Ecological Insights and First Confirmed Record of *Ooencyrtus nezarae* (Hymenoptera: Encyrtidae) on Eggs of *Megacopta cribraria* (Hemiptera: Plataspidae) in Tennessee and Georgia[1]

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Abstract The kudzu bug, *Megacopta cribraria* (F.) (Heteroptera: Plataspidae), is native to Asia, was first detected in Georgia, USA, in 2009, and has since been recognized as a damaging pest of soybean (*Glycine max* (L.) Merrill; Fabales: Fabaceae) in several southeastern states. *Megacopta cribraria* dispersed rapidly from 2009 to 2013, after which its spread rate declined sharply. Despite this decline, established populations have remained stable. This decline may be partially attributed to natural enemies of kudzu bug. For example, the exotic egg parasitoids *Ooencyrtus nezarae* Ishii (Hymenoptera: Encyrtidae) and *Paratelenomus saccharalis* (Dodd) (Hymenoptera: Platygastridae) have been occasionally detected in the United States since 2016 and 2013, respectively. We recovered *O. nezarae* from *M. cribraria* eggs collected from wild patches of kudzu (*Pueraria montana* Lour. (Merrill) var. *lobata* (Willd.)) throughout summer 2017. Although the occurrence of *O. nezarae* in southern Georgia has been suggested based on exit holes from kudzu bug eggs, to our knowledge, this report is the first to document and confirm recovery of *O. nezarae* from kudzu bug eggs in both Tennessee and Georgia. In addition, at the time of collection in 2017, this recovery was the first confirmation of this species from kudzu in North America. This early-season natural enemy combined with the later-occurring entomopathogen *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin may reduce and maintain kudzu bug densities, which could lessen economic impacts on soybean producers.

Key Words kudzu bug, biological control, egg parasitoid, kudzu, soybean

The kudzu bug, *Megacopta cribraria* (F.), native to Asia, was first found in the United States in Georgia in 2009 (Eger et al. 2010, Hosokawa et al. 2014). By 2017, it had spread to 14 states as well as the District of Columbia (Megacopta Working Group 2023). *Megacopta cribraria* dispersed rapidly from 2009 to 2013, after which its spread rate declined sharply (109 to 20 km/yr from 2013 to 2014) (Britt 2016, Liang et al. 2019, Megacopta Working Group 2023). Despite this sharp

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decline, established populations have remained stable (Megacopta Working Group 2023). Kudzu bugs can successfully complete development on both kudzu (*Pueraria montana* Lour. (Merrill) var. *lobata* (Willd.)) and soybean (*Glycine max* (L.) Merrill), as well as many documented alternate hosts (Eger et al. 2010; Medal et al. 2013, 2016; Zhang et al. 2012). Although kudzu bug is an occasional pest of soybean in its native range (Suiter et al. 2010), it quickly became an economic pest of soybean in the southeastern United States, where yield losses reached 59% in experimental cage studies (Seiter et al. 2013). On a practical scale, the combined cost of targeted treatment and loss of crop yield to kudzu bug across seven southern states was estimated at >US$4 million in 2016, with >$1.5 million in Tennessee alone (Musser et al. 2017). Since then, the cost and loss of yield to kudzu bugs in established areas have only increased, reaching an estimate of >$7.9 million for 2022 in Tennessee (Musser et al. 2023).

The invasive kudzu bug originated in Japan (Hosokawa et al. 2014, Jenkins and Eaton 2010, Jenkins et al. 2010), where biological control agents have been heavily scouted for possible introduction into the United States. Two species of parasitic wasps have been the focus of these efforts: *Ooencyrtus nezarae* Ishii (Hymenoptera: Encyrtidae) and *Paratelenomus saccharalis* (Dodd) (Hymenoptera: Platygastridae). Both *O. nezarae* and *P. saccharalis* parasitize eggs of *Megacopta* spp. in kudzu and soybean in Japan (Hoshino et al. 2017, Takagi and Murakami 1997). However, *O. nezarae* also has been documented as a parasitoid of eggs belonging to members of several other hemipteran families in its native range (Hirose et al. 1996, Zhang et al. 2005). These alternate hosts include several soybean pests, such as the southern green stink bug, *Nezara viridula* (L.) (Hemiptera: Pentatomidae), and *Riptortus clavatus* (Thunberg) (Hemiptera: Alydidae). Researchers in Brazil investigated *O. nezarae* for potential release as a biological control agent of several stink bug pests in the 1980s (Jones 1988, Kobayashi and Cosenza 1987) and found promising results for its use in integrated control of stink bugs. However, quarantine specialists found its host range too broad to consider its release against kudzu bug within the United States (Ruberson et al. 2013).

