Neotropical Entomology
https://doi.org/10.1007/s13744-022-00948-9

SCIENTIFIC NOTE

Distinguishing Symbiotic Partners of *Acropyga* Ants from Free-Living Soil Inhabitants

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Received: 1 December 2021 / Accepted: 31 January 2022

Abstract

The fruitful study of associations between ants and scale insects yields insight into the mechanisms that shape these symbioses. Field collections provide the basic information linking partnered species, and as such it is critical that collection techniques from the field reflect true species-to-species partnerships in the published literature. It is equally critical that such practices limit the potential for mistaking free-living "neighbors" for symbiotic partners and publishing erroneous associations. This article describes a protocol for collecting subterranean scale insects and associated *Acropyga* Roger ants, which relies upon the activity of worker ants to sort and distinguish symbionts from free-living scale insects that happen to live near the colony. By collecting samples of ants and scales into nest boxes and allowing a resting period of several hours, worker ants will gather symbiotic partners into dense, protected clusters in which symbionts are actively tended. Free-living scale insects neighboring the colony can be collected from soil along with colony samples, but these free-living individuals are excluded from protective clusters and ignored by workers. Following confirmation of ant attendance, true symbiotic partners can be confidently collected, preserved, and recorded for future study. We illustrate the value of employing this collection protocol using a case study from Peru.

Keywords Coccoidea · Coccomorpha · mealybug · Rhizoecidae · trophobiosis · Xenococcidae

Introduction

Several groups of ants specialize in pastoral mutualisms by caring for and herding populations of phytophagous insects like livestock. Ants receive nutritive rewards from their partner in exchange for providing services, such as protective dwellings and defense from natural enemies. Collectively, these relationships have been referred to as trophobioses (Delabie 2001; Hölldobler and Wilson 1990).

The trophobiotic relationships that transpire between *Acropyga* Roger ants and scale insects are an intriguing case study in the evolution of mutualisms. The primary group of associates, root mealybugs belonging to the family Xenococcidae Tang (Schneider and LaPolla 2011; Williams 1998, 2004b), have a shared history of obligate association with *Acropyga* extending back 15–30 million years based on fossil records (Johnson et al. 2001; LaPolla 2005) and divergence dating estimates (Blaimer et al. 2016). The two insect lineages are co-distributed across the tropics and some subtropical regions of the world (LaPolla 2004). The xenococcids, as their name implies, are highly modified morphologically and are seemingly well adapted to life with ants. For example, they have lost the wax-producing structures that are a defining feature of scale insect ecology (Gullan and Kosztarab 1997). The bond uniting ant and scale partners is reinforced through their unique mode of colony foundation involving vertical transmission of scales across generations, referred to as trophophoresy (LaPolla et al. 2002). An alate *Acropyga* queen will transport a gravid female scale insect, taken from the natal colony, on her nuptial flight and subsequently to their new nest (LaPolla and Spearman 2007). Both the ant queen and female scale are foundresses, each establishing a new colony comprised of their offspring. The fidelity between

Edited by Heraldo Vasconcelos

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these partners has given rise to a unique and long-lasting mutualism.

But partner fidelity is rarely absolute, and a few other secondary lineages of scale insects have come to be associated with *Acropyga* ants (Schneider and LaPolla 2020; Williams 1998). Most belong to another family of root mealybugs, the Rhizoecidae Williams, while some few are more distantly related, such as the ortheziid, *Acropygorthezia williamsi* LaPolla & Miller (LaPolla et al. 2008). The origins of these independent associations began with a horizontal transmission event (Page 2003), in which a novel scale insect partner was acquired from a free-living ancestor and their offspring were subjected to the same process of vertical transmission over generations. Interesting information can be gleaned from these independently evolved associations: the frequency of horizontal shifts, the phylogenetic limits of successful shifts, radiations of lineages following association and the impact on diversification rates, speciation rates, anatomical adaptations, and so forth.

However, to further complicate the matter, other free-living hypogaeic scale insects are often found to reside alongside *Acropyga* nests (Schneider and LaPolla 2020). For the purposes of this article, we refer to them as neighbors. These "neighbors" are likely to be common soil inhabitants, and they may gain an indirect benefit from the defensive services and niche construction that an *Acropyga* colony offers their direct associates (trophobionts). Thus, they may be facultatively commensal to the relationship between the ant colony and their trophobionts. But the neighbors are also potential associates themselves; they produce honeydew just like associated populations, although perhaps of lesser quality or appeal to the ants (Fischer and Shingleton 2001; Völkl et al. 1999). And such neighboring scale communities were likely the source of contemporary secondarily associated lineages (e.g., those belonging to Rhizoecidae), acquired through past horizontal transmission events.

