

## Research



**Cite this article:** Ward AKG, Khodor OS, Egan SP, Weinersmith KL, Forbes AA. 2019 A keeper of many crypts: a behaviour-manipulating parasite attacks a taxonomically diverse array of oak gall wasp species. *Biol. Lett.* **15**: 20190428.

<http://dx.doi.org/10.1098/rsbl.2019.0428>

Received: 5 June 2019

Accepted: 3 September 2019

### Subject Areas:

behaviour, ecology, evolution

### Keywords:

host specificity, behavioural manipulation, extended phenotype, *Euderus set*, gall wasp

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Electronic supplementary material is available online at <https://doi.org/10.6084/m9.figshare.c.4662122>.

# A keeper of many crypts: a behaviour-manipulating parasite attacks a taxonomically diverse array of oak gall wasp species

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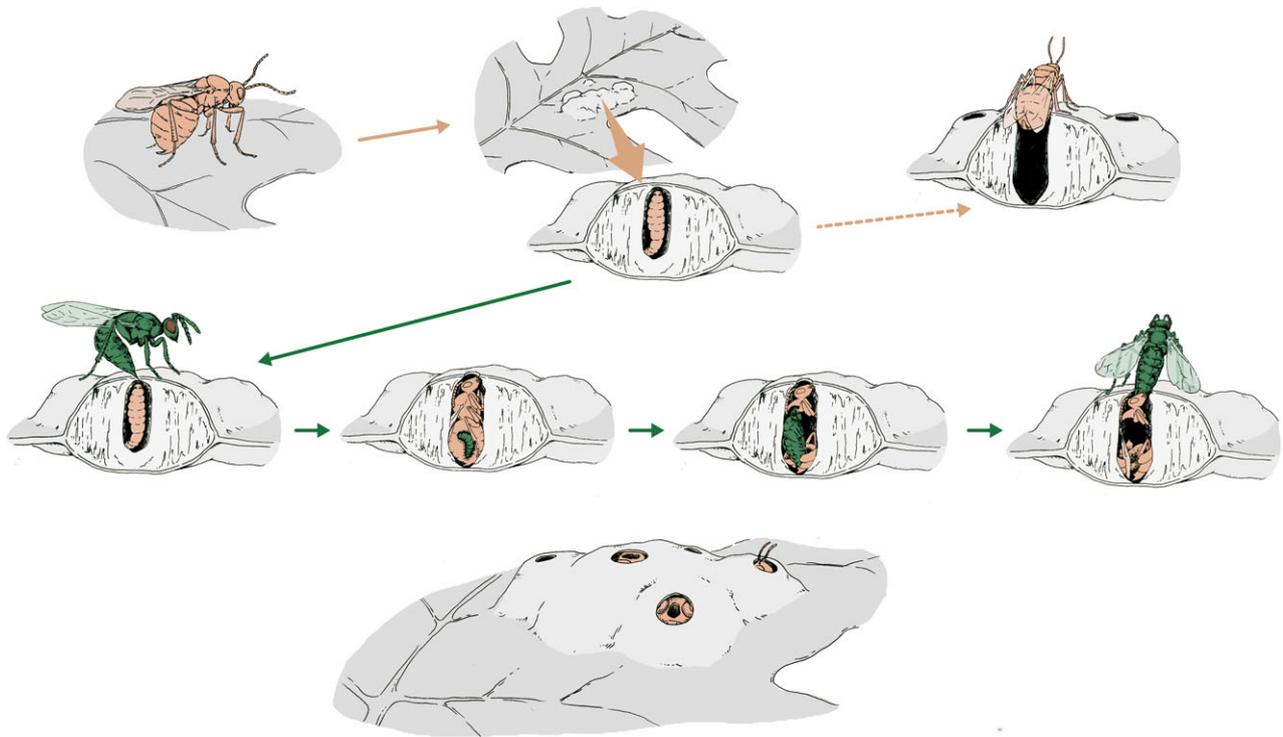
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Parasites of animals and plants can encounter trade-offs between their specificity to any single host and their fitness on alternative hosts. For parasites that manipulate their host's behaviour, the added complexity of that manipulation may further limit the parasite's host range. However, this is rarely tested. The recently described crypt-keeper wasp, *Euderus set*, changes the behaviour of the gall wasp *Bassettia pallida* such that *B. pallida* chews a significantly smaller exit hole in the side of its larval chamber and 'plugs' that hole with its head before dying. *Euderus set* benefits from this head plug, as it facilitates the escape of the parasitoid from the crypt after it completes development. Here, we find direct and indirect evidence that *E. set* attacks and manipulates the behaviour of at least six additional gall wasp species, and that these hosts are taxonomically diverse. Interestingly, each of *E. set*'s hosts has converged upon similarities in their extended phenotypes: the galls they induce on oaks share characters that may make them vulnerable to attack by *E. set*. The specialization required to behaviourally manipulate hosts may be less important in determining the range of hosts in this parasitoid system than other dimensions of the host–parasitoid interaction, like the host's physical defences.