Paratelenomus saccharalis has a much more restrictive host range and thus was considered a better candidate for classical biological control of kudzu bug in the United States (Ruberson et al. 2013). This species had been under investigation for potential release as a biological control agent of kudzu bug by the USDA-ARS, National Biological Control Laboratory in Mississippi before separate, serendipitously introduced populations were found in Georgia and Alabama in 2013 (Gardner et al. 2013). *Paratelenomus saccharalis* has been shown to overwinter in Alabama, Georgia, and Florida (Ademokoya 2016, Medal et al. 2015, Tillman et al. 2016), which implies that they would be capable of persisting in the field without requiring annual reintroductions.

Since its initial detection in the United States, *P. saccharalis* has been explored as a control tactic in organic soybean production in Georgia (Tillman et al. 2016). However, the sustainability of *P. saccharalis* as a biological control agent is dubious given low, intermittent recoveries of this parasitoid from kudzu bug eggs in kudzu patches in years following initial detection (Gardner and Olson 2016). Although reduced kudzu bug population densities and oviposition play a role in detection of this parasitoid (Knight et al. 2017), *P. saccharalis* were recovered from kudzu bug eggs in northern Georgia less frequently than from kudzu bug eggs in southern Georgia despite statistically similar egg densities over the course of a 3-yr study (Gardner and Olson 2016). No egg parasitoids of kudzu bug were recovered from either yellow pan traps or *M. cribraria* eggs in a 2015 survey of kudzu in east Tennessee (Britt 2016). These observations together suggest that *P. saccharalis* may be viable only in warmer, more tropical regions. Thus, a multi-year research project was initiated to assess presence and distribution of parasitoids of kudzu bug eggs in eastern Tennessee and northern Georgia.

Materials and Methods

Collection of kudzu bug egg masses. Kudzu bug egg masses were collected from kudzu patches at 13 localities in east Tennessee and one locality in northern Georgia (Fig. 1) from June to September in 2016 and 2017. These kudzu patches, which were used in a previous study by Britt (2016) to monitor kudzu bug populations and survey for natural enemies, were located in both urbanized (Knox and Blount counties [TN]) and rural areas (Monroe, McMinn, Polk [all TN], and Murray [GA] counties). In all cases, kudzu patches were located within 1 km of major local highways. No parasitoids were recovered from kudzu bug eggs collected in 2016, so this paper focuses only on the parasitoid recoveries in 2017.

Kudzu bug egg masses were collected from 7 June 2015 to September 2017. Three egg masses containing at least 10 eggs each were collected from each locality on each collection date from 07 June 2017 to 24 August 2017. Collections made from 29 August 2017 to 15 September 2017 were solely opportunistic due to reduced oviposition by *M. cribraria*. Subsequent collections of kudzu bug egg masses were made at the same sites (Fig. 1) beginning on 23 May 2018 to determine whether *O. nezarae* can overwinter in Tennessee and northern Georgia.

All egg masses from the same locality and date were placed in a Petri dish (100 $\times$ 15 mm) containing filter paper moistened with three drops of deionized water and sealed with Parafilm® (Amcor Co., Zurich, Switzerland). Parasitoids present at the time of collection were removed by aspirator to prevent further oviposition and stored in vials containing 95% ethanol. Petri dishes were then stored in an open cardboard box on a bench in the laboratory at ambient temperature (24 $\pm$ 1°C). Dishes were observed a minimum of twice per week until a period of 2 weeks had passed with no parasitoid or kudzu bug emergence. During heavy emergence periods, dishes were monitored daily. Any parasitoids observed were removed by aspirator and stored in 95% ethanol. Total number of eggs, number of parasitoids, number of kudzu bugs, and number of nonproductive (e.g., nonhatched) eggs were recorded.

Parasitoid identification and assessment of sex ratios. Specimens were identified to family by using the key of Goulet and Huber (1993) and to species by using the key of Zhang et al. (2005). Whole specimens of recorded adult parasitoids were then photographed in ethanol by using a dissecting scope. One female and one male each were partially cleared using Andre's agent I before being dissected and mounted onto slides by using Hoyer's medium to facilitate identification of internal structures (Schauff 2001). One additional male and female were photographed using scanning electron microscopy for more detailed images. Voucher specimens were deposited in the Insect Museum at the University of Tennessee, Knoxville.

