Life history of an endangered prairie butterfly: Insights from an *ex situ* population of Poweshiek skipperling in Manitoba, Canada

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ABSTRACT

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Species recovery plans benefit from a complete understanding of an organism's biology. The Poweshiek skipperling, *Oarisma poweshiek* (Parker) (Lepidoptera: Hesperiidae), is an endangered butterfly native to tallgrass prairies in North America. Following rapid, range-wide population crashes, Poweshiek are now reared *ex situ* in Manitoba, Canada to augment the remaining wild population. Although this species has been the focus of research since their "Endangered" listing in 2014, there are knowledge gaps regarding Poweshiek skipperling life history. I measured head capsule widths of larvae of two Poweshiek cohorts to determine the overwintering instar. I analysed six years of *ex situ* data to assess trends in the timing of flight period, degree day accumulations, and protandry. I estimated this species' lower developmental threshold using dates for developmental milestones. Head capsule measurements showed that Poweshiek typically overwinter in the fourth instar. Peak flight period varied among years by up to three weeks, while degree day accumulations were similar between years. I found evidence that this species is protandrous. An information theoretical approach using Akaike Information Criterion was used to assess lower developmental thresholds. This approach indicated different lower developmental thresholds for larvae and pupae; the AICc scores also demonstrated that larvae enter winter dormancy at different stages of development. These findings will inform conservation decision-making and may allow for greater accuracy in predicting the adult flight period.

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“For those of you who think you don’t belong in science – stay. Stay, and make your contribution.” (Dr. Esther Ngumbi)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The purpose of this study is to broaden our understanding of the endangered Poweshiek skipperling's growth and development in Manitoba, Canada. My thesis is sectioned into four parts, beginning with a literature review (Chapter One) followed by research manuscripts on Poweshiek larval growth (Chapter Two) and predicting adult flight period (Chapter Three), and ending with general conclusions and implications of my research (Chapter Four). In Chapter Two, I monitor the development of two cohorts of *ex situ* Poweshiek larvae at Assiniboine Park Zoo in Winnipeg, Manitoba. Detailed data are available for all individuals in the *ex situ* population, including dates for egg hatch, overwintering, pupation, and eclosion. In Chapter Three, I use these data to examine trends in adult emergence from 2018 to 2022, and to estimate the species' lower developmental threshold.

The objectives of this thesis are to:

i. document the number of instars, overwintering instar, and stage-specific degree day requirements of *ex situ* Poweshiek larvae,
ii. summarise past trends in adult emergence by year and sex, and
iii. estimate the lower developmental threshold to improve annual emergence predictions

Using the published literature reviewed in Chapter One, I predict that Poweshiek in Manitoba overwinter as fourth instars, and that degree day requirements will increase through successive instars. I also predict that adult emergence dates will vary according to annual weather variation. Lastly, I predict that the lower developmental threshold will vary by developmental stage, with larvae having a lower threshold compared to pupae.

CHAPTER 1: LITERATURE REVIEW

1.1 Butterfly conservation

The butterflies (Lepidoptera: Papilionoidea) as a group comprise more than 18,000 described species and are among the most popular and thoroughly studied insects (Heikkila *et al.* 2012). Due to their abundance, relative ease in rearing, and short generation times, butterflies are very popular model organisms and have been used to investigate concepts such as sexual selection (Fagerström and Wiklund 1982; Nylin *et al.* 1993; Zwaan *et al.* 2008), responses to climate change (Altermatt 2010; Kerr *et al.* 2020; Fitchett *et al.* 2022), and evolution (Cong *et al.* 2015; Usami *et al.* 2021). The phylogeny of family-level relationships within Papilionoidea has long been the subject of debate, with Hesperiidae ("skippers") and Hedylidae ("nocturnal butterflies") formerly placed in separate superfamilies due to their unique morphology and life history traits (Scott 1984; Beck and Fiedler 2009). Compared to other butterfly families, adult hesperiids have larger eyes, stockier bodies, a unique basking posture, and hooked antennae (Scott 1984; Cong *et al.* 2015). In addition, the hesperiid flight style is characterised by rapid wing beats and it is from this that they derive their common name, "skippers" (Cong *et al.* 2015). Despite these differences, phylogenomic studies have demonstrated strong support for the inclusion of both Hesperiidae and Hedylidae within Papilionoidea alongside the other butterfly families: Nymphalidae, Papilionidae, Lycaenidae, Riodinidae, and Pieridae (Heikkila *et al.* 2012; Kawahara and Breinholt 2014; Espeland *et al.* 2018).

Butterflies have widespread appeal and have been aptly described as "charismatic microfauna" (Daniels *et al.* 2015). Popular flagship species, such as the Monarch butterfly, *Danaus plexippus* (Linnaeus, 1758) (Nymphalidae) encourage public engagement in conservation actions and can draw attention to broader conservation issues (New 1997; Preston *et al.* 2021). Despite their popularity in the public sphere and their use in scientific research, butterflies, like many other insect groups, are currently experiencing global declines in abundance and diversity (Thomas *et al*. 2009; Wagner 2020; Forister *et al*. 2021). Univoltine species (i.e. those that produce a single generation per year) and habitat specialists have shown particularly negative population trends (Wagner 2020; Wepprich *et al*. 2019). These trends are often linked to the degradation and fragmentation of suitable habitat (Beyer and Schultz 2010), as is the case for butterflies in prairie-oak regions on the Pacific coast (Schultz *et al*. 2011) and tallgrass prairies in the Midwest (Swengel and Swengel 2015; Dupont-Morozoff *et al*. 2022; Post van der Burg *et al*. 2023).

Among the invertebrates, butterflies receive the most attention from conservationists (New 1997). While conservation programs focus first and foremost on habitat restoration and other *in situ* actions (Schultz *et al.* 2008), *ex situ* management may be necessary for species that require more significant interventions to recover (Crone *et al.* 2007; Grow *et al*. 2015). *Ex situ* management is a widely used conservation strategy to slow or reverse populations declines, and usually involves rearing in human care, along with reintroductions and/or translocations (Schultz *et al.* 2008; McGowan *et al*. 2017). Due in part to the widespread appeal of their subjects, butterfly *ex situ* rearing programs have become an increasingly popular conservation strategy (Daniels *et al*. 2015).

Successful conservation of endangered butterflies relies on detailed knowledge of their biology and behaviour (Schultz *et al*. 2011; Thomas *et al*. 2009; Demarse *et al.* 2023). This information is important for maintaining suitable habitat *in situ* (Daniels *et al*. 2015), and it is also crucial for *ex situ* rearing programs to meet the species' particular needs. For example, preliminary efforts in *ex situ* rearing of threatened Taylor's checkerspot, *Euphydryas editha* *taylori* (W.H. Edwards, 1888) (Nymphalidae) involved identifying host plants and suitable diapause conditions (Schultz *et al*. 2011). The absence of species-specific information can reduce the effectiveness of conservation actions. For instance, a lack of understanding of the larval development and eclosion timing has hindered endangered Mardon skipper, *Polites mardon* (W.H. Edwards, 1881) (Hesperiidae) *ex situ* rearing efforts (Hatfield *et al*. 2015). It is therefore important to address key knowledge gaps with empirical data to inform conservation decision-making (Grow *et al*. 2015).

Gathering detailed information on rare species in the field can be difficult due to the low abundance of individuals (Lu and Samways 2001). Butterfly larvae may be especially challenging to locate, as they are generally much less conspicuous than the winged adults. Consequently, the immature stages of butterflies tend to be understudied compared to the adults (Klockmann and Fischer 2017). In cases where *in situ* studies are not feasible, insights into an organism's biology can be obtained through studying *ex situ* populations (Lu and Samways 2001; Koi and Daniels 2015; Williams *et al*. 2023). Given that most butterflies, especially those that undergo winter dormancy, spend most of their life as larvae (Dennis 2020) research focused on the growth and development of the larval stage is critical.

1.2 Insect growth

All insects that undergo complete metamorphosis, including butterflies, have four major life stages: egg, larva, pupa, and adult (Layberry *et al.* 1998). The larval stage is further subdivided into a number of instars. Many butterfly larvae develop through five instars (Minno 1994; Layberry *et al.* 1998), but intraspecific variation in instar number can occur due to host plant quality (Penz and Araújo 1991; Abarca *et al.* 2020), sexual dimorphism (Esperk *et al.*

2007a), or photoperiod (Garcia-Barros 2006). Instar durations typically increase with each successive instar (Hutchinson *et al.* 1997; Du Plessis *et al.* 2020). Each instar is followed by a short period of moulting, also known as ecdysis, with the final moult resulting in the beginning of the pupal stage (Layberry *et al.* 1998). In order for insects to grow, the thin cuticle that covers the head and body of the insect must be shed. The cuticle covering the body is somewhat flexible, while the cuticle on the head and mouthparts is rigid, or sclerotized (Hutchinson *et al.* 1997). Due to the size limitations imposed by their sclerotized cuticle, insect larvae exhibit stepwise growth, meaning that while their mass increases throughout an instar, the size of their head capsule only increases during ecdysis (Gaines and Campbell 1935; Hutchinson *et al.* 1997).

Larval age can be determined by measuring the width of the head capsule and assigning it to an instar, based on the regular size progression documented in many insect species (Dyar 1890; Calvo and Molina 2008). Dyar's rule describes the geometric progression in larval head capsule widths, wherein the width of the head capsule increases by a species-specific, constant ratio over the course of the larval stage (Dyar 1890; Hutchinson *et al.* 1997; Wu *et al.* 2013). Dyar's rule has been used to determine whether all instars are represented in a sample, such as when insects have been collected from the field without prior knowledge of the species' instar number (Logan *et al.* 1998; Calvo and Molina 2008). Support for Dyar's rule is variable, with some insects reportedly adhering to it (Godin *et al*. 2002; Richardi *et al.* 2013) while others do not (Gaines and Campbell 1935; Jobin *et al.* 1992; Hutchinson *et al.* 1997). However, if head capsule widths for each instar form non-overlapping (or minimally overlapping) distributions, larvae can be sorted into the correct instar regardless of whether Dyar's rule is obeyed (Gaines and Campbell 1935; Castañeda-Vildózola *et al.* 2016).

Insect growth and development is highly temperature dependent. As ectotherms, insects' internal temperature typically adheres to that of their environment (Tauber *et al.* 1986). Consequently, the annual cycles in environmental conditions characteristic of temperate regions cause a variety of seasonal responses in insects, with the most common being dormancy (Tauber *et al.* 1986; Belozerov 2009). Dormancy, an umbrella term that encompasses both winter diapause and non-diapause quiescence, is defined as the suppression of development to escape adverse environmental conditions (Tauber *et al.* 1986; Danks 1987). "True" diapause is a physiologically controlled response that begins before adverse conditions commence and continues for a period of time even after conditions become favourable (Tauber *et al.* 1986; Danks 1987). In contrast, quiescence is a direct and immediate response that is mediated by environmental cues (i.e. temperature and photoperiod) but is quickly reversible when conditions improve (Danks 1987; Belozerov 2009). Determining whether an insect undergoes diapause requires monitoring hormone levels (Tauber *et al.* 1986; Ragland *et al.* 2019) or analysing gene expression (Fremdt *et al.* 2014).

Temperature is one of the parameters that influences many insect life history traits, including the termination of diapause, the timing of the adult flight period, and the number of generations per year, also known as voltinism (Danks 1987; Altermatt 2010; Roy *et al*. 2015; Kerr *et al*. 2020). For example, adult insect emergence can vary widely depending on annual variation in temperature, with earlier emergence during warmer years (Cayton *et al.* 2015; Demarse *et al.* 2023). It is therefore important to consider a species' thermal requirements when building phenological models (Rebaudo and Rabhi 2018). Insect phenological models have many applications, such as managing pest outbreaks (Soroka *et al*. 2020), predicting voltinism under climate change (Altermatt 2010; Kerr *et al.* 2020), and assessing geographic patterns in local adaptations to climate (Roy *et al.* 2015). Accurately predicting adult flight period is also of interest when surveying for endangered insect species (Dearborn and Westwood 2014; Demarse *et al.* 2023).

Insect development largely occurs over the range of temperatures between their upper and lower developmental thresholds, with the highest growth rate occurring at a species-specific optimal temperature (Allen 1976; Koda and Nakamura 2012; Davídková and Dolezal 2019). At "moderate" temperatures, the rate of development is proportional to temperature; however, this relationship becomes non-linear as temperature approaches the species-specific upper or lower thresholds (Collier and Finch 1985; Lamb 1992). The lower developmental threshold (LDT) is typically determined experimentally, by rearing larvae at a range of constant temperatures, monitoring their growth rate, and using linear regression to extrapolate the temperature value at which the developmental rate is predicted to be zero (Sanborn *et al.* 1982; Davídková and Dolezal 2019). However, Lamb (1992) questioned the validity of this approach due to the non-linearity of the relationship between temperature and development at very low and very high temperatures. Many non-linear methods for modeling temperature-dependent development have been developed (e.g. Lactin *et al.* 1995; Briere *et al.* 1999).