## 1. Introduction

Trade-offs present limits to adaptation [1], as resources are finite and maximal optimization of all traits at once is impossible. For example, when energy is dedicated towards growth, it may not be available for reproduction [2]. One common trade-off observed in nature is that when parasites adapt to attack one group of hosts, their adaptations may reduce their ability to attack other hosts. This trade-off has been used to explain, in part, the tendency for parasites to specialize on a subset of available hosts (e.g. herbivorous insects on plant hosts or blood feeding insects on animal hosts) [3]. However, few studies quantify the host ranges of behaviour-manipulating parasites, and this is particularly true for parasitoids (insects that parasitize and eventually kill their hosts), which constitute perhaps the most speciose group of all animals [4].

Two recent papers [5,6] describe the discovery and life history of the parasitoid 'crypt keeper wasp' (*Euderus set*), so named because of its parasitism and behavioural manipulation of the 'crypt-gall wasp' (*Bassettia pallida*) in live oaks (*Quercus virginiana* and *Q. geminata*). Oviposition by a *B. pallida* female into a young stem induces the formation of a swollen internal gall (known as a 'crypt') inside the



**Figure 1.** Life cycles of an oak gall wasp (in this case, *Melikaiella ostensackenii*; pink), and the crypt keeper wasp, *E. set* (green). It is not known whether the larvae of *E. set* are ecto- or endoparasitic, so this illustration is intentionally ambiguous. Also, although the stage of adult attack is shown to be at the host's late larval stage, it is not yet known exactly when attack occurs. Artwork by Mona Luo.

stem, where the developing larval wasp will then feed and grow. Unparasitized adult *B. pallida* later chew a small exit hole in their gall—made of woody plant material—and then fly away. However, when parasitized by *E. set*, *B. pallida* chew significantly smaller exit holes, do not (or cannot) leave the gall and stop moving with their heads blocking (or ‘plugging’) the exit hole. The parasitoid then feeds on the disabled body of the host wasp, and, upon maturing into an adult wasp, chews through the ‘head plug’ and exits the gall (figure 1). In the absence of information on mechanisms, we are unable to determine if *E. set* is a ‘simple’ manipulator that debilitates/reduces host activity in a specific favourable context or a more ‘complex’ manipulator (e.g. one that interacts with central nervous system functions) [7,8]. Because *B. pallida* itself manipulates oaks to develop galls in their young branches, *E. set* is also a ‘hypermanipulator’—a rarely quantified phenomenon in which a parasite manipulates a parasite that itself manipulates its host. Though many species of *Euderus* have previously been described [9], *E. set* represents the first definitive example of behavioural manipulation in this genus.

Given the potential trade-off between specialization and host range, does this strategy of behavioural manipulation limit the host range of *E. set*? The parasitoid communities of most oak gall wasps are understudied or unknown, such that it is premature to assert that the manipulation of *B. pallida* by *E. set* is a unique relationship. Indeed, Weinersmith *et al.* [6] report additional examples of ‘head plugging’ in the gall wasp *Bassettia ligni* on the host plants *Q. lobata* and *Q. douglasii*. They also report evidence of head-plugging in an unidentified cynipid on *Q. nigra* in southeast Texas [6], which has since been keyed to genus *Bassettia* (probably *B. aquatica*; A.A.F. 2018, unpublished data). Further, our own parasitoid-rearing studies have yielded several unidentified *Euderus* from non-*Bassettia* galls (A.K.G.W. & A.A.F. 2018, unpublished data). These findings suggest the possibility that *E. set* may have a

