NATURAL HISTORY AND CONSERVATION GENETICS OF THE FEDERALLY ENDANGERED MITCHELL'S SATYR BUTTERFLY, *NEONYMPHA MITCHELLII MITCHELLII*

By

Christopher Alan Hamm

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ABSTRACT

NATURAL HISTORY AND CONSERVATION GENETICS OF THE FEDERALLY ENDANGERED MITCHELL'S SATYR BUTTERFLY, *NEONYMPHA MITCHELLII MITCHELLII*

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The Mitchell's satyr butterfly, *Neonympha mitchellii mitchellii*, is a federally endangered species with protected populations found in Michigan, Indiana, and wherever else populations may be discovered. The conservation status of the Mitchell's satyr began to be called into question when populations of a phenotypically similar butterfly were discovered in the eastern United States. It is unclear if these recently discovered populations are *N. m. mitchellii* and thus warrant protection. In order to clarify the conservation status of the Mitchell's satyr I first acquired sample sizes large enough for population genetic analysis I developed a method of non-lethal sampling that has no detectable effect on the survival of the butterfly. I then traveled to all regions in which *N. mitchellii* is known to be extant and collected genetic samples. Using a variety of population genetic techniques I demonstrated that the federally protected populations in Michigan and Indiana are genetically distinct from the recently discovered populations in the southern US. I also detected the presence of the reproductive endosymbiotic bacterium *Wolbachia*, and surveyed addition Lepidoptera of conservation concern. This survey revealed that *Wolbachia* is a real concern for conservation managers and should be addressed in management plans. Finally, I examined the variation in wing patterns among *Neonympha* taxa using geometric morphometrics and multivariate statistics. This methodology allows researchers to empirically examine qualitative traits by placing landmarks at homologous position and quantify the variation among taxon. Analysis of wing patterns revealed that the endangered taxa

could be clearly and consistently distinguished from congeners. I conclude this dissertation with an outlook for the Mitchell's satyr and a call to action for the protection and recovery of this endangered species.

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CHAPTER 1

NATURAL HISTORY AND CONSERVATION STATUS OF THE ENDANGERED MITCHELL'S SATYR BUTTERFLY: A SYNTHESIS AND UPDATE OF OUR KNOWLEDGE REGARDING *NEONYMPHA MITCHELLII MITCHELLII* FRENCH 1889

Abstract

The Mitchell's satyr butterfly, *Neonympha mitchellii mitchellii* French 1889 is a federally-listed endangered species found in parts of the eastern United States of America. Because of its endangered status considerable research efforts have been devoted to understanding its biology, ecology, and its conservation. Despite these efforts, information about *N. m. mitchellii* has not been summarized for more than a decade. Here we summarize and expand upon the work conducted by governmental and not-for-profit agencies that have produced reports that are not easily accessible to researchers or interested lepidopterists. In addition to summarizing the literature, we present data from feeding trials and also demonstrate that microclimates exist that may be exploited by larvae. We conclude by identifying key areas of needed research and describe steps imperative to the recovery the Mitchell's satyr.

Introduction

The Mitchell's satyr butterfly, *Neonympha mitchellii mitchellii* French 1889 (Nymphalidae: Satyrinae) is a federally-listed endangered species found in parts of the eastern United States of America. As one of only 60 endangered insects, and one of only 20 federally endangered Lepidoptera in the US, it is of particular interest to conservation organizations and butterfly enthusiasts alike (US Fish and Wildlife Service 2011). As a result, a considerable amount of research effort has been devoted to understanding its biology and ecology, as well as studies that inform its conservation. Adding to the uniqueness of *N. m. mitchellii* as a target of conservation, is the fact that its sister taxon, the Saint Francis' satyr, *N. m. francisi*, is also endangered in the US. Morphologically similar to Mitchell's satyr, *N. m. francisi* is known from only one small region of North Carolina on the Fort Bragg Military Reservation. While the Saint Francis Satyr has recently received a thorough treatment of its natural history and population biology by Kuefler et al. (2008), information about *N. m. mitchellii* has not been summarized for more than a decade (Shuey 1997). In that time, considerable new findings have emerged that fundamentally shift our understanding of the species and the prospects for its long-term conservation.

One key discovery that has changed the way we perceive Mitchell's Satyr is the discovery of new populations that greatly expand its known geographic range and habitat use patterns. Discovered in Virginia, Alabama and Mississippi in 1998-2004 (Roble et al. 2001; Hart 2004), these new populations are designated as *N. mitchellii* and do not share the federally endangered status of *N. m. mitchellii* populations found in Michigan and Indiana [hereafter we use *N. mitchellii* to refer to the species in the broad sense, i.e. including both the northern, and southern populations but excluding the Saint Francis' satyr, and *N. m. mitchellii* to refer

specifically to the protected northern populations]. These new populations offer unique opportunities to explore the natural history of the Mitchell's satyr throughout a larger portion of its historic range and to conduct new investigations that may inform its conservation more broadly. While the new populations may increase options for recovery, within Michigan and Indiana there is evidence that *N. m. mitchellii* populations are being lost at an alarming rate, emphasizing the pressing need for effective recovery plans based on sound conservation science (Landis et al. 2011).

Given its protected status, governmental and not-for-profit conservation organizations have spent thousands of person hours investigating aspects of *N. mitchellii* biology. Unfortunately, many of the reports generated by these efforts enter the so-called "grey literature," and are not easily accessible to academic researchers or lepidopterists. During many conversations with both researchers and butterfly enthusiasts it has become clear that much confusion surrounds the biology of Mitchell's satyr, sometimes with little distinction between lore and published data. As such, one goal of this paper is to synthesize and update the literature surrounding *N. mitchellii*. In addition, by pointing to critical gaps in our knowledge, we hope to prioritize future research needs for effective conservation of this endangered species.

Physical Description

The following physical descriptions represent those typical of *N. mitchellii* and are not absolute descriptions. All traits are variable and when there are major deviations from the typical forms they are noted as such. The eggs of *N. mitchellii* appear light to pale lime green (see McAlpine et al. 1960 for detailed line drawings of all immature stages) with their color imparted by the developing embryo, since the chorion itself is transparent (C. Hamm pers. obs). The egg is spherical in shape with a diameter between 0.7 and 1.0 mm and covered with an alveolate

sculpturing (Harris 1979). Within two days before hatching, the developing head capsule is visible as a dark spot within the egg (McAlpine et al. 1960; Legge and Rabe 1996; C. Hamm pers. obs.).

First instar larvae have a conspicuous dark brown head capsule and bilobed projections that are common to satyrine larvae (Wagner 2005). First instars range in length from 3 to 4 mm (McAlpine et al. 1960; Szymanski 1999) and are cylindrical in shape, with the tip of the abdomen terminating abruptly. All subsequent instars (total of 5) have a green head capsule and retain the bilobed shape, with the abdomen terminating in a bifurcated process. These later instars, which are 6 – 12 mm in length, also possess two raised white ridges on the dorsum that traverse the antero-posterior axis from the prothoracic segment to the tip of the abdomen. Additionally, later instars are covered with irregular white papillae. All larvae are inconspicuous and extremely difficult to locate in the field (Darlow 2000). Observations on the size of *N. mitchellii* larvae may be upwardly biased since they were based on individuals reared in captivity under conditions that may not approximate those in nature (McAlpine et al. 1960; Wilsman and Schweitzer 1991; Legge and Rabe 1996; Darlow 2000; B. Bergman pers. comm., M. Nielsen pers. comm.). The pale green chrysalis is suspended from the cremaster in the head down orientation typical of many satyrine butterflies (Mosher 1916; DeVries 1987), and is between 10 and 15 mm in length. As with the egg, it is the developing imago that imparts color to the pupa, the actual integument being translucent and smoky in color (McAlpine et al. 1960; C. Hamm pers. comm.). Approximately 48 hrs prior to eclosion the chrysalis begins to transition its color from light green to medium brown.

The adult Mitchell's satyr butterfly was described based on a series of six males and four females collected by J.N. Mitchell, a professor at the University of Michigan (French 1889). The

type series was collected in Cass County, Michigan from an "upland dry meadow," but these butterflies likely originated from the nearby tallgrass prairie fen (French 1889; McAlpine et al. 1960; Shuey 1997). The original name of the Mitchell's satyr, *Neonympha mitchellii*, was later changed to *Euptychia mitchellii* and then *Cissia mitchellii*, but the original genus name of *Neonympha* is currently valid (Dyer 1902; Hemming 1937; Lewis 1974; Hamm 2007).

Imagos of *N. mitchellii* are medium-sized brown butterflies that resemble many of the other members of the Satyrinae. Male *N. mitchellii* have a wingspan of roughly 2.5 cm while females are larger, with a wingspan of approximately 3.0 cm (Hamm et al. 2010). The Mitchell's satyr was originally described with a medium brown dorsal wing surface and lighter brown ventral wing surface, with females darker than males (French 1889). Subsequent research has noted that both sexes are darker when they first emerge from the chrysalis and may even have a 'sheen' to them, which wears off within hours of eclosion (Barton and Bach 2005). In addition, *N. mitchellii* color appears to vary throughout its range, may be polyphenic (from tan to a dark brown) and associated with the hydrology of sites (Brakefield 1996; Hamm 2009). We have observed that, in general, sites with high levels of water are associated with darker butterflies (Hamm 2009), although this observation remains to be quantified. Similar observations have been made for other butterflies, including satyrs (Brakefield 1996). Color polyphenism is thought to provide an advantage by correlating the color of the butterfly more closely with its habitat (Brakefield 1996). High water levels support more lush plant growth, against which a light colored butterfly would stand out. By being darker when there are higher levels of ground water, the butterfly is presumably able to blend in more effectively. Adult *N. m. mitchellii* are rather short lived, with the average male living between two to five days and the average female two to four days (Szymanski et al. 2004).

One of the most conspicuous characters noted in the descriptions of *N. mitchellii* is the prominent border ocelli on the ventral surface of the wings. Border ocelli, sometimes mistakenly referred to as eye spots (Nijhout 1991), are situated in cells between wing veins in the postmedial area of the wings (Figure 1). Females have the same number of border ocelli as males but they tend to be larger (C. Hamm unpub. data). On the forewing, border ocelli may be found in the cells M_1, M_2, M_3 and Cu_1, and on the hindwing the border ocelli may be found in the cells R_5, M_1, M_2, M_3, Cu_1 and Cu_2. Based on preliminary data from over 300 museum specimens, each *N. mitchellii* male forewing usually has three and each hindwing has six ocelli (C. Hamm unpub. data).

Each ocellus appears as two concentric rings of pigment, with an outer ring of buff yellow and an inner ring of black, centered on a silver focus (Figure 1). In contrast to the original description, which holds that all border ocelli are circular (French 1889), ocelli actually range in shape from circular to oval (C. Hamm unpub. data). A pair of bands surrounding the border ocelli often converge at the leading and trailing edges of the wings. These bands correspond to the proximal and distal bands of the central symmetry system (Nijhout 1991) and range in color from light orange to brown. The thorax and walking legs of *N. mitchellii* are densely covered with setae and scales similar in color to that of the wings, though the setae projecting off of the prolegs are often a dark brown.

Distribution

Our understanding of the range of Mitchell's satyr has continued to evolve over time. After French's description was published, the Mitchell's satyr was subsequently found in fens throughout the Battle Creek-Kalamazoo and Jackson glacial interlobate regions (areas where ice sheets were in contact) of Michigan (Figure 2) (Wolcott 1893; Siepmann 1936; Moore 1939,

1960; Landis et al. 2011). The influential *Butterfly Book* (Holland 1898) also noted the Mitchell's satyr in Morris and/or Sussex Counties of northern New Jersey. The Mitchell's satyr was next confirmed in Portage County in eastern Ohio (Pallister 1927) and LaGrange County of northern Indiana (Badger 1958).

Several subsequent reports of *N. mitchellii* have been called into question for various reasons. One such report is that of *N. mitchellii* from Anne Arundel County, Maryland. During World War II two brothers collected a butterfly from a "military marsh" in the vicinity of Fort Meade and shipped the specimens home, where they were subsequently lost (Opler and Malikul 1998; P. Opler pers. comm.). The lack of a voucher specimen should warrant skepticism, but in this case some authors are convinced that the sighting was accurate (P. Opler pers. comm.). Arnett (2000) referenced Mitchell's satyr from Pennsylvania but no details were given beyond the state level reference. Rutkowski (1966) stated that it was highly likely the butterfly existed in Pennsylvania and he encouraged lepidopterists to search for it, no specimen of Mitchell's satyr from Pennsylvania is known to exist.

In 1983, a single population of butterflies, which appeared phenotypically similar to the Mitchell's satyr, was discovered on the Fort Bragg Military Reservation in North Carolina. Further exploration uncovered a number of additional occupied sites, all were restricted to Fort Bragg (Parshall and Kral 1989; Kuefler et al. 2008). Citing phenotypic differences, such as the shape of the male valvae, and ecological differences, such as voltinism (these populations are bi-voltine), the *N. mitchellii* in North Carolina were described as a new sub-species, *Neonympha mitchellii francisi*, the Saint Francis' satyr (Parshall and Kral 1989).

In 1998, during a 4th of July Butterfly Count, observers discovered a population of what appeared to be *N. mitchellii* in Floyd County, Virginia, approximately 200 km from Fort Bragg, North Carolina (Roble et al. 2001). Subsequent searching revealed additional sites that harbored *N. mitchellii* populations within Virginia, although only within Floyd County. In June of 2000, a population of *N. mitchellii* was discovered in the Oakmulgee Ranger District of the Talladega National Forest in central Alabama (Glassberg 2000, 2001). Since this discovery, researchers have identified approximately 20 sites within the Oakmulgee Ranger district as well as sites along the Natchez Trace Parkway in northeastern Mississippi that contained *N. mitchellii* (Hart 2004; Hamm 2008). As noted earlier, the recently discovered populations (Virginia, Alabama and Mississippi) are treated as *N .mitchellii* and not as either the Mitchell's (*N. m. mitchellii*) or the Saint Francis' satyr (*N. m. francisi*), hence they have no subspecies designation. Research is underway to determine the taxonomic status of these recently discovered populations.

A number of *N. m. mitchellii* populations have apparently been extirpated leading to the elimination of the species in parts of its former range. The Mitchell's satyr was extirpated from Ohio sometime in the 1950's and it was last seen in New Jersey in 1988 (Shuey 1997; Hamm 2008). High collecting pressure has been implicated in the extirpation of at least one New Jersey population due to a collector returning daily over successive seasons to the site (Glassberg 1999). While examining the Strecker collection in the Field Museum of Natural History, a part of the entomology collection not accessioned with the rest of the material, CAH found *N. mitchellii* with collection labels indicating they were taken from Wisconsin. These specimens were donated by E.T. Owen, who removed Strecker's original labels and replaced them with his own (J. Boone pers. comm.); any date or locality information have apparently been lost, though southeastern Wisconsin has a number of the tallgrass prairie fens that may provide suitable habitat. We are

unaware of any surveys in Wisconsin that have searched for *N. m. mitchellii*, but we suspect that it may be extirpated from Wisconsin and the surrounding region.

Habitats

With the discovery of these new populations of Mitchell's satyr, our understanding of its habitat usage patterns has also expanded. *Neonympha mitchellii* was first described from specimens collected near a "bog" (French 1889), although we now know that this habitat was a tallgrass prairie fen (Spieles et al. 1999; Kost et al. 2007). Tallgrass prairie fens are groundwater fed, sedge-dominated wetlands, whereas a "bog" is a basin that has no net outflow of water (Pielou 1991). Conditions leading to the formation of fens were a result of the Pleistocene glaciation (Pielou 1991) and prairie fens are concentrated in the interlobate regions of the Laurentide ice sheet (Landis et al. 2011). All *N. m. mitchellii* sites in Michigan and Indiana were subsequently determined to be tallgrass prairie fens (Shuey 1997). Previous workers have suggested that these wetlands provide microhabitat, which allows Mitchell's satyr to escape the high heat that characterizes these sites during the summer (Darlow 2000). Indeed, recent evidence suggests that there are significant differences between the ground level and air temperatures (Figure 3; C. Hamm unpub. data). During the winter (Figure 3a) the ground is significantly warmer than the air (t-test, $P < 0.01$), likely due to insolation of the sedge tussocks. In the early spring there is no significant difference between the ground and air temperatures Figure 3b)(t-test, $P = 0.28$), while during the early summer the ground is significantly cooler than the air Figure 3c)(t-test, $P < 0.01$).

Neonympha mitchellii populations located south of the glacial maximum are not found in tallgrass prairie fens, but rather in other sedge dominated wetlands such as the edges of beaver ponds and groundwater seepage slopes (Roble 2001; Hart 2004). Sites with *N. mitchellii* in

Alabama and Mississippi tend to occur on the periphery of beaver ponds, on the edge of pocosin swamps, in areas where roads culvert create a buildup of water, or in proximity to seepage slopes (Hart 2004). In these habitats, it appears that hydrological disturbance creates the necessary conditions for a sedge-wetland to exist, if even for a short time (Hart 2004; Bartel 2010). The Alabama and Mississippi sites were initially surveyed for *N. mitchellii* in 2002 and 2003, during which time sites on the periphery of beaver ponds had high numbers of *N. mitchellii* (Hart 2004). However, when revisited in 2008 and 2009 *N. mitchellii* was absent from all such sites (Hamm 2008; Hamm and Hart, unpub. data). As beavers had absconded the pond filled with silt, which allowed shrubs to encroach on the banks, and left few sedges (Hamm 2008). Immediately upstream from these ponds (approx. 800 m) were recently constructed beaver ponds (approx. 2-3 y.o.; C. Ragland pers. comm.) that had a high number of *N. mitchellii*. This scenario of site loss and colonization was observed at five sites in Alabama and one site in Mississippi (C. Hamm and B. Hart; pers. obs.). This pattern fits into the metapopulation model of Hanski (1994) and suggests that, in Alabama and Mississippi, *N. mitchellii* historically existed in a metapopulation structure with regular movement along riparian corridors. This pattern of utilizing temporally available habitats has also been suggested as the population structure that describes Saint Francis' satyr populations (Kuefler et al. 2008; Bartel et al. 2010).

A number of Alabama sites were also found on the edges of seepage slopes or along the margins of impoundments created by road culverts (Hart 2004). Unlike the populations associated with beaver ponds, these sites had maintained *N. mitchellii* populations when surveyed six and seven years later (Hamm 2008; Hamm and Hart unpub. data). It appears that these sites avoid shrub encroachment though hydrological disturbance, though again, we have only observational data to support these postulations. The culverts and seepage slopes were

imbedded within a matrix of fire dependent habitat, often a considerable (3 km) distance from the nearest actively populated beaver pond, which indicates the possibility that these sites may experience other forms of disturbance. Sites with *N. mitchellii* in Virginia are all found in close proximity to groundwater seepage slopes. These sites are very open compared with other *N. mitchellii* sites and are often used as pasture for cattle and other livestock (Roble et al. 2001). Management of the sites for cattle (i.e. the removal of shrubs and prevention of overgrazing) appears to simultaneously manage for *N. mitchellii* as these sites had high population density estimates (C. Hamm, unpub. data).

While there are many apparent differences among sites with *N. mitchellii* there are a number of commonalities that unite these habitats. All habitats, whether beaver pond, seepage slope, pasture or prairie fen, are sedge-dominated, early successional wetlands. Another commonality is that changes in hydrology and shrub encroachment are commonly associated with population extinctions, although the process by which this occurs remains unclear. The postglacial radiation of sedge wetlands northward from what is now the southern US following the Pleistocene glaciation provides a plausible explanation for the current distribution of *N. mitchellii* (Landis et al. 2011). Initially postulated based on distribution maps (Shapiro 1970; 1977) researchers have only recently begun to test these hypotheses (Emerson et al. 2010).

Vagility and Dispersal

Neonympha mitchellii exhibits low vagility relative to many other butterflies. As has commonly been reported for most Satyrinae, *N. mitchellii* has a low and jerky flight with an up and down bobbing motion for each wingbeat (Scott 1986). Males tend to fly through the habitat (between sedges and grasses) rather than over it and they generally fly below the height of the dominant vegetation, perhaps to avoid predators (see below). Individual male flights are short,

lasting an average of ten seconds (range: 1 sec to 1 min) (Sferra and Aguiar 1993). Female flight is even shorter, averaging five seconds though this distribution is extremely skewed (range: 1 sec to 19 min). When ovipositing, females approach potential sites and hover a few seconds before alighting (Sferra and Aguiar 1993). Males appear to spend the majority of time (~70%) patrolling, whereas females spend much of their time resting (~60%) early in the flight period, but later females spent 70% of their time flying in search of oviposition sites (Sferra and Aguiar 1993; Barton and Bach 2005).

While males fly with high frequency they appear to have small home ranges (Brussard et al. 1974). After examining two sites in southwest Michigan, Szymanski et al. (2004) reported that *N. m. mitchellii* did not disperse long distances. The mean daily distance moved for males was 18 m and for females was 11 m (Szymanski et al. 2004). Concordant with these observations, the mean minimum home range for the butterflies were small, with males occupying ~0.04 ha and females occupying ~0.01 ha (Szymanski et al. 2004). However, the sites where these data were recorded were relatively small (2.3 ha and 1.6 ha) and suffered from shrub encroachment, which may have biased the estimates. The size of surveyed habitats can produce a downward bias because habitat size may constrain movement. Using similar protocols at a larger site (12 ha), Barton and Bach (2005) reported larger home ranges for males (0.22 ha) and females (0.07 ha) and higher means for the daily distance moved (males: 35 m; females: 33 m). Overall, the data from both Szymanski et al. (2004) and Barton and Bach (2005) suggest that *N. m. mitchellii* does not disperse very far and thus falls into the sedentary mobility class of Pollard and Yates (1994). Sedentary butterflies are categorized by a movement rate between 10 and 200 m per day with colonization occurring up to 1 km away from natal habitat (Thomas 2000). At

present there are no data on the vagility of *N. mitchellii* populations in Virginia, Alabama and Mississippi and studies are needed to examine vagility among these populations.

Individual dispersal events for individual *N. mitchellii* are not well characterized. Habitat corridors of 200 m and 400 m length have been created to connect tallgrass prairie fens at two sites in Michigan and *N. m. mitchellii* have been observed in both. However, without mark-release-recapture (MRR) studies it is not clear if these individuals were transiting or were resident in the corridor. The longest distance recorded between subsequent captures in MRR studies was recorded by Barton and Bach (2005) and was 510 m for a male and 344 m for a female. A male in Virginia was observed at two different sites along a creek that were 1 km apart (S. Roble, pers. comm.), and this stands as the longest recorded distance for *N. mitchellii* dispersal.