Since insect growth is temperature-dependent, development is best described by units of physiological, rather than chronological, time (Allen 1976; Caicedo *et al*. 2012). Degree days are a widely used method of quantifying the amount of heat accumulated in a 24 h period when the ambient temperature is 1 °C above the insect's lower developmental threshold (Wu *et al.* 2015). Each insect life stage requires a certain number of degree days for completion, but the relationship between growth and temperature is not always constant within a species (Wu *et al*. 2015; Rebaudo and Rabhi 2018). For instance, it is common for the lower developmental threshold to vary by instar (Wardlaw *et al.* 2022) and by geographical region as an adaptation to local climate (Lamb 1992; Rebaudo and Rabhi 2018). Despite the non-linear relationship between development and temperature at extreme temperatures, linear methods for estimating degree days are most common for practical applications (Caicedo *et al.* 2012; Rebaudo and Rabhi 2018).

Head capsule measurements are sometimes used in conjunction with degree day estimates to monitor larval growth (Wardlaw *et al.* 2022). In most cases head capsule measurements are made on deceased larvae; however, measurements are also possible without the need for destructive sampling (Garcia-Barros 2006; Calvo and Molina 2008). Both degree day estimation and head capsule measurements are simple, non-invasive techniques, yet they can be highly effective for gaining insight into insect phenology. For example, head capsule measurements have been used to investigate plasticity in instar number (Garcia-Barros 2006; Abarca *et al*. 2020). Degree day models are widely used to predict the emergence of pest species (Soroka *et al.* 2020; Rebaudo and Rabhi 2018), but they have also been used to investigate changes in butterfly emergence over time (Westwood and Blair 2010). In addition, the flight period of habitat specialists may be easier to predict via the degree day method than species with more ecological flexibility (Cayton *et al.* 2015). The non-invasive nature of head capsule measurements and degree day modeling makes these methods well suited to studying endangered insects.

1.3 Introduction to Poweshiek skipperling

The species-rich butterfly subfamily, Hesperiinae (Hesperiidae), also known as the "grass skippers", contains at least 35 species-at-risk (Beyer and Schultz 2010; Cong *et al.* 2015).

Included in this group is the Poweshiek skipperling, *Oarisma poweshiek* (Parker, 1870) (Figure 1) , an endangered, univoltine butterfly endemic to tallgrass prairie and prairie fens (COSEWIC 2014). Since at least the early-2000s, the Poweshiek skipperling has undergone rapid, range-wide population declines and is consequently at high risk of extinction throughout their range (USFWS 2014; COSEWIC 2014). The reasons for their decline are uncertain, but it is likely that habitat loss and habitat degradation are contributing factors (Swengel and Swengel 2015; Saarinen *et al.* 2016; Belitz *et al.* 2020).

More than 160 million ha of tallgrass prairies once extended across the Great Plains in North America, but nearly all of it was cleared for urbanization or converted to agricultural land by the mid-20th century (Samson and Knopf 1994; Belitz *et al.* 2020). Less than 1% of native tallgrass prairie remains in Canada (Dupont-Morozoff *et al.* 2022). More recent threats to the tallgrass prairie include the encroachment of woody shrubs due to the absence of regular disturbance from fires and grazing (Collins and Calabrese 2012; Henderson *et al.* 2018). The remaining tallgrass prairie is highly fragmented, yet it is a diverse ecosystem that provides critical habitat for Poweshiek skipperling and other rare endemics, such as the regal fritillary, *Argynnis idalia* (Drury, 1773) (Nymphalidae) (Henderson *et al.* 2018) and the western prairie fringed orchid, *Platanthera praeclara* Sheviak & M.L. Bowles (Ross and Travers 2015).

Poweshiek were once found in at least nine U.S. states, primarily in Minnesota, Iowa, and South Dakota (USFWS 2014). Manitoba is the sole Canadian province included in Poweshiek's historical range (COSEWIC 2014). Currently, Poweshiek skipperling is restricted to remnant prairie patches and the two remaining populations, located in Manitoba and Michigan, are small (< 1000 individuals combined) and separated by more than 1000 km (COSEWIC 2014; Belitz *et al.* 2020; Dupont-Morozoff *et al.* 2022). The importance of large, continuous tracts of prairie is reflected in the historically high levels of gene flow between the extant Poweshiek populations, despite their current geographical isolation (Saarinen *et al.* 2016).

Figure 1. Adult Poweshiek skipperling at Tall Grass Prairie Preserve, 80 km southeast of Winnipeg, Manitoba. Photo by Kirstyn Eckhardt.

Following the Poweshiek skipperling's "Endangered" listing in 2014 (USFWS 2014; COSEWIC 2014), international efforts commenced to prevent the extinction of the species. Annual surveys for Poweshiek are conducted at the remaining populated sites within the Tall Grass Prairie Preserve (TGPP) (49.15°, -96.72°) in southeastern Manitoba (Dearborn and Westwood 2022; Burns *et al.* 2022). In addition, *ex situ* rearing programs were established by Minnesota Zoo and Assiniboine Park Zoo (APZ) (49.87, -97.24) in Winnipeg, Manitoba (NCC 2021). The *ex situ* program at APZ focuses primarily on head-starting, a conservation strategy in which eggs are collected from wild female Poweshiek and the resulting offspring are reared through their year-long life cycle. Late-stage pupae are returned to the field to eclose (i.e. emerge from their chrysalids) before the flight period, which is in late June and early July (Assiniboine

Park Zoo 2024). Between 2018 and 2023, the *ex situ* program at APZ has successfully reared and released over 300 adult Poweshiek (L. Burns, pers. comm.) and greater numbers of Poweshiek have been recorded in TGPP sites with population augmentation compared to control sites (Burns *et al.* 2022).

Like other grass skippers, Poweshiek larvae feed on several species of prairie grasses (Poaceae) (Henault and Westwood 2022) and are cryptically coloured (Figure 2) (Layberry *et al.* 1998; Beyer and Schultz 2010). Hesperiinae in general are challenging to locate *in situ* due to their camouflage and small size (Layberry *et al.* 1998). Poweshiek larvae are nearly impossible to locate in large numbers *in situ* due to their rarity and small size, combined with the fact that (unlike many other Hesperiinae) they do not construct conspicuous leaf shelters (Minno 1994; Belitz *et al.* 2019). Henault and Westwood (2022) documented the degree day requirements of several Poweshiek instars *in situ* and hypothesized that the fourth instar overwintered. However, their sample size was small (n=3), and they were unable to detect larvae after the third instar (Henault and Westwood 2022).

Figure 2. *Ex situ* Poweshiek skipperling larva reared at Assiniboine Park Zoo in Winnipeg, Manitoba. Mature larvae, as pictured here, are approximately 2 cm long. Photo by Kirstyn Eckhardt.

Information on Poweshiek skipperling life history is currently incomplete. Poweshiek skipperling spend roughly 11 months as larvae (COSEWIC 2014), yet there is limited information regarding their growth and development. The Canadian population has received less study compared to southern populations located in the midwestern United States (Dupont-Morozoff *et al.* 2022). Geographically isolated insect populations often show differences in their phenology (Scriber 1994), as well as their instar number and their overwintering stage (Verspagen *et al.* 2023). If geographic differences exist for the remaining two Poweshiek populations, then the information available for the U.S. population may not apply to the Canadian population.

My research aims to fill in knowledge gaps surrounding Poweshiek growth and development in Canada, with the intention that this information will contribute to the ongoing recovery efforts.

CHAPTER TWO: OVERWINTERING INSTAR AND DEGREE DAY REQUIREMENTS OF AN *EX SITU* POPULATION OF POWESHIEK SKIPPERLING BUTTERFLY

2.1 Abstract

The Poweshiek skipperling, *Oarisma poweshiek* (Parker, 1870), is an endangered, univoltine grassland butterfly with extant populations in Michigan, United States and Manitoba, Canada. Larvae of this species are cryptic and have proven challenging to observe *in situ*. Features of Poweshiek life history, including the overwintering instar and stage-specific degree day requirements, have not yet been documented in Manitoba. In this study, I used macro photography to measure head capsule widths of Poweshiek skipperling larvae reared *ex situ* at Assiniboine Park Zoo in Winnipeg, Manitoba. With one exception, all reared Poweshiek skipperling larvae overwintered in the fourth instar and completed a total of six instars. I produced degree day estimates for each instar, as well as overall accumulations for pre- and post-overwintering immature stages. My results address knowledge gaps related to Poweshiek biology that will benefit ongoing *ex situ* rearing efforts. In addition, the non-invasive methods presented here could be adapted for studying the growth and development of other endangered insects.

2.2 Introduction

Rare species can be challenging to study in the wild due to their small populations and consequently low detectability (Williams *et al.* 2023). Researching animals managed in human care can provide insights into the lives of cryptic species. Zoos may assist in the conservation of species-at-risk using a variety of methods, including managed breeding, population monitoring, habitat restoration, and through the development of species recovery plans (Olive and Jansen 2017). *Ex situ* conservation programs are one practice that zoos have adopted in response to the growing number of species threatened with extinction (McGrath *et al.* 2017). Zoos provide unique opportunities to study endangered species in captivity and fill in knowledge gaps surrounding species' behavioural patterns (Williams *et al.* 2023), reproductive physiology (Durgavich *et al.* 2023), and food preferences (McGrath *et al.* 2017). This detailed information on biology and life history is critical for species-at-risk recovery planning (SARA 2002).

The Poweshiek skipperling, *Oarisma poweshiek* (Parker, 1870) (Lepidoptera: Hesperiidae) is a small butterfly native to tallgrass prairie and prairie fens (COSEWIC 2014). Once common across the midwestern United States and in southeastern Manitoba, Canada, populations have sharply declined and now fewer than 1000 mature individuals are thought to exist globally (Royer 2020). As a result, the Poweshiek skipperling is listed as "Critically Imperiled" globally (NatureServe 2013) and designated as "Endangered" in both the United States and Canada (USFWS 2014; COSEWIC 2014). Currently, isolated populations exist in Michigan, USA and Manitoba, Canada (Belitz *et al.* 2020). In the ten years since their "Endangered" listing (COSEWIC 2014), research has been conducted on Poweshiek skipperling habitat requirements (Pogue *et al.* 2016; Westwood *et al.* 2020; Dupont-Morozoff *et al.* 2022), host plant preferences (Henault and Westwood 2022), and flight period (Dearborn and Westwood 2014). However, crucial aspects of Poweshiek biology and life history remain unknown, in part due to the cryptic nature of the larvae (Westwood *et al.* 2020).

Poweshiek are univoltine, spending more than 11 months as immatures before emerging as adults in late June or July (COSEWIC 2014). Partially grown larvae are believed to overwinter under the snowpack near the base of prairie grasses from late September through late

May (Henault and Westwood 2022). It has been assumed that Poweshiek overwinter in the fifth instar (Belitz *et al.* 2020; COSEWIC 2014). This assumption is based solely on an account by McAlpine (1972), who collected Poweshiek eggs and reared two larvae in his home near Grand Rapids, Michigan nearly thirty years prior to publishing his account. McAlpine (1972) observed seven instars for Poweshiek, noting that the fifth instar was inactive outdoors from September to April. However, deviations from this timeline may be found for the Manitoba population due to temperature and regional climate. Henault and Westwood (2022) hypothesized that Poweshiek in Manitoba overwinter in the fourth instar, due to the limited number of growing degree days remaining in the autumn. They were unable to test this theory, as no larvae could be located later in the autumn (Henault and Westwood 2022).

Insect growth is highly temperature-dependent, with development generally accelerating as temperature increases (Allen 1976). Other factors, including food quality and humidity can influence growth rate, but these are typically minor in comparison to the effect of temperature (Wu *et al.* 2015). The relationship between insect growth and temperature is linear only at "moderate" temperatures between the insect's upper and lower developmental thresholds (Lamb 1992). Development is non-linear at temperatures above and below the species-specific maximum and minimum thresholds (Lamb 1992). Although growth is non-linear at extreme temperatures, linear models are still frequently used to predict insect development due to their comparative simplicity over non-linear models (Rebaudo and Rabhi 2018).

Degree days are a common method of measuring developmental time in insects (Wu *et al.* 2015). Degree day modeling predicts physiological events, such as adult insect emergence, better than chronological time due to the relationship between insect growth and temperature (Caicedo *et al.* 2012). Each insect life stage requires a certain number of degree days for completion (Rebaudo and Rabhi 2018). Degree days can be calculated following several models, including triangle methods and sine waves (Caicedo *et al.* 2012). All these approaches assume a linear relationship between temperature and developmental rate (University of California Natural Resources 2012). One popular technique is the double-sine method, which fits a sine wave to the daily maximum and minimum temperatures and then calculates the area under the curve (Allen 1976). While degree day estimates are widely used in pest management (Régnier *et al.* 2022), they have also been used to predict the flight period of endangered butterflies, including Poweshiek skipperling (Dearborn and Westwood 2014). Degree day estimates have been calculated for the first three Poweshiek skipperling instars *in situ* (Henault and Westwood 2022), but the degree day requirements for the other instars are unknown.

