host-associated populations of a cynipid gall wasp. Here, we examined the generality of this mechanism by testing the hypothesis of cascading temporal isolation for five additional gall-formers and three natural enemy species associated with these same oak species. The timing of adult emergence from galls differed significantly between host-associated populations for all nine species and parallels the direction of the phenological differences between host plants. Differences in emergence timing can reduce gene flow between host-associated populations by diminishing mating opportunities and/or reducing the fitness of immigrants due to differences in the availability of ephemeral resources. Our study suggests that cascading temporal isolation could be a powerful 'biodiversity generator' across multiple trophic levels in tightly coupled plant–insect systems.

1. Introduction

Understanding the origins of biodiversity presents an enduring challenge for biologists. Sequential speciation posits that speciation events within one lineage can have cascading effects across an entire community, by promoting divergence and speciation across multiple species [1–4]. Emerging from this perspective, *cascading reproductive isolation* describes the process whereby trait divergence that generates reproductive isolation (RI) between populations in one species transcends trophic levels to generate population divergence within interacting species [5]. The diversity of host-specific insect herbivores with their close ties to host plants offers opportunities to test whether cascading RI is a common mechanism promoting divergence [5–8].

Phenological difference between host plants is a key selective force that promotes phenological differences and initial population divergence of host-specific insects with life histories tied to host plants (e.g. *Rhagoletis* fruit flies [9]; *Eurosta* gall flies [10] and *Enchenopa* treehoppers [11]; reviewed in [12,13]). For example, Hood *et al.* [5] demonstrated that temporal RI between two sympatric sister species of live oaks, *Quercus virginiana* (*Qv*), and *Q. geminata* (*Qg*) cascades as a reproductive barrier among populations of the host-specific, gall-former, *Belonocnema treatae*. Divergence in the timing of budbreak, which is linked to male flower production, generates temporal RI between the two sister plant species [14]. Simultaneous temporal RI between *B. treatae* populations associated with each oak species is also generated as budbreak leads to the formation of new leaves that the wasps require at an early developmental

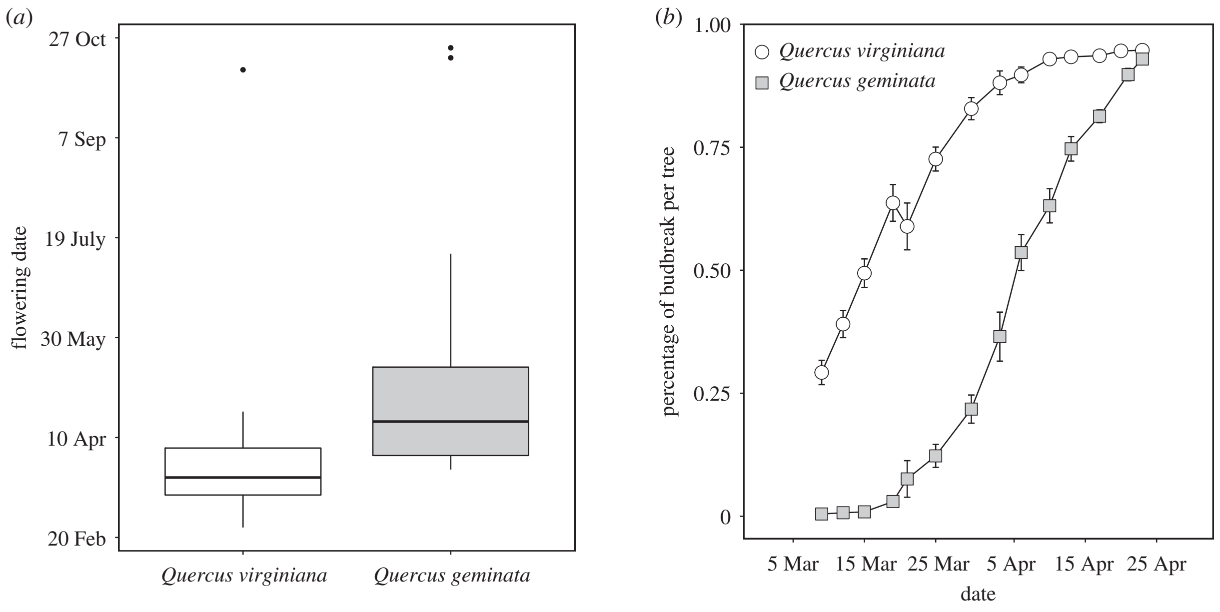

Figure 1. Phenological differences between host plants *Q. virginiana* (*Qv*) and *Q. geminata* (*Qg*). (*a*) Boxplot of the average flowering date by host illustrating the median, 25th and 75th percentiles, and the 95% confidential intervals displayed. The dots outside of the boxes are outliers. (*b*) Cumulative frequency of budbreak of *Qv* and *Qg* (mean % budbreak per tree ± s.e.). Panels modified from Hood *et al.* [5].

