

# **FINAL PERFORMANCE REPORT**



**Federal Aid Grant No. F14AF01227 (T-82-1)**

**A Biological Survey of the Linda's Roadside Skipper Butterfly  
(*Amblyscirtes linda*)**

**Oklahoma Department of Wildlife Conservation**

**Grant Period: April 1, 2015 – March 31, 2020**

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**Principal Investigator:** Dr. Katrina Menard, Assistant Staff Curator of Recent Invertebrates, Sam Noble Oklahoma Museum of Natural History, University of Oklahoma.

### ABSTRACT:

We conducted field surveys in seven counties in eastern Oklahoma to document Linda's Roadside Skippers (*Amblyscirtes linda*). We obtained 196 adult Hesperiidae butterflies of which at least 37 are *Amblyscirtes linda* or its sister taxon *A. belli*. Each specimen that we collected was properly prepared, labeled and accessioned into the Sam Noble Oklahoma Museum of Natural History's Recent Invertebrates Collection. Many of these specimens represented species placed in our collection for the first time including the first curated specimens of *A. linda* collected from Oklahoma. We collected DNA vouchers from each of the butterfly species that we collected and successfully extracted DNA from these specimens, amplified it using Polymerase Chain Reaction, sequenced the CO1 gene, and uploaded the genetic information into the Barcode of Life Database. Each butterfly specimen was imaged to coordinate with its DNA barcode records. Genitalia also were imaged to aid in species identification and verification. We obtained samples of the presumed larval host plant, Indian Woodoat (*Chasmanthium latifolium*), from most sites where we collected butterflies and deposited these into the OU Herbarium for future study. Despite repeated attempts, however, we could not confirm the use of *Chasmanthium* as a larval food plant by *A. linda*.

### OBJECTIVES:

- 1.) To estimate the distribution and abundance of *Amblyscirtes linda* in Eastern Oklahoma by counting adult or larval individuals by aerial nets or hand counting off their grass host plants at each collecting site each year for 4 years to determine current ranges and distributions.
- 2.) Collect voucher specimens of the butterflies for species identification and site verification to deposit at the SNOMNH.
- 3.) Obtain tissue samples of the butterflies for the COI barcode gene for investigations into the population genetic structure and estimation of butterfly dispersal and population mixing.
- 4.) Obtain host plant Indian Wood Oat grass samples with the butterflies are found and woodland areas nearby to investigate why the butterflies need the grasses grown in woodlands and not the grass across its entire range.

**NEED:**

The Linda's Roadside Skipper (*Amblyscirtes linda*) is a small dark butterfly in the family Hesperiidae whose range is limited to the south-central United States in the region encompassing Arkansas, Kansas, Missouri, and Oklahoma. This species is classified as "Vulnerable" by the Xerces Society (Vaughan and Shepard 2005) based primarily upon its rarity and limited geographic range. It is also considered to be a species of greatest conservation need and included in the Oklahoma Comprehensive Wildlife Conservation Strategy for these same reasons (ODWC 2015). This project was undertaken to learn more about the distribution, abundance, and habitat associations of the Linda's Roadside Skipper because future actions to conserve and protect this species cannot be undertaken without first establishing a baseline condition. This study also sought to examine the population-level genetic diversity and genetic flow between populations of this butterfly in Oklahoma. Finally, we tested the assumption that the Linda's Roadside Skipper's caterpillars are dependent upon a single grass host – Indian Woodoat (*Chasmanthium latifolium*).

The known life cycle of the Linda's Roadside Skipper is based upon a study of one population and suggests that this species includes two generations per year between April and July (Heitzman and Heitzman 1969). The adults feed on nectar from various woodland plants after the grass-feeding larval stage. The species is identified from three very similar roadside skipper species that overlap in habitat and range (*A. belli*, *A. vialis*, and *A. samoset*) by the male genitalia and the wing coloration (Heitzman and Heitzman 1969). As recently as 2005, the Xerces Society estimated that the Linda's Roadside Skipper occurred as approximately 20 metapopulations (Vaughan and Shepard 2005). In contrast to its apparent rarity and limited distribution, this species is assumed to depend upon the widespread and abundant Indian Woodoat as the host plant for its caterpillars. This grass has a much wider distribution than the butterfly and occurs across most of the eastern and central United States. Despite how common and widely distributed the host grass is in counties across Oklahoma, this butterfly has been found only in a fraction of them and is considered to be very difficult to find (Opler et. al 2014, Nelson 2014). One hypothesis about their contrasting distributions is that the Linda's Roadside Skipper may use Indian Woodoat plants that grow only under specific ecological conditions such as forested riparian areas; however, this has never been empirically tested.