I aim to address knowledge gaps regarding the larval stages of Poweshiek skipperling in Manitoba. While observations of larvae in the field are possible (Henault and Westwood 2022), Poweshiek larvae are small (< 2 cm long) and extremely well-camouflaged, making it challenging and incredibly time-consuming to study larvae in the field. In 2017, Assiniboine Park Zoo (APZ) in Winnipeg, Manitoba joined an international *ex situ* rearing program to prevent the extinction of the butterfly (NCC 2021). Since then, APZ has reared, bred, and released adult Poweshiek back into the wild to augment natural populations in Manitoba (NCC 2021). The *ex situ* rearing program provides an opportunity for an intimate look into the cryptic larval stage of this insect. My objectives here are to: 1) investigate the overwintering instar, and 2) determine the degree day requirements for each instar.

2.3 Methods

2.3.1 Study population, rearing protocol at APZ

Poweshiek skipperling reared at Assiniboine Park Zoo (APZ) in Winnipeg, Manitoba (49.87°, -97.24°) are each given a unique identifier to differentiate individuals. Since this species is univoltine, hatch year is one year before the year the adults are released (e.g., the 2021 cohort was released in 2022). To simulate winter conditions larvae would experience under snowpack, overwintering larvae are maintained in an Isotemp incubator at -4 °C from approximately mid-October until early May. In the spring, larvae are transferred to individual potted grasses and moved to an outdoor hoop house (Figure 3). Each potted grass is fitted with a wire frame and covered by a thin, beige nylon sock. The hoop house is in at least partial shade for most of the day, and the mesh walls and ceiling allow wind and precipitation to enter.

Figure 3. *Ex situ* rearing hoop house at Assiniboine Park Zoo. Each potted grass contains a Poweshiek larva.

Larvae pupate in mid-June, after which most late-stage pupae are transported to the Tall Grass Prairie Preserve (TGPP) in southeastern Manitoba (49.15°, -96.72°) and eclose in the field.

Some adult butterflies are retained at the zoo for breeding purposes. Eggs are collected from both wild-caught butterflies and *ex situ* individuals, and hatch in July. Larvae are reared on prairie dropseed, *Sporobolus heterolepsis* G. (Poaceae), a native prairie grass. Further details on the *ex situ* rearing protocol can be found in Assiniboine Park Zoo (2024).

My data collection began in May 2022, when the 2021 cohort was removed from overwintering chambers at APZ. I monitored a subsample of 25 post-overwintering larvae to adult eclosion. I then selected another subsample (n=25) from the 2022 cohort when the eggs hatched in July. I followed the 2022 cohort through their entire life cycle.

2.3.2 Head capsule measurements

Width of the head capsule is a better predictor of larval instar than body length (Calvo and Molina 2008). Head capsule measurements were used to assess 1) the overwintering instar and 2) the duration of each instar, which is important for degree day calculations. In addition to measurements, the presence of exuviae or shed head capsules provided further evidence a moult had occurred. Green plastic plant trays were installed around the base of the grass to facilitate locating exuviae.

Over the course of the larval stage, I monitored larvae for major changes in head capsule width that would be indicative of a new instar. Small changes in width within an instar were assumed to be due to human error rather than growth, as head size within an instar is constant (Dyar 1890). My measurement error rates were typically less than 1% and did not exceed 3%. Increases in head capsule width greater than 10% were indicative of a new instar. Head capsule width measurements were tested for adherence to Dyar's rule, which states there is a tendency for geometric progression in head capsule widths between instars, assuming that all instars are present in a sample (Dyar 1890). Dyar's rule can be tested by taking the mean width of any given instar and dividing it by the previous instar's mean width (Gaines and Campbell 1935). The resulting growth ratios are constant from one instar to the next if Dyar's rule applies.

To measure head capsule width, larvae were photographed on their grasses using a Nikon D7500 camera fitted with an 85 mm macro lens, and an external camera flash. A ruler, held parallel to the larva, served as a scale in each photograph. This procedure was adapted from Assiniboine Park Zoo (2024). Photos were analysed with ImageJ software (Schneider *et al.* 2012), where a straight line was drawn across the ruler to provide a known distance. A second line was then drawn across the widest point of the larva's head capsule (Figure 4). ImageJ then converted the width from pixels into millimetres. Multiple measurements were taken for an individual on separate days. To avoid pseudoreplication, I averaged the measurements within an instar for each larva.

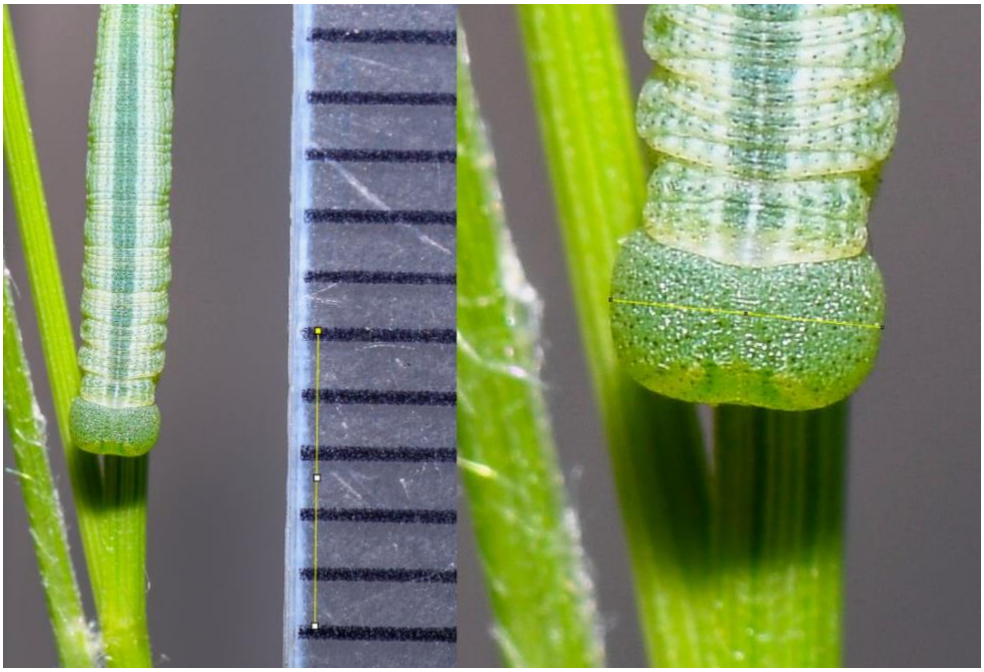

Figure 4. Head capsule measurement procedure for Poweshiek skipperling larvae reared at Assiniboine Park Zoo. Yellow lines were drawn in the computer program, ImageJ (Schneider *et al.* 2012).

During the 2022 season, larvae were photographed twice per week. In 2023, larvae were checked daily until at least the fifth instar, and then every 1–2 days until pupation. Although larvae in 2023 were checked daily, they were photographed only when evidence of a moult was detected via change in head capsule colouration or when exuvia were found in the plant trays. A head capsule measurement was then taken to confirm the larva had moulted. Due to natural mortality, or in cases where larvae were not found for several weeks in a row, the total number of larvae in my samples varied slightly. I replaced "not found" or deceased larvae with others from the *ex situ* population to retain the same or similar number of individuals. Replacement larvae were selected at random. A total of seven larvae were replaced (two individuals from cohort 2021 and five from cohort 2022). The replacement larvae were categorized into an instar based on their head capsule width; in all cases, the individual's head capsule width fell well within the expected range of a given instar. Larvae that died or were not found for > 5 weeks were excluded from analysis in the instar in which they died/were not found.

2.3.3 Degree day estimates

To estimate degree day (dd) requirements for Poweshiek skipperling, I sourced hourly temperature and relative humidity data throughout the insect's life cycle. The weather data source used to compute degree days was based on the location of the larvae. Larvae are reared at APZ in Winnipeg until pupation. Weather data were downloaded from the Richardson International Airport (RIA) weather station (49.91°, -97.24°) (ECCC 2023) while the larvae were at APZ. After pupating, larvae were transferred to release sites near Tolstoi, Manitoba to complete their development. In 2022, I downloaded data from the Emerson weather station (49.00°, -97.24°) (ECCC 2023) located 35 km west-southwest of the release sites. In 2023, I installed a HOBO U-30 NRC weather station at the Tall Grass Prairie Interpretive Centre (49.15°, -96.73°) several kilometres from the release sites to provide weather data that would more closely approximate the conditions experienced by the wild population. However, there was a significant relationship between data from the Emerson station and data from the HOBO U-30 NRC ($R^2 = 0.95$). Therefore, I computed degree days using data from the Emerson station for both cohorts.

Degree days were calculated using the degreedays function from the package "TrenchR" (v1.1.1; Buckley *et al.* 2023) using R Statistical Software (v4.2.2; R Core Team 2022) in RStudio (v2022.02.1; RStudio Team 2022). I used the double sine method, as this has been used in annual predictions of wild Poweshiek skipperling emergence (Dearborn and Westwood 2014). The double sine method requires daily maximum and minimum temperatures, as well as lower and upper developmental thresholds (University of California Natural Resources 2012). I selected 6 °C as the lower threshold and 32 °C as the upper threshold to compare my results to *in situ* degree day estimates for Poweshiek skipperling (Dearborn and Westwood 2014). On rare occasions the degree day formula would produce a "not a number" warning, due to a floating-point error within the arcsin term. To obtain the correct degree day value in those instances, I manually input the correct arcsin value using the package source code (https://rdrr.io/cran/TrenchR/src/R/DDFunctions.R).

I calculated cumulative degree days for each instar, as well as total degree days accumulated from the time larvae were removed from their overwintering chambers until adult eclosion. To test for differences in total degree day accumulation between cohorts, I conducted a two-sample *t*-test using the t.test function from the R "stats" package (R Core Team 2022).

2.4 Results

2.4.1 Pre- and post-overwintering behaviour, body colouration

Poweshiek skipperling larvae are light green with a central, longitudinal dark green stripe. One to two days before moulting, the entire head capsule became translucent, and the longitudinal body stripe no longer extended to the head capsule (Figure 5). Head colour varied from light green to brown, usually becoming brown preceding a moult. Some individuals also exhibited a brown line directly behind the head and/or a brown terminal abdominal segment. Individuals with such colouration returned to green directly after a moult, and the brown pigment was visible on the exuvia (Figure 5). Larvae that hatched in the summer ceased moulting by early to mid-September, apart from one individual that moulted on or shortly after September 23, 2022. The following spring, most larvae began moving within minutes of being removed from their overwintering chambers. However, they did not begin feeding until seven to ten days after removal from cold storage and required an average of 16 days to moult to the next (usually, fifth) instar.

Figure 5. Poweshiek skipperling larva body colouration. **a)** 1–3 d prior to moulting. The longitudinal body stripe does not extend to head capsule. This individual has brown colouration on the head and terminal abdominal segment. **b)** < 1 d after moulting. The head and terminal body segment are green, and brown pigment is visible on the exuvia.

2.4.2 Overwintering instar

Instars lasted between 9 and 15 days, apart from the overwintering instar (Table 1). The 2022 cohort hatched in July 2022 and completed instars one through three. Fourth instars were moved into overwintering chambers in mid-October. In May 2023, this cohort was removed from the overwintering chambers, completed instars four through six, and pupated in June. All larvae overwintered in the fourth instar, with the exception of P220403. This individual moulted to the fifth instar during the last week of September 2022.

Head capsule widths ranged from 0.53 mm to 2.28 mm and formed five distinct peaks (Figure 6). Head capsule widths formed separate distributions for instars one, two, five, and six, while instars three and four had slightly overlapping distributions (Figure 6). Growth ratios from one instar to the next varied between moults (Table 2). There were greater size increases in head capsule widths between early instars than between instars three and four. The greatest growth ratios were observed for instars five and six (Table 2). Due to the variable growth ratios between instars, Poweshiek larval growth was therefore inconsistent with Dyar's rule.

Larva P220403 followed a unique growth trajectory, completing seven moults in total instead of the expected six (Figure 6). Head capsule widths for instars one to four fell within the expected ranges, but the remainder of this individual's head capsule widths differed from the rest of the cohort. The head capsule widths for instars five (1.35 mm) and six (1.59 mm) were abnormally small, while the head capsule width for instar seven (2.28 mm) fell at the upper range of a "normal" instar six. Despite completing an extra, seventh instar in the spring of 2023, larva P220403 pupated (on June 12) and eclosed as a healthy adult female (on June 26) in synchrony with the rest of the 2022 cohort.

2.4.3 Degree day estimates

I calculated cumulative degree days for all instars of cohort 2022, and for the final three instars of cohort 2021. Degree day accumulations for the post-overwintering instars exclude accumulations prior to winter (e.g., the cumulative total for “pupa” includes only the post-overwintering degree day accumulations). Degree day estimates post-overwintering differed based on cohort (Figures 7–8). Post-overwintering degree day accumulations differed significantly between cohorts, $t(41) = 3.92$, $P < 0.001$, with cohort 2021 accumulating a greater number of degree days than cohort 2022. Prior to overwintering, larvae in cohort 2022 accumulated a mean of 794.7 dd (Figure 9).

Table 1. Instar durations for the 2022 cohort of Poweshiek skipperling. Larvae were checked twice weekly pre-overwintering (instars 1–4) and daily post-overwintering (instars 4–pupa).