Therefore, it is crucial to carefully distinguish between scale populations that are living in direct association with *Acropyga* and local communities that simply neighbor their colonies. Everything that is known about *Acropyga*/scale relationships began with careful fieldwork. Determining which species are involved in direct mutualism with *Acropyga* informs the patterns and processes of mutualism discussed above, but erroneous records are misleading in these studies. Unfortunately, several speculative and potentially erroneous associations have already been reported in the literature (discussed in detail in Schneider and LaPolla 2020). The purpose of this paper is to provide a field protocol for collecting *Acropyga* colonies and verifying direct species-to-species associations. This protocol aims to prevent erroneous records of trophobiosis from being introduced into the literature. This work acknowledges and emphasizes the critical role that basic field collection plays in descriptive studies of mutualisms.

Methods

Locating colonies

Certain environmental conditions seem to be favorable to *Acropyga* as nesting sites; focusing on these parameters can help narrow the search for their subterranean colonies. *Acropyga* workers are often captured in leaf-litter samples (Ward 2000), which could be a useful first step in identifying the approximate location of a colony. In forests, nests are frequently located along the sides of large tree buttresses, under shallowly buried rocks, and within felled decomposing logs that are partially buried and permeated with roots. In grasslands, nests can be found under rocks or on rootlets of large tufts of grass, by digging around the grass crown and removing the tuft. Nests are often located near the soil surface, roughly 5–30 cm deep (Kishimoto-Yamada et al. 2005; LaPolla et al. 2002; Schneider and LaPolla 2020). However, nest depth varies with overall soil moisture. In dry conditions, *Acropyga* tend to nest deeper in the ground as the top layers of soil dry out, making it quite difficult to locate colonies. Even in tropical rainforests, *Acropyga* nests are harder to find during dry periods. Nest chambers tend to be diffuse, with just a few workers, brood, and scales present in each chamber. Occasionally, however, their nests can be densely concentrated with many ants and scales present in a small area. Scales are both housed in nest chambers and located along roots.

Agricultural fields are often excellent habitat for finding *Acropyga* nests, which have been collected in areas of cultivated banana, cacao, coconut, coffee, grapevines, and sugarcane (Balachowsky 1957; Beardsley 1970; Caballero et al. 2019; Williams 1970, 2004a; Williams and Granara de Willink 1992). Coffee and cacao plantations are especially productive places to search for *Acropyga* in the Neotropics, particularly in plantations that have preserved some large native trees and contain a dense leaf litter layer (JSL and SAS personal observation). In India, Deepthy et al. (2017) found numerous collections of *Xenococcus annandalei* Silvestri associated with *Acropyga acutiventris* Roger on tender roots of 18 cultivated crops in mixed agricultural fields. They considered *X. annandalei* to be a devastating pest within these fields.

Mating swarms are the ideal collection for determining direct association as there is no doubt regarding the association between a queen and the scale insect she carries in her mandibles. Unfortunately, the collection of mating swarms depends heavily upon seasonality and serendipity. Reproductive male and female ants typically emerge after a period of rainfall. LaPolla et al. (2002) surmised that the thin cuticle of *A. epedana* Snelling indicates the alates are quite susceptible to desiccation, and so the timing of release is likely dependent upon relative humidity (see also Smith et al. 2007). Mating swarms of *A. arnoldi* Santschi were also collected following heavy rainfall (LaPolla and Spearman 2007).

Collection and observation

The distinction between this protocol and current common practice is the incorporation of an observational period. This is generally applied in the collection of arboreal ants and symbionts (see for example, Dill et al. 2002), but has been less broadly adopted in the collection of hypogaeic ants, where interacting partners are more difficult to observe.