stage (less than 72 h old) to induce gall formation [5,15]. Temporal isolation reduces gene flow between host-associated gall-former populations by decreasing mating opportunities and lowering immigrant fitness in non-natal host plant environments (i.e. immigrant inviability) due to a mismatch in timing of ephemeral host resources [15]. Thus, budbreak acts as a 'multitrophic, multi-effect trait,' whereby differences in budbreak phenology generates RI between both the oak species and their obligately dependent insect herbivores [5].

These sister oak species share a diverse community of gall-formers and their associated insect natural enemies are similarly dependent on plant phenology [16,17]. Thus, the stage is set to test whether differences in host plant phenology drive temporal isolation across multiple species and trophic levels. To accomplish this, we monitor the emergence phenology of five additional gall-former species and three natural enemy species found on the two oaks [18–20].

2. Material and methods

(a) Study system

Gall-formers are specialist insect herbivores that induce three-dimensional outgrowths of nutritive plant tissues (i.e. 'galls') within which larvae feed and develop, and from which adults emerge [21]. Many species within the major gall-forming lineage Cynipidae (Hymenoptera) exhibit cyclical parthenogenesis, wherein alternating sexual and asexual generations develop on different plant tissues to complete a bivoltine life cycle [21–23]. Both generations require newly formed plant tissues as oviposition sites to successfully induce galls. Oviposition into these tissues at the wrong time, often by only a week or two, can preclude gall formation [15]. Thus, the timing of tissue availability is critical for gall formation and insect survival [15,22].

The gall-former community of *Qv* and *Qg* includes five common cynipid species: *Andricus quercuslanigera*, *A. quercusfoliatus*, *B. treatae*, *Callirhytis quercusbatatoides* and *Disholcaspis quercusvirens*; and the gall-forming midge, *Arnoldiola atra* (Diptera: Cecidomyiidae) [18]. The geographical ranges of *Qv* and *Qg* overlap in the southeastern USA, but the production of new flowers and leaves takes place an average of three weeks earlier in the spring for *Qv* (figure 1). Cavender-Bares & Pahlich [14] suggested that this phenological difference is a response to selection against hybridization. The development of these six gall-formers depends on the new spring growth of plant tissues [19,20,24]. Sexual generation *B. treatae* and *A. atra* emerge and oviposit into the new leaves and shoots, respectively [24,25]. Similarly, asexual generation *A. quercuslanigera* and *D. quercusvirens* oviposit into developing buds inducing galls that harbour the sexual generations on catkins and manipulated buds, respectively [19,20]. Lastly, while the sexual generations of *A. quercusfoliatus* and *C. quercusbatatoides* are unknown, asexuals emerge from rapidly developing bud and stem galls, respectively. The reproductive life stage of gall-formers is typically brief, with adults surviving 2–7 days in ideal laboratory conditions, and oviposition beginning immediately upon emergence [5,20,22,24]. Therefore, differences in the timing of ephemeral resources between alternative host plant species likely results in divergent selection on the timing of emergence and oviposition [5,15,21,22].

Gall-formers are attacked by a diverse community of natural enemies, including inquilines that compete for plant resources within galls and often negatively impact gall-formers [10,20]. In this study, we focused on gall-formers and their inquilines because both are directly dependent on host plant resources, the timing of which differs between host plants [26]. The dominant inquilines of galls on live oaks are *Synergus* spp. (Cynipidae: Synerginae) [17,27]. Inspection of the *COI* and *cytb* regions in the mtDNA genome (approx. 1000 bp total) showed that *Synergus* emerging from galls induced by *A. quercuslanigera*, *C. quercusbatatoides* and *D. quercusvirens* on *Qv* and *Qg* represent distinct evolutionary lineages based on sequence divergence, which ranged from 11% to 17% between groups (electronic supplementary material, figure S1). Therefore, we also tested whether phenological differences between host plant species cascade to phenological differences in emergence timing within the *Synergus* natural enemy community.

