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Quarantine host range of *Bikasha collaris*, a potential biological control agent of Chinese tallowtree (*Triadica sebifera*) in North America

Gregory S. Wheeler[1]*, M. Sedonia Steininger[2] & Susan Wright[3]

[1]*USDA/ARS Invasive Plant Research Lab, Ft Lauderdale, FL 33314, USA,* [2]*USDA/ARS Center for Veterinary and Medical Entomology, Gainesville, FL 32608, USA, and* [3]*USDA/ARS Invasive Plant Research Lab, Gainesville, FL 32608, USA*

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Abstract

Chinese tallowtree, *Triadica sebifera* (L.) Small (Euphorbiaceae), is one of the worst invasive weeds of the southeastern USA impacting coastal wetlands, forests, and natural areas. Traditional mechanical and chemical controls have been unable to limit the spread, and this invasive species continues to expand its range. A proposed biological control candidate, the flea beetle *Bikasha collaris* (Baly) (Coleoptera: Chrysomelidae), shows high specificity for the target weed Chinese tallowtree. Results from a series of no-choice and choice feeding tests of *B. collaris* adults and larvae indicated that this flea beetle was highly specific to Chinese tallowtree. The larvae of *B. collaris* feed by tunneling in the roots, whereas the adults feed on the leaves of Chinese tallowtree. A total of 77 plant taxa, primarily from members of the tallow plant family Euphorbiaceae, were tested in numerous test designs. Larval no-choice tests indicated that larvae completed development only on two of the non-target taxa. Of 80 *B. collaris* larvae fed roots of *Hippomane mancinella* L. and 50 larvae fed roots of *Ricinus communis* L., two and three larvae completed development, respectively. The emerging adults of these five larvae died within 3 days without reproducing. Larval choice tests also indicated little use of these non-target taxa. Adult no-choice tests indicated little leaf damage by *B. collaris* on the non-targets except for *Ditrysinia fruticosa* (Bartram) Govaerts & Frodin and *Gymnanthes lucida* Sw. When given a choice, however, *B. collaris* adults consumed much less of the non-targets *D. fruticosa* (7.4%) and *G. lucida* (6.1%) compared with the control leaves. Finally, no-choice oviposition tests indicated that no eggs were produced when adults were fed all non-target taxa, except those fed *G. lucida*. These *B. collaris* adults fed *G. lucida* leaves produced an average of 4.6 eggs compared with 115.0 eggs per female when fed Chinese tallowtree. The eggs produced from adults fed *G. lucida* were either inviable or the emerging larvae died within 1 day. These results indicate that the flea beetle *B. collaris* was unable to complete its life cycle on any of the non-target taxa tested. If approved for field release, *B. collaris* will be the first biological control agent deployed against Chinese tallowtree in the USA. This flea beetle may play an important role in suppressing Chinese tallowtree and contribute to the integrated control of this invasive weed.

Introduction

Chinese tallowtree [*Triadica sebifera* (L.) Small (Euphorbiaceae), hereafter 'tallow'] is one of the most damaging invasive weeds in the southeastern USA, impacting wetlands, forests, and natural areas (Bruce et al., 1997). The native range of tallow includes parts of China, Japan, and northern Vietnam (Bingtao & Esser, 2008). In China, tallow occurs mostly in provinces south of the Yellow River (Zheng et al., 2005). In its invaded range in the USA, tallow infests 185 000 ha of southern forests, stranded swamps, flatwoods, and ruderal communities where it has invaded areas of 10 states that border the Gulf of Mexico and California (Rawlins et al., 2014; www.invasive.org,

*Correspondence: Gregory S. Wheeler, USDA/ARS Invasive Plant Research Lab, Ft Lauderdale, FL 33314, USA.
E-mail: greg.wheeler@ars.usda.gov

2015). Tallow is now a prohibited noxious weed in Florida, Louisiana, Mississippi, and Texas (USDA/NRCS, 2016). The projected economic impact of this invasive weed over the next 20 years in forestlands of Texas, Louisiana, and Mississippi, in terms of survey, timber losses, and control costs range from $200 to $400 million (Wang et al., 2012a). Chemical and mechanical control measures have been used with short-term success. Permanent cost-effective maintenance programs that integrate several control methods are required to prevent regrowth and recruitment (Jubinsky & Anderson, 1996). Classical biological control can provide an ecologically sound, cost-effective, and sustainable management solution to protect native plants in these habitats (Wheeler & Ding, 2014).

The native sources of the USA introductions of tallow are best matched genetically to several western and southern Chinese populations (Lieux, 1975; Bruce et al., 1997; Dewalt et al., 2011). Since its introduction, the weed has been reported primarily in 10 states: North Carolina, South Carolina, Georgia, Florida, Alabama, Mississippi, Louisiana, Arkansas, Texas, and California (Rawlins et al., 2014; USDA/NRCS, 2016). Tallow primarily invades temperate areas, apparently unable to infest more subtropical or tropical areas. In Florida, tallow occurs primarily north of Tampa, Orlando, and Daytona Beach (Wunderlin & Hansen, 2008; Rawlins et al., 2014). Additionally, its range extends west along the Gulf coast to south of Houston, Texas. These infestations extend north through Louisiana to southern Arkansas. A separate infestation occurs mostly in riparian areas of four counties of the Central Valley of California (Butte, Los Angeles, Sacramento, and Yolo) (Bower et al., 2009; Rawlins et al., 2014; USDA/NRCS, 2016).

One factor contributing to the success of tallow in its invaded range is the historical lack of specialized herbivores that exert population-level regulation (Harcombe et al., 1993; Bruce et al., 1997). The implementation of classical biological control presents a potentially safe and cost-effective option that can be a component of an integrated pest management program. As this species was cultivated for centuries in China, many pests are known (Zheng et al., 2005). The specialists of these species are candidates for biological control of tallow that can be screened for possible release in the USA. Three fungal pathogens and 115 species of arthropods have been reported to damage tallow and related members of the *Triadica* genus. Many of these species are generalist defoliators but a few are specialists. These specialist species are candidates for biological control of tallow that can be screened for possible release in the USA. At least three species showed promise following tests conducted in China (Wang et al., 2009, 2012b; Huang et al., 2011).

