PAST, PRESENT, AND FUTURE MONITORING OF INSECTS SENSITIVE TO CLIMATE CHANGE

by

Cayley Riana Faurot-Daniels

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Biological Sciences

MONTANA STATE UNIVERSITY
Bozeman, Montana

December 2024

DEDICATION

To all appreciators of small things

.

ACKNOWLEDGEMENTS

There are many people who have left indelible marks on me as a person and as a scientist who deserve my heartfelt thanks. Thank you to my parents, Ellen and Dan Faurot-Daniels, for encouraging me to play in the dirt, past and present, and their unwavering support in all forms. Thank you to my grandma, Margaret Faurot, for her encouragement in art and writing, and to my grandpa, Rodgers Faurot, for emulating curiosity about the world around him and kindness for those who are in it. This work would have been much harder without my partner, Allen Krabill, his ability to see good in all things, and his open, listening ears. Thank you to my friends, for the hikes, paddles, long days in the field with smiles at the end, and perspective.

For many years, I worked on field ecology projects in the summers and molecular genetics in the winters, and in this master's research I was finally able to meld the two together. Getting the opportunity to do that while being advised by two women who are leaders in their field, Dr. Diane Debinksi and Dr. Michelle Flenniken, has truly been a privilege. Thank you, Diane and Michelle, for striking the balance of guidance and supporting independent discovery. Thank you as well to my committee members, Dr. Laura Burkle and Dr. Adam Sepulveda, for their insight, and to Adam and Patrick Hutchins for sharing space and resources in their laboratory. Thanks to Steve Kohler for his help with butterfly identification and Chris Fields for sharing bioinformatic code and expertise. I could not have completed this work without the many miles walked and run in the pursuit of butterflies by technicians Sagan Simmons and Kaylee Guajardo, field housing and research support from Glacier National Park and Tara Carolin, and financial support from the National Science Foundation Graduate Research Fellowship and Montana State University Research Expansion Grant.

TABLE OF CONTENTS

TABLE OF CONTENTS CONTINUED

LIST OF TABLES

Table Page

LIST OF TABLES CONTINUED

LIST OF TABLES CONTINUED

LIST OF FIGURES

Figure Page

LIST OF FIGURES CONTINUED

ABSTRACT

Methods that can provide expediency in biodiversity and ecosystem assessments are becoming increasingly important in a shifting conservation landscape due to climate and land use change. Insects provide vital ecosystem services, yet the full scope, causes, and future implications of insect decline have yet to be fully understood. In this work, butterflies are utilized as ecological indicators of an ecosystem sensitive to climate change and as indicators for the development of novel molecular genetic techniques of surveying terrestrial insect biodiversity. Wildflower-associated environmental DNA (eDNA) has recently been used to identify insects and may provide an efficient and non-invasive alternative for monitoring terrestrial insect biodiversity**.** A resurvey effort of butterfly diversity in Glacier National Park, Montana using traditional netting surveys was conducted to assess the 2022 and 2023 butterfly community in comparison to survey data obtained 35 years earlier in 1988 and 1989. Information from the resurvey effort was used in comparative analyses to determine if molecular methods can detect butterflies at a similar or better resolution to traditional surveys and whether insect DNA sequence abundance correlates with known butterfly visitation. Comparison of the butterfly community in Glacier National Park from the past survey (1988-1989) to the present (2022-2023) revealed that butterfly species richness and community composition did not change overall compared to 35 years ago, though considerable variation between the two present study years and the presence of distinct butterfly communities in habitats sensitive to climate change warrants further investigation and monitoring. High-quality and abundant insect sequences were obtained from wildflower-associated eDNA samples, and six unique butterfly taxa were identified, five of which were verified as present at the survey sites using traditional netting surveys on the same day as wildflower collection. Traditional survey methods captured 43 additional butterfly species that were not identified by eDNA, and butterflies that were physically placed or observed on the flower before collection were not detected. This work indicates the importance of continued traditional butterfly monitoring efforts while working in parallel with controlled experiments to further develop the protocols to reliably detect butterflies and other insects with flower-associated eDNA.

CHAPTER ONE

INTRODUCTION

The famed ecologist, Edward O. Wilson, once called insects "the little things that run the world" (Wilson, 1987). That, in fact, was the title of an address in 1987 at the National Zoological Park in Washington, D.C. where he ended by saying,

> "A hundred years ago few people thought of saving any kind of animal or plant. The circle of concern has expanded steadily since, and it is just now beginning to encompass the invertebrates. For reasons that have to do with almost every facet of human welfare, we should welcome this new development."

Research in the years since that address has continued to document the value of insects, where it is estimated that 87.5% of flowering plant species and 35% of the world's food production depend upon insect pollination (Ollerton et al., 2011; van der Sluijs & Vaage, 2016). The economic value of the ecosystem services that wild, non-cultivated, insects provide such as dung burial, pest control, wildlife nutrition and pollination is worth $57 billion dollars annually in the U.S. alone (Losey & Vaughan, 2006). However, research in recent decades has also continued to document decline in many insect populations across the globe, with estimates ranging from 20-75% decline in insect abundance, biomass or diversity at both local and global scales (Dalton et al., 2023). The magnitude and scope of insect biodiversity loss is of some debate, with some naming the phenomena an "insect apocalypse" (Jarvis, 2018; Owens et al., 2020) and others calling for a more nuanced view pointing to areas of increase along with declines (van Klink et al., 2020; Crossley et al., 2021). Meanwhile, only a fraction of insect diversity has been described by science, around one million insect species have been named while it is estimated that millions more are undescribed (Stork, 2018), and factors impacting insect populations, such

as land use and climate change, continue to progress (Halsch et al., 2021; Winkler et al., 2021; IPCC, 2023). Thus, insect conservation efforts are working on an issue that is both not fully defined and continually evolving.

In a shifting conservation landscape, methods that can provide expediency in biodiversity and ecosystem assessments are becoming increasingly important. Indicator taxa, those that can be easily monitored and reflect changes in their environment, and novel molecular techniques of identifying species from DNA in the environment (eDNA) can facilitate investigations of community composition in tandem with environmental changes (Siddig et al., 2016; Deiner et al., 2017; Ruppert et al., 2019). Insects have short generation times and are sensitive to abiotic conditions, therefore insect populations may serve as indicators of inter-annual environmental change. Butterflies are relatively easy to capture, handle, and identify by morphological characters in the field compared to other insects (Debinski et al., 2011) and have long been used as indicator taxa, meaning that population changes can reflect environmental change due to their sensitivity to abiotic conditions (Debinski et al., 2013; Gerlach et al., 2013; Hill et al., 2021). In chapter two butterflies were utilized as indicator taxa in a high-elevation, high-latitude ecosystem where impacts of climate change are expected to be high (Hall & Fagre, 2003; Pederson et al., 2010; Pepin et al., 2015). A resurvey effort of butterfly diversity in Glacier National Park, Montana was conducted to assess the 2022 and 2023 butterfly community in comparison to survey data obtained in 1988 and 1989 in the context of climate change.

The same qualities that make butterflies easy to study as ecological indicators are also useful characteristics for testing novel molecular methods of identifying terrestrial insects. The advancement of high throughput sequencing (HTS) of trace DNA from the environment (eDNA)

can provide information about a whole community of organisms, including rare and cryptic species, in a rapid, cost-effective, and non-invasive manner. This utility of eDNA has translated into high demand in contemporary bioassessments, conservation, and management, although methods development and standardization are ongoing (Ruppert et al., 2019; Deiner et al., 2021). While the isolation and sequencing of DNA from the environment has been utilized for microbial investigations for several decades (Ogram et al., 1987), the identification of macro-organisms from eDNA is more recent; amphibians were identified from aquatic eDNA in 2008 (Ficetola et al., 2008). The intervening years have shown an exponential increase in macro-organismal studies using eDNA, for which the majority concern eDNA from freshwater sources, and the transition to terrestrial sources of eDNA is even more recent (Beng & Corlett, 2020). In 2019, wildflowers were used as a source of DNA to detect insects for the first time (Thomsen & Sigsgaard, 2019) indicating the potential for characterizing and monitoring pollinator communities that are critically important to both wild and cultivated systems (Gamonal Gomez et al., 2023; Harper et al., 2023; Avalos et al., 2024). Butterflies are frequent flower visitors (Rader et al., 2016) and their ease of capture and identification provided an opportunity to compare results obtained using traditional surveys, and known butterfly-flower interactions, to those obtained from analyses of wildflower-associated eDNA samples. In chapter three, information from the resurvey effort of butterfly diversity in Glacier National Park was used in comparative analyses to determine if molecular methods can detect butterflies at a similar or better resolution to traditional surveys and whether insect DNA sequence abundance correlates with known butterfly visitation.

Several decades after E.O. Wilson's 1987 address, the "circle of concern" continues to fill in for invertebrates, though the full scope, causes, and future implications of insect decline have yet to be fully understood. In this work, butterflies are utilized as ecological indicators of an ecosystem sensitive to climate change and as indicators for the development of novel molecular genetic techniques of surveying terrestrial insect biodiversity. Together, the following chapters span the past, present, and future of monitoring critically important insects.

References

Avalos, G., Trott, R., Ballas, J., Lin, C. H., Raines, C., Iwanowicz, D., Goodell, K., & Richardson, R. T. (2024). Prospects of pollinator community surveillance using terrestrial environmental DNA metagenetics. *Environmental DNA*, *6*(1), e492. https://doi.org/10.1002/edn3.492

Beng, K. C., & Corlett, R. T. (2020). Applications of environmental DNA (eDNA) in ecology and conservation: Opportunities, challenges and prospects. *Biodiversity and Conservation*, *29*(7), 2089–2121. https://doi.org/10.1007/s10531-020-01980-0

Crossley, M. S., Smith, O. M., Berry, L. L., Phillips-Cosio, R., Glassberg, J., Holman, K. M., Holmquest, J. G., Meier, A. R., Varriano, S. A., McClung, M. R., Moran, M. D., & Snyder, W. E. (2021). Recent climate change is creating hotspots of butterfly increase and decline across North America. *Global Change Biology*, *27*(12), 2702–2714. https://doi.org/10.1111/gcb.15582

Dalton, R. M., Underwood, N. C., Inouye, D. W., Soulé, M. E., & Inouye, B. D. (2023). Long-term declines in insect abundance and biomass in a subalpine habitat. *Ecosphere*, *14*(8), e4620. https://doi.org/10.1002/ecs2.4620

Debinski, D. M., Moranz, R. A., Delaney, J. T., Miller, J. R., Engle, D. M., Winkler, L. B., McGranahan, D. A., Barney, R. J., Trager, J. C., Stephenson, A. L., & Gillespie, M. K. (2011). A cross-taxonomic comparison of insect responses to grassland management and land-use legacies. *Ecosphere*, *2*(12), art131. https://doi.org/10.1890/ES11-00226.1

Debinski, D. M., Caruthers, J. C., Cook, D., Crowley, J., & Wickham, H. (2013). Gradient-based habitat affinities predict species vulnerability to drought. *Ecology*, *94*(5), 1036–1045. https://doi.org/10.1890/12-0359.1

Deiner, K., Bik, H. M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., Creer, S., Bista, I., Lodge, D. M., de Vere, N., Pfrender, M. E., & Bernatchez, L. (2017). Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. *Molecular Ecology*, *26*(21), 5872–5895. https://doi.org/10.1111/mec.14350

Deiner, K., Yamanaka, H., & Bernatchez, L. (2021). The future of biodiversity monitoring and conservation utilizing environmental DNA. *Environmental DNA*, *3*(1), 3–7. https://doi.org/10.1002/edn3.178

Ficetola, G. F., Miaud, C., Pompanon, F., & Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biology Letters*, *4*(4), 423–425. https://doi.org/10.1098/rsbl.2008.0118

Gamonal Gomez, N., Sørensen, D. H., Chua, P. Y. S., & Sigsgaard, L. (2023). Assessing flower-

visiting arthropod diversity in apple orchards through metabarcoding of environmental DNA from flowers and visual census. *Environmental DNA*, *5*(1), 117–131. https://doi.org/10.1002/edn3.362

Garrido-Sanz, L., Senar, M. À., & Piñol, J. (2022). Relative species abundance estimation in artificial mixtures of insects using mito-metagenomics and a correction factor for the mitochondrial DNA copy number. *Molecular Ecology Resources*, *22*(1), 153–167. https://doi.org/10.1111/1755-0998.13464

Gerlach, J., Samways, M., & Pryke, J. (2013). Terrestrial invertebrates as bioindicators: An overview of available taxonomic groups. *Journal of Insect Conservation*, *17*(4), 831–850. https://doi.org/10.1007/s10841-013-9565-9

Hall, M. H. P., & Fagre, D. B. (2003). Modeled Climate-Induced Glacier Change in Glacier National Park, 1850–2100. *BioScience*, *53*(2), 131–140. https://doi.org/10.1641/0006-3568(2003)053[0131:MCIGCI]2.0.CO;2

Halsch, C. A., Shapiro, A. M., Fordyce, J. A., Nice, C. C., Thorne, J. H., Waetjen, D. P., & Forister, M. L. (2021). Insects and recent climate change. *Proceedings of the National Academy of Sciences*, *118*(2), e2002543117. https://doi.org/10.1073/pnas.2002543117

Harper, L. R., Niemiller, M. L., Benito, J. B., Paddock, L. E., Knittle, E., Molano-Flores, B., & Davis, M. A. (2023). BeeDNA: Microfluidic environmental DNA metabarcoding as a tool for connecting plant and pollinator communities. *Environmental DNA*, *5*(1), 191–211. https://doi.org/10.1002/edn3.370

Hill, G. M., Kawahara, A. Y., Daniels, J. C., Bateman, C. C., & Scheffers, B. R. (2021). Climate change effects on animal ecology: Butterflies and moths as a case study. *Biological Reviews*, *96*(5), 2113–2126. https://doi.org/10.1111/brv.12746

IPCC. (2023). *Climate Change 2023: Synthesis Report. Contribution of Working Groups I, II and III to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change* (H. Lee, J. Skea, P. Shukla, P. Zhai, V. Masson-Delmotte, A. Pirani, W. Moufouma-Okia, C. Péan, R. Pidcock, S. Connors, J. B. R. Matthews, M. Chen, Y. Sokona, M. I. Aguilar, C. Mintenbeck, A. Okem, B. Rama, J. Wilcock, W. Maycock, & M. Tignor, Eds.). IPCC. https://doi.org/10.59327/IPCC/AR6-9789291691647

Jarvis, B. (2018, November 27). The Insect Apocalypse Is Here. *The New York Times*. https://www.nytimes.com/2018/11/27/magazine/insect-apocalypse.html

Losey, J. E., & Vaughan, M. (2006). The Economic Value of Ecological Services Provided by Insects. *BioScience*, *56*(4), 311–323. https://doi.org/10.1641/0006-3568(2006)56[311:TEVOES]2.0.CO;2

Ogram, A., Sayler, G. S., & Barkay, T. (1987). The extraction and purification of microbial DNA from sediments. *Journal of Microbiological Methods*, *7*(2), 57–66. https://doi.org/10.1016/0167-7012(87)90025-X

Ollerton, J., Winfree, R., & Tarrant, S. (2011). How many flowering plants are pollinated by animals? *Oikos*, *120*(3), 321–326. https://doi.org/10.1111/j.1600-0706.2010.18644.x

Owens, A. C. S., Cochard, P., Durrant, J., Farnworth, B., Perkin, E. K., & Seymoure, B. (2020). Light pollution is a driver of insect declines. *Biological Conservation*, *241*, 108259. https://doi.org/10.1016/j.biocon.2019.108259

Pederson, G. T., Graumlich, L. J., Fagre, D. B., Kipfer, T., & Muhlfeld, C. C. (2010). A century of climate and ecosystem change in Western Montana: What do temperature trends portend? *Climatic Change*, *98*(1), 133–154. https://doi.org/10.1007/s10584-009-9642-y

Pepin, N., Bradley, R. S., Diaz, H. F., Baraer, M., Caceres, E. B., Forsythe, N., Fowler, H., Greenwood, G., Hashmi, M. Z., Liu, X. D., Miller, J. R., Ning, L., Ohmura, A., Palazzi, E., Rangwala, I., Schöner, W., Severskiy, I., Shahgedanova, M., Wang, M. B., … Mountain Research Initiative EDW Working Group. (2015). Elevation-dependent warming in mountain regions of the world. *Nature Climate Change*, *5*(5), Article 5. https://doi.org/10.1038/nclimate2563

Rader, R., Bartomeus, I., Garibaldi, L. A., Garratt, M. P. D., Howlett, B. G., Winfree, R., Cunningham, S. A., Mayfield, M. M., Arthur, A. D., Andersson, G. K. S., Bommarco, R., Brittain, C., Carvalheiro, L. G., Chacoff, N. P., Entling, M. H., Foully, B., Freitas, B. M., Gemmill-Herren, B., Ghazoul, J., … Woyciechowski, M. (2016). Non-bee insects are important contributors to global crop pollination. *Proceedings of the National Academy of Sciences*, *113*(1), 146–151. https://doi.org/10.1073/pnas.1517092112

Ruppert, K. M., Kline, R. J., & Rahman, M. S. (2019). Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. *Global Ecology and Conservation*, *17*, e00547. https://doi.org/10.1016/j.gecco.2019.e00547

Siddig, A. A. H., Ellison, A. M., Ochs, A., Villar-Leeman, C., & Lau, M. K. (2016). How do ecologists select and use indicator species to monitor ecological change? Insights from 14 years of publication in Ecological Indicators. *Ecological Indicators*, *60*, 223–230. https://doi.org/10.1016/j.ecolind.2015.06.036

Stork, N. E. (2018). How Many Species of Insects and Other Terrestrial Arthropods Are There on Earth? *Annual Review of Entomology*, *63*(Volume 63, 2018), 31–45. https://doi.org/10.1146/annurev-ento-020117-043348

Thomsen, P. F., & Sigsgaard, E. E. (2019). Environmental DNA metabarcoding of wild flowers

reveals diverse communities of terrestrial arthropods. *Ecology and Evolution*, *9*(4), 1665–1679. https://doi.org/10.1002/ece3.4809

van der Sluijs, J. P., & Vaage, N. S. (2016). Pollinators and Global Food Security: The Need for Holistic Global Stewardship. *Food Ethics*, *1*(1), 75–91. https://doi.org/10.1007/s41055-016-0003-z

van Klink, R., Bowler, D. E., Gongalsky, K. B., Swengel, A. B., Gentile, A., & Chase, J. M. (2020). Meta-analysis reveals declines in terrestrial but increases in freshwater insect abundances. *Science*, *368*(6489), 417–420. https://doi.org/10.1126/science.aax9931

Wilson, E. O. (1987). The Little Things That Run the World (The Importance and Conservation of Invertebrates). *Conservation Biology*, *1*(4), 344–346.

Winkler, K., Fuchs, R., Rounsevell, M., & Herold, M. (2021). Global land use changes are four times greater than previously estimated. *Nature Communications*, *12*(1), 2501. https://doi.org/10.1038/s41467-021-22702-2

CHAPTER TWO

SIMILARITIES AND DIFFERENCES IN ALPINE BUTTERFLY COMMUNITY COMPOSITION OVER 35 YEARS

Contribution of Authors and Co-Authors

Manuscript(s) in Chapter(s) [1]

Author: Cayley R. Faurot-Daniels

Contributions: contributed to methodology, data collection and curation, formal analysis, writing – original draft, visualization, funding acquisition

Co-Author: Michelle L. Flenniken

Contributions: writing - original draft preparation, writing - review and editing, visualization, funding acquisition

Co-Author: Diane M. Debinski

Contributions: conceptualization, methodology, formal analysis, visualization, writing - original draft preparation, writing - review and editing, visualization, supervision, project administration, funding acquisition

Manuscript Information

Cayley Faurot-Daniels, Michelle Flenniken, Diane Debinski

Journal of Insect Conservation

Status of Manuscript:
✓ Prepared for submission to a peer-reviewed journal
☐ Officially submitted to a peer-reviewed journal
☐ Accepted by a peer-reviewed journal
☐ Published in a peer-reviewed journal

Springer Nature

Abstract

Declines in insect diversity, abundance and biomass threaten vital ecosystem services that insects provide to both wild and human systems. Butterflies are one of the most well-studied insect groups due to their ease of capture, species identification, and sensitivity to climatic conditions allowing for efficient monitoring to track responses to changing environmental conditions. This study provided a temporal snapshot of a butterfly community in a high latitude, high elevation, protected area where impacts of climate change are expected to be observed. Shifts in butterfly species richness and community composition in Glacier National Park, Montana, were assessed by resurveying of butterfly monitoring sites that were established in 1987 through 1989 and resurveyed in 2022 and 2023. Butterfly species richness and community composition did not change overall compared to 35 years ago. Butterfly species richness was lower in 2022 than all other years but similar in 2023 compared to the past study years. Distinct butterfly communities were found in different habitat types across the park in all study years, with mesic meadows supporting the highest number of species. With considerable variation between the two present study years, additional years of monitoring may be useful in identifying an overall trend with more confidence while also tracking distinct communities that are likely to shift with increasing stress from climate change.

Implications for Conservation

In a world of changing climate and land use, protected areas can serve as refugia for plants and animals and provide the opportunity to disentangle impacts of climate change from land use change. Using the same methods, sampling locations, and sampling effort as the past survey provided us with the opportunity for a robust comparison of a butterfly community at two

temporal snapshots three and half decades apart and provided a critical contemporary baseline for future monitoring in an area particularly sensitive to climate change.