Population Structure

The population structure of Mitchell's satyr is influenced by habitat isolation, flight phenology and within-habitat spatial preferences. In Michigan and Indiana today, tallgrass prairie fens are typically highly isolated from one another and there is no evidence for *N. m. mitchellii* dispersal among them. In contrast, analysis of historic data on the distribution of tallgrass prairie fens indicated that these habitats may once have been nearly contiguous and would have allowed for increased dispersal among sites (MacKinnon and Albert 1996; Landis et al. 2011). Mitchell' satyr occupied sites in Virginia, Alabama, and Mississippi are typically much closer together, and dispersal among sites in these states has been observed (Roble 2002, 2003; Hart 2004; Hamm unpub. data).

Flight phenology and patterns of within-patch habitat preference may also contribute to population structure. Overall, *N. mitchellii* is protandrous with males emerging one to two days

before the first female. As a result, during the first week of flight the sex ratio is male biased, after which there is a three to four day period of approximately equal sex ratio followed by a female biased sex ratio as the flight progresses (Barton and Bach 2005). This pattern of shifting sex ratios within the flight season is often used to infer the progress of the flight period. The detection probability of males is generally higher than that of females and is probably due to patrolling behavior making males more conspicuous (Szymanski et al. 2004; Barton and Bach 2005). Within sites, *N. m. mitchellii* are not uniformly distributed throughout the available habitat but the location of these aggregations fluctuates from year to year (Szymanski et al. 2004). For an as yet unknown reason, *N. m. mitchellii* are often found near habitat margins, especially at the interface of tallgrass prairie fen and upland areas (Barton and Bach 2005; Hamm unpub. data).

Several techniques have been used to attempt to estimate the population size of *N. m. mitchelli*. In 1997, Pollard walks were conducted at three sites in southern central Michigan, but the data generated from these walks were not analyzed and may not have had enough samples to generate parameter estimates (Summerville 1997). Mark release recapture (MRR) methods have also been used in several instances. In all cases, the pattern of *N. mitchellii* adult distribution within habitats is complex, which complicates population size estimates. For two sites in southwestern Michigan, Szymanski et al. (2004) used MRR techniques to estimate population size in 1997 and 1998. They found that each site contained no more than 80 *N. m. mitchellii* per day and had a total population of no more than 380 individuals. MRR studies were also conducted at one site in southern central Michigan during the 2003, 2005, 2007 flight periods of *N. m. mitchellii* (Barton 2008). During the 2003 survey, the maximum daily population estimate was approximately 1100 individuals and was approximately 3000 during 2007. That population

estimates varied a great deal from year to year is indicative of the stochasticity inherent with insect populations (Brown and Boyce 1998). In addition, short-lived study organisms complicate the use of MRR based methods for population estimation and may have influenced the results. In Michigan, the total population of Mitchell's satyr is informally estimated to be less than 10,000 individuals (Barton and Bach; D. Cuthrell and D. Hyde pers. comm.) but the uncertainty around this estimate reveals the need for standardized methods to more accurately assess the size of Mitchell's satyr populations.

Population size estimates are also poorly known for the southern populations of *N. mitchellii*. Sites with *N. mitchellii* outside of Michigan and Indiana have only been the focus of attention since 2000 (Roble et al. 2001; Hart 2004) with approximately 20 known *N. mitchellii* sites in Virginia, 15 in Alabama, and four in Mississippi. The estimates of total population size have been compiled from governmental reports, biological surveys, and our own experience. No statewide survey has been conducted on Mitchell's satyr or *N. mitchellii* in Virginia, Alabama or Mississippi (but see Haddad et al. 2008). Estimates of the total population size of Virginia *N. mitchellii* are roughly 8,000 individuals (Roble 2005). Researchers in Virginia have irregularly visited sites with *N. mitchellii* in Alabama and Mississippi since Hart (2004), but in that time many of these sites have become overgrown by shrubs since they were first surveyed and no butterflies have subsequently been observed (Hamm 2008). At least 15 sites are extant in the Talladega National Forest and are estimated to contain 1,500 individuals total (B. Hart pers. comm.). The three sites in the Natchez Trace Parkway of Mississippi have been surveyed for *N. mitchellii*, and were only found at one of these sites (the others having been overgrown by shrubs). We estimate fewer than 100 individuals occur at this occupied site (C. Hamm and B. Hart pers. obs.). Recently, additional populated sites have been discovered in the same area of

Mississippi and there are unconfirmed reports of additional sites in Alabama (S. Surette and P. Hartfield, pers. comm.: Turner 2007).

The populations of *N. mitchellii* in Alabama and Mississippi are bivoltine, with the first flight parenting the second. The first flight begins in early June and the second flight in mid August, and all flights last approximately three weeks. In contrast, all populations of *N. m. mitchellii* are univoltine and begin flying in late June in Michigan and Indiana. The *N. mitchellii* in Virginia are also univoltine and their flight begins in late July. Voltinism in *N. mitchellii* appears to be controlled by accumulated degree-days as a second generation can be induced in Michigan and Indiana populations by rearing them at higher temperatures (Shuey 1997; P. Tolson and C. Ellsworth, pers. comm.; C. Hamm unpub. data). Similarly, a single generation can be induced in Alabama populations of *N. mitchellii* by rearing them under cool conditions (C. Hamm unpub. data).

Host Plants

A variety of host plants have been associated with the Mitchell's satyr, but there are surprisingly few records of observed larval feeding. Based largely on the work of McAlpine et al. (1960) the sedge *Carex stricta* (Cyperaceae) was assumed to be the host of *N. mitchellii* because it was found at all Michigan and Indiana sites. Further observations, in both the field and artificial conditions, have demonstrated that *N. mitchellii* feeds on Cyperaceae and some graminoids as well (Table 1). It also appears that *N. mitchellii* rarely oviposits onto its sedge host plants (Table 2). One common observation among all oviposition reports is that female *N. mitchellii* generally deposit eggs close to ground level (Hyde et al. 2000; Darlow et al. 2000). We have observed eggs that were deposited singly and in groups of up to six (C. Hamm, unpub.

data), which contradicts the commonly accepted theme that satyrs only lay eggs single (Opler and Krizek 1984).

To address questions of host plant specialization an experiment was conducted using sedges from different regions of the *N. mitchellii* distribution. *Carex mitchelliana*, *C. lurida*, and *C. stricta* were collected from North Carolina, Alabama, and Michigan, respectively. These species were selected because they were endogenous to one or two sites but not present at all three. After collection, plants were grown in a 90:10 mixture Fafard 3B soilless potting medium (Conrad Fafard Inc., Agawam, MA) and calcined clay (Diamond Pro, Dallas, TX) in thee quart pots. Sedges were initially grown under greenhouse conditions at Michigan State University and were watered *ad libidum* using a 19-4-23-2 Ca fertilizer (Greencare Fertilizers, Chicago, IL) with H_2SO_4 added to counteract the high alkalinity of the well. Plants were then transferred to environmental growth chambers (Percival I-35LLVL) to simulate environmental conditions in Michigan and Alabama. Two females from Alabama and two from Michigan were collected for oviposition. The females were moved to 0.5 m^3 mesh cages with potted sedge (*C. lurida* for AL females and *C. stricta* for MI females) and allowed to oviposit for 48 hrs, each female laid 30 – 35 eggs. Eggs were then placed into treatment groups based on the experimental design outlined in Table 3.

Environmental conditions in growth chambers were set to simulate those encountered at *N. mitchellii* sites when the eggs were collected. Temperature, humidity and photoperiod were adjusted weekly based on data acquired from weather stations nearest the appropriate collection sites. Plants were placed in environmental chambers one week before the addition of *N. mitchellii* larvae. Once larvae were added, the plants were enclosed is mesh cages to prevent escape. Individuals were moved by hand to new plants as needed and mortality noted daily. The

total number of survivors to pupation, by treatment, was noted. Survivors from Michigan grown under Alabama conditions were allowed to mate and produce a second generation while the remaining individuals were sampled for DNA extraction. Voucher specimens were deposited in the Albert J. Cook Arthropod collection at Michigan State University. Logistic regression was used to compare all survival against all two-way interactions in the statistical program R (R Core Development Team 2011) against a significance value of $\alpha = 0.05$.

Adults emerged after approximately 900 degree days (base 50) accumulated. Michigan collected individuals reared under Alabama conditions went through a second generation after an additional 900 degree days accumulated. The photoperiod in Alabama was shorter than that of Michigan, which suggests that photoperiod does not play a role in voltinism for *N. mitchellii*. Logistic regression revealed no difference among treatment for survival. Due to permitting restrictions, only two females were sampled. As a result, this experiment did not have high genotypic diversity among treatments. Lastly, this study did not quantify growth rates among treatments, though the final size of adults is not significantly different from other wild caught specimens (C. Hamm unpub. data). These results, while preliminary, indicate no difference in host plant performance, and serve as proof of concept that such rearing experiments can be successfully undertaken.

Predators

An eclectic group of predators has been observed to prey on Mitchell's satyr. In the course of various oviposition studies researchers have reported numerous accounts of larval predation by spiders (Arachnida: Araneae) (C. Ellesworth and B. Barton, pers. comm.). During an enclosure experiment to test the effects of fire on larval survival, a group of researchers collected gravid females and placed them in enclosures that covered *C. stricta* tussocks. The

experiment was quickly abandoned due to high levels of predation by spiders (Barton 2008). Additionally, we have observed a number of predators attack adult *N. mitchellii* (Table 4) in the course of research. When a male *N. mitchellii* patrols an area he tends to fly through the sedges rather than over them. All aerial predation events (birds and insects such as robber flies and dragonflies) we observed occurred when a male flew over sedges and was thus exposed.

Conservation Status

Effective conservation of *N. mitchellii* into the future depends on a combination of biological, ecological and social factors. For example, the taxonomic uncertainty of *N. mitchellii* in Virginia, Alabama, and Mississippi will impact the federal conservation status of *N. m. mitchellii* more broadly. Currently, these populations are not included in the endangered species listing but they are protected by other measures. The State of Virginia considers their populations of *N. mitchellii* to be endangered at the state level and many of the sites are protected by conservation easements (S. Roble, pers. comm.). Many of the *N. mitchellii* sites in Alabama and Mississippi are located on U.S. Forest Service and National Park Service lands, thus affording them some level of protection.

The future of the northern protected populations of *N. m. mitchellii* and tallgrass prairie fens on which they depend is also uncertain (reviewed in Landis et al. 2011). Preliminary data suggest that the water feeding these sites may enter the aquifer many kilometers away from the fen decades ago (H. Abbas unpub. thesis). For example, the water coming out of the ground today may have entered the aquifer 50 years ago. We do not know the impact that contemporary levels of water consumption and groundwater extraction will have on the future of these sites. Field observations have noted that when fens dry out shrubs move in and as a result the biodiversity is apparently reduced (C Hamm pers. obs.).

The reproductive parasite *Wolbachia* in both *N. m. francisi* and *N. m. mitchellii* raises serious issues for conservation (Hamm et al. in review). *Wolbachia* is a common intracellular bacterium that is found in 20% of arthropods and 66% of insects (Hilgenboecker et al. 2008). This bacterial endosymbiont manipulates its host's reproduction to facilitate its own and is of major importance for the management of insects (Nice et al. 2009). *Wolbachia* can feminize males, kill male embryos, induce parthenogenesis or, in its most common form, induces cytoplasmic incompatibility (Werren et al. 2008). Cytoplasmic incompatibility only results in successful mating between the same strains of *Wolbachia*, of which there are currently over 200 known strains (Baldo et al. 2006; Stahlhut et al. 2010). *Wolbachia* imparts a reproductive advantage to infected individuals and is spread through maternal transmission, so when a population becomes infected it will pass through a bottleneck until infection rates are high (Werren et al. 2008; Nice et al. 2009; Hamm et al. in review). While the identity of a strain may be deduced from molecular sequence data, the induced phenotype can only be determined by controlled breeding experiments. Demographic models suggest that if differently infected individuals are mixed the consequences for small populations will be catastrophic (Nice et al. 2009; Hamm et al. in review).

Federal Actions

The Mitchell's satyr was first petitioned for listing under the endangered species act in November of 1974 by a private citizen; however in May of 1975 the USFWS judged that listing was not warranted due to insufficient data (49 FR 2485). In 1984 the USFWS listed *N. mitchellii* within category 3C in their Animal Notice of Review (49 FR 21664), indicating that it was considered too abundant to be considered for protected status. However, in 1989, the USFWS upgraded the species to category 2 and thus made *N. mitchellii* a candidate for listing under the Endangered Species Act (ESA) (54 FR 554). In 1989 a new subspecies was recognized that

altered the taxonomic status of *N. mitchellii.* The newly discovered Saint Francis' satyr was found on the Fort Bragg military reservation in North Carolina and given the trinomial *Neonympha mitchellii francisi* (Parshall and Krall 1989). With this split, the Mitchell's satyr became the nominate subspecies *Neonympha mitchellii mitchellii* (Parshall and Krall 1989).

A 1991 report issued to the USFWS described the rangewide status of *N. m. mitchellii* (Wilsmann and Schweitzer 1991). The authors noted that the Mitchell's satyr was once known from approximately 30 sites in four states (Michigan, Indiana, Ohio, and New Jersey) but at the time of the report, was known from only 15 sites in two states (Michigan and Indiana) (Wilsmann and Schweitzer 1991). This report recommended that the USFWS list *N. m. mitchellii* as endangered, which led to an emergency listing on 25 June 1991 (56 FR 28825). The emergency listing provided 240 days of protection and on 11 September 1992, the USFWS formally proposed a rule to fully protect the Mitchell's satyr under the ESA (56 FR 46273). The final ruling that listed the Mitchell's satyr as an endangered species was published in May 1992 (57 FR 21564). Note that while the ESA considers a "species" to be any taxonomically recognized subspecies, this does not apply to insects (section 4.(15) of the ESA). Cited among the reasons that the Mitchell's satyr deserved protection were: destruction and modification of its habitat, overutilization for commercial purposes, inadequacy of existing regulatory mechanisms, and other man-made factors affecting its continued existence (i.e. habitat loss due to anthropogenic forces).

The Mitchell's satyr received additional attention in the early 1990's as preparations to extend the US-31 freeway in southern Michigan through a fen were being put into motion. The original 1981 Final Environmental Impact Statement (FEIS) identified Blue Creek fen as a site where the Mitchell's satyr was present, but a 1991 report by the Michigan Department of

Transportation (MDOT) mistakenly reported that the site contained the *Lycaeides melissa samuelis*, the Karner Blue butterfly (Lepidoptera: Lycaenidae) and not the Mitchell's satyr (MDOT 1981; MDOT 2004). With the 1981 FEIS no longer accurate, the USFWS required MDOT to revise the path for the freeway. Negotiations between the USFWS and MDOT, ultimately resulted in the freeway being rerouted around the wetland complex and today an easement has been negotiated that allows biologists access to survey for the Mitchell's satyr.

In 1998 the Mitchell's satyr Recovery Team, a group of key stakeholders representing various state and federal governmental agencies and conservation organizations, submitted a recovery plan to the USFWS. This plan described the sites where extant and historical populations of *N. m. mitchellii* were found and noted that many of the original descriptions of those habitats were inaccurate (USFWS 1998). Additionally, the report outlined conservation measures that should be taken to aid in the recovery of the Mitchell's satyr. These included; range-wide surveys for the satyr, host plant identification and general study of the life history and ecology of the satyr, land acquisition and the development of habitat management plans, and the securing of easements with private property owners. The report also outlined the criteria that must be met for the Mitchell's satyr to be have its status changed (USFWS 1998):

> "1. For reclassification from endangered to threatened a total of 16 geographically distinct and viable populations or metapopulations must exist and these populations may be extant, established via translocation, or discovered. 12 of these sites must occur in Michigan, two in Indiana, one in Ohio and one in New Jersey and at least half of these sites must be protected in some form (i.e. conservation easement or under the ownership of a conservation organization).

2. For delisting to occur a total of 25 distinct and viable populations must be exist and remain viable for five years after delisting. At least 15 of these sites must have legal protection and we should note that the recovery team may modify or change the recovery criteria if new information becomes available."

In March of 2009 the USFWS began a five-year review of the Mitchell's satyr to determine if the species was still in need of protection (74 FR 11600). In April of 2009 the, the Mitchell's satyr was identified by the USFWS as a "Spotlight Species" and an action plan was instituted that brought additional resources to bear on the butterfly's recovery.

Discussion

While much is already known about *N. mitchellii,* this manuscript highlights the need for prioritized research in key areas. One critical need is for the development and use of standardized methods to estimate demographic parameters such as population size. While the currently used method of timed meander surveys is reasonably standardized, it is not quantitative with respect to area and thus does not yield a population density. Such density estimates are critically needed before any management practice can be tested robustly. Without such baseline data we cannot compare treatments let alone determine if populations are in decline. Methods that do not require handling the butterflies, such as distance methods, may be ideal for *N. mitchellii* work and have already been used with butterflies (Brown and Boyce 1998; Isaac et al. 2011).

Natural history forms the foundation for all biological work and without the data contained herein, any inferences based on molecular data could be out of context. Knowledge of the evolutionary history of *N. mitchellii* can aid in the recovery of the species by informing us about the relationship among populations at the regional and state level. Determining if and how the Virginia, Alabama, and Mississippi populations of *N. mitchellii* are related to the northern

populations of *N. m. mitchellii* with have an impact on the recovery criteria of the species. These inferences can be made both with morphology and with DNA-based evidence. For example, Parshal and Krall (1989) cited morphological character differences between the Saint Francis' satyr and Mitchell's satyr. Using methods such as geometric morphometrics we can test if these differences are robust to statistical testing and may serve to distinguish taxa. The use of DNA technology will allow us to directly compare populations when the time since divergence is not great enough to allow morphological characters to diverge. Using two mitochondrial DNA markers, Goldstein et al. (2004) surveyed a number of *N. mitchellii* from throughout its range. Their findings suggested that the Saint Francis' satyr was distinguishable from other *N. mitchellii*, however, the populations from Michigan, Virginia, and Alabama could not be resolved as unique (Goldstein et al. 2004). These results, while interesting, may be compromised by the presence of the reproductive endosymbiont *Wolbachia*, which is transmitted maternally in the same manner as mitochondria (Nice et al. 2009; Hamm et al. in review). Once *Wolbachia* is corrected for, molecular methods will allow us to test proposed routes of post-glacial radiation that these butterflies undertook (Shapiro 1977).

New research is also need to determine the full implications of the recently discovered infections of the reproductive parasite *Wolbachia* (Hamm et al. in review). Examination of the prevalence and strain type of *Wolbachia* should be conducted before any individuals are moved among populations. This is perhaps the single most pressing need for research because the introduction of a new Wolbachia strain into a population could result in population extinction. Once the strain is "typed" its effects must be determined experimentally so that any future introductions can be monitored for the effects of *Wolbachia*.

We must also continue to quantify aspects of *N. mitchellii* biology and habitat ecology. Replicated experiments to compare host plant performance among populations could reveal local adaptation, which if found, may counterindicate the movement of individuals among populations. Finally, understanding the hydrology of tallgrass prairie fen habitat will better allow us to manage these sites by telling us where the groundwater is coming from and thus prevent the loss of these habitats.

The goal of the Endangered Species Act is to recover species that were placed in peril by anthropogenic forces. No insect has ever been removed from the endangered species list due to recovery; rather they have been removed due to extinction. If sustained recovery is the goal, then quantifiable research must be conducted to address the major obstacles that face *N. mitchellii* conservation. Conservation organizations must partner with academic researchers to design critical experiments and research thrusts that will directly benefit *N. mitchellii*. The Mitchell's satyr is at a critical juncture, this butterfly presents an amazing opportunity to successfully recover the first insect species if steps are taken immediately. If these steps are not taken immediately, populations will likely continue to decline and surveys will no longer be necessary.

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APPENDICES

Table 1.1. Plant species fed upon by *Neonympha mitchellii* with literature reference and type of observation (field or artificial conditions).

Food plant	Family	Reference	Field
Carex alopecoidea	Cyperaceae	1	N
Carex atlantica	Cyperaceae	5	Y
Carex cephalophora	Cyperaceae	1	N
Carex lasiocarpa	Cyperaceae	2	Y
Carex leptalea	Cyperaceae	7	N
Carex lurida	Cyperaceae	6	Y
Carex mitchellii	Cyperaceae	8	N
Carex stricta	Cyperaceae	2, 3, 4, 5, 7, 9	Y
Carex tetanica	Cyperaceae	3, 7	Y
Cyperus esculentus	Cyperaceae	8	N
Rhynchospora capillaceae	Cyperaceae	7	N
Scripus atrovirens	Cyperaceae	1	N
Poa pratensis	Poaceae	8	N

References: [1]McAlpine et al. 1960; [2]Legge and Rabe 1996; [3]Szymanski and Shuey 2002; [4]Roble 2005 [5]Roble 2006; [6]Hart 2006; [7]Tolson 2008; [8]B. Bergman, unpub. data; [9]Hamm, unpub data.

Table 1.2. Plants on which *Neonympha mitchellii* oviposited, listed by family and reference (nomenclature follows Reznicek et al. 2011).

Plant species	Family	Reference
Eupatorium maculatum	Asteraceae	2
Solidago spp.	Asteraceae	2
Symphyotrichum ontarionis	Asteraceae	1
Carex bromoides	Cyperaceae	3
Scripus expansus	Cyperaceae	4
Juncus effuses	Cyperaceae	3, 5
Pycnanthemum virginuanum	Lamiaceae	1
Thalictrum dasycarpum	Ranunculaceae	1, 2
Galium boreale	Rubiaceae	1
Thelypteris palustris	Thelypteridaceae	2
Viola nephrophylla	Violaceae	1, 5

References: [1]Legge and Rabe 1996; [2]Darlow 2000; [3]Hart 2004; [4]Roble 2005; [5]Hamm unpub. data.

Table 1.3. Experimental design for larval rearing experiment. All treatments began with 10 larvae, the data presented here indicate the number of survivors for each treatment. Treatments are listed by environmental conditions and the state of origin for *Carex* (L to R): *C. mitchelliana* (NC), *C. lurida* (AL), and *C. stricta* (MI).

		Environmental Conditions					
		Alabama Conditions Sedges from:			Michigan Conditions Sedges from:		
		NC	AL	MI	NC	AL	MI
Larvae from:	AL	5	4	4	5	5	3
	MI	3	5	4	4	4	5

Table 1.4. Observed predators of *Neonympha mitchellii*.

Taxon	Common name	Family	Notes
Erythimis simplicicolis	Eastern Pondhawk	Odonata : Libellulidae	Aerial predation
	Robber Fly	Diptera: Asilidae	Aerial predation
	Ambush bug	Homoptera: Reduviidae	Nymph on *Rudbeckia*
Formica spp.	Wood ant	Hymenoptera: Formicidae	Landed on aphid tended plant
Bombycilla cedorum	Cedar Waxwing	Aves: Bombycillidae	Attempted aerial predation
Tyrannus tyrannus	Eastern Kingbird	Aves: Tyrannidae	Aerial predation

Figure 1.1. Dorsal (left) and ventral (right) wing pattern from the right wing of a *N. mitchellii* specimen collected at the Kellogg Biological Station in 1953. This population was extirpated in the 1960s. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Figure 1.2. Map highlighting the locations of *N. mitchellii* including both extant and extirpated populations. Extirpated populations are found in Wisconsin, Ohio, and New Jersey.