Biological control screening of potential agents for tallow began in 2006 with foreign surveys initiated by the Wuhan Botanical Garden, Chinese Academy of Science, in collaboration with USDA/ARS Invasive Plant Research Laboratory (IPRL). These surveys discovered several species of insects and conducted preliminary testing of three, *Heterapoderopsis* (= *Apoderus*) *bicallosicollis* Voss (Coleoptera: Attelabidae), *Bikasha collaris* (Baly) (Coleoptera: Chrysomelidae), and *Gadirtha fusca* Pogue (Lepidoptera: Nolidae). These preliminary studies showed all three species had high specificity to the target weed (Wang et al., 2009, 2012b; Huang et al., 2011). Following these Chinese studies, all three species were imported and tested in quarantine at the USDA/ARS/IPRL facility. Upon testing North American non-target species, the leaf rolling weevil *H. bicallosicollis* was rejected due to broad specificity (Steininger et al., 2013). Testing of *G. fusca* is ongoing and indicates this will be a promising candidate for biological control of tallow. Quarantine testing of the flea beetle, *B. collaris* began in 2010 and concluded in 2016. The larvae of *B. collaris* are root feeders whereas the adults feed on tallow leaves. Our goal here was to examine the host range of both feeding stages of *B. collaris* to determine its suitability for field release as a classical biological control agent of tallow.

Materials and methods

Non-target test plants

To predict the host range of *B. collaris*, a test plant list was compiled with those species most closely related to tallow given highest priority. However, plant species were also selected from diverse phylogenetic groups (Wheeler & Ding, 2014). The prioritization of plant species to be tested generally followed the phylogeny of the plant family to which tallow is assigned. Priorities were based upon the centrifugal phylogenetic method recommended by Wapshere (1974) with modifications (Briese & Walker, 2008; Wheeler & Ding, 2014).

These test plant taxa were grouped into seven categories based upon several criteria, including their phylogenetic relatedness to tallow and environmental and recovery (e.g., endangered/threatened) status: category 1, genetic types of the weed; category 2, species in the same genus; category 3, species in other genera in the same family; category 4, threatened and endangered species in the same family; category 5, species in other families in the same order; category 6, species in other orders; and category 7, any plant on which the proposed biological control agent or its close relatives have been previously found (TAG-BCAW-Manual, 2016). The test plant list for this target weed was compiled using USA, Caribbean, and Mexican flora.

Organization of test plant taxa in these different categories mostly followed the phylogeny of the weed and its relatives. Tallow is assigned to the large family Euphorbiaceae in the Malpighiales by the angiosperm phylogeny group III (Stevens, 2011). Other authorities place the Euphorbiaceae in its own order, Euphorbiales (USDA/NRCS, 2016). The phylogeny follows that of Wurdack et al. (2005), Wurdack & Davis (2009), Govaerts et al. (2015), and Riina & Berry (2013). In the USA, there are 60 genera (including the genera of Phyllanthaceae and Putranjivaceae) in the family and 596 accepted taxa (USDA/NRCS, 2016). Included here are the genera of the now distinct families Phyllanthaceae and Putranjivaceae, as they were previously included in the Euphorbiaceae (Stevens, 2011). The family Euphorbiaceae is organized into four subfamilies, of which only Acalyphoideae, Crotonoideae, and Euphorbioideae occur in the invaded range of tallow (Wurdack et al., 2005; Stevens, 2011). The Euphorbioideae subfamily has five tribes and 54 genera. In tallow's invasive range, only two tribes occur, Hippomaneae and Euphorbieae. The tribe Hippomaneae contains a single subtribe Hippomaninae to which tallow is assigned. The tribe Euphorbieae also has a single subtribe in tallow's invaded range, Euphorbiinae. The taxa thought to be most vulnerable to non-target damage by biological control agents are the close relatives, those assigned to the tribe Hippomaneae. Although the susceptibility of these taxa was the focus of host testing, representatives distributed throughout the family were also tested.

The *Triadica* taxon is a small genus, endemic to eastern and southeastern Asia (Esser, 2002). The genus is well-circumscribed, with only three accepted taxa, and very probably monophyletic (Esser et al., 1997). Tallow was previously placed in the *Sapium* genus and upon revision reassigned to the Asian *Triadica* genus (Esser, 2002). No members of *Triadica* are native to the New World. The closest relatives in North America are members of the same subtribe, Hippomaninae, which include *Ditrysinia* (= *Sebastiania*) *fruticosa* (Bartram) Govaerts & Frodin, *Gymnanthes lucida* Sw., *Hippomane mancinella* L., *Sebastiania bilocularis* S. Watson, and *Stillingia sylvatica* L. (USDA/NRCS, 2016). Two Caribbean taxa assigned to this subtribe, outside the invaded tallow range, include *Sapium laurifolium* (A.Rich.) Griseb. and *Sapium laurocerasus* Desf. (USDA/NRCS, 2016). Species of the Euphorbiaceae with some agricultural or ornamental significance include *Codiaeum variegatum* var. 'Mammy' and var. 'Petra' (L.) Rumph. ex A. Juss. (Garden Croton), *Jatropha gossypifolia* L. (bellyache bush), *Manihot esculenta* var. 'variegata' Crantz (cassava), and *Euphorbia* (= *Poinsettia*) *pulcherrima* Willid. ex Klotzch (Poinsettia).

Insect source and life history

In its native range, the flea beetle *B. collaris* has a temperate to subtropical distribution and was collected in Hubei, Ghizhou, Guangxi, and Hunan provinces, ranging from 31.6°N to 24.8°N. Quarantine colonies of *B. collaris* were established from two shipments made November 2008 and October 2009 from Wuhan Botanical Garden, Wuhan, Hubei, China. The collections contained 250 and 179 *B. collaris* adults, respectively. Upon arrival in the USA, the *B. collaris* collections were introduced into the quarantine laboratory at the USDA/ARS/IPRL (Gainesville, FL, USA) where all laboratory studies were conducted.