Introduction

Of all the animal species that have been described on earth, more than half are insects, with millions more estimated to be awaiting discovery (Mora et al., 2011; Stork, 2018). Insects are a diverse group of organisms that provide vital ecosystem services including plant pollination, decomposition, biocontrol, and as a food resource to other organisms (Losey & Vaughan, 2006; Rader et al., 2016). Declines in insect diversity, abundance and biomass have been reported across the globe and these losses threaten vital ecosystem services that insects provide to both wild and human systems (Dirzo et al., 2014; Hallmann et al., 2017; Forister et al., 2019). Land use and climate change are key drivers in insect diversity and abundance loss (Forister et al., 2010; Halsch et al., 2021; Uhler et al., 2021). As of 2019, over a third of global land area had been affected by land use change, and global temperatures under current implemented policies for greenhouse gas emissions are predicted to warm by 3.2°C by 2100, well above the 1.5°C degree threshold set by the Paris Climate Agreement (Winkler et al., 2021; IPCC, 2023).

In a world of changing climate and land use, protected areas can serve as refugia for plants and animals and aid in conservation of biodiversity (Selwood & Zimmer, 2020; Mi et al., 2023;). Studies in protected areas also can serve as a baseline, providing the opportunity to disentangle the impacts of climate change from land use change, which is information that can enhance conservation efforts (Forister et al., 2021). While the overall global temperature rise is a commonly referenced estimate, temperatures are not expected to increase uniformly across the

globe. High latitude and high elevation areas are warming at faster rates than lower latitudes and elevations (Pepin et al., 2015; Pepin et al., 2022; Rantanen et al., 2022). Therefore, monitoring of insect populations in high latitude and high elevation protected areas can be particularly useful as bellwethers of ecosystem change in areas more sensitive to climate change.

As the most diverse animal group on earth, insects are difficult to study. Major criticisms of recent meta-analyses of changes to insect populations on the global or large regional scale arise from difficulties in collating studies with varying methods, sampling effort, and scale in space and time (Wagner, 2020; Welti et al., 2021), meaning studies that can replicate methods and sampling effort across time are particularly valuable. Given the difficulty in assessing many insect species and that the full breadth of insect diversity is yet to be described for many taxa, single well-studied taxa are often used as ecological indicators. Butterflies are one of the most well-studied insect groups due their ease of capture, species identification, and sensitivity to climatic conditions (Thomas, 2005; Debinski et al., 2011a; Gerlach et al., 2013; Hill et al., 2021) allowing for efficient monitoring to track responses to changing environmental conditions. Declines in butterfly diversity and abundance have been documented in Europe (Warren et al., 2021) and in North America (Wepprich et al., 2019; Forister et al., 2021) over the past several decades though areas of increases have also been observed in North America (Crossley et al., 2021). Butterfly responses to climate change also have been documented along ecological gradients, including shifts upward in elevation in concert with increasing temperatures (Molina-Martínez et al., 2016; Kerner et al., 2023) and shifts in abundance along hydrological gradients, where species adapted to xeric (dry) habitats showed increases in response to drought whereas species adapted to hydric (very wet) habitats decreased. (Debinski et al., 2013). Quantifying

butterfly species distribution changes across time and space can therefore allow us to document the impacts of climate change.

Glacier National Park in Northwest Montana, USA is a high elevation, high latitude protected area with a long history of monitoring climate change. The area of western Montana, including Glacier National Park, is warming at two times the global rate (Pederson et al., 2010; Whitlock et al., 2017), and documented declines of glaciers and perennial snow of over 80% since 1850 have been attributed to warming temperatures (Pederson et al., 2010). Glacier National Park is also home to a diverse butterfly community with 116 butterfly species documented historically in the park (Chinn, 2014). Natural ecological gradients of elevation and moisture exist, where elevation ranges from 980 meters to 3,190 meters and meadows range in moisture from very wet (hydric), to moderately wet (mesic) and dry (xeric), allowing hypotheses of butterfly species richness and community change to be tested along these ecological gradients.

Temporal shifts in butterfly species richness and community in Glacier National Park were assessed through resurveying of butterfly monitoring sites that were established in a study conducted from 1987 through 1989 (Debinski & Brussard, 1994; Garzella & Debinski, 2020). We resurveyed these monitoring sites 35 years later in 2022 and 2023 using the same methods and with one of the same investigators as in the earlier surveys. In this study, we sought to test whether butterfly species richness and community composition has changed over time in the context of documented climate change. We hypothesized that a comparison between the 2022-2023 to 1988-1989 survey data, would indicate 1) a decline in species richness, 2) community composition differences at a parkwide scale, 3) an increase in species richness at higher elevations, and more specifically 4) an increase in species richness at alpine meadows, decrease

at mesic meadows, and increase or no change at xeric meadows. To facilitate future monitoring efforts, quantitative vegetation surveys were added to the present study which allowed testing of an additional hypothesis 5) that butterfly species richness would be positively correlated with plant richness and total floral resources in a site. One family of butterflies, Hesperiidae (skippers), was not assessed in the past study but was included in the present study. While skippers are not quantitatively assessed herein, they are discussed separately and included in the raw data to provide a full baseline assessment of all butterfly species found presently. Using the same methods, sampling locations, and sampling effort as the past survey provided us with the opportunity for a robust comparison of a butterfly community at two temporal snapshots three and half decades apart and provided a critical contemporary baseline for future monitoring in an area particularly sensitive to climate change.

Methods

Study Area

Butterfly diversity was surveyed in Glacier National Park, Montana, U.S.A which, established in 1910, protects over 4,000 square kilometers immediately south of the U.S. – Canadian border in Northwest Montana. Butterfly diversity monitoring sites were established in a study from 1987 through 1989, hereafter referred to as 'past' study, where 24 sites ($1km^2$ in size) were selected along an elevation and moisture gradient to capture the butterfly community across the park, and in varying habitats and elevations (Debinski & Brussard, 1994; Garzella & Debinski, 2020). Higher elevation site types were characterized as 'sub-alpine', 'alpine', or 'high-alpine', ranging from 1,632 to 2,251 meters (m), whereas lower elevation sites, ranging from 981 m to 1422 m were also characterized by moisture; 'hydric', meadows have standing

water throughout the summer season, ‘mesic’, meadows are wet in the early season and dry in later season, ‘xeric’, meadows are dry throughout the summer season, and ‘riparian’, are areas of mixed vegetation with trees, shrubs, grasses and forbs along a river corridor. The elevation of all sites ranged from 981 m to 2,251 m. There were 24 sites from the past study designated as butterfly monitoring sites, and nine fall into the greater alpine category (four high-alpine, three alpine and two sub-alpine). The two sub-alpine sites were redesignated in the present analysis as alpine and high alpine based on elevation and treeline, resulting in two alpine categories where high alpine included five sites above treeline (> 2,000 m) and alpine included four sites at treeline (1,600 - 2,000 m). Seven sites were characterized as mesic, four xeric and one each of hydric and riparian (Figure 1). These butterfly monitoring sites were resurveyed for butterfly diversity in 2022 and 2023, hereafter referred to as ‘present’ study; 23 of the 24 original sites were resurveyed. One site was not suitable for survey in the present study due to dense post-fire regrowth (named Route 8 Burnscar in past study).

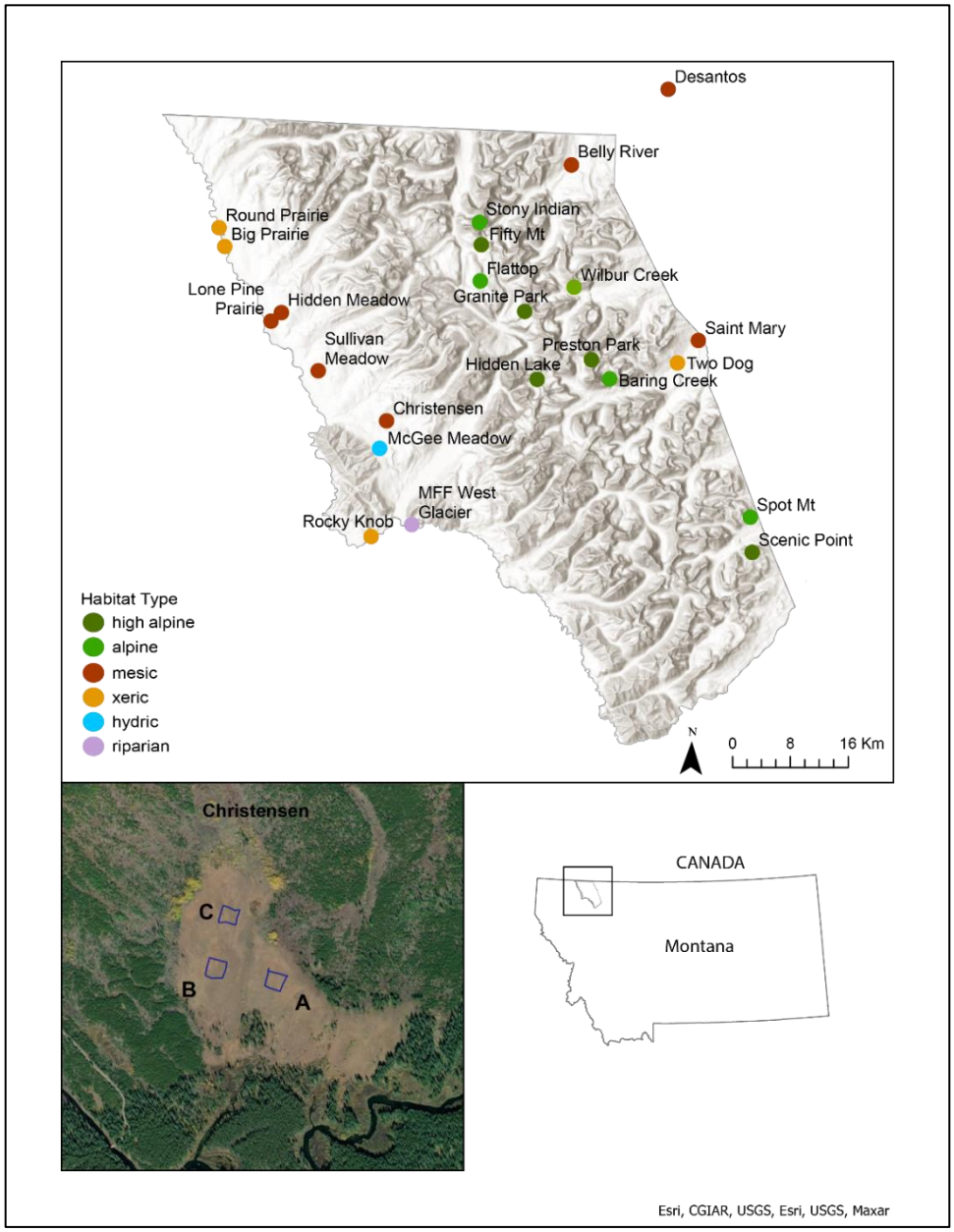


Fig 1. Butterfly monitoring sites in Glacier National Park, Montana. Butterfly diversity was resurveyed in 2022 and 2023 at 23 sites across Glacier National Park that were surveyed with the same methods in 1988 and 1989. One site (Desantos) was located outside of Glacier National Park on private land near Lee Creek in Alberta, Canada. Each site was surveyed in three 50-meter by 50-meter plots, except for McGee Meadow and MFF West Glacier, linear habitats which were surveyed as three transects. An example of three plot locations within a site is shown from Christensen meadow with GPS-tracked outlines of the plot perimeters. Sites were arrayed along an elevation gradient (985m to 2,251m) and habitat types. Alpine sites were at tree line, high alpine sites were above tree line, mesic sites were characterized by moderate moisture, and xeric sites were characterized by low moisture.

The 1 km^2 sites were sampled for butterflies using three 50-meter by 50-meter plots. While the historic 1 km^2 site locations were identified on 7.5-minute quadrangle maps, plot locations from the past study were not available because accurate GPS locations were not widely accessible in 1987. As such, site locations for the present survey were determined using locations from the 7.5-minute quadrangle maps and descriptions from the past survey (Garzella & Debinski, 2020) and precise GPS locations of each plot were established during the 2022 field season. Three 50 m by 50 m plots were set with perimeters 70-100 m apart in each site. Each plot was marked with a waypoint that became the bottom-left corner of the plot and 50-meter tapes were then run following 90-degree bearings from the waypoint. Variable topography and meadow edges made it difficult to have one rule for plot orientation across sites, however plot orientation was consistent for all three plots within a site whenever possible (Supplemental Table 1).

Field Methods

Butterfly Surveys. Butterfly species were captured during timed aerial netting surveys of 30 person-minutes during ideal conditions for butterfly movement; between the hours of 09:30 and 16:30, when ambient air temperature was between 21° to 32° C, the sun was unobscured by clouds, and when wind speed was less than 16 km/h. In the past study, timed surveys were either 15 minutes if there were two observers in one plot, or 30 minutes if there was one observer in the plot. In the present study, 30-minute surveys with one observer were consistent for all surveys. Two surveys for each site were attempted in each survey year, targeting two major emergence periods (Debinski & Brussard, 1994), though weather conditions did not allow for this in all

sites. All surveys and their associated dates analyzed in this study are listed in Supplemental Table 2.

During the past and present timed netting surveys, butterflies were captured and placed in glassine envelopes and the time of capture was noted. In the present surveys, the behavior of the individual when captured (F: flying, N: nectaring, O: ovipositing, C: chasing or R: resting) and the associated substrate or plant genus, if other than flying, was recorded in addition to the time. In both past and present surveys, species richness estimates rather than abundance estimates were the goal, so a species that had not yet been captured during a particular survey was targeted for capture rather than species that had already been caught. Timed surveys were conducted in each plot and at the end of all plot surveys, individual butterflies were either identified and released or collected as voucher specimens. Physical specimens of some butterflies were retained as vouchers in the past surveys, though this associated collection was not retained in one location by the authors and the majority of the specimens were not accessible as a reference for this study. With the availability of digital photography in the present study, photo vouchers were taken of all species and individuals for all but the most common and easily identified species; 78% of all butterfly individuals were photographed. Individuals were photographed on dorsal and ventral sides for later identification, then released. No voucher specimens were collected.

Floral Resources. In the past biodiversity surveys, floral resources were not assessed from a quantitative perspective, but plant species in flower were noted in the comment section of the data sheet. For the present surveys, floral resources were assessed twice during each summer sampling season on the same visit as butterfly surveys. A 60-meter diagonal transect was run at a 45-degree bearing from each subplot waypoint (Supplemental Table 1) and counts of each

flowering species were conducted. Each transect was divided into three 20-meter sections and all reproductive ramets within one meter of the right side of the transect were counted (modified from Lyon et al., 2021). Reproductive ramets were generally counted as flowering stalks originating from the soil, with some exceptions; when stalks originating from soil were too difficult to distinguish, flower heads were counted as the reproductive unit. Definitions of the unit of flowering ramet counted for each plant genus are listed in Supplemental Table 3. While identifications to species were made whenever possible, analysis of floral resources was conducted at the genus level to ensure accuracy. The total floral resources for each genus at the site were summed across the three 20-meter sections of transects and the three transects for each site survey.

Butterfly Identification and Naming. Butterflies caught during the present surveys were identified based on morphological characters either in the field or from photo vouchers of the dorsal and ventral sides. Several taxonomic revisions have taken place since the past study (Zhang et al., 2020, 2022) and scientific names for data related to both studies were updated to present accepted taxonomy in accordance with The Butterflies of America online catalogue (Warren et al., 2024). To mitigate uncertainty in identifications between past and present studies of species that are difficult to distinguish in the field, several species were combined for analysis. Any individuals that were identified as either *Euphydryas anicia* or *Euphydryas editha* were combined into the species name *Euphydryas anicia_editha*, two species of *Phyciodes*, *Phyciodes cocyta* and *Phyciodes tharos* were combined into *Phyciodes_cocyta_tharos*, two *Plebejus* species, *Plebejus idas* and *Plebejus melissa* were combined into *Plebejus_idas_melissa*, two species of *Tharsalea*, *Tharsalea dorcas florus* and *Tharsalea helloides* were combined into

Tharsala_florus_helloides, and finally, *Argynnis atlantis* and *Argynnis hesperis* were combined into *Argynnis_atlantis_hesperis*. This meant that our species richness tallies were slightly underestimated, but it reduced the potential of taxonomic issues confounding our assessment of changes in the butterfly species composition.

Site Related Growing Degree Days

Site-related temperature data were obtained from the Oak Ridge National Laboratory DAYMET Version 4 data, which has daily surface climate variables estimated on a $1km^2$ grid scale for North America from 1980 to present day (Thornton et al., 2021). For all sites, the coordinates of plot B were used as the central coordinates representing the $1km^2$ butterfly monitoring plot (Supplemental Table 1) because plot B was either the most central plot in the site or no one plot was more central than the other two plots. The daily minimum and maximum temperatures (° C) related to these site coordinates were accessed and summarized using Google Earth Engine (Gorelick et al., 2017). To facilitate comparisons of conditions affecting butterfly emergence between studies, the cumulative growing degree days (cGDD), defined as the sum of daily mean temperatures above a threshold 4.4° C, was calculated from March 15^{th} of the survey year to each survey date of the study (Supplemental Table 2). March 15^{th} was determined by the authors as a date typically associated with spring warm-up in the study area.

Filtering Survey Data for Even Comparisons Between Studies

To compare even effort between past and present studies, two of the three past study years (1988 and 1989) were considered for analysis to match the two years of the present study (2022 and 2023); 1987 was a pilot year and past study notes indicated the season started late and

may have missed early season butterflies (Debinski & Brussard, 1994). Two full surveys, where a full survey was defined as three timed butterfly surveys in three 50-meter by 50-meter plots, were attempted for each site in each survey year to target two peaks in butterfly emergence: Survey 1 = early summer (~June 1st ~ July 15th) and Survey 2 = late summer (~July 1st ~ August 31st). The two survey periods overlap due to differences in seasonal conditions along the elevation gradient. While equal survey effort at each site was the goal in each study year, seasonal conditions or other factors affecting timing created uneven surveys in some cases and in the past study some sites had more than two surveys. To ensure even comparisons between studies as much as possible, surveys were "filtered" out from both studies to ensure equal survey effort in each of the comparisons. Filtering of survey data was first based on survey conditions noted in the survey metadata; for instance, if there were more than two surveys for a site from the past study, a survey was removed if the ambient temperature, wind speed, or cloud cover was noted to be outside the ideal survey condition parameters (between the hours of 09:30 and 16:30, ambient air temperature between 21° C and 32° C, sun unobscured by clouds, and wind speed less than 16 kmh). In cases where a site could not be surveyed for a second time in one survey year, the second survey was removed from all other years to create even effort comparisons. Three high alpine sites were not surveyed in late summer (Survey 2) in either 1989 or 2022 (Stony Indian, 50 Mt., and Hidden Lake). Two other alpine sites were not surveyed twice in each year; Flattop was only surveyed once (early summer) in 1988, 1989, and 2022, and 2023 was the only year it was surveyed twice; Preston Park was surveyed twice in both years of the present study but only once (early summer) in 1989. One mesic site, St. Mary, was only surveyed once in 1988 and twice in the other three years, and the riparian site, MFF West Glacier, was not

surveyed at all in 1989. Therefore, to conduct analyses using equivalent survey effort between years and sites, surveys with incomplete replication were removed prior to data analysis resulting in 22 early summer surveys and 16 late summer surveys from each survey year included in analysis (Supplemental Table 2).

Statistical Analysis

Butterfly survey data were converted to binary presence (1) or absence (0) rather than abundance for all analyses. While multiple individuals of the same species may have been caught during butterfly surveys and a relative abundance may have been estimated, surveys were designed to target new species to the survey rather than estimate abundance. Given the uncertainty in how multiple individuals of the same species were targeted (or not) between the past and present surveys, binary species data was the most appropriate for these analyses. All analyses were conducted in R version 4.3.2 (R Core Team, 2023), data manipulation was done using tidyverse (Wickham et al., 2019) and graphics were created using ggplot2 (Wickham, 2016), unless otherwise noted.

Species presence values were combined across plots and surveys for each site of each study year. Species richness (the number of unique species), at the site level for each year was calculated using the specnumber() function in the vegan package (Oksanen et al., 2022). Comparisons of species richness and cGDD between the same sites at different time points were tested with paired t-tests using the rstatix package (Kassambara, 2023). The effects of habitat type, year, and elevation on species richness were tested using generalized linear mixed effects models (glmer) including all study years. Elevation values were standardized to have a mean of zero and standard deviation of one. To test whether species richness varied by year across the

elevation gradient and habitat types, generalized linear mixed effects models were used to test the effects of an interaction of year and elevation (Model 1: *Species richness* ~ YEAR + ELEVATION + YEAR x ELEVATION) and, separately, year and habitat type (Model 2: *Species richness* ~ YEAR + HABITAT + YEAR x HABITAT) on observed species richness values from each site and year. The models were fit with a random effect for site (LOCALITY) and a Poisson distribution with a log-link function. Habitat type is related to elevation, so these models were run separately. Two additional models were fit to the data that did not assume any interactions of habitat or elevation across years, again with habitat and elevation fit separately (Model 3: *Species richness* ~ YEAR + ELEVATION and Model 4: *Species richness* ~ YEAR + HABITAT). Model fit was assessed by comparing Akaike Information Criterion (AIC) scores.

Floral resources were assessed quantitively for the present study only, so models including plant generic richness (i.e., richness at the genus level) and total floral resources were conducted separately for present study years (2022 and 2023) using generalized linear models (glm). Counts of the total number of reproductive ramets (TotalInf) and the number of unique plant genera (VegGenRich), of each site were included as predictors in a generalized linear model of butterfly species richness data from the two present study survey years, 2022 and 2023, where floral resources were identified to plant genus and quantitatively assessed (counts of reproductive ramets) at each survey visit. Continuous predictors were standardized to have a mean of zero and standard deviation of one. The model was fitted with a Poisson distribution and log-link function (*Species richness* ~ YEAR + HABITAT + TotalInf + VegGenRich). Models were conducted using the glmer() and glm() functions in the lme4 package (Bates et al., 2015) and effects plots were generated using ggeffects (Lüdecke, 2018).