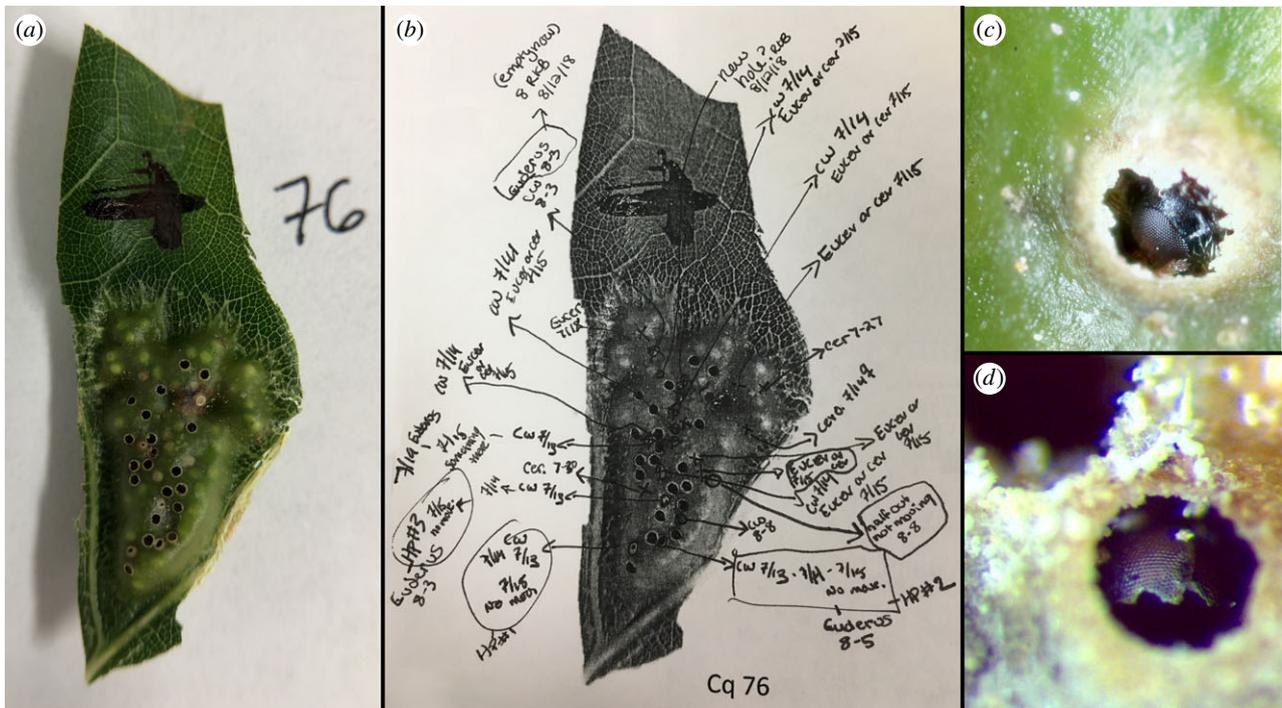
wide host range, but requires assessment of whether these *Euderus* are all the same species and whether they are inducing the same behavioural changes in their gall hosts.

Here, we ask whether the host head-plugging manipulation by *E. set* is (i) limited to the *B. pallida* host and its close relatives or (ii) found across multiple gall wasp species. If (ii), then what properties of hosts make them vulnerable to *E. set*? We identify hosts and non-hosts of *E. set* using wide-ranging collections (approx. 100 species of oak gall wasp) and DNA barcoding. We assess associations between *Euderus* infection and ‘head-plugging’ behaviour in *Euderus* hosts. This study addresses a critical question and a gap in our current knowledge regarding how manipulation of insect behaviour by parasitoids is (or is not) translatable across disparate hosts.

## 2. Material and methods

### (a) Collections and discovery of new *Euderus*/galler associations

From August 2015 to August 2018, we collected more than 23 000 galls from more than 10 oak species (electronic supplementary material, table S1), with a focus on maximizing the diversity of gall wasp species and on collecting mature galls that were most likely to have been parasitized. All galls were North American, and most collections (approx. 60%) were made in Midwestern states, but at least some collections extended farther afield, including to, for example, New England, North Carolina and Texas. Details on rearing conditions are provided in the electronic supplementary material. We identified *Euderus* to species using Yoshimoto [9] and Egan *et al.* [5]. We extracted DNA from two to six individual *Euderus* reared from each of the six different hosts using a CTAB/PCI method based on Chen *et al.* [10]. For each sample, we sequenced a segment of the mitochondrial cytochrome oxidase I (mtCOI) gene (see electronic supplementary material).