Beetles as biological control agent are among the most effective herbivores at reducing weed populations (Stiling & Cornelissen, 2005; Clewley et al., 2012). The tallow potential agent, *B. collaris* is a small beetle, adults are about 2 mm long, with an extended adult longevity, and relatively high fecundity. Adults feed on young and old tallow leaves, and live for about 266 days. Females produce on average 637.4 ± 52.4 (mean ± SE) eggs per female. Eggs are oviposited in clutches (Figure 1) at a rate of one clutch every 3 days with 24.6 ± 2.4 eggs per clutch. In the laboratory, females oviposit on multiple surfaces: leaves, stems, damp sand, cotton, and filter paper. In nature, females oviposit on the soil surface at the base of plants. The general life cycle of *B. collaris* under laboratory conditions fed tallow was about 45 days from egg to adult (9 days from egg to larva, 21 days from larva to pupa, and 15 days for pupa to adults; Figure 1). Females began oviposition 17 days after eclosion and continued to produce eggs for 6 months.

Quarantine host range tests

A major goal of the testing protocol was to determine whether the mobile larval and adult feeding stages of *B. collaris* can feed and develop on any of the non-target taxa. No-choice, or starvation, tests are considered the most rigorous and conservative test design to define a candidate's fundamental or physiological host range (van Klinken, 2000; Schaffner, 2001). Both adults and larvae were tested in no-choice feeding trials in quarantine. The primary criticism of these tests is that they are too conservative and they provide results that potentially lead researchers to overlook candidates that would be safe to release (Cullen, 1990; Schaffner, 2001). Because of this concern, dual-choice tests on adults and larvae were conducted as they complement no-choice tests to better simulate more natural conditions (Harley, 1969), and may be

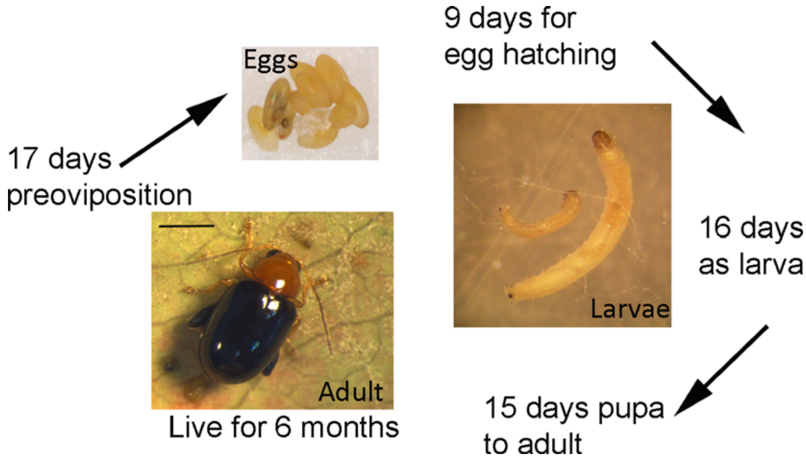

Figure 1 Life history stages of the flea beetle *Bikasha collaris* reared on roots and leaves of *Triadica sebifera* in quarantine USDA/ARS/IPRL (Gainesville, FL, USA). Eggs are usually laid in clusters in soil at the base of plant. Early and late instars tunnel in roots. Scale bar = 1 mm. [Colour figure can be viewed at wileyonlinelibrary.com]

better predictors of risk than other testing methods (Cullen, 1990). Dual-choice tests were conducted on those plant taxa on which adult or larvae fed or were able to survive for prolonged time periods. To add confidence to our results, species closely related to the target were also included in choice tests. The dual-choice tests conducted here were the 'normal choice tests' as they simultaneously exposed the target weed and a single test species (Schaffner, 2001). Furthermore, we conducted oviposition tests in no-choice conditions with naïve adults that had not yet begun to feed. Like with dual-choice tests, the oviposition tests were conducted on a subset of the total non-target plant list, those that were fed on in earlier no-choice tests, or taxa of special concern.

Adult no-choice consumption and longevity test. Consumption of test plants and longevity of *B. collaris* were assessed by no-choice tests. Beetles were offered leaves individually of each of the 77 non-target taxa or a tallow leaf as a paired control. The experimental unit was an adult pair placed in a 30-ml transparent plastic cup containing one cut leaf of each taxon. Five replicates of each taxon were generally included, 10 for the taxa that were close phylogenetic relatives. For taxa with small leaves, more than one leaf may have been used (e.g., *Euphorbia hypericifolia* L.). Adults used for these tests were generally 2 weeks old and had previously fed freely on tallow leaves. Each cup had a layer of moistened sand (10 ml) covered with round filter paper. The petiole of each leaf was inserted into the moistened sand along the edge of the cup. Each cup was sealed with a lid provisioned with a small hole plugged with cotton. Cut leaves were replaced as needed, generally every 2–3 days, and survivorship was recorded 3× each week. All cups were held in the laboratory under ambient conditions of 27 °C, 50% r.h., and L16:D8 photoperiod. Flea beetle consumption was estimated by measuring area consumed after each test. Leaf area was measured with a portable digital microscope (40–140×; IPM Scope Mega Pixel, Spectrum Technologies, Aurora, IL, USA). Each photographic image was processed with Adobe Photoshop Elements which converted the consumption results to mm^2. To ensure the validity of the results, adults fed the tallow leaves continued to be monitored at least 2 weeks after all adults had died on non-target leaves. For comparison, newly emerged control adults ($n = 50$) were fed tallow until death and these longevity results were compared with the longevity of those fed non-targets by a Student's t test with a Bonferroni adjustment. Adult consumption of those fed non-target leaves, that consumed at least 0.5% (0.4 mm^2) of the tallow leaves, were compared by individual one-way ANOVAs to the paired tallow control ($\alpha = 0.05$).