Prior to this study, all records and sightings of *A. linda* in Oklahoma had been visual confirmations; there were no specimens of the butterfly deposited in the Sam Noble Oklahoma Museum of Natural History (SNOMNH) or the Oklahoma State University's K.C. Emerson Entomology Museum to confirm these observations. Having no specimen records in Oklahoma museums is another impediment to future conservation efforts and attempts to study this species. One of the priorities for this project was to collect and properly voucher specimens to confirm their presence and store them in perpetuity for other scientific study. Future conservation efforts also should consider how the apparently fragmented populations of *A. linda* are structured and interact across the species' range. A leg from each specimen can yield sufficient DNA information to sequence the cytochrome oxidase 1 gene (CO1), which is used commonly for population genetics and identification through the Barcode of Life Database (BOLD) (Ratnasingham and Hebert 2007). Currently, there is no DNA information for this butterfly in the BOLD database or in the National Center for Biotechnology Information (NCBI) Gene Database. This was remedied through this project by collecting DNA samples from each of the butterfly specimens that were collected, depositing tissue into the SNOMNH tissue collection, and sequencing the CO1 gene and entering those data into the Barcode of Life Database.

## **APPROACH:**

### Field Surveys:

We conducted field surveys between May and July in 2015, and between April and May in 2016, 2017, 2018 and 2019. The surveys were conducted in eastern Oklahoma (Adair, Atoka, Delaware, Latimer, LeFlore, McCurtain, Pushmataha, and Sequoyah counties) and focused on riparian sites and riparian-adjacent sites where the presumed host grass was found. Most of the survey sites were located on public lands such as state parks, wildlife management areas, and the Ouachita National Forest. A timed-search survey was conducted at each site, with one or two observers at each location walking through habitats within and adjacent to forested riparian zones that supported the host grass for the larvae and suitable nectar sources for the adults. Most survey areas were long and linear; GPS locations were recorded at the closest and farthest points. Searches were conducted for adults on host grasses and nectar-producing plants and adult butterflies were captured with a hand net and placed in a large plastic terrarium for counting. Voucher specimens were taken from each site with butterflies and any remaining butterflies were released. Where possible, we vouchered up to two adult male butterflies per site to confirm the species identification. At each site, searches were conducted also of patches of Indian Woodoat plants for butterfly eggs and larvae. When larvae were detected, they were hand-counted on the plants to estimate population size, and at least one was collected and returned to the lab for rearing to the adult stage. Most of the locations that were surveyed between 2015 and 2018 were surveyed in two separate years to confirm the presence or absence of Linda's Roadside Skipper more accurately. At each location surveyed between 2015 and 2017, where we documented butterfly larvae, we collected samples of the host grass in forested riparian and non-riparian sites for analysis of the plants. These were deposited as specimens in the University of Oklahoma's Bebb Herbarium.

### Specimen Preparation and DNA Sequencing:

Voucher specimens were prepared, photographed, and deposited into the SNOMNH's collection of Recent Invertebrates. The adult butterflies were prepared and spread according to the SNOMNH's standards for long-term preservation. Samples of DNA were obtained from a leg of each specimen before preservation to store the tissue for subsequent DNA sequencing to allow for the study of population genetics and to submit these data to the Barcode of Life Database. DNA samples were extracted from each skipper leg and a PCR reaction was used to amplify the CO1 gene. The final sequencing reaction and machine run was conducted at the University of Oklahoma's Biology Core Molecular Lab.

## **RESULTS AND DISCUSSION:**

### **OBJECTIVE 1)**

Field surveys were conducted at 78 sites distributed across eight counties as follows: Adair (1), Atoka (3), Delaware (3), Latimer (3), LeFlore (25), McCurtain (33), Pushmataha (9) and Sequoyah (1). Many of these sites were surveyed twice, either within the same field season or across different years, to improve the probability of detecting *A. linda* if it were present. Over the course of five spring field seasons, 196 butterflies from the family HesperIIDae (skippers) were collected. These represent a combination of adults and caterpillars that were collected from host plants and subsequently reared to metamorphosis. Additional caterpillars were collected that could not be reared to metamorphosis because they had been parasitized by wasps prior to our collection. We observed a particularly heavy frequency of parasitism in 2016.