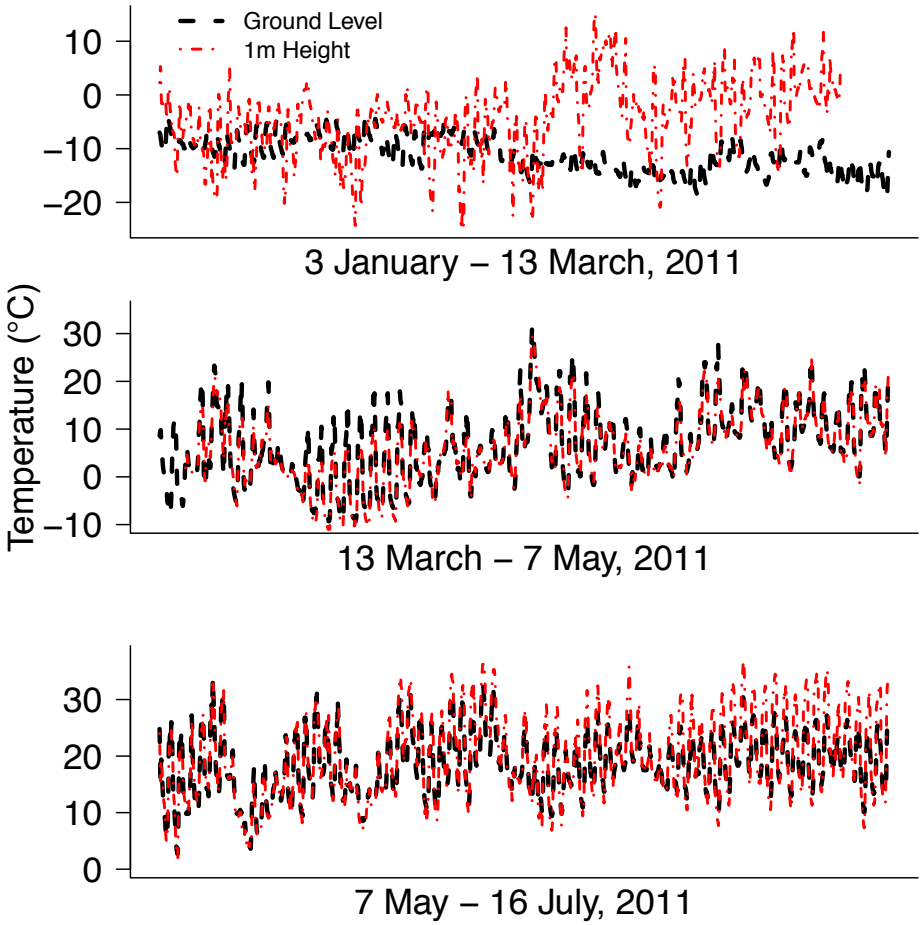

Figure 1.3. Temperature data depicting ground level and air temperature in a Michigan tallgrass prairie fen. A: during the winter (top plot) the ground (black dotted line) was significantly warmer than the air (grey dotted line) (t-test, $P < 0.01$), B: during the spring (middle plot) the temperatures were not significantly different (t-test, $P = 0.28$), C: during the early summer (bottom plot) the air was significantly warmer than the ground (t-test, $P < 0.01$).

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CHAPTER 2

EVALUATING THE IMPACT OF NON-LETHAL DNA SAMPLING ON TWO BUTTERFLIES, *VANESSA CARDUI* AND *SATYRODES EURYDICE*

Abstract

Genetic sampling of endangered species can inform conservation management and potentially aid the long-term survival of a species. However, when dealing with very small populations of rare species, the sacrifice of whole animals may not be desirable or permitted. We set out to develop a technique that demonstrably non-lethal of obtaining DNA from the federally-endangered Mitchell's satyr butterfly, *Neonympha mitchellii mitchellii*. Because of its endangered status we developed our methods on related species. In greenhouse and fields trials, we demonstrate that removal of small amounts of hind wing (2-3mm^2) has no significant impact on the behavior or survival of *Vanessa cardui* and *Satyrodes eurydice*. Based on these studies we were successful in obtaining a permit from the US Fish and Wildlife Service to sample DNA from *N. m. mitchellii* populations. We suggest that our results can be extended to the sampling of other rare butterfly species.

Introduction

Genetic sampling is commonly used to inform management decisions regarding rare or endangered species (Allendorf and Luikart 2007). Information gained from such studies can be critical for determining levels of genetic variation relating to population subdivision (Hanski et al. 1995), exposing potential cases of inbreeding depression (Saccheri et al. 1998), and for determining units of conservation management (Vogler and DeSalle 1994). For taxa with larger body sizes, birds, mammals etc., nonlethal sampling of small amounts of fluid or tissues is normal (Taberlet and Waits 1998). In studies where arthropod populations are large, whole individuals are commonly sacrificed to obtain DNA (Williams 2002; Gompert et al. 2006). However, when populations are small, sacrifice of individuals in numbers sufficient for reliable statistical inference may not be permitted due to the potentially negative impact on population viability. In such cases, a sampling technique is required that can yield sufficient quantities of DNA without causing harm to the individual.

A number of techniques have been proposed to non-lethally sample insect DNA (Table 1). These include: hemolymph sampling (Gerken et al. 1998), tarsal clipping (Holehouse et al. 2003), removal of tibia (Fincke and Hadrys 2001), and wing clipping (Rose et al. 1994; Lushai et al. 2000). Larval *Panorpa vulgaris* (Mecoptera: Panorpidae) appeared to tolerate hemolymph removal well, as 96% of treated larvae later emerged into adults (Gerken et al. 1998). While studying the fitness of the Neotropical damselfly *Megaloprepus coerulatus* (Odonata: Pseudostigmatidae), Finke and Hadrys (2001) removed tibia and reported that the treatment did not qualitatively alter behavior. Tibia were also removed from *Polistes* (Hymenoptera: Vespidae) wasps by Starks and Peters (2002), who found that treated wasps performed the same tasks as untreated wasps, though they were observed on nests at a significantly reduced rate. Holehouse

et al. (2003) showed that DNA could be successfully extracted from *Bombus terrestris* (Hymenoptera: Apidae) tarsi, and further demonstrated that the technique did not impact the survival of the insect over its lifespan.

Several studies have reported taking small fragments of lepidpteran wing for DNA extraction (Rose et al. 1994; Lushai et al. 2000), however, they provided no measure of the impact the technique had on survival. Other studies have examined the influence of wing clipping on butterfly behavior without the goal of DNA sampling. Experimentally reducing the wing surface area of *Pontia occidentalis* (Lepidoptera: Pieridae) butterflies increased wingbeat frequencies during hovering, but did not impact flight activity or the survival of treated butterflies (Kingsolver 1999). In these studies, 1mm of wing area was uniformly trimmed from both the forewing and hindwing margins of chilled butterflies, a technique not easily applied to field-work.

Mitchell's satyr *Neonympha mitchellii mitchellii* (Lepidoptera: Nymphalidae) is a federally endangered butterfly, which currently occurs in Michigan and Indiana. Formerly known from Wisconsin, Ohio and New Jersey, Mitchell's Satyr has apparently been extirpated in those states. In Michigan and Indiana habitat loss, invasive species, and the suppression of succession retarding processes has led to the extinction of many populations of this butterfly (Barton and Bach 2005). All remaining populations in Michigan and Indiana occur in prairie fen, itself a global rare community, and are considered isolated populations without the potential to interbreed. Many of these populations face imminent threat of extinction and will likely be lost in the future without supplementary translocations of individuals from larger populations. Before this can be accomplished the genetic structure of these populations must be understood.

Recently, morphologically similar populations of butterflies have recently been discovered in Alabama, Mississippi, and Virginia and are putatively treated as *N. mitchellii* (Roble et al., 2001; Hart, 2004). While widely disjunct from Mitchell's satyr's historic range the newly discovered populations appear to be more closely related to *N. m. mitchellii* than to the Saint Francis satyr, *N. m. francisi* Parshall and Kral (Goldstein et al. 2004). As part of an effort to understand the population genetic structure, taxonomy and phylogeography of these populations we required DNA samples from extant populations of *N. mitchellii*. Because the Endangered Species Act prohibits harming a listed species, a demonstrably non-lethal technique was required.

The Mitchell's Satyr Recovery Working Group is composed of key stakeholders representing federal, state and local organizations with an interest in preserving this butterfly. In early discussions with the working group, tarsal sampling was discouraged as this nymphalid butterfly uses only four legs for walking. Destructive sampling (requiring the sacrifice of entire organisms) would not be permitted in numbers sufficient for high levels of statistical power, and noninvasive sampling (in which the source of the DNA is left by the organism) was deemed impractical because noninvasive techniques often yield only low quantities of poor quality DNA (Taberlet et al. 1999) and pupal skins are rarely observed by researchers (C. Hamm, pers. obs.). Hemolymph removal was ruled out, as it would require chilling larvae and using a fine gauge needle to pierce the cuticle. This is not easily applied to field work with *N. m. mitchellii* because the larvae have never been observed in numbers sufficient for a population level genetic analysis.

In contrast, Mitchell's satyr adults are readily observed in the field and the working group favored wing clip sampling. However, concerns were raised about potential impacts on Mitchell's satyr flight and predator avoidance. In particular, members voiced concern about any

disruption of hind wing border ocelli that may serve in predator escape (Wourms and Wasserman 1985). In preliminary tests using *Vanessa cardui* (Lepidoptera: Nymphalidae) wing we found that only when the sample passed through a wing vein did it consistently yield amplifiable DNA. Given these limitations, our objective was to develop a demonstrably non-lethal wing clipping technique that could be administered to *N. m. mitchellii* in the field, yield sufficient DNA for analysis, and not alter butterfly flight or survival. Here we describe studies used to develop this technique using two surrogate species, the Painted Lady, *V. cardui* and the Eyed Brown, *Satyrodes eurydice* (Lepidoptera: Nymphalidae).

Methods

Study Species

We utilized *V. cardui* for initial greenhouse experiments due to its year-round commercial availability. We used *S. eurydice* for subsequent field studies because it is closely related to *N. m. mitchellii*, shares very similar habitat requirements and wing aspect ratios with *N. m. mitchellii*, and is more common and can be collected in sufficient numbers for experimentation.

Greenhouse Study

Initial studies with *V. cardui* were conducted in the Michigan State University 4-H Children's Greenhouse. The facility is open to the public and annually hosts over 5,000 children on field trips; approximately 150 children visited the greenhouse over the course of our experiment in Summer 2007 (J. Albright, pers. comm.). The greenhouse measured approximately 15m long by 8m wide and had a pitched roof, the highest point of which was 6m. The unit was filled with foliage and flowering plants as well as decorative and educational structures. This provided a complex free-flight arena in which *V. cardui* could naturally fly, nectar, and exhibit

avoidance behaviors. During the course of the study the average temperature in the greenhouse during the day was 34°C and at night was 20°C. *Vanessa cardui* pupae were purchased from a commercial supplier (Berkshire Butterflies, Chuluota, Florida.). Upon receipt, the pupae were placed into 0.3 x 0.3 x 0.6m rectangular cages (~15 pupae per cage) and held in the greenhouse until emergence.

After eclosion new adults were given 24 hours for their wings to dry and harden, then 70 *V. cardui* adults were placed randomly into each of five treatment groups (14 per treatment): control (no wing removal), 3mm^2 of wing removed, ¼ wing removal, ½ wing removal, and full wing removal. All manipulations occurred on the right metathoracic wing. The 3mm^2 treatment group had a section of wing removed from the anal angle (Figure 1). All wing sections were removed using a pair of fine tipped forceps or fine tipped scissors, all other treatments were taken according to Figure 1. Once each butterfly received its clipping treatment it was individually marked with a unique number on the opposite hind wing using a fine-tipped Sharpie® (Sanford Corporation, Oak Park, Illinois) and released into the greenhouse.

For subsequent surveys the greenhouse was divided into 12 equal sections, each of which contained nectar sources (*Buddleia* sp., *Eupatorium purpureum, and Rudbeckia hirta*) and oviposition plants (*Gylcine max*). Using a random number generator the observer moved between each section of the greenhouse so that each section was visited only once per observation period. Each observation period lasted 36 minutes with the observer spending 3 minutes in each section. Observations were conducted three times a day and each day was treated as one replicate. Butterflies were identified by their unique alphanumeric code (by the unaided eye or with the assistance of binoculars), and their behavior (nectaring, flying, mating, ovipositing) was noted along with section in which they were observed. Observations began on

16 July 2007 and were repeated until no individual *V. cardui* were seen for three consecutive days (3 August).

Field Study

Field studies were conducted in a prairie fen at the Michigan State University MacCready Reserve in Jackson County, Michigan. Individual *S. eurydice* were captured from the fen using a 0.4m wide insect net with butterfly mesh (BioQuip Products, Inc., Rancho Dominguez, California). Two treatment groups were established, a control with no wing removal (n=21) and an experimental group with a $2mm^2$ wing section removed (n=21). Every butterfly received a unique alphanumeric marking using the technique described above. Data recorded upon initial capture include wing condition (using the scale of Lederhouse, 1982), gender, and behavior when first observed. This experiment was conducted toward the end of the *S. eurydice* flight period. All butterflies were initially sampled, beginning 16 July, 2007 (day 0), for three hours each day, weather permitting, until no individual *S. eurydice* were seen for three consecutive days. The last *S. eurydice* was seen on 26 July, 2007 and the experiment was concluded on 29 July, 2007 (day 13). Butterflies were observed by noting the alphanumeric code on each butterfly with the unaided eye, binoculars, or occasionally captured with an insect net (Hein and Myers, 2000). Behavior of the individual was recorded when it was recaptured. The same observers were used throughout the course of this study to minimize observer bias (Hein and Myers, 2000).

To further understand the use of *S. eurydice* as a proxy for *N. m. mitchellii* we compared the wing aspect ratios and total wing surface area of museum specimens collected from Michigan. Aspect ratio (AR) is an important determinant of the properties of flying objects and is defined as $AR = 4R^2/S$, where R is the wing length and S is the total surface area (Ellington, 1984; Dudley, 2000). The loss of approximately 5% of wing surface area is thought to resemble

normal wing wear, while a loss of 10% or greater is thought to alter flight performance (Dudley, 2000; R. Dudley, pers. comm.). Digital images of 40 museum specimens were taken (10 per sex per species) from individuals in the Albert J. Cook Arthropod Collection at Michigan State University. Each image included a reference scale and was edited in Adobe Photoshop CS (Adobe Systems, Inc., San Jose, CA) to trace with wing outline and convert the image to black and white. This image was then imported into Scion Image (Scion Corporation, Frederick, Maryland) where the relevant wing metrics were measured. Wing surface areas were calculated to represent normal flight configurations as opposed to total surface area.

Molecular Analysis

Wing samples from *S. eurydice* which passed through the anal vein (n=4) and samples which did not pass through the anal vein (n=4) were processed for DNA extraction using a QIAGEN DNeasy Blood & Tissue kit (Qiagen Inc. Valencia, California). Excess wing membrane and scales were removed from around the anal veins to improve yield by reducing the amount of PCR inhibiting compounds. The purified DNA was amplified for part of the Cytochrome Oxidase I gene using conserved primers (Hebert et al. 2004). The resulting products were cleaned using ExoSAP-IT® (USB Corporation, Cleveland, Ohio) and sequenced at the Genomics Core Facility at Michigan State University. The generated sequences were compared against known sequences using the BLASTn search feature of GenBank.

Statistical Analysis

For both the greenhouse and field experiment analyses we considered the recapture probability for individuals in each treatment group a measure of survivorship. To generate preliminary data we first tested our greenhouse and field data using one-way ANOVAs. Because simple ANOVAs may not be sensitive enough for mark-recapture analysis, we subsequently

tested our data using the Generalized Linear Model (Lebreton et al. 1992; Kingsolver 1999). We followed standard model testing protocols for our analysis though we were primarily interested in the impact of treatment on recapture probability (Bolker 2008). Once the optimal model was chosen, we derived P values from that model and tested the null hypothesis that the treatment had no effect on the recapture probability of an individual butterfly.

Greenhouse data variables included in the models were: treatment (five levels), sex (two levels), and day. Since these were collected over a temporal period (i.e. longitudinal data) there was also a nested effect, which was butterfly nested in treatment and sex. The outcome was binary, thus in SAS v9.1 (SAS Institute Inc., Cary, North Carolina) the distribution was set as "BINOMIAL" and link as "LOGIT" in the model code. With ρ denoting the probability that a butterfly was recaptured, the full model was: Log (ρ/1-ρ) = mean + treatment + sex + day + treatment x sex + treatment x day + sex x day + treatment x sex x day + butterfly(treatment x sex) + error.

We first analyzed the full model with all interaction terms, then removed non-significant terms and analyzed the reduced models. In total, seven models were analyzed and Akaike's Information Criterion (AIC) scores calculated for later comparison (Akaike 1974) (Table 3). Akaike's Information Criterion is a goodness of fit test that compares the amount of information lost in a statistical model when that model is compared to the data. For each model an AIC score is calculated and the model with the lowest AIC score is considered to be the best model. In this way the AIC methodology attempts to find the optimal model for goodness of fit that also has the fewest number of parameters.

Statistical models to analyze the field experiments were also derived. Field data variables included treatment (two levels), sex (two levels), wing condition (four levels), and day of

recapture. Field data models were analyzed following the same general procedure as the greenhouse models with individual butterflies nested in treatment by sex and wing condition. With ρ denoting the probability that a butterfly was recaptured, the full model was: Log (ρ/1-ρ) = mean + treatment + sex + day+ wingcondition + treatment x sex + treatment x day + treatment x wing condition + sex x day + sex x wing condition + treatment x sex x day + treatment x sex x wing condition + sex x day x wing condition + treatment x sex x day x wing condition + butterfly(treatment x sex x wing condition) + error.

We first analyzed the full model with all interaction terms, then removed non-significant terms and analyzed the reduced models. In total, four models were analyzed and AIC scores calculated for later comparison. Despite a high recapture rate in the field, some of the reduced models from the field data failed to converge likely as a result of days that had a low number of butterfly sightings (Table 4).

All greenhouse and field data were tested against $\alpha = 0.05$ with a general mixed model using PROC GLIMMIX procedure (Breslow and Clayton 1993). The aspect ratio and surface areas for museum specimens of *N. m. mitchellii* and *S. eurydice* were analyzed in SAS for differences between species and sexes using PROC GLIMMIX.

Results

Greenhouse Study

During the greenhouse experiment adult *V. cardui* exhibited a variety of behaviors typically associated with butterflies. Within five minutes of receiving the experimental treatment *V. cardui* individuals from all treatment groups were observed flying and nectaring. On all subsequent days there was no observable impact of treatment on butterfly behavior (P=0.06). Individuals from each treatment group were seen flying between greenhouse sections, nectaring,

and resting. Butterflies from the treatment groups appeared remarkably resilient and as vagile as control butterflies. For example, butterfly #24 (a female from the $3mm^2$ treatment group), was observed three different times on day four of the study; first in Section 1 nectaring on *Buddleia* sp., one hour later it was observed 15m from the initial sighting ovipositing on *G. max*, and one hour after the second observation 9m away nectaring on *Buddleia* sp.

The mean butterfly lifespan, inferred from the average number of days that members of a treatment group were recaptured, did not differ between groups based our GLIMMIX model ($p = 0.94$) (Figure 2). The best model for the greenhouse data set, as indicated by lowest AIC score, was model #5 (Table 3). While a number of variables were identified as significant, in no model was the wing clipping treatment identified as having a significant impact on the probability of recapturing a butterfly.

Field study

We expected field conditions to differ from the controlled nature of the greenhouse. Despite numerous stochastic weather events, our recapture rate of 38% (n = 16, 9 control and 7 treatment recaptures) was relatively high for a field study of butterflies (Morton 1982). We observed no behavioral differences between control and treatment group butterflies. Male butterflies, from both the experimental and control groups, were observed to establish and defend territories. Also, a female from the experimental group was observed *in copulo* with an unmarked male.

Over the course of 14 days of observation, three thunderstorms passed through the study site (on the nights preceding days 1, 6, and 8). On the days following the thunderstorms no butterflies were observed (Figure 3). These days were all overcast with cool temperatures (~15ºC), light rain and high relative humidity (>70%) as measured by a Kestrel®3000 (Nielsen

Kellerman Inc., Boothwyn, Pennsylvania) or a local NOAA weather station. Such conditions are likely below the thermal optima for these butterflies, resulting in reduced butterfly activity (Heinrich 1993). However, butterflies were always observed on subsequent days. Statistical modeling of field study data revealed no difference in the recapture probability between the control and treatment groups (i.e. wing clipping had no significant impact on recapture).

The aspect ratios of *N. m. mitchellii* and *S. eurydice* butterflies were not significantly different between species when compared using a Generalized Linear Model ($p = 0.87$) (Figure 4). Sexual dimorphism exists in both *N. m. mitchellii* and *S. eurydice*. Wing surface area is statistically significantly different among the sexes for both species ($p = <0.001$) (Figure 5). This sexual dimorphism is more pronounced in *N. m. mitchellii*, resulting from females having much larger wings than males, though they exhibit similar aspect ratios. As such, any reduction in wing surface area will likely have a greater potential impact on males than females.

Molecular Analysis

All samples taken through the anal veins yielded PCR amplifiable DNA. Samples not passing through the anal vein did not yield amplifiable DNA. A BLASTn search conducted with the *S. eurydice* sequences showed 100% similarity (E value = 0.0) to the reference sequences on GenBank.

Discussion

In the greenhouse study, adult *V. cardui* butterflies showed no significant difference in recapture probability regardless of treatment. Butterflies with zero to full hind wing removal showed no difference in daily probability of recapture. In the field study, adult *S. eurydice* butterflies with small amounts of wing ($2mm^2$) removed showed no significant difference in

recapture probability when compared to the control group, again suggesting no impact on the probability of recapture.

It is perhaps not surprising that the removal of small amounts of lepidopteran hind wing has no impact on recapture probability as butterflies have some of the lowest wing loading rates (wing loading = body mass/total wing area) in the animal kingdom (Kingsolver 1999; Dudley 2000). Many lepidopterists have observed butterflies in the field with tattered wings or beak marks on their wings (Edmunds 1974). The removal of $2mm^2$ of wing from *S. eurydice* and *N. m. mitchellii* females equates to less than 3% for both species (Table 2). Even with the smaller wings of male by *N. m. mitchellii* and *S. eurydice*, the removal of $2mm^2$ of tissue is less than 4% of the wing surface area (Table 4). The loss of 5% of the wing surface area is considered approximate to normal wear (Dudley 2000; R. Dudley, pers. comm.). In contrast, wing clipping may negatively affect organisms with higher wing loading. Previous studies on *Bombus* sp. indicated a correlation between wing wear and increased mortality (Rodd et al 1980; Carter 1992). In another study, experimental reduction in *B. terrestris* wing surface area increased wingbeat frequency, though did not significantly increase metabolic flight costs, nor was it considered a direct factor in the increased mortality rate in bumblebees (Hedenström et al. 2001). Alternatively, increased wing wear may reduce the maneuverability of bumblebees and thus their susceptibility to predation (Hedenström et al. 2001). Thus, for lepidopterans with higher wing loading, i.e. skippers, wing clipping may not be appropriate.