Changes in the butterfly community over time were assessed using temporal beta diversity indices (TBI). Jaccard dissimilarity coefficients among sites between two time points (T1 and T2) were used as the TBI indices and found using the TBI() function in the adespatial package (Dray et al., 2023). Temporal beta diversity indices are composed of two parts, B = species gains and C = species losses, and the mean of differences between B and C across all sites in the study area were tested for significance using paired permutation t-tests (Legendre & Condit, 2019). Differences in species gains and losses for each site were visualized in 'B-C plots'; TBI indices and plots were assessed between all pairwise comparisons of the four study years.

Associations of the butterfly community from each year with site habitat types were assessed using canonical correspondence analysis (CCA) in vegan. The choice of CCA rather than redundancy analysis (RDA) was supported by results of a detrended correspondence analysis (DCA) and the length of the first DCA axis, where a length less than three suggests a linear relationship of species data with environmental predictors leading towards RDA analysis, a length greater than four suggests a unimodal relationship of species data to gradients of environmental predictors leading towards CCA analysis, and a length in between three and four indicates either analysis can be used (Borcard et al., 2011). Species data from three of four years had a DCA axis 1 length greater than three, the exception was 1989, so CCA was used for all years for consistency (Supplemental Table 4). Significance of constraining predictors were tested using post-hoc permutation tests by the anova.cca() function in vegan.

Results

Butterfly Species Lists from Past (1988-1989) and Present (2022-2023) Studies in Glacier National Park

A similar number of species were found between past (1988-1989) and present (2022-2023) surveys across 23 sites in Glacier National Park, Montana, but several were unique to each survey (Table 1). Butterfly species lists were created from all surveys from the 23 sites in the four years of study (i.e. prior to filtering of surveys to compare results between years); 74 species were identified in the past survey, 72 species in the present survey and 63 species were found in both studies. Eleven species were only found in the past survey, including 16 *Boloria chariclea* (Arctic Fritillary) individuals, nine *Nymphalis vaualbum* (Compton Tortoiseshell) individuals and four *Celastrina* (Azures) individuals, all found in both 1988 and 1989. An additional eight species unique to the past survey were found in either 1988 or 1989, but not both years. There were nine butterfly species unique to the present survey, but only *Colias christina* (Christina Sulphur) was found in both 2022 and 2023 (four individuals). Four individuals of *Chlosyne damoetas* (Rockslide Checkerspot) were found in 2023 only and the one to two individuals of the remaining six species were found either in 2022 or 2023. A similar number of species were found in each year, with 63 species captured in 1988, 60 species in 1989, 59 species in 2022, and 65 species in 2023. The total number of butterflies caught were higher in 2023 than any other year, with 1,068 individuals caught in 2023, 663 in 2022, 747 individuals in 1989 and 683 individuals in 1988. In the present study, an additional 13 species of skippers (Hesperiidae) were captured and identified between the 2022 and 2023 survey years (Supplemental Table 5), though skippers are not included in further analysis because this family was not identified during surveys in the past study.

Table 1. Butterfly species between past (1988-1989) and present (2022-2023) surveys in Glacier National Park, Montana. Butterfly species lists were created from all surveys (before filtering for even effort) from the 23 sites in the four years of study and after combining species that were difficult to distinguish in the field. Values indicate the number of individuals captured in each year of study. The total number of species and the total number of individuals across all species for each year of study are given at the top of the table, with species lists for each year following. Values depict the number of individuals captured across all sites in the study year. The list is ordered first by species that were unique to the past survey, then by species unique to the present survey, and species that were shared between both past and present are ordered by decreasing number of individuals in 1988.

			1988	**1989**	**2022**	**2023**
		total species	**63**	**60**	**59**	**65**
		total individuals	**683**	**747**	**663**	**1068**
Species Unique to Past	1	*Boloria chariclea*	13	3	0	0
	2	*Nymphalis vaualbum*	8	1	0	0
	3	*Celastrina argiolus*	3	1	0	0
	4	*Argynnis aphrodite*	0	3	0	0
	5	*Limenitis weidemeyerii*	2	0	0	0
	6	*Limenitis arthemis*	0	2	0	0
	7	*Oeneis uhleri*	2	0	0	0
	8	*Vanessa annabella*	2	0	0	0
	9	*Boloria astarte*	0	1	0	0
	10	*Satyrium saepium*	0	1	0	0
	11	*Neophasia menapia*	1	0	0	0
Species Unique to Present	12	*Chlosyne damoetus*	0	0	0	4
	13	*Colias christina*	0	0	2	2
	14	*Argynnis coronis*	0	0	0	2
	15	*Colias alexandra*	0	0	0	2
	16	*Tharsalea editha*	0	0	0	2
	17	*Euptoieta claudia*	0	0	2	0
	18	*Argynnis edwardsii*	0	0	2	0
	19	*Callophrys augustinus*	0	0	1	0
	20	*Polygonia oreas*	0	0	0	1
Species Shared Past and Present	21	*Cercyonis oetus*	42	36	35	36
	23	*Plebejus idas_melissa*	40	13	19	57
	24	*Colias interior*	33	22	13	22
	25	*Pontia occidentalis*	30	40	32	53
	26	*Argynnis atlantis_hesperis*	30	19	10	27

Table 1 Continued

Species Shared Past and Present	27	*Aglais milberti*	28	21	6	18
	28	*Parnassius smintheus*	28	14	31	13
	29	*Icaricia saepiolus*	27	29	4	15
	30	*Euphydryas anicia_editha*	26	29	25	68
	31	*Coenonympha california*	25	29	28	25
	32	*Erebia epipsodea*	18	31	50	38
	33	*Phyciodes cocyta_tharos*	17	24	73	116
	34	*Phyciodes pulchella*	15	38	27	65
	35	*Vanessa cardui*	15	2	0	16
	36	*Boloria epithore*	14	15	11	2
	37	*Argynnis zerene*	14	1	1	1
	38	*Pieris marginalis*	12	14	14	7
	39	*Tharsalea mariposa*	11	1	10	11
	40	*Chlosyne palla*	10	12	8	30
	41	*Polygonia faunus*	10	10	4	1
	42	*Argynnis hydaspe*	10	3	2	1
	43	*Limenitis lorquini*	10	1	2	3
	44	*Pieris rapae*	9	6	2	2
	45	*Tharsalea heteronea*	8	10	16	19
	46	*Agriades glandon*	8	2	8	19
	47	*Papilio rutulus*	7	44	5	8
	48	*Colias eriphyle*	7	41	22	114
	49	*Nymphalis antiopa*	7	5	4	1
	50	*Oeneis chryxus*	6	16	5	1
	51	*Callophrys eryphon*	6	16	2	1
	52	*Euchloe ausonides*	6	12	8	9
	53	*Polygonia gracilis*	6	3	1	4
	54	*Icaricia acmon*	6	0	6	27
	55	*Papilio eurymedon*	5	19	6	6
	56	*Papilio zelicaon*	5	17	3	5
	57	*Boloria selene*	5	8	7	27
	58	*Tharsalea florus_helloides*	5	0	12	28
	59	*Cercyonis pegala*	4	9	21	22
	60	*Nymphalis californica*	4	0	0	1
	61	*Colias eurytheme*	3	47	7	10
	62	*Colias meadii*	3	2	12	15
	63	*Cupido amyntula*	3	2	4	5
	64	*Polygonia satyrus*	3	2	1	1

Table 1 Continued

Species Shared Past and Present	65	*Tharsalea nivalis*	3	0	6	1
	66	*Lycaena cupreus*	3	0	1	2
	67	*Euphydryas gillettii*	2	6	0	2
	68	*Icaricia icarioides*	1	26	45	45
	69	*Argynnis egleis*	1	3	4	1
	70	*Anthocharis sara*	1	2	2	0
	71	*Colias nastes*	1	1	2	1
	72	*Tharsalea hyllus*	1	0	3	10
	73	*Callophrys sheridanii*	1	0	1	0
	74	*Parnassius clodius*	1	0	1	0
	75	*Euphilotes ancilla*	1	0	0	2
	76	*Glaucopsyche piasus*	1	0	0	2
	77	*Glaucopsyche lygdamus*	0	4	5	3
	78	*Pterourus canadensis*	0	4	3	8
	79	*Satyrium titus*	0	4	0	2
	80	*Argynnis cybele*	0	1	2	1
	81	*Vanessa atalanta*	0	1	1	0
	82	*Lycaena phlaeas*	0	1	0	4
	83	*Callophrys polios*	0	1	0	1

Growing Degree Days Associated with Each Survey Between Past and Present Studies

To compare the conditions of each survey between years, the site-related cumulative growing degree days (cGDD) between March 15th of each survey year up until the survey date were calculated and assessed (Supplemental Figure 1) (Cayton et al., 2015). The mean difference in cGDD between paired sites of Survey 1 was different between consecutive years of each study period (1988 v 1989 paired t-test: $t = 5.11$, df = 21, Bonferroni adjusted p-value < 0.001 and 2022 v 2023 paired t-test: $t = -3.61$, df = 21, Bonferroni adjusted p-value = 0.010) and between 1989 and 2023 (paired t-test: $t = -3.49$, df = 21, Bonferroni adjusted p-value = 0.013). There was no difference between survey years of the past study and 2022 or between 1988 and 2023 (Bonferroni adjusted p-values for remaining comparisons > 0.100). For Survey 2, the mean

difference in cGDD of paired sites was different between 1988 and 1989 (t = 9.27, df = 15, Bonferroni adjusted p-value < 0.001) but there was no difference between any other survey years (Bonferroni adjusted p-values for remaining comparisons > 0.100). All statistics for pairwise comparisons are in Supplemental Table 6.

Butterfly Species Richness and Community at the Site Level Between All Survey Years

To investigate whether butterfly species richness changed overall between the past and present survey years (Hypothesis 1), the number of unique species found at each monitoring site (excluding MFF West Glacier) across surveys within each survey year were compared (Figure 2). The mean difference in species richness of sites did not differ between 1988 and 1989 (paired t-test: t = 0.098, df = 21, Bonferroni adjusted p-value = 1) or between the past survey years with 2023 (1988 v 2023: t = 0.490, df = 21, Bonferroni adjusted p-value = 1, 1989 v 2023: t = 0.401, df = 21, Bonferroni adjusted p-value = 1). The mean difference in species richness between paired sites in 2022 differed from 1988 and 1989 (1988 v 2022: t = 4.280 df = 21, Bonferroni adjusted p-value = 0.002; 1989 v 2022: t = 3.860, df = 21, Bonferroni adjusted p-value = 0.005). Species richness of paired sites in the two present study years, 2022 and 2023, also differed from each other (t = -3.250, df = 21, Bonferroni adjusted p-value = 0.023) (Supplemental Table 7). Overall, the mean species richness was less than 10 in 2022 and ranged from 12 to 14 in other years.

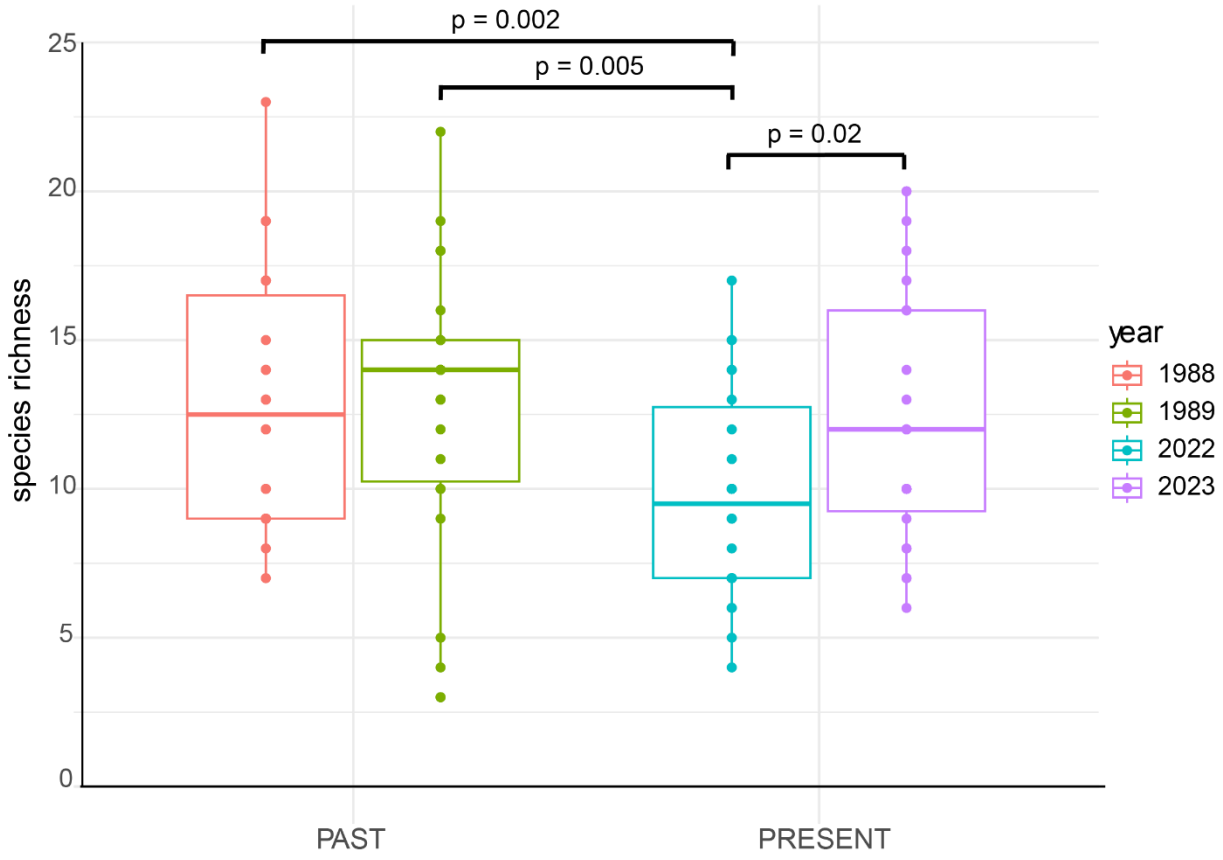


Fig 2. Site level species richness for each year of study. Butterfly species richness was less in 2022 compared to past study years 1988 and 1989, but not in 2023. Butterfly species were captured and identified at 23 sites in four survey years, 1988 and 1989 (PAST study) and 2022 and 2023 (PRESENT study). Butterfly species richness at each site was plotted as the number of butterfly species found across surveys within each year of both studies. One site, MFF West Glacier, was removed because it was not surveyed in all years. The mean difference in species richness of paired sites did not differ between 1988 and 1989 or between the past survey years with 2023 (Bonferonni adjusted p-values = 1 for all comparisons). The mean difference in species richness between paired sites in 2022 differed from both past study years (Bonferroni adjusted p-values < 0.001) and between 2023 (Bonferroni adjusted p-value = 0.0230).

Table 2: Species richness for each site across four years of study. Butterfly species richness values associated with each of 22 sites included in analysis comparing four years of study, 1988 and 1989 from the past study, and 2022 and 2023 in the present study. The table is ordered in decreasing values of species richness in 1988. Species richness values are based on all unique species found from three plots surveyed at each site and two temporal replicates at each site and year, except for those listed in Supplemental Table 2 where a late summer survey was not accomplished and only one survey in the year was considered.

	Locality	**Habitat**	**1988**	**1989**	**2022**	**2023**
1	Belly River	mesic	23	19	17	18
2	Christensen	mesic	19	22	15	20
3	Spot Mt	alpine	19	18	15	12
4	Baring Creek	alpine	17	13	7	8
5	Granite Park	high alpine	17	14	11	6
6	Hidden Meadow	mesic	17	14	6	16
7	Big Prairie	xeric	15	10	9	12
8	Lone Pine	mesic	14	14	14	19
9	Two Dog	xeric	14	12	10	9
10	Sullivan	mesic	13	16	13	20
11	Wilbur Creek	alpine	13	14	14	16
12	Flattop	alpine	12	3	7	10
13	Stony Indian	alpine	12	13	10	17
14	Desantos	mesic	10	18	9	12
15	Scenic Point	high alpine	10	14	7	10
16	Hidden Lake	high alpine	9	10	12	13
17	McGee	hydric	9	11	7	7
18	Preston Park	high alpine	9	5	4	14
19	Rocky Knob	xeric	9	15	10	10
20	St Mary	mesic	9	9	8	7
21	50Mt	high alpine	8	4	6	8
22	Round Prairie	xeric	7	15	5	10

To assess whether there were differences in the butterfly community composition between past and present survey years (Hypothesis 2), temporal beta diversity indices between each site and year were calculated as the differences in species gains (C) and species losses (B) between the same sites at two different points in time (years) (Figure 3). Estimates of B and C were based on Jaccard dissimilarity coefficients between sites and the mean differences between B and C were tested with paired permutation t-tests for each year comparison (Table 4). The

mean difference in butterfly species C (gains) – B (losses) from 22 sites was different from zero in all comparisons with 2022. Species losses dominated gains for sites in 2022 compared to both past study years (1988 v 2022: Mean C-B = -0.181, t = -4.574, p-value = 0.004; 1989 v 2022: Mean C-B = -0.155, t = -3.083, p-value = 0.004) and species gains dominated losses in 2023 compared to 2022 (Mean C-B = 0.155, t = 3.083, p-value = 0.006). Temporal beta diversity did not differ between any other year comparisons (p-values > 0.10) (Table 3). At the site level, several sites had either consistent gains or losses in comparisons between years (Supplemental Table 8). Between all year comparisons, species gains were greater than losses at Hidden Lake, a high alpine site, and species losses were greater than gains at Granite Park, an alpine site. Baring Creek, another alpine site, was characterized by species losses in all comparisons between present and past years, and gains were only greater than losses when comparing 2022 and 2023. When comparing 2023 to the two past study years, which were similar in species richness comparisons, Preston Park, Stony Indian, 50 Mt., and Wilbur Creek (all alpine or high alpine sites) and two mesic sites, Sullivan and Lone Pine, had species gains greater than losses.

Because there was a difference in species richness and community composition in one present study year (2022) compared to the two past study years, the effects of habitat type and elevation on species richness across years were investigated to address hypotheses 3 and 4.

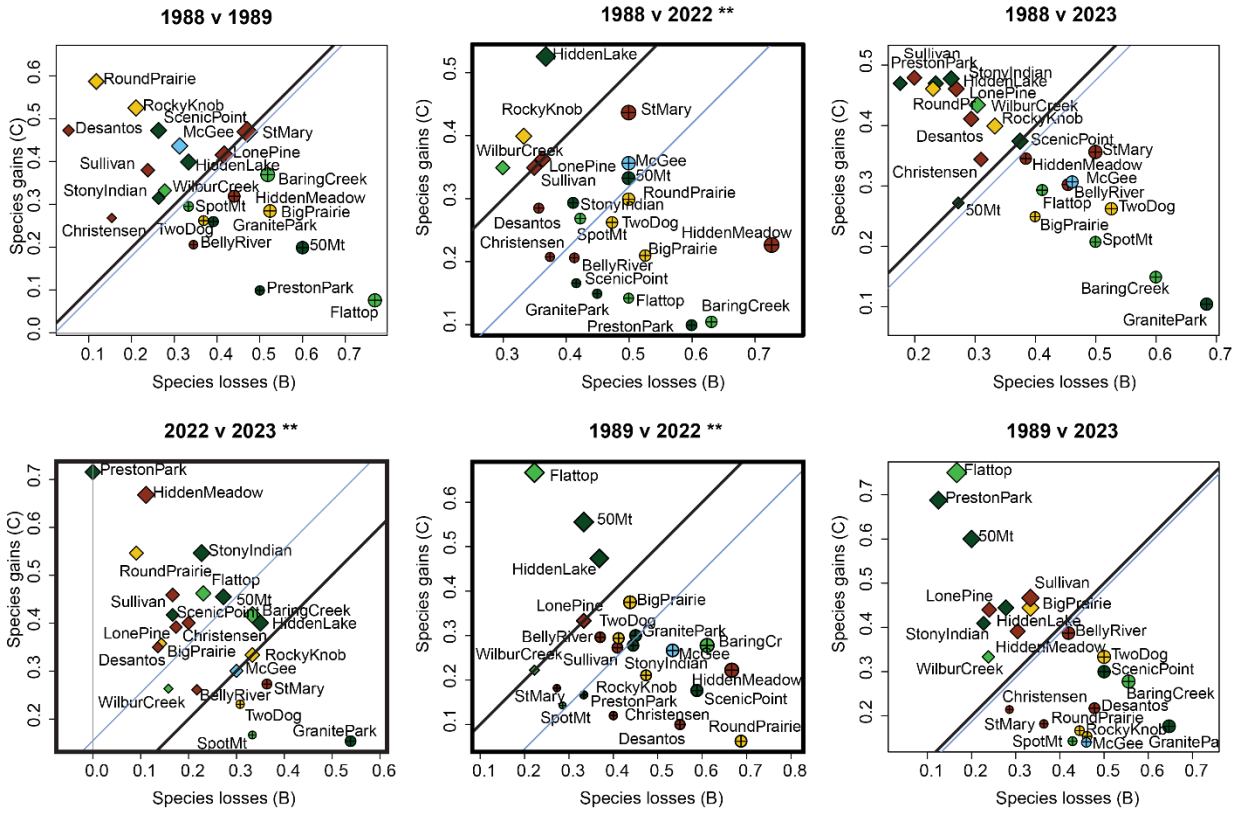


Figure 3: Temporal beta diversity B-C plots comparing butterfly species losses and gains at all sites in each study year. Temporal beta diversity indices were calculated for each of 22 sites between each pairwise year comparison and decomposed into species gains (C) and species losses (B). Temporal beta diversity differed between 2022 and all other survey years. Within each plot, the thick black line, with a slope of 1, indicates where species gains equal species losses. The thinner grey line is drawn parallel to the black line and passes through the centroid of the points. Its position below the black line indicates that, on average, species losses dominated gains between the two study years and its position above the black line indicates that, on average, species gains dominated losses between the two years. Diamonds indicate sites where C (gains) were greater than B (losses) and circles represent sites where B (losses) were greater than C (gains). Colors represent the five habitat types across the 22 sites and correspond to Figure 1: dark green = high alpine, lighter green = alpine, dark red = mesic, yellow = xeric, and blue = hydric. One site (MFF West Glacier = riparian) was removed because it was not surveyed in all four years. Plots with thick black borders and asterisks indicate statistical significance of differences in temporal beta diversity from paired permutation tests (Table 3).