(b) Sample collection

From November to December 2014–2018, we collected and husbanded galls potentially housing the six gall-formers and the three *Synergus* species from *Qv* and *Qg* from 72 sites in Florida and Alabama where the geographical ranges of the host plants

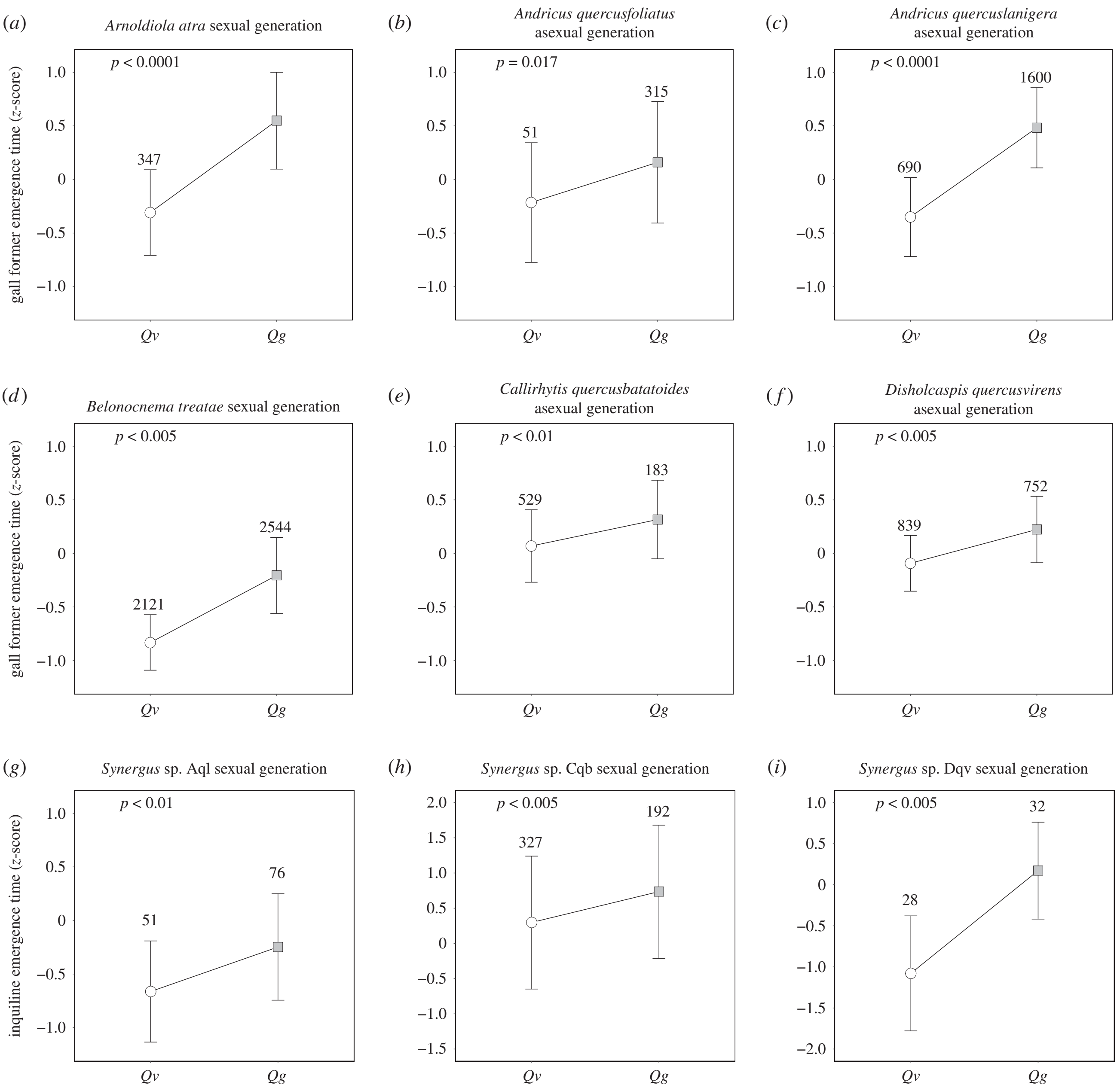

Figure 2. (*a–i*) The timing of adult emergence (standardized as *z*-scores) for six gall-formers and three *Synergus* associated with three gall-formers (Aql: *A. quercuslanigera*; Cqb: *C. quercusbatatoides* and Dqv: *D. quercusvirens*). The values above each confidence interval indicate sample sizes per host plant. Least squared means of emergence time were displayed with 95% confidence intervals, calculated from linear mixed models with host plants and sampling years as fixed factors. All *p*-values from one-tailed hypothesis testing.

overlap. Galls of each species were placed in separate 1 l glass jars that were covered by filter paper and misted twice weekly. In 2014–2016 and 2018, galls were reared under ambient environmental conditions at ≈23°C in the laboratory. In 2017, galls were maintained outdoors under natural conditions in Houston, TX, USA. Each year, the numbers of emergent individuals were monitored once every 2 days through mid-May. In total, we identified 10 249 gall-formers and 706 *Synergus* that emerged from galls induced by *A. quercuslanigera*; *C. quercusbatatoides* and *D. quercusvirens* (see electronic supplementary material).