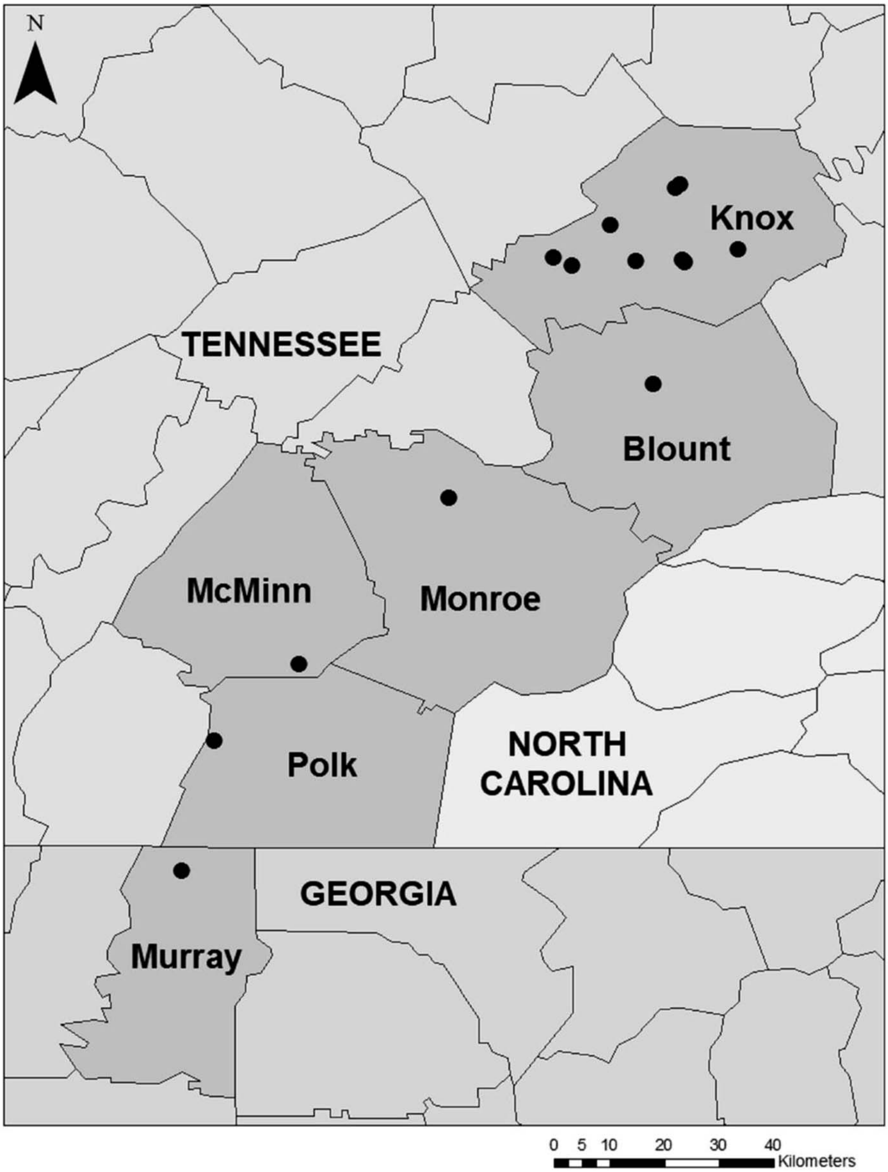

Fig. 1. Localities ($n = 14$) of kudzu bug egg collections from kudzu in eastern Tennessee and northern Georgia, 2017.

Female and male wasps were counted separately to determine a sex ratio for each locality. Whenever possible, sex was determined using sexually dimorphic antennal morphology. However, the heads of several wasps were separated from the body during handling or storage. In these cases, specimens were partially cleared to determine the presence or absence of an ovipositor. A clearing solution of 85% lactic acid (1 ml) was placed into an open-topped glass shell vial (3.7-ml capacity) and heated to 62°C by using a Fisherbrand™ Isotemp hot plate (Fisher Scientific, Waltham, MA). All headless specimens from the same sample locality and date were heated in the solution for 5 min and 30 s before rinsing in deionized water and examined under a dissecting microscope. Because clearing via lactic acid does not progress following removal from heat and rinsing, partially cleared specimens were returned to their sample of origin and stored along with the uncleared specimens in 95% ethanol.

DNA was nondestructively extracted from one female specimen by using the DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. Extraction, polymerase chain reaction amplification, purification, sequencing, and sequence verification/assembly were performed using the protocols outlined in Liu and Mottern (2017). A 592-bp fragment of the large subunit 28S rDNA expansion region D2 (GenBank OR766105) was amplified using the following primer combination: 28S D2-3551-F, 5′-CGG GTT GCT TGA GAG TGC AGC-3′ (modified from Campbell et al. 2000) and 28S D2-4039-R, 5′-CTC CTT GGT CCG TGT TTC-3′ (Mottern and Heraty 2014). All molecular work was performed at the USDA-APHIS, National Identification Services Insect Molecular Diagnostics Laboratory in Beltsville, MD.