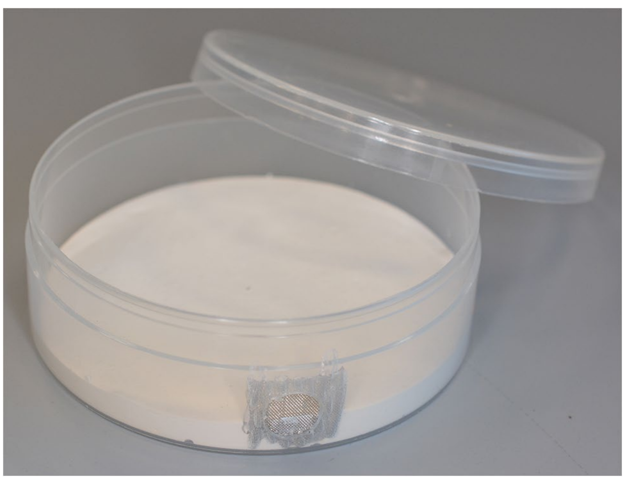
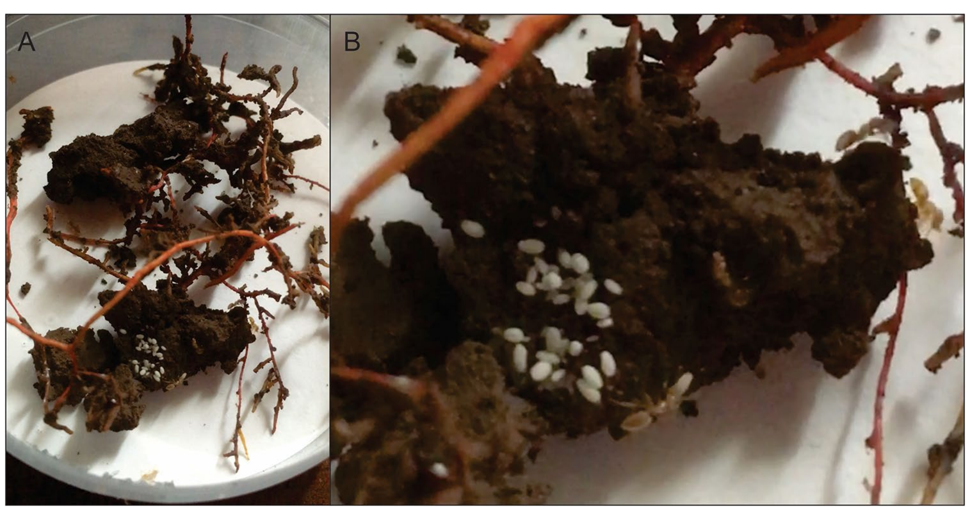


Fig. 1 A nest box for the collection of *Acropyga* colonies and scale insects, a necessary tool for properly vetting direct association between trophobiotic partners through observation

A nest box (Fig. 1) is required for collecting colonies in the field as this facilitates observation of worker and scale interactions. Nest boxes can be constructed from a Nucons round two-piece clear plastic container (LA Container, Yorba Linda, CA, USA) and modified by adding a small hole covered with fine wire mesh affixed to the side for air exchange, and dental cement added to the basin to manage moisture. Prior to a collecting trip, a few drops of water should be added to the bottom of the nest box to moisten the plaster and prevent the nest from desiccating. Species identification is aided by collecting representatives of all life stages. Ants and scales that are found in chambers can be aspirated. Scales found feeding on rootlets can be collected by transferring the rootlet segment into the nest box. Keeping the nest boxes away from direct light with additional soil and rootlets is useful for reducing agitation of the ants and allows the root mealybugs to continue feeding.

It is critical to allow time for the colony to settle into the nest box before making behavioral observations. For a minimum of 2 h, place the colony in a darkened space (e.g., a desk drawer, a bookshelf) and leave the nest box undisturbed. During this period, *Acropyga* workers will actively gather trophobionts into protective clusters or chambers in loose soil (Fig. 2) and they will ignore or exclude non-associated scales from such clusters. Workers may still be observed carrying trophobionts in their mandibles within the nest box after a few hours as well. These behaviors signify direct association and are critical for distinguishing trophobionts from non-associated neighbors of the colony, which may be superficially similar in appearance. Nest boxes can be observed under low-light conditions without the use of equipment or preferably using a dissecting microscope. Small portable options are available for remote fieldwork, such as a Nikon 7314 20 × Field Microscope Mini (Nikon USA, Melville, NY).