Larval no-choice survivorship test. To determine survival of *B. collaris* larvae fed the roots of each of the 77 non-target taxa, recently emerged (<12 h old) neonates were tested in no-choice studies. Eggs were monitored visually to ensure that all had hatched within the previous 12 h. Larvae fed each non-target taxon were paired with control larvae fed tallow roots. Generally at least five replicates of each taxon were assessed. Tests for plant taxa that were close phylogenetic relatives, members of the Hippomaninae subtribe, were replicated 10×. Each replicate or experimental unit consisted of five larvae placed together in a Petri dish (5.5 cm diameter) lined with a moistened absorbent cotton pad, over which lay a moistened filter paper. In a few examples [e.g., *Euphorbia conferta* (Small) B.E. Sm.], due to plant scarcity, we were unable to cultivate and test all the planned replicates. Each Petri dish contained three sections of roots, that were cut from a single plant. Only apical roots of about 2.5 cm long that did not appear woody or damaged were used. Roots with a similar diameter as larvae were used as these seemed to be preferred. Each Petri dish top was sealed to its base with a cotton string that maintained moisture and prevented larval escape. All dishes were placed in a darkened container in the laboratory (25–27 °C, 55–65% r.h.) to simulate underground light conditions.

Each test was checked 3× per week to ensure that fresh roots were available. New root sections were added and old root sections discarded each time the test was checked. Tests were monitored until all larvae died or until adults emerged. Percent survival to adult was calculated. The paired control larvae were followed concurrently and the same data were collected. Survival data for larvae fed non-target roots were compared with data from the paired tallow-fed larvae with logistic regression. Those non-target taxa that were visibly damaged by root feeding, where larval development occurred, or in some cases taxa closely related to the target, were later tested in dual-choice larval tests.

Adult dual-choice feeding test. The purpose of this test was to determine how naïve adults would feed given a choice between the leaves of each non-target and tallow. Adults tested in no-choice (see below), previously fed on tallow, fed on two non-target species (*D. fruticosa* and *G. lucida*) and to a lesser extent on *H. mancinella*, *E. hypericifolia*, and *M. esculenta*.

The general methods of this dual-choice adult test were similar to the adult no-choice test described above. The experimental unit consisted of clear plastic square containers (300 ml) that held two 30-ml cups, one for the non-target and one for tallow. Each 30-ml cup was lined as before with moistened sand that was covered with a piece of filter paper. A single leaf was inserted into each container so that the petiole projected down between the edge of the filter paper and the wall of the cup and into the moistened sand. Each test arena had a lid with two small holes plugged with cotton for access and ventilation. One naïve male and female pair was added to each container and allowed to select between the two leaves. Each adult choice test was replicated at least 5×.

Data were collected on consumption by each adult pair as described above. The test was ended 2 weeks after the initiation date if no feeding occurred on the non-target. If beetles fed on the non-target leaves, the test was extended to 4 weeks after the initiation date. These termination times were selected to expedite testing as adults are relatively long-lived. The amount of tallow and the non-target leaf consumed was compared with individual ANOVA for each non-target taxon.

Larval dual-choice feeding test. Few larvae survived to the adult stage in previous no-choice tests when fed non-target taxa (see Results). The non-target taxa *H. mancinella* and *Ricinus communis* L. were further tested in dual-choice larval tests. Additional taxa selected for dual-choice testing included eight non-targets, *C. variegatum*, *Drypetes lateriflora* (Sw.) Krug & Urb., *G. lucida*, *Heterosavia bahamensis* (Britton) Petra Hoffm., *Hura crepitans* L., *Jatropha podagrica* Hook., *Manihot grahamii* Hook., and *S. bilocularis.*

Similar methods as for the larval no-choice tests were used for larval dual-choice tests. As previously, a Petri dish was the experimental unit and each was replicated 5×. Each Petri dish contained five newly emerged neonates which were presented with a choice between roots of tallow and a non-target. Each Petri dish was split in half by a line drawn on the filter paper. One side contained three 3-cm-long sections of roots from the control and the other half contained a similar amount of roots from the non-target. Each Petri dish half was provisioned with roots from an individual plant. All dishes were placed in a darkened container in the laboratory to simulate underground lighting conditions (25–27 °C, 55–65% r.h.). Tests were checked every 2–3 days when the roots were replaced and data were collected on larval location and feeding. At each observation the larvae were counted on the tallow side and on the non-target side of the Petri dish. Larval root feeding damage was scored as absent (0), few feeding spots (nibbling; 1), minor tunneling (2), or extensive tunneling (3). The position and feeding damage for larvae fed tallow and the non-target roots were compared with individual one-way ANOVA for each non-target taxon.

Adult no-choice oviposition/multiple generation test. The nutritional value of plant taxa, specifically non-targets, may limit the ability of adult females to feed, mature, and produce eggs (Awmack & Leather, 2002). The ability of adult females to produce eggs when fed only non-target plants was assessed. In addition, the number of *B. collaris* generations that could be sustained on non-target taxa was observed. Oviposition was assessed on the same non-target plants used in the adult choice tests. Possibly naïve females, those that had not fed on tallow previously, were unable to mature eggs and oviposit in a no-choice situation with non-targets. Above, no-choice tests were conducted with 2-week-old adults previously fed tallow. The no-choice oviposition/multiple generation test follows the methods described above for adult no-choice tests but used a male-female pair of recently emerged naïve adults. These adults were placed in a 30-ml cup with an excised leaf. A cup was the experimental unit and was replicated 5× per non-target taxon. The number of eggs produced was monitored 3× per week until all adults died in the non-target leaf treatment. Cut leaves were replaced as needed generally every 3 days. All cups were held in the lab under ambient conditions (27 °C, 50% r.h., and L16: D8 photoperiod). Adults were monitored in the tallow control containers for 2 weeks after all individuals died in non-target containers. The number of eggs produced on tallow and the non-target leaves was compared with individual one-way ANOVA for each non-target taxon.

Results

Adult no-choice consumption and longevity test

With few exceptions, none of the non-target taxa were significantly consumed by adult *B. collaris*. These no-choice tests were conducted with 2-week-old adults that had previously been fed tallow leaves. Overall consumption in tests by adults fed control tallow leaves was 126.8 ± 15.6 mm^2 (mean ± SE) and adults consumed only a few non-target taxa (Table 1). The exceptions included eight taxa: *Breynia disticha* J.R. Forst. & G. Forst., *D. fruticosa*, *Euphorbia cyathophora* Murray, *E. hypericifolia*, *G. lucida*, *Jatropha multifida* L., *M. esculenta*, and *R. communis*. Individual comparisons with their paired control indicated that *B. collaris* adult consumption of these non-targets was significantly less than that of tallow for all non-targets except for those fed *G. lucida* (Table 2).