Results

In total, 511 kudzu bug egg masses containing 10,282 eggs were collected throughout the duration of this study. *Megacopta cribraria* egg masses were readily available for collection, and egg densities remained consistent from 07 June 2017 to 24 August 2017, whereas numbers of both egg masses and individual eggs decreased dramatically beginning 29 August 2017 (Fig. 2). The number of eggs per mass also remained relatively consistent throughout the season, with a slight increase in eggs per mass from 18 July 2017 to 01 August 2017. This increase was similar to an increase in the number of individual eggs during the same period. The number of eggs per mass declined slightly beginning on 29 August 2017, which coincided with a decrease in the number of egg masses and individual eggs. *Paratelenomus saccharalis* were not recovered from kudzu bug eggs collected during this study. Instead, another parasitoid species emerged from field-collected kudzu bug eggs. This parasitoid was identified as a single species of sexually dimorphic wasps, *Ooencyrtus nezarae* Ishii (Hymenoptera: Encyrtidae). Images of the adult female and male, antennal features, and other morphological characteristics are shown in Fig. 3. The first record of this species in North America was reported in 2016, when it was recovered from kudzu bug eggs collected from soybean in Auburn, AL (Ademokoya et al. 2018). Molecular analysis confirmed that the 28S D2 gene of specimens recovered from Tennessee exactly matched the same gene from *O. nezarae* specimens (GenBank KY965821.1 and KY965822.1) collected in Auburn (Ademokoya et al. 2018), with 100% sequence similarity with the exception of a single ambiguous base call ("N") in the Tennessee specimen.

Ooencyrtus nezarae were recovered from kudzu bug eggs collected from all 14 localities in six counties in Georgia and Tennessee. Parasitoids were first recovered

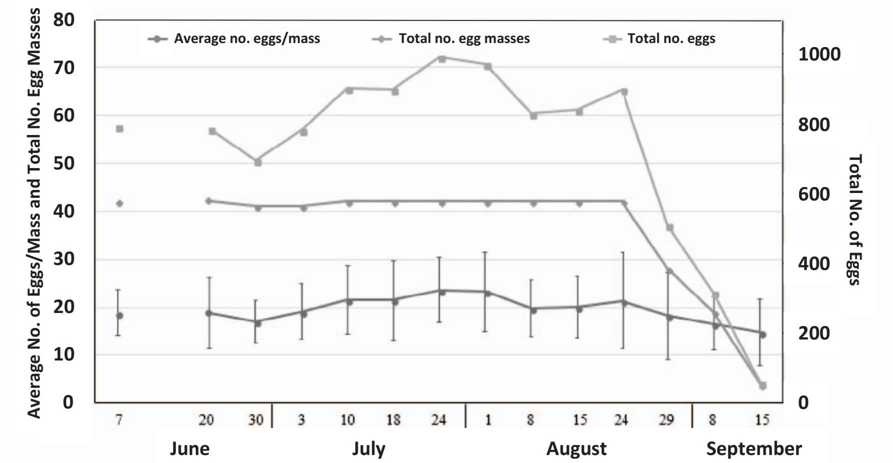

Fig. 2. Average number of kudzu bug eggs (*n* = 10,282) and egg masses (*n* = 511) collected per week from kudzu across all 14 localities in eastern Tennessee and northern Georgia, 2017.

on 07 June 2017 from four counties in Tennessee and one county in Georgia and were recovered on 22 June 2017 from the remaining county (Polk in TN). No other species of kudzu bug egg parasitoids were recovered throughout the duration of this study. In addition, no parasitoids of any species were recovered from limited collections of kudzu bug eggs in a small companion survey conducted in soybean at the West Tennessee Research and Education Center in Jackson (S.D.S. unpubl. data). Our collections in June 2017 represent the first confirmed report of this parasitoid species on kudzu bug eggs in kudzu in North America, as well as a first confirmed recovery of this species in Georgia and Tennessee. Although Basili and Merwin (2019) sampled for parasitoids of kudzu bug eggs in several southern states, including Georgia, in 2016, they did not confirm the identity of these parasitoids to be *O. nezarae*. They based their findings on the location of the parasitoid emergence hole from the egg, which is a general characteristic of *O. nezarae*. In addition, they pooled the Georgia samples with collections from several other states, which made it difficult to determine the original source of recovered parasitoids. Since our initial collections in 2017, *O. nezarae* also has been recovered from kudzu bug eggs on kudzu in northern Florida and southern Georgia (Diedrick 2018; Diedrick et al. 2020, 2023; Goltz et al. 2020; Morain 2021).