(c) Statistical analysis

To test the hypothesis that gall-formers and their natural enemies emerge earlier on *Qv* than those on *Qg*, linear mixed model analysis was conducted in each individual species using the *nlme* package in R v. 3.5.2, with standardized *z*-scores of transformed emergence times (Julian dates) in each year as the response variable, host plant and collection year as fixed effects, and collection site as a random effect [28]. We also analysed models with latitude as a fixed effect, but results were similar (electronic supplementary material, table S1). We report least squared means of *z*-scores, (calculated using *emmeans* package in R, [29]) to illustrate differences in emergence between *Qv*- and *Qg*-associated populations across years for each species. We also report differences in average (±s.e.) emergence time in days to show biologically relevant values by back-transforming the least square mean *z*-score from the linear mixed model into the average emergence date for each host-associated population each year. Finally, to test the generality of cascading temporal isolation in this system, we compliment individual analyses with a standard sign test to compare the least square means of emergence date from our linear mixed model across all nine species.

(d) Temporal isolation

Temporal isolation between host-associated populations for all nine species was estimated as the percent of individuals for

Table 1. Results of linear mixed model analysis with host plant as a fixed effect and temporal isolation between populations of nine species developing on different host plants.

				effect of host plant in LMM				estimate of temporal isolation				
species	generation	tissue gall forms on	expected longevity (days)	coefficient	d.f.	*t*-value	*p*-value	year 2015	year 2016	year 2017	year 2018	average across years
Arnoldiola atra	sexual	Bud	5	0.61	35	4.88	*<0.0001*	0.53	1	0.25	0.69	0.62
A. quercusfoliatus	asexual	Bud	5	0.27	31	2.26	*0.012*	—	—	0.39	0.9	0.65
A. quercuslanigera	asexual	bud	5	0.59	42	11.59	*<0.0001*	1	0.59	0.52	0.34	0.62
B. treatae	sexual	root	3	0.44	1	18.1	*0.018*	—	—	0.47	—	0.47
C. quercusbatatoides	asexual	stem	5	0.18	28	2.52	*0.006*	—	—	0.31	—	0.31
D. quercusvirens	asexual	stem	7	0.22	46	2.36	*0.009*	1	0.96	0.05	0.15	0.54
Synergus spp. from *A. quercuslanigera*	sexual	n.a.	5	0.29	21	2.48	*0.007*	—	—	0.62	0.58	0.6
Synergus spp. from *C. quercusbatatoides*	sexual	n.a.	5	0.31	15	2.79	*0.003*	—	—	0.36	0.47	0.42
Synergus spp. from *D. quercusvirens*	sexual	n.a.	5	0.7	13	2.51	*0.008*	—	—	1	0.88	0.94

Note: italic indicates *p*-value < 0.05; 0–1 in temporal isolation means the population completely overlapped (0) to not overlapped at all (1).

each host-associated population that overlaps temporally based on emergence date and average adult longevity [19,20,30]. Estimated longevity of *B. treatae* (3 days) and *D. quercuesvirens* (7 days) were adopted from Hood *et al.* [5] and Bird *et al.* [20]. In the remaining seven species, we used the average adult longevity of *B. treatae* and *D. quercusvirens* (i.e. 5 days) to calculate temporal overlap (5 days) was used to calculate temporal overlap. We quantified temporal isolation (TI) between *Qv*- and *Qg*-associated populations following Feder *et al.* [30]:

$$\mathrm{TI} = 1 - \frac{\sum x_i y_i}{\sqrt{\sum x_i^2 y_i^2}}.$$

Here, x_i and y_i are the percentage of wasps from populations x and y alive on day i based on emergence time and probabilities of survival to day i based on average longevity estimates.

Temporal isolation estimates RI and ecological isolation for sexual and asexual generations emerging in the spring, respectively.

