Environment and Natural Resources Trust Fund

Research Addendum for Peer Review

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Project Title: **Prairie Butterfly Conservation, Research and Breeding Program**

Project number: 017-A

Project Summary

Prairies and their native wildlife are an important part of Minnesota's natural and cultural heritage. With only 1% of that native prairie remaining however, many prairie plant and animal species – including several species of once prevalent butterflies – have dramatically declined and are threatened with extinction. This collaborative project between the Minnesota Zoo and the Minnesota Department of Natural Resources (DNR) seeks to prevent the extinction of Minnesota-native prairie butterflies, provide current estimates of their status, research mechanisms for their declines, inform future management strategies, and provide public education opportunities about them and their rich but imperiled ecosystems. We will accomplish these goals through the following five activities. The Minnesota Zoo will expand its newly established conservation breeding program for several of these species (Activity 1), conduct foundational conservation genetics research (Activity 2), assess the effects of common agricultural pesticides on butterfly survival and fitness (Activity 4), and produce two free publications about Minnesota's prairie butterflies and prairie habitats (Activity 5). Finally, the Minnesota DNR (Activity 3) will develop an intensive monitoring protocol across multiple sites in western and southern Minnesota to track the presence and/or abundance of prairie butterflies and evaluate the impacts of various management activities on species populations. The research processes for each Activity are outlined below, with the exception of Activity 5 which is a non-scientific endeavor.

Activity 1: Minnesota Zoo breeding conservation program for imperiled prairie butterflies

Abstract

The Minnesota Zoo's prairie butterfly conservation program is working to prevent the extinction of at least two Minnesota native prairie butterflies, the Poweshiek skipperling and Dakota skipper. We are doing this by establishing a conservation breeding program at the Zoo to preserve as much genetic diversity as possible. Funding from ENTRF will allow for much needed expansion of our operations and allow us to test a variety of methodological approaches to optimize breeding success.

Background

The Minnesota Zoo's prairie butterfly conservation program was launched in 2012 following consultations with the U.S. Fish and Wildlife Service (USFWS) and Minnesota Department of Natural Resources (DNR) on the need to establish conservation breeding populations for endangered, threatened, and imperiled Minnesota-native prairie butterflies whose wild populations have experienced catastrophic recent declines and face the risk of global extinction. Two of these species, the Poweshiek skipperling (*Oarisma poweshiek*) and Dakota skipper (*Hesperia dacotae*) are currently listed in Minnesota as Endangered and were recently proposed for federal listing as Endangered and Threatened (respectively) in October 2013 (U.S. Fish and Wildlife Service. October 23, 2013. Endangered and Threatened Wildlife and Plants; Designation of Critical Habitat for Dakota Skipper and Poweshiek Skipperling. Federal Register Vol. 78, No. 206: 63625-63745). Both have disappeared from the majority of their historic ranges (90+% for Poweshiek, 50+% for Dakota) in recent decades. Dakota skippers may only remain in one Minnesota location. Poweshiek, sometimes referred to as the "Most Minnesotan Butterfly" because half of its historic range was the state, was once one of the most abundant butterflies on Minnesota's prairies, but has not been seen in Minnesota since 2007. It has also disappeared in North Dakota, South Dakota and Iowa between 2001 and 2008. Intensive 2013 surveys across the remaining isolated populations in Michigan, Wisconsin, and Manitoba indicate that fewer than 500 Poweshiek skipperlings likely remain globally, making them at least three times rarer than wild giant pandas and one of the most endangered animals on earth.

Our breeding program seeks to utilize the recognized organizational capacity and experience of the Minnesota Zoo for the managed breeding of endangered species to establish breeding populations of Poweshiek skipperlings and Dakota skippers at the Zoo as insurance against the risk of extinction, not only in Minnesota but globally. Our efforts are international, involving over a dozen partner U.S., Canadian, and tribal agencies and organizations. In consultations with our partners, we have established safeguards to ensure that our efforts protect wild population integrity. If it is deemed by the network of partners that these efforts may pose a risk to the demographic and/or genetic viability of a population, this will be suspended, potentially on short notice. No wild adults are permanently removed from populations. Instead, we temporarily hold adult females from the largest populations, up to 20% of that year's censused population size, for up to two days in secured individually-labelled cups with honey water feeding solutions and caterpillar hostplants where they can lay eggs. After two days, the females are released back into the wild at their location of initial capture so that they may continue to lay eggs in the wild. The eggs and resulting caterpillars are then transported back to the Minnesota Zoo for rearing under a variety of methods we are in the process of testing. We constructed an outdoor butterfly breeding facility for this program in 2012 with built-in multi-level containment capabilities, but so far have lacked stable indoor space in which we can control temperature and lighting.

Hypotheses

The Zoo-based conservation breeding program may investigate a range of questions and employ a variety of methodologies depending on the availability of individuals and resources. The primary motivation is to maximize our ability to establish large, genetically robust conservation breeding populations at the Minnesota Zoo. As such, our hypotheses and associated methodologies are adaptive and context-dependent and seek to minimize mortality sources and maximize reproduction. We have already made progress on these aspects. Among the remaining questions we are interested in addressing include:

1) How does larval hostplant affect larval growth rates and survivorship?
2) What is the lower temperature threshold for winter hibernation survival?
3) What conditions provide the greatest success for mating?

Methodology

Progress to date:

We regularly consult with our partner regulatory agencies, especially the U.S. Fish and Wildlife Service and the Minnesota Department of Natural Resources, to determine steps that we may or may not take to ensure that our activities are only beneficial and do not have detrimental impacts on the demographics of wild populations. This includes the suspension of breeding operations if it is deemed suitable. All of our operations are detailed in annual reports to our partner agencies that can be made available upon request.

To date, we have conducted rearing operations with Poweshiek skipperlings from Michigan and Wisconsin in 2012, Manitoba in 2013, and Dakota skippers from tribal lands in South Dakota in 2013. Wild population numbers were too low in Michigan and Wisconsin in 2013 to overcome minimum protection measures. We have tested several methods for rearing these species, and have significantly improved our protocols. Our most successful protocol for Poweshiek skipperlings utilizes 50mL laboratory plastic tubes into which live blades of larval hostplants like prairie dropseed are strung. Larvae are placed in the tubes to feed normally and the tubes are sealed with a cube of porous foam. The slow-growth rates of Poweshiek skipperling caterpillars and a 3-4 day cleaning schedule reduce the potential for disease. After the initial critical first few days after egg hatching, the majority of mortality resulted from caterpillar's failing to shed their skins properly or other random accidents. Using this method, we have reared Poweshiek skipperlings from egg to adulthood. We believe the Live Grass Tube Method possess significant potential with Poweshiek skipperlings.