Adult beetles lived significantly longer when fed tallow leaves (80.8 ± 14.1 days) than when fed any of the non-target leaves (Table 2). Adults fed *G. lucida* leaves lived the longest (37.6 days) of all non-target taxa (Table 1).

Larval no-choice survivorship test

With only two exceptions, all larvae died within 5 days when fed the non-target taxa (Table 1). These two exceptions included larvae fed roots of *H. mancinella*, where three larvae of 80 emerged as adults, and *R. communis*, where two larvae of 50 emerged. These five adults all died within 3 days and did not reproduce. The surviving larvae

Table 1 Mean adult consumption (mm^2), adult longevity (days), and larval survival (%) of *Bikasha collaris* when fed non-target or target Chinese tallowtree (tallow) leaves in no-choice tests. Each adult replicate represents a male and female pair in a 30-ml plastic cup with an excised leaf. Each larval test included five larvae per replicate fed excised roots

Plant species	Adult consumption (mm^2)			Adult longevity (days)[3]	Larval survival (%)		
	n	Non-target	Tallow		n	Non-target	Tallow
Category 1 – Genetic types of tallow found in North America							
Malpighiales							
Euphorbiaceae							
Triadica sebifera (L.) Small	–	–	126.8[2]	80.8			
Category 2 – Species in the same (or closely related) genus as tallow							
Malpighiales							
Euphorbiaceae: subfamily Eurphorbioideae, tribe Hippomaneae, subtribe Hippomaninae							
Sapium laurifolium (A. Rich.) Griseb.	10	0	88.2	11.1	10	0	30.0
Sapium laurocerasus Desf.	10	0	158.4	12.3	10	0	37.1
Category 3 – Species in other genera in the same family as tallow							
Malpighiales							
Euphorbiaceae: subfamily Euphorbioideae, tribe Hippomaneae, subtribe Hippomaninae							
Ditrysinia (= *Sebastiania*) *fruticosa* (Bartram) Govaerts & Frodin	12	50.3[1]	251.7	12.3	10	0	48.0
Gymnanthes lucida Sw.	11	406.5	257.4	37.6	10	0	60.0
Sebastiania bilocularis S. Watson	10	0	97.2	9.3	10	0	51.1
Stillingia sylvatica L.	12	0.2	246.1	14.0	10	0	74.0
Euphorbiaceae: subfamily: Euphorbioideae, tribe Euphorbieae, subtribe Euphorbiinae							
Euphorbia (= *Chamaesyce*) *conferta* (Small) B.E. Sm.	2	0	150.4	6.5	1	0	80.0
Euphorbia (= *Poinsettia*) *cyathophora* Murray	5	1.0[1]	116.8	10.2	5	0	48.0
Euphorbia graminea Jacq.	5	0	99.4	8.4	5	0	84.0
Euphorbia graminea Jacq. 'Diamond Frost'	5	0	113.8	9.1	5	0	56.0
Euphorbia (= *Poinsettia*) *heterophylla* L.	5	0	93.8	9.7	5	0	52.0
Euphorbia (= *Chamaesyce*) *hirta* L.	5	0	164.1	7.6	5	0	56.0

Table 1. Continued

Plant species	Adult consumption (mm²) n	Adult consumption Non-target	Adult consumption Tallow	Adult longevity (days)[3]	Larval survival (%) n	Larval survival Non-target	Larval survival Tallow
Euphorbia (= *Chamaesyce*) *hypericifolia* L.	10	14.4[1]	190.1	10.2	5	0	56.0
Euphorbia (= *Chamaesyce*) *hyssopifolia* L.	5	0	97.1	9.6	4	0	68.0
Euphorbia (= *Chamaesyce*) *maculata* L.	5	0	116.4	10.2	4	0	68.0
Euphorbia milii Des Moul.	5	0	137.5	9.2	5	0	80.0
Euphorbia (= *Chamaesyce*) *pinetorum* Small	5	0	88.5	11.0	5	0	65.0
Euphorbia (= *Poinsettia*) *pulcherrima* Willd. ex Klotzch	5	0	124.1	8.5	5	0	68.0
Euphorbia tirucalli L.	5	0	113.1	9.1	5	0	45.0
Euphorbia (= *Pedilanthus*) *tithymaloides* L.	5	0	90.3	8.5	5	0	40.0
Tribe Hureae							
Hura crepitans L.	5	0	146.0	8.0	5	0	68.0
Subfamily Acalyphoideae, tribe Acalypheae, subtribe Acalyphinae							
Acalypha arvensis Poepp.	5	0	122.7	10.8	5	0	52.0
Acalypha chamaedryfolia (Lam.) Mull. Arg.	5	0.2	122.8	11.3	5	0	52.0
Acalypha gracilens A. Gray	5	0	170.7	8.2	5	0	44.0
Acalypha (= *reptans*) *herzogiana* Pax & K. Hoffm.	5	0	116.1	8.4	5	0	66.7
Acalypha ostryifolia Riddel ex J.J. Coult	5	0	104.8	10.0	5	0	52.0
Acalypha wilkesiana (= *amentacea* ssp. *wilkesiana*) Mull. Arg.	5	0	129.1	9.2	5	0	52.0
Subtribe Ricininae							
Ricinus communis L.	12	3.8[1]	213.0	15.3	10	4[4]	54.0
Caperonia castaneifolia (L.) A. St.-Hil.	5	0.3	97.0	6.5	5	0	76.0
Caperonia palustris (L.) A. St.-Hil.	5	0	70.7	7.0	5	0	64.0
Tribe Plukenetieae, subtribe Dalechampiinae							
Dalechampia scandens L.	5	0	97.0	8.1	5	0	52.0
Subfamily Crotonoideae, tribe Aleuritideae, subtribe Aleuritinae							
Vernicia (= *Aleurites*) *fordii* (Hemsl.) Airy Shaw	5	0.2	127.6	9.1	5	0	52.0
Tribe Codiaeae							
Codiaeum variegatum (L.) Rumph. ex A. Juss. 'Mammy'	5	0	156.8	9.1	5	0	64.0
Codiaeum variegatum (L.) Rumph. ex A. Juss. 'Petra'	5	0	95.6	9.6	5	0	68.0
Tribe Crotoneae							
Croton alabamensis E.A. Sm. ex Chapm.	5	0	137.1	9.4	5	0	44.0
Croton argyranthemus Michx.	5	0	227.1	10.5	5	0	60.0
Croton glandulosus L.	5	0	124.0	12.5	5	0	44.0
Croton linearis Jacq.	5	0	151.0	10.5	5	0	40.0
Croton punctatus Jacq.	5	0	101.0	8.8	6	0	60.0
Tribe Jatropheae							
Jatropha curcas L.	5	0	129.8	9.1	5	0	64.0
Jatropha gossipyfolia L.	5	0	91.0	9.0	5	0	48.0
Jatropha integerrima Jacq.	5	0	97.7	9.9	5	0	68.0
Jatropha multifida L.	5	0.4[1]	84.5	9.9	5	0	55.0
Jatropha podagrica Hook.	5	0	97.2	8.3	5	0	56.0
Tribe Manihoteae							
Cnidoscolus urens (= *stimulosus*) (L.) Arthur	5	0	100.8	9.7	5	0	68.0
Manihot esculenta Crantz	5	2.2[1]	79.7	10.0	5	0	52.0
Manihot grahamii Hook.	5	0	79.8	10.0	5	0	64.0
Category 4 – Threatened and endangered species in the same family as tallow							
Hippomane mancinella L.	10	0.1	118.3	9.9	15	4[4]	69.3
Euphorbia telephioides Chapm.	5	0	68.8	7.3	5	0	40.0