**Figure 2.** Details of methods for the *M. ostensackeni*/*Euderus* emergence study. (a) Photograph of an *M. ostensackeni* gall 1 h after collection, showing both intact chambers (light green circles) and galls from which an insect had emerged prior to the gall's collection (dark circles). Very light tan-coloured circles with a small pinprick of black in the centre are animals that were either actively chewing out of their gall or had stopped chewing and were already 'head plugs'. (b) Example of notations made across the course of the study. Any changes to the gall were noted daily, such that all emergent animals found in the cup on any given day could be associated with the individual chamber from which they emerged. This particular gall had four *Euderus* emerge from gall chambers for which chewing (CW) and/or a 'head plug' (HP) had previously been observed. Other notation refers to dates of observations, initials of the observer (e.g. 'RKB') or the genus of the emergent animal (e.g. 'Eucer' for the inquiline *Euceroptres*); (c) close-up of a 'head plug' where a *M. ostensackeni* galler has chewed a partial hole and then stopped moving; (d) close-up of *M. ostensackeni* head after emergence of a *Euderus* parasitoid. (Online version in colour.)

### (b) Observations of *Euderus* behaviour and emergence phenology

We chose one galler species for a focused study of the natural history, behaviour and phenology of *Euderus* host manipulation in a non-*Bassettia* gall. From 15 June to 20 July 2018, we collected *Melikaiella ostensackeni* galls weekly from a single, heavily infested pin oak (*Quercus palustris*) tree in Iowa City, IA, USA. Galls of *M. ostensackeni* manifest as parenchymal thickenings that project on both sides of the leaf. Each gall contains anywhere from a dozen to upwards of a hundred larval chambers (figure 2a). Each week we collected galls representing from 319 to 1040 larval chambers (electronic supplementary material, table S2). Upon collection, we cut each multi-chambered gall from its leaf, assigned it a number, photographed it from above using a Canon EOS Rebel T1i camera with an MP-E 65 mm f/2.8 1-5x Macro Lens (Canon USA, New York, NY, USA) mounted on a StackShot automated macro rail (Cognisys Inc., Traverse City, MI, USA) and placed it into an individual closed container. Individual chambers in each gall were visible as light green sub-circles, and, especially in later collections, dark circles with an exit hole indicated that a galler, parasitoid or inquiline had emerged (figure 2a). Twice daily, we checked galls for (i) emergent animals in cups, (ii) appearance of new emergence holes, (iii) signs of active chewing/movement at the gall surface, (iv) apparent head plugs, and (v) previously identified head plugs that had been chewed through or otherwise destroyed. Head plugs were usually obvious, and defined as when gallers had chewed incomplete holes and had then stopped moving (figure 2c,d). We softly poked putative head plugs with the blunt end of a 0.20 mm pin (BioQuip, Rancho Dominguez, CA, USA) and only recorded an observation as a head plug when the insect made no movement in response. All observations were recorded daily on printed

black and white photographs of each gall (figure 2b), such that newly emergent animals (gallers or parasites) could be traced back to a specific gall chamber or head plug. After *Euderus* had ceased emerging, we dissected six of the remaining head plugs to capture details of *Euderus* interactions with its host while inside the gall. In two chambers, we found gall wasps along with an apparently dead larva (electronic supplementary material, figure S3). We extracted DNA from these larvae and sequenced mtCOI as above.

## 3. Results

### (a) Collections and discovery of new *Euderus*/galler associations

Between 2015 and 2018, we collected more than 23 000 galls representing approximately 100 oak gall wasp species (electronic supplementary material, table S1) and subsequently reared more than 15 000 individual parasitoids, inquilines and hyperparasitoids (A.K.G.W. & A.A.F. 2018, unpublished data). Among these collections, we reared *Euderus* wasps from six different gall wasp host species (table 1): *Andricus quercuspetiolicola* ( $n=68$ ), *Callirhytis flavipes* (14), *Callirhytis quercusscutula* (34), *M. ostensackeni* (157, including the 44 below), *Melikaiella tumifica* (10) and *Neuroterus quercusbatatus* (22). All *Euderus* keyed morphologically to *E. set*.