We also tested this tube method with Dakota skipper caterpillar rearing in 2013, but had greater success in terms of larval mass by transferring partially grown caterpillars from the tubes to potted hostplants inside fine-mesh screen cages. The potted plant method reduces our ability to track survivorship and growth rates compared to the tube method, but it also significantly reduces the weekly cleaning and maintenance workload.

Winter hibernation conditions also contribute to larval mortality, and we have begun work on some aspects of this. We have kept individual caterpillars of multiple species inside protected containers both inside a controlled refrigerator/freezer and outside where they are exposed to variable temperatures. During the winter of 2012-2013, we had greater survivorship of Poweshiek skipperlings and two other prairie butterfly species inside the refrigerator/freezer than ambient outside conditions. Acclimation to winter conditions is a likely necessity, but no data exists on the conditions necessary. We have been incorporating pre-hibernation acclimation into our rearing operations with endangered skippers. For example, we transfer caterpillars of Poweshiek skipperlings and Dakota skippers that had been reared under indoor controlled conditions to protected outdoor enclosures in autumn to allow them to experience cooler nights and shortening diurnal photoperiod. This also allows the host plants that they are utilizing to senesce, which further acclimates caterpillars to the decreasing moisture and nutritional content of their food. As average temperatures approach freezing in autumn and early winter, these caterpillars are removed from their hosts and transferred into a refrigerator/freezer. Temperature in the refrigerator/freezer is gradually decreased to mimic the average outdoor temperatures. Temperatures are then held at a constant level similar to those experienced under the insulating effects of a persistent snowpack for the duration of winter. Similar acclimation strategies will be employed in our proposed research.

Remaining Questions:

1) Poweshiek skipperlings and Dakota skippers both feed on native prairie grasses as caterpillars, and spend the majority of their lives as caterpillars. Most grasses are similar biochemically, so any potential differences in hostplant suitability are likely to be due to differences in plant structure, plant

texture, and/or conferred microclimate differences. We have successfully reared both of these species on several species of grasses including prairie dropseed, little bluestem, and big bluestem, as well as Pennsylvania sedge (Poweshiek skipperling only). We have observed that Poweshiek skipperling caterpillars seem to prefer prairie dropseed and struggle to eat the coarser little bluestem as very young hatchlings, but are able to digest all grass species offered at later stages. Dakota skippers appear to be a bit more robust and can feed on tougher plants at younger stages. While Dana (1989) did not find a statistically significant effect of different host species in Dakota skipper larval mass at winter diapause, it is not known if host specific differences in mass and/or survivorship become more pronounced at later stages that may affect lifetime fitness. Our ability to perform these tests with the endangered species is contingent on having large, stable breeding populations, and adaptive rearing techniques may take priority over experimental arrays in the short-term to maximize survivorship.

Theoretically, experimental tests on the potential effects of different host plants on larval growth rates, larval survivorship, and adult viability would be structurally similar to the proposed Activity 4 research (see below). Multiple caterpillars would be randomly distributed across replicated pots of multiple host plant species. These potential species could include both likely native hosts (prairie dropseed, little bluestem, big bluestem, sideoats grama, porcupine grass, for example) and introduced hosts (smooth brome and Kentucky bluegrass). Plant species would serve as the treatment, and each treatment plant species would have multiple pot replicates (at least five pots). Ten to 15 caterpillars would be placed on each potted plant and allowed to behave as "wild" caterpillars. All caterpillars would be periodically censused and weighed to allow for estimations of survivorship and growth rate. Hibernation mass, pupal mass, and adult survivorship would also be recorded. Analyses would be conducted in an ANOVA framework with percent surviving/pot and average mass/pot serving as test variables. Pots would be replicates.

2) Both Poweshiek skipperlings and Dakota skippers hibernate through Upper Midwest winters as partially grown caterpillars at the base of their hostplant grasses. Snow can provide a layer of insulation, but snow free periods can expose them to potentially wide temperature fluctuations and very cold temperatures. We have begun assessing the insulation potential of snow over the winter of 2013-2014 using paired automated temperature and humidity data loggers placed strategically on the ground and above the snowpack at the Minnesota Zoo. Preliminary results support the well-documented insulation provided by snowcover, with conditions as much as 45°F warmer under the snow. We hope to couple this data gathering in future years with assessments of winter larval survivorship inside secured containers under variable snow depths at the Minnesota Zoo. Until large populations of the imperiled species can be established, this work will likely first be conducted using non-endangered surrogate species of related grass-feeding skippers that have similar ecological habits and needs (see Activity 4 below). We are ultimately interested in researching the conditions that contribute to overwintering mortality, especially under wild conditions. For example, what are the effects of snow-free periods during on hibernating caterpillars in terms of temperature and humidity variation? We are also interested in testing the effects of periods of temperatures above freezing on hibernating caterpillars, a condition that is expected to occur under warming climate change scenarios. The effect of these freeze-thaw cycles may be substantial. We also hope to conduct controlled condition temperature tolerance tests with surrogate species by exposing hibernating caterpillars to periods of extreme cold to determine the likely effects of occasional periods of temperatures far below freezing in the absence of insulating snowcover. It is expected that cold-climate adapted butterflies possess antifreeze chemicals in their hemolymph that prevent the formation of potentially lethal intra-cellular ice crystals at sub-freezing temperatures, but the lower temperature threshold at which those chemicals become ineffective is not known. Testing for this threshold can be conducted using commercial freezers. The Minnesota Zoo possesses -20°C and -40°C freezers that could be used for this purpose. If enough individuals are available, stratified arrays that vary both temperature and exposure time would be ideal.

3) As noted above, the breeding program has lacked stable indoor space in which we could control both lighting and temperature. Optimization of these factors that also controls for extreme temperatures that may limit adult activity is critical for successful adult breeding. For example, Poweshiek skipperling females are known to lay unfertilized eggs when temperatures exceed about 90°F. The construction of a breeding chamber dedicated exclusively to the Prairie Butterfly Conservation Program will allow us to control temperature cycles that mimic natural conditions, and to be able to maintain them at ideal levels

as necessary. We can also control lighting duration, timing, intensity, and spectra to mimic or maintain ideal natural conditions for adult breeding. This will also be an adaptive process.

Results and Deliverables

Knowledge of which potential hostplants confer the greatest viability to larvae is highly applicable to habitat management and prairie restoration activities. These activities will also help us optimize breeding protocols for these endangered species, as will understanding temperature and lighting conditions most suitable for breeding. Similarly, temperature regimes and winter snowpack timing and duration are likely to change under future climate change scenarios and our efforts to research tolerance thresholds can help inform habitat management activities for these butterflies. Our results of these activities will be communicated to our partners, and may be submitted for publication in peer-reviewed scientific journals.