Table 1. Continued

Plant species	Adult consumption (mm²)			Adult longevity (days)[3]	Larval survival (%)		
	n	Non-target	Tallow		n	Non-target	Tallow
Ditaxis argothamnoides (= *Argythamnia blodgettii*) (Bertero. ex Spreng.) Radcl.-Sm. & Govaerts	4	0	69.0	7.3	4	0	50.0
Tragia saxicola Small	5	0	126.9	8.9	5	0	70.0
Croton humilis L.	5	0	70.7	7.0	5	0	64.0
Manihot walkerae Croizat	5	0	70.7	7.0	5	0	68.0
Heterosavia (= *Savia*) *bahamensis* (Britton) Petra Hoffm.	5	0	118.3	10.6	6	0	53.3
Drypetes diversifolia (Sw.) Krug & Urb.	5	0	97.0	10.2	5	0	36.0
Category 5 – North American or introduced species in other families in the same order that have some phylogenetic, morphological, or biochemical similarities to tallow							
Phyllanthaceae: tribe Bischofieae							
Bischofia javanica Blume	5	0	118.6	10.6	6	0	50.0
Tribe Phyllantheae							
Breynia disticha J.R. Forst. & G. Forst.	5	1.4[1]	190.1	6.8	5	0	48.0
Tribe Phyllantheae, subtribe Flueggeinae							
Flueggea virosa (Roxb. ex Willd.) Royle	5	0	181.2	11.0	5	0	60.0
Glochidion puberum (L.) Hutch.	5	0	181.2	11.2	5	0	72.0
Phyllanthus acidus (L.) Skeels	5	0	147.6	7.2	5	0	48.0
Phyllanthus amarus Schumach. & Thonn.	5	0	159.5	11.3	5	0	40.0
Phyllanthus pentaphyllus C. Wright ex Griseb.	5	0	83.7	9.6	5	0	72.0
Phyllanthus tenellus Roxb.	5	0	114.7	10.3	5	0	64.0
Phyllanthus urinaria L.	5	0	113.1	10.9	5	0	44.0
Tribe Poranthereae							
Phyllanthopsis (= *Leptopus*) *phyllanthoides* (Nutt.) Voronts. & Petra Hoffm.	5	0	146.0	6.9	5	0	70.0
Putranjivaceae							
Drypetes lateriflora (Sw.) Krug & Urb	5	0	91.2	9.3	5	0	60.0
Category 6 – North American or introduced species in other orders that have some phylogenetic, morphological, or biochemical similarities to tallow							
Rosales							
Rosaceae							
Prunus caroliniana Aiton	5	0	115.9	9.1	5	0	44.0
Eriobotrya japonica (Thunb.) Lindl	5	0	181.2	11.4	5	0	76.0
Sapindales							
Rutaceae							
Citrus × *aurantium* L.	5	0	104.8	8.2	5	0	64.0
Citrus jambhiri Lush.	5	0	104.8	8.9	5	0	56.0
Myricales							
Myricaceae							
Morella (= *Myrica*) *cerifera* (L.) Small	5	0	113.4	8.6	4	0	76.0
Cyperales							
Poaceae							
Saccharum officinarum L.	5	0	110.0	9.1	5	0	56.0
Lamiales							
Verbenaceae							
Vitis rotundifolia Michx.	5	0.3	123.8	8.9	5	0	52.0
Myrtales							
Lythraceae							
Lagerstroemia indica L.	5	0	138.9	8.7	5	0	80.0
Lagerstroemia (*indica* × *fauriei*) 'Natchez'	5	0	131.3	10.2	5	0	60.0

Table 1. Continued

Plant species	Adult consumption (mm^2) n	Non-target	Tallow	Adult longevity (days)[3]	Larval survival (%) n	Non-target	Tallow
Euphorbiales							
Buxaceae							
Pachysandra procumbens Michx.	5	0	181.2	14.1	5	0	76.0
Illiciales							
Illiciaceae							
Illicium parviflorum Michx. ex Vent.	5	0	135.3	10.1	5	0	52.0

[1]*Bikasha collaris* adult consumption significantly different from the control. See Table 2 for ANOVA results.
[2]Overall mean adult consumption averaged over all tests.
[3]Longevity of adults fed non-target taxa was compared with tallow-fed adults.
[4]Survival of larvae fed *H. manchinella* (χ^2 = 40.2) and *R. communis* (χ^2 = 18.5, both d.f. = 1, P<0.0001) were different from those fed tallow.