COI sequences also suggest that all *Euderus* wasps in this study were *E. set*. The 16 adult and two larval *Euderus* sequenced in this study had mtCOI sequences that were 95–100% identical to one another (electronic supplementary

**Table 1.** Description of oak gall wasps, tree habitats and geographical locations from which *E. set* has been reared and identified.

cynipid oak gall-wasp	oak host(s) species and section	locations	description of gall	timing of galls collections and <i>Euderus</i> emergences	record
<i>Andricus quercuspetiolicola</i>	<i>Q. alba</i> ; <i>Q. bicolor</i> (section: <i>Quercus</i> )	IA	multichambered integral leaf gall, swelling of leaf petiole and sometimes basal midrib projecting on both sides of leaf	galls collected May–August, most <i>E. set</i> emergences June–July	this study
<i>Bassettia pallida</i>	<i>Q. geminata</i> ; <i>Q. virginiana</i> (section: <i>Quercus</i> )	FL, GA, LA, MS, TX	integral stem gall (a ‘crypt’)	galls collected July–October, most <i>E. set</i> emergences February–March	[11,12]
<i>Callirhytis flavipes</i>	<i>Q. macrocarpae</i> (section: <i>Quercus</i> )	IA	integral leaf gall, elongated swelling of the midrib	galls collected May–July, most <i>Euderus</i> emergences June–July	this study
<i>Melikaella ostensackenii</i>	<i>Q. palustris</i> <i>Q. rubra</i> (section: <i>Lobatae</i> )	IA, MO	integral leaf gall, parenchyma of leaf swelling, projecting on both sides, multiple chambers	galls collected May–August, most <i>E. set</i> emergences July–August	this study
<i>Callirhytis quercusscutula</i>	<i>Q. imbricaria</i> (section: <i>Lobatae</i> )	IA, MO	swelling of stem at the base of where new leaves attach	galls collected June, most <i>E. set</i> emergences June–July	this study
<i>Melikaella tumifica</i>	<i>Q. coccinea</i> (section: <i>Lobatae</i> )	PA	integral leaf gall, swelling of midrib on the bottom third of leaf	galls collected May–June, most <i>E. set</i> emergences July	this study
<i>Neuroterus quercusbatatus</i>	<i>Q. bicolor</i> (section: <i>Quercus</i> )	IA	integral leaf gall	galls collected June, most <i>E. set</i> emergences June–July	this study

material, figure S1). Though some sequences were relatively different (approx. 5%) from one another, percentage sequence similarity should not alone be used to define species [13], and wasps with some of the most divergent mtCOI sequences in the dataset were reared from the same collections, suggesting these are all the same species with variable mtCOI haplotypes. The alternative—that two very closely related *Euderus* species attack the same gall wasps on the same trees and attack no other gall wasps in our widespread collections—remains possible, but seems unlikely.

### (b) Observations of *Euderus* behaviour and emergence

Across 128 *M. ostensackeni* galls, we reared 291 adult *M. ostensackeni* gallers, 44 *Euderus* wasps and 649 other parasitoids and inquilines (electronic supplementary material, table S3 and figure S2). During the course of the study, we observed 63 *M. ostensackeni* ‘head plugs’. Thirty-nine emergent *Euderus* were conclusively linked to a specific head plug that had been noted during a previous observation period and that, after *Euderus* emergence, had a visible hole in the gall wasp’s head. The 24 head plugs from which no *Euderus* emerged did not produce any other adult parasitoids, suggesting that *Euderus* may have died in an early stage of development as in the two we found during dissections (electronic supplementary material, figure S3). In five cases, a *Euderus* emerged from a gall where we had not previously observed a head plug, indicating either a failure of detection or a genuine lack of a plug. No non-*Euderus* parasitoid emerged from any gall previously characterized as a head plug.

For the five additional non-*Bassettia* gall wasp species from which *Euderus* emerged in the laboratory (table 1), we inspected a subset of post-emergence galls for evidence of head plugs, chewed heads or other signs of hypermanipulation previously described in the *B. pallida*/*E. set* interaction. We observed more than 10 head plugs, both intact and eviscerated, on *A. quercuspetilicola* galls and found one or more incidences of head plugs in each of the other four additional hosts (electronic supplementary material, figure S4). Head plugs were not observed in gall collections from which *Euderus* did not emerge, as was the case in the previous study [6].