Instar	*n*	Mean and SD instar duration (days)
1	23	10.3 ± 1.9
2	22	9.0 ± 3.6
3	23	10.2 ± 2.2
4	28	247.5 ± 18.8
5	27	9.5 ± 2.0
6	27	12.0 ± 3.7
Pupa	24	14.8 ± 0.9

Table 2. Head capsule widths of Poweshiek skipperling. Cohorts 2021 and 2022 are combined for instars 4–6.

Instar	*n*	Mean width (mm)	Range (mm)	Growth ratio
1	25	0.53	0.51–0.58	
2	24	0.71	0.66–0.75	1.3
3	24	0.93	0.86–1.01	1.3
4	55	1.07	0.98–1.26	1.2
5	54	1.48	1.36–1.65	1.4
6	52	2.11	1.98–2.25	1.4

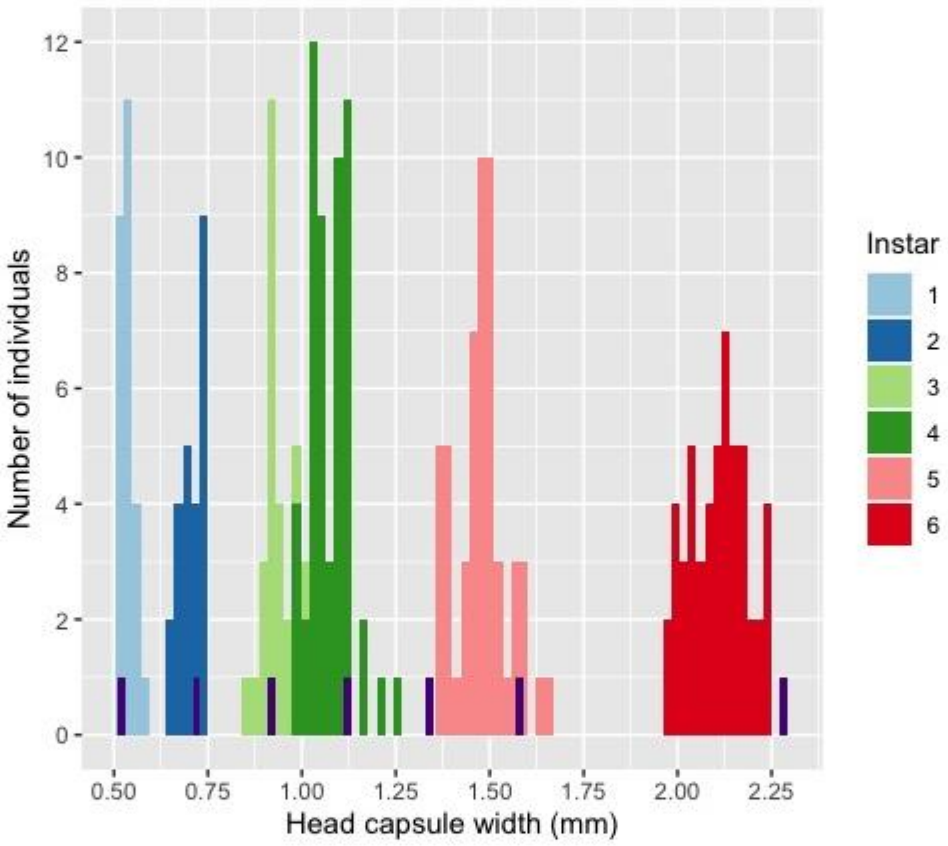

Figure 6. Frequency distribution of head capsule widths of Poweshiek skipperling. Instars 1–3 are represented by individuals from the 2022 cohort. Instars 4–6 include data from cohorts 2021 and 2022. Individual P220403 (indicated by dark purple bars) followed a seven-instar developmental pathway.

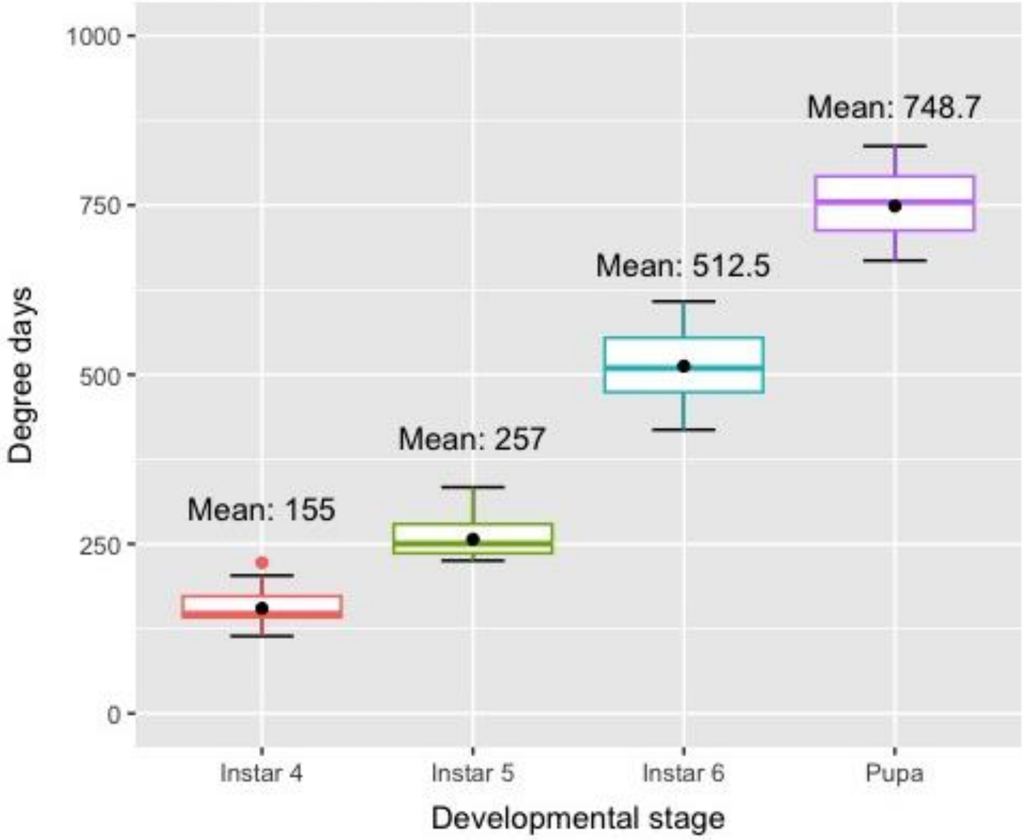

Figure 7. Cumulative post-overwintering degree days since removal from overwintering chambers for the 2021 cohort of Poweshiek skipperling. Degree days were calculated using a lower threshold of 6 °C. Instar means indicate the mean total degree day accumulation at the end of an instar. The means are calculated from cumulative degree day accumulations (e.g. the mean for instar six degree days accumulated in instars four through six).

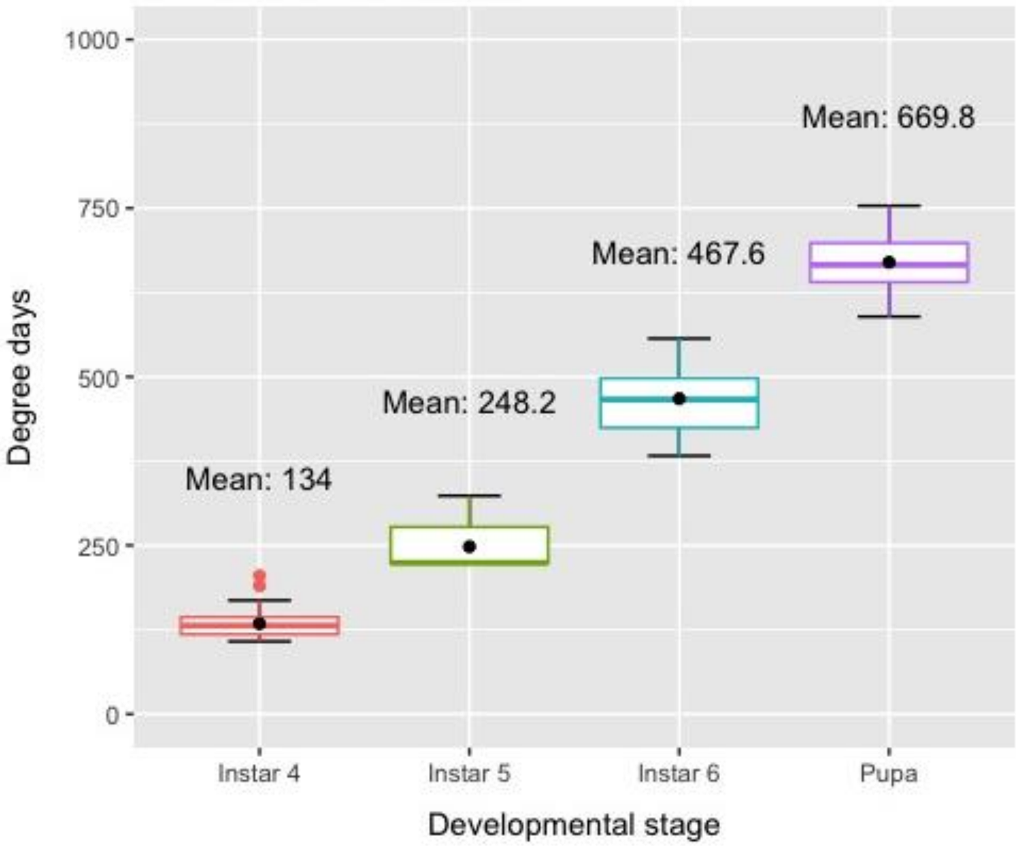

Figure 8. Cumulative post-overwintering degree days since removal from overwintering chambers for the 2022 cohort of Poweshiek skipperling. Degree days were calculated using a lower threshold of 6 °C. Instar means indicate the mean total degree day accumulation at the end of an instar. The means are calculated from cumulative degree day accumulations (e.g. the mean for instar six degree days accumulated in instars four through six).

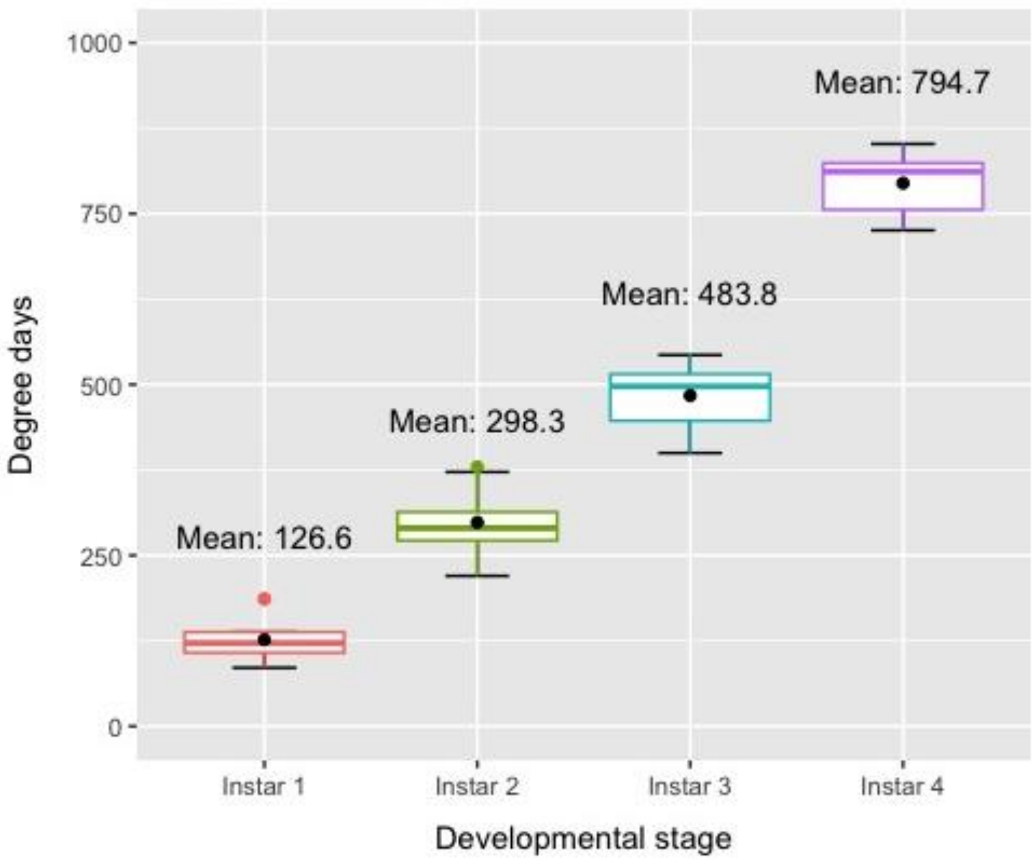

Figure 9. Cumulative pre-overwintering degree days for the 2022 cohort of Poweshiek skipperling. Degree days were calculated using a lower threshold of 6 °C. Instar means indicate the mean total degree day accumulation at the end of an instar. The means are calculated from cumulative degree day accumulations (e.g. the mean for instar six degree days accumulated in instars one through four).

2.5 Discussion

In this study, I observed *ex situ* Poweshiek skipperling larvae to address unanswered questions regarding their growth and development. By monitoring the 2022 cohort through their entire year-long life cycle, I found that larvae typically overwinter in the fourth instar. I estimated degree day requirements and documented differences in post-overwintering total degree day accumulations between the two cohorts.