We set out to investigate a non-lethal technique to acquire DNA from butterflies in sufficient quantities for application of molecular techniques. In both greenhouse and field trials we were able to take small amounts of wing material without significantly altering survival or behavior. This finding is concordant with the prior studies showing that removal of small

amounts of wing material had negligible impact on behavior and survival (Kingsolver 1999). Thus, we conclude that the removal of small amounts of metathoracic wings of butterflies is a viable non-lethal technique that can produce amplifiable DNA without significantly impacting their survival. Based on this study we applied for and were subsequently granted a US FWS permit (TE 175852) to sample DNA to investigate the biogeography and population genetic structure of *N. m. mitchellii.*

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APPENDICES

Table 2.1. Chronological list of studies using non-lethal means to study survival or obtain DNA samples from terrestrial arthropods.

Method	Citation	Taxon/Taxa	Quantification of impact[a]	DNA extracted
Wing clipping	Rose et al., 1994	Lepidoptera: Hesperiidae and Lycaenidae	No	Yes
Hemolymph	Gerken et al., 1998	Mecoptera: Panorpidae	No	Yes
Wing clipping	Kingsolver, 1999	Lepidoptera: Pieridae	Yes	N/A
Wing clipping	Lushai et al., 2000	Lepidoptera: Papilionidae	No	Yes
Wing clipping	Hedenström et al., 2001	Hymenoptera: Apidae	Yes	N/A
Tibia removal	Fincke and Hadrys, 2001	Odonata: Pseudostigmatidae	No	Yes
Tibial removal	Starks and Peters, 2002	Hymenoptera: Vespidae	No	Yes
Tarsal clipping	Holehouse et al., 2003	Hymenoptera: Apidae	Yes	Yes
Wing clipping and tarsal removal	Châline et al., 2004	Hymenoptera: Apidae	Yes	Yes
Pupal exuviae	Petersen et al., 2007	Araneae: Theraphosidae	N/A	Yes
Leg removal	Longhorn et al., 2007	Araneae: Theraphosidae	No	Yes

[a]I considered quantification of impact as the use of statistical inference, with appropriate controls, to assess the treatment effect on the research organism

Table 2.2. Percent reduction in wing surface flight area (S) that would result from the removal of $2mm^2$.

Species / sex	% reduction in S ± SE
S. eurydice/ female	2.09 ± .08
S. eurydice/ male	2.31 ± .06
N. m. mitchellii/ female	2.78 ± .13
N. m. mitchellii/ male	3.84 ± .08

Table 2.3. Greenhouse recapture probability models with Akaike's Information Criterion (AIC) scores and significant terms affecting recapture probability.

Model	AIC score	Significant terms
1. Log (ρ/1-ρ) = mean + treatment + sex + day + treatment x sex + treatment x day + sex x day + treatment x sex x day + butterfly(treatment x sex) + error	4020.34	Day
2. Log (ρ/1-ρ) = mean + treatment + sex + day + treatment x sex + treatment x day + sex x day + butterfly(treatment x sex) + error	4060.38	Day, sex, day x sex
3. Log (ρ/1-ρ) = mean + treatment + sex + day + treatment x day + sex x day + treatment x sex x day + butterfly(treatment x sex) + error	3999.96	Day
4. Log (ρ/1-ρ) = mean + treatment + sex + day + treatment x day + sex x day + butterfly(treatment x sex) + error	4094.56	Day, sex, day x sex
5. Log (ρ/1-ρ) = mean + treatment + sex + day + sex x day + butterfly(treatment x sex) + error	3917.12[a]	Day
6. Log (ρ/1-ρ) = mean + treatment + sex + day + treatment x sex + butterfly(treatment x sex) + error	4014.70	Day
7. Log (ρ/1-ρ) = mean + treatment + sex + day + butterfly(treatment x sex) + error	3919.06	Day

[a]Denotes best model as selected by lowest AIC score.

Table 2.4. Field recapture probability models with AIC scores and significant terms affecting recapture probability.

Model	AIC score	Significant terms
1. Log (ρ/1-ρ) = mean + treatment + sex + day+ wing condition + butterfly(treatment x sex x wing condition) + error	4992.44[a]	No significant terms
2. Log (ρ/1-ρ) = mean + treatment + sex + day+ wing condition +treatment x sex + treatment x day + treatment x wing condition + sex x day + sex x wing condition + butterfly(treatment x sex x wing condition) + error	6515.76	No significant terms

[a]Denotes best model as selected by lowest AIC score.

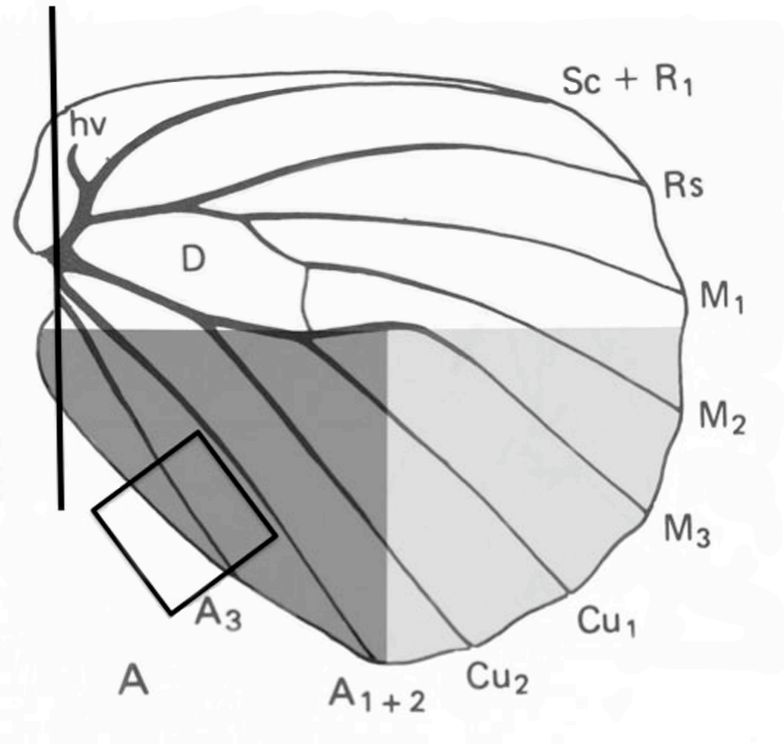

Figure 2.1. Typical nymphalid hindwing showing wing clipping treatments. For the 3mm^2 treatment (*V. cardui*) or 2mm^2 (*S. eurydice*) wing was removed from the boxed area. For other *V. cardui* treatments: ¼ wing, the light shaded area was removed, ½ wing, both shaded areas were removed, the full wing was removed at the solid line.

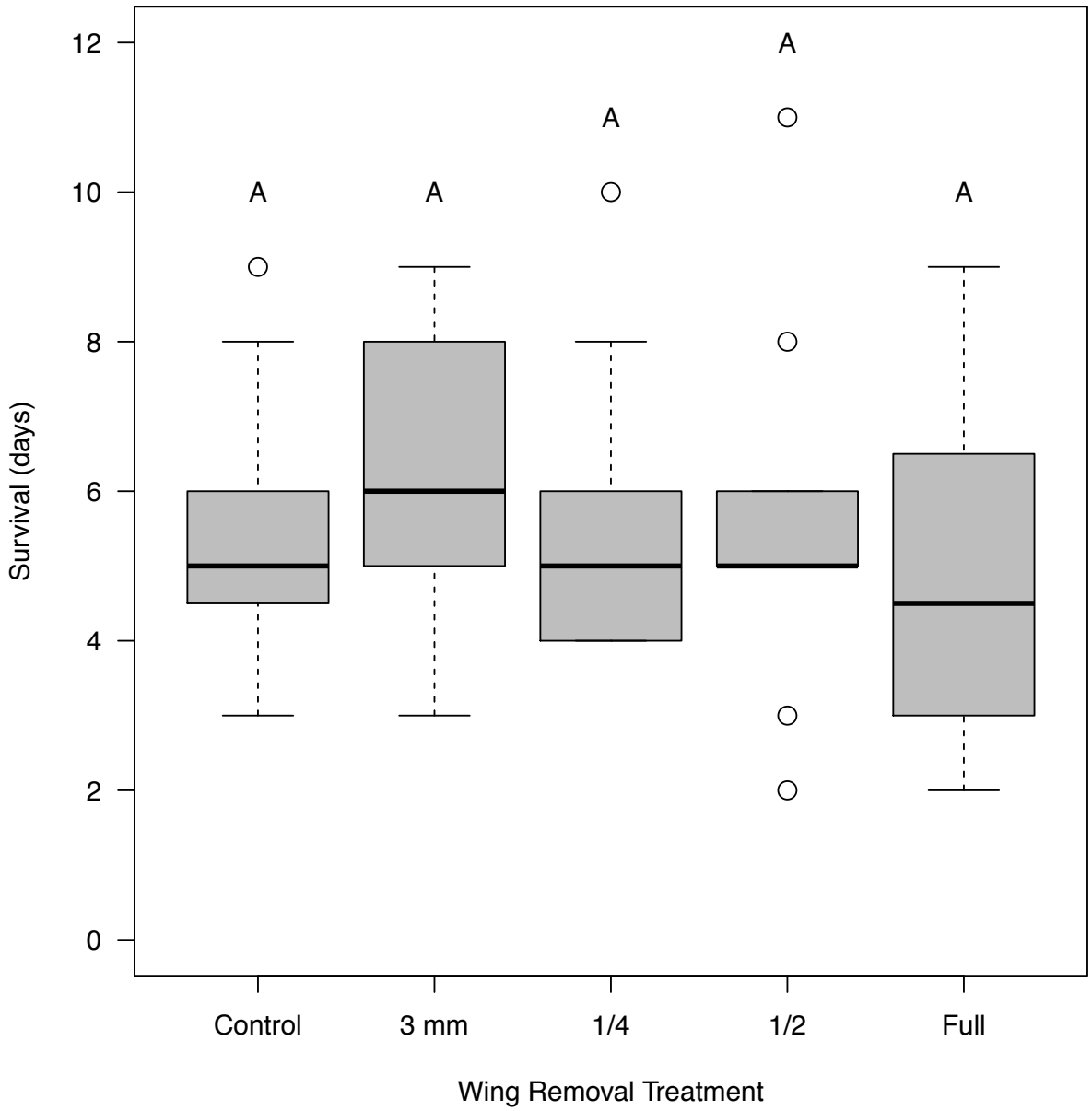

Figure 2.2. Average lifespan by treatment inferred from recapture history of *V. cardui* over the 13 days of the greenhouse experiment. Mean lifespan extrapolated from the number of days an individual was recaptured per treatment. Treatments with the same letter denote mean lifespans not significantly different from each other when compared using one-way ANOVA ($\alpha = 0.05$).

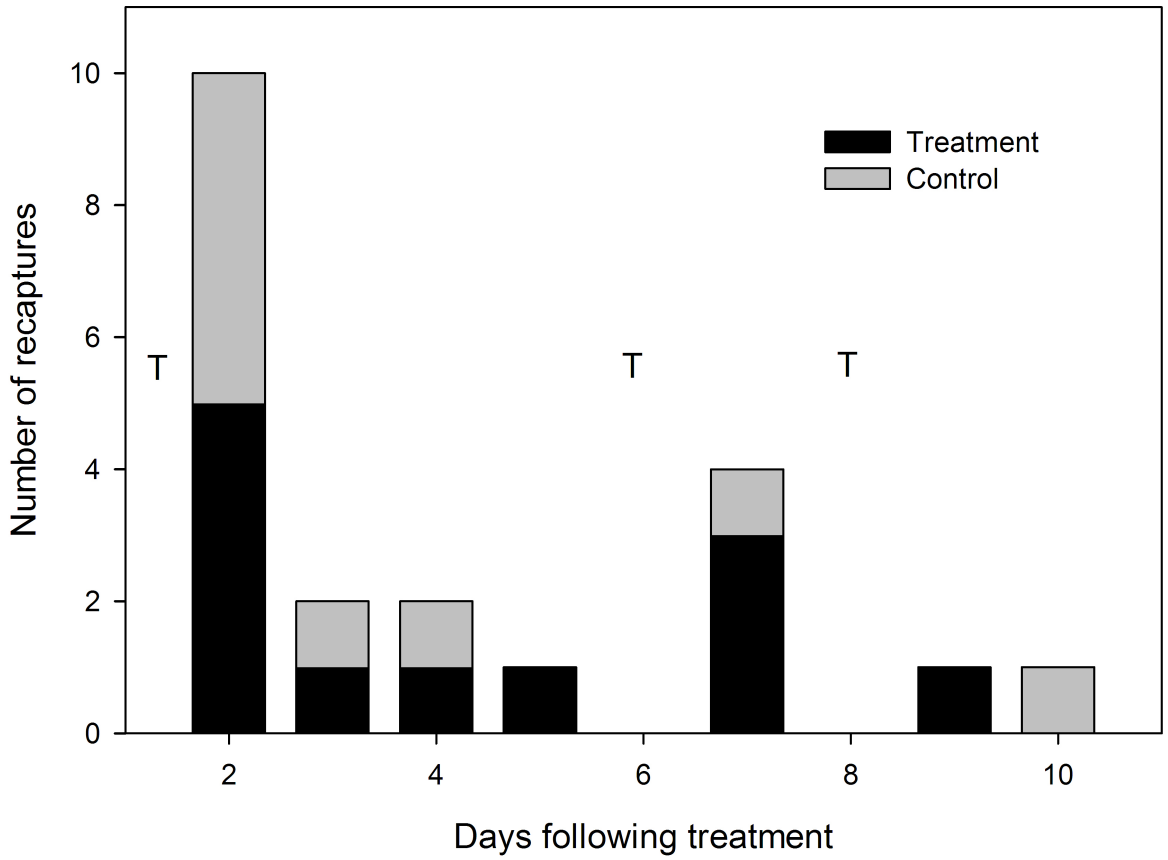

Figure 2.3. Recapture history of *S. eurydice* from field experiment. Treatment group butterflies had a 2mm^2 section of hind wing removed. Recapture rate = 38% (n = 16, 9 control and 7 treatment recaptures). No butterflies were observed on days 1, 6, and 8 due to thunderstorms (T) the previous evening.

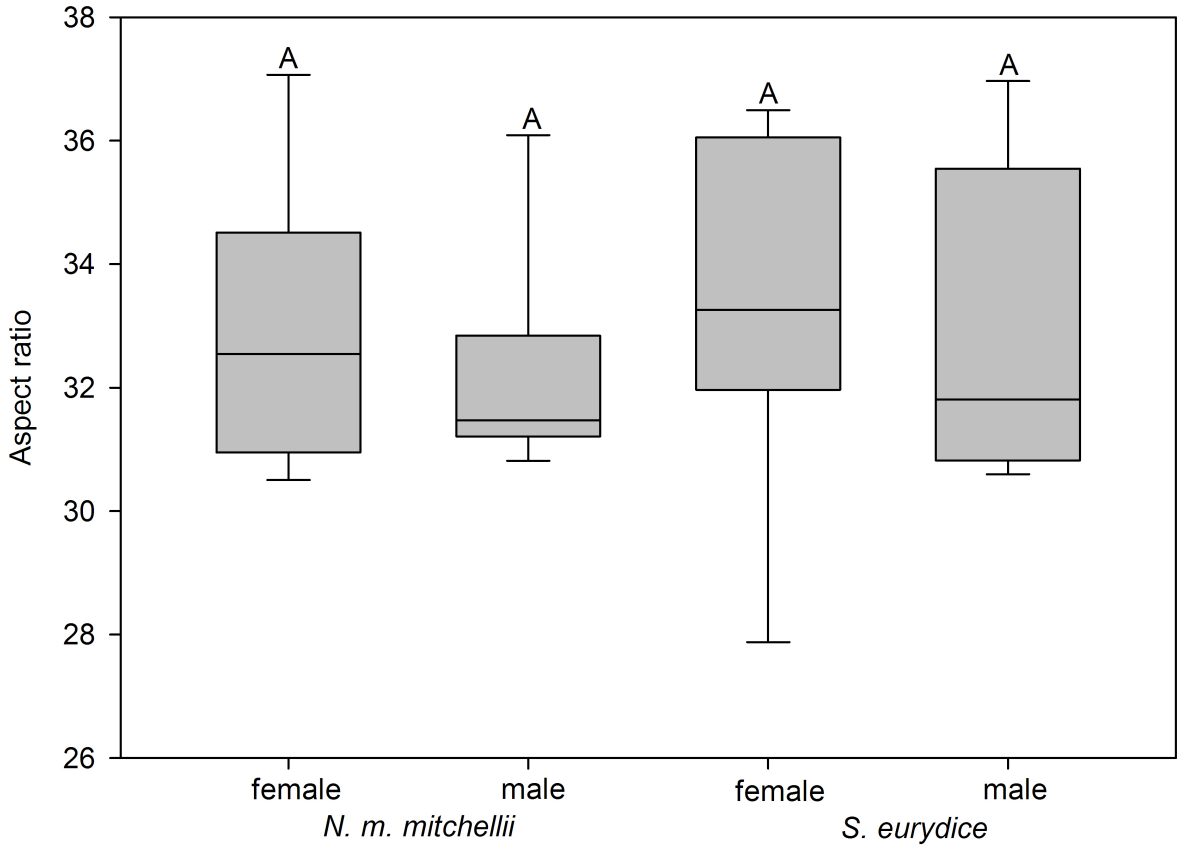

Figure 2.4. Box plots showing median, quartiles, and extreme values for aspect ratio (=$4R^2/S$, where R = wing length, S = wing surface area) of each species and sex (10 butterflies measured per column). Plots with the same letter denote aspect ratios not significantly different when compared using one-way ANOVA ($\alpha = 0.05$).

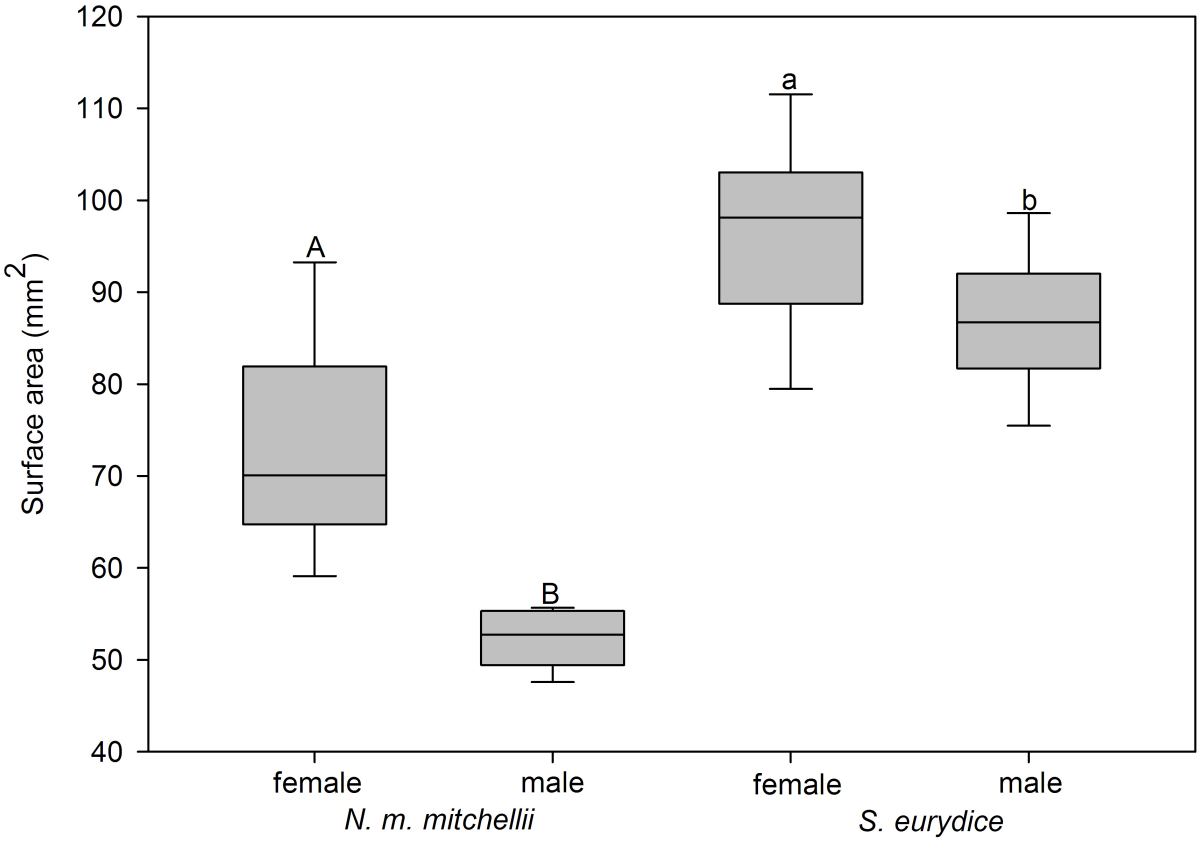

Figure 2.5. Box plots showing median, quartiles, and extreme values for wing surface area (mm^2) of each species and sex (10 butterflies measured per column). Surface areas were significantly different between sexes and species. Plots with the same letter denote aspect ratios not significantly different when compared using one-way ANOVA ($\alpha = 0.05$) with LSMEANS

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CHAPTER 3

CONSERVATION GENETICS AND THE IMPLICATION FOR RECOVERY OF THE ENDANGERED MITCHELL'S SATYR BUTTERFLY

Abstract

Modern delineation of taxonomic groups is often guided by analyses of molecular data, which can also help inform conservation biology. Two subspecies of the butterfly *Neonympha mitchellii* are classified as federally endangered in the United States, *N. m. mitchellii*, the Mitchell's satyr, and *N. m. francisi*, the Saint Francis' satyr. The relatively recent discovery of additional disjunct populations of *N. mitchellii* in the southeastern US could have important implications for both legal and management decisions. The goal of our work was to elucidate the relationships among *N. mitchellii* populations using mitochondrial and nuclear sequence data and a variety of analytical frameworks to clarify the conservation implications. Maximum likelihood and Bayesian concordance phylogenetic analysis resulted in moderately supported clades that corresponded with the geographic region where samples originated. Clustering analyses resulted in three groups, all with high assignment probabilities, wherein the two named subspecies formed separate clusters and the recently discovered populations formed the third cluster. Coalescent analyses rejected a splitting time of zero between *N. m. mitchellii* and all other populations, but failed to reject divergence among *N. m. francisi* and the recently discovered populations. Hence, the two-preexisting subspecies are clearly different from one another, but the recently discovered populations cannot be completely distinguished from *N. m. francisi* or each other. This result is likely due to incomplete lineage sorting present among these populations, which clouds pairwise comparisons in a coalescent framework. These results suggest that *N. m. mitchellii* and *N. m. francisi* should continue to be managed as endangered species. A genome-wide data set may be required to resolve the relationships among all *N. mitchellii*.