Table 2 ANOVA statistics for consumption and longevity of *Bikasha collaris* adults examined in no-choice tests for those fed non-targets and Chinese tallowtree leaves

Species	Adult consumption F	d.f.	P	Adult longevity t	P
Bryenia disticha	83.13	1,8	<0.0001	121.11	<0.0001
Ditrysinia fruticosa	21.31	1,22	<0.0001	62.65	<0.0001
Euphorbia cyathophora	102.74	1,8	<0.0001	53.69	<0.0001
Euphorbia hypericifolia	48.15	1,18	<0.0001	40.01	<0.0001
Gymnanthes lucida	2.13	1,20	0.16	5.02	<0.0001
Jatropha multifida	311.04	1,8	<0.0001	73.00	<0.0001
Manihot esculenta	61.06	1,8	<0.0001	59.84	<0.0001
Ricinus communis	136.35	1,22	<0.0001	22.53	<0.0001

on *H. mancinella* and *R. communis* developed slower – 48.3 and 61.5 days to reach the adult stage, respectively – compared with those fed the tallow control roots (39.0 days). No statistical analysis was conducted on these development time results as the number of surviving individuals on the non-targets was very small. Additionally, some larval nibbling was seen on roots of *D. fruticosa*, *G. lucida*, *S. bilocularis*, and *S. sylvatica* but no larvae completed development to the pupal or adult stages on these non-targets (Table 1).

Adult dual-choice feeding test

When given a choice, naïve *B. collaris* adults selected tallow for feeding and consumed little of the non-target leaves (Figure 2). Adult consumption was significantly greater on tallow (125.1–195.8 mm^2) than on any of the non-target leaves (0–14.6 mm^2). The greatest amount of non-target leaves consumed was on *D. fruticosa* and *G. lucida* but consumption was 7.4 and 6.1% of the amount eaten on the corresponding control leaves, respectively. On average, the amount eaten of *H. mancinella*, *E. hypericifolia*, and *M. esculenta* leaves was <1% of the amount eaten of control leaves.

Larval dual-choice feeding test

Naïve larvae were significantly more frequently found on the tallow side compared with the non-target side of each Petri dish (Figure 3A). Overall, 3.2 ± 0.2 (mean ± SE) of the five larvae were found on the tallow side, compared with 0.3 ± 0.1 on the non-target side. Feeding categories on the control tallow roots in all tests were minor or extensive tunneling (larval root feeding damage score: 2.7 ± 0.1). However, larvae avoided roots or nibbled on non-target taxa (0.1 ± 0.1; Figure 3B). The larval feeding scores were significantly greater on tallow than on any of the non-target taxa (Figure 3B).

Adult no-choice oviposition/multiple generation test

When *B. collaris* were fed tallow, egg production ranged from an average of 45.6 to 115.0 eggs per

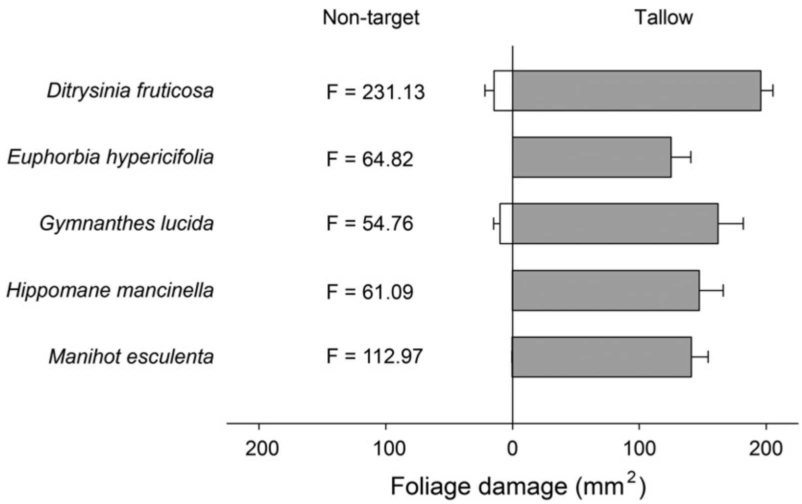

Figure 2 Mean (+ SE; n = 5) adult *Bikasha collaris* consumption (mm^2) from choice tests between leaves of Chinese tallowtree (tallow) and each of five non-target species. Adult consumption was significantly greater on tallow in each comparison (ANOVA: all d.f. = 1,8 and P<0.0001).