Timetable

<u>Late 2014 to early 2015</u>: Construction and outfitting of the indoor breeding chamber.
<u>Winters of 2014, 2015, and 2016:</u> Testing of snow cover insulation effects and temperature tolerance thresholds.
<u>May 2014 – June 2015:</u> Initiation of breeding operations of surrogate skipper species at the Zoo.
<u>September 2015:</u> Rearing and breeding protocols with Poweshiek skipperlings will be finalized.
<u>September 2016:</u> Rearing and breeding protocols with Dakota skippers will be finalized.
<u>Continuing:</u> Analyses and submission of findings to collaborators and scientific journals.

Budget

The Minnesota Zoo's portion of the ENTRF budget is $380,000 over three years (see attached). Of this, $335,400 is apportioned to support the breeding operations. This includes 100% of the salary and benefits for the Project Manager, Dr. Erik Runquist, as well as funding to support 25% time for a new temporary worker who will support butterfly rearing and husbandry operations as well as portions of Activities 3 and 4. Legacy Amendment funding to the Zoo will support the remaining 75% of this temporary worker position for FY15 (and perhaps beyond). ENTRF funding for this Activity will also support construction and outfitting costs for the breeding chamber in FY15, and travel costs to current and historic population locations to support egg collection.

Credentials

Dr. Erik Runquist is the Program Manager for this ENTRF Project 017-A. He has managed the Prairie Butterfly Conservation Program at the Minnesota Zoo since its inception in February 2012. He has a B.S. with Honors in Wildlife Ecology and Management and a Minor in Zoology from the University of Florida. He received a Ph.D. in Ecology (Emphasis: Conservation) from the University of California, Davis in 2012 (Thesis: Patterns and mechanisms of divergence in butterflies across spatial scales).

Dissemination and Use

Our results of these activities will be communicated to our partners, and may be submitted for publication in peer-reviewed scientific journals.

References

Dana, R. P. 1991. Conservation Management of the Prairie Skippers *Hesperia dacotae* and *Hesperia ottoe*: Basic Biology and Threat Mortality during Prescribed Burning in Spring. Unversity of Minnesota, Minnesota Agricultural Experiment Station, Station Bulletin 594-1991 (AD-SB-5511-S).

Activity 2: Conservation genetics research on imperiled prairie butterflies

Abstract

The Minnesota Zoo has established a conservation genetics laboratory and also formed a partnership with a professor from the University of Michigan-Dearborn to conduct critically needed foundational studies of intra- and inter-population genetic diversity and divergence in imperiled prairie butterflies. Populations will also be screened for a bacterial endosymbiont that can pose significant challenges to populations if they become infected by novel strains. This research will inform both management activities of both wild populations and the Minnesota Zoo's prairie butterfly conservation breeding program.

Background

Successful conservation management of both *in situ* and *ex situ* populations of endangered species requires knowledge of both existing genetic variation within populations and the degree of differentiation between populations and regions of those species. The primary goal of the Minnesota Zoo's prairie butterfly conservation breeding program is to establish large, genetically robust *ex situ* populations at the Zoo that can serve as an "insurance policy" against the risk of regional and global extinction of endangered species like the Poweshiek skipperling and Dakota skipper. These *ex situ* Zoo populations may also serve as reservoirs from which potential supplementations to wild populations and reintroductions to historic or potentially suitable sites may be drawn. These potential needs and the role of the Minnesota Zoo to achieve these goals are highlighted in the recent federal Endangered and Threatened species listing proposals for Poweshiek skipperlings and Dakota skippers (USFWS 2013). The Minnesota Zoo has established a conservation genetics laboratory under the supervision of Program Manager Dr. Erik Runquist and formed a collaborative relationship with Dr. Emily Saarinen (Assistant Professor, University of Michigan-Dearborn). Using non-ENTRF funding, Dr. Saarinen's lab will perform DNA extractions and conduct "next-generation" sequencing, isolation, and identification of micro-satellite genetic markers for estimates of population-level genetic diversity. Dr. Saarinen will provide these DNA extractions to Dr. Runquist who will use ENTRF funds to 1) sequence several additional known genetic markers for which evolutionary rates are better understood to estimate evolutionary divergence and 2) screen populations for the presence of *Wolbachia*, an intracellular bacterial endosymbiont that has the potential to sterilize or kill infected male butterflies when populations become infected with incompatible strains. There is significant need to screen for and identify *Wolbachia* strains that may be infecting populations of endangered species. Any *ex situ* conservation breeding program with these species must not allow individuals of differing infection status to breed due to the expected decreases in fitness. Furthermore, any individuals derived from conservation breeding programs that may be used for re-introduction or supplementation programs must not be introduced to populations of differing infection status (Nice *et al*. 2009).

Hypotheses

We will be examining the following null hypotheses: 1) there are not significant differences in genetic composition between different populations or regions of the same species and 2) co-occurring common and endangered species do not have different levels of genetic diversity at the local level. We are interested in addressing the following questions:

> How much genetic diversity remains within populations of Poweshiek skipperlings, Dakota skippers, and Regal Fritillaries?
>
> Within each of these species, how genetically divergent are different populations and regions?
>
> Do population-level diversity metrics significantly differ between these endangered species and abundant co-occurring species that have similar ecological habits?

Are populations of prairie butterflies like the Poweshiek skipperling, Dakota skipper, and Regal fritillary infected with *Wolbachia*, and do infected populations vary in their strains?

Methodology

Using standard alcohol extraction methods, Dr. Saarinen's lab has and will continue to extract genomic DNA of Poweshiek skipperlings, Dakota skippers, and other prairie butterflies, including the Regal Fritillary (*Speyera idalia*; a Minnesota Species of Special Concern) collected from small tissue samples using non-lethal methods by many partners under numerous permits. Her lab is utilizing the PacBio and Illumina "next-generation" platforms to generate enormous amounts of genome-wide data. This data will be used for microsatellite identification and optimization. The goal is to identify at least 20 microsatellites unique to each species that can provide estimates of population genetic diversity. Composition and differences in frequencies of microsatellite variants between individuals within populations allow for estimates of local genetic diversity and effective population breeding sizes. We hope to perform these analyses for about 30 individuals per population.

Aliquots of these extractions are being provided to the Minnesota Zoo for the ENTRF-funded portion of this Activity. Dr. Runquist will used already established facilities and molecular genetics equipment at the Minnesota Zoo to amplify and sequence several known genes and other molecular markers for which evolutionary rates are better approximated than the largely anonymous microsatellites. These include mitochondrial genes like *cytochrome oxidase I* and *II*. Data from these and other known genes will also allow for estimates of evolutionary divergence between populations and regions using standard phylogenetic and phylogeographic methods. The Zoo will also screen 25-30 individuals oer population (as numbers allow) for *Wolbachia* and identify strains following the protocols outlined in Nice *et al.* (2009) and the *Wolbachia* MLST database (Baldo *et. al* 2006). It is expected that all, or nearly all, individuals within a population will be infected with *Wolbachia* if it is present in the population. Therefore, *Wolbachia* screening will be performed on a random subset of individuals (~10%) from each population, similar to the protocols in Nice *et al.* (2009).