Fig. 2 Colony of *Acropyga* and root mealybugs in a nest box after a resting period of several hours. **A** Full-view image of nest box; **B** enlarged view of area where workers have gathered scales into a dense protective cluster alongside a small segment of soil



Acropyga seemingly form aggregates of separate colonies near root systems (Kishimoto-Yamada et al. 2005). Disjunct clusters of scales and signs of aggression among workers may indicate that members of two colonies are present in the nest box along with their respective trophobionts. Conspecific workers from separate nests will often bite and spray when they encounter one another; thus, it is important to observe for these behaviors. Interestingly, in our experience, when two different *Acropyga* species are placed in a nest box, aggression is not usually observed but dominance behaviors may be seen (e.g., one species picking the other up and moving it out of the way, but with no biting or spraying). When more than one colony is present in a nest box, take care in associating their trophobionts to be certain the correct species are collected together.

Preservation

After worker ants have sorted root mealybugs into groups and behavioral observations have been completed, individuals can then be preserved. Collect any suspected non-associated scales into a separate vial; this includes individuals that were left unattended by ants or outside of protective chambers. Collect members belonging to separate colonies, if more than one is present in the nest box, each into their own vials. This assures that only directly associated partners are paired in collection vials and the resulting reports of associations between species are accurate and have been properly vetted.

Case study results and discussion

This protocol was implemented by SAS and JSL on a field collecting trip to Madre de Dios, Peru, in June 2019. Sampling was conducted at sites in Las Cruces, Pantiacolla, and Cocha Cashu Biological Station in Parque Nacional del Manú. The Las Cruces site represented mature cloud forest at higher elevation (1500 m) whereas the Pantiacolla and Cocha Cashu sites were in mature broadleaf rainforest at lower elevations (370–450 m and 300–370 m, respectively). Nests were located mainly along the sides of large tree buttresses. The resulting collections of 25 nests, comprising six species of *Acropyga* and 15 species of scale insects, are summarized in Table 1.

We documented direct association of 10 scale insect species with *Acropyga* colonies and prevented five free-living species from being introduced as erroneous records of trophobiosis. Free-living scales were collected into nest boxes along with ants and their trophobionts from four separate nests (Table 1; PER01-02, PER02-01, PER04-01, PER13-01). If specimens had been collected and directly preserved, without first conducting observations in a nest box, new erroneous records of trophobiosis would have been introduced into the published literature, involving species of *Coccidella*, *Rhizoecus*, *Ripersiella* (Rhizoecidae), and *Jermycoccus* (Ortheziidae). The accumulation of improperly vetted associations in the literature artificially inflates the breadth and frequency of trophobioses reported between *Acropyga* and secondary groups of scale partners (those falling outside of Xenococcidae). Additionally, these records would falsely indicate the presence of multiple trophobiont lineages in a single nest.

Most *Acropyga* colonies (22 of 25) associated with a xenococcid partner in the genus *Neochavesia* Williams & Granara de Willink. A minority of colonies (4 of 25) were confirmed to associate with rhizoecid trophobionts, including at least one colony each of *A.* (poss.) *decedens* (Mayr), *A. fuhrmanni* Forel, and *A. goeldii* Forel (species group) (Table 1). Rhizoecids were the only associates in three of these nests (PER15-01, PER24-01, PER25-01) but a fourth nest contained both rhizoecid and xenococcid trophobionts (PER21-01). Every rhizoecid we collected, with adult females available for species-level identification, represented an undescribed species. We also discovered a new species of *Acropyga* associating with a new *Neochavesia* from a nest in Las Cruces, Peru (PER01-01,-02), and a new species of *Neochavesia* associating with *A. smithii* Forel from a nest at Cocha Cashu (PER09-01).

Overall, our collections indicate high partner fidelity in this region. For example, *A. fuhrmanni* was found associating with *Neochavesia caldasiae* (Balachowsky) in six out of seven nests we collected, and *A. smithii* associated with *N. trinidadensis* (Beardsley) in all nine nests collected (Table 1). However, one of these nests (PER09-01) included a second trophobiont species, a new member of *Neochavesia* (near *caldasiae*). Colonies of *Acropyga guianensis* Weber associated with *N. cephalonodus* Schneider & LaPolla at Pantiacolla, but at Cocha Cashu they associated with *N. weberi* (Beardsley). These records overlap with what has been previously recorded in the literature (Table 2), but one-to-one relationships clearly vary across the geographic range of a species, as demonstrated by *A. fuhrmanni* also associating with *N. eversi* (Beardsley) and *N. trinidadensis* in other areas of its range (Johnson et al. 2001; Williams 2004b).