Table 3. Temporal beta diversity comparisons between each year were tested for significance with paired permutation t-tests; the mean difference in community composition between each of 22 sites between years was statistically significant for all comparisons with 2022 where species losses were greater than gains when comparing 1988 and 1989 with 2022 and species gains were greater than losses with comparing 2022 and 2023.

Comparison	Mean (C-B)	*t*	p-parametric	p-permutation
1988 v 1989	-0.0410	-0.6302	0.5353	0.537
1988 v 2022	**-0.2130**	**-4.9915**	**0.0001**	**0.001**
1988 v 2023	-0.0407	-0.8119	0.4260	0.425
1989 v 2022	**-0.1671**	**-3.3503**	**0.0030**	**0.008**
1989 v 2023	-0.0093	-0.1263	0.0901	0.895
2022 v 2023	**0.1684**	**2.8226**	**0.0102**	**0.014**

Effects of Elevation and Habitat Type on Species Richness

To test whether species richness varied by year across the elevation gradient and habitat types (Hypotheses 3 and 4), generalized linear mixed effects models were used to test the effects of an interaction of year and elevation and, separately, year and habitat type on observed species richness values from each site and year. There was no evidence of an interaction in either model so two additional models were fit to the data that did not assume any interactions of habitat or elevation across years, again with habitat and elevation fit separately (Supplemental Table 9). Comparison of AIC scores between Model 1 and Model 3, which included elevation as a predictor, indicated the model without an interaction was more supported (AIC Model 1 = 490.004 and AIC Model 3 = 486.099). Similarly for the two models that included habitat as a predictor, Model 4 without an interaction term had a lower AIC score than Model 2 with an interaction (AIC Model 2 = 478.854 and AIC Model 4 = 464.180). In both cases the difference in AIC scores was greater than two, so the models without interactions were selected to describe effects of elevation and habitat on species richness.

Species richness followed a similar pattern among habitat types and years of study, where species richness was highest overall in mesic habitats but lowest across all habitat types in the year 2022 (Figure 4). Species richness in mesic habitats was predicted to be 1.35 times that of the reference habitat level (xeric), or 35% higher, after accounting for different years of study (Table 4, 95% CI: 1.04 to 1.77, p-value =0.027). After accounting for different habitat types, species richness was predicted to be 0.76 times, or 24% lower, in 2022 compared to the reference year of 1988 (95% CI: 0.63 to 0.91, p-value = 0.002). No evidence was found for differences in species richness related to other study years or habitat types. The model predictors explained 28.0% (Marginal $R^2 = 0.280$) of the variation in observed species richness and 45.9% (Conditional $R^2 = 0.459$) of the variation was explained by predictors and the random effect of site. While species richness was predicted to have a slight decreasing trend with increasing elevation, 0.91 or 9% less with each unit of elevation compared to baseline, 95% confidence intervals overlapped zero (95% CI = 0.81 to 1.02, p-value = 0.092), so no significant effect of elevation on species richness was found (Table 5).

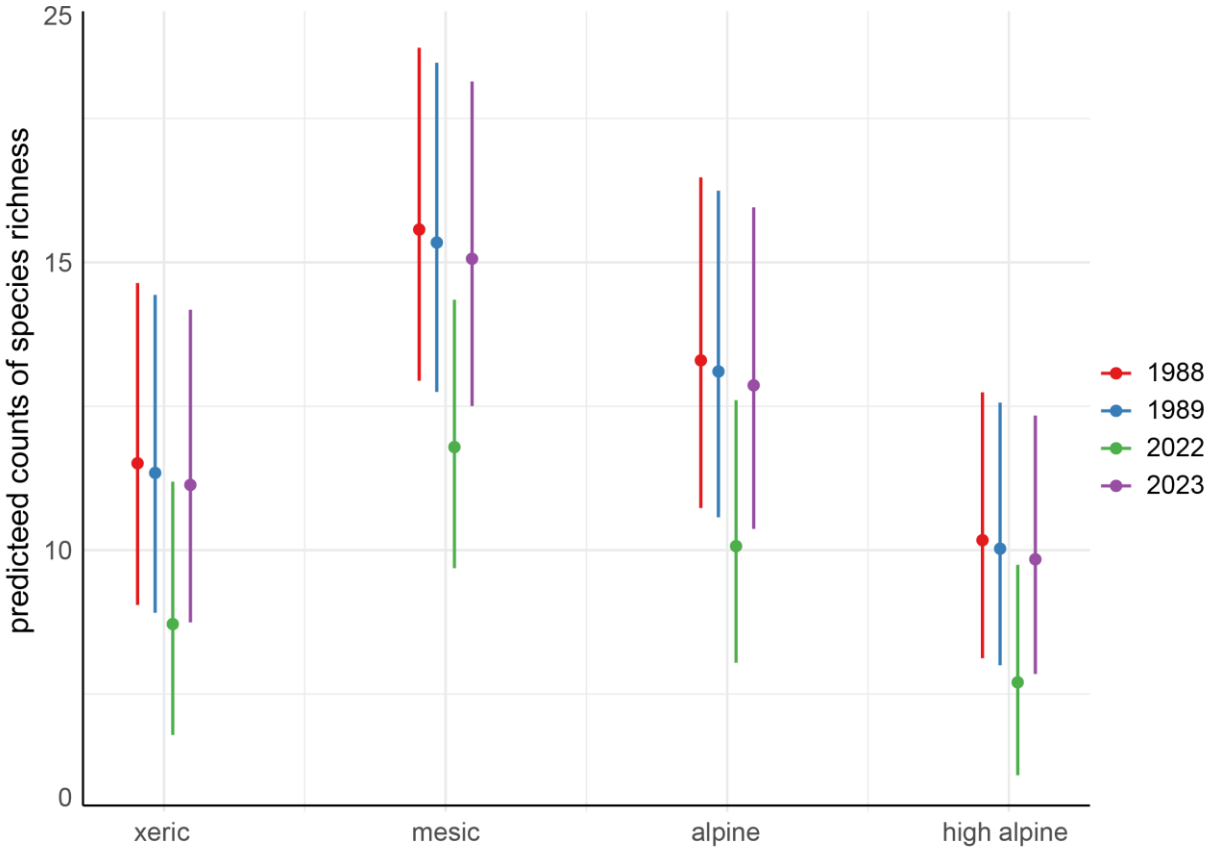


Fig 4. Marginal effects of habitat type within each study year on predicted butterfly species richness. Predicted butterfly species richness was higher in mesic habitats and lower in 2022 in a generalized linear mixed effects model of observed site-level species richness from each of four years of study. The effects plot shows the predicted species richness for each habitat type and year after accounting for the random effect of site (LOCALITY). Points represent the mean species richness prediction and bars represent 95% confidence intervals. There were four habitat types from 21 sites; xeric, mesic, alpine and high alpine surveyed for butterfly species in four separate years, 1988 and 1989 (past study) and 2022 and 2023 (present study).

Table 4. Model output from generalized linear mixed effects model testing effects of habitat and study year on butterfly species richness. The fixed effects of habitat and study year on observed species richness from 21 sites surveyed in four years were tested with a generalized linear mixed effects model including a random effect of site (LOCALITY). Two sites were removed before running the model, MFF West Glacier (riparian) because it was not surveyed in all four years and McGee (hydric) because the hydric habitat characterization was unreplicated, leaving 21 sites of four habitat types (xeric, mesic, alpine, high alpine) surveyed in four years (1988, 1989, 2022 and 2023). Parameter estimates transformed from the log-link function to incidence rate ratios (IRR), 95% confidence intervals (CI) and p-values are listed for each level of predictor, with 1988 and xeric habitat sites as the baseline levels.

Predictors	IRR	95% CI	p
(Intercept)	11.51	9.05 - 14.63	**<0.001**
Year [1989]	0.99	0.83 - 1.16	0.864
Year [2022]	0.76	0.63 - 0.91	**0.002**
Year [2023]	0.97	0.82 - 1.14	0.699
Habitat [mesic]	1.35	1.04 - 1.77	**0.027**
Habitat [alpine]	1.16	0.87 - 1.54	0.327
Habitat [high alpine]	0.88	0.66 - 1.19	0.415
Random Effects			
σ2	0.08		
τ00 LOCALITY	0.03		
ICC	0.25		
N	^{21}LOCALITY		
Observations	84		
Marginal R2 / Conditional R2	0.280 / 0.459		

Table 5. Model output from generalized linear mixed effects model testing effects of elevation and study year on butterfly species richness. One site was removed before running the model, MFF West Glacier (riparian) because it was not surveyed in all four years. Parameter estimates transformed from the log-link function to incidence rate ratios (IRR), 95% confidence intervals (CI) and p-values are listed for each level of predictor, with 1988 as the reference year. The residual variance (σ^2), random intercept variance (τ00 LOCALITY), and intraclass correlation coefficient (ICC, proportion of variance explained by random effects) for the model are also listed.

Predictors	IRR	95% CI	p
(Intercept)	12.59	10.86 - 14.61	**<0.001**
Year [1989]	0.99	0.84 - 1.17	0.933
Year [2022]	0.76	0.64 - 0.90	**0.002**
Year [2023]	0.96	0.81 - 1.13	0.641

Table 5 Continued

Elevation	0.91	0.81- 1.02	0.092
Random Effects			
σ2	0.08		
τ00 LOCALITY	0.05		
ICC	0.37		
N	^{22}LOCALITY		
Observations	88		
Marginal R2 / Conditional R2	0.149 / 0.468		

Species-habitat Relationships for Each Study Year

To investigate the effect of habitat type on butterfly community composition, canonical correspondence analysis (CCA) was used to test the amount of variation in butterfly species data that could be explained by different habitat types for each individual study year. Habitat was a significant predictor of the butterfly community in all four years (1988: $F_{3,11} = 1.499$, p-value = 0.001, 1989: $F_{3,11} = 1.782$, p-value = 0.001, 2022: $F_{3,11} = 1.817$, p-value = 0.001, 2023: $F_{3,11} = 1.503$, p-value = 0.001) and explained a similar proportion of variation in all years, 20.9% in 1988, 23.9% in 1989, 24.3% in 2022 and 21.0% in 2023 (Table 6). Associations of sites and species with the first two CCA axes were visualized with a CCA biplot for each year (Figure 5). Sites formed distinct groupings by habitat type across years indicating similar species compositions associated with xeric, mesic, alpine and high alpine habitats. Several species were particularly associated with each habitat type across multiple years, assessed visually by those that had species arrows with a length greater than 0.5 drawn towards the same habitat type in multiple years; *Callophrys eryphon* (Pine Elfin), *Argynnis cybele* (Great Spangled Fritillary), and *Papilio eurymedon* (Pale Swallowtail) were associated with xeric sites, *Boloria selene* (Silver-

bordered Fritillary), *Cercyonis pegala* (Common Wood-nymph), and *Cupido amyntula* (Western Tailed Blue) were particularly associated with mesic sites, *Tharsalea heteronea* (Blue Copper) was most associated with alpine sites, and *Agriades glandon* (Arctic Blue) was particularly associated with high alpine sites. Additionally, *Argynnis egleis* (Great Basic Fritillary), *Colias meadii* (Mead's Sulphur), *Aglais milberti* (Milbert's Tortoiseshell) and *Parnassius smintheus* (Rocky Mountain Parnassian) were associated with either alpine or high alpine sites.

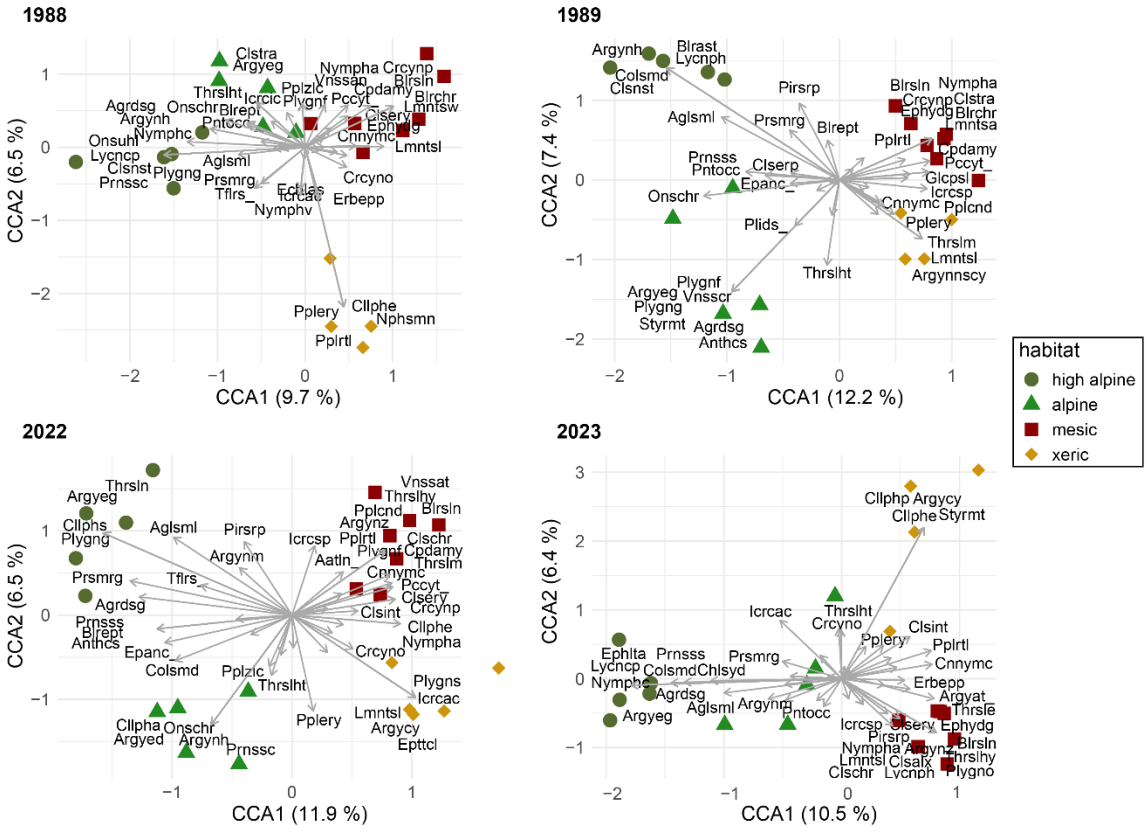


Fig 5. Canonical correspondence analysis (CCA) biplots associating habitat and the butterfly community in each year of study. Associations of sites with the first two CCA axes are plotted as points with shapes and colors representing the four habitat types. Species associations are depicted by arrows with the length of the arrow indicating the strength of the association. Species names are abbreviated from those listed in Table 1. The proportion of variation explained by each axis for all four plots (years) is represented as a percentage.

Table 6: Results from canonical correspondence analysis (CCA) associated habitat and the butterfly community in each year of study. The effect of habitat type on butterfly community composition was tested using canonical correspondence analysis (CCA) with habitat as a constraining variable for each year of study. Post-hoc permutation tests were used to test the significance of the constraining variable (habitat) and the CCA axes for each year. The proportion of variation explained by each variable and axis, F-statistics and p-values are listed. P-values less than 0.05 are bolded.

Year	Variable	Proportion Explained	F3,11	p
1988	Habitat	0.209	1.499	**0.001**
1989	Habitat	0.239	1.782	**0.001**
2022	Habitat	0.243	1.817	**0.001**
2023	Habitat	0.21	1.503	**0.001**
CCA Axes				
1988	Proportion Explained	F1,17	p	
CCA1	**0.097**	**2.083**	**0.001**	
CCA2	0.066	1.415	0.112	
CCA3	0.046	0.997	0.492	
1989	Proportion Explained	F1,17	p	
CCA1	**0.122**	**2.721**	**0.001**	
CCA2	**0.074**	**1.658**	**0.009**	
CCA3	0.043	0.967	0.534	
2022	Proportion Explained	F1,17	p	
CCA1	**0.119**	**2.672**	**0.001**	
CCA2	0.065	1.453	0.065	
CCA3	0.059	1.325	0.071	
2023	Proportion Explained	F1,17	p	
CCA1	**0.105**	**2.254**	**0.001**	
CCA2	0.064	1.372	0.148	
CCA3	0.041	0.884	0.729	

Effect of Floral Resources on Butterfly Species Richness

To understand whether the amount of floral resources in a site affected butterfly species richness (Hypothesis 5), counts of the total number of reproductive ramets and the number of unique plant genera (plant generic richness), of each site were assessed in relation to butterfly species richness in the two present study survey years, 2022 and 2023. Plant richness at the genus level affected butterfly species richness after accounting for different habitat types and study year, where it was estimated that butterfly richness was 1.13 times, or 13% higher, for each one-unit increase in plant generic richness (Figure 6, Table 7, 95% CI: 1.01 to 1.26, p-value = 0.037). There was no effect of the total number of reproductive ramets on butterfly species richness after accounting for habitat type, year, and plant generic richness (p-value = 0.825). Additionally, butterfly species richness in 2023 was predicted to be 1.41 times, 41% more, than the reference year of 2022, after accounting for habitat type and floral resources (95% CI: 1.16 to 1.73, p-value = 0.001).

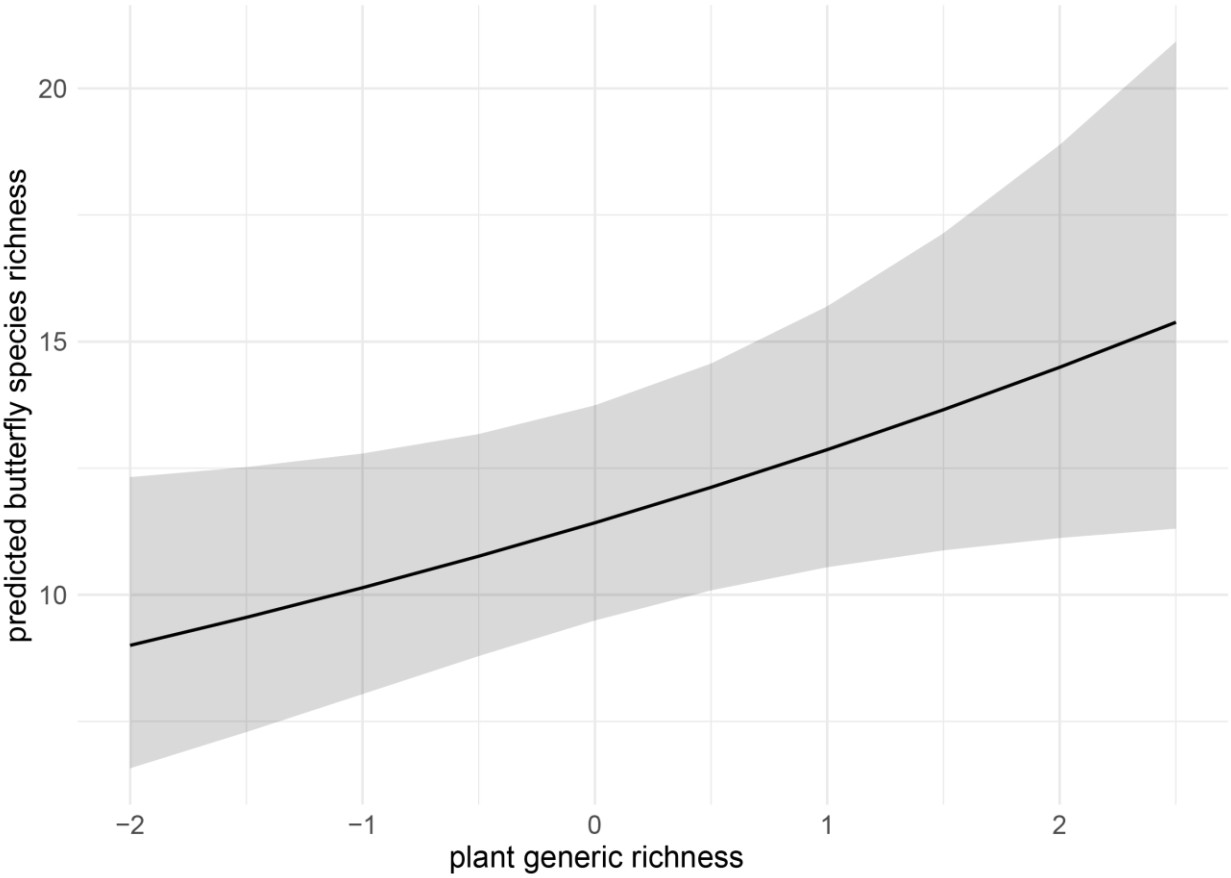


Fig 6. Effect of floral generic richness on predicted butterfly species richness. The predicted butterfly species richness increases with increasing plant generic richness. The effect of plant generic richness on butterfly species richness was modelled with a generalized linear model for two years (2022 and 2023) of butterfly surveys at 21 sites and four habitat types (xeric, mesic, alpine and high alpine). All numeric covariates were standardized to have a mean of zero and standard deviation of one before incorporating them in the model. The line represents the estimate of butterfly species richness with each unit increase in plant generic richness after accounting for habitat type, study year and total reproductive ramets and the shaded grey areas represent the 95% confidence interval.

Table 7: Model output from generalized linear model testing impact of floral resources on butterfly species richness. Floral resources were quantitively assessed in the present study (2022 and 2023) and the effects of plant generic richness and total reproductive ramets on butterfly species richness were tested with a generalized linear model after accounting for study year and habitat types. Parameter estimates transformed from the log-link function to incidence rate ratios (IRR), 95% confidence intervals (CI) and p-values are listed for each level of predictor, with 2022 and xeric habitats as the reference levels.