Introduction

The delineation of taxonomic groups is often guided by the analysis of molecular data (Avise 1989). One important application of such delineation is the classification of threatened and endangered species, particularly in the United States where legal protection tends to be focused on taxonomic designations (e.g. the Endangered Species Act) rather than community or habitat assemblages (Scott et al. 2006). Molecular methods have been used for some time to identify genetically distinct populations, and more recently to estimate historic gene flow and the timing of vicariance events (Hedrick and Miller 1992; Forister et al. 2011). While molecular methods should not be used to replace studies of natural history and ecology, their application to certain problems may help resolve otherwise intractable issues (Forister et al. 2011).

The Mitchell's satyr, *Neonympha mitchellii mitchellii* French 1889, and the Saint Francis' satyr, *Neonympha m. francisi* Parshall & Kral 1989, are two endangered species of butterfly found in the eastern United States of America. The Mitchell's and Saint Francis' satyrs were originally classified into different subspecies based on ecological and phenotypic differences (Parshall and Kral 1989; Hamm 2007). Due to the wording of the Endangered Species Act, endangered invertebrate subspecies are treated as endangered species wherever they are found, thus for the remainder of this work we will use the term 'species' when referring to *N. m. mitchellii* and *N. m. francisi*. The Mitchell's satyr is currently found at 18 isolated sites across the states of Michigan and Indiana, though it historically also occurred in Ohio, New Jersey, Wisconsin, and possibly Maryland (Figure 1) (Hamm et al. in revision); the Saint Francis' satyr has only been known from one small site (260 km^2) in North Carolina (Figure 1) (Kuefler et al. 2008). Each endangered species has a recovery plan that outlines the minimum number of "viable populations" required before the species are no longer protected (USFWS 1996; 1998). If

additional populations of either species were to be discovered it could thus affect their protected status.

In 1998 a butterfly, identified as *N. mitchellii*, was observed in southwestern Virginia (Roble et al. 2001) and subsequent field surveys identified 17 distinct sites where the butterfly was present. These sites are ~200 km from the *N. m. francisi* site in North Carolina, and they are separated by watersheds and geophysical features that would have precluded recent migration given this species propensity for short range dispersal (<1 km) (Figure 1; Hamm *et al.* in revision). During the summer of 2000 a lepidopterist observed *N. mitchellii* butterflies in central Alabama (Glassberg 2000), which prompted the US Fish and Wildlife to commission a survey that ultimately found 15 occupied sites in Alabama and three in northeastern Mississippi (Hart 2004). These recently discovered *N. mitchellii* have not been formally assigned to a taxonomic group below the species level, but have had federal protection extended to them until their rank can be firmly established. Preliminary molecular work (using mtDNA) has been conducted on *N. mitchellii* from throughout its range, but the results were inconclusive (Goldstein et al. 2004). If any of the recently discovered *N. mitchellii* populations were assigned to either of the protected species, the recovery criteria (of 25 viable populations for *N. m. mitchellii* [USFWS 1998]; and three additional metapopulations for *N. m. francisi* [USFWS 1996]) for those species could possibly be considered fulfilled, and federal protection under the ESA removed.

In this work, we sought to determine the taxonomic status of both previously recognized subspecies as well as all recently discovered populations based on the analysis of genetic variation from one mitochondrial and five nuclear loci. We specifically addressed three questions: 1) do molecular characters validate the recently discovered populations as *N. mitchellii*; 2) do the endangered species form evolutionarily distinct clades and/or clusters; and

3) can we reject a divergence time of zero between *N. m. mitchellii* and all other regional populations of *N. mitchellii*?

Methods

Collection and preparation of samples

Genetic samples were collected from extant *N. mitchellii* populations located in the Eastern United States of America. The samples (n=48) were collected during the 2008 and 2009 flight periods from locations representing all regions where *N. mitchellii* is extant: Alabama (n=4), Indiana (n=2), Michigan (n=25), Mississippi (n=3), North Carolina (n=7) and Virginia (n=6) (Figure 1). All samples were collected according to conditions specified in USFWS permit TE-175852 or other agency permits (Supplement 1). To obtain genetic material from this protected species we employed a non-lethal sampling method, in which 2 mm^2 of wing vein was removed from field caught butterflies (Hamm *et al.* 2010). Samples from *Neonympha areolata*, a sister taxon to *N. mitchellii*, were obtained from Alabama for use as an outgroup. Wing vein samples were placed directly into 95% EtOH and DNA were extracted within 48 hours using a DNEasy® Blood & Tissue Kit (Qiagen Inc., Valencia, CA) or a ZR Tissue & Insect DNA Kit™ (Zymo Research Corporation, Irvine, CA) and then stored at -80°C.

PCR amplification DNA sequencing

We sequenced six loci for all 48 *N. mitchellii* individuals and the outgroup taxon, *N. areolata*: the mitochondrial gene cytochrome oxidase I (COI); the nuclear genes elongation factor 1α (EF-1α), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein S5 (RpS5); and two anonymous single copy nuclear loci AL15_16, and AL20_21 (Hamm 2011) (Table 1). PCR products were visualized by gel electrophoresis and prepared for direct sequencing with ExoSAP-IT® (USB Products, Santa Clara, California) and directly sequenced

on both strands using an ABI Prism 3730 (Life Technologies Corporation, Carlsbad California) at the Research Technology Support Facility at Michigan State University. All anonymous loci and some highly heterozygous sequences were cloned into pGEM vector (Promega Corp. Madison, Wisconsin), then transformed into *E. coli* DH5α and were then sequenced using M13 primers. Contigs were assembled using the Geneious v5.5 program (Drummond et al. 2012) and heterozygous positions were called only if both forward and reverse reads were in agreement. Sequences were used as queries for BLASTn searches (Altschul et al. 1990) within the 'nr' database at NCBI to confirm orthology. Sequences were then aligned using the program Clustal W (Thompson et al. 1994) and the assemblies were visually inspected for evidence of linkage and recombination.

Phylogenetics

Two phylogenetic approaches, maximum likelihood and Bayesian concordance, to distinguish major phylogenetic groups from one another. All loci were examined for neutrality against $\alpha = 0.05$ using Tajima's D in the program DnaSP 5.1 (Librado and Rozas 2009). We used the program MrModeltest 2.3 (Nylander 2004) to select models of molecular evolution for each locus (partition) within the data set and optimal models were selected based on Akaike's information criterion (Posada and Buckley 2004) (Table 1). We used the program RAxML v7.2 (Stamatakis 2006) to construct a partitioned maximum likelihood tree. The most likely tree was determined based on 2,000 independent starting trees and branch support was assessed based on 10,000 bootstrap pseudo-replicates.

We examined the concordance of gene trees under a Bayesian framework. First, we explored the tree space of each locus using the program MrBayes 3.2 (Ronquist and Huelsenbeck 2003) using two MCMC simulations with four chains of two million generations each, the results

of which were analyzed after the first 25% were removed as burn-in. We considered the runs to have converged once the standard deviation of the split frequencies was below 0.01 and the two runs produced identical topologies. Concordance among the resulting gene trees was determined using the program BUCKy (Larget *et al.* 2010) with one million MCMC simulations and four chains across a range of α priors (0.1, 1, 100).

Population assignment

To prepare the data for population level analysis we used the program PHASE 2.1.2 (Stephens et al. 2001) to estimate haplotypes for genotypes that were heterozygous across multiple sites, and invoked the –d1 argument to accommodate multi-allelic data without the stepwise mutation model. We used the Bayesian algorithm implemented in the program STRUCTURE 2.3 (Hubisz et al. 2009) to assign individuals to clusters using data from the four loci that were variable within *N. mithcellii*. We tested clustering models for admixture with both correlated and uncorrelated allele frequencies and a model with no admixture and uncorrelated allele frequencies, the latter of which we considered to be the most biologically plausible. We determined the optimal number of clusters (K), by conducting MCMC simulations for 100,000 iterations with a 10,000 iteration burnin for $K = 1$ through $K = 10$ and each simulation was conducted 10 times. We determined the optimal K for each model using the ΔK method of Evanno *et al.* (2005), which uses the rate of change of log probability for successive values of K to estimate the optimal number of clusters. While each MCMC simulation was run under the same parameters, some stochasticity, such as label switching, is expected (Stephens 2000). To account for label switching the assignment probabilities from the ΔK best model were averaged over the 10 runs using the "FullSearch" option in the program CLUMPP 1.1.2 (Jakobsson & Rosenberg 2007).

To investigate the population genetic history of *N. m. mitchellii* we used the program IMa2 (Hey 2010) to estimate splitting times between regional populations using the isolation with migration model of Hey and Nielsen (2004). We conducted pairwise coalescent simulations between *N. m. mitchellii* and the clades and clusters identified by the previously described analyses. We ran each comparison using an exponential migration prior (-j7 option) and 20 MCMC chains with geometric heating (heating parameters: $a = 0.96$, $b = 0.9$) and at least 20 M steps with at least 100,000 genealogies saved after a burnin of 100,000. We confirmed a lack of pattern in the parameter estimates over time by visually inspecting trend plots from each run to ensure that sufficient chain mixing had occurred. Priors were adjusted after initial runs to ensure that the posterior distributions of parameter estimates were not sensitive to the priors.

Results

Genetic diversity

We sequenced a total of 3,011 base pairs across six loci for all 48 *N. mitchellii* individuals and one *N. areolata* (Table 1). No insertions or deletions were observed in the data and the sequences were easily aligned. Nucleotide BLAST results exhibited strong sequence identity (E value = 0.0) to putative butterfly orthologs, all failed to reject neutrality using Tajima's D ($D \approx 0$; $P > 0.05$). Estimates for the number of haplotypes, number of variable sites, nucleotide diversity, Watterson's theta, and the results of AIC model selection are presented in Table 1. Polymorphism varied greatly by locus: for example two of the loci (RpS5 and AL20_21) were invariant within *N. mitchellii*, while each of the remaining loci had at least nine variable sites. Nucleotide diversity varied from 0.15×10^{-3} to 8.53×10^{-3} and correlated with the number of variable sites for each locus. For all loci, inferred haplotypes were consistent across replicated runs in PHASE.

Phylogenetics

Based on maximum likelihood analyses we found that *N. mitchellii* formed a monophyletic group and that individuals were assigned to clades based on the geographic region from which they were sampled, though with varying levels of bootstrap support (Figure 2a). Bayesian concordance methods generated the same phylogenetic relationships as were observed in the maximum likelihood tree, though with low levels of concordance among the species trees (Figure 2a). Both methods support *N. m. francisi* as the sister-clade to the *N. mitchellii* clade; and these data also support monophyly for three regional clades (Alabama / Mississippi, Virginia, and Michigan / Indiana), although the order of branching was not clear.

Population assignment

The program STRUCTURE produced similar patterns of clustering across all models and values of *K*. The optimal number of clusters was $K = 3$ (Fig 2b) for all models using the ΔK method of Evanno et al. (2005). All three models examined through STRUCTURE resulted in the same pattern of clustering with high assignment probabilities. This consistent pattern was such that all individuals from North Carolina (*N. m. francisi*) clustered together, all individuals from Michigan and Indiana (*N. m. mitchellii*) clustered together, and individuals from Virginia, Alabama and Mississippi clustered together (Figure 2c).

Coalescent analysis in IMa2 between *N. m. mitchellii* and *N. m. francisi* revealed divergence times that did not overlap with present day (Fig 3a). Similarly, the divergence time posteriors from comparison between populations from Michigan and Indiana with those from Alabama and Mississippi did not overlap with zero. Comparison between *N. m. mitchellii* and *N. mitchellii* from Virginia generated a distribution that partially overlapped with zero (Fig 3b), though the 95% HPD did not include zero. Pairwise comparisons among the remaining regional populations supported the phylogenetic and clustering results, though some had low posterior probability (C. Hamm unpub. data).

Discussion

The recently discovered populations clearly fell within the species *N. mitchellii*, but complicated inference of the evolutionary history among regional populations. Although the *N. m. mitchellii* was distinct from all other regional populations, the relationships among the remaining regional populations were not clear across all analytical methods. Results from concordance and coalescent analyses, variation in signal among loci, and low power indicated that divergence among regional populations was relatively recent and has resulted in incomplete lineage sorting among alleles. The molecular data confirm that all recently discovered

populations are members of *N. mitchellii*. Even the loci that were invariant within *N. mitchellii* had fixed differences relative to the outgroup species *N. areolata*, the putative sister taxon (Fig 2A). Because these recently discovered populations are *N. mitchellii* they should be included in all subsequent studies concerning the population structure and conservation status of the species as a whole.

When we restrict our interpretation of the data exclusively to the endangered species, *N. m. mitchellii* and *N. m. francisi*, we conclude that they are evolutionarily distinct. Phylogenetic, concordance tree, clustering and coalescent methods all indicated that the two endangered species are different (Figs. 2 & 3a), which is important because the initial distinction was made using largely qualitative data and a limited sample size (Parshall and Kral 1989). Our data are more compelling in support of the taxonomic status for these subspecies. Indeed, the phylogenetic, concordance and coalescent analyses indicted that these subspecies were among the most distantly related populations examined. It is important to note the statistical support for clades was moderate but the posterior probabilities for divergence time from coalescent estimates did not overlap with zero.

When we interpreted our data in light of the newly discovered populations, the taxonomic distinctions among regional populations were more complex. One consistent result from the analyses was that the *N. m. mitchellii* clade was distinct from all other regional populations. The inconsistencies included the separation of *N. m. francisi* from Alabama/Mississippi based on coalescent methods (Figure 3b), where divergence times overlapped with zero. Finally, the phylogenetic and concordance methods differentiated between the Alabama/Mississippi and Virginia regional populations (Figure 2a), whereas clustering and coalescent methods did not (Figs. 2c & 3b). We further note that posterior probability of splitting time between Virginia and

N. m. francisi partially overlapped with zero, but was extremely flat and indicated that our data lacked the power to address this pairwise comparison in a coalescent framework.

We suspect that the three regional populations characterized by clustering methods (*N. m. mitchellii*, *N. m. francisi*, and Virginia plus Alabama/Mississippi) (Figure 2c) are distinct evolutionary units, but that historical demographic factors combined with recent reductions in the number and size of populations resulted in the observed inconsistencies and/or low statistical support among methods of genetic analyses. Each of the methods has obvious differences in the type of historical signal that is detected and these data appear to have been particularly exemplary in highlighting those differences. For example, statistical support from phylogenetic and concordance methods relies more heavily on the presence of shared derived characters and their prevalence within the data set (Felsenstein 2004). Since some of the loci had few informative characters (there were 48 variable sites within *N. mitchellii* among 3011 characters, though two loci were invariant), branch support and concordance values were low, despite a clear signal in the few informative characters present. The lack of informative sites resulted in many bootstrapped datasets without information for subsets of branches and resulted in low bootstrap support. Similarly, many of the trees sampled during the MCMC analyses were unresolved with respect to some branches, which resulted in low concordance values. Alternatively, had the data been consistent with strong support for alternative topologies across loci or sites, the results would have provided moderate or low support for those alternative topologies (Rokas et al. 2003). Clustering analyses do not rely on synapomorphies, but are more sensitive to multi-locus genotypes and their frequencies in the sample (Falush et al. 2003). Clustering separated *N. m. mitchellii* and *N. m. francisi* from the recently discovered populations, but did not detect any structure between these remaining groups (Figure 2b).

The variance in signal across loci was also a likely contributor to the differences in results across methods. The lack of variation in two of the loci (GAPDH and AL20_21), which exhibited standard levels of divergence from the sister species, combined with the relatively low haplotype variation at the mitochondrial locus (COI), relative to other butterflies (de Jong et al. 2011), is consistent with an historically small effective population size. The informative sites present in COI exhibited the lowest levels of genetic diversity in *N. m. mitchellii*, despite the much larger sample size (n=25; Table 1). Mitochondrial loci have a smaller effective population size than nuclear loci in animals due to uniparental inheritance and very low levels of recombination. This reduced effective population size should, on average, result in greater sensitivity of levels of genetic variation to population bottlenecks. Further complicating interpretation of the signal from COI is the fact that the bacterial endosymbiont *Wolbachia* is present in *N. mitchellii* at unknown levels (Hamm *et al.* unpub. data). We suspect that this is why the *N. m. mitchellii* exhibited the lowest levels of genetic variation at COI, and given the derived position of that regional population on the topology, may be indicative of small colonizing populations in the formerly glaciated states of Michigan and Indiana. The nuclear loci exhibited shared haplotypes among the regional populations, which combined with the evidence of recent divergence among all clades leads us to infer that these populations are likely exhibiting incomplete lineage sorting among alleles. Although we were able to infer some consistent patterns from these data, we also suspect that stronger statistical support for the postulated relationships and historical demography among all of the regional populations will require further study.

Based on our results, we believe that current conservation practices should continue such that *N. m. mitchellii* and *N. m. francisi* are managed separately as endangered species. Indeed, the

one consistent result across all analytical methods is that *N. m. mitchellii* is different from all other *N. mitchellii*. While these populations are clearly not *N. m. mitchellii*, their relationship to *N. m. francisi* is ambiguous when compared using pairwise coalescent analyses. Phylogenetic and clustering methods suggest they are separate from *N. m. francisi* but coalescent analyses failed to differentiate these populations. We believe the results from the pairwise coalescent analysis were influenced by incomplete lineage and had low power to resolve any differences. Thus, until a more powerful genome wide study can be conducted, we recommend the *N. mitchellii* found in Virginia, Alabama, and Mississippi should not be considered either of the endangered species.

Acknowledgments

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APPENDICES

Table 3.1. Descriptive information and statistics per locus used to generate sequence data for all *Neonympha* butterflies for this study. S is the number of variable sites π is the nucleotide diversity.

Locus	Primers (f / r)	Length (bp)	# Haplotypes	S	π	AIC best model[8]
COI	CI-J-2183 / Pat2[1] LCO 1490 / HCO 2198[2]	689	9	15	3.35×10^{-3}	HKY + I
EF1α	ef44 / ef51r[3] ef51.9 / efrcM4V[3]	590	11	9	1.94×10^{-3}	SYM
GAPDH	GAPDH_F1 / GAPDH_R2[4]	635	6	9	2.74×10^{-3}	SYM
RpS5	RpS5_F1 / RpS5_R1[4]	249	2	1	0.16×10^{-3}	K80
AL15_16	2-H9-F1 / 2-H9-R1[5]	576	10	19	8.53×10^{-3}	F81 + I
AL20_21	2-E12-F1 / 2-E12-R1[6]	272	2	1	0.15×10^{-3}	F81

[1]Simon et al. 1994; [2]Folmer et al. 1994; [3]Monteiro and Pierce 2001; [4]Based on Wahlberg and Wheat 2008 though modified for this study; [5]Hamm 2011; [6]Developed for this study, using methods described in Hamm 2011. [7]Parameters estimated in the program DnaSP (Librado and Rozas 2009), [8]AIC model comparisons conducted in the program MrModeltest 2.3 (Nylander 2004).

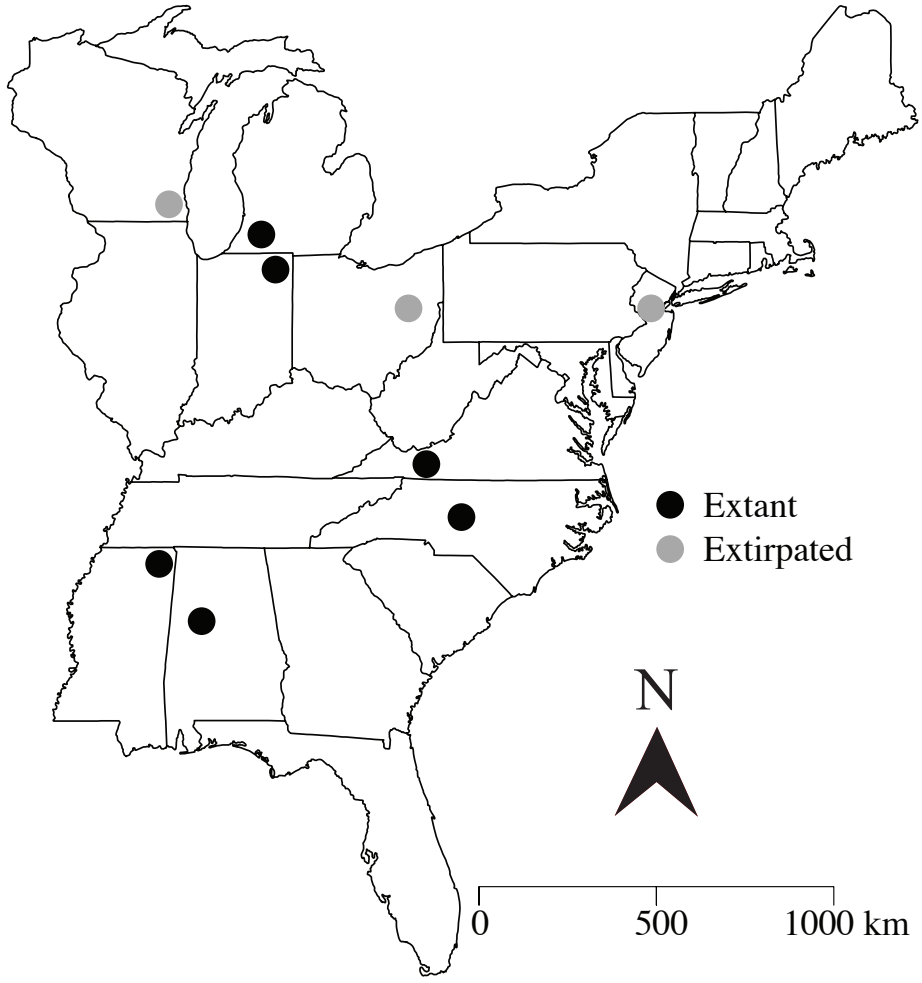

Figure 3.1. Map of known *Neonympha mitchellii* populations in the United States. Black dots indicate areas with extant populations and grey dots indicate areas with extirpated populations.