Our collections include the first confirmed records, to our knowledge, of *Acropyga* colonies tending two scale species in a single nest (Table 1; PER09-01, PER21-01), where no signs of aggression among workers were noted. This observation has interesting implications. They may represent novel partner acquisition events from the local community of neighboring scales, each a potential precursor to new horizontal transmission events. Alternatively, the observation of multiple directly associated scales in one nest may suggest that *Acropyga* colonies are polygynous; some colonies may comprise a blend of multiple trophobiont lineages, each

Table 1 Summary of collections from Madre de Dios, Peru, June 2019. Each nest is labeled with a unique identifier that indicates the country, site number, and nest number (ex: PER01-01). Nests that contained more than one species of scale insect are indicated in bold, with the observation of association indicated as either "trophobiont" or "free-living (neighbor)" also indicated in bold

Ant species	Nest ID	Scale family	Scale species	Association	Latitude/longitude
Acropyga fuhrmanni	PER07-01	Xenococcidae	*Neochavesia caldasiae*	Trophobiont	S11° 53.241, W71° 24.427
	PER08-01	Xenococcidae	*Neochavesia caldasiae*	Trophobiont	S11° 53.24, W71° 24.42
	PER08-02	Xenococcidae	*Neochavesia caldasiae*	Trophobiont	S11° 53.24, W71° 24.42
	PER18-01	Xenococcidae	*Neochavesia caldasiae*	Trophobiont	S11° 53.231, W71° 24.442
	PER20-01	Xenococcidae	*Neochavesia caldasiae*	Trophobiont	S11° 53.486, W71° 24.520
	PER21-01	Rhizoecidae	*Ripersiella* near *andensis* (i)	**Trophobiont**	S11° 53.564, W71° 24.547
		Xenococcidae	*Neochavesia caldasiae*	**Trophobiont**	
	PER26-01	Xenococcidae	*Neochavesia caldasiae*	Trophobiont	S11° 53, W71° 24
Acropyga goeldii group	PER15-01	Rhizoecidae	*Rhizoecus* undescribed	Trophobiont	S11° 53.926, W71° 23.951
	PER25-01	Rhizoecidae	*Ripersiella* undescribed (i)	Trophobiont	S11° 53, W71° 24
Acropyga guianensis	**PER02-01**	Ortheziidae	*Jermycoccus* undescribed	**Free-living (neighbor)**	S12° 39.382, W71° 13.936
		Xenococcidae	*Neochavesia cephalonodus*	**Trophobiont**	
	PER04-01	Rhizoecidae	*Rhizoecus* sp. undet	**Free-living (neighbor)**	S12° 39.382, W71° 13.939
		Xenococcidae	*Neochavesia cephalonodus*	**Trophobiont**	
	PER11-01	Xenococcidae	*Neochavesia weberi*	Trophobiont	S11° 54.010, W71° 24.049
	PER12-01	Xenococcidae	*Neochavesia weberi*	Trophobiont	S11° 54.017, W71° 24.086
Acropyga poss. *decedens*	PER24-01	Rhizoecidae	*Ripersiella* near *andensis* (ii)	Trophobiont	S11° 53, W71° 24
Acropyga smithii	**PER09-01**	Xenococcidae	*Neochavesia* near *caldasiae*	**Trophobiont**	S11° 53, W71° 24
		Xenococcidae	*Neochavesia trinidadensis*	**Trophobiont**	
	PER10-01	Xenococcidae	*Neochavesia trinidadensis*	Trophobiont	S11° 53.269, W71° 24.396
	PER13-01	Rhizoecidae	*Rhizoecus* sp. undet	**Free-living (neighbor)**	S11° 54.033, W71° 24.072
		Rhizoecidae	*Ripersiella* undescribed (ii)	**Free-living (neighbor)**	
		Xenococcidae	*Neochavesia trinidadensis*	**Trophobiont**	
	PER16-01	Xenococcidae	*Neochavesia trinidadensis*	Trophobiont	S11° 53.232, W71° 24.435
	PER17-01	Xenococcidae	*Neochavesia trinidadensis*	Trophobiont	S11° 53.236, W71° 24.443
	PER19-01	Xenococcidae	*Neochavesia trinidadensis*	Trophobiont	S11° 53.481, W71° 24.510
	PER22-01	Xenococcidae	*Neochavesia trinidadensis*	Trophobiont	S11° 53.145, W71° 24.323
	PER23-01	Xenococcidae	*Neochavesia trinidadensis*	Trophobiont	S11° 53.148, W71° 24.334
	PER27-01	Xenococcidae	*Neochavesia trinidadensis*	Trophobiont	S11° 53, W71° 24
Acropyga undescribed	PER01-01	Xenococcidae	*Neochavesia* undescribed	Trophobiont	S13° 03.3, W71° 32.64
	PER01-02	Rhizoecidae	*Coccidella* near *boliviana*	**Free-living (neighbor)**	S13° 03.3, W71° 32.64
		Xenococcidae	*Neochavesia* undescribed	**Trophobiont**	