Predictors	IRR	95% CI	p
(Intercept)	8.81	6.87 - 11.15	**<0.001**
Year [2023]	1.41	1.16 - 1.73	**0.001**
Habitat [mesic]	1.3	1.01 - 1.68	**0.046**
Habitat [alpine]	1.24	0.88 - 1.73	0.208
Habitat [high alpine]	1.07	0.75 - 1.52	0.705
plant generic richness	1.13	1.01 - 1.26	**0.037**
total reproductive ramets	1.01	0.90 - 1.13	0.825
Observations	42		
R2	0.617		

Discussion

This study provided a temporal snapshot of a butterfly community in a high latitude, high elevation, protected area where impacts of climate change are expected to be observed. Contrary to our predictions, the butterfly community in Glacier National Park, Montana appears to be relatively unchanged in the present snapshot (2022 and 2023) compared to a snapshot taken 35 years ago (1988 and 1989). However, considerable variation in the two present study years warrants further investigation and monitoring.

Most of the butterfly species that were found in the past survey were also found in the present; 63 of the 83 total species observed in both studies were shared (Table 1). While many of the species that were only found in the present or past survey were singletons, traits associated with species that were more common in the past and not found in the present may indicate true shifts worthy of more discussion and study. *Boloria chariclea* (Arctic Fritillary) was found in

both past study years (16 individuals) but none were found in the present study. *Boloria chariclea* is a species associated with moist meadows or bogs (Layberry et al., 1998) and was found in mesic meadows in the past study. Host plants for *Boloria chariclea* include *Salix* (willow), *Polygonum* (bistorts), and some reports of *Viola* (violets) and *Vaccinum* (blueberries) (Scott, 1986). These plant species were not found in flower during floral surveys at mesic sites in the present study, although it is possible those plants were present but not counted since plant surveys were only focused on counts of species in flower. Mesic meadows and their butterfly communities could be particularly sensitive to warming temperatures and drying landscapes because intermittent moisture that is characteristic of mesic meadows leads to high interannual variability (Debinski et al., 2000, 2013). The genus *Celastrina* (Azures) was also observed in both years of the past study (4 individuals) but not in the present survey. *Celastrina* species are some of the first species to emerge in the spring, with peaks in emergence generally in May in the Rocky Mountains (Scott, 1986). Butterfly surveys in both studies began in June, so it possible that surveys in the past study were capturing the tail-end of emergence and even a slight shift in phenology to earlier emergence over the past 35 years would result in not capturing this species in the present survey. Phenological shifts are a documented and expected response of species to climate change (Forister & Shapiro, 2003) so our observations may indicate a phenological shift for *Celastrina.* However, it is also possible that species which were found discordantly between the two surveys were present but not detected. Detection probability can vary by species, with smaller and less conspicuous butterflies being more difficult to detect, and behavior, where flying individuals are more often caught than those that are resting or nectaring (Kral-O'Brien et al., 2020). Detection probabilities were not incorporated into this analysis, so

false-negative observations cannot be discounted. There were two species that were noted as a particular concern for being impacted by future climate change in the past study, the mesic-adapted *Euphydryas gillettii* (Gillette's Checkerspot) and the high alpine-adapted *Colias nastes* (Labrador Sulphur) (Debinski & Brussard, 1994), and it is encouraging that both of these species were still found in the present survey. Only two individuals of *Euphydryas gillettii* were captured in one present survey year while a total of eight individuals were captured over both years of the past study which could indicate cause for additional monitoring. This study targeted species richness rather than abundance and abundance was not assessed quantitatively, so more concerted study over more sampling years would be needed to reveal a true trend for this species and others.

While there was some indication of differences at the species level between past and present studies, species richness and community composition did not differ overall at the park-wide scale between the past and present. However, the two present study years did differ with respect to both species richness and community composition. Species richness in 2022 was significantly lower than both past study years (1988 and 1989) and lower than 2023, while species richness in 2023 was not different from the past study years (Figure 2). Similarly, community composition was different between 2022 and all other study years, with species losses dominating gains when comparing both past study years, and gains dominating losses when comparing 2022 and 2023 (Figure 3). Community composition in 2023 did not differ between the two past study years. While a habitat level trend in temporal beta diversity was not apparent, several sites did show either consistent gains or losses in species between years,

indicating that factors other than the broad habitat characterizations used in this study be affecting temporal beta diversity.

Although specific climatic conditions were not assessed for this study, two very different survey seasons were observed between 2022 and 2023. Late spring snow led to spring flooding in June of 2022 and butterfly surveys did not begin until the end of June. These conditions contrasted sharply with those of 2023, where spring conditions were observed to be much warmer and drier, and surveys began early to mid-June, which was similar to beginning survey dates in 1988 and 1989 (Supplemental Table 2). However, analysis of cumulative growing degree days (cGDD) associated with each site and the date it was surveyed in all years did not indicate a significant difference between survey dates in 2022 with past survey years for either the early summer (Survey 1) or late summer (Survey 2) surveys. Indeed, cGDD was more variable for both surveys between 1988 and 1989 than it was between 2022 and 2023. Growing degree days have recently been used to predict butterfly emergence (Cayton et al., 2015) so although survey dates differed in 2022, comparisons of cGDD in 2022 with other years indicated we were surveying at similar conditions known to affect butterfly emergence across years. It is therefore unlikely that differing survey dates explain all the variation in 2022 compared to other years, and further analysis of climate and conditions in 2022 may be needed to explain this outlier year. The similarity in butterfly species richness and community composition in 2023 compared to both past study years indicated that overall, butterfly species richness and community composition has not shifted significantly from past to present. However, the total number of individuals caught in 2023 was higher than all other survey years; over 1,000 individuals were caught in 2023 compared to 600-700 in the three other survey years (Table 1). It is not clear why more

individuals were caught in 2023; methods were unchanged, and one of two observers was the same in both 2022 and 2023. It may be that warmer spring conditions in 2023 allowed for more broods in multivoltine species and increased the flight duration, and thus the number of individuals caught, of some species (Diamond et al., 2011; Hill et al., 2021). Further analysis of species-specific trends and traits in relation to climatic conditions could reveal an explanation for differences in 2022 and 2023.

The natural elevation and moisture gradients in Glacier National Park provided the opportunity to test predictions of butterfly species richness along these ecological gradients between past and present studies. Although there was evidence for a difference in species richness in one present study year (2022) compared to the past study years at the park-wide scale, no evidence was found for a difference in species richness at the habitat level or along the elevation gradient that was dependent upon year. Generalized linear mixed effects models did not support evidence for interactive terms between year and either habitat or elevation (Supplemental Table 9). Instead, results supported that, although species richness was lower overall in all habitats in 2022, relationships between species richness and habitat types were consistent across years. Mesic habitats supported the highest number of species (Figure 4) and species richness did not differ along the elevation gradient (Table 5). Butterfly community composition among habitat types also formed distinct groupings that remained distinct in both present study years, though xeric and mesic sites in 1989 and 2022 were more similar in composition (closer together on the CCA biplots) than they were in 1988 and 2023; alpine and high alpine sites had more similar community composition in 1988 and 2023 than in 1989 and 2022 (Figure 6). Habitat was a significant predictor of the butterfly community in each year but only explained ~ 20% of the

variation in community composition (Table 6), suggesting that other predictors may explain additional or more of the observed variation in butterfly composition than the habitat characterizations used in this study. Future research could examine whether site-specific remotely sensed climate and soil moisture characterizations may reveal patterns in the butterfly data that are not apparent with broad habitat types alone.

In the present study, floral resources were assessed quantitatively and were added to models predicting butterfly species richness for data from 2022 and 2023. Plant richness at the genus level had a positive relationship with butterfly species richness (Figure 7) and positive relationships between butterfly and plant richness and abundance have been supported in other studies (Kitahara et al., 2008; Wix et al., 2019; Han et al., 2021). Though assessment of floral resources does not allow inference of host plant presence or abundance that are not in flower, decreasing nectar supply has also been correlated with butterfly decline (Wallisdevries et al., 2012) suggesting that butterfly species richness may indicate habitat quality. Continued assessment of floral resources will be important to future butterfly monitoring efforts.

While differences in species richness and community composition were not observed overall in this study, responses to climate change may be more species-specific (Diamond et al., 2011; Habel et al., 2023). Recent work has found that butterflies belonging to the families of Lycaenidae (Blues) and Hesperiidae (Skippers) may be more at risk to climate change than others (Forister et al., 2023). Analysis of species-specific occurrence with data from this study would be a likely next step in analysis and may elucidate changes from past to present that are not apparent when considering butterfly richness and composition overall. However, it is also possible that high-latitude, high-elevation butterflies may respond more slowly to climate stress

because they have wider thermal tolerances than their lower-latitude and elevation counterparts (Deutsch et al., 2008). Declines in abundance may also be an initial signal of change, and declines in abundance of formerly common butterflies and moths have been documented (Conrad et al., 2006; Forister et al., 2021). Changes in abundance could not be reliably assessed with the data in this study but future monitoring efforts could benefit from shifts in study design that would allow reliable abundance estimates at the species level.

This study provided a contemporary baseline of a high-elevation, high-latitude, butterfly community protected from land use change, by which future changes due to impacts of climate change can, and should, be monitored through continued survey efforts. It is promising that this initial assessment of the butterfly community in Glacier National Park did not reveal widespread change from 35 years ago, though comparison of only two years of data on either end of this window is truly only a snapshot in time. The two present study years did vary in species richness and community composition, so additional monitoring is needed to assess whether there is an actual trend. Further research with these and related data sets could provide additional insights into species-specific trends for individual protected areas or ecosystems of interest. Factors besides habitat type or growing degree days may potentially affect variation in the butterfly community observed in these past and present windows and may reveal more nuanced change than was apparent in our assessment of overall species richness and community composition. We did find distinct butterfly communities among each of the habitat types examined and mesic sites supported the highest number of species. Given that mesic meadows are expected to be vulnerable to climate change, these sites should be high priorities for future monitoring.

References

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*, *67*(1), 1–48. https://doi.org/10.18637/jss.v067.i01

Borcard, D., Gillet, F., & Legendre, P. (2011). *Numerical Ecology with R*. Springer. https://doi.org/10.1007/978-1-4419-7976-6

Cayton, H. L., Haddad, N. M., Gross, K., Diamond, S. E., & Ries, L. (2015). Do growing degree days predict phenology across butterfly species? *Ecology*, *96*(6), 1473–1479. https://doi.org/10.1890/15-0131.1

Chinn, B. (2014). *Lepidoptera of Glacier National Park* (Third). Project 4 Publishing.

Conrad, K. F., Warren, M. S., Fox, R., Parsons, M. S., & Woiwod, I. P. (2006). Rapid declines of common, widespread British moths provide evidence of an insect biodiversity crisis. *Biological Conservation*, *132*(3), 279–291. https://doi.org/10.1016/j.biocon.2006.04.020

Crossley, M. S., Smith, O. M., Berry, L. L., Phillips-Cosio, R., Glassberg, J., Holman, K. M., Holmquest, J. G., Meier, A. R., Varriano, S. A., McClung, M. R., Moran, M. D., & Snyder, W. E. (2021). Recent climate change is creating hotspots of butterfly increase and decline across North America. *Global Change Biology*, *27*(12), 2702–2714. https://doi.org/10.1111/gcb.15582

Debinski, D. M., & Brussard, P. F. (1994). Using Biodiversity Data to Assess Species-Habitat Relationships in Glacier National Park, Montana. *Ecological Applications*, *4*(4), 833–843. https://doi.org/10.2307/1942012

Debinski, D. M., Caruthers, J. C., Cook, D., Crowley, J., & Wickham, H. (2013). Gradient-based habitat affinities predict species vulnerability to drought. *Ecology*, *94*(5), 1036–1045. https://doi.org/10.1890/12-0359.1

Debinski, D. M., Jakubauskas, M. E., & Kindscher, K. (2000). Montane Meadows as Indicators of Environmental Change. In S. S. Sandhu, B. D. Melzian, E. R. Long, W. G. Whitford, & B. T. Walton (Eds.), *Monitoring Ecological Condition in the Western United States: Proceedings of the Fourth Symposium on the Environmental Monitoring and Assessment Program (EMAP), San Franciso, CA, April 6–8, 1999* (pp. 213–225). Springer Netherlands. https://doi.org/10.1007/978-94-011-4343-1_18

Debinski, D. M., Moranz, R. A., Delaney, J. T., Miller, J. R., Engle, D. M., Winkler, L. B., McGranahan, D. A., Barney, R. J., Trager, J. C., Stephenson, A. L., & Gillespie, M. K. (2011). A cross-taxonomic comparison of insect responses to grassland management and land-use legacies. *Ecosphere*, *2*(12), art131. https://doi.org/10.1890/ES11-00226.1

Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C., & Martin, P. R. (2008). Impacts of climate warming on terrestrial ectotherms across latitude. *Proceedings of the National Academy of Sciences*, *105*(18), 6668–6672. https://doi.org/10.1073/pnas.0709472105

Diamond, S. E., Frame, A. M., Martin, R. A., & Buckley, L. B. (2011). Species' traits predict phenological responses to climate change in butterflies. *Ecology*, *92*(5), 1005–1012. https://doi.org/10.1890/10-1594.1

Dirzo, R., Young, H. S., Galetti, M., Ceballos, G., Isaac, N. J. B., & Collen, B. (2014). Defaunation in the Anthropocene. *Science*, *345*(6195), 401–406. https://doi.org/10.1126/science.1251817

Dray, S., Bauman, D., Blanchet, G., Borcard, D., Clappe, S., Guénard, G., Jombart, T., Larocque, G., Legendre, P., Madi, N., & Wagner, H. H. (2023). *adespatial: Multivariate Multiscale Spatial Analysis*. https://CRAN.R-project.org/package=adespatial

Forister, M. L., Grames, E. M., Halsch, C. A., Burls, K. J., Carroll, C. F., Bell, K. L., Jahner, J. P., Bradford, T. A., Zhang, J., Cong, Q., Grishin, N. V., Glassberg, J., Shapiro, A. M., & Riecke, T. V. (2023). Assessing risk for butterflies in the context of climate change, demographic uncertainty, and heterogeneous data sources. *Ecological Monographs*, *93*(3), e1584. https://doi.org/10.1002/ecm.1584

Forister, M. L., Halsch, C. A., Nice, C. C., Fordyce, J. A., Dilts, T. E., Oliver, J. C., Prudic, K. L., Shapiro, A. M., Wilson, J. K., & Glassberg, J. (2021). Fewer butterflies seen by community scientists across the warming and drying landscapes of the American West. *Science*, *371*(6533), 1042–1045. https://doi.org/10.1126/science.abe5585

Forister, M. L., McCall, A. C., Sanders, N. J., Fordyce, J. A., Thorne, J. H., O'Brien, J., Waetjen, D. P., & Shapiro, A. M. (2010). Compounded effects of climate change and habitat alteration shift patterns of butterfly diversity. *Proceedings of the National Academy of Sciences*, *107*(5), 2088–2092. https://doi.org/10.1073/pnas.0909686107

Forister, M. L., Pelton, E. M., & Black, S. H. (2019). Declines in insect abundance and diversity: We know enough to act now. *Conservation Science and Practice*, *1*(8), e80. https://doi.org/10.1111/csp2.80

Forister, M., & Shapiro, A. (2003). Climatic trends and advancing spring flight of butterflies in lowland California. *Global Change Biology*, *9*, 1130–1135. https://doi.org/10.1046/j.1365-2486.2003.00643.x

Garzella, C., & Debinski, D. (2020). *Using Biodiversity Data to Assess Species-Habitat Relationships in Glacier National Park, Montana* (Version 8). Environmental Data Initiative. https://doi.org/10.6073/pasta/222532f38cb2e996244a60be7d048261

Gerlach, J., Samways, M., & Pryke, J. (2013). Terrestrial invertebrates as bioindicators: An overview of available taxonomic groups. *Journal of Insect Conservation*, *17*(4), 831–850. https://doi.org/10.1007/s10841-013-9565-9

Gorelick, N., Hancher, M., Dixon, M., Ilyushchenko, S., Thau, D., & Moore, R. (2017). Google Earth Engine: Planetary-scale geospatial analysis for everyone. *Remote Sensing of Environment*, *202*, 18–27. https://doi.org/10.1016/j.rse.2017.06.031

Habel, J. C., Ulrich, W., Gros, P., Teucher, M., & Schmitt, T. (2023). Butterfly species respond differently to climate warming and land use change in the northern Alps. *Science of The Total Environment*, *890*, 164268. https://doi.org/10.1016/j.scitotenv.2023.164268

Hallmann, C. A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., Stenmans, W., Müller, A., Sumser, H., Hörren, T., Goulson, D., & Kroon, H. de. (2017). More than 75 percent decline over 27 years in total flying insect biomass in protected areas. *PLOS ONE*, *12*(10), e0185809. https://doi.org/10.1371/journal.pone.0185809

Halsch, C. A., Shapiro, A. M., Fordyce, J. A., Nice, C. C., Thorne, J. H., Waetjen, D. P., & Forister, M. L. (2021). Insects and recent climate change. *Proceedings of the National Academy of Sciences*, *118*(2), e2002543117. https://doi.org/10.1073/pnas.2002543117

Han, D., Zhang, C., Wang, C., She, J., Sun, Z., Zhao, D., Bian, Q., Han, W., Yin, L., Sun, R., Wang, X., & Cheng, H. (2021). Differences in Response of Butterfly Diversity and Species Composition in Urban Parks to Land Cover and Local Habitat Variables. *Forests*, *12*(2), Article 2. https://doi.org/10.3390/f12020140

Hill, G. M., Kawahara, A. Y., Daniels, J. C., Bateman, C. C., & Scheffers, B. R. (2021). Climate change effects on animal ecology: Butterflies and moths as a case study. *Biological Reviews*, *96*(5), 2113–2126. https://doi.org/10.1111/brv.12746

IPCC. (2023). *Climate Change 2023: Synthesis Report. Contribution of Working Groups I, II and III to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change* (H. Lee, J. Skea, P. Shukla, P. Zhai, V. Masson-Delmotte, A. Pirani, W. Moufouma-Okia, C. Péan, R. Pidcock, S. Connors, J. B. R. Matthews, M. Chen, Y. Sokona, M. I. Aguilar, C. Mintenbeck, A. Okem, B. Rama, J. Wilcock, W. Maycock, & M. Tignor, Eds.). IPCC. https://doi.org/10.59327/IPCC/AR6-9789291691647

Kassambara, A. (2023). *rstatix: Pipe-Friendly Framework for Basic Statistical Tests*. https://CRAN.R-project.org/package=rstatix

Kerner, J. M., Krauss, J., Maihoff, F., Bofinger, L., & Classen, A. (2023). Alpine butterflies want to fly high: Species and communities shift upwards faster than their host plants. *Ecology*, *104*(1), e3848. https://doi.org/10.1002/ecy.3848

Kitahara, M., Yumoto, M., & Kobayashi, T. (2008). Relationship of butterfly diversity with nectar plant species richness in and around the Aokigahara primary woodland of Mount Fuji, central Japan. *Biodiversity and Conservation*, *17*(11), 2713–2734. https://doi.org/10.1007/s10531-007-9265-4

Kral-O'Brien, K. C., Karasch, B. M., Hovick, T. J., Moranz, R. A., & Harmon, J. P. (2020). Morphological traits determine detectability bias in North American grassland butterflies. *Ecosphere*, *11*(12), e03304. https://doi.org/10.1002/ecs2.3304

Layberry, R., Hall, P., & Lafontaine, D. (1998). *The Butterflies of Canada*. University of Toronto Press. https://doi.org/10.3138/9781442623163

Legendre, P., & Condit, R. (2019). Spatial and temporal analysis of beta diversity in the Barro Colorado Island forest dynamics plot, Panama. *Forest Ecosystems*, *6*(1), 7. https://doi.org/10.1186/s40663-019-0164-4

Losey, J. E., & Vaughan, M. (2006). The Economic Value of Ecological Services Provided by Insects. *BioScience*, *56*(4), 311–323. https://doi.org/10.1641/0006-3568(2006)56[311:TEVOES]2.0.CO;2

Lüdecke, D. (2018). ggeffects: Tidy Data Frames of Marginal Effects from Regression Models. *Journal of Open Source Software*, *3*(26), 772. https://doi.org/10.21105/joss.00772

Lyon, N. J., Stein, D. S., Debinski, D. M., Miller, J. R., & Schacht, W. H. (2021). Responses of Flowering Plant and Butterfly Communities to Experimental Herbicide and Seeding Treatments for Native Grassland Restoration. *Ecological Restoration*, *39*(3), 168–181. https://doi.org/10.3368/er.39.3.168

Mi, C., Ma, L., Yang, M., Li, X., Meiri, S., Roll, U., Oskyrko, O., Pincheira-Donoso, D., Harvey, L. P., Jablonski, D., Safaei-Mahroo, B., Ghaffari, H., Smid, J., Jarvie, S., Kimani, R. M., Masroor, R., Kazemi, S. M., Nneji, L. M., Fokoua, A. M. T., … Du, W. (2023). Global Protected Areas as refuges for amphibians and reptiles under climate change. *Nature Communications*, *14*(1), 1389. https://doi.org/10.1038/s41467-023-36987-y

Molina-Martínez, A., León-Cortés, J. L., Regan, H. M., Lewis, O. T., Navarrete, D., Caballero, U., & Luis-Martínez, A. (2016). Changes in butterfly distributions and species assemblages on a Neotropical mountain range in response to global warming and anthropogenic land use. *Diversity and Distributions*, *22*(11), 1085–1098. https://doi.org/10.1111/ddi.12473

Mora, C., Tittensor, D. P., Adl, S., Simpson, A. G. B., & Worm, B. (2011). How Many Species Are There on Earth and in the Ocean? *PLoS Biology*, *9*(8), e1001127. https://doi.org/10.1371/journal.pbio.1001127

Oksanen, J., Simpson, G. L., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., Solymos, P., Stevens, M. H. H., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., Caceres, M. D., Durand, S., … Weedon, J. (2022). *vegan: Community Ecology Package*. https://CRAN.R-project.org/package=vegan

Pederson, G. T., Graumlich, L. J., Fagre, D. B., Kipfer, T., & Muhlfeld, C. C. (2010). A century of climate and ecosystem change in Western Montana: What do temperature trends portend? *Climatic Change*, *98*(1), 133–154. https://doi.org/10.1007/s10584-009-9642-y

Pepin, N., Bradley, R. S., Diaz, H. F., Baraer, M., Caceres, E. B., Forsythe, N., Fowler, H., Greenwood, G., Hashmi, M. Z., Liu, X. D., Miller, J. R., Ning, L., Ohmura, A., Palazzi, E., Rangwala, I., Schöner, W., Severskiy, I., Shahgedanova, M., Wang, M. B., … Mountain Research Initiative EDW Working Group. (2015). Elevation-dependent warming in mountain regions of the world. *Nature Climate Change*, *5*(5), Article 5. https://doi.org/10.1038/nclimate2563

Pepin, N. C., Arnone, E., Gobiet, A., Haslinger, K., Kotlarski, S., Notarnicola, C., Palazzi, E., Seibert, P., Serafin, S., Schöner, W., Terzago, S., Thornton, J. M., Vuille, M., & Adler, C. (2022). Climate Changes and Their Elevational Patterns in the Mountains of the World. *Reviews of Geophysics*, *60*(1), e2020RG000730. https://doi.org/10.1029/2020RG000730

R Core Team. (2023). *R: A Language and Environment for Statistical Computing* [Computer software]. R Foundation for Statistical Computing. https://www.R-project.org/

Rader, R., Bartomeus, I., Garibaldi, L. A., Garratt, M. P. D., Howlett, B. G., Winfree, R., Cunningham, S. A., Mayfield, M. M., Arthur, A. D., Andersson, G. K. S., Bommarco, R., Brittain, C., Carvalheiro, L. G., Chacoff, N. P., Entling, M. H., Foully, B., Freitas, B. M., Gemmill-Herren, B., Ghazoul, J., … Woyciechowski, M. (2016). Non-bee insects are important contributors to global crop pollination. *Proceedings of the National Academy of Sciences*, *113*(1), 146–151. https://doi.org/10.1073/pnas.1517092112

Rantanen, M., Karpechko, A. Y., Lipponen, A., Nordling, K., Hyvärinen, O., Ruosteenoja, K., Vihma, T., & Laaksonen, A. (2022). The Arctic has warmed nearly four times faster than the globe since 1979. *Communications Earth & Environment*, *3*(1), 1–10. https://doi.org/10.1038/s43247-022-00498-3

Scott, J., A. (1986). *The Butterflies of North America A Natural History and Field Guide*. Stanford University Press.