Widespread, near-record spring flooding occurred during two of our five field seasons (2015 and 2019). In both years, riparian areas were difficult to access in many places and the sampling season was prolonged due to problems of access. In 2015, the survey period was pushed back several weeks to mid-May through early July. Contrary to the literature, we did not find evidence of a second generation of *A. linda* at least in the Ouachita Mountains region of southeastern Oklahoma. In southeastern Oklahoma, this species appears to have only a single generation with the adults flying during a relatively short period between late March through late May. In 2016, 2017 and 2018, most surveys were conducted in April and the first half of May which resulted in greater success in locating adult *Amblyscirtes*. In 2019, flooding hampered the April surveys and most surveys occurred in May. This reduced the survey success rate and only two confirmed *A. linda* were located.

During the 2015, 2016 and 2017 field seasons, a substantial effort was placed into collecting live caterpillars from the presumed host grass (*Chasmanthium latifolium*). Nearly 100 HesperIIDae caterpillars were returned to the lab for rearing to metamorphosis. A small number of these were *Amblyscirtes* species; however, none of the caterpillars metamorphosed into butterflies that could be confirmed as *Amblyscirtes linda* or its sister taxon *A. belli*. Most of the caterpillars were Tawny-edged Skippers (*Polites themistocles*), cloudywings (*Thorybes* sp.), Southern Broken Dash Skippers (*Wallengrenia otho*) and Little Glassywing Skippers (*Pompeius verna*). We reared one caterpillar of the uncommon Byssus Skipper (*Problema byssus*) from a site in Delaware County. Despite rearing many caterpillars from *Chasmanthium latifolium*, we were not able to confirm that this is the host plant for *Amblyscirtes linda* in Oklahoma.

Among the nearly 200 adult HesperIIDae butterflies that we collected, were at least 37 that were either *Amblyscirtes linda* or *A. belli*. Eighteen (18) *Amblyscirtes* were genetically confirmed to be *A. linda*. These specimens were collected from ten sites in McCurtain County (primarily on the Ouachita National Forest on all sides of Broken Bow Reservoir), six locations on the Ouachita National Forest in Le Flore County, and two locations on the Pushmataha Wildlife Management Area in Pushmataha County. The remaining 19 *A. linda/A. belli* specimens came from sites within the same three-county area plus Latimer County, but the majority were located in LeFlore County. These specimens included several that were morphologically identical to *A. linda* but were genetically more similar to *A. belli*.

Our experience with this species indicates that the Linda's Roadside Skipper differs from other species of *Amblyscirtes* within Oklahoma in that it appears to have only a single generation each year. Adults emerge early in the spring, mate and lay their eggs typically before the end of May. Mid-March through late-April appear to be the most productive weeks for conducting field surveys for this species. Although roadside skippers are capable of feeding from a wide range of blooming plants, we found adult *Amblyscirtes* primarily on blackberry (*Rubus* sp.) plants, and this appears to be one of the most common nectar plants for this genus in the early spring.

## **OBJECTIVE 2)**

All of the specimens that were collected from the field were properly prepared and labeled. These are now housed in the Sam Noble Oklahoma Museum of Natural History's Recent Invertebrates Collection. Several of these specimens represent species placed in the collection for the first time and/or new county records. Photographic images were taken of all the butterflies to coordinate with their DNA barcode information (see Objective 3 results). Their

genitalia also were imaged to aid in species identification and verification by Lepidoptera expert Nick Grishin at the University of Texas.

### **OBJECTIVE 3)**

We collected DNA vouchers from all of the butterfly specimens that were collected. A sample of DNA from each specimen was extracted and amplified using the Polymerase Chain Reaction so that the cytochrome oxidase 1 gene could be sequenced and uploaded into the Barcode of Life Database. We uploaded CO1 sequences for 16 *Amblyscirtes linda* specimens from Oklahoma representing the first sequences for this species.

One of the greatest challenges that we encountered during this project was determining the species limits for *Amblyscirtes linda*. The genus *Amblyscirtes* contains several nearly identical taxa and three of these (*A. linda*, *A. belli*, and *A. vialis*) occur widely in eastern Oklahoma and overlap one another in their habitat associations. We identified at least 37 specimens that were either *A. linda* or *A. belli*, but only 18 of these could be clearly assigned to *A. linda*. Another 19 specimens possessed genetic and phenotypic traits that were indicative of both *A. linda* and *A. belli*. We developed a phylogenetic analysis of the *Amblyscirtes* species that are found together in southeastern Oklahoma to provide a discussion of and a key to their species limits because without a clear definition of what *A. linda* is in a phylogenetic and taxonomic context, studying the species' population structure would be mute. We collaborated with Dr. Nick Grishin at the University of Texas who is working on an extensive phylogenetic assessment of the family HesperIIDae based upon over 250 species representing each of the recognized genera within the family. Dr. Grishin confirmed the challenges of defining species limits within this large family. Our work supported his hypothesis that the traditional use of DNA from the mitochondrial CO1 gene may not be as indicative of species limits within the HesperIIDae as nuclear DNA (personal communication). Our phylogenetic analysis of the *Amblyscirtes* species, based upon the collections made during the first four field seasons, indicate that the species-level identification of *A. linda* versus its sister species *A. belli* may be subjective. Specimens that were identified as *A. linda* by expert Nick Grishin based on morphology - including species-specific characters of the male genitalia - were found to share the same CO1 haplotypes as specimens of *A. belli* that were collected in Cleveland County in central Oklahoma, indicating that the two species may be morphologically cryptic. Several haplotypes appear to be regionally distributed in small areas of the Ouachita Mountains, although these haplotypes are morphologically indistinguishable. We completed a preliminary phylogenetic analysis of the *Amblyscirtes* species in the study area, which is shown in Appendix 1.