Overall parasitism averaged across all 14 localities for the duration of the season was 51.97%, and average parasitism across all localities ranged from 11.15% in the week of 07 June 2017 for the first collection to 60.85% in the week of 15 September 2017 for the last collection (Fig. 4). The highest average percent parasitism near the beginning and end of the study, from late June to early July and mid-August to late September, occurred in Knox Co., TN (Fig. 4). Similar patterns were observed in Blount, Polk, and Murray counties, whereas Monroe Co. had a

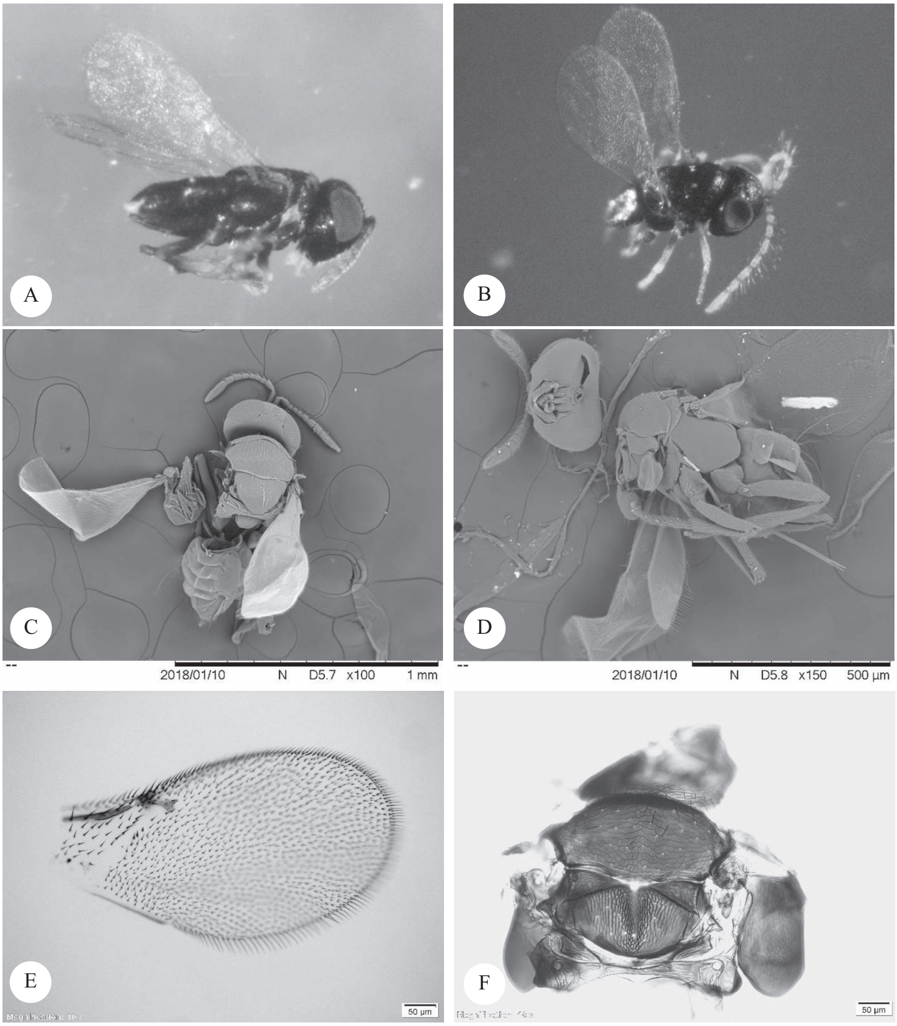

Fig. 3. ***Ooencyrtus nezarae*. (A) Lateral view of female habitus. (B) Lateral view of male habitus. Note the dimorphic antennal morphology. (C, D) Scanning electron microscopy images detailing the female dorsal (C) and lateral (D) sclerites. (E) Female forewing. (F) Female dorsal mesosoma and detail of ultrastructure.**

delayed first peak on 20 July and McMinn Co. had the highest percent parasitism of any locality throughout July. In Knox Co., more kudzu bugs than parasitoids emerged from kudzu bug eggs on 07 June as well as on 10 July–8 August.