Selwood, K. E., & Zimmer, H. C. (2020). Refuges for biodiversity conservation: A review of the evidence. *Biological Conservation*, *245*, 108502. https://doi.org/10.1016/j.biocon.2020.108502

Stork, N. E. (2018). How Many Species of Insects and Other Terrestrial Arthropods Are There on Earth? *Annual Review of Entomology*, *63*(Volume 63, 2018), 31–45. https://doi.org/10.1146/annurev-ento-020117-043348

Thomas, J. A. (2005). Monitoring change in the abundance and distribution of insects using butterflies and other indicator groups. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *360*(1454), 339–357. https://doi.org/10.1098/rstb.2004.1585

Thornton, P. E., Shrestha, R., Thornton, M., Kao, S.-C., Wei, Y., & Wilson, B. E. (2021). Gridded daily weather data for North America with comprehensive uncertainty quantification. *Scientific Data*, *8*(1), Article 1. https://doi.org/10.1038/s41597-021-00973-0

Uhler, J., Redlich, S., Zhang, J., Hothorn, T., Tobisch, C., Ewald, J., Thorn, S., Seibold, S., Mitesser, O., Morinière, J., Bozicevic, V., Benjamin, C. S., Englmeier, J., Fricke, U., Ganuza, C., Haensel, M., Riebl, R., Rojas-Botero, S., Rummler, T., … Müller, J. (2021). Relationship of insect biomass and richness with land use along a climate gradient. *Nature Communications*, *12*(1), 5946. https://doi.org/10.1038/s41467-021-26181-3

Wagner, D. L. (2020). Insect Declines in the Anthropocene. *Annual Review of Entomology*, *65*(Volume 65, 2020), 457–480. https://doi.org/10.1146/annurev-ento-011019-025151

Wallisdevries, M. F., Van Swaay, C. A. M., & Plate, C. L. (2012). Changes in nectar supply: A possible cause of widespread butterfly decline. *Current Zoology*, *58*(3), 384–391. https://doi.org/10.1093/czoolo/58.3.384

Warren, A. D., Davis, K. J., Stangeland, E. M., Pelham, J. P., Willmott, K. R., & Grishin, N. V. (2024, September 3). *Illustrated Lists of American Butterflies*. http://butterfliesofamerica.com

Warren, M. S., Maes, D., van Swaay, C. A. M., Goffart, P., Van Dyck, H., Bourn, N. A. D., Wynhoff, I., Hoare, D., & Ellis, S. (2021). The decline of butterflies in Europe: Problems, significance, and possible solutions. *Proceedings of the National Academy of Sciences*, *118*(2), e2002551117. https://doi.org/10.1073/pnas.2002551117

Welti, E. A. R., Joern, A., Ellison, A. M., Lightfoot, D. C., Record, S., Rodenhouse, N., Stanley, E. H., & Kaspari, M. (2021). Studies of insect temporal trends must account for the complex sampling histories inherent to many long-term monitoring efforts. *Nature Ecology & Evolution*, *5*(5), 589–591. https://doi.org/10.1038/s41559-021-01424-0

Wepprich, T., Adrion, J. R., Ries, L., Wiedmann, J., & Haddad, N. M. (2019). Butterfly abundance declines over 20 years of systematic monitoring in Ohio, USA. *PLOS ONE*, *14*(7), e0216270. https://doi.org/10.1371/journal.pone.0216270

Whitlock, C., Cross, W., Maxwell, B., Silverman, N., & Wade, A. A. (2017). *2017 Montana Climate Assessment*. Montana State University ScholarWorks. https://doi.org/10.15788/M2WW8W

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York. https://ggplot2.tidyverse.org

Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L. D., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J., Kuhn, M., Pedersen, T. L., Miller, E., Bache, S. M., Müller, K., Ooms, J., Robinson, D., Seidel, D. P., Spinu, V., … Yutani, H. (2019). Welcome to the tidyverse. *Journal of Open Source Software*, *4*(43), 1686. https://doi.org/10.21105/joss.01686

Winkler, K., Fuchs, R., Rounsevell, M., & Herold, M. (2021). Global land use changes are four times greater than previously estimated. *Nature Communications*, *12*(1), 2501. https://doi.org/10.1038/s41467-021-22702-2

Wix, N., Reich, M., & Schaarschmidt, F. (2019). Butterfly richness and abundance in flower strips and field margins: The role of local habitat quality and landscape context. *Heliyon*, *5*(5), e01636. https://doi.org/10.1016/j.heliyon.2019.e01636

Zhang, J., Cong, Q., Shen, J., Opler, P. A., & Grishin, N. V. (2020). Genomic evidence suggests further changes of butterfly names. *The Taxonomic Report of the International Lepidoptera Survey*, *8*, 7.

Zhang, J., Cong, Q., Shen, J., Song, L., Gott, R. J., Boyer, P., Guppy, C. S., Kohler, S., Lamas, G., Opler, P. A., & Grishin, N. V. (2022). Taxonomic discoveries enabled by genomic analysis of butterflies. *The Taxonomic Report of the International Lepidoptera Survey*, *10*(7), 1–59. https://doi.org/10.5281/zenodo.7160429

CHAPTER THREE

TESTING ENVIRONMENTAL DNA METABARCODING OF WILDFLOWER-ASSOCIATED INSECT DNA: A CASE STUDY OF BUTTERFLIES

Contribution of Authors and Co-Authors

Manuscript(s) in Chapter(s) [1]

Author: Cayley Faurot-Daniels

Contributions: conceptualization, methodology, data collection and curation, formal analysis, writing—original draft preparation, visualization, funding acquisition

Co-Author: Diane Debinski

Contributions: conceptualization, methodology, writing—original draft preparation, writing—review and editing, visualization, funding acquisition

Co-Author: Michelle Flenniken

Contributions: conceptualization, methodology, formal analysis, investigation, resources, data curation, writing—original draft preparation, writing—review and editing, visualization, supervision, project administration, funding acquisition

Manuscript Information

Cayley Faurot-Daniels, Diane Debinski, Michelle Flenniken

Molecular Ecology Resources

Status of Manuscript:
✓ Prepared for submission to a peer-reviewed journal
☐ Officially submitted to a peer-reviewed journal
☐ Accepted by a peer-reviewed journal
☐ Published in a peer-reviewed journal

John Wiley & Sons Ltd

Abstract

Rapid and accurate assessment of insect communities are becoming increasingly important in the context of climate change and biodiversity loss. Wildflower-associated environmental DNA (eDNA) has recently been used to infer arthropod occurrence and has potential as an efficient and non-invasive method for insect pollinator biomonitoring. This study utilized traditional butterfly netting methods in concert with the collection of wildflowers for eDNA metabarcoding, to test whether butterfly species and their abundance can be accurately inferred/estimated using eDNA collected from wildflowers. Of nearly 300 million sequences obtained from eDNA samples in this study, over 278 million or 93.75% were identified as insect sequences, and 23% were identified as belonging to Lepidoptera (butterflies and moths). DNA from six butterfly genera and four species were identified from wildflower-associated eDNA, and five were consistent with butterfly species captured during traditional netting surveys on the same day as wildflower collections. However, eDNA detections were not in accordance with butterflies that were physically placed or naturally observed on flowers, and traditional surveys identified 42 butterfly species that were not captured by eDNA. The most prevalently detected insect DNA from wildflower samples were of small-bodied insects that are more closely associated with flowers. Much more experimentation and methods development will be needed before wildflower-associated eDNA sampling can be an accurate method of surveying butterfly species.

Introduction

Reported declines in insect biodiversity and abundance continue to mount across the globe (Forister et al., 2010; Dirzo et al., 2014) threatening vital ecosystem services like pest control (Bale et al., 2008), decomposition (Seibold et al., 2021), supplying a food resource in the basis of food webs (Lister & Garcia, 2018), and pollination, where over 85% of flowering plants depend on animal pollinators (Ollerton et al., 2011). While awareness of biodiversity loss is long-standing, with calls for concern over insect loss as early as 1936 (Patch 1938; Carson, 1962; Soulé, 1991; Hughes et al., 1997; Wilson & Peter, 1988), novel methods of characterizing and monitoring biodiversity are emerging. Rapid advancement of detecting macro-organisms from DNA in the environment (eDNA), since first implementation in 2008, has enabled characterization of a particular species or whole communities of organisms from eDNA in an increasingly accurate and cost-effective manner (Ficetola et al., 2008). These novel molecular methods can facilitate detection of rare, cryptic or invasive species and provide broad ecosystem assessment with non-invasive techniques which are becoming increasingly important in the context of climate change and biodiversity loss across the globe (Deiner et al., 2017).

Environmental DNA methods for macro taxonomic identification originated in freshwater systems and to date the majority of studies are still associated with eDNA collected from water (Beng & Corlett, 2020). Analyzing macro taxonomic eDNA originating from terrestrial sources is relatively new, with recent studies utilizing soil (Leempoel et al., 2020; Kirse et al., 2021; Ariza et al., 2023), spider webs (Toju & Baba, 2018; Gregorič et al., 2022;), feces (Walker et al., 2016; Kaunisto et al., 2017; Nørgaard et al., 2021), pollen (Pornon et al., 2017; Ronca et al., 2023; Alvanou et al., 2024), and wildflowers as natural aggregates of eDNA to identify

arthropods (Thomsen & Sigsgaard, 2019; Gomez et al., 2023; Harper et al., 2023; Johnson et al., 2023; Avalos et al., 2024), as well as plants, birds, mammals, and other organisms. The ecology of eDNA, defined as the origin, deposition, degradation, and transport, varies by substrate and species (Barnes & Turner, 2016; van der Heyde et al., 2022) providing a need to benchmark results of eDNA detections against tandem observational studies and with controlled experiments.

Pollinators have received increasing attention in global insect conservation efforts as their critical role in pollinating both commercial and wild plants creates broad implications for pollinator loss to impact anthropogenic and natural systems (Losey & Vaughan, 2006; Ollerton et al., 2011; Rader et al., 2016). The first study to utilize wildflowers to detect insect DNA was in 2019, where DNA from 67 different insect families was identified from nine plant genera. This study used citizen science data from the study area to corroborate eDNA results, and found that several pollinators, including several bumble bee and butterfly species, were documented in the study area where eDNA of those species was also recovered (Thomsen and Sigsgaard, 2019), although direct comparison with observational data was not included. Several studies since the seminal work in 2019 that have compared observational pollinator data directly with flowers collected for eDNA metabarcoding have found discrepancies in identifications from traditional methods to eDNA. Most authors of these studies have observed that eDNA provides the potential in detecting DNA from nocturnal, cryptic, or phytophagous, or plant-eating, insects whereas traditional methods capture diurnal, showy, and perhaps less frequent flower visitors better than eDNA (Harper et al., 2023; Johnson et al., 2023: Avalos et al., 2024). These results indicate potential in characterizing flower-associated insect species that are more difficult to study,

although incongruence between observational and eDNA results creates uncertainty in the ability of eDNA methods in their current state to characterize whole terrestrial insect communities.

Further development of molecular methods utilizing wildflower-associated eDNA can benefit from focusing on insect groups that are well-studied with both traditional and molecular methods. Butterflies are one of the most studied insect groups due to their conspicuous morphological characters and relative ease of capture that aid reliable species identification and community assessment (Debinski et al., 2011b). Where most insects require a taxonomic expert and a microscope for identification, which is time consuming and costly, the ability to easily capture and identify butterflies in a field setting creates an opportunity to test molecular detections from wildflower eDNA against tandem observational data in a cost-effective, efficient, and noninvasive manner. Genetic information from butterfly species is also well-represented in molecular databases.

Detecting species from eDNA relies on amplifying a genetic region, or barcode, that is common among all organisms of interest but variable enough to sufficiently differentiate between species. For animals, a 658-base pair (bp) region of cytochrome oxidase subunit I (COI), a subunit of the mitochondrial cytochrome C oxidase (COX1) gene, has been utilized for species identification (Hebert, Ratnasingham, et al., 2003; Lear et al., 2018). The COX1 protein is involved in energy production, and mutations in the COI encoding region may hinder energy production or cause cell death. Therefore, there is strong selective pressure to maintain conserved amino acids and the DNA sequence that encodes those critical amino acids (Pentinsaari et al., 2016). The 219 amino acids of the COI region in animals has 23 conserved and 99 highly variable amino acids (Pentinsaari et al., 2016). DNA sequencing of the COI encoding region

demonstrated that the variation in this 'barcode region' is sufficient for the identification of nearly all animal phyla, except Cnidarians (i.e., jellyfish, corals, etc.) (Hebert, Ratnasingham, et al., 2003). Although Lepidoptera (butterflies and moths) are one of the most diverse animal groups and they have less sequence divergence in the COI barcode region than other diverse orders including Coleoptera (beetles), the COI barcode has been reliably utilized for Lepidopteran species identification (Hebert et al., 2003; Hajibabaei et al., 2006). The utility of the COI DNA barcode for species identification has resulted in its wide adoption and an extensive reference database of COI barcode sequences housed on the Barcode of Life Database (BOLD) (Ratnasingham & Hebert, 2007). As of March 2024, there were over 10.7 million COI barcode sequences in the BOLD database, and more than 1.3 million of those were Lepidopteran sequences representing 107,879 species (BOLD Systems, 2024).

Collection of eDNA samples enables detection of many different species in a single sample after amplification and sequencing of the COI barcode. The power of eDNA sampling and sequencing is further enhanced by DNA metabarcoding, a method where COI primers for polymerase chain reaction (PCR) amplification of the target region contain additional 6-8 unique nucleotides at the 5' end to uniquely tag products amplified with that primer. Use of varying combinations of 5'-tagged COI primer pairs for amplification of different eDNA samples facilitates sequencing of pooled samples (Taberlet et al., 2012). Nested metabarcoding, which integrates unique tags into primers used for a second PCR reaction to incorporate 5'adapter sequences required for sequencing, can further increase the number of samples included in one sequencing run. For instance, each DNA sample on a 96-well PCR plate can be amplified with a unique combination of forward and reverse indexes (or tags) in the COI primers (PCR1). Then

all products from one PCR plate can be pooled for the second PCR amplification (PCR2) with unique tags, and PCR2 products can be pooled into one sequencing library representing several hundred eDNA samples (Taberlet et al., 2012; Kitson et al., 2019), though care must be taken in experimental design and analysis to account for potential ambiguity created by sequencing errors when reusing indexes in a combinatorial design (Schnell et al., 2015; Guenay-Greunke et al., 2021) .

Standardized workflows for eDNA metabarcoding of terrestrial samples have yet to be realized (Hassan et al., 2022), and there is considerable variation in all steps including collection, processing, and data analyses (Lear et al., 2018; Ruppert et al., 2019). While many studies report that eDNA techniques either compliment or provide higher taxonomic resolution compared to traditional survey methods (Deiner et al., 2017), the potential for eDNA data to be quantitative for community analysis requires further investigation. Uncertainty created by variation of eDNA production and degradation in different states and environments (Barnes & Turner, 2016; Kirtane et al., 2023), the potential taxa-specific amplification bias of some primer sets (Clarke et al., 2014; Elbrecht & Leese, 2015), and variation in bioinformatic processing and analyses of sequencing data (Alberdi et al., 2018; Hakimzadeh et al., 2023), complicate DNA sequence abundance inferences of realized species abundance. The ongoing establishment and validation of eDNA methods to identify and quantify taxa will require studies that include observational survey data in tandem with eDNA sequencing data, and mesocosm and controlled experiments to advance the scope of inferences that can be made with eDNA metabarcoding data.

This study utilized a resurvey effort of butterfly diversity in Glacier National Park, Montana, U.S.A. using traditional butterfly netting methods in concert with the collection of

wildflowers for eDNA analysis to test two main questions; 1) Can butterfly genetic material be identified from eDNA obtained from wildflowers at a resolution that is similar or better than traditional surveys and 2) Can the abundance of butterfly DNA sequences be correlated with increasing butterfly flower visits? To address these questions, wildflowers were collected at butterfly monitoring sites directly after traditional butterfly surveys and an experiment where butterflies were placed on flowers in varying numbers of contrived visits was conducted at one field site. DNA was isolated from wildflower washes and used as template for amplification of two different segments of COI using primer sets established for their ability to distinguish Lepidoptera: MLepF1/LepR1 (Primer Set 1) and LepF1/MHMR1 (Primer Set 2) (Hajibabaei et al., 2006). To assess whether resulting sequence data could be analyzed quantitatively, samples amplified with each primer set included a dilution series of a positive control sample that contained DNA from five insect species, and a subset of high priority wildflower eDNA samples were amplified and sequenced in three technical replicates. As the potential of eDNA to reliably detect terrestrial insects may hold more advantages for more cryptic and elusive species than the often-conspicuous butterflies, another goal of the study was to provide baseline data of all insects detected in association with wildflower collections.

2 Materials and Methods

2.1 Study Site

Wildflower eDNA samples were collected in Glacier National Park, Montana, U.S.A., a federally protected area of over 4,000 square kilometers at the U.S. – Canadian border in Northwest Montana. Wildflower collections were in association with an observational study using traditional netting surveys to estimate butterfly diversity at 23, 1km2 sites across Glacier

National Park. Sites were arrayed along an elevation gradient (998 m to 2,251 m) and by habitat types, where sites were characterized as xeric (low moisture), mesic (moderate moisture) or alpine/high alpine (at/above tree line). A subset of nine sites, three of each habitat type, were selected for wildflower eDNA collection; Big Prairie, Rocky Knob, and Two Dog Flats were selected as the xeric sites, Sullivan, Christensen and Belly River were selected as the mesic sites, and Spot Mountain, Baring Creek, and Preston Park were selected as the alpine sites (Figure 1). The 1km2 sites were assessed using three 50 m x 50 m plots for butterfly diversity surveys but wildflower collections were conducted in just one plot. The most central plot to the site was chosen for wildflower collection unless wildflower abundance was too low to collect all high-quality wildflower samples; in that case the subplot with highest abundance of wildflowers was chosen. A tenth site (Saint Mary – mesic) was sampled in a side experiment to test contrived butterfly visits onto flowers.

2.2 Wildflower Collection

Wildflowers were collected directly following traditional netting surveys of butterflies in Glacier National Park, Montana (Chapter 1). Sample collection dates ranged from July 1st to August 16th, 2022, to target peak flowering and butterfly activity and were conducted on dates at least 2-3 days after the most recent precipitation to limit the possibility of eDNA removal from the flowers due to rain (Valentin et al., 2021; Johnson et al., 2023). Thirty flowers from two to three dominant flower species with high butterfly visitation were collected at each site. To minimize contamination, flower samples were obtained using sterile gloves with gloves changed between each flower species. Flowers were plucked by hand from the base of the flower head, minimizing direct contact with the flower head and collection of vegetative material. Flower

collections radiated from the center of a 50 m^2 plot and one mature inflorescence was collected from plants at least one meter apart from each other. One meter was selected as the minimum distance between flower collections to increase the chances of different individual butterflies having been present on the collected flower. Flowers with observed butterfly visitation were opportunistically collected, and the butterfly genus, or species when possible, was recorded for individual flower samples. Flowers were placed in a 2 x 3-inch envelope then in a 3 x 4-inch plastic bag containing 15-20 mL of silica (Dry & Dry). Flower samples were then frozen at -20° C upon return from the field and the time in hours between collection and freezer was noted, ranging from three to 24 hours. A total of 267 flowers of 12 genera were collected from nine sites (Supplemental Table 1), with the tenth site described below. Each wildflower collection event included a field negative control where a sterile gauze pad was opened in the field and handled with fresh sterile gloves for 10-15 seconds, equivalent to the time flower samples were handled, then preserved and stored in the same manner as flower samples.