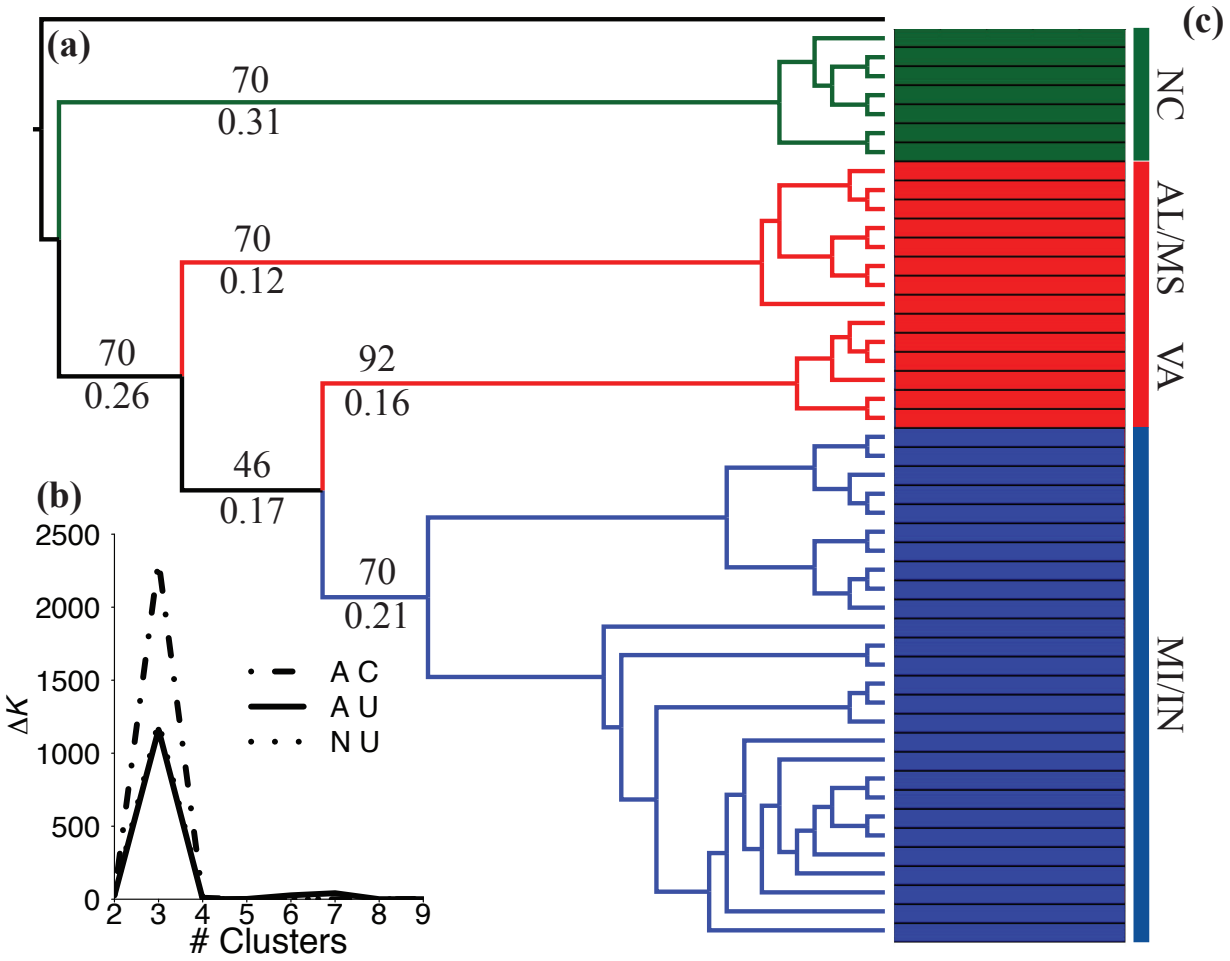

Figure 3.2. (A) Phylogenetic tree depicting the relationships among *Neonympha mitchellii* butterflies. Numbers above the branches represent support values based on 10,000 bootstrap pseudoreplicates on the ML tree generated in the program RAxML, and numbers below the branches represent Bayesian concordance values generated by the program BUCKy. The colors of the branches correspond to the clusters described in (C).

(B) Plot of Δ*K* values for the three models of population structure examined: population admixture with correlated allele frequencies (AC), population admixture with uncorrelated allele frequencies (AU), and no population admixture with uncorrelated allele frequencies (NU).

(C) Barplot depicting assignment probability from STRUCTURE analysis using a model with no admixture and uncorrelated allele frequencies and k = 3 clusters, though all models tested generated the same clustering pattern. Each barplot represents the proportion of that individual's genome derived from a certain cluster.

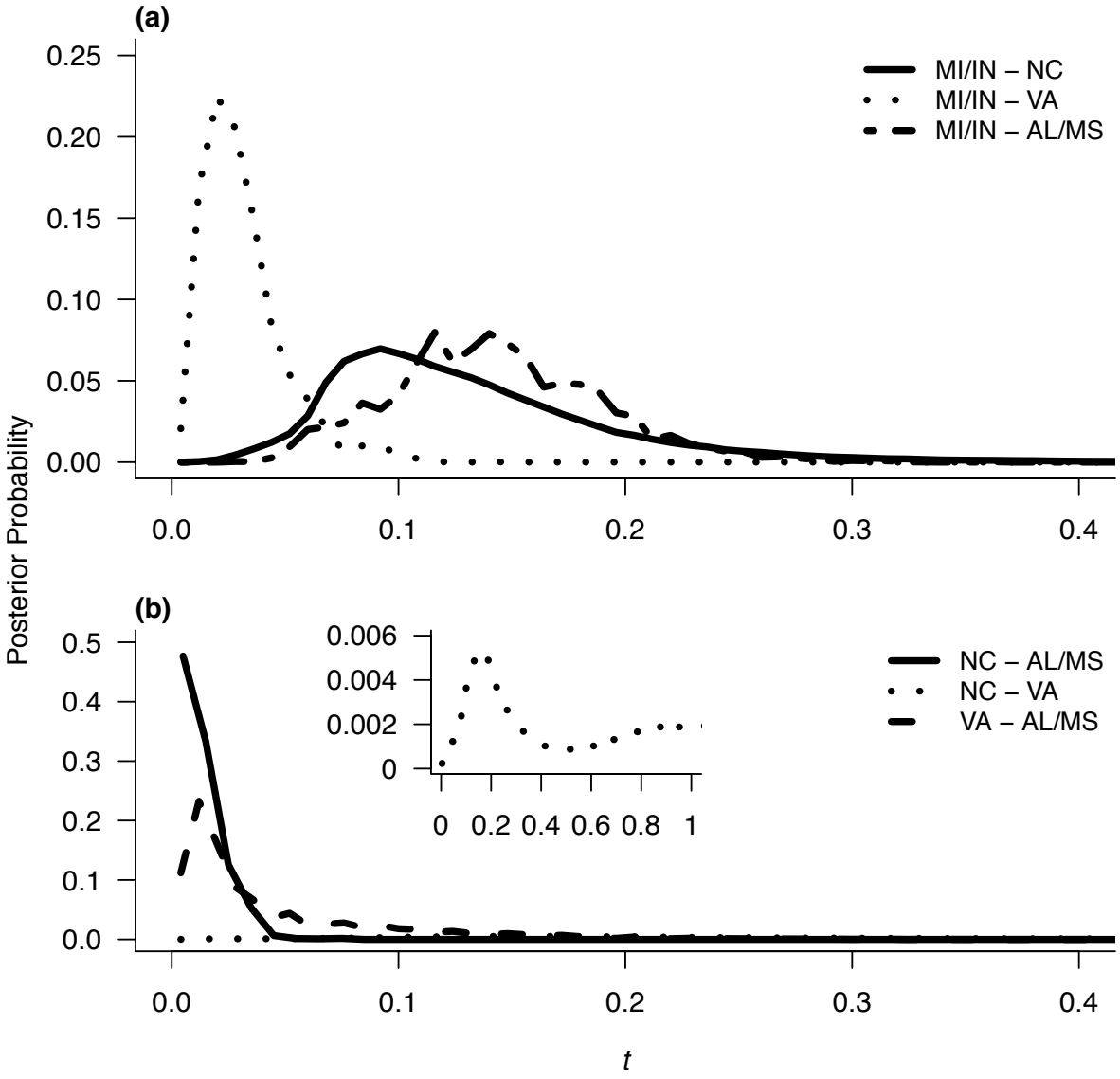

Figure 3.3. Posterior probability distributions of splitting times for: A) *N. m. mitchellii* (MI/IN) comparisons to *N. m. francisi* (NC), and the recently discovered populations in AL/MS and VA; and B) *N. m. francisi* to the recently discovered populations. Inset reflects the expanded NC – VA comparison.

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CHAPTER 4

PREVALENCE OF *WOLBACHIA* INFECTION IN SOME LEPIDOPTERA OF CONSERVATION CONCERN

Abstract

Conservation of at-risk species requires multi-faceted, carefully considered, management approaches to be successful. For arthropods, the presence of endosymbiotic bacteria, such as *Wolbachia*, may exacerbate these challenges. *Wolbachia* poses a substantial and underappreciated threat to the conservation of arthropods because infection may induce a number of phenotypic effects, most of which are considered deleterious to the host population. I examined the prevalence of *Wolbachia* infection in lepidopteran species of conservation concern. Using standard molecular techniques, I screened 22 species of Lepidoptera and identified 18 that were infected. This rate is comparable to that observed in insects as a whole. However, this is likely an underestimate because geographic sampling was not extensive and may not have included infected segments of the species' ranges. *Wolbachia* infections may be particularly problematic for conservation management plans that incorporate captive propagation or translocation. Inadvertent introduction of *Wolbachia* into uninfected populations or introduction of a new strain may put these populations at greater risk for extinction. Further sampling to investigate the geographic extent of *Wolbachia* infections within species of conservation concern, and experiments designed to determine the nature of the infection phenotype(s), are necessary to manage the potential threat of infection.

Introduction

Conservation managers often adopt active management strategies when confronted by complex and interconnected problems. Captive propagation and translocation programs are increasingly being incorporated into management plans to augment endangered populations or repopulate formerly occupied habitats (Crone et al. 2007). These programs present their own challenges and must be carefully designed to minimize the possibility of disease transmission and maintain genetic diversity (Snyder et al. 1996; Van Oosterhout et al. 2007). When working with arthropods of conservation concern an additional and under-appreciated challenge arises in the form of endosymbiotic bacteria that may manipulate the reproductive biology of their hosts (Werren et al. 2008; Nice et al. 2009).

A diverse assemblage of manipulative endosymbiotic bacteria are associated with arthropods, including representatives from the Bacteroidetes (*Cardinium hertigii*, *Flavobecterium* sp.), Mollicutes (*Spiroplasma* spp.), γ-proteobacteria (*Arsenophonus nasoniae*), and α-proteobactiera (*Rickettsia* sp., *Wolbachia pipientis*) (Duron et al. 2008, Engelstadter & Hurst 2009, Himler et al. 2011). *Wolbachia,* likely the most prevalent and best studied of these, is estimated to occur in 20% of arthropods and 66% of insects (Hilgenboecker et al. 2008). There are currently over 200 known strains of *Wolbachia* (Baldo et al. 2006; Stahlhut et al. 2010), which can induce at least four distinct phenotypes in their hosts. These include the following: (1) Feminization occurs when a *Wolbachia* infection transforms genetically male embryos into fully functional females, leading to production of progeny that are all functionally female, (2) Male-killing strains eliminate all male embryos so only female progeny are produced, (3) Parthenogenesis occurs in species with haplo-diploid sex determination, infected females do not need to mate and produce only female progeny from the unfertilized eggs, and, (4) Cytoplasmic

incompatibility (CI) prevents infected males from reproducing with uninfected females as well as females infected with a different strain of *Wolbachia* (Figure 1; Werren et al. 2008). The association between arthropods and *Wolbachia*, and the consequent manipulation of the hosts' biology, might have been occurring for extended periods of evolutionary time. The major lineages of *Wolbachia* are quite old, with divergence between the A and B groups approximately 58 - 67 MYA (Werren et al. 1995). Indeed, the association between *Wolbachia* and its hosts is likely a long and complicated one, as evidenced by the diversity of interactions between *Wolbachia* and host and the varied outcomes. For example, *Aedes aegypti* (Diptera: Culicidae) populations do not have naturally occurring *Wolbachia* infections, but genome analysis has identified *Wolbachia* genes in the *A. aegypti* genome, remnants of an ancient infection (Xi et al. 2005; Klasson et al. 2009). Some *Wolbachia* infections appear to confer some benefit to their host, such as increased resistance to viral infection (Hedges et al. 2008; Teixeira et al. 2008) and increased reproductive rates (Dedeine et al. 2001; Weeks et al. 2007; Kambris et al. 2009). Additionally, many *Wolbachia* strains appear to be uniquely adapted to their host's genome, and the phenotype induced by a particular strain may change when transfected to another host species (Fujii et al. 2001).

Divergence estimates among strains within the major *Wolbachia* groups are considerably smaller, between 0 - 1.6 MYA, than divergence between groups (Werren et al 1995). Such small divergence times are likely due to frequent horizontal gene transfer events between *Wolbachia* strains (Werren et al. 1995; Heath et al. 1999) which complicate estimates of the age of infections. Coalescent simulations of the minimum time since infection estimate that *Drosophila innubila* may have acquired *Wolbachia* between 15,000 - 700,000 years ago (Jaenike and Dyer 2008). Thus *Wolbachia* has been associated with at least some insect hosts for extended periods.

Despite these long associations and the opportunity for the evolution of avirulence, *Wolbachia* is generally considered a reproductive parasite because of the deleterious impacts infection has on host population biology (Werren et al. 2008).

Because *Wolbachia* is transmitted maternally, all of the reproductive phenotypes mentioned above increase *Wolbachia'*s ability to spread by creating more infected females in the population. However, these same modifications also skew host sex ratios, by eliminating members of the host population (usually males) or creating infertile matings. While CI-inducing strains are most frequently encountered in insects and cause the most obvious negative effects on their hosts by creating reproductive barriers, all four of the induced phenotypes might adversely affect populations of threatened or endangered species by potentially reducing the effective population size (Hoffman and Turelli 1997; Dyson and Hurst 2004; Werren et al. 2008). A reduction in the effective population size is especially problematic for populations that are already small, as is often the case for species of conservation concern. As populations shrink, the magnitude of demographic stochasticity increases, thus increasing the probability of extirpation. Furthermore, reduction in genetic diversity associated with a genetic bottleneck can reduce the ability of a population to persist in light of increased environmental stochasticity, such as that predicted by the Intergovernmental Panel on Climate Change (Lande and Shannon 1996; IPCC 2007). Thus, if a new *Wolbachia* infection or novel strain is introduced into a population it may quickly reduce the effective population size and induce a genetic bottleneck. This impact on population size is, of course, a major concern for conservation managers (Figure 2; Nice et al. 2009).

Wolbachia is especially problematic for recently infected populations because it can spread rapidly and can be difficult to detect by demographic observations (Weeks et al. 2007).

Sex-ratio distorting phenotypes can be detected by close examination of the offspring, but the CI-inducing phenotype has no impact on the sex ratio and no apparent consequences to health and vigor of individuals, making it more difficult to detect. Mating experiments are ultimately required to determine the induced phenotype of a *Wolbachia* infection.

Another impact of a *Wolbachia* infection relates to its potentially confounding effect on the geographic pattern of mitochondrial DNA (mtDNA) variation (Gompert et al. 2006). Because *Wolbachia* are maternally inherited, as are mitochondria, mtDNA variants in linkage disequilibrium with *Wolbachia* infection may be swept along as the infection spreads (Turelli et al. 1992; Jiggins 2003; Rasgon et al. 2003; Hurst and Jiggins 2005). This is essentially a form of indirect selection that favors the mitochondrial variant that is fortuitously associated with the initial *Wolbachia* infection. The resulting mitochondrial sweep can homogenize mtDNA variation over large geographic areas (Turelli et al. 1992; Gompert et al. 2008). This homogenization has even been demonstrated to cross taxonomic barriers, as in the case of the federally endangered Karner Blue Butterfly (*Lycaeides samuelis*, formerly *L. melissa samuelis*) (Gompert et al. 2006; Nice et al. 2009, Forister et al. 2011). This ultimately compromises the utility of mtDNA markers for diagnosing evolutionarily significant units or units of conservation (Forister et al. 2008; Crandall et al. 2009).

While the threat posed by spread of *Wolbachia* might be serious, I do not know its prevalence among endangered arthropods. While previous work has estimated the infection rate of insects at ~66% (Hilgenboecker et al. 2008), I have no empirical estimate of the infection rate in taxa of conservation concern. Here, I address this lack of knowledge by surveying Lepidoptera of conservation concern for *Wolbachia* infection. I tested 22 lepidopteran species from the United States of America, including federal and state listed threatened and endangered taxa and

other species of concern (Table 1). I chose to focus effort on the Lepidoptera because: (1) they are over-represented on the endangered species list, (2) they are the focus of numerous conservation efforts and (3) their taxonomy and natural history are relatively well-known compared to other invertebrates. This survey provides, to the best of our knowledge, the first glimpse into the prevalence of *Wolbachia* infection in endangered species and will help form the basis for assessing the risk of this infection in species of conservation concern.

Methods

A total of 150 individuals from 22 at-risk lepidopteran species were sampled (Table 1). These taxa were chosen because the material was either at our immediate disposal, or was kindly donated by colleagues. Genomic DNA (gDNA) was extracted from all individuals following standard methods (Hillis et al. 1996; Brookes et al. 1997). Screening for *Wolbachia* in gDNA samples required two polymerase chain reactions (PCR) and followed the methods used by Nice et al. (2009). Primers for the 16S rDNA gene were used to detect *Wolbachia* and were run in concert with arthropod-specific 28S rDNA primers to act as a positive control for each *Wolbachia* 16S reaction. Standard positive and negative controls were run simultaneously during the *Wolbachia* screens. PCR products were visualized on a 1% agarose gel and scored for the presence or absence of *Wolbachia*. When a positive reaction was scored the length of the band was compared against the known size for that amplicon. Detected infections were not genotyped following the Multilocus Strain Typing (MLST) protocols for *Wolbachia* (Baldo et al. 2006) for two reasons: 1) many taxa have only a single representative and knowledge of strain type in an individual would not alter our conclusions regarding infection frequency, and, (2) knowledge of strain type cannot be used to infer the induced phenotype. The nature of the induced phenotype can only be determined through experimental breeding. Any reactions that failed to produce a

band for the 28S rDNA reaction were subjected to re-amplification using a dilution series ranging from one part gDNA: ten parts water to one part gDNA: 200 parts water (Werren and Windsor, 2000). These dilutions were necessary to reduce the concentration of any reagents that might interfere with the PCR.

While the use of *Wolbachia*-specific PCR remains the standard method for the detection of *Wolbachia* (Baldo et al. 2006) certain primers may be prone to cross amplification of other bacteria (Simoes et al. 2011). To minimize the likelihood of false positives, I used additional primers to amplify FbpA (a protein coding gene) on a subset of 16 individuals that were positive for 16S rDNA (Table 1). For FbpA amplification I followed the PCR protocols outlined in Simoes et al. (2011) using GoTaq© DNA Polymerase (Promega, Madison, Wisconsin).

I next conducted a restriction digest on the PCR products of both 16S and FbpA reactions using the 4-cutter *MseI* (New England Biolabs, Ipswich, Massachusetts) using the manufacturers protocols. To estimate the band size generated *MseI* I conducted a BLAST search (Altschul et al. 1990) on GenBank (http://www.ncbi.nlm.nih.gov/) of 16S rDNA and FbpA sequences. The *MseI* enzyme should have two cut sites on 16S rDNA, both located at the ends of the sequence, which should produce one bandf of ~300bp. The FbpA gene has two cuts sites for *MseI*, both located at the ends of the sequence, and should produce one band of approximately ~350 bp. The product of the restriction digests was visualized on a 2% agarose gel to confirm that band sizes matched those predicted.

Finally, eight individuals (Table 1) were sequenced forward and reverse for both 16S rDNA and FbpA at the Research Technology Support Facility at Michigan State University on an ABI 3730 Genetic Analyzer (Applied Biosystems Inc., Carlsbad, California). The resulting

sequences were submitted for a BLAST search to confirm the amplification of *Wolbachia* sequences.

Results

18 of the 22 species screened had at least one individual score positive for *Wolbachia* infection (Table 1). Of these, all individuals from 13 species scored positive. Five positive species had at least one individual score positive.

All individuals that were positive for 16S rDNA were also positive for FbpA. The bands produced by the restriction digest were ~300 bp for 16S rDNA and ~350 for FbpA. All individuals sequenced for 16S rDNA and FbpA had BLAST hits matching appropriate *Wolbachia* sequences. The 16S rDNA sequences showed ~100% pairwise identity (E value = 0.0) with other *Wolbachia* 16S rDNA sequences on Genbank. The FbpA sequences showed 99% pairwise identity (E value = 0.0) with other *Wolbachia* FbpA sequences on GenBank. All resulting sequences were deposited in GenBank.

Discussion

I have documented the presence or absence of *Wolbachia* in 22 lepidopteran species of conservation concern and I have demonstrated that our PCR products are in fact *Wolbachia*. Of those screened, 18 of 22 (82%) had one or more infected individual. This percentage is slightly higher than infection rates found in surveys by other researchers (Werren et al. 1995; 2008; West et al. 1998; Werren and Windsor 2000; Hilgenboecker et al. 2008). It should be noted that our method of *Wolbachia* detection is conservative (Nice et al. 2009; Simoes et al. 2011). A meta-analysis of *Wolbachia* infection suggested that infection rates are rarely observed at intermediate levels, that is, populations are either mostly infected or uninfected (Hilgenboeker et al. 2008). I asked if our observed infection rate was significantly higher than the estimate of Hilgenboecker

et al. (2008) using a probability mass function for the binomial distribution with user-generated code in R (R Core Development Team) and a significance level of $\alpha = 0.05$. The proportion of infection I detected was higher that that of Hilgenboecker et al. (2008) but not statistically significantly higher (P=0.056). I urge caution when interpreting this analysis because our samples were not drawn randomly. As such, I cannot conclude that Lepidoptera of conservation concern have a higher infection rate of *Wolbachia* than do other insects. But it is clear that lepidopteran taxa of conservation concern are certainly no less susceptible to *Wolbachia* infection than other groups of insects.

A consequence of the small sample sizes for each taxon is that some species, which were scored as negative, might, in fact, be infected. It is known that infection rates are low during the initial stages of infection in a population, or can persist at low levels in some populations, so it is possible that infected individuals were missed in our sample.

Introduction of *Wolbachia* into an uninfected population may have serious consequences. Simulation models suggest that the spread of a CI-inducing *Wolbachia* strains into an uninfected population could reduce the effective population size and small populations are at an increased risk of extirpation due to stochastic events (Figure 3). This is perhaps ironic since small populations are most likely to be the recipients of supplemental individuals from captive rearing or translocation programs. Extreme care must be taken when choosing the source population for such programs to avoid introducing a *Wolbachia* infection.