Some of the kudzu bug eggs collected from kudzu did not hatch or produce a parasitoid. Interestingly, in Knox Co., approximately 36.27% of kudzu bug eggs did not hatch. It is unknown whether these eggs had been parasitized and the parasitoid did not develop or whether the eggs were simply infertile. The number of

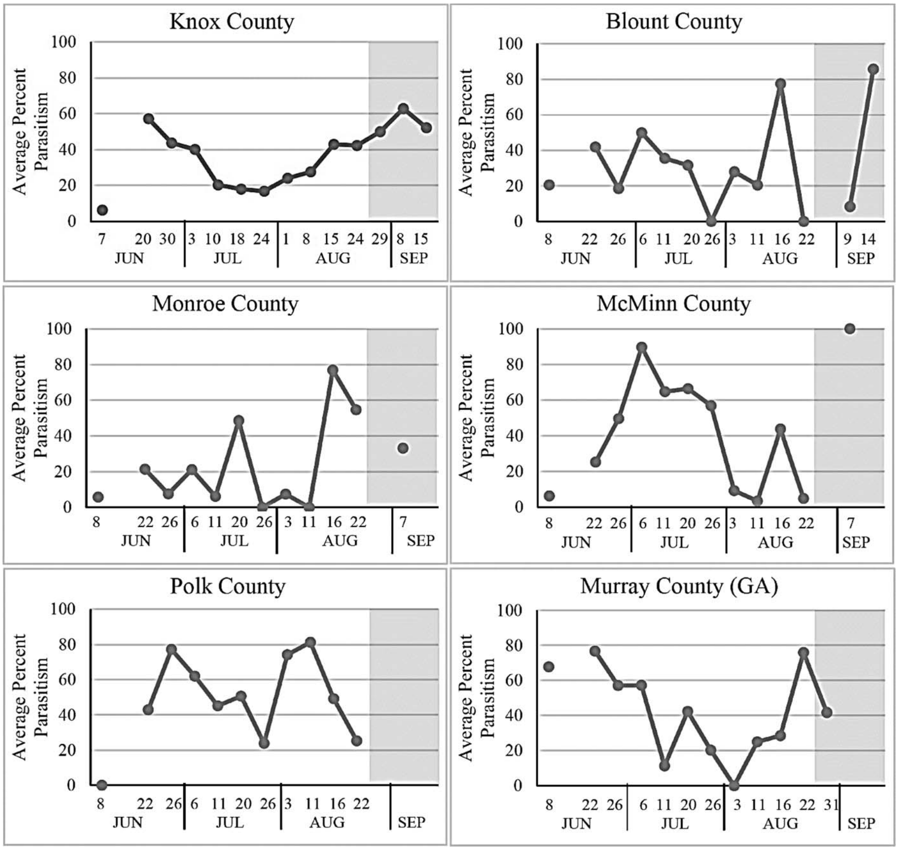

Fig. 4. Average percent parasitism of kudzu bug eggs collected from kudzu in all counties, 2017. Shaded portion of graphs indicates opportunistic collections of kudzu bug eggs due to lower numbers of ovipositing kudzu bugs and is presented for conceptual purposes only.

eggs that produced neither a kudzu bug nor a parasitoid was highest from mid-July to early August in Knox Co. (Fig. 5). *Ooencyrtus nezarae* were also successfully recovered from kudzu bug eggs for several weeks while kudzu bug oviposition was in decline, with percent parasitism ranging from 8.29 to 100% per sample (usually three egg masses with at least 10 eggs each) across all localities during this time frame (Fig. 4).

Throughout the duration of sampling, 655 males and 1,511 females were recovered across all localities, for an overall sex ratio of 1:2.3 (male:female). Female *O. nezarae* were recovered at higher numbers than males at every week during sampling (Fig. 6). In particular, females composed a much higher proportion of the population in samples collected from 07 June to 15 August. After this time, fewer *M. cribraria* eggs and lower numbers of *O. nezarae* were recovered from the field and the numbers of male and female collected were relatively similar (Figs. 2, 6).

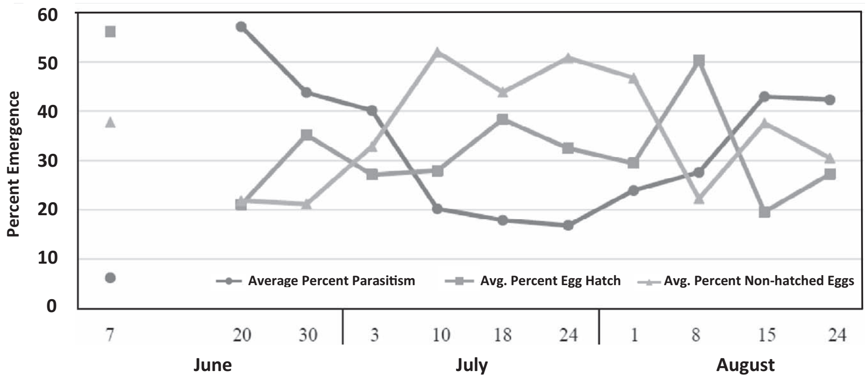

Fig. 5. Comparison of kudzu bug eggs collected from kudzu that hatched and failed to hatch, relative to percent parasitism of kudzu bug eggs by *O. nezarae*, Knox Co., TN, 2017.