Post-collection, the cumulative precipitation (mm) and solar radiation (W/m2) for the week prior to wildflower eDNA sampling was determined using daily estimates from Daymet v4 (Thornton et al., 2022) which is at 1km^2 resolution. Precipitation and solar radiation values for the site coordinates for the seven days prior to the collection date were summarized at the site level using Google Earth Engine (Gorelick et al., 2017) and the sum of daily values were added as a covariate to the sample metadata file (Supplemental File 1).

2.3 Contrived Flower Visits

A secondary experiment in which butterfly visits were contrived was carried out to (1) ensure that butterfly DNA could be detected and identified from visited flowers, and (2)

investigate whether eDNA assessment may provide insight about butterfly activity by assessing whether the number of visitations would be reflected in the abundance of butterfly sequences. Two individual butterflies were caught at the St. Mary site on August 12th, 2022: one mid-sized Nymphalid butterfly, *Speyeria mormonia* (Mormon Fritillary), and one small Lycaenid butterfly, *Plebejus melissa* (Melissa Blue), both common to the study area and well represented in the COI barcode database. It should be noted that the genus name for *Speyeria* was revised to *Argynnis* (Zhang et al., 2020) but *Speyeria* is used herein to match record names in the COI database. Each butterfly was placed on flowers of *Erigeron caespitosus* in a series of manipulated visits. One visit was contrived by holding the butterfly by enclosing its wings with butterfly forceps and physically placing the butterfly on the flower for five seconds. A series consisting of 1, 5 and 10 visits was conducted for each butterfly with n=5 flowers for each category, with the exception of the 10-visit category for *Speyeria mormonia* where the individual escaped and only n=3 flowers were collected. Three no-visit control flowers were also collected at the time of sampling to infer DNA already present on the flowers. Flowers were collected and stored as described above.

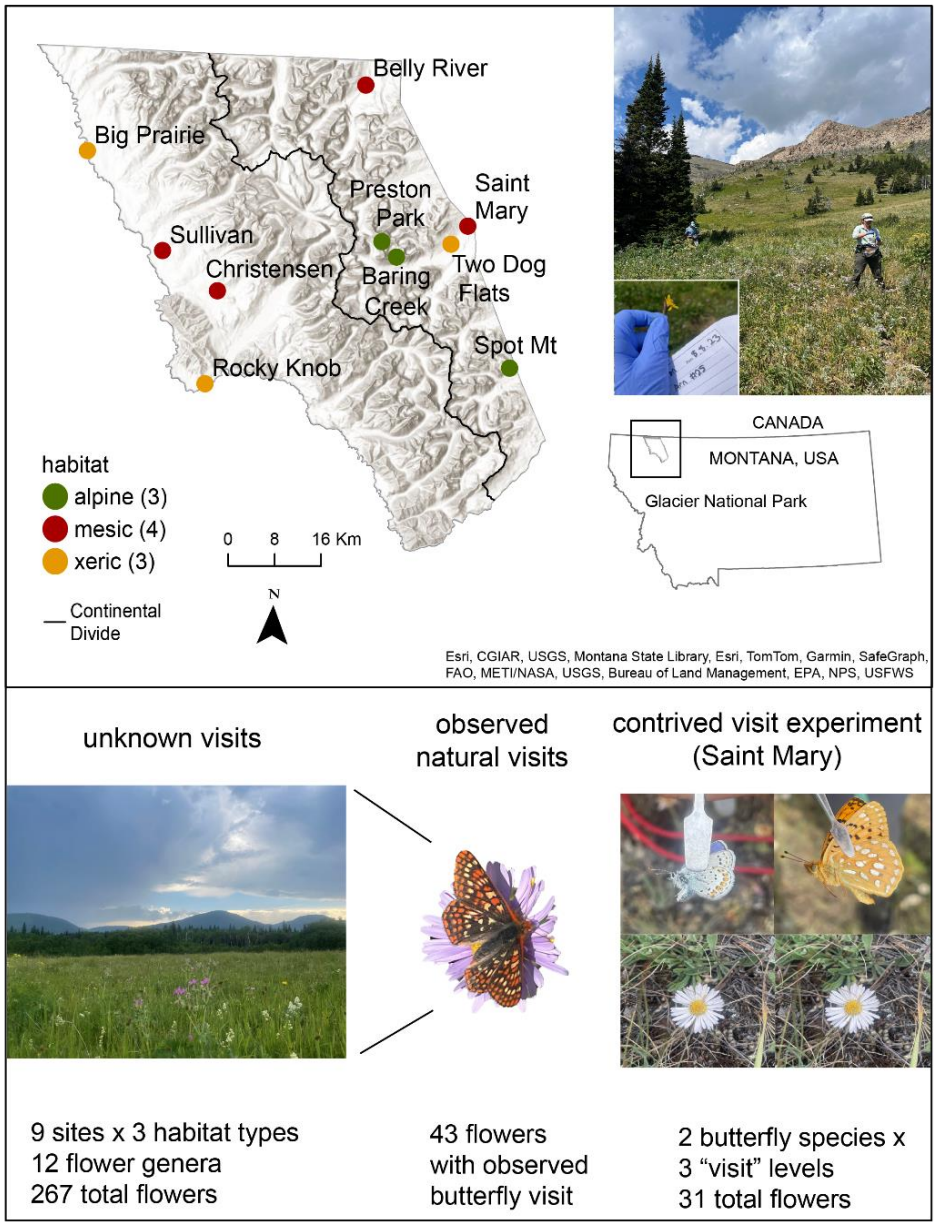


Figure 1. Summary of sites and wildflowers samples for environmental DNA (eDNA) analyses. Wildflowers were collected for eDNA metabarcoding at ten sites in Glacier National Park, Montana immediately following traditional netting surveys for butterfly species identification. The ten sites were comprised of three habitat types, alpine (at or above tree line), mesic (moderate moisture) and xeric (low moisture). For nine sites (three of each habitat type) mature wildflower heads were collected from three dominant genera at each site and twelve genera were collected across all nine sites for a total of 267 individual wildflowers, 43 of which had an observed visit of an identified butterfly before collection. An additional 31 wildflowers were collected at a 10th site (Saint Mary) in a field experiment where two butterfly species were physically placed on one flower species in a series of contrived visits.

2.4 eDNA Extraction and Metabarcoding

Wildflower eDNA samples were processed in a designated low-template laboratory (USGS Northern Rocky Mountain Science Center, Bozeman, MT) and DNA metabarcoding of the COI barcode was conducted using a nested metabarcoding strategy (Figure 2), described in detail below.

DNA Extraction. Each individual wildflower head was submerged in cell lysis buffer and agitated in an incubated shaker for three hours, after which DNA was extracted from the buffer using the Qiagen DNEasy Blood and Tissue Kit with a modified protocol as in previous studies extracting arthropod DNA from wildflowers (Thomsen & Sigsgaard, 2019; Harper et al., 2023). A buffer only negative control was added to each DNA extraction batch at the cell-lysis and incubation stage and carried throughout remaining analysis and sequencing. DNA concentration (ng/µL) was quantified for each sample using an Invitrogen Qubit 4.0 fluorometer and the 1x dsDNA High Sensitivity (HS) Assay Kit (Supplemental File 1).

Specifically, flowers were first transferred to sterile 50 mL tubes, taking care to orient flower heads towards the bottom of the tube. Buffer ATL and proteinase K (ProK, 600 m/AU) were added to the tubes in volumes specified by the size of the flower so that flowers were fully submerged in cell lysis buffer (small: 540 µL ATL and 60 µL ProK, medium: 900 µl ATL and 10 µL ProK, large: 1200 µl ATL and 130 µl Prok), vortexed 10 seconds, and placed in an incubated shaker at 56° C and 200 rpm for three hours. A buffer only negative control was also included at this step with 180 µL ATL and 20 µL ProK in a 2.0 mL tube included in incubation and all downstream steps through DNA extraction, PCR amplification and sequencing.

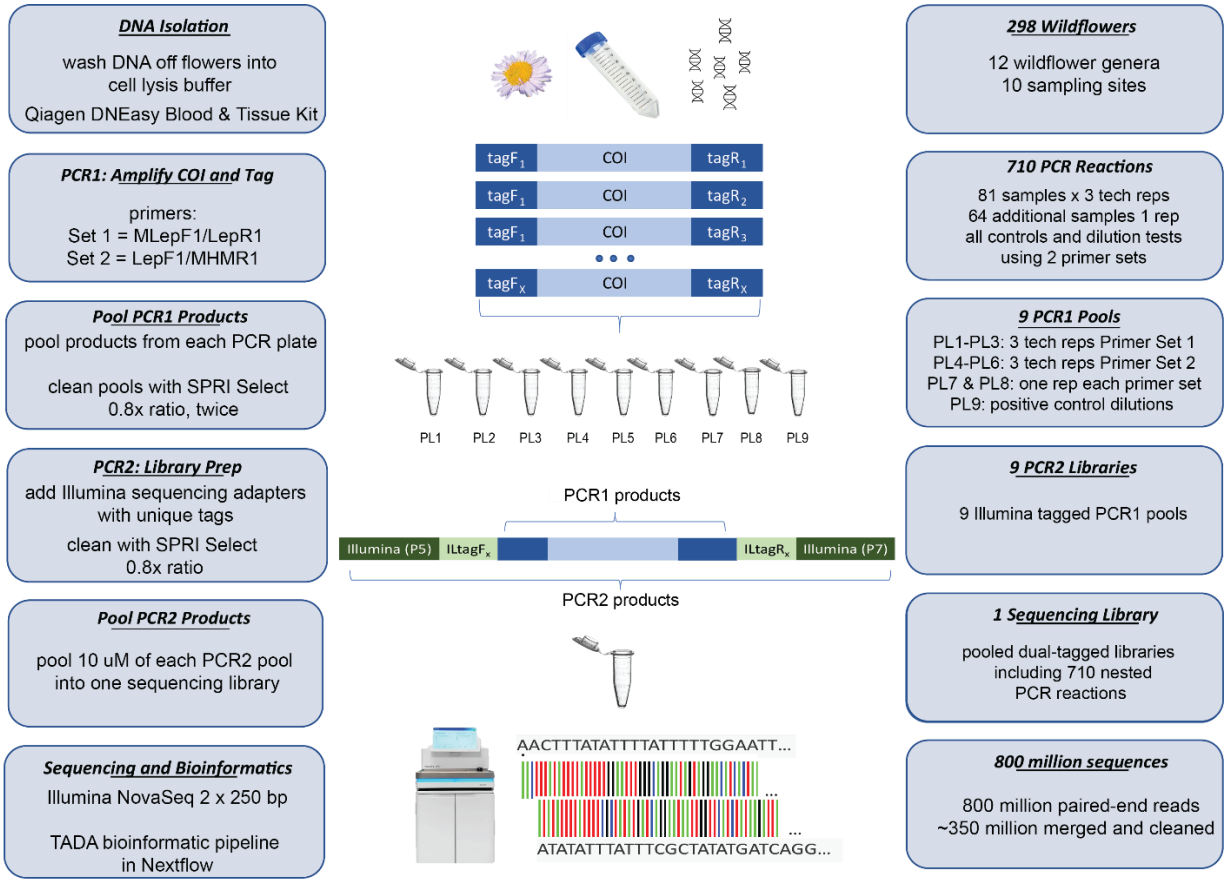


Figure 2. Nested eDNA metabarcoding and sequencing workflow. DNA obtained from flower and control samples was prepared for high throughput sequencing (Illumina NovSeq) by amplifying two *cytochrome c oxidase I* (COI) gene regions using two primer pairs (Primer Set 1 = MLepF1/LepR1 and Primer Set 2 = LepF1/MHMR1) (PCR1). Select samples were amplified and tagged in three independent technical replicates with each primer set (Figure 3). Other samples and positive control dilutions were amplified and tagged in single reactions. The PCR1 products were generated with unique tag combinations, such that the amplicons generated on each 96-well plate could be pooled for a second PCR amplification step (PCR2) that added Illumina sequencing adapters and unique dual indexes (tags). The nine PCR2 libraries were diluted to 10 uM and pooled into one library for high throughput sequencing with an Illumina NovaSeq that resulted in ~ 800 million paired-end sequences. After bioinformatic processing using the targeted amplicon diversity analysis (TADA) pipeline, including quality filtering, read merging, denoising, and chimera removal, approximately 350 million sequences were retained for analyses.

Samples were vortexed for 10 seconds each hour. After incubation, the buffer was transferred to fresh 1.5 mL tubes and equal volumes of buffer AL and molecular grade ethanol

(EtOH) were added. All volume was then spun through a spin column at 10,000 xg for one minute. Columns were washed with 600 µl each of AW1 and AW2 buffers and spun at 10,000 xg for one minute after each addition. Columns were then placed in fresh 1.5 mL tubes and DNA was eluted in two steps of 60 µL dH20 with incubation at 37° C for 15 minutes, for a final volume of 120 µL soluble DNA.

Summary of Controls Used in the Study. In addition to field negative controls from each wildflower collection event and buffer negative controls from each DNA extraction, all PCR plates included two PCR positive controls and one no-template control (NTC). Positive controls consisted of equal amounts of mixed insect DNA from five species: two bees, *Bombus rufocinctus* (red-belted bumble bee) and *Apis mellifera* (honey bee), one ambush bug *Phymata americana* (jagged ambush bug), one beetle *Coccinella transversoguttata* (transverse lady beetle), and one butterfly *Pieris rapae* (cabbage white). A stock of 100 ng/µl of each of the five insect positive controls was diluted in molecular grade water (Invitrogen) and then serial diluted to 5 ng/µl. A stock of mixed DNA from flowers was also made to reflect background DNA; equal volumes (10 µl) of DNA from 10 samples representing wildflower genera of manipulated and known-visit wildflowers (*Arnica, Erigeron, Potentilla, Senecio, Solidago*) were mixed in a 1.5 mL tube. One µL of 5 ng/µL positive control was either added alone to the PCR reaction or to a PCR reaction also including 3 µL background DNA, which was the average volume of DNA template added. All controls once implemented were included in all downstream steps of analysis.

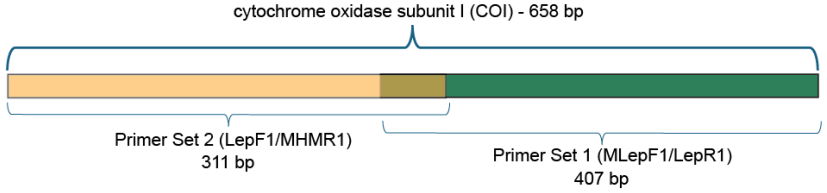
Samples. For PCR amplification, samples were categorized based on butterfly visitation prior to flower collection. Thirty-one samples were a part of the contrived visit experiment and

43 of the 267 site collected wildflowers had a known butterfly visit before collection. Contrived and known visit flowers were analyzed individually to validate molecular detections of butterflies associated with a particular flower. The remaining 224 site-collected flowers did not have a known visit of a butterfly before collection, hereafter referred to as 'unknown visit' flowers. DNA of unknown visit flowers were pooled to maximize samples included in analysis, referred to as "unknown pools". Samples were pooled within flower species and by site so that 10 ng each of three DNA samples were pooled, although in cases where like samples were not divisible by three, two or four DNA samples were pooled. The final concentration (ng/µl) of the pool was calculated (Supplemental Table 2) and 5 ng of each individual or pooled DNA was used as template in PCR, though if DNA concentration was too low to reach 5 ng in 10 µL for the PCR reaction a total of 10 µL of template was added.

Samples were further categorized into priority and non-priority samples so that priority samples could be sequenced in three technical replicates (sequencing replicate) with two primer sets. Remaining capacity due to financial constraints meant that all other samples were sequenced in one replicate with each primer set (Figure 3). All 31 DNA samples from the contrived visit flowers, a subset of DNA samples from the known visit flowers (n=25), and a subset of DNA samples from the pooled unknown visit flowers (n=25) were included as priority samples. Priority samples were amplified in three technical replicates with each primer set (Plates (PL) 1, 2 and 3 for Primer Set 1 and PL 4, 5 and 6 for Primer Set 2). Non-priority samples, 18 remaining known-visit flowers, 45 unknown visit pools, all nine field negative controls, and nine of 14 buffer negative controls were amplified with each primer pair in one replicate (PL7 for Primer Set 1 and PL8 for Primer Set 2).

The remaining five buffer negative controls and tests using a dilution series of the positive control were amplified with both primer sets on plate 9 (PL9). Dilution series consisted of serial dilutions from the 5 ng/µL five-species positive control for a resulting series of 5.0 ng, 2.5 ng, 1.25 ng and 0.625 ng per µL in either molecular grade water alone (Invitrogen) or the wildflower DNA background stock. The 2.5 ng/µL stock was also added to DNA representing each wildflower genus alone (Ex: 10 µl each of the 10 *Arnica* DNA samples used in the mixed wildflower background DNA).

A)



B) priority samples
3 technical replicates

Contrived Visit (CV)

# of visits	Speyeria (N)	Plebejus (N)
1	5	5
5	5	5
10	3	5
genus total	13	15
	0 visit (control)	3
	experiment total	**31**

Known Visit (KV)

flower genus	butterfly genus	# flower butterfly pair
Erigeron	*Speyeria*	3
Symphyotrichum	*Tharsalea*	3
Senecio	*Pontia*	3
Erigeron	*Phyciodes*	2
Erigeron	*Colias*	2
Erigeron	*Thymelicus*	2
Solidago	*Tharsalea*	2
Arnica	*Boloria*	1
Arnica	*Phyciodes*	1
Erigeron	*Cercyonis*	1
Erigeron	*Pontia*	1
Erigeron	*Aglais*	1
Erigeron	*Hesperia*	1
Senecio	*Aglais*	1
Senecio	Colias	1
	total	**25**

Unknown Pool (UNP)

flower genus	# pools	# flowers represented
Arnica	9	28
Erigeron	6	19
Achillea	3	10
Solidago	3	8
Potentilla	2	7
Senecio	2	5
total	**25**	**77**

C) remaining samples
1 replicate

Known Visit (KV)

flower genus	butterfly genus	N flower-butterly pair
Arnica	*Tharsalea*	1
Arnica	*Speyeria*	1
Arnica	*Thymelicus*	1
Erigeron	*Speyeria*	4
Erigeron	*Phyciodes*	1
Erigeron	*Tharsalea*	1
Erigeron	*Pontia*	1
Symphyotrichum	*Tharsalea*	7
Geranium	*Plebejus*	1
	total	**18**

Unknown Pool (UNP)

flower genus	# pools	# flowers represented
Dasiphora	6	18
Eriogonum	9	29
Packera	3	10
Geranium	9	28
Senecio	3	10
Drymocallis	3	10
Achillea	3	10
Potentilla	3	10
Arnica	6	20
total	**45**	**145**

Negative Controls

type	N
field negative	9
buffer negative	14

D) positive control dilutions
1 replicate

Positive Control Species	Stocks
Phymata americana	5.0 ng/ul
Pieris rapae	2.5 ng/ul
Coccinella transversoguttata	1.25 ng/ul
Apis mellifera	0.625 ng/ul
Bombus rufocinctus	

Dilution	Background (BG)
5.0 ng	dH20
2.5 ng	dH20
1.25 ng	dH20
0.625 ng	dH20
5.0 ng	mixed floral
2.5 ng	mixed floral
1.25 ng	mixed floral
0.625 ng	mixed floral
2.5 ng	*Arnica* floral
2.5ng	*Erigeron* floral
2.5 ng	*Potentilla* floral
2.5 ng	*Senecio* floral
2.5 ng	*Solidago* floral

Figure 3. Target regions of the *cytochrome c oxidase I* (COI) gene with two primer sets and sample categorizations for PCR amplification. A) Two fragments of the standard 658-base pair (bp) barcode from the *cytochrome c oxidase I* (COI) gene were targeted with two primer sets: Primer

Set 1 (MLepF1/LepR1) targets a 407 bp fragment ending on the 3' end of the barcode region and Primer Set 2 (LepF1/MHMR1) targets a 311 bp fragment beginning on the 5' end of the barcode region. The two primer sets amplify fragments that overlap ~60 bp of the COI barcode. Wildflower eDNA samples and controls were amplified with both primer sets. B) Priority samples were amplified in three technical replicates and included all contrived visit experiment flowers, 25 known visit flowers, and 25 unknown visit pools. C) The remaining samples were amplified in one replicate with each primer set and included 18 known visit flowers, 45 unknown visit pools, 9 field negative controls and 14 buffer negative controls. D) Positive control dilution tests were conducted in one replicate with each primer set. The positive control consisted of equal amounts of DNA from five insect species: one plant bug (*Phymata americana*), one butterfly (*Pieris rapae*), one beetle (*Coccinella transversoguttata*), and two bees (*Bombus rufocinctus* and *Apis mellifera*). The mixed insect DNA positive control was serial diluted into stocks of 5.0 ng, 2.5 ng 1.25 ng and 0.625 ng per microliter. Sequencing libraries were generated from each positive control dilution in either a background (BG) of molecular grade water, a solution of mixed floral DNA that included DNA from five plant genera (*Arnica, Erigeron, Potentilla, Senecio* and *Solidago*) mixed together in equal volumes (5ul each from 10 of each genera). Floral backgrounds for each of the five plant genera were also created from the 10 wildflowers of each genus that were included in the mixed wildflower DNA background and 2.5 ng of positive control was added to each floral background in separate reactions.