To provide a context on the effects of isolation experienced by endangered species, these same methods and analyses are being and will be performed on non-endangered common species that co-occur with and have similar ecological habits as the endangered species. For example, population genetic diversity of Poweshiek skipperlings in Michigan will be compared with the co-occurring common Mulberry wing (*Poanes massasoit*). The Long Dash (*Polites mystic*) or Tawny-edged skipper (*Polites themistocles*) are the most likely species for Minnesota comparisons. The Regal Fritillary may also provide a metric for the potential effects of isolation on small skippers due to its higher dispersal abilities.

Results and Deliverables

Prior to this Activity, there have been no population genetic studies with Poweshiek skipperlings, and only minor studies with Dakota skippers using outdated genetic allozyme methods (Britten and Glasford 2002). Slightly more work has been conducted with Regal Fritillaries (Williams 2002; Williams *et al.* 2003), but none using whole genome "next-generation" methods. Therefore, our work will be foundational. Within the scope of this project, we expect to be able to quantify genetic diversity within each known extant population of the Poweshiek skipperling. Dr. Saarinen's lab has extracted DNA from about 120 Poweshiek skipperlings, mostly from the Michigan and Wisconsin populations, but also including Manitoba and dozens more await extraction. Her lab has already optimized 13 microsatellite markers for this species and has begun preliminary estimates of the effective population size for one population in Michigan using this data. Similar efforts are underway with both Dakota skippers and Regal Fritillaries, and we expect to be able to assess genetic diversity at the regional level (i.e. "west central Minnesota", "northeast South Dakota", "central North Dakota", etc.) for both of them. More populations are known to be extant for Dakota skippers and Regal Fritillaries than Poweshiek skipperlings, so more effort will be required for Dakota skippers and Regal Fritillaries.

For every individual, this microsatellite data will be paired with Zoo-generated sequence data from known genetic markers. These latter genes will allow for estimates of genetic divergence between populations, such as the average pairwise number of nucleotides differences and percentages within a given sequence that are different between individuals and populations. Estimates of inter-population and inter-region genetic distance are extremely important in identifying local areas of endemism that may be reflective of adaptations to local conditions. Local endemism may reflect histories of selection from local conditions that may not exist in other populations or regions. Signatures of local endemism may also arise due to isolation through local bottlenecks and drift due, even if local divergence was not present before the isolation event. Local endemism may or may not be due to selection, and the evolutionary source of that endemism is not always known. In either case, the preservation of local genetic diversity should be a goal for management plans.

We expect Poweshiek skipperlings and Dakota skippers to have more pronounced signatures of reduced local genetic diversity (lower heterozygosity) than related non-endangered species and highly dispersive species like Regal Fritillaries. Genetic divergence between populations and/or regions may also be more pronounced for the two endangered skipper species, but the effects of isolation may not be as pronounced in this regard because the reduction of the historically vast tallgrass prairies occurred within relatively recent evolutionary time and there may not have been significant local or regional endemism before its fragmentation. Comparisons of genetic diversity and divergence between endangered and non-endangered species will provide information on the evolutionary capacity of isolated populations.

Any prescriptions for future re-introductions or population supplementations of the endangered butterfly species must account for potential differences in 1) population and/or regional genetic endemism and 2) *Wolbachia* infection and strain status between populations and/or regions. The introduction of novel genetic identities and/or *Wolbachia* strains can severely harm destination population demographics. The primary goal of the Minnesota Zoo's prairie butterfly conservation breeding program is to provide an "insurance policy" that protects the genetic diversity remaining within threatened and endangered Minnesota prairie butterfly species. This genetic data collection will also be performed on every individual introduced into the Zoo's breeding population. The ancestry and genetic background of every individual in the breeding population will be tracked so that informed breeding prescriptions can be made to sustain the genetic integrity of the breeding populations that is also reflective of wild population genetic composition.

Timetable

<u>Ongoing:</u> Extraction of DNA from tissue samples as they become available.
<u>June-September 2014</u> – Collection of tissue samples for DNA extraction as necessary.
<u>October 2014-March 2015</u> – Sequencing of known markers to test for population-level divergence for Poweshiek skipperlings. Screening for and strain identification of *Wolbachia* strains in Poweshiek skipperlings. Assessment of genetic diversity of any Zoo-bred Poweshiek skipperlings and/or Dakota skippers for *ex situ* breeding prescriptions.
<u>June-September 2015</u> – Collection of tissue samples for DNA extraction as necessary.
<u>October 2015-March 2016</u> – Sequencing of known markers to test for population-level divergence for Dakota skippers. Screening for and strain identification of *Wolbachia* strains in Dakota skippers. Assessment of genetic diversity of any Zoo-bred Poweshiek skipperlings and/or Dakota skippers for *ex situ* breeding prescriptions.
<u>June-September 2016</u> – Collection of tissue samples for DNA extraction as necessary.
<u>October 2016-March 2017</u> – Final sequencing and analyses for remaining individuals and species. Assessment of genetic diversity of any Zoo-bred Poweshiek skipperlings and/or Dakota skippers for *ex situ* breeding prescriptions.
<u>April 2017-June 2017</u> – Preparation of results and submission to peer-reviewed scientific journals for publication.

Budget

The Conservation Genetics Laboratory at the Minnesota Zoo has already obtained all necessary equipment and machinery (thermocycler, freezer, pipettors, gel readers, etc.) through previously allocated Minnesota Zoo Legacy Amendment funds. The Minnesota Zoo has been quoted $1.50/read by a respected sequencing company. Building in associated shipping costs and laboratory reagent and equipment expenses, we anticipate the ability to produce about 2,000 sequences per year using this allocation. Following initial work using existing Legacy funds in late FY14, it is anticipated that the bulk of the sequencing needs will occur in FY15 and FY16.

Credentials

The Principal Investigator for this work will be Program Manager, Dr. Erik Runquist (see Activity 1). His Ph.D. employed a wide-range of phylogenetic, population genetic, and phylogeographic analyses using molecular genetic data he generated to test evolutionary and ecological questions associated with the effects of population isolation. Selected publication: Runquist, E.B., M.L. Forister, A.M. Shapiro. 2012. Phylogeography at large spatial scales: incongruent patterns of population structure and demography of Pan-American butterflies associated with weedy habitats. Journal of Biogeography 39(2): 382-396.