While CI-inducing phenotypes are frequently observed in insects, its presence has not been confirmed in this study and other phenotypes are possible. If present, feminization and male-killing *Wolbachia* strains pose an even greater threat than CI-inducing strains. While CI-inducing strains are most deleterious during their spread across a population, the negative effects

of other phenotypes that skew the sex ratio continue even after infections become fixed (Charlat 2003). Therefore, determining the induced phenotype associated with any infection is critical to understanding the threat that *Wolbachia* poses to a host species.

Accurately determining the presence of infection and its induced phenotype requires the use of multiple methods. While necessary to detect the presence of infection, molecular genetic tools alone cannot identify the induced phenotype because induced phenotypes are not monophyletic and the same strain might induce different phenotypes in different species (Zhou et al. 1998; Baldo et al. 2006). Molecular methods, in conjunction with demographic observations, can allow managers to detect feminization, male-killing and parthenogenesis phenotypes. However, confirming infection by a CI strain is more intensive, requiring experimental crosses between infected and uninfected individuals.

There are two additional concerns when managing *Wolbachia*: (1) the possibility that the induced phenotype is suppressed (Hornett et al. 2006) and (2) that there are multiple strains occurring sympatrically in the same population (Hiroki et al. 2004). In the case of the former, some populations may appear uninfected because they have been able to ameliorate the phenotypic effects of infection, though they remain infected. These populations have been able to suppress the infection phenotype, and while *Wolbachia* are still detectable using molecular methods, the infection phenotype is absent (Hornett et al. 2006). Transmission of *Wolbachia* from a suppressing population to an uninfected population may result in the expression of the induced phenotype and all of the subsequent consequences. While suppression might allow populations to escape the consequences of infection, the concealed presence of *Wolbachia* increases the likelihood of inadvertently introducing an infection through captive management or

translocation. In the case of the latter, multiple strains of *Wolbachia* might infect a species (Reuter and Keller, 2003; Hiroki et al. 2004), and each strain might induce a different phenotype.

For these reasons, management programs should screen a representative subset of individuals propagated in captivity to verify that they are free of infection or ensure that they are infected with the same strain as the recipient population. This study may serve as a foundation for examining other at-risk arthropods, many of which have captive propagation programs. Screening of these species is necessary to determine the extent of *Wolbachia* infection within populations and across species. Future studies should also seek to determine the nature of the *Wolbachia*-induced phenotypes in these species. Information on the prevalence, geographic extent and phenotypic effects of *Wolbachia* might prove critical for effective management of threatened and endangered arthropods.

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APPENDICES

Table 4.1. Results of a survey of *Wolbachia* infections in 150 lepidopteran individuals, representing 22 species of conservation concern. 18 species were represented by one or more infected individual. County of collection was not available for some individuals. Total number screened is presented with the number testing positive for *Wolbachia* presence given parentheses.

Taxon	Locality	# Screened 16S (# Present)	*Wolbachia* Infection Status	# Screened FbpA (# Positive)	Conservation Status[1]
FAMILY: LYCAENIDAE					
Callophrys (*Mitoura*) *muiri*	California	8(0)	Not Detected	0	SC(CA), C(Federal)
Callophrys (*Incisalia*) *irus*	Allegan Co., MI	1(1)	Positive	0	E(DE, MD, NH, OH); T(CT, MI, NJ, NY, WI); SC(MA, RI)
Callophrys (*Mitoura*) *gryneus thornei*	San Diego Co., CA	5(0)	Not Detected	0	SC(Federal), E(CA)
Euphilotes pallescens arenamontana	Sand Mountain, Churchill Co., NV	12(3)	Positive	0	C(Federal)
Plebejus (*Lycaeides*) *idas nabokovi*	Schoolcraft Co., MI	2(1)	Positive	1(1)	T(MI), SC(MN)
Plebejus saepiolus	White Mountains, Mono Co., CA	10(2)	Positive	0	SC(Federal), E(CA)
FAMILY: NYMPHALIDAE					
Speyeria diana	North Carolina	1(1)	Positive	1(1)	SC(NC)

Table 4.1 (con't)

Speyeria idalia	Buena Vista prairie, WI	1(1)*	Positive	1(1)*	E(MI, NY, OH, WI); T(IL); SC(IA, MN, MO, OK, PA)
Speyeria idalia	Hog Back prairie, WI	1(1)	Positive	1(1)	E(MI, NY, OH, WI); T(IL); SC(IA, MN, MO, OK, PA)
Speyeria idalia	Thompson Prairie, WI	1(1)	Positive	1(1)	E(MI, NY, OH, WI); T(IL); SC(IA, MN, MO, OK, PA)
Phyciodes batesii	Alger Co., MI	1(0)	Not Detected	0	SC(MI)
Neonympha mitchellii francisi	Fort Bragg, NC	4(4)	Positive	3(3)	E(Federal)
Neonympha mitchellii mitchellii	Jackson County Central, MI	30(3)*	Positive	3(3)*	E(Federal, MI); T(VA)
FAMILY: HESPERIIDAE					
Pyrgus ruralis lagunae	San Diego Co., CA	7(1)	Positive	0	E(Federal)
Hesperia ottoe	Allegan Co., MI	1(1)*	Positive	1(1)*	T(IL, MI, MN); SC(IA, MT)
Polites sabuleti albomontana	White Mountains, Mono Co., CA	53(25)	Positive	0	C(Federal), E(CA)
Polites mardon	Jackson Co., OR	1(1)	Positive	0	C(Federal), E(CA)

Table 4.1 (con't)

Euphyes dukesi	Wayne Co., MI	1(1)*	Positive	1(1)*	T(MI)
Atrytonopsis hianna	Newaygo Co., MI	1(1)	Positive	0	SC(MI, WI)
Atrytonopsis n. sp.1	Fort Macon, NC	5(5)	Positive	0	SC(Federal)
FAMILY: NOCTUIDAE					
Papaipema beeriana	Washtenaw Co., MI	1(1)	Positive	1(1)	SC(MI)
Papaipema sciata	Jackson Co., MI	1(1)*	Positive	1(1)*	SC(MI)
Papaipema silphii	Washtenaw Co., MI	1(1)	Positive	1(1)	T(MI)
Schinia lucens	Ransom Co., ND	1(1)	Positive	0	E(MI)

[1]Correspoding codes:
T= threatened
E= endangered
SC = species of special concern
C = candidate for protection
* = sequenced

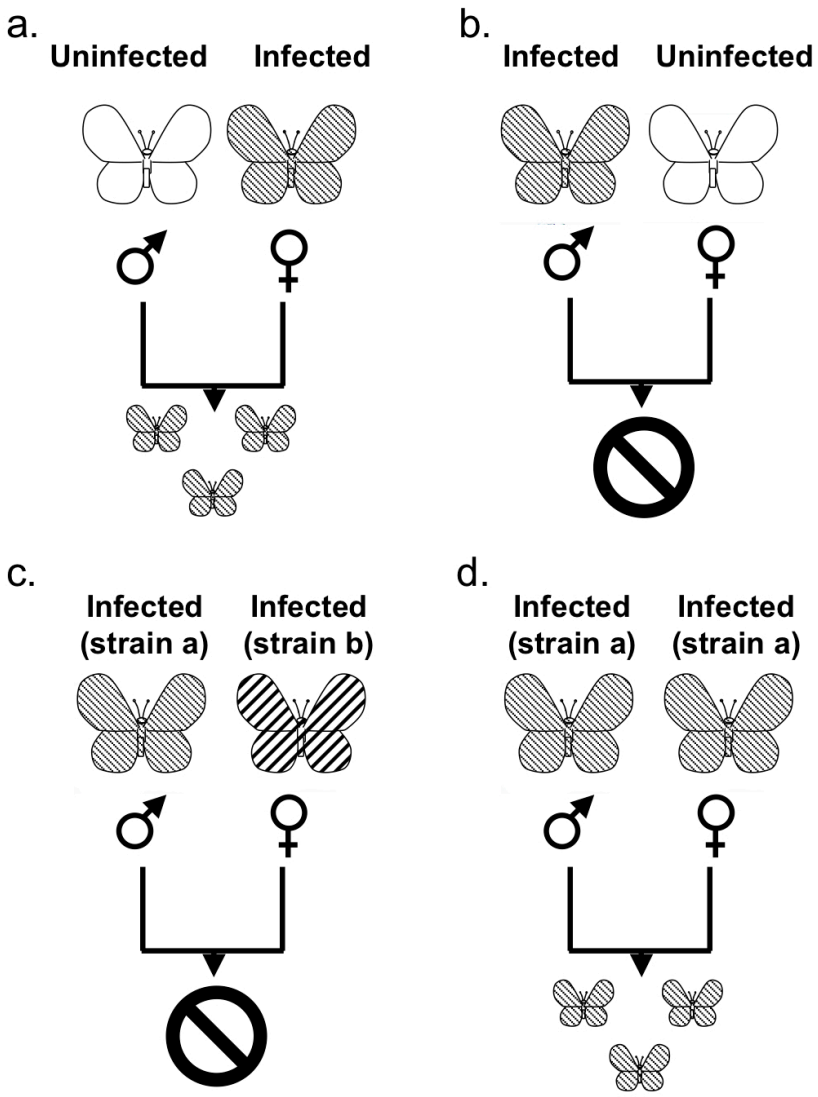

Figure 4.1. Phenotypic effects of a Cytoplasmic Incompatibility (CI) – inducing *Wolbachia* strain. A) Matings between infected females and uninfected males produce infected offspring, but B) matings between infected males and uninfected females fail, thus infected females have a reproductive advantage over uninfected females. C) In populations infected with multiple CI *Wolbachia* strains, matings between males and females infected with different strains also fail, however, D) matings between males and females infected with the same strain produce infected offspring.

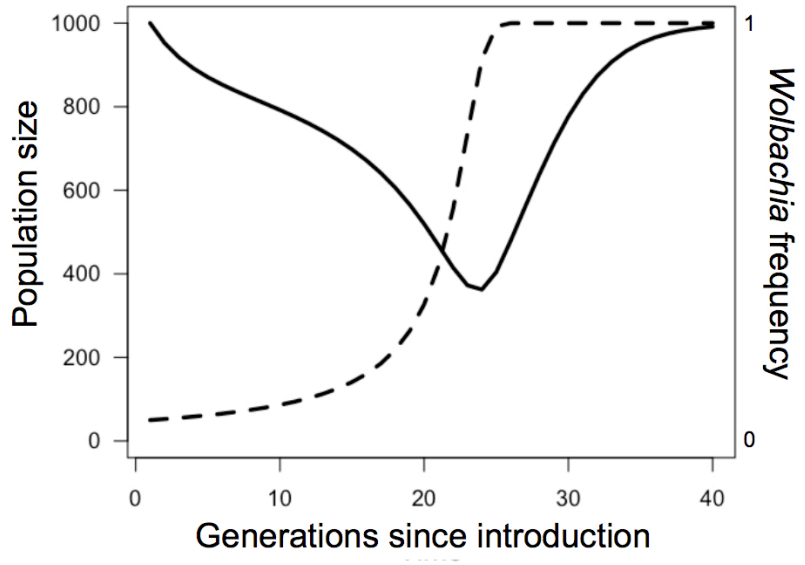

Figure 4.2. The impact of the introduction of a CI-inducing *Wolbachia* strain on population size. Population size (solid line) declines following the onset of infection until the infection rate (dashed line) nears 100%, after which, population size recovers. This "bottleneck" might increase the probability of extinction in populations that are initially small (For model details, see Nice *et al.* 2009).

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LITERATURE CITED

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CHAPTER 5

DISCRIMINATING WING PATTERN DIFFERENCES AMONG *NEONYMPHA* (LEPIDOPTERA: NYMPHALIDAE) USING GEOMETRIC MORPHOMETRICS

Abstract

Butterflies of the genus *Neonympha* have been known from the eastern United States since the late 18th century. The major differences reported for the taxa of the genus were wing pigmentation patterns on the hind wing. Often, these descriptions were based on small sample sizes and attempted to classify continuous traits as discrete ones. Given that two *Neonympha* taxa are federally protected, it is important to know that the traits used to describe and distinguish these taxa actually discriminate them. Using the approaches of geometric morphometrics I investigated wing pattern shape and how that shape varies. By placing landmarks at homologous positions on a large number of samples it is possible to determine if there is any variation among the described taxa. In this study, I applied geometric morphometrics to the question of *Neonympha* taxonomy. I applied landmarks to 26 locations on the ventral side of hind wings on 221 male *Neonympha* butterflies. After landmarks were placed, I used multivariate statistical procedures to determine if the differences reported by authors do distinguish *Neonympha* taxa. The two endangered species were clearly and consistently distinguished from other *Neonympha* and one another. The remaining *Neonympha* could not reliably be distinguished from one another, which is not surprising given that these taxa have historically been considered the same species. The application of geometric morphometrics to taxonomy has thus validated the major taxonomic distinctions within *Neonympha.*

Introduction

The species concept is central to biology despite over 20 different species concepts existing (Bock 2005). Mayr (1942) defined biological species as 'groups of interbreeding natural populations which are reproductively isolated from one another' and many consider this the gold standard of species concepts (Bock 2005). While the Biological Species Concept may be an ideal definition, it is often difficult to test in practice (Mallet 1995). Taxonomists often delimit species using methods that identify discontinuities among groups, and frequently cite morphological or molecular data to substantiate their hypotheses (Agapow et al. 2004). While modern molecular methods are generating important data at a rapid rate, most species descriptions follow the morphological species concept, which holds that species are uniquely identifiable based on phenotype and separated from other species by discontinuities in phenotype (Isaac et al. 2004; de Queiroz 2005; Vogler and Monoghan 2007).

Species diagnostic characters measured to capture variation of each character underlie an informative morphology-based species description (Chapman 2005; Dahdul et al. 2010). In the butterfly enthusiast community species descriptions are sometimes based on qualitative descriptions of ambiguous characters and often with a limited sample size. The species description of many butterflies describes general features of the taxon such as color or the position of pigment bands. For example, in the description of the butterfly *Neonympha mitchellii*:

> "Both wings are crossed by four transverse brownish-yellow stripes… occupying the same position as the same lines in its ally, *N. Areolatus* [sic], the first and second uniting by a rounded end about a tenth of an inch from the inner margin of hind wings… In *Areolatus* [sic] these lines do not unite" (French 1889).

These verbal descriptions treat what may by a continuous character as though it were discrete, and many butterfly species descriptions have been called into question due to this (Descimon and Mallet 2009).

What is needed is a method that allows researchers to place quantitative values on verbal descriptions, one such method is geometric morphometrics. Contemporary geometric morphometrics are based on the configuration of landmarks, which are "discrete, homologous anatomical loci" that are "points of correspondence" within and among groups of samples (Zeldtich et al. 2004). Specimens are digitally photographed and then landmarks identifying homologous positions are placed onto the images. The raw coordinates of these landmarks are then "superimposed" on one another and mathematically adjusted until all that remains are differences in shape (Zelditch et al. 2004). Shape is defined as "all the geometric information that remains when location, scale and rotational effects are filtered out from an object" (Kendall 1977). After superimposition multivariate statistics can be applied to the data set. Recently, geometric morphometrics has been applied to taxonomic questions in the Lepidoptera (Mutanen 2005; Dapporto 2008a, 2008b) because subjective and qualitative descriptions of taxonomic differences were often found to be lacking. In the case of pigment bands described above, landmarks could be placed where these band overlap with wing veins and could thus be used to quantitatively determine if two taxa are distinguishable based on certain characters.

The North American butterfly genus *Neonympha* contains three species that are presently recognized, *Neonympha areolatus* (Smith 1797), *N. helicta* (Hübner 1808), and *N. mitchellii* French 1889. The Mitchell's satyr was described from specimens collected in the northern United States, but was given the subspecific name *N. m. mitchellii* when a variant from the southern US, the Saint Francis' satyr, was described and named *N. m. francisi* Parhsal & Krall 1989. While the delimitation of species is always important, it is essential in cases where taxonomic names carry with them legal protection. Both *N. m. mitchellii* and *N. m. francisci* are federally endangered species and are legally protected wherever they occur (Hamm et al.

accepted). Current descriptions of these taxa are largely qualitative and focus on differences in the pattern of pigmentation on the ventral side of the hind wing (Davis 1924; Gatrelle 1999), and authors have disagreed as to the significance of those characters to the point where these taxa have been combined and split a number of times (Pelham 2008). Indeed, *N. areolatus* and *N. helicta* exhibit large areas of sympatry and qualitatively appear very similar phenotypically, so much so that many authors have treated them as the same entity (Scott 1986), and there has been a call for corroboration of this separation (Pelham 2008).

The purpose of this study was to quantify the pigmentation patterns on *Neonympha* butterflies and determine if the qualitative differences reported by previous authors correspond to the presently recognized taxa and thus validate the characters. Additionally, because of the protected status of *N. m. mitchellii* and *N. m. francisci*, any collector that takes a specimen could face federal prosecution. Ensuring that *Neonympha* taxa are distinguishable would remove any excuse regarding taxonomic uncertainty.

Methods

A total of 221 male *Neonympha* butterflies representing four taxa from 11 states (Table 1) were digitally imaged from entomology collections (United States National Museum, American Museum of Natural History, University of Michigan, Michigan State University, and the University of Florida). The images were then landmarked using the software TPSdig v2.1 (Rohlf 2006) at 26 locations where pigmentation bands overlapped with wing veins (Fig. 1), which corresponded to type II landmarks, which are defined in terms of specific local features relative to a specific structure (Zelditch et al. 2004). The pigment bands landmarked were the distal band of the central symmetry system, both the proximal and distal bands of the border symmetry system, and the marginal band (Nijhout 1991). The landmark coordinates were then imported

into the program MorphoJ v1.05a (Klingenberg 2010), where a generalized least squares (GLS) Procrustes superimposition was conducted, in which the data were centered, rotated, and scaled to a standard size. A major problem for the GLS Procrustes superimposition is that it yields a full complement of variable coordinates, which is four more than the number of dimensions of the shape space for two dimensional images (Zelditch et al. 2004). To avoid this problem, I conducted a principle component analysis (PCA) on the Procrustes coordinates and used the first 48 PC scores (each coordinate has an X and Y component) as shape variables in subsequent analyses.

Variation in shape among taxa was examined using the linear discriminant analysis (LDA), using maximum likelihood to estimate mean and variance, from the MASS package (Venables & Ripley 2002) in the statistical software R 2.14.2 (R Development Core Team 2012). A linear discriminant analysis (LDA) finds the linear combination of features that separates groups in a fashion similar to a principle components analysis in that it finds the axes that account for the most variation possible (Venables & Ripley 2002). The model examined was:

Neonympha taxa ~ PC

Where PC represented the first 48 principle components from the GLS Procrustes transformed data. Means for groups are based on the prior probability of a sample belonging to a group; these priors are estimated based on the number of samples assigned to a certain group. The scores from the discriminant analysis were then used to predict the probability of correctly assigning a sample to the correct taxon. The predicted group membership for each sample was determined by calculating the sample mean, and then determining which group mean it was closest to; if the closest group mean was from the taxon from which the sample originated then the sample was correctly assigned (Zelditch et al. 2004). Note that this test cannot be used to determine the

statistical significance of differences among groups; rather it is used to describe differences among group means. To determine if the shape variables examined in this study were able to discriminate among the taxa I conducted a MANOVA using the following model:

PC ~ *Neonympha* taxa

Additionally, I calculated a multivariate R^2 (Procrustes variance; code from W. Pitchers) by taking the sum of the diagonal components of the variance-covariance matrix for the fitted variance, and divided that by the sum of diagonals from the fitted and residual variance (e.g. total variance).

Results

The wing patterns of *N. areolatus* and *N. helicta* clustered separately from those of *N. m. francisci* and *N. m. mitchellii* (Figure 5.2). The taxa that have been combined and split repeatedly in the literature, *N. areolatus* and *N. helicta*, overlapped considerably with one another (Fig 5.3). Inspection of the plotted means (Fig. 5.3) reveals that landmarks 4 & 5 and 6 & 7 appear to be moving in opposite directions, relative to the rest of the landmarks, between *N. areolatus* / *N. helicta* and *N. m. mitchellii* / *N. m. francisci*. The result is that the bands of the border symmetry system in *N. m. mitchellii* and *N. m. francisci* are often joined at the leading edge of the wing, while in *N. areolatus* and *N. helicta* that are rarely joined.

The assignment test concurs with the results of the LDA, as most samples were assigned to the proper taxon (Fig. 4). Both *N. m. francisci* and *N. m. mitchellii* were correctly assigned the >90% of the time (Figs. 5.4C & D; Table 5.2), which stands in contrast to *N. areolatus* and *N. helicta*, which were correctly assigned to the proper taxon with lower frequency (Figs. 5.4A & B). Multivariate patterns of shape change among *Neonympha* taxa was highly significant ($F_{3, 217} = 4.5$, $P < 0.001$) and accounted for 23% ($R^2 = 0.23$) of the variance in the model.

Discussion

Analysis of wing pattern using geometric morphometrics clearly distinguished the endangered taxa, *N. m. mitchellii* and *N. m. francisci*, from one another and other *Neonympha* (Figs. 5.2, 5.4; Table 5.2). These results parallel ongoing genetic work, which demonstrate that *N. m. mitchellii* and *N. m. francisci* are reciprocally monophyletic but diverged some time during the Pleistocene (Hamm et al. unpub. data). Given that phenotypic characters are thought to evolve more slowly than molecular characters, this estimate may need to be reexamined. A Pleistocene divergence could still be possible if these wing pattern characters were undergoing strong selection, which has been demonstrated for similar characters in other butterflies (Hines et al. 2011). Alternatively, these wing pattern characters could be plastic and reflective of the environments in which they were reared (Schlichting and Pigliucci 1998), thought this is unlikely because the taxa studied here are often sympatric and were sampled from the same regions (Table 1).

Though the protected species can be correctly classified with high accuracy, there are no accepted limits as to what degree of classification is acceptable for species of conservation concern. This is important because the legal status of endangered insects is dependent on their taxonomic status, and if a non-threated species was often assigned incorrectly to a threatened taxon then considerable legal problems could result. Analysis of the taxa that have been repeatedly combined and split, *N. areolatus* and *N. helicta*, demonstrated the difficulty faced by taxonomists, as they appeared highly similar. Indeed, these taxa had considerable overlap in the plotted LDA (Fig. 5.2) and were often misclassified by the permutation test (Table 5.2). Should either taxon be proposed for protection some difficulty might arise in determining exactly which taxon a specimen belongs to (assuming they are distinct species).

By placing landmarks on characters that were described qualitatively, this study has demonstrated the utility of geometric morphometrics when applied to taxonomic problems. Future work with *Neonympha* butterflies can greatly expand upon this initial study by examining how other wing traits, such as the border ocelli, covary with wing size and shape. It would also be interesting to explore how the structure of the wing changes among taxa, as differences in these characters (i.e. vein intersections) have also been qualitatively described (Gatrelle 1999).