## 4. Discussion

Given that trade-offs present limits to adaptation, we hypothesized that for parasites that manipulate their host’s behaviour, the complexity of that manipulation would limit the parasite’s host range. Thus, we predicted that *E. set*-like wasps from different hosts should be genetically and morphologically distinct lineages tightly coevolving with their hosts. In contrast with our prediction, the combination of natural history observations, with morphological and genetic data imply that all *Euderus* wasps reared in this study are *E. set*, the species described previously from *B. pallida*. We now, therefore, have direct (2 gall wasp species) and indirect (5 species) evidence that *E. set* attacks and manipulates at least seven gall wasp hosts spanning five genera (table 1). Notably, many potential gall wasp hosts were collected from the same tree host species at the same time as the *E. set*-associated gallers, and yet were not apparently manipulated or infected by *E. set*. Our collections (electronic supplementary material, table S1) represent

approximately 1/7 of the approximately 700 described species of Nearctic oak gall wasps [14]. So, while *E. set* is oligophagous, widespread (Iowa to Pennsylvania; south to Texas and Florida) and attacks representatives of five different genera, it is also not a broad generalist on all oak gall wasps. This pattern of host use is unexpected. Many insect parasites of plants and animals are taxonomically specialized [4,15,16], and for parasites that manipulate the behaviour of their hosts, the symbiotic intimacy implied by behavioural control might be expected to further restrict host range—though the literature to date is equivocal on this point [7,11,17].

Remarkably, each of *E. set*’s hosts show similar extended gall phenotypes suggesting that behavioural manipulation may be less important to its host range than other dimensions of the host–parasitoid interaction, such as host’s physical defences. The Enemy Hypothesis states that galls provide defence against natural enemies and, in turn, pressures from those natural enemies have led to a wide diversity of defensive gall structures [12,18]. Even though gall wasps are often heavily attacked by parasites, this hypothesis is supported by findings showing correlations between gall characteristics and their corresponding parasitoid communities, suggesting that gall phenotypes may limit host ranges for parasitoids [19].

Consistent with the Enemy Hypothesis, all of *E. set*’s hosts induce integral galls (i.e. enclosed within the epidermis; gall is not detachable without causing significant damage to the plant tissue). All known hosts also lack the baroque structural defences found on many other galls, which can include spines, fuzz or larval cells suspended deep inside otherwise empty chambers [20]. These defences grow more substantial as the gall grows, which may render many galls inaccessible to *Euderus* and other parasitoids of gall wasp pupae. *Euderus* are late-instar larval or pupal parasitoids [9,21], and lack the long, exerted ovipositors typical of some other genera of late-attacking galler parasitoids [22], such that they may be limited to attacking hosts that are not buried deep within a gall nor protected by complex defence structures. Closer study of the phenology of attack in the *M. ostensackeni* system supports the hypothesis that *E. set* successfully attacks only at this late stage of development (i.e. at a stage of development where the host’s body nearly fills the chamber, and is easier to find by the parasitoid’s ovipositor) (electronic supplementary material, figure S5).

We have discovered that the parasitoid wasp *E. set* is specialized on a taxonomically diverse subset of available gall wasp hosts, a finding that contrasts with our expectation that this behaviour-manipulating insect would have a taxonomically limited host range. Further, all of the known hosts of *E. set* induce galls with similar characteristics, suggesting that the extended phenotypes of gall wasps better explain *E. set*’s host range than does its ability to manipulate host behaviour.

**Data accessibility.** All data are available as electronic supplementary material.

**Authors’ contributions.** A.K.G.W. and A.A.F. conceived of the study. A.K.G.W. and O.S.K. conducted the *M. ostensackeni* study. All authors contributed to gall collections, insect rearing and writing of this paper. All authors agree to be held accountable for the content and approve the final version of the manuscript.

**Competing interests.** We have no competing interests.

**Funding.** This study was funded by University of Iowa’s Center for Global and Regional Environmental Research (CGRER).

**Acknowledgements.** Robin Bagley, Will Carr, Sara Devine, Rachel Erikson, Leo Gastel, Alaine Hippee, Emily Manders, Danny McGarry, Moe Shakally, Joseph Verry and Caleb Wilson assisted with gall collections and rearing and preservation of insects. Thanks to

Robbins Park Environmental Education Center in Ambler, PA, USA, for permission to collect *M. tumifica* galls from a scarlet oak on their property. Figure 1 was illustrated by Mona Luo (<http://www.monaluo.com/>).

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