Providing a significant collaborative role for this Activity is Dr. Emily Saarinen, Assistant Professor of Biological Sciences at the University of Michigan-Dearborn. She has a M.S. and Ph.D. in Entomology from the University of Florida where she conducted foundational conservation genetics research on the endangered Miami Blue butterfly. Her research focuses on environmental science and the evolutionary potential of populations of conservation that are small or declining. Selected Publication: Saarinen, E.V., J. Daniels, and J. Maruniak. *In press.* Local extinction despite high levels of gene flow and genetic diversity in the federally-endangered Miami blue butterfly. *Conservation Genetics.*

Dissemination and Use

All genetic data produced will be deposited on relevant public databases (Genbank, MLST *Wolbachia* database, Dryad, etc.). Findings will be communicated to collaborators, relevant agencies, and submitted for publication in peer-reviewed scientific journals. The findings will help inform both *in situ* and *ex situ* management prescriptions of the endangered species.

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Activity 3: DNR Butterfly Status Monitoring

Abstract

This component of the project will establish long-term monitoring of the status of 12 butterfly species that are restricted to native prairie habitats in Minnesota. The nearly complete destruction of their habitat and the highly fragmented condition of what remains places these animals at a high risk of extirpation in the state. The failure to recognize catastrophic declines in two species until they had disappeared or almost disappeared from the state highlights the need to detect declines before they become irreversible. The first year of the project will initiate presence-absence monitoring in about 40 sites distributed throughout western MN. This will be repeated the second year. If sites with extant populations are located during the initial year, more intensive abundance monitoring phase will begin in these sites the second year.

Background

Survey projects targeting the Dakota skipper and the Poweshiek skipperling, two butterflies that require native prairie habitat and that historically have been more common in MN than elsewhere in their limited ranges, have recently made it apparent that both have declined catastrophically. The Poweshiek skipperling may no longer be present in MN and the Dakota skipper may be on the verge of following. The first is currently proposed for federal listing as Endangered by the USFWS, the second for listing as Threatened (USFWS 2013). Both species are currently listed as Endangered in Minnesota. Until recently efforts to monitor the status of these two species were sporadic and limited in scope, and even recent work has left room for significant uncertainties. Numerous factors contribute to the difficulty of detecting the presence of these butterflies and the only currently feasible method of dealing with these is to increase the intensity of sampling. It remains possible that these two species are present in MN but have escaped detection because of very low numbers.

Discovery of survivors is important for several reasons, a salient one being to provide foundation stock for the MN Zoo's captive breeding project for these two species. The butterflies from more distant parts of the range may differ genetically from those in MN in ways that will affect the success of reintroductions here. Furthermore, survivors may be resistant to whatever has caused the decline, and their use for captive breeding and reintroduction could accelerate adaption to the new stressors. More generally, preserving as much of the genetic diversity of these species as possible will be fundamental for the survival of the species in a changing world. The MN Zoo is investigating the genetics of these skippers, and obtaining tissue samples from Minnesota will provide crucial data (see Activity 2). Site characteristics where skippers survive may also provide clues to other possible factors in the decline (e.g., isolation from cropland and associated pesticide drift).

In addition to the two skippers just discussed there are 10 other butterfly species in Minnesota that require native prairie habitat: Dusted skipper (*Atrytonopsis hianna*), Garita skipperling (*Oarisma garita*), Iowa beardgrass skipper (*Atrytone arogos iowa*), Ottoe skipper (*Hesperia ottoe*), Leonard's skipper (*Hesperia leonardus leonardus*, but atypical "blend zone" populations), Pawnee skipper (*Hesperia leonardus pawnee*), Melissa blue (*Plebejus melissa melissa*), Gorgone checkerspot (*Chlosyne gorgone*), and Regal fritillary (*Speyeria idalia*). Of these, only the Regal fritillary seems to be holding its own; its size, distinctive appearance, and long adult flight period make it the most readily detectable of these specialist butterflies, resulting in reports of its presence from even non-expert observers. Uhler's arctic (*Oeneis uhleri*), a dry grassland species, has not been seen in the state for more than 25 years; this butterfly was documented from only a small area in MN, at the eastern edge of its range, and habitat loss probably doomed it. What data we have for the other prairie butterflies suggest declines for most, but survey efforts targeting them have been limited (for some, none).

Hypothesis

No hypothesis in the usual sense is being tested, unless it is that the two skipper species discussed above are still present in the state, and that the other species are not declining. The intent of the project is to design and implement a monitoring approach that will have reasonable power to answer those questions.

Methodology

Approximately 40 sites distributed throughout western MN will be surveyed. These will include all of the sites having historical records indicating strong populations of Poweshiek skipperling and Dakota skipper, as well as sites without such documentation but having attributes that should support such populations. The other 10 species have been recorded in most of these sites, and sites will not be included for these species where either of the two highest priority skippers have not been recorded. Below, Figure 1 displays probable monitoring site locations.

The initial phase of this work will be essentially a structured search of the selected sites. In sites where the presence-absence survey finds a threshold number of individuals of a species, abundance monitoring of that species in that site will subsequently be initiated. For the presence-absence survey, timed visual search of potential habitat in each site will be conducted by an experienced surveyor during the flight period of each target species. Search effort will be limited to the period of the day and weather conditions when adults are normally active. The standards usually applied are those developed by Pollard (1971), but for search (as opposed to monitoring), these can be loosened somewhat as long as the surveyor feels that the target species is likely to be active enough to be detected. In order to standardize effort the potential habitat area to be surveyed will be estimated and the maximum amount of time allowed for a survey of the site will be based on this, at 1 hr/100 ac. Surveyors will follow a roughly delineated route taking them through most or all of the appropriate habitat in a site, but adjusting the precise route as their judgment of conditions (e.g. locations of nectar flowers, host plants) indicates would be productive. They will employ GPS units to help guide them and record track logs of actual survey route taken. Search for a target species in a site will terminate when 10 individuals of that species are encountered or when the alloted time is up, whichever comes first. If possible two presence-absence surveys of each site will be conducted in a year, especially if conditions during the first search were suboptimal. If 10 individuals of a target species are recorded during the first survey of a site, no additional search of that site for the species is necessary the same year, Recording 10 individuals of a species during the presence-absence search of a site is the threshold for subsequently conducting abundance monitoring of the species in that site. It is expected that abundance monitoring will begin the season following the presence-absence survey that triggers it.