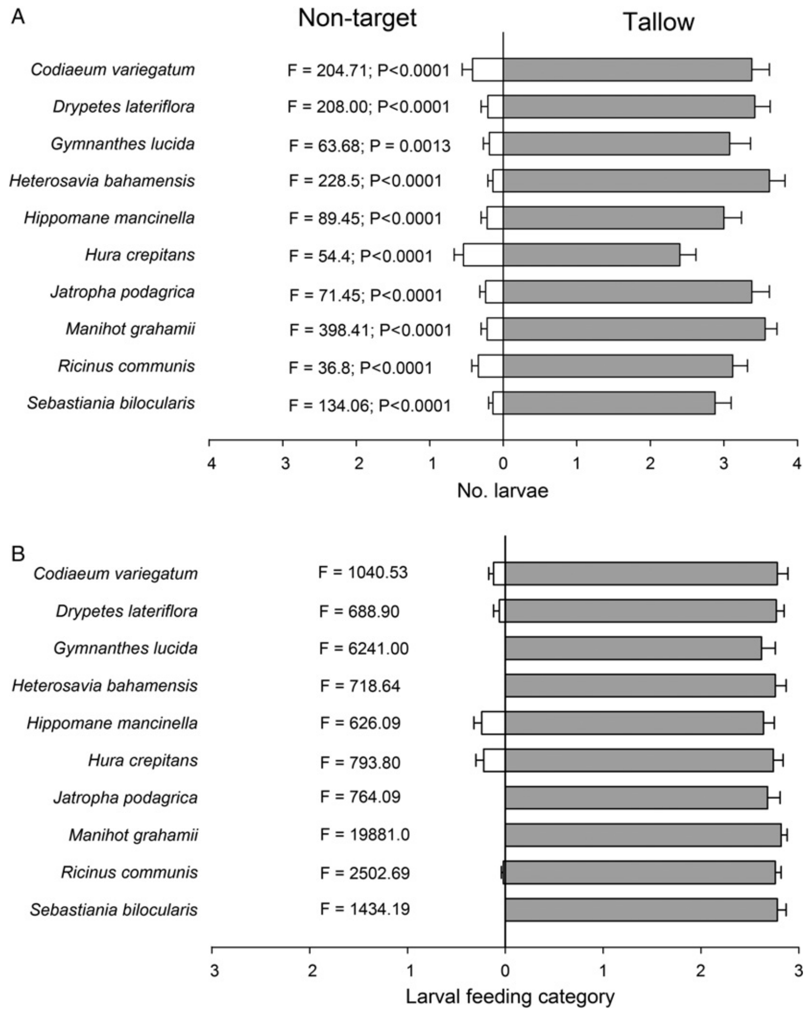

Figure 3 Mean (+ SE; n = 5) (A) number of *Bikasha collaris* larvae recorded on each side of Petri dishes and (B) feeding damage category [0, no feeding; 1, nibbling (few feeding spots); 2, minor tunneling; 3, extensive tunneling] from choice tests between roots of Chinese tallowtree (tallow) and each of 10 non-target species. Significantly more larvae (ANOVA: all d.f. = 1,8) and greater damage (ANOVA: all d.f. = 1,8 and P<0.0001) were found on the tallow roots than on the non-target roots.

female (Table 3). When naïve adults were fed any of the non-target leaves, only one pair produced eggs and that pair were fed *G. lucida* leaves. Of the 23 eggs produced, only eight hatched. The eight emerging larvae all died after 1 day. Hence, successive generations could not be sustained.

Table 3 Mean (± SE; n = 5) number of eggs oviposited by *Bikasha collaris*-naïve adults in no-choice tests on excised young leaves of Chinese tallowtree (tallow) or a non-target taxon

	Oviposition		ANOVA	
Non-target species	Non-target	Target (tallow)	$F_{1,8}$	P
Ditrysinia fruticosa	0	98.0 ± 50.9	117.15	<0.0001
Gymnanthes lucida	4.6 ± 4.6	96.2 ± 52.4	11.60	0.0093
Hippomane mancinella	0	90.4 ± 54.2	22.87	0.0014
Euphorbia hypericifolia	0	115.0 ± 61.0	20.33	0.002
Manihot esculenta	0	45.6 ± 10.9	130.13	<0.0001

Discussion

In total 77 plant taxa were tested in USA quarantine to determine the host range of *B. collaris*. The plant taxa included seven species from Hippomaninae, the subtribe that includes tallow, and 23 taxa from the Euphorbioideae subfamily (to which the Hippomaninae belong). Moreover, we tested numerous taxa from the other two major subfamilies, Crotonoideae and Acalyphoideae. In larval and adult no-choice (starvation) tests the flea beetle demonstrated a high degree of specificity toward the target weed, tallow. Complete larval development only occurred on *H. mancinella* (three of 80 larvae matured) and *R. communis* (two of 50 larvae matured). However, the five emerging adults from these two non-target taxa only lived a few days and did not reproduce. No-choice tests of young adults that had previously fed on tallow ate the leaves of *D. fruticosa* and *G. lucida*. When the adults were given a choice between tallow and these non-targets, a small amount of feeding, less than 7.5% of that on the control, occurred on *D. fruticosa* and less occurred on *G. lucida* (6.1% of the control). The no-choice oviposition test indicated that eggs were laid primarily after feeding on the weed (on average 45 eggs or more) and a few (on average 4.6 eggs) were laid after feeding on *G. lucida*. However, only a few of these eggs hatched and the resulting larvae died while eating the roots of *G. lucida*. These results indicate that although a small amount of adult feeding may occur on *D. fruticosa* and *G. lucida*, *B. collaris* cannot sustain a population on any of the non-target taxa tested. These results confirm the specificity of *B. collaris* found from laboratory and field host-range tests conducted in China (Huang et al., 2011).

The results suggest that, if this flea beetle species is approved for release, the greatest threat to native plants may be adult feeding on *D. fruticosa* and *G. lucida*. The threat to *G. lucida* is minimal as this tropical species is out of the geographic range of tallow (Rawlins et al., 2014; USDA/NRCS, 2016). Moreover, the threat toward *D. fruticosa* and non-targets in general will be temporary as *B. collaris* needs tallow to complete development. Additional research was conducted to examine the ability of *B. collaris* adults to spillover and damage non-targets with and without a previous tallow meal. These studies supported the results presented here and demonstrated that *B. collaris* adults were dependent upon a tallow meal for longevity, egg production, and survival (Wheeler et al., 2017).

The two most damaged non-target taxa by *B. collaris* adults were *D. fruticosa* and *G. lucida*. Both non-target taxa are close relatives of tallow and are natives of the southeastern USA. Another important close relative, *H. mancinella*, was not damaged by adults but three of 80 larvae matured when fed roots of this non-target species. This south-Florida native was tested repeatedly here as it is a state listed endangered species (Coile & Garland, 2003; Weaver & Anderson, 2010). The ranges of both *G. lucida* and *H. mancinella* (south Florida and the Caribbean) are more than 300 km distant from the invasive range of tallow (Rawlins et al., 2014; USDA/NRCS, 2016). Another species slightly damaged by adults, *R. communis*, is an invasive exotic species in the USA. A combined summary of these results indicated that none of the non-target taxa constitute an alternate host for *B. collaris*.

Bikasha collaris is anticipated to have little direct negative impact on native and economic flora in the invaded range of tallow, while having the potential to severely decrease the ability of tallow to regenerate and spread. Adult flea beetle feeding decreased above-ground plant biomass while larval feeding decreased below-ground tallow biomass (Huang et al., 2012). Above-ground damage by adults may increase below-ground larval damage and together decrease the ability of tallow to compete with native vegetation (Huang et al., 2013). Results reported here from a series of no-choice and choice feeding and oviposition tests indicate that this flea beetle is highly specific to the target weed. If approved, this will be the first biological control agent released against tallow in the USA. This flea beetle may play an important role and contribute to the integrated control of this invasive weed.

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