Discussion

Multiple species of kudzu bug egg parasitoids have been found in the United States since the initial detection of *M. cribraria* (Ademokoya et al. 2018; Diedrick 2018; Diedrick et al. 2020, 2023; Dhammi et al. 2016; Gardner et al. 2013). The use of morphological and molecular techniques in this study confirms the presence of *O. nezarae* in southeastern Tennessee and northern Georgia. Similar to the populations observed by Ademokoya et al. (2018), specimens recovered in this study are smaller on average than the holotype, but are not morphologically similar to other species of *Ooencyrtus* (Zhang et al. 2005). Congeneric parasitoids were

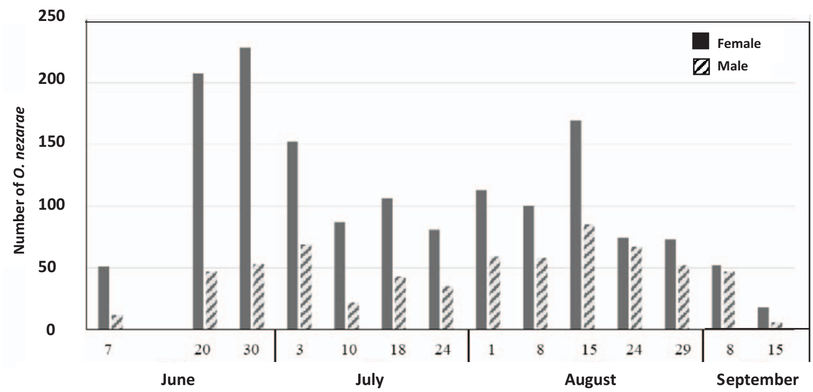

Fig. 6. Total number of female and male *O. nezarae* that emerged from kudzu bug eggs collected from kudzu across all 14 localities each week, 2017.

recovered from kudzu bug eggs in Virginia in 2015 (Dhammi et al. 2016), although they appear morphologically dissimilar to *O. nezarae* (Ademokoya et al. 2018). Although only *O. nezarae* were recovered during this study, it is possible that both populations of *Ooencyrtus* spp. may coexist in northeastern Tennessee. Further collections are necessary to determine the composition of kudzu bug egg parasitoids present in Tennessee.

These data suggest that *O. nezarae* exhibits two peaks of parasitism on kudzu bug eggs each year in eastern Tennessee (Fig. 4), peaks that coincide roughly with the time frame that adult kudzu bugs complete development, mate, and begin to oviposit (Britt 2016). Seasonal fluctuations in the percentage of unhatched kudzu bug eggs align closely with observations made by Britt (2016) in 2015, during which no parasitoids were recovered from kudzu bug eggs ($n = 43{,}707$). Interestingly, no parasitoids were recovered again in 2016. However, in 2017, 51.97% of kudzu bug eggs (10,282 eggs) were parasitized. These results illustrate the importance of continued assessment of parasitism and establishment.

In both this study and that of Britt (2016), successful kudzu bug hatch rates were highest at the beginning of June and then dropped before returning to similar levels in August. This decline in hatch rates suggests that kudzu bug fecundity is typically lower later in June and throughout July, and it could be that the reduction in parasitism by *O. nezarae* during this time follows the reduced availability of viable kudzu bug eggs. The mid-June–early July and mid-August parasitism peaks do not entirely match with studies conducted in soybean and kudzu in Japan (Hirose et al. 1996, Hoshino et al. 2017); this disparity may be due to regional climate differences or a relative lack of competition with other natural enemies of kudzu bugs.

Although densities of kudzu bug eggs declined in late August, *O. nezarae* were recovered into September at all localities except Polk Co. (Figs. 2, 4). The decline in kudzu bug eggs also corresponds with a shift toward a less female-biased sex ratio (Fig. 6). This shift could be due to a behavioral response to declining host availability; however, *O. nezarae* has a wide host range that may provide a larger array of available eggs later in the season. The reduction in numbers and shift toward males could also be a prediapausal response triggered by reduced day length and temperatures (Numata 1993, Teraoka and Numata 2000). The shift in sex ratio and lack of kudzu bug egg availability does not seem to deter oviposition by the remaining females, because *O. nezarae* exhibited parasitism levels as high as 100% in the final weeks of the study (Fig. 4). These later-season detections indicate that this parasitoid species may be a sustainable, effective biological control agent even when *M. cribraria* population densities are low.