I measured head capsule sizes of live larvae using macro photography. Typically, head capsule measurements involve destructive sampling in which the insect specimen is slide-mounted and measured with either an ocular micrometer or digital photography (Gaines and Campbell 1935; Richardi *et al.* 2013; Castañeda-Vildózola *et al.* 2016). However, to investigate individual growth trajectories larvae must be measured alive and allowed to develop through all instars (Garcia-Barros 2006). Observing the same individuals throughout their development was important in my study, as P220403 underwent an extra instar that may not have otherwise been detected. Macro photography was precise enough to minimize measurement error rates within an instar and made it possible to easily detect size increases between instars within an individual. The non-invasive method for head capsule measurements presented here would be suitable for other insect larvae when destructive sampling is not possible.

Growth ratios between instars varied, decreasing toward winter dormancy and increasing again the following spring (Table 2). Therefore, Dyar's rule of geometric growth did not apply to Poweshiek skipperling (Dyar 1890). Other studies have similarly documented non-static growth rates in insect larvae. In cases where larval development is inconsistent with Dyar's rule, growth ratios usually decrease with increasing instar (Gaines and Campbell 1935; Jobin *et al.* 1992; Calvo and Molina 2008). For Poweshiek skipperling, the decrease in growth ratios resulted in overlap between third and fourth instar head widths when all individuals were plotted as a frequency distribution (Figure 6). This overlap represents actual variation, rather than statistical uncertainty, because I documented individual growth histories.

The overlap between instars three and four was minimal, and most Poweshiek larvae of unknown age could be correctly categorized if their measurements fell in the third to fourth instar range. It would be difficult to categorize larvae with supernumerary instars, since these individuals usually have head capsules intermediate in size compared to "normal" larvae (Abarca *et al.* 2020). For example, P220403 underwent an extra instar and had fourth and fifth instar head capsules intermediate to other individuals in the respective instars. Consistent with other research (Garcia-Barros 2006), P220403 also had a larger final instar head capsule than larvae exhibiting typical development. Larval head capsule widths formed discrete size categories with non-overlapping ranges, or with minimally overlapping ranges in the case of instars three and four (Table 2). Assuming the seven-instar developmental pathway is very uncommon for Poweshiek in Manitoba, since it occurred only once in my sample, most individuals of unknown instar could be sorted based on their head capsule measurement alone. The ability to determine larval age could be relevant for the conservation program at APZ, for example in determining whether a larva is developmentally synchronized with the rest of their cohort.

With one exception, Poweshiek skipperling larvae at APZ developed through a total of six instars, plus pupa, and entered winter dormancy in the fourth instar. McAlpine (1972) previously reported larvae in Michigan overwintered as fifth instars and completed seven instars before pupating. Dormancy can occur in different instars within the same species (Danks 1987; Belozerov 2009). Latitudinal differences in insect phenology can be due to the greater number of degree days available for development in southern latitudes (Scriber 1994; Cayton *et al.* 2015). For example, intraspecific variation in the overwintering instar has been documented in the Glanville fritillary butterfly, *Melitaea cinxia* Linnaeus, 1758 (Lepidoptera: Nymphalidae), wherein larvae from northern latitudes overwintered in an earlier instar than their southern counterparts (Verspagen *et al.* 2023). Overwintering instar may likewise be plastic in Poweshiek, varying according to geographic location. Differences in instar number between populations are widespread across insect taxa (Esperk *et al.* 2007b) and can indicate adaptation to local climate

(Danks 1987; Verspagen *et al.* 2023). Knowledge of the overwintering instar may be beneficial for species recovery planning. If larvae in Manitoba overwinter in a different instar than those in Michigan, then exchanging individuals could result in phenological mismatch. The developmental schedule of Poweshiek larvae in Michigan should therefore be assessed.

Larva P220403 exhibited a unique developmental trajectory, overwintering in the fifth instar and completing seven instars in total. Intraspecific variability in the number of larval instars is not uncommon in insects (Esperk *et al.* 2007b) and can occur for a variety of reasons including rearing temperature, photoperiod, and food quality or quantity (Abarca *et al.* 2020; Esperk *et al.* 2007b). All Poweshiek larvae were reared on the same host species at the same location, making significant differences in rearing conditions unlikely. Variation in instar number in the small heath butterfly, *Coenonympha pamphilus* (Linnaeus, 1758) (Lepidoptera: Nymphalidae) was found to be a response to environmental conditions and diapause (Garcia-Barros 2006). Individuals that continued to feed in late summer due to mild temperatures, and then underwent diapause, were significantly more likely to develop an extra instar (Garcia-Barros 2006). Abiotic factors such as temperature can directly impact developmental rates, even within individuals of the same species (Danks 1987). For unknown reasons, P220403 moulted to the fifth instar before winter dormancy. Post-winter, developing through an extra, seventh instar may have been a compensatory strategy to achieve adequate size for pupation (Nijhout and Williams 1974).

Post-overwintering, Poweshiek larvae in cohorts 2021 and 2022 accumulated an average of 748.7 and 669.8 dd, respectively. I calculated total post-overwintering degree days (i.e. from removal from the overwintering chambers to adult eclosion), as this is the developmental period used to predict the timing of wild Poweshiek flight each year (Dearborn and Westwood 2014).

Annual surveys for adult Poweshiek skipperling are coordinated using degree-day estimations to predict the rate of larval development *in situ* (Dearborn and Westwood 2014). From 2006–2022, the standard deviation of average degree days at adult emergence was 635–725 dd (Dearborn *et al.* 2014–2023). My estimate of 669.8 dd for the *ex situ* 2022 cohort is in line with these findings. However, degree day estimates for the 2021 cohort are significantly higher than those for the 2022 cohort. In Chapter Three, I address potential causes for the unusual degree day accumulations by the 2021 cohort.

The biofix date, or date at which degree day accumulations begin, used to predict the wild population flight period is March 1 (Dearborn and Westwood 2014). My degree day accumulations began when larvae at APZ were removed from their overwintering chambers and placed outdoors. These dates varied from May 3 to May 9 depending on the year and individual. Despite excluding potential degree day accumulations in March and April, the total degree day accumulations in my study were comparable to *in situ* estimates (Dearborn and Westwood 2014; Dearborn *et al.* 2014–2023). My degree day estimates may be artificially high due to microclimate differences in the rearing environment for *ex situ* larvae compared to individuals in the field. Fine-scale variations in microclimate can result in meterological air temperature readings that do not accurately reflect the temperatures experienced by the insect (Lactin and Holliday 1994; Pincebourde and Woods 2020). Larvae at APZ are shaded by a large tree, the hoop house netting, and the nylon sock covering each plant. This shading may have resulted in cooler conditions within the larval enclosures compared to temperature readings from the airport weather station. Consequently, larvae may have accumulated fewer degree days than expected by the temperature data.

I found that even when temperatures were above the estimated lower developmental threshold and host plants were available, *ex situ* Poweshiek larvae did not resume feeding until one to two weeks after removal from their overwintering chambers. This corresponded to the second or third week of May in both of my study years. Henault and Westwood (2022) hypothesized that because Poweshiek host plant species were not abundant in tallgrass prairie sites until May 30, larvae *in situ* likely do not resume activity until the latter part of May. My direct observations of post-overwintering larvae suggest *ex situ* larvae are likely on the same developmental schedule as those *in situ*, both resuming feeding during the last two weeks of May. As shown in Chapter Three, flight dates are closely correlated between the two populations, suggesting that both emerge at approximately the same time. The initial inactivity after removal from overwintering chambers could be explained by overwintering physiology. If larvae are in a state of post-diapause quiescence, they may be unable to resume activity until temperatures rise (Danks 1987). However, since I did not measure hormone levels (Tauber *et al.* 1986; Ragland *et al.* 2019) or analyse gene expression (Fremdt *et al.* 2014), I was unable to determine whether Poweshiek exhibit true diapause.

Understanding the biology of endangered butterflies can aid in their conservation (Belitz *et al.* 2019). My study has addressed previously unanswered questions regarding Poweshiek skipperling biology, in the hopes that this information will support ongoing *ex situ* rearing efforts. I documented the developmental stages for the entirety of Poweshiek skipperling's year-long life cycle at APZ. I found that larvae typically overwinter in the fourth instar and complete six instars total. I documented one case of a larvae moulting to the fifth instar before winter, demonstrating variability in this species' development. Only one larva deviated from "normal" development, suggesting that most of the time, conditions are not favourable for the completion of the fourth instar prior to overwintering. The head capsule measurements provided here for Manitoba larvae will allow future comparisons of Poweshiek larval development in Michigan. I found that larvae require a mean of 669.8 dd from the end of winter dormancy to adult eclosion, though this varied by cohort. Future work could focus on detailing the microclimate differences between *ex situ* and *in situ* Poweshiek microhabitats, in order to better understand the variation in degree day accumulation between the two groups.

CHAPTER THREE: PREDICTING THE FLIGHT PERIOD OF ENDANGERED POWESHIEK SKIPPERLING BUTTERFLY

3.1 Abstract

The ability to accurately predict an insect’s flight period is necessary for concentrating survey efforts. In the case of endangered insects, conservation initiatives also require understanding the species’ phenology. The Poweshiek skipperling, *Oarisma poweshiek* (Parker, 1870), is a critically endangered butterfly that has been reared *ex situ* at Assiniboine Park Zoo since 2017 to augment the existing wild population in Manitoba, Canada. This species has a short flight period that must be predicted every year using degree day accumulations. Annual emergence predictions could be improved by estimating the lower developmental threshold (LDT), which is currently unknown. Due to the very small wild population size, many more Poweshiek currently exist *ex situ*. I analysed six years of *ex situ* data to calculate degree day accumulations, estimate the LDT for different life stages, and assess sex-related differences in phenology. A generalized linear model demonstrated that Poweshiek are protandrous. There was considerable annual variation in flight date, but degree day requirements were similar between years. The optimal LDTs for larvae (5 °C) and pupae (13 °C) were determined by comparing AICc scores. Results from this study can help refine degree day estimates and improve annual emergence predictions.

3.2 Introduction

Understanding the phenology, or timing of life history events, of endangered butterflies is important for implementing conservation actions. The timing of reintroductions, field surveys, and habitat management practices depend on in-depth knowledge of when different life stages are active (Schultz *et al.* 2008; Swengel and Swengel 2015). The winged adult is the stage in which Lepidoptera are typically most conspicuous and easiest to survey *in situ*. Field surveys must be conducted during the flight period, which is the number of days between the first and last observations of an adult butterfly (Bubova *et al.* 2016). Many threatened butterflies have relatively short flight periods, underscoring the need for highly accurate predictions of adult emergence (Kotiaho *et al.* 2005). Butterfly flight periods can vary substantially from one year to the next due to annual variation in weather. For example, from 2019 to 2021 the peak flight season of endangered mottled duskywing, *Erynnis martialis* (Scudder, 1870) (Lepidoptera: Hesperiidae) varied by up to four weeks (Demarse *et al.* 2023). This annual fluctuation in emergence timing makes calendar dates a less reliable method of predicting flight period (Cayton *et al.* 2015). Instead, temperature-based models are frequently used to predict phenological events such as first emergence and peak flight period (Legg *et al.* 2002; Andreassen *et al.* 2010; Mo and Stevens 2021).

Constructing phenological models requires knowledge of a species' thermal requirements for development. The three major components of phenological models are: a biofix date, species-specific degree day requirements, and a lower developmental threshold (Legg *et al.* 2002; Dearborn and Westwood 2014). A biofix date is the date on which to begin degree day accumulations (University of California Natural Resources 2012). This can be a fixed date, or it may reflect a biological event such as egg hatch (Legg *et al.* 2002). Degree days (dd) quantify the number of heat units required for an insect to develop between life stages (Allen 1976). Most degree day calculation methods assume a linear relationship between temperature and development; however, this relationship becomes non-linear as temperatures approach the species' maximum and minimum tolerances (Lamb 1992). Degree day calculations require a species-specific lower developmental threshold (LDT), which is the minimum temperature at which development can occur (Allen 1976). This threshold may vary for different life stages (Danks 1987). The LDT is typically investigated experimentally by rearing insects at a range of constant temperatures (Wu *et al.* 2015; Davídková and Dolezal 2019; Régnier *et al.* 2022; but see Legg *et al.* 2002).

Although phenological modeling is predominantly used in pest management to time control measures (Rebaudo and Rabhi 2018; Soroka *et al.* 2020), the same principles of degree day analysis can be applied to predict the emergence of endangered lepidoptera (Koda and Nakamura 2012; Dearborn and Westwood 2014). The Poweshiek skipperling, *Oarisma poweshiek* (Parker, 1870) (Lepidoptera: Hesperiidae) is a critically endangered butterfly that has experienced dramatic population declines since the early 2000s (Belitz *et al.* 2018). This species is endemic to native tallgrass prairie and prairie fens and was once distributed across the upper mid-western United States (Belitz *et al.* 2019). As of 2023, two populations exist on the fringes of the species' historical range—one in Michigan, USA and one in southeastern Manitoba, Canada (Dupont-Morozoff *et al.* 2022). Loss of habitat connectivity and habitat degradation are hypothesized to have contributed to this species' decline, but the exact causes are unknown (Saarinen *et al.* 2016; Belitz *et al.* 2020). Poweshiek are univoltine and overwinter as partially grown larvae (COSEWIC 2014). The flight season is brief, occurring during a two-week period between late June and mid-July in Canada (Dearborn and Westwood 2014; Dupont-Morozoff *et al.* 2022). Unlike many grass skippers (subfamily Hesperiinae), adult male Poweshiek lack a stigma (Klassen *et al.* 1989) and differences between the sexes are subtle (Figure 10).