Table 2 Comparison of field collected associations and records of association from the literature

Ant species	Peru 2019 trophobionts	Published trophobionts	References
Acropyga fuhrmanni	*Neochavesia caldasiae; Ripersiella* near *andensis* (i) (**new record**)	*Neochavesia caldasiae; Neochavesia eversi; Neochavesia trinidadensis*	Johnson et al. 2001; Williams 2004b
Acropyga guianensis	*Neochavesia cephalonodus; Neochavesia weberi* (**new record**)	*Neochavesia cephalonodus; Neochavesia linealuma*	Schneider and LaPolla 2011
Acropyga smithii	*Neochavesia trinidadensis* (**new record**); *Neochavesia* near *caldasiae* (**new record**)	*Neochavesia caldasiae; Neochavesia eversi*	Caballero et al. 2019; Johnson et al. 2001

having been introduced by a different queen via trophophoresy. There is limited and conflicting evidence of polygyny vs. monogyny in the *Acropyga* literature. *Acropyga* nests are often found to have numerous dealate queens residing within (LaPolla et al. 2002; Smith et al. 2007; JSL pers. obs.), which is highly suggestive of polygyny. LaPolla and

Spearman (2007) noted anecdotal evidence of multiple foundresses of *A. arnoldi* establishing nests together as well. But Kishimoto-Yamada et al. (2005) regarded colonies of *A. sauteri* Forel to be monogynous with multiple small colonies forming aggregates around root systems. Our observations from Peru are more suggestive of *Acropyga* colonies being polygynous rather than monogynous. Population-level genetic studies can definitively resolve these unsettled questions. Previously, Schneider and LaPolla (2011) reported the presence of two trophobionts in a nest of *Acropyga lauta* Mann from the Solomon Islands, but it cannot be ruled out that they came from separate but closely situated nests.

Conclusion

Field collections are fundamental to the study of trophobiosis. The protocol described here incorporates a process for vetting trophobioses involving *Acropyga*, or other hypogaeic ants. By allowing workers time to sort their own nest associates, collectors can confidently distinguish between symbionts of the ants and neighboring communities of free-living hypogaeic scale insects. This simple protocol requires minimal equipment but a significant time investment for the collection of nests. However, the overall impact, in terms of quality of the data gathered from field collections and results of subsequent investigations, is worth the investment of time. We strongly encourage anyone interested in *Acropyga* and their symbionts to follow these recommendations when collecting. As illustrated by our sampling in Peru, false records of association with free-living neighbors can quickly accumulate and obscure true patterns of symbiosis unless they are carefully vetted.

Acknowledgements We thank Dr. Roxana Arauco Aliaga for accommodation at Cocha Cashu Biological Station and for assistance with permitting (SERFOR #003620). Special thanks to Dr. Harald Beck (Towson University) and the 2019 Tropical Field Ecology class for allowing SAS and JSL to join in on their trip to Peru. Support for this research was provided by the National Science Foundation (award number 1754242 to JSL and SAS). The research was supported also in part by the U.S. Department of Agriculture, Agricultural Research Service. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture; USDA is an equal opportunity provider and employer.

Author contribution All authors contributed to the study conception and design. Material preparation and data collection were performed by SAS and JSL. The first draft of the manuscript was written by SAS and JS and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Declarations

Conflict of interest The authors declare no competing interests.

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