3. Results

Across all nine species, we observed consistent differences in emergence timing, with *Qv*-associated populations emerging significantly earlier, on average, than *Qg*-associated populations (figure 2 and table 1; sign test: $z = 3.0$, $p = 0.0027$). This suggests that a community-wide difference of gall-formers and natural enemies is associated with the observed differences in host plant phenology (figure 1). However, there is significant variation in the difference in emergence times between hosts across gall-former species: *A. atra* = 16.5 ± 5.5 days, *A. quercusfoliatus* = 7.0 ± 0.8 days, *A. quercuslanigera* = 18.1 ± 4.9, *B. treatae* = 9.3 days, *C. quercusbatatoides* = 4.3 days and *D. quercusvirens* = 6.9 ± 1.3 days, *Synergus* from *A. quercuslanigera* = 10.1 ± 2.3 days, *Synergus* from *C. quercusbatatoides* = 5.2 ± 0.6 days and *Synergus* from *D. quercusvirens* = 72 ± 27 days.

Estimated temporal isolation between host-associated populations of each of the nine species across years ranges from a low of 0.31 in *C. quercusbatatoides*, to a high of 0.94 in *Synergus* attacking *D. quercusvirens* (table 1).

4. Discussion

Cascading RI could be an important evolutionary mechanism to promote divergence and RI between populations within species across adjacent trophic levels [4]. Our results demonstrate that herbivore and natural enemy populations of each species on *Qv* emerged earlier than populations on *Qg*, consistent with the phenological differences in plant tissue growth between the live oak species. Even though the overall pattern is consistent across the community, there is remarkable variation in the phenological differences among species, from just 4 days in *C. quercusbatatoides* to 72 days for the *Synergus* sp. attacking *D. quercusvirens*. This suggests that there are likely many important natural history details underlying the degree to which temporal host plant RI cascades.

Our data suggest that the observed phenological differences among host-associated populations are a result of individuals specializing on each host plant rather than a generalist bet-hedging strategy, as a generalist strategy should require either that individuals emerge over a broad length of time or live long enough to oviposit on either host plants. Both assumptions contradict our observations in this and previous studies [5]. The emergence time differences between populations of sexual generations in the gall-formers *A. atra* and *B. treatae*, and the three *Synergus* species directly translates into temporal RI. Temporal RI across these sexual lineages ranges from 0.42 to 0.94, which represents a significant direct reduction in the opportunity for gene flow. Differences in emergence time between populations in the four asexual lineages may also promote RI indirectly since galling insects require developing plant tissues with a narrow time window to induce and form galls (e.g. [13]). Thus, the time mismatch between the emergent gall-formers and the ephemeral plant tissues from an alternative host plant could translate into reduced immigrant fitness [13]. There are 10–30 different natural enemy species that attack the gall-former in this system, many of which are host-specific [5,9,10]. Future research will explore the extent to which temporal isolation continues to cascade across these natural enemy species and how phenological divergence contributes to the total RI between populations throughout this oak-gall wasp community.

How common is cascading temporal isolation in nature? Our study shows that this phenomenon is common among this guild of host-specific herbivores, where the difference in ephemeral tissue resources presents strong selection on the phenology of herbivores, and eventually cascades to the third trophic level. Cascading temporal isolation occurs in two key steps: (i) temporal isolation evolves in one focal (keystone) species and (ii) temporal isolation extends to other species at different trophic levels that rely on the traits of the focal species. Accumulating case studies suggest that temporal isolation initiates divergence and promotes speciation in a diverse range of taxa including plants, insects and coral reefs, thus building a case that it is a widespread phenomenon [31–33]. The second step of cascading temporal isolation requires temporal isolation in one species to exert strong selection on timing of other interacting species in the community. This situation is likely in systems with highly specialized interactions, such as parasitism or mutualism, when one of the interacting species is closely tied with the focal organism's reproduction, or when the development of the resources the interacting species depends on is strongly linked with the focal organism's reproduction time. Since specialists compose a majority of the biodiversity on Earth [34], this highlights the possible generality of cascading temporal isolation in promoting species diversity.

Data accessibility. Data are available from the Dryad Digital Repository: https://dx.doi.org/10.5061/dryad.h44j0zpf9 [35].

Authors' contributions. L.Z., G.R.H., J.R.O. and S.P.E. designed the study and all authors collected data. L.Z. analysed the data and wrote the manuscript. All authors edited and approved the final version. All authors agree to be held accountable for the work performed.

Competing interests. We declare we have no competing interests.

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