Figure 10. Comparative morphology of adult female (top) and male Poweshiek skipperling. Females have annulated antennae, a white wing fringe (indicated by the arrow), and a thicker abdomen. Male antennae are solid orange, the wing fringe is black, and the abdomen is thinner. Photo credits: female by Evelien de Greef, male by Kirstyn Eckhardt.

Field survey data for Poweshiek do not always include the sex of the individuals, which has prevented drawing conclusions about whether this species is protandrous (Dearborn and Westwood 2014). Protandry, a reproductive strategy in which adult males emerge between several days to two weeks earlier than females, is common among butterflies (Wiklund and Fagerstrom 1977). One field study found consistent sex ratios throughout Poweshiek flight period, which suggests an absence of protandry; however, sex was not recorded for one quarter of the individuals (Belitz *et al.* 2019). Populations with very low densities are at risk of mate-finding failure due to low encounter rates between conspecifics, and protandry may exacerbate this problem (Calabrese *et al.* 2004). Adult Poweshiek are short-lived, likely surviving less than one week in the wild. Protandry may further reduce Poweshiek reproductive success if a significant number of males expire before females emerge (Bubova *et al.* 2016; Belitz *et al.* 2019). It is therefore important to examine potential sex-based differences in phenology for this species.

The aim of this study is to improve flight period predictions for Poweshiek skipperling in Manitoba by analysing past emergence trends and estimating the lower developmental threshold. Poweshiek at the remaining populated sites in Manitoba have been surveyed annually since 2006, and first occurrence records have been used to produce degree day estimates to predict the Poweshiek skipperling's emergence (Dearborn and Westwood 2014; Dearborn *et al.* 2014–2023). An LDT of 6 °C is used in these degree day calculations; however, this value is based on other lepidopterans that overwinter as larvae in the region, and the LDT for Poweshiek skipperling remains untested (Dearborn and Westwood 2014). Annual flight period predictions are usually accurate to within several days of skipper emergence (Dearborn *et al.* 2014–2023), but a better understanding of the butterfly's LDT could further improve predictions and help maximize survey efforts.

Conducting research on wild Poweshiek skipperling has been challenging due to difficulty in locating substantial numbers of individuals in the field (Belitz *et al.* 2019). Since 2017, Assiniboine Park Zoo (APZ) in Winnipeg, Manitoba has managed an *ex situ* population of Poweshiek skipperling to increase the population size in Canada (NCC 2021). Immature stages are reared at APZ in an outdoor hoop house (Assiniboine Park Zoo 2024). Adult butterflies are released into native prairie habitat at Tall Grass Prairie Preserve (TGPP), where the remaining wild population is located. Studying the *ex situ* population provides an opportunity to draw statistically robust conclusions, as far greater numbers of individuals are available *ex situ* than can be observed in the wild. In addition, sex ratios and dates for major developmental milestones are recorded as part of the *ex situ* program. This information is crucial for estimating the LDT.

The standard method of investigating the LDT (e.g. Régnier *et al.* 2022) was not possible for Poweshiek skipperling, as rearing immatures at temperatures near their minimum tolerance would likely result in high mortality rates. Non-experimental approaches to determine LDT are also possible. In the "least variability" method used by Sanborn *et al.* (1982), degree day accumulations were calculated at a variety of LDTs. The temperature closest to the true LDT was the one with the smallest coefficient of variation (Sanborn *et al.* 1982). Mo and Stevens (2021) indirectly determined threshold temperatures by evaluating the degree day model's ability to predict emergence. I used an information theoretic approach.

My objectives are to 1) analyse emergence trends between *ex situ* cohorts, as well as compare *ex situ* emergence to that of the *in situ* population, and 2) estimate the LDT. I expect yearly variation in emergences dates due to annual variation in weather, and I expect degree day requirements to be constant between cohorts. I expect the lower developmental threshold to increase with life stage.

3.3 Methods

3.3.1 Study population

Poweshiek skipperling larvae are reared from eggs to pupae at Assiniboine Park Zoo in Winnipeg, Manitoba (49.87°, -97.24°). Eggs are laid in July, and each larva is reared on an individual Prairie dropseed (*Sporobolus heterolepsis* G.) (Poaceae) potted plant covered by a thin nylon sock. The plants are then placed in an outdoor mesh-walled hoop house, which allows wind and precipitation to enter but helps prevent entry of potential predators (e.g. birds, predatory arthropods). From approximately mid-October until the first week of May, fourth instars (see Chapter Two) are stored in Isotemp incubators (hereafter “overwintering chambers”) at -4 °C to simulate winter conditions. The date larvae are placed into and removed from overwintering chambers varies by year, based on when mean daily temperatures are consistently below 0°C in the autumn and above that threshold again in the spring (Assiniboine Park Zoo 2024). Time spent in overwintering chambers for most cohorts is between 190–200 days.

Larvae resume growth in the spring and pupate at APZ in early- to mid-June. Pupae then eclose at a release site in TGPP near Tolstoi, Manitoba (49.15°, -96.72°) approximately 80 km southeast of Winnipeg. Individuals are sexed before release. Since this species is univoltine, hatch year is one year before the year adults are released (i.e. butterflies from the 2017 cohort eclosed in 2018). For further information on the *ex situ* rearing protocol, refer to Assiniboine Park Zoo (2024).

3.3.2 Sampling design

A total of 328 larvae belonging to *ex situ* cohorts 2017–2022 were included in my analysis, with the greatest number of individuals from cohorts 2020, 2021, and 2022. Sex ratios were approximately equal, with 167 females and 161 males. Larvae are closely monitored by Zoo staff during their development and exact dates for major developmental milestones are recorded. I used dates for hatching, storage in overwintering chambers, pupation, and eclosion in my analysis. Individuals that died prior to eclosion were removed from analysis, as were two individuals whose sex was not determined prior to their release. Three developmentally delayed larvae were reared in an Isotemp incubator at a constant 28°C to accelerate their development. These individuals were excluded from the analysis, as they did not experience the same rearing conditions and eclosed considerably later than the rest of the cohort.

Ex situ Poweshiek skipperling have been released annually in Manitoba from 2018–2023. To assess synchronicity in flight period between the *ex situ* and *in situ* populations, I used annual *in situ* flight survey data from 2018–2023. These data consist of the first sighting of a wild Poweshiek skipperling each year in TGPP. Surveys are conducted several days prior to the anticipated emergence to avoid missing the beginning of the flight period (R. Westwood, pers. comm.).

3.3.3 Flight period trends

All analyses were performed using R Statistical Software (v4.2.2; R Core Team 2022) in RStudio (v2022.02.1; Rstudio Team 2022). To calculate cumulative degree days (dd) for larvae and pupae, I used the double sine method in the degreedays function from the R package "TrenchR" (v1.1.1; Buckley *et al.* 2023). On several occasions the degree day formula produced a "not a number" warning, caused by a floating-point error in the arcsin term. To obtain the correct degree day value in those instances, I manually input the correct arcsin value using the package source code (https://rdrr.io/cran/TrenchR/src/R/DDFunctions.R). Weather data for degree day calculations were sourced from the Richardson International Airport weather station (49.91°, -97.24°) (ECCC 2023) when the larvae were housed at APZ. Once the larvae had been transported to the field site to complete their development, I used weather data from the Emerson station (49.00°, -97.24°) (ECCC 2023) located approximately 35 km west-southwest of the release site.

Degree day (dd) accumulations are computed annually to predict the first occurrence of Poweshiek *in situ* and optimize survey efforts (Dearborn *et al.* 2014–2023). I used the same lower and upper developmental thresholds (6 °C and 32 °C, respectively) as Dearborn and Westwood (2014) to enable comparison between my *ex situ* degree day estimates and those for the wild population. Degree day calculations for the *in situ* population use a biofix date of March 1, since few degree days accumulate prior to this date *in situ* (Dearborn and Westwood 2014). My degree day calculations began when larvae were removed from their overwintering chambers at APZ in the spring (i.e. "post-overwintering") and continued until adult emergence.

To assess whether the start of the flight period *in situ* is correlated to the emergence of *ex situ* individuals, I linearly regressed the *ex situ* eclosion dates against *in situ* first flight dates (i.e. the date wild individuals were first encountered at TGPP each year). To test for differences in post-overwintering degree day accumulations between sexes and cohorts, I used a generalized linear model with cumulative degree days as the response variable. Degree days were selected as the response variable because they represent the total accumulation at the "event" of interest, in this case eclosion (Legg *et al.* 2002). Degree day accumulations for all cohorts were compared to cohort 2020 in the generalized linear model.

3.3.4 Lower developmental threshold estimation

There is evidence that lower thresholds can vary for different life stages, sometimes increasing during pupal development (Sanborn *et al.* 1982; Davídková and Dolezal 2019). I therefore estimated the LDT using degree day accumulations for three periods of larval development: egg hatch to pupation, post-overwintering to eclosion, and pupation to eclosion. To determine the optimal LDT for each developmental period, my explanatory variables were sex and degree day accumulations at a given LDT. To create predictive models that would allow forecasting eclosion or pupation dates in future years, the models excluded year as an explanatory variable, since year effects cannot be evaluated for future events. For all models, the response variable was Julian date of either pupation or eclosion.

Models were tested using LDTs between 0 °C and 22 °C, in increments of 1–2 °C. Model fit was assessed using Akaike's information criterion with small sample size correction (AICc), using R package "MuMIn" (v1.47.5; Bartoń 2023). In some cases, models with very low LDTs (< 0 °C) were selected as the optimal fit due to a statistical artifact. While AICc scores indicate the best statistical model, they may not always accurately reflect actual biological processes (Régnier *et al.* 2022). Bahar *et al.* (2014) excluded models with LDTs below 4 °C based on biological implausibility. Régnier *et al.* (2022) dismissed any LDT below 5 °C due to high mortality rates below this threshold. I chose to exclude LDTs below 5 °C from consideration.

3.4 Results

3.4.1 Flight period trends

Eclosion dates varied by year (Figure 11). Notably, the 2021 cohort eclosed in mid-late July in 2022 (July 11–July 26), while the 2022 cohort emergence began three weeks earlier in 2023 (June 19–July 5). The other cohorts generally eclosed in early July (Figure 11). There was a significant relationship between *ex situ* Poweshiek skipperling emergence dates and *in situ* first occurrence dates, $R^2 = 0.90$, $F(1, 4) = 49.49$, $P < 0.05$. In most years, the first Zoo-reared individual eclosed within one to two calendar days of the first wild Poweshiek sighting (Table 3). Ranges in eclosion dates were greater for cohorts 2020–2022 compared to cohorts 2017–2019 (Table 3).

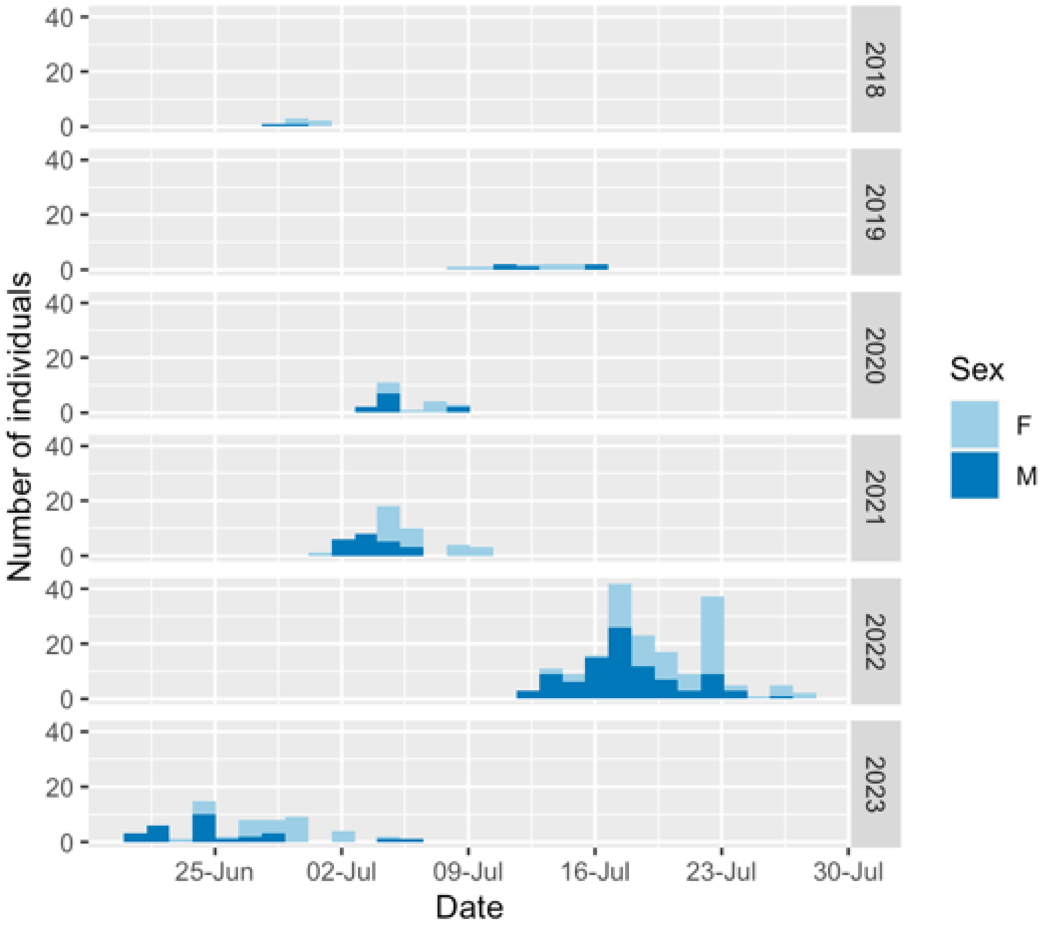

Figure 11. Histograms of *ex situ* Poweshiek skipperling eclosion dates by release year and sex. Release year is one year ahead of cohort (e.g., cohort 2017 was released in 2018).