Presence-absence surveys will be repeated in the second year (2015) for all species in all sites except in cases where the results of this survey trigger initiation of abundance monitoring. Because the project cannot begin until 1 July 2014, the two species whose flight periods are over by this date, Dusted skipper, Garita skipperling, will have their first presence-absence survey in 2015. For these two species, any abundance monitoring will wait until the final year (2016). In normal years, Poweshiek skipperling and Dakota skipper flights begin the latter part of June and continue into July, If there is a cooler than normal spring/early summer in 2014 it may not be possible to survey for them this first year, and if 2016 has a warmer than normal early season, it may not be possible to conduct a second season search or abundance monitoring for them before the end of June, Consequently, if the first presence-absence survey for these two species is delayed until 2015, abundance monitoring for either will be initiated immediately upon observation of the threshold number in a site during the presence-absence search. In the abbreviated 2016 field season (May-June) the two early-season butterflies will be targeted for their second round of presence-absence survey, with abundance monitoring initiated for a species in sites where the 2015 survey met the threshold of 10 observations of that species.

The presence-absence monitoring can establish presence with certainty, but absence can only be estimated as a likelihood. We will not attempt to provide quantitative estimates of absence, which would require modeling that is beyond the scope of our knowledge about the butterflies at this point.

The trigger for abundance monitoring of 10 individuals is somewhat arbitrary, chosen as a convenient marker of the presence of a large enough population in the site to warrant this more intensive monitoring. When abundance monitoring is conducted for a species in a site, other target species whose flight period coincides with it will recorded in that site with this protocol as well. Otherwise, the other species will continue to be monitored for presence only. The methodology to be used for abundance monitoring has not been decided upon yet. This will likely be some version of the “Pollard Walk” approach (Pollard & Yates 1993), essentially counts of observations along transects, standardized by either distance or time (therefore, an observational rate). The modification developed by Debinski et al. (2000) for the National Park Service is particularly attractive, as it allows for standard statistical analysis. This involves the use of multiple short transects of equal length, similar to the point count methodology used for bird surveys

Tissue samples for the genetics and Wolbachia research components of this project will consist of one middle leg removed from each butterfly sampled. Butterflies will be netted and temporarily anesthetized using CO2 before leg-removal, and held until recovery before release. While anestetized these will be marked with a felt-tip marker on the hindwing underside so that subsequent recapture can be avoided.

Results and Deliverables

The first year’s work, assuming normal seasonal development will either determine that there are sites in MN where one or both of the two highest priority skippers still occur, or it will add to the evidence that they are extirpated. These data will be quickly available, sometime in Fall 2014. If survivors are found, the presence-absence data will be analyzed for patterns in site characteristics that could point to possible agents causing the declines. Preliminary analysis would be done during Fall-Winter-Spring between the 2014 and 2015 field seasons. Managers of sites where either of these skippers are found will be informed so that modifications to planned management activities that could joepardize their survival can be made to reduce the threat. Tissue samples obtained would be transferred almost immediately to the researchers conducting the conservation genetics and *Wolbachia* studies of Activity 2.

These results will also be the initial data long-term presence-absence monitoring of the other summer prairie species at a large sample of sites.

The second year’s work will in most aspects supplement the results from the first year, with initial presence-absence monitoring of the early-season species and abundance-monitoring, if any, being the major addition.

A report detailing the presence-absence results and analysing the pattern if there is any presence will be completed by the end of the funded project . If any abundance monitoring occurs, these data and analyses will also be presented in the report. A database will be created to maintain all monitoring data.

Budget

The entire DNR allocation for this Project of $245,000 will go to supporting the expenses associated with the monitoring work as outlined in the attached budget.

Credentials

Principal investigator for this component: Robert P. Dana.

Ph.D. 1989. University of Minnesota. Entomology
Thesis: Biologies of the prairie skippers *Hesperia dacotae* and *H. ottoe* (Lepidoptera: Hesperiidae), and factors affecting mortality in prescribed burning of their habitat in spring

Employed by MN DNR since 1985, most of that time as a plant and insect ecologist with the MN Biological Survey, with substantial focus on native prairie. Extensive work surveying for native prairie remnants and collecting and analyzing vegetation data. Occasional insect survey work, principally butterflies, but some moth survey as well. Several prairie butterfly monitoring studies since 2006. Much involvement with insect conservation issues in DNR, The Nature Conservancy, and U.S. Fish and Wildlife Service.

Selected publications: Conservation management of the prairie skippers, *Hesperia dacotae* and *Hesperia ottoe*: basic biology and threat of mortality during prescribed burning in spring. Station Bulletin 594-1991. University of Minnesota, Agricultural Experiment Station, St. Paul, MN. 1991.

Dissemination and Use

Occurrence data will be entered into the DNR Rare Features Database. A major use of the data in the database is environmental review. Occurrences in sites managed by DNR, USFWS, and private conservation organizations (TNC) will be made known to those entities. The data may be useful to researchers investigating causes of declines or more generally factors important in population dynamics. The abundance-monitoring data will provide a more sensitive means of detecting declines. This monitoring may be useful for evaluating the effects of management activities on these insects so that management can be adjusted to assure that it does not adversely affect these butterflies. Other prairie-dependent invertebrates will probably also benefit from application of such knowledge to prairie management. Tissue samples will be used by the MN Zoo investigators conducting other components of the research in this project. The discovery of survivors in MN will enable the Zoo's *ex situ* conservation breeding program to include MN populations (if population sizes are deemed to be large enough following consultations with partners), preserving greater genetic diversity and local adaptations.