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APPENDICES

Table 5.1. Sample size for each *Neonympha* taxon by state.

Taxon	N	State	N
N. areolatus	118	Alabama	10
		Florida	34
		Georgia	16
		Louisiana	10
		Mississippi	6
		New jersey	13
		North Carolina	14
		Texas	6
		Virginia	9
N. helicta	37	Florida	10
		Georgia	3
		Mississippi	6
		New Jersey	11
		Virginia	7
N. m. francisci	15	North Carolina	15
N. m. mitchellii	51	Indiana	1
		Michigan	50

Table 5.2. MANOVA summary for the model: PC ~ Taxon, where PC represents the first 48 principle components of GLS Procrustes transformed data and taxon represents the four *Neonympha* butterfly taxa in this study. df, degrees of freedom; Den. df, denominator degrees of freedom; Prob *F*, probability of *F*.

Source	df	*F*-value	Den. df	Prob *F*
Taxon	32	4.5	516	2.2E-16
Residual	217			

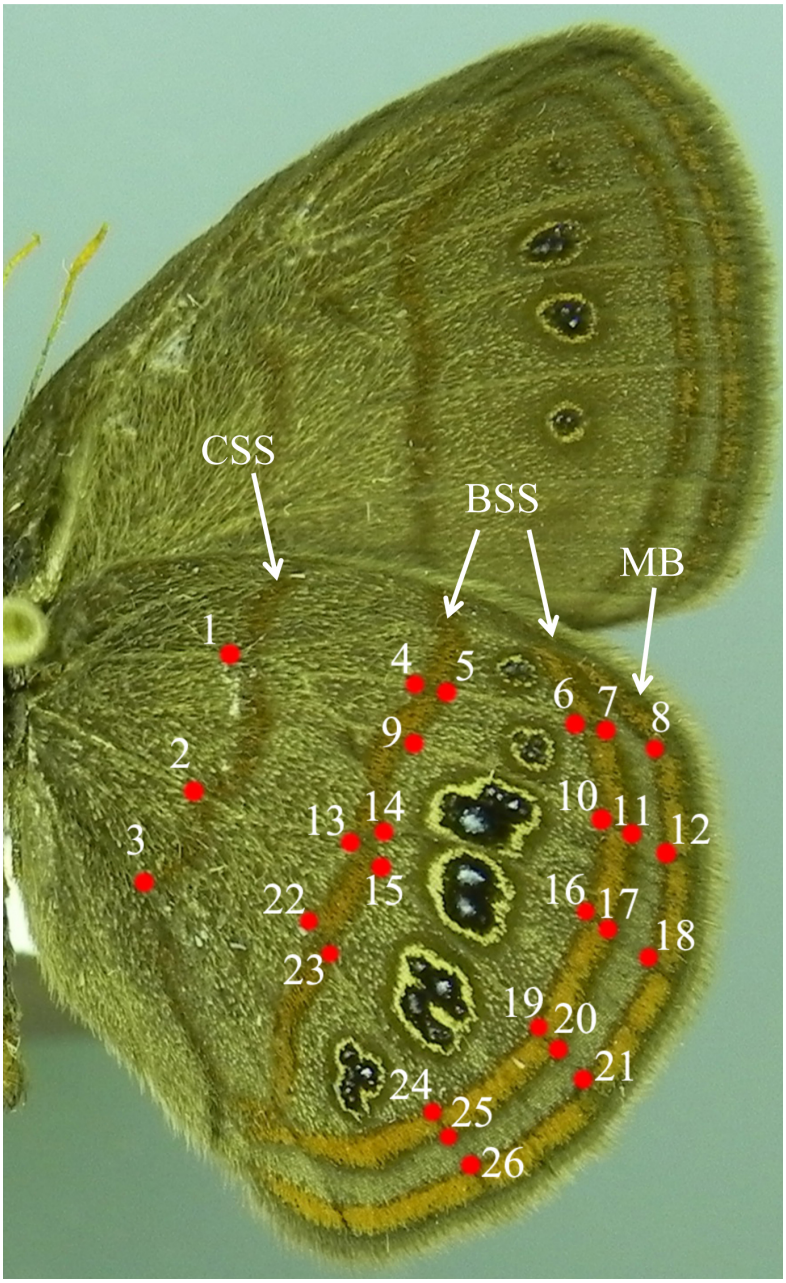

Figure 5.1. Photograph of the left ventral side of *N. m. francisci* with red points indicating the placement of type II landmarks (where pigmented lines of the central symmetry system, border symmetry system, and marginal bands overlap with wing veins. The forewing is included in the image for perspective. CSS, central symmetry system; BSS, border symmetry system; MB, marginal band.

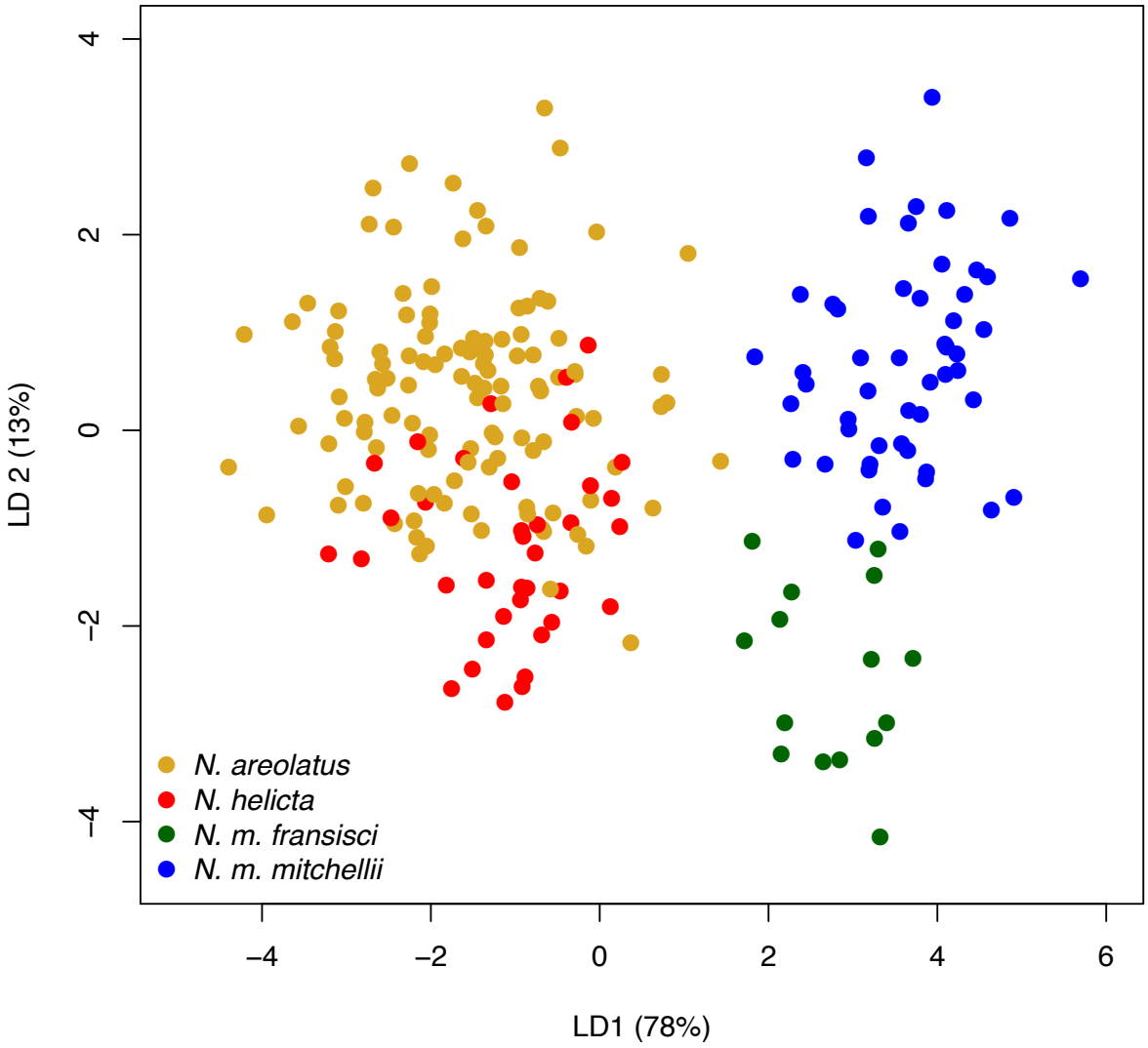

Figure 5.2. Plot of discriminant analysis results on *Neonympha* butterflies against the first discriminant axis (LD1), which explained 78% of the variance, and the second discriminant axis (LD2), which explained 13% of the variance. The third discriminant axis (not depicted) explained 9% of the variance.

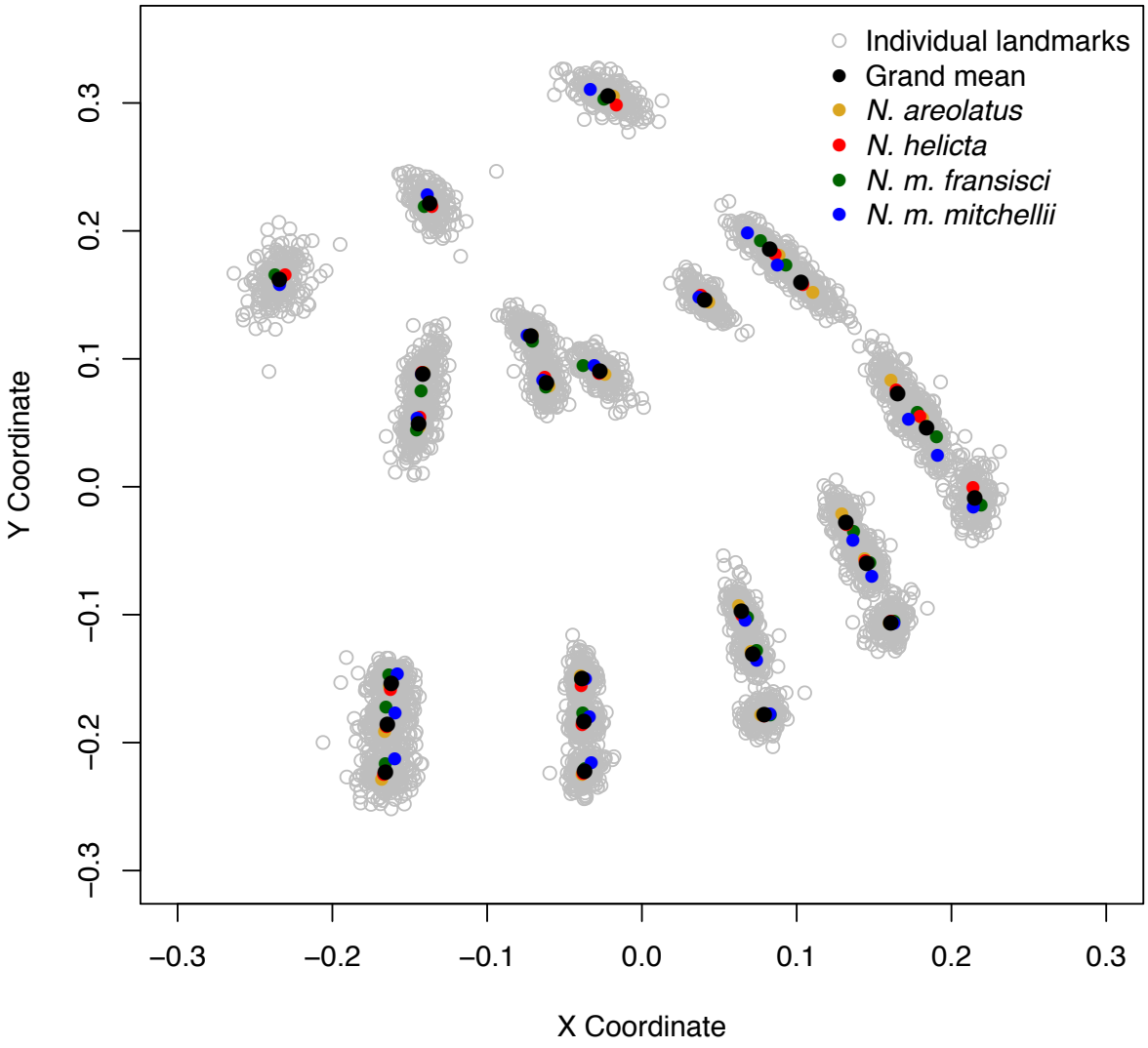

Figure 5.3. Depiction of type II landmarks from digitized *Neonympha* butterflies after generalized least squares Procrustes transformation with the grand mean and taxon means. Landmarks correspond to those depicted in Figure 1.

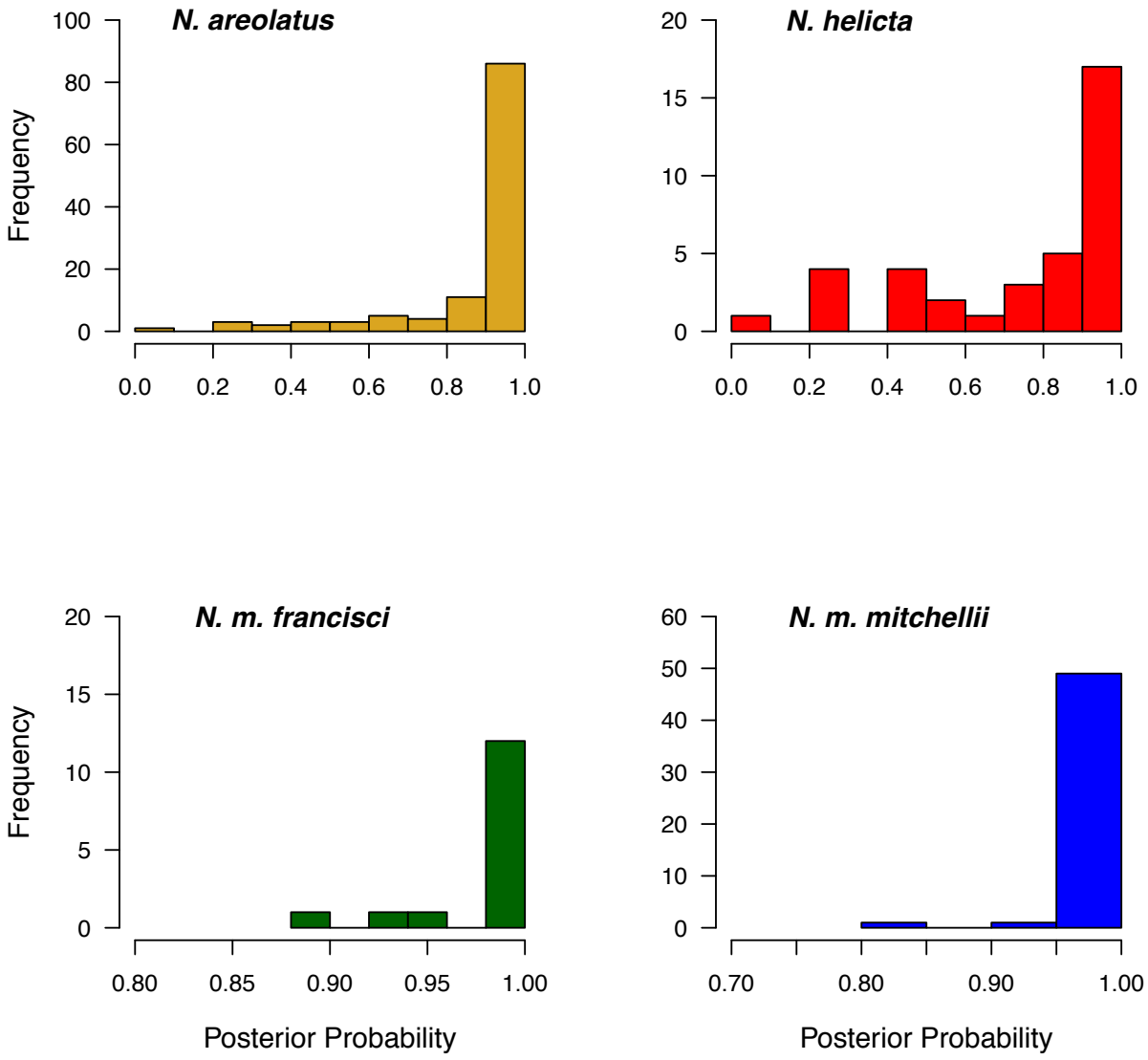

Figure 5.4. Histograms of posterior probabilities from assignment tests for *Neonympha* taxa. A "1" indicates that a particular individual was correctly assigned its taxon. A: *Neonympha areolatus*, B: *N. helicta*, C: *N. mitchellii francisci*, D: *N. m. mitchellii*.

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CHAPTER 6

SUMMARY AND INSIGHT

The Mitchell's satyr butterfly, *Neonympha mitchellii mitchellii* was initially listed as an endangered species in the early 1990's under an emergency ruling because a significant number of populations had been extirpated over the course of a few decades. This butterfly lives in wetlands that seem to have had a repellent effect on lepidopterists and biologists alike, the result being that there were significant gaps in our knowledge regarding this species and has left a number of unanswered questions. Chief among these questions was "what is the Mitchell's satyr?" Since its description in 1889, the Mitchell's satyr was known to occur in Michigan, Indiana, Wisconsin, Ohio, New Jersey and likely Maryland (OH, WI, NJ and MD were extirpated by 1988). In recent years, populations of a similar looking butterfly were found in North Carolina, Alabama, Mississippi, and Virginia. Though initial attempts were made to address their identity little progress was made apart from determining they were *N. mitchellii*.

The first step to address the question of identity was to examine the relevant literature concerning *N. m. mitchellii*. I found a wide range of reports from non-profit agencies, governmental departments, articles from Lepidoptera enthusiasts, and the peer reviewed literature, some of which required significant effort to acquire. Previous works on the natural history of the Mitchell's satyr needed to be updated, and so I synthesize and updated the natural history of this butterfly, incorporating these data into a single manuscript. In the course of this research I found that a number of the commonly held beliefs about the Mitchell's satyr, which were prevalent among conservation workers, were not based on solid evidence. For example, many workers reported that the butterfly would only oviposit and feed on *Carex stricta*, which is not the case. In fact, the Mitchell's satyr will oviposit and feed on a range of Cyperaceae and Poaceae. In fact, preliminary feeding experiments on *N. mitchellii* from different regions revealed no performance differences for larvae reared on different species of *Carex*, including

plants not native to the areas the larvae were collected from. Many of the experiments were preliminary but were aimed to demonstrate that the butterfly could be reared in captivity and that it was amenable to experimentation.

I decided to use molecular genetics techniques to examine difference among the regions in which *N. mitchellii* was found. In order to conduct the genetic analysis I would have to acquire DNA, which, in butterfly studies, was normally taken from whole specimens collected in the field. However, the very fact that the Mitchell's satyr was endangered would limit my ability to conduct research on it. A method that allowed for large sample size collection without unduly harming the butterfly was needed. While conducting my review of the relevant literature on sampling techniques I realized that no study had examined the effect of non-lethal sampling (wherein enough tissue was collected for genetic analysis) on the survivability of butterflies. Even if a sampling technique did not kill the butterfly, if the procedure lowered their chances for survival it would ultimately have the same effect. I conducted field and greenhouse experiments that demonstrated that removing a small piece of wing from a butterfly did not affect its survival. As a consequence of this research I was given state and federal permits to acquire genetic material from an endangered species.

With the DNA in hand I was able to determine if the endangered *N. m. mitchellii* and *N. m. francisci*, the sister taxon to the Mitchell's satyr) were genetically distinct from the recently discovered *N. mitchellii* from Alabama, Mississippi, and Virginia. Using a suite of molecular markers, many of which were uniquely tailored for this research, I determined that the two endangered species, *N. m. mitchellii* and *N. m. francisci*, were genetically distinct from the recently discovered populations in Alabama, Mississippi, and Virginia. This was an important discovery because the taxonomic status of these populations would have impacted the recovery

status of the endangered species. In fact, these recently discovered populations were being treated as endangered (despite a lack of evidence).

During the genetic analysis I became aware of the importance that the reproductive parasite *Wolbachia* may play in insect conservation. This bacterium manipulates its hosts' reproduction to facilitate its own, and provides a fitness benefit to infected individuals who can successfully mate only with similarly infected individuals. Because of this reproductive asymmetry the infected population undergoes a bottleneck as the infection spreads to fixation. Movement of infected individuals into uninfected populations could cause a bottleneck. This bacterium is present in the Mitchell's satyr, though at low to moderate levels, and appears fixed in the St. Francis' satyr. I surveyed additional Lepidoptera of conservation concern for *Wolbachia* and found that it is present in many of them, though at unknown frequencies. These data suggest that insects of conservation concern be screened for reproductive parasites before translocation be considered.

As I conducted my research on *N. m. mitchellii* I became interested with the taxonomy of the genus *Neonympha*. Most of the species descriptions were based on small sample sizes and characters that seemed ambiguous. Descriptions of the width or curvature of pigment bands on the hind wing were often cited as differences among species. These authors seemed to take continuous variables and convert them to discrete variables without quantifying any differences. I applied geometric morphometrics and multivariate statistics to the problem and found that, once again, both endangered species (*N. m. mitchellii* and *N. m. francisci*) were distinct from their congeners, yet the remaining congeners (*N. areolatus* and *N. helicta*) could not readily be distinguished from one another. Historically, *N. areolatus* and *N. helicta* had been classified as the same species, or subspecies within the same taxon, so this was not surprising.

After five years of working full time on *N. m. mitchellii* I have learned a great deal but recognize that there are still significant research needs that must be filled. The first and most pressing need is for evidence-based conservation. I sincerely hope that the natural history synthesis is a first step towards correcting this. Along these lines, the need for fundamentally sound research into the biology of this butterfly is paramount. One important step towards this goal is collaboration between academic researchers and conservation workers. To this end, I hope that my work will demonstrate the value of academic collaboration and the value in cost this represents. When a graduate student works on a project they are fully committed to it, whereas many conservation workers are spread too thin and cannot dedicate themselves exclusively to one task.

The goal of the Endangered Species Act is the recovery of species that are threatened with extinction. The goal is recovery. Recovery requires action, and inaction will kill endangered species. The few species that have ever been removed from the endangered species list have had significant, focused, and proactive recovery efforts guided by rigorous science. In order for the Mitchell's satyr to recover and thrive similar efforts are needed immediately.

Significant research efforts are needed to investigate: the phenotype of *Wolbachia* and its prevalence in *N. m. mitchellii*; the impact of global climate change on larval and host plant phenology; the suitability of northern fens as refugia; the state of the groundwater that feeds prairie fens in Michigan and Indiana. For the Mitchell's satyr to recover a significant investment must be made by the conservation community, academic researchers, and politicians. This investment must include rigorous research that leads directly to evidence based conservation. If this investment is made soon then I feel that the Mitchell's satyr has a future, if not I fear it is doomed to extinction.