Results from the generalized linear model indicated that degree day accumulations from post-overwintering to eclosion did not differ between cohorts 2017, 2018, 2019 and 2020 ($P >$ 0.05 for all years) (Table 4). Degree day accumulations differed significantly for cohort 2021 ($P < 0.001$) and, to a lesser degree, cohort 2022 ($P < 0.05$) (Table 4). The 2021 cohort accumulated a greater number of degree days both pre- and post-overwintering, compared to all other cohorts (Table 5). *Ex situ* cohorts accumulated 734–1057 dd (mean = 949) prior to overwintering (Table 5). Post-overwintering, cohorts accumulated 641– 760 dd (mean = 716) (Table 5). Males eclosed after 40 fewer degree days compared to females ($P < 0.001$) (Table 4).

Table 3. Emergence comparison between *in situ* and *ex situ* Poweshiek skipperling by year.

Release year	*In situ* first occurrence	*Ex situ* first eclosion	*Ex situ* peak eclosion	*Ex situ* eclosion range (days)
2018	26 June	27 June	28 June	4
2019	11 July	8 July	11 July	8
2020	2 July	1 July	3 July	6
2021	30 June	30 June	3 July	10
2022	8 July	11 July	18 July	16
2023	21 June	19 June	25 June	17

Table 4. Generalized linear model results for year and sex effects on post-overwintering degree day accumulation of Poweshiek skipperling. Significant *P*-values are shown in bold (α = 0.05).

	Estimate	Std. error	*t* value	*P*-value
Intercept	673.12	6.13	109.89	< 0.001
Cohort2017	-10.32	17.70	-0.58	0.56
Cohort2018	-15.85	13.16	-1.20	0.23
Cohort2019	-10.63	10.65	-1.00	0.32
Cohort2021	108.35	6.56	16.53	**< 0.001**
Cohort2022	22.82	7.87	2.90	**0.004**
SexMale	-39.96	4.54	-8.80	**<0.001**

Table 5. Degree day (dd) accumulations for *ex situ* Poweshiek skipperling using a lower developmental threshold of 6 °C. Pre-overwintering includes the period from egg hatch until larvae were placed in overwintering chambers. Post-overwintering includes removal from overwintering chambers until eclosion.

Cohort	*n*	Mean and SD pre-overwintering dd	Mean and SD post-overwintering dd
2017	6	859.62 ± 12.43	649.46 ± 18.97
2018	12	865.91 ± 26.43	640.61 ± 37.01
2019	21	734.20 ± 22.72	641.55 ± 30.28
2020	50	892.19 ± 36.55	655.53 ± 36.51
2021	180	1057.35 ± 38.21	760.59 ± 47.52
2022	59	769.52 ± 52.72	677.65 ± 53.05
Pooled mean and SE	328	949.09 ± 7.34	715.61 ± 3.75

Table 6. Degree day (dd) accumulations for *ex situ* Poweshiek skipperling larvae (egg hatch to pupation) and pupae (pupation to eclosion) using the highest-ranking lower developmental threshold for the respective developmental periods. These values were generated from a development model that includes sex as a predictor.

Cohort	*n*	Mean and SD dd for larva, LDT 5 °C	Mean and SD dd for pupae, LDT 13 °C
2017	6	1433.53 ± 30.01	104.95 ± 5.00
2018	12	1415.48 ± 40.99	106.98 ± 9.07
2019	21	1259.73 ± 46.61	115.37 ± 7.55
2020	50	1471.30 ± 54.74	104.13 ± 7.10
2021	180	1720.56 ± 68.10	129.55 ± 12.21
2022	59	1339.96 ± 78.47	105.73 ± 10.58
Pooled mean and SE	328	1568.18 ± 10.36	119.21 ± 0.88

3.4.2 Lower developmental threshold estimation

The models with the lowest AICc scores were LDT 5 °C, 5 °C, and 13 °C for developmental periods: egg hatch to pupation, post-overwintering to eclosion, and pupation to eclosion, respectively. For all models, there was no significant interaction between degree day accumulations and year ($P > 0.05$). Degree day accumulations were calculated for each cohort using the highest-ranking LDT models for larvae (egg hatch to pupation) and pupae (pupation to eclosion) (Table 6). Mean degree day accumulations for larvae were 1568 dd while pupae accumulated an average of 119 dd (Table 6).

The roughly parallel lines of data in the post-overwintering to eclosion model suggest that degree day accumulations during this developmental period varied between years (Figure 12). Model predictions for pupation and eclosion dates were most accurate for the egg hatch to pupation period ($R^2 = 0.75$), and least accurate for pupation to eclosion ($R^2 = 0.61$) (Figures 13–14). The egg hatch to pupation model predicted pupation dates that differed from the observed dates by an average of 2–5 days for 2018, 2021, 2022, and 2023 (Figure 13). In 2019 and 2020, observed pupation dates averaged 9–10 days later than model predictions (Figure 13). Observed eclosion dates were later than predicted for nearly all individuals in 2019 and 2022 (Figure 14).

3.5 Discussion

As expected, first occurrence and first eclosion dates of Poweshiek skipperling varied by year, with peak eclosion varying by up to three weeks over the six years of the *ex situ* program (June 25 in 2023 and July 18 in 2022). As ectotherms, butterfly development time is inversely related to temperature (Allen 1976). The years 2022 and 2023 presented opposite extremes in weather during the time when Poweshiek skipperling larvae were completing their development.

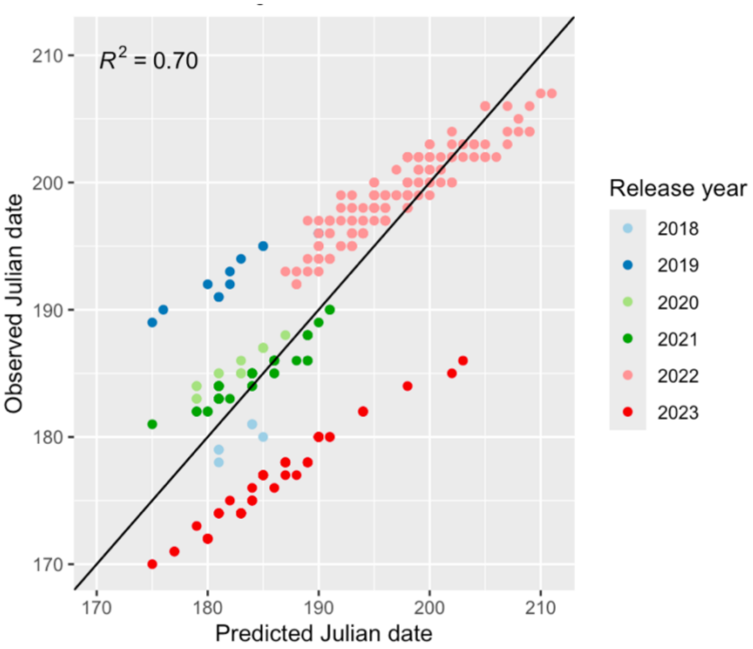

Figure 12. Observed and predicted Julian dates of eclosion for the post-overwintering to eclosion developmental period. Predictions were generated from a forecast development model that includes sex as a predictor variable, with a lower threshold of 5 °C. The diagonal line represents perfect agreement of observed and predicted values.

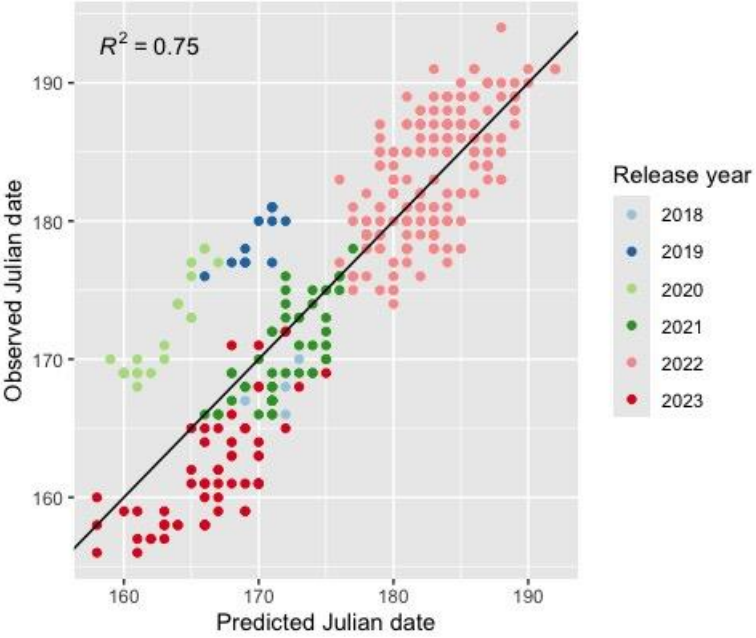

Figure 13. Observed and predicted Julian dates of pupation for the egg hatch to pupation developmental period. Predictions were generated from a forecast development model that includes sex as a predictor variable, with a lower threshold of 5 °C. The diagonal line represents perfect agreement of observed and predicted values.

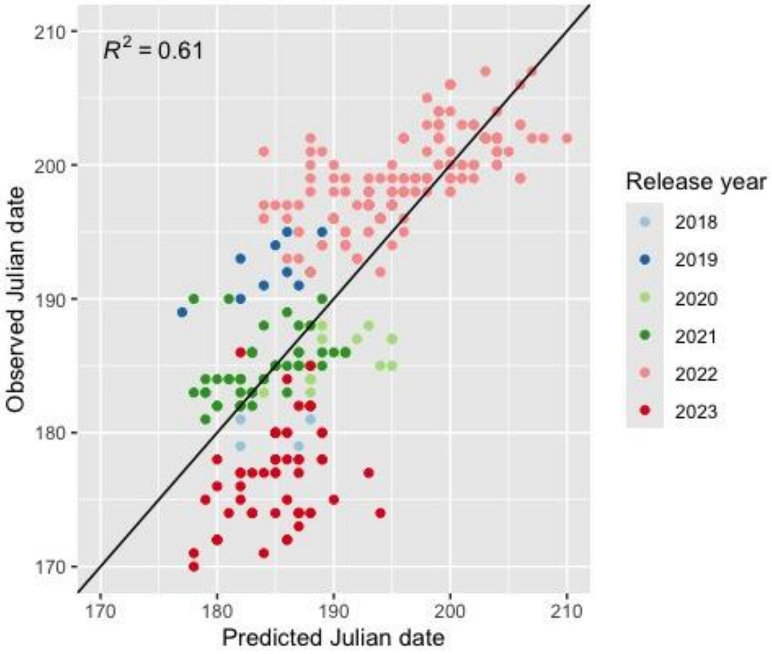

Figure 14. Observed and predicted Julian dates of eclosion for the pupation to eclosion developmental period. Predictions were generated from a forecast development model that includes sex as a predictor variable, with a lower threshold of 13 °C. The diagonal line represents perfect agreement of observed and predicted values.

Spring in 2022 was colder and wetter than normal in southern Manitoba, with a mean daily temperature of 14.5 °C for May and June (ECCC 2023). In comparison, mean temperature over the same months was 15.9 °C in 2021 and 18.4 °C in 2023 (ECCC 2023). Differences in the timing of adult flight due to annual variation in temperature is well-documented in insects, with earlier flight periods occurring in response to warmer temperatures (Roy and Sparks 2000). The delayed emergence of Poweshiek skipperling in 2022—with some individuals eclosing as late as July 26—can largely be explained by unseasonably cold spring and early summer temperatures.