Higher percent parasitism observed toward the end of the egg-laying season may reduce the number of second-generation kudzu bug adults and therefore reduce the occurrence of adult kudzu bugs acting as a nuisance pest in the fall when they frequently invade homes and other structures (Britt et al. 2016b). Furthermore, the fungus *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Ascomycota: Hypocreales: Cordycipitaceae) has not been commonly observed to exhibit entomopathogenicity in *M. cribraria* before mid- to late August in kudzu patches in east Tennessee (Britt et al. 2016a, Hollabaugh 2022). The early June–mid-September activity for *O. nezarae* also provides biological control of kudzu bug earlier in the season. The presence of this early-season natural enemy could reduce and maintain kudzu bug densities below levels that are economically damaging to soybean producers.

Despite its potential as a biological control agent, several concerns about *O. nezarae* require further investigation. Some of these concerns are lessened when considering the life history and parasitism of the other egg parasitoid, *P. saccharalis*. Although researchers have demonstrated that parasitism of kudzu bug eggs by *P. saccharalis* on kudzu is approximately 41% (slightly less than our findings of ca. 52% parasitism of eggs by *O. nezarae*), its parasitism on kudzu bug eggs on soybean was only 10% (Miller 2015). The study concluded that egg parasitism by *P. saccharalis* was significantly impacting overall kudzu bug populations (even though parasitism levels in soybean were low). Although little is known about the ability of *O. nezarae* to parasitize kudzu bug eggs in soybean, it also should sufficiently reduce overall kudzu bug populations on kudzu to lessen the impact of kudzu bug on soybean. Thus, their parasitism on kudzu bug in soybean may not be as important. Unfortunately, that scenario applies only to soybean fields adjacent, or close, to soybean fields. It is unknown whether this parasitoid will survive in areas without kudzu. Another major consideration in the use of biological control agents is their ability to overwinter in their introduced range. The 2016–2017 winter preceding the detection of *O. nezarae* was relatively mild compared with the 2017–2018 winter in our sampling range. The average minimum temperature from 01 November 2016 to 31 March 2017 was approximately 3.78°C, with only 41 d where the minimum temperature was $<$0°C (NOAA 2018). The 2017–2018 winter had an average minimum temperature of 1.88°C and 55 d with a minimum temperature $<$0°C (NOAA 2018). *Paratelenomus saccharalis* has been recovered at fairly low levels and only in states to the south of Tennessee since its initial discovery, which may be due to a lack of cold tolerance (Gardner and Olson 2016, Hoshino et al. 2017, Ruberson et al. 2013). Populations of kudzu bug, however, have become established much farther north (Grant et al. 2014, Megacopta Working Group 2023). *Ooencyrtus nezarae* has been shown to withstand exposure to temperatures as low as $-$5°C (Numata 1993), near the lowest temperatures that invasive populations of kudzu bugs have been observed to survive (Grant and Lamp 2017). Thus, *O. nezarae* may be a more viable biological control agent than *P. saccharalis* throughout the colder regions that *M. cribraria* inhabits in the United States.

Ooencyrtus nezarae were recovered from kudzu bug eggs collected at three sites in Knox Co. TN, on 06 June 2018 and the site in Murray Co., GA, on 07 June 2018, demonstrating that *O. nezarae* can overwinter in both states, and recurs at a similar time of year even when the preceding winter is colder. Additional surveillance is needed in following years to determine whether *O. nezarae* can continue to overwinter in Tennessee or survive in states with colder winters and provide biological control beyond the southeastern range of *M. cribraria*.

Furthermore, *O. nezarae* were first found in a location (Alabama) where *P. saccharalis* has been consistently found. Although it is yet unknown how either parasitoid was introduced into the United States, it is important to understand how these populations may interact. *Ooencyrtus nezarae* and *P. saccharalis* coexist in their native range. Hoshino et al. (2017) have found evidence of interspecific competition between these wasps, the most worrisome of which is that *O. nezarae* can hyperparasitize final-instar *P. saccharalis* within kudzu bug eggs. Although the resulting egg parasitoid that emerges can offer control of *M. cribraria* regardless, this potential for competition may be a consideration for those planning to release *P. saccharalis* as a part of their pest management program. Although these observations provide key initial insights into the interactions between *O. nezarae* and *M. cribraria* in kudzu, continued research is needed to investigate the capacity of *O. nezarae* to provide biological control of kudzu bugs in soybean fields in the United Staes and any potential impact this parasitoid species may have on native, nontarget species.

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