The examination of nuclear DNA as opposed to mitochondrial DNA ultimately may prove more useful in defining species limits in this genus of skippers. We attempted to explore this during the final year of the project; however, this work was cut short. In mid-June 2019, the Principal Investigator was notified that her position at the SNOMNH was one of 75 throughout the University of Oklahoma that was chosen to be eliminated because of budget cuts and staff reductions. Only eight weeks advanced notice was provided and the final weeks of the project were focused on extracting, amplifying and sequencing the DNA from the CO1 gene for the specimens that were collected during the spring of 2019 in order to identify potential *A. linda*.

### **OBJECTIVE 4)**

Between 2015 and 2018, we examined the initial hypothesis that host plant differences between growing conditions (woodlands versus open habitats) determined their use as a larval food for

*Amblyscirtes linda*. We obtained Indian Woodoat samples from each of the sites at which we collected butterflies, caterpillars or eggs and deposited them into the OU Herbarium for further study. However, through all four years we found no instances of the presumed host grass being utilized by *A. linda* despite multiple attempts to rear eggs and caterpillars found on Indian Woodoat plants. We currently doubt the literature about this butterfly's host specificity and have found no confident association with the assumed host grass. On a cautionary note, our caterpillar collections to date have occurred primarily during May and June. It is possible that later caterpillar instars may be utilizing this plant in mid-summer such that searches for *Amblyscirtes* larvae on *Chasmanthium* later in the year are recommended as a future study.

**SIGNIFICANT DEVIATIONS:**

The initial hypothesis of host plant differences between *Chasmanthium* growing under different environmental conditions was pursued during the first few years of the grant, but because no instances were found of the presumed host grass being utilized by *A. linda*, it seemed misleading to continue in that direction. Specimens of *Chasmanthium latifolium* were collected in areas where potential *Amblyscirtes* larvae or adults were present in Years 1 through 3, but few specimens were collected in Years 4 and 5.

Because of the difficulty encountered in defining the genetic limits for the species *Amblyscirtes linda*, we were unable to accurately address the genetic structure of populations or estimate the frequency of genetic mixing between populations in Oklahoma.

PI Menard was informed on June 13<sup>th</sup>, 2019 that her position was being terminated on August 14<sup>th</sup>, 2019 due to University of Oklahoma layoffs. This significantly changed the timeline for what could be accomplished in Year 5 of the grant and reduced the focus of the grant to what could be done in the 60-day notice. We focused on completing the molecular work since it was the most expensive, and reliant on software and equipment available with Menard's position as curator. Our hope was to use the molecular data that we had already collected in previous years in addition to what we could accomplish during the final few months of the grant to finish some publications if possible.

**PREPARED BY:** Dr. Katrina Menard  
Assistant Staff Curator, Sam Noble Oklahoma Museum of Natural History

**DATE:** 2 June 2020

**APPROVED BY:** Russ Horton, Assistant Chief of Wildlife Division  
Oklahoma Department of Wildlife Conservation

Andrea Crews, Federal Aid Coordinator  
Oklahoma Department of Wildlife Conservation

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Appendix I. Strict Consensus Tree of the COI gene in populations of *Amblyscirtes* species sampled from southeastern Oklahoma (Atoka, Latimer, LeFlore, McCurtain, and Pushmataha counties) 2015 - 2018. A specimen of *Amblyscirtes aenus* was included from central Texas as a possible outgroup. Two major morphological characters used to separate species are highlighted – the male aedagus and the patterning of the forewing. Support values of the nodes are indicated in color for values above 50%.

Strict Consensus of 4 equally parsimonious trees of 157 steps using Tree Analysis using New Technology Search Algorithms.

Symmetric Resampling (P=33)  
 Jackknifing (P=36)  
 Standard Bootstrap  
 Poisson Bootstrap