Despite yearly variation in the start of Poweshiek flight period, the *ex situ* first eclosion date was significantly related to the *in situ* first occurrence, suggesting that development is synchronized between the two populations. In addition, degree day estimates from post-overwintering to eclosion for the *ex situ* population were in line with those for the *in situ* population. After winter dormancy, adult emergence of Poweshiek skipperling *in situ* occurs after 635–725 dd (Dearborn *et al.* 2014–2023), whereas *ex situ* emergence occurred after a mean of 716 dd (Table 5). A critical component of *ex situ* rearing is reproducing natural conditions as much as possible to ensure that individuals in human care develop at the same rate as their wild counterparts (Aardema *et al.* 2011). The fact that *ex situ* Poweshiek eclose at the same time wild individuals begin their flight, and accumulate similar degree days, suggests that rearing conditions at APZ approximate conditions larvae experience in the wild (Cayton *et al.* 2015).

I found evidence that Poweshiek are protandrous, with males emerging after significantly fewer degree days compared to females (Table 4). On average, males eclosed 2–3 calendar days earlier than females in cohorts 2020, 2021, and 2022 (Figure 11). Sex-based differences in eclosion date were most apparent in these three cohorts, likely due to larger sample sizes compared to cohorts 2017–2019. Protandry is common in butterflies, and more pronounced protandry has been documented in another skipper, with males emerging two weeks prior to females (Demarse *et al.* 2023). Although the degree of protandry I observed for Poweshiek skipperling appears minor in comparison, two or three days may comprise a significant portion of an adult Poweshiek's lifespan, which is thought to be about one week in the wild (R. Westwood, pers. comm.). The asynchrony in male and female Poweshiek skipperling emergence may therefore prohibit some individuals from finding a mate (Calabrese and Fagan 2004). However, wild-caught Poweshiek females brought to APZ for egg collection typically produce fertilized eggs (L. Burns, pers. comm.), which suggests that most females are able to encounter mates.

The 2021 cohort accumulated an unexpectedly high number of degree days both pre- and post-winter dormancy compared to the other cohorts (Table 5). Since degree days are a measure of physiological time (Caicedo *et al.* 2012), species-specific degree day requirements are not expected to differ significantly, regardless of annual temperature variation. Rearing procedures at APZ did not change for this cohort, nor did the sources of the weather data used to calculate degree days. One possible reason for the abnormal degree day accumulations is if temperatures approached or exceeded the hypothesized upper developmental threshold of 32°C (Dearborn and Westwood 2014) during the larval stage. Heat injury, which causes physiological damage, can occur when insect larvae are exposed to sub-lethal temperatures above their thermal optimum (Iltis *et al.* 2021; Ma *et al.* 2021). Exposure to extremely high temperatures can decrease development rate (Ma *et al.* 2021; Régnier *et al.* 2022) and reduce immune function (Iltis *et al.* 2021). Poweshiek skippering cohorts 2018 and 2021 experienced more days above 30 °C during their early development (14 d and 18 d, respectively) than any other cohort (ECCC 2023). Peak eclosion for cohorts 2018 and 2021 was also later than any other *ex situ* cohort (Table 3), and nearly all individuals in these cohorts eclosed later than model predictions (Figure 12). Potentially, the intense heat during the summers of 2018 and 2021 resulted in heat injury, interrupted larval development, and delayed adult emergence.

It is also possible that extensive rainfall during the spring of 2022 inhibited the larval growth of the 2021 cohort of Poweshiek skipperling. Winnipeg received over 160 mm of rain in May 2022, nearly three times the monthly average (ECCC 2023). Rain can alter an insect’s microclimate by decreasing the temperature of the leaf surface, sometimes by up to 5 °C, and temperatures may not return to baseline for more than an hour after rainfall ceases (Chen *et al.* 2019). This cooling can significantly prolong larval development (Chen *et al.* 2019). Heavy rain may also disrupt larvae from feeding, further supressing their growth (Kamata and Igarashi 1994). If microclimate within Poweshiek enclosures was frequently cooled by rain, larvae may have accumulated fewer degree days than predicted by temperature data from the airport weather station. The hot weather in the fall of 2021, combined with cold, wet weather in the spring of 2022 may explain the unusually high degree day accumulations for the 2021 cohort.

Adult emergence *in situ* is currently estimated by summing degree days beginning on March 1 each year (Dearborn *et al.* 2014–2023). My results indicate that larvae enter winter dormancy after variable amounts of development within instar four (Figure 12), or even in different instars (see Chapter Two). Because larvae are in different stages of development post-winter dormancy, predictions of adult emergence should instead be based on degree day accumulations beginning at egg hatch. This would ensure that larvae are at same level of development when degree day summations begin. Directly observing egg hatch *in situ* every year is impractical but could be estimated based on the end of the flight season and the known duration of egg development (Henault and Westwood 2022; Burns *et al.* 2022).

An LDT of 5 °C was the best-fitting threshold for the larval stage and is similar to the 6 °C threshold currently used to produce degree day predictions (Dearborn and Westwood 2014). This threshold is reasonable, given that insect species at higher latitudes tend to have lower LDTs as an adaptation to developing in colder environments (Bahar *et al.* 2014; Buckley *et al.* 2017). I found that Poweshiek skipperling pupal development was best described by an LDT of 13 °C. It has previously been reported that insect life stages may have different lower developmental thresholds (Sanborn *et al.* 1982; Danks 1987; Davídková and Dolezal 2019). Daily mean temperature in southern Manitoba is 5.6 °C higher during the period Poweshiek skipperling pupate (typically, mid-June), compared to when larvae resume growth in the spring (ECCC 2023). Critical life history events, such as adult emergence, often have much higher temperature thresholds to help synchronize emergence at the correct time of year (Danks 1987).

Therefore, an LDT of 13 °C is not unexpected given the daily mean temperature in June is 17 °C (ECCC 2023).

Predictions from the forecast models were most accurate for the developmental period egg hatch to pupation using an LDT of 5 °C. This model predicted pupation dates that, in most years, differed from observed pupation dates by several days. However, for 2019 and 2020 the predicted pupation dates were more than one week earlier than observed (Figure 13). Despite no significant interaction between year and degree days, the imperfect fit when “year” is excluded in the forecast models (Figures 12–14) demonstrates that years vary in some unknown, unmeasured variable. There are abiotic factors other than temperature that can affect insect development. For example, drought can decrease host plant quality by reducing the amount of nitrogen in plant tissues (Carnicier *et al.* 2019). Insects feeding on drought-exposed plants may have reduced body mass or lower survival rates, and early-instar larvae may be particularly susceptible due to their limited mobility (Salgado and Saastamoinen 2019; Portman *et al.* 2021). Similarly, low humidity can negatively impact early life stages by increasing the risk of desiccation (Klockmann and Fischer 2017). A combination of these factors may have contributed to the “year” effect reported here and warrant further investigation.

Climate change has the potential to alter the distribution and phenology of insect species, with many examples in Lepidoptera (Westwood and Blair 2010; Roy *et al.* 2015; Fitchett *et al.* 2022). While I was unable to assess potential climate-related changes in Poweshiek skipperling flight due to the limited number of years of *ex situ* data, future work could focus on comparing phenological trends for this species over time. Given that climate change may affect the phenology of less mobile species to a greater degree (Kharouba *et al.* 2014), this could be particularly relevant to Poweshiek skipperling due to their limited dispersal capabilities and specialist habitat requirements (Belitz *et al.* 2020). Changes in autumn temperatures that extend the growing season may result in this species developing beyond the larval stage before winter dormancy, creating a lost generation (Kerr *et al.* 2020). An estimation of the lower developmental threshold and more accurate degree day predictions will hopefully support future research into the effects of climate change on Poweshiek phenology, as well as inform conservation actions for this endangered species.

In conclusion, wild Poweshiek skipperling emergence appears to be synchronized with that of the *ex situ* individuals. This developmental synchronicity is critical for species recovery efforts. I found evidence that Poweshiek are protandrous, which could be helpful to APZ staff in determining when to capture females for egg collection. I estimated this species’ lower developmental threshold based on *ex situ* degree day accumulations. My results show that a lower threshold of 5 °C best describes Poweshiek larval development, while a threshold of 13 °C is appropriate for pupal development. Using these thresholds, Poweshiek larvae require 1570 dd to pupate, and pupae require 120 dd to eclose. I recommend that annual Poweshiek emergence predictions be based on degree day accumulations beginning at egg hatch, as I found evidence that larvae overwinter at different stages of physiological development.

CHAPTER 4: GENERAL DISCUSSION AND CONCLUSIONS

In this study, I made observations on the larval development (Chapter Two) and flight period trends (Chapter Three) of *ex situ* Poweshiek skipperling in Manitoba. My objectives were (i) document the overwintering instar and stage-specific degree day requirements of larvae, (ii) summarize trends in adult emergence, and (iii) estimate the species' lower developmental threshold from *ex situ* data. The purpose of this project was to clarify aspects of Poweshiek development and to improve annual emergence predictions.

I met my first objective by measuring head capsules of two cohorts of Poweshiek skipperling larvae; this included monitoring one cohort through the entirety of their year-long life cycle. From these observations, I produced a guide that will allow researchers at Assiniboine Park Zoo (APZ) to categorize larvae into age groups based on their head capsule width. I estimated the degree day requirements and documented the durations of all six instars, adding to the existing observations made by Henault and Westwood (2022). I determined that Poweshiek in Manitoba usually overwinter in the fourth instar and complete six instars in total. This is contrary to the existing literature regarding this species' instar number and overwintering instar (McAlpine 1972). Further work should be done to determine whether Poweshiek larvae in the U.S. overwinter in the same instar as larvae in Manitoba. Differences in overwintering instar or degree day requirements may be relevant if individuals need to be translocated as part of the species' recovery.

I met my second objective by analyzing data belonging to over 300 Poweshiek larvae reared *ex situ* at APZ. I found that from 2018–2023, Poweshiek peak flight varied by as much as three weeks due to annual weather variation. A generalized linear model demonstrated that Poweshiek are protandrous, with males emerging 2–3 days earlier than females. I calculated degree days using the same developmental period (post-overwintering to eclosion) as Dearborn *et al.* (2014–2023) to enable comparisons between my findings for the *ex situ* cohorts and the wild population. I found that post-overwintering larvae in most *ex situ* cohorts required an average of 641–678 dd to eclose, similar to the 635–725 dd expected for larvae *in situ* (Dearborn *et al.* 2014–2023). Degree day accumulations for cohort 2021 were unexpectedly high, which I hypothesized was due to weather anomalies during their development. I found evidence that *ex situ* individuals are developmentally synchronized with the wild population, as annual emergence dates were similar between the two populations. This synchronism is essential to the success of *ex situ* rearing programs and suggests that rearing conditions at APZ are similar to those experienced *in situ*.

One of my most significant findings was that larvae overwinter after completing different amounts of development within the fourth instar (Chapter Three) or in different instars (Chapter Two). In support of this, forecast models that used degree day accumulations beginning post-winter dormancy had poorer predictive power ($R^2 = 0.70$) than models using degree day summations from egg hatch ($R^2 = 0.75$). It may therefore be beneficial to base annual emergence predictions on the degree day accumulations beginning at egg hatch rather than post-winter dormancy. The environmental cues that trigger winter dormancy should be investigated to determine why some larvae overwinter at different stages of physiological development. It has been assumed that Poweshiek larvae diapause (McAlpine 1972), but I was unable to confirm this through observational studies alone. Insects in temperate regions primarily rely on photoperiod cues to initiate diapause (Danks 1987; Bale and Hayward 2010). However, if warmer-than-normal fall temperatures allow Poweshiek to continue development regardless of photoperiod, it is expected that some individuals would develop beyond the appropriate overwintering stage

(Bale and Hayward 2010; Kerr *et al.* 2020). While in one instance a fifth instar successfully overwintered (Chapter Two), it is unlikely that pupae could do the same (Tauber *et al.* 1986). It is therefore essential to understand the factors regulating Poweshiek dormancy.

I modeled the lower developmental threshold (LDT) for Poweshiek skipperling, meeting my third objective. By comparing model AICc scores, I found that the optimal LDT for larvae (5 °C) is substantially lower than for pupae (13 °C). The 5 °C threshold is similar to the 6 °C threshold currently used to estimate degree day accumulations for annual emergence predictions (Dearborn and Westwood 2014). Given these thresholds, I found that larvae accumulate an average of 1570 degree days from egg hatch to pupation, and pupae require a further 120 degree days to eclose. This information may be helpful to refine emergence predictions and plan field surveys. I used non-invasive methods to deduce the LDT for Poweshiek, which was necessary due to the species' "Endangered" status and the limited number of *ex situ* individuals. However, if the *ex situ* program continues to grow, it may be possible in the future to conduct traditional rearing experiments at fixed temperatures to confirm the LDT (Davídková and Dolezal 2019; Régnier *et al.* 2022).

The disjunct wild Poweshiek populations may eventually require translocations to expand their range and retain sufficient levels of genetic diversity, as has been recommended for other butterfly *ex situ* programs (Schultz *et al.* 2011). The Canadian population of Poweshiek skipperling contains several distinct alleles, which could be used to revive genetic diversity in the U.S. portion of the species' range (Saarinen *et al.* 2016). But before translocations can be made, it will be important to understand any biological differences between the disjunct populations. My thesis has shown that there are valuable insights to be gained from studying *ex* *situ* Poweshiek skipperling. The new life history information I present here addresses knowledge gaps and will hopefully aid in conservation decision-making.

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