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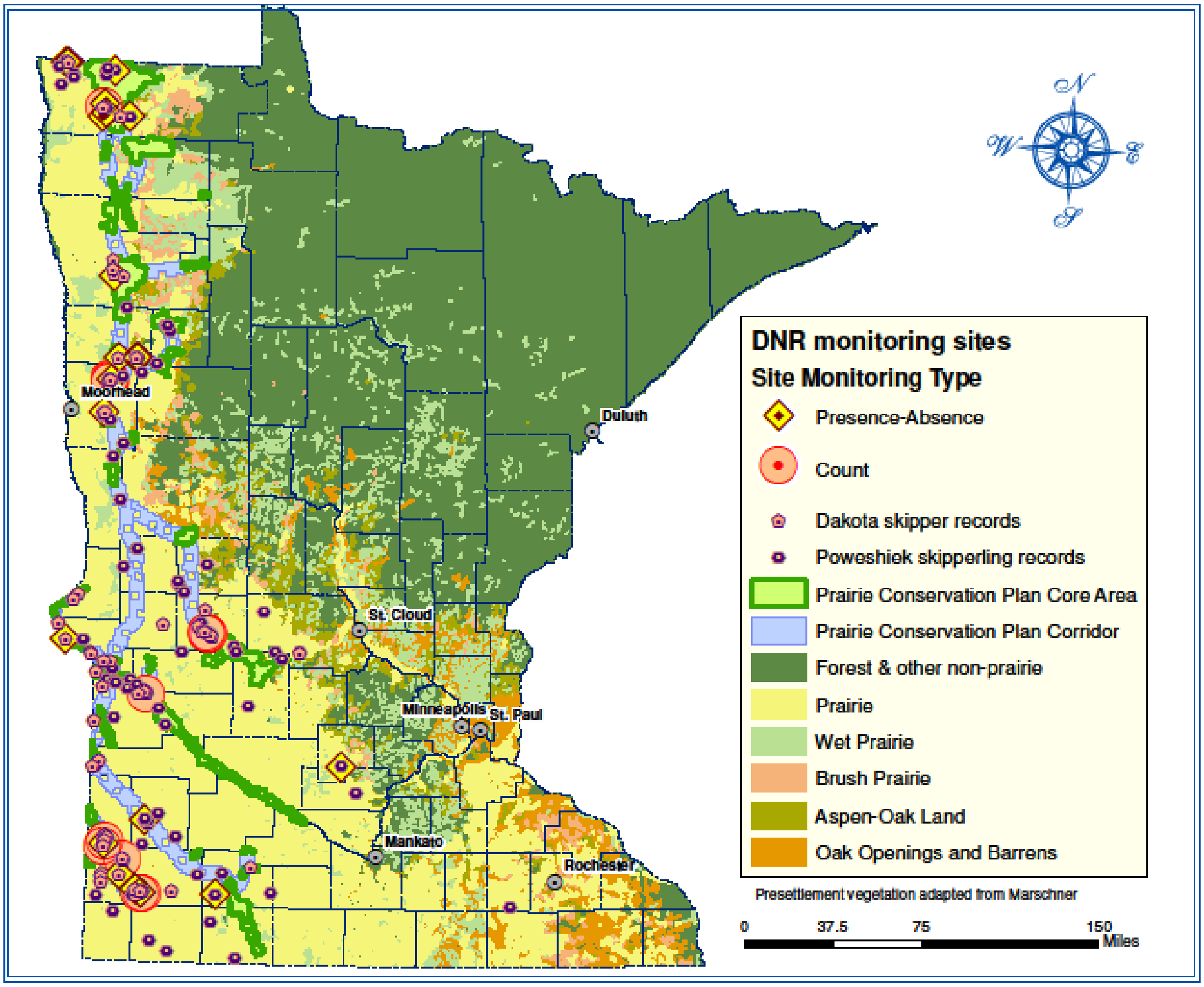
Figure 1: Probable sites for Activity 3, the Minnesota DNR's monitoring survey efforts for Poweshiek skipperlings, Dakota skippers, and other prairie butterflies.

2014 LCCMR Proposal: Prairie Butterfly Conservation, Research, and Breeding Program.
DNR Monitoring Sites in Relation to MN Prairie Plan



Activity 4: Pesticides-related Mortality Research

Abstract

The proximity of agricultural fields to remnant native tallgrass prairie habitats has led to speculation that non-target insecticide exposures from agricultural operations may have contributed to the decline and apparent disappearance of many Minnesota native prairie butterflies. Using experimental arrays of larval hostplants treated with variable concentrations of a common neonicotinoid insecticide, we will test for differences in growth rates and survivorship in non-endangered surrogate species that have similar habits to endangered Minnesota native endangered skipper butterflies. We will pair this research with assays of field collected plant tissues to see if this insecticide is detectable on wild Minnesota prairies. This research will help inform pesticide management in the prairie-agriculture interface, as well as in horticultural settings were plants are commonly treated with the insecticide.

Background

The historically vast tallgrass prairies of the Upper Midwest have been dramatically reduced and fragmented, with the vast majority of the historic acreage now converted to intensive row crop agriculture. The close proximity of agricultural lands to prairie remnants that formerly or may still retain populations of threatened and endangered prairie butterflies suggests the potential that drift from agricultural pesticide applications near prairie fragments may have indirect effects on these imperiled and other prairie species (Longey and Sotherton 1997). Neonicotinoids have become one of the most important groups of agricultural and horticultural insecticides since their development in the 1990s. Their use has increased as an alternative to previously widespread applications of pyrethroid, carbamate, and organophosphate insecticides due to their lower binding potential to mammalian neural receptors and correspondingly lower human health risks. These insecticides can be applied as a foliar spray, a soil treatment, and as a seed coat powder. This systemic pesticide becomes incorporated into plant tissues, nectar, and pollen and can persist and accumulate in soil and water for years (Goulson 2013). Numerous studies have documented the negative influence of neonicotinoids on non-target organisms (Mason *et al.* 2013), including beneficial insects like honey bees (Pettis *et al.* 2013) and aquatic macroinvertebrates (Van Dijk *et. al* 2013). Seed coat applications of neonicotinoids can also become airborne as dust during planting operations that can coat adjacent non-crop plants with powder that can have lethal and sub-lethal effects (Marzaro *et. al* 2011; Krupke *et. al* 2012; Tapparo *et. al* 2012).

The USFWS proposal to list Poweshiek skipperlings and Dakota skippers under the U.S. Endangered Species Act (USFWS 2013), as well as at the Northern Tallgrass Prairie Lepidoptera Conservation Conference (Minnesota Zoo 2013) identified the need for further data on the effects of neonicotinoids on non-target invertebrates and on endangered species. To begin addressing this research need, we propose an experiment that tests the effects of varying concentrations of thiamethoxam applications on growth rates and survivorship of grass skipper butterfly caterpillars. Thiamethoxam is one of the primary neonicotinoids used in Minnesota for corn and soybean seed treatments, and is therefore a likely potential source of exposure. Its usage also became common in Minnesota and the agricultural Upper Midwest at roughly the same time that Poweshiek skipperling and other prairie butterfly populations experienced steep in the early-mid 2000s (Pesticide National Synthesis Project, accessed February 2014). Grass skippers spend the majority of their lives as caterpillars, and potential pesticide effects are expected to be greatest on caterpillars. Comparable experiments to our proposed work with Monarchs (*Danaus plexippus*) and Painted Ladies (*Vanessa cardui*) demonstrate strong effects on larval survivorship but non-significant effects on the nectar feeding adults by the biochemically comparable neonicotinoid imidacloprid (Krischik, in prep). We will perform the experimental tests using non-endangered surrogate species of related grass skippers that are similar in terms of their natural history and ecological associations to mitigate the cost of conducting these experiments with endangered species. No experiments on the effects of thiamethoxam on butterflies like these skippers have been conducted to date. Paired with this experimental research, we will test for the presence of thiamethoxam residues (and potentially other neonicotinoids if possible) that may be present on non-target native prairie remnants adjacent to agricultural fields.

Hypotheses

We hypothesize that small grass-feeding skipper butterfly caterpillars are susceptible to thiamethoxam exposure, and that the effects will be more pronounced than on them than on larger species of butterflies. We are interested in addressing the following questions:

1) How do varying concentrations of thiamethoxam affect larval growth rates and survivorship in grass-feeding skippers? Are there subsequent differences in fitness and survivorship during the pupal and/or adult stages?

2) Can thiamethoxam be detected in wild plants and in soil samples on native prairie remnants adjacent to agricultural fields?

Methodology

1) To test our first set of questions, we will build upon and use the resources already dedicated for the Minnesota Zoo's conservation breeding program (Activity 1) to initiate breeding with several potential surrogate species in 2014. These might include the Long Dash (*Polites mystic*), Peck's skipper (*Polites peckius*), Least skipper (*Ancyloxypha numitor*), or European skipper (*Thymelicus lineola*). Like Poweshiek skipperling and Dakota skipper, all of these species are small Hesperiine skippers that also feed on grass hostplants as caterpillars. These butterflies are relatively common and often co-occur on prairie fragments and other grassy areas throughout Minnesota. Final assessment of the species to be used for the experiment will depend on population number for each species. The additional costs of rearing these species will be minimal and largely only constitute personnel hours.

We will apply three concentrations of thiamethoxam to the soil of established potted grasses that serve as larval hostplants for the test surrogate skippers. Plants in Treatment 1 will be applied a 1X concentration according to manufacturer instructions. Plants in Treatment 2 will be applied a 2Xconcentration, Treatment 3 plants will have a 0.25X concentration (but see methods Question 2 below), and Treatment 4 plants will serve as a control with no thiamethoxam and only a comparable volume of water as Treatments 1 -3 be applied to them. Once plants are provided enough time to uptake the thiamethoxam, approximately 10 caterpillars will be placed on each of 15 potted plants per treatment. The caterpillars will be allowed to feed as they would naturally on the potted plants under controlled conditions inside fine mesh screen cages. All caterpillars will be weighed prior to placement on the plants, and caterpillar survivorship and weights will be censused on all plants approximately weekly. Pupal weight will be recorded as well as adult weight and forewing length one day post emergence. Survivorship and weight measures within each pot will be averaged across individual butterflies. We will test for differences in these metrics between the four treatments in an ANOVA framework, with the number of pots (n=15) as the within treatment sample size. To ensure consistency across all aspects of this Activity, we will repeat the entire experiment during the same season of the second year and test for differences between years.

Concentrations of thiamethoxam present in the potted plants will likely be consistent across pots, but we will collect leaf tissue samples from three randomly chosen plants per treatment per year for imidacloprid residue concentration testing. The samples will likely be sent to the USDA National Science Laboratory in Gastonia, NC for testing. This lab is the only U.S. laboratory certified to conduct these residue concentration tests.

2) We will test for residues of thiamethoxam (and likely other neonicotinoids) existing in non-target plants on native prairie remnants by collecting samples of young leaf tissues of the same species of prairie grasses used in our first set of experiments (likely prairie dropseed, *Sporobolus heterolepis*). Soil samples may also be collected. We will consult with the Minnesota Department of Agriculture and other partners to identify suitable prairie remnants adjacent to agricultural fields where thiamethoxam has been applied. We will collect at least 35 samples per year at variable distances from the agricultural field over two years. These samples will also be sent to the certified laboratory for residue testing. If thiamethoxam is detected in wild plants, we may consider modifying the application concentration of Treatment 3 plants to more precisely measure the potential effects of that exposure to wild caterpillars.

Results and Deliverables

Our first experiment will produce the following variable metrics to assess the effects of thiamethoxam on grass skipper survivorship and viability: Larval survivorship, larval growth rates, pupal mass, adult mass, and adult size. Our experiment will also provide novel information on the degree to which native prairie grasses may take up thiamethoxam, as well as the potential exposure levels to thiamethoxam on native prairie fragments. This research will not only inform the risk presented by thiamethoxam to small butterflies like skippers under wild conditions, but also the risks that horticultural applications of thiamethoxam and other neonicotinoids to native butterflies on urban and suburban landscapes.

Timetable

May 2014 – June 2015: Breeding of surrogate species at the Zoo and selection of species to be used in testing.
July 2014- March 2015: Collection of wild plant tissue and soil samples for residue analysis.
May 2015: Application of treatments to potted test plants.
June 2015- September 2015: Placement of caterpillars on plants and weekly monitoring of their survivorship and growth.
September 2015 – June 2016: Data analyses. Continuation of breeding program with surrogate species.
May 2016: Application of treatments to new potted test plants.
July – September 2016: Collection of additional plant tissue and soil samples for residue analysis. Placement of caterpillars on plants and weekly monitoring of their survivorship and growth.
September 2016 – June 2017: Data analyses and submission to peer review journal(s) for publication.

Budget

The USDA National Science Laboratory in Gastonia, NC is currently the only U.S. laboratory certified to conduct the pesticide residue concentration tests. The current per sample cost is $166/sample (as of December 2013). Therefore, we estimate that the cost to assess pesticide residue concentrations for 24 potted plants (3 randomly chosen from each of the 4 treatments x 2 replicate years) from our controlled experiment to be about $4,000. We also estimate another $12,000 for residue testing of about 70 plant samples collected from native prairie remnants. Therefore, we are budgeting $16,000 for the residue analyses. Another $1,600 will likely be needed to purchase potted plants for experimental testing, pesticides, and associated additional equipment and expenses. This results in non-personnel costs of about $17,600 for this research, the majority of which will be conducted in FY16 and FY17. A portion of the temporary worker's time is allotted to assist in operations of this research.

Credentials

The primary investigator will be Dr. Erik Runquist, Butterfly Conservation Biologist at the Minnesota Zoo. See qualifications above. He is also serving on the Minnesota Department of Agriculture's Pollinator Best Management Practices working group on Prairie-Agricultural land interface issues.

Cooperating in the research is Dr. Vera Krischik, tenured Associate Professor of Landscape Ecology in the Department of Entomology, College of Food, Agricultural and Natural Resource Sciences at the University of Minnesota. Dr. Krischik is a leading researcher on the effects of imidacloprid on non-target insects, including butterflies, and can provide equipment, facilities, and advice for this Activity. She is also director of CUES: Center for Sustainable Urban Ecosystems that promotes natural resource management (www.entomology.umn.edu/cues). Dr. Krischik received an LCCMR grant "Mitigating Pollinator decline" in 2010. From the 2010 LCCMR grant 5 research papers will be produced. An outreach poster on bee-friendly plants, a protecting bee bulletin, pesticide and bee bulletin, and an online workshop are available at http://www.entomology.umn.edu/cues/pollinators/index.html.

Dissemination and Use

Results will be prepared and submitted to peer-reviewed scientific journals for publication. Our findings will be communicated to policy makers, including those at the federal (EPA) and state-levels (MN Department of Agriculture) evaluating neonicotinoid pesticide best practices. Program Manager Dr. Erik Runquist is serving on the Pollinator Best Management Practices working group, and communication between the activities of the working group and the Zoo's ENTRF research will also be advanced.

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