PCR and Multiplexing. Two sub-regions of the standard 658-bp COI barcode were targeted using two different primer sets established for their ability to distinguish Lepidopteran species (Hajibabaei et al., 2006). MLepF1 (5'-GCTTTCCCACGAATAAATAATA-3') – LepR1 (5'-TAAACTTCTGGATGTCCAAAAAATCA-3'), referred to as 'Primer Set 1', targets a 407-bp amplicon ending on the 3' end of the barcode region and LepF1 (5'-ATTCAACCAATCATAAAGATATTGG-3') – MH-MR1 (5'-CCTGTTCCAGCTCCATTTTC-3'), referred to as 'Primer Set 2', targets a 311-bp amplicon beginning on the 5' end of the barcode region (Figure 3). The two primer regions overlap ~60 bp of the 658 bp COI barcode region. For all PCR work, reagents were added in a template-free clean room underneath a biosafety hood and DNA template was added in a separate PCR room.

DNA samples were amplified and multiplexed with a 2-step PCR nested metabarcoding method (Kitson et al., 2019; Guenay-Greunke et al., 2021). In the first PCR (PCR1), fusion

primers (Integrated DNA Technologies) were used to add a unique 8-nucleotide sequence (internal index) for sample tagging, a 0-6-bp variable spacer to increase sequence heterogeneity, and an extension sequence to bind sequencing adapters in the second PCR (PCR2). Twelve forward and eight reverse primers with unique internal indices were used to tag a full 96-well PCR plate with unique combinations of indices on forward and reverse primers (Supplemental Table 3). However, 12 tag combinations were not used in the entire study to facilitate tracking of index hopping after sequencing, thus 84 PCR reactions could be performed on each PCR plate. Each PCR plate also included two PCR positive controls (PosCtrl_noBG and PostCtrl_wBG), described above, and one no-template control (NTC), or dH20 alone added as template.

The first PCR was conducted in a 25 µL reaction with 12.5 µL AmpliTaq Gold™ 360 Master Mix (Applied Biosystems), 1.25 µL each of 10 µM forward and reverse primers, 5 ng of DNA template, and dH20 to 25 µL. Reactions were cycled on a Thermal Cycler with the following profile: 95° C for 10 minutes, 35 cycles of 95° C 10 seconds/46° C for 30 seconds/72° C for 1 minute, followed by a five minutes at 72° C (Clarke et al., 2017).

PCR product concentration (ng/µL) was quantified for each sample using an Invitrogen Qubit 4.0 fluorometer and the 1x dsDNA High Sensitivity (HS) Assay Kit (Fisher Scientific). With each PCR reaction consisting of a unique combination of tags allowing product differentiation after sequencing, 30 ng of each reaction from the PCR plate was pooled in a 1.5 mL tube (PCR1 pools). Immediately after pooling, PCR1 pools were cleaned and size selected with SPRISelect (Beckman Coulter) magnetic beads. A 0.8x bead to sample ratio was used for a left-sided size selection of products above 200 bp. Briefly, 40 µL of SPRISelect beads were added to 50 µL of PCR1 pool, bound on a magnetic stand, washed with freshly diluted 85%

EtOH, and the cleaned products were eluted with 50 µL dH20. The eluate was then cleaned a second time with the same protocol and the final cleaned PCR1 pool was eluted in 20 µL dH20.

A second PCR (PCR2) was used to add Illumina sequencing adapters and a second set of unique dual indices (Illumina Index) on each forward and reverse primer. Nine forward and reverse primers (IDT) with unique indices were used on the nine PCR1 pools (Supplemental Table 3). PCR2 consisted of 50 µL reactions with 25 µL AmpliTaq Gold™ 360 Master Mix, 1.0 µL each of 10 µM forward and reverse primers, 5 µL PCR1 pool template, and dH20 to 50 µL. Reactions were cycled on a Thermal Cycler with the following profile: 95° C for 10 minutes, 10 cycles of 95° C 10 seconds/55° C for 30 seconds/72° C for 1 minute, followed by a five minutes at 72° C (Clarke et al., 2017).

PCR2 reactions, representing all samples pooled from a PCR plate, were cleaned with one round of 0.8x SPRISelect as described above. The size and concentration of cleaned PCR2 reactions, referred to as sub-libraries, were then obtained using an Agilent Bioanalyzer and the Agilent High Sensitivity DNA kit. Sub-libraries were pooled together in a final sequencing library to 10 µM, where each pre-library was diluted to a 5 µL stock at 10 µM, apart from pre-library 8 which was of too low concentration to obtain 10 µM and instead equal volume (5 µL) was added to the final pool (Supplemental Table 4). The final wildflower-associated eDNA sequencing library was quantified on the Agilent Bioanalyzer with the High Sensitivity DNA kit.

2.5 High Throughput Sequencing and Bioinformatic Processing

The wildflower-associated eDNA sequencing library was sent to the University of Illinois Roy J. Carver Biotechnology Center for 2 x 250-bp paired-end sequencing on the Illumina NovaSeq SP flowcell. Sequence data (806,792,426 reads) was de-multiplexed by Illumina

indices (i.e., sub-libraries) by the University of Illinois. All further computation efforts were performed on the Tempest High Performance Computing System, operated and supported by University Information Technology Research Cyberinfrastructure at Montana State University. First, sequences were further de-multiplexed by internal indices using Cutadapt v4.2 (Martin, 2011) with allowed errors set to 2 (0.25 error rate), resulting in sequences associated with the sample of origin. All possible combinations of indices were given in the Cutadapt search, including 12 primer index combinations that were not used in this study. Unused index combinations were used to derive (or calculate) an index hopping rate by comparing unexpected tag combinations to those that are expected (Schnell et al., 2015; Guenay-Greunke et al., 2021). Total sequences for all index combinations were found using SeqKit v2.7.0 (Shen et al., 2016) and proportions of unmatched and matched sequences were calculated in R v4.3.2 (R Core Team, 2023).

Demultiplexed files were renamed with their sample associated names in R and primers were removed using Cutadapt. All further bioinformatic processing was conducted using the DADA2 bioinformatic pipeline (Callahan et al., 2016) via the Targeted Amplicon Diversity Analysis (TADA) workflow implemented in Nextflow v22.10.8.5859 (Lennard et al., 2020) and Java v17.0, which includes quality filtering, sample denoising, read merging, and chimera removal. The TADA pipeline outputs amplicon sequence variants (ASVs), or sequences that vary by one or more nucleotides.

2.6 Taxonomic Assignment

Taxonomic assignment of unknown sequences obtained from eDNA relies on aligning unknown sequences to a reference database comprised of the COI barcode regions of the COXI

gene. The Barcode of Life Database (BOLD) is the main repository for COI reference sequences with over 9 million vouchered specimens. For clarity, sequences associated with COI databases will be referred to as "records" and sequences obtained from wildflower eDNA will retain the term "sequences."

COI Reference Databases. The full BOLD database was downloaded from the March 2023 versioned database and contained 9,237,843 records (BOLD, 2023). Several initial quality control and filtering steps were conducted from the Linux command line, including removing records associated with plants and markers other than COI, removing duplicated records, and removing records containing non-IUPAC characters (ACTG), which resulted in 8,287,203 records. Sequences associated with these records and accompanying taxonomy were imported into QIIME 2 v2024.2.0 (Bolyen et al., 2019) where sequences were culled further and dereplicated with RESCRIPt, a QIIME 2 plugin (Ii et al., 2021) leaving 3,747,990 records. Finally, records associated only with the United States and Canada were retained for a final database, hereafter referred to as 'bold-full-USCAN', representing 1,114,806 COI barcodes of animal species from North America (Supplemental Files 2 and 3).

Additionally, a North American butterfly specific COI database was downloaded by accessing the project name DS-USCANLEP which consists of 14,286 records representing the 835 butterfly species known from Canada and the United States (D'Ercole et al., 2021). A Glacier National Park butterfly specific database was created by filtering the DS-USCANLEP database using a species list created from butterflies that were observed in the traditional butterfly diversity study (Chapter 1) or could be expected in the study area based on cross-referencing citizen science identifications (iNaturalist) with the Montana Field Guide (Montana

Natural Heritage Program) relative density and recency plots pertaining to a species. The resulting list included 126 species of butterflies which corresponds to 6,345 records in the DS-USCANLEP database (Supplemental Table 5), hereafter referred to as DS-USCANLEP-GNPSps (Supplemental Files 4 and 5).

Taxonomic assignment of ASVs from each primer pair was conducted in QIIME 2 using a global alignment tool, VSEARCH (Rognes et al., 2016), where query sequences were aligned to the reference databases described above. The threshold for alignment of unknown sequences to the reference database was set to 95% similarity. This value was based from COI barcode sequence alignments from all records in the DS-USCANLEP-GNPSps database. The FASTA formatted sequences associated with these records were imported into Geneious Prime (2023.0.1) and aligned using Muscle 5.1 with the Super5 alignment algorithm (Edgar, 2004). All 6,345 records from the DS-USCANLEP-GNPSps database were aligned and had an average of 87.1% similarity, indicating the VSEARCH default alignment threshold of 80% would be insufficient for elucidating target butterfly species-specific differences. Several species-specific alignments were done where all records associated with a species were aligned. Records of three of the species with the highest representation in the database and representing different butterfly families were aligned; 279 records of *Boloria chariclea* (Nymphalidae) shared 99.3% similarity, 242 records of *Glaucopsyche lygdamus* (Lycaenidae) shared 98.2% similarity, and 210 records of *Burnsius communis* (Hesperiidae) shared 99.9% similarity. The two species used in manipulated visits, *Speyeria mormonia* (Nymphalidae) and *Plebejus melissa* (Lycaenidae), were 99.1% (95 records) and 99.3% (41 records) similar, respectively. Records from *Plebejus idas* which is closely related to *Plebejus melissa* and difficult to distinguish in the field shared 96.3% similarity

(122 records) and the 93 records of the butterfly species used in the positive control, *Pieris rapae* (Pieridae), shared 98.7% similarity. Species with fewer records in the database followed a similar pattern, where 5 records of *Pterourus multicaudata* (Papilionidae) shared 99.8%, 11 records of *Tharsalea nivalis* (Lycaenidae) shared 99.9% and 15 records of *Euchloe olympia* (Pieridae) shared 97.3% similarity. Because the within-species similarity varied from 96.3% to 99.9% and the percent similarity between butterfly species expected in Glacier was 87.1%, it was determined that a 95% similarity threshold would best facilitate assignment of unknown sequences in the wildflower-eDNA library described herein.

The VSEARCH global alignment tool was implemented in QIIME 2 using the q2-feature-classifier (Bokulich et al., 2018) and classify-consensus-vsearch (Rognes et al., 2016) plugins where after alignment of query sequences to the reference database, a consensus taxonomy is assigned by selecting the top hit among a maximum of accepted hits ('maxaccepts' set to 100) of which more than 50% share consensus with the top hit ('minconsensus', default 0.51). Output tables of taxonomic assignment results include the ASV feature identifier, the consensus taxonomy and the consensus score. These tables were imported into R v4.3.2 for the remainder of analysis.

Final Cleaning and Filtering Steps. Several quality control and filtering steps were applied to sequences during the TADA bioinformatic pipeline and two more filtering steps, filtering out likely contaminants and applying a sequence length filter, were applied before analysis. There were 7,466 amplicon sequence variants (ASVs), or sequences that differ by one or more nucleotides, output from the TADA bioinformatic pipeline for Primer Set 1 (MLepF1/MHMR1) and 2,681 ASVs for Primer Set 2 (LepF1/MHMR1). Subsequent filtering

and analyses were conducted using R v4.3.2 (R Core Team, 2023) and packages phyloseq v1.46.0 (McMurdie & Holmes, 2013) and decontam v1.22.0 (Davis et al., 2018). Data organization (or sorting) was done using phyloseq and tidyr/dplyr (v1.3.1/v1.1.4). The ASV tables, sample metadata and taxonomy files were merged using phyloseq. Likely contaminants were removed using the 'frequency' function of the decontam package that considers sequence abundance and DNA concentration of input template to recognize likely contaminates using the property that contaminant sequence abundance will scale linearly with the concentration of DNA, while true sequence abundance will be independent of DNA concentration (Davis et al., 2018). Dilution experiment samples (PL9) were analyzed separately and not filtered using decontam since DNA concentrations varied by design and would introduce bias using a frequency-based definition of contaminants. From all remaining samples (PL1 – PL8), twenty-five contaminants were identified by decontam for Primer Set 1 data and nine contaminants were identified for Primer Set 2 (Supplemental Table 6). The contaminant ASVs, as well as any ASVs with less than five associated sequences, were removed using phyloseq. An additional length filter was applied to remove sequences that were 100 bp shorter than the expected size for each primer pair (i.e., less than 310-bp for Primer Set 1 and less than 210-bp for Primer Set 2, Supplemental Table 7). This resulted in 122 fewer ASVs for the Primer Set 1 and 54 fewer ASVs for Primer Set 2. Finally, ASVs identified to any of the five positive control insect species were removed from wildflower-associated samples after finding two positive control species, *Phymata americana* and *Pieris rapae*, in negative control samples and in 136 of 672 (20.3%) and 142 of 672 (21.1%) of wildflower-associated samples, respectively. The final ASV and taxonomy table contained 8,147 ASVs between both primer sets (Supplemental File 6).

2.7 Data Analysis and Summary

The cleaned and filtered sequence abundance values from each ASV were used for assessments of positive and negative controls and Lepidopteran (butterflies and moths) sequences associated with contrived visits. Abundance values were then converted to presence (1) if greater than zero and absence (0) values. To assess continuity between technical replicates of each primer set, accumulation curves of ASVs and unique taxa were created using vegan (v2.6.4). Analyses of observed richness (number of unique taxonomic identifications) or detections (sum of presence values) by taxonomic rankings were conducted using presence or absence values across all non-control samples, where samples with technical replicates were merged so that a presence value in any of the three replicates was considered presence and absence would be considered as absence from all three technical replicates. Comparisons of taxonomic richness of the same samples between both primer sets were tested using paired Wilcoxon ranked sign tests.

3 Results

3.1 Wildflower-associated eDNA Samples and Sequencing

A total of 298 wildflowers were collected from ten sites in Glacier National Park directly after traditional netting surveys. Forty-three flowers had an observed butterfly visit where the butterfly was identified to genus or species before collection and 31 flowers were involved in a contrived visit experiment (Figure 1, Supplemental Table 1). Contrived visitation consisted of physically placing two butterfly species, *Speyeria mormonia* and *Plebejus melissa*, on *Erigeron caespitosus* flowers in series of one, five, or ten, five-second “visits”. Wildflower-associated DNA was extracted from wildflower washes of all individual flowers and the 74 flowers that had

known butterfly contact with the flower were kept as individual samples throughout analysis. The remaining 224 wildflower-associated eDNA samples were pooled with equal amounts of DNA from 2-4 flowers of the same species and site (Supplemental Table 2) so that identifications of butterflies from eDNA samples could be compared to known butterfly species presence from traditional surveys. A dilution series of a five-insect species positive control sample was also included in analysis to test whether sequencing data in this sample cohort could be analyzed quantitatively (Figure 3).

Wildflower-associated eDNA samples were amplified with two primer sets that targeted different segments of COI (Primer Set 1 = MLepF1/LepR1 and Primer Set 2 = LepF1/MHMR1). The nested metabarcoding scheme utilized in this study tagged PCR products from each wildflower eDNA sample with unique combinatorial indexes integrated into the amplification primers (PCR1), allowing all PCR products from a plate (PL) to be pooled into "sub-libraries" (Figure 2). Twelve index combinations were unused in the entire study to facilitate estimates of index hopping after sequencing. A select cohort of samples consisting of all contrived-visit samples (n=31) and subsets of DNA from flowers with an observed natural visit (n=25) and pooled samples (n=25) were amplified in three technical replicates with each primer set (Primer Set 1: PL1, PL2, PL3 and Primer Set 2: PL4, PL5, PL6). All remaining samples and positive control dilution series were amplified in one replicate with each primer set (Primer Set 1: PL7, Primer Set 2: PL8, Dilutions: PL9) (Figure 3). Sub-libraries were prepared for sequencing using a second PCR reaction (PCR2) that added Illumina sequencing adapters and unique dual indexes, so that sub-libraries could be pooled into one sequencing library (Supplemental Table 4). The final library for high throughput sequencing contained the nine sub-libraries that included a total

of 710 tagged PCR1 reactions from wildflower-associated eDNA samples and all positive and negative controls that were amplified with two COI primer sets.

Sequencing yielded over 800 million paired-end sequences and after all bioinformatic processing and quality control steps, ~350 million sequences were retained for taxonomic assignment and analysis. Estimates of index hopping, calculated by dividing the total sequences that had unexpected tag combinations by the total sequences associated with expected tag combinations for each sub-library, ranged from 0.008% to 0.822% for PL1 through PL8 which contained all wildflower-associated eDNA samples. The library containing the dilution tests (PL9) had a higher percentage of unexpected tag combinations at 9.5% (Supplemental Table 8). The data generated using Primer Set 1 (MLepF1/LepR1) resulted in ~223 million retained sequences for analysis, whereas amplification with Primer Set 2 (LepF1/MHMR1) resulted in ~124 million sequences retained for analyses. The number of sequences per sample differed for each primer pair. Specifically, the mean number of sequences generated using Primer Set 1 was 524,030 sequences per wildflower eDNA sample and the range was 0 to 4,289,400 (SD = 903,735) sequences. The mean number of sequences generated using Primer Set 2 was 381,296 sequences per sample, with a range of 0 to 9,459,852 (SD = 1,319,378) sequences (Table 1). Six wildflower eDNA samples amplified with Primer Set 1 and 49 samples amplified with Primer Set 2 had fewer than 10 sequences. The majority of these samples (i.e., 52 of 55) were pooled eDNA samples obtained from flowers for which butterfly visitation was unknown (i.e., not observed). Only two of these low-yield samples were shared between primer pairs, both were pools of *Geranium* flowers (Supplemental Table 9). More than half of the samples that had less than 10 sequences from Primer Set 2 (i.e. 28 of 49) originated from plate eight (PL8) which had

very low product yield after the cleaning step during the final sequencing library preparation (Supplemental Table 4). The remaining 21 samples were three technical replicates of seven samples that had zero or only one sequence associated with them.

Table 1: Environmental DNA (eDNA) sample sequence data overview. The mean number of sequences for wildflower eDNA samples and each type of positive (pos ctrl) and negative control (neg) for both primer sets is summarized after all filtering and quality controls steps. For individual sample data see Supplemental Table 9. The PCR plate (PL) numbers that correspond to each category of sample listed in the table are given in superscripts. Samples that were included in PL9, which included positive control dilution series and five of the 14 buffer negative controls, are reported separately due to high abundance in the no-template control (NTC) for PL9 compared to PL1 through PL8 for Primer Set 1. Positive controls on PL1 and PL2 were missed, and reactions were sequenced without positive control template. For each category the number of samples (N) and the total, mean, min, max and standard deviation (SD) of sequences are reported.

Primer Set 1 (MLepF1/LepR1)

	N	Total	Mean	Min	Max	SD
wildflower eDNA samples	306	160,352,788	524,030	0	4,289,400	903,735
positive controls						
pos ctrl floral BG [PL3, PL7]	2	10,199,071	5,099,536	5,093,689	5,105,382	8,268
pos ctrl dH_20 BG [PL3, PL7]	2	26,144	13,072	8,821	17,323	6,012
pos ctrl dilutions [PL9]	13	46,661,683	3,589,360	2,543,248	4,832,526	745,989
negative controls						
field negs [PL7]	9	20,021	2,225	0	14,650	4,817
buffer negs [PL7]	9	46,687	5,187	0	20,858	7,821
buffer negs [PL9]	5	1,789,510	357,902	24	1,202,282	536,125

Table 1 Continued

NTC [PL1-3, PL7]	4	523	131	2	249	101
NTC [PL9]	1	1,273,389	-	-	-	-
missed pos ctrl floral BG [PL1-2]	2	2,173,046	1,086,523	1,036,859	1,136,187	70,235
missed pos ctrl dH_20 BG [PL1-2]	2	220,174	110,087	98,328	121,846	16,630
total	355	222,763,036				

Primer Set 2 (LepF1/MHMR1)

	N	Total	Mean	Min	Max	SD
wildflower eDNA samples	306	116,676,621	381,296	0	9,459,852	1,319,478
positive controls						
pos ctrl floral BG [PL4-6, PL8]	4	3,720,358	930,090	216,879	2,129,572	849,673
pos ctrl dH20 BG [PL4-6, PL8]	4	3,797,014	949,254	353,917	1,682,103	558,093
pos ctrl dilutions [PL9]	13	43,060	3,312	0	12,163	3,691
negative controls						
field negs [PL8]	9	20,003	2,223	0	19,488	6,476
buffer negs [PL8]	9	97	11	0	34	13
buffer negs [PL9]	5	10	2	0	7	3
NTC [PL4-6, PL8]	4	4,567	1,142	29	2,490	1,014
NTC [PL9]	1	5	-	-	-	-
total	355	124,261,735				

3.2 Mixed Insect DNA Dilution Series and Sequencing Summary

To evaluate the molecular biology and bioinformatic analyses utilized in this study to provide accurate, and potentially quantitative, taxonomic data, positive control samples containing known amounts of insect DNA sample were generated and sequenced (Figure 3). Primer Set 1 was expected to bind and amplify all five species in the positive control while Primer Set 2 was expected to bind and amplify only *Pieris rapae* (Supplemental Figure 1). The total sequence count per mixed positive control sample, and the number and the percentage of sequences for each positive control species are summarized in Supplemental Table 10. Three out of five positive control species were identified using Primer Set 1, *Phymata americana, Coccinella transversoguttata,* and *Pieris rapae.* The majority, 97.12% to 99.78%, of the dilution series sequences were identified as *Phymata americana*. *Pieris rapae* was the only species identified using Primer Set 2. Positive control insect DNA was also amplified in floral DNA backgrounds that included five plant genera: *Arnica, Erigeron, Potentilla, Senecio,* and *Solidago*. The percent of total sequences identified to *Pieris rapae* varied between floral backgrounds, 10.16% of the total sequences from *Solidago,* 29.47% in *Potentilla,* and more than 90% in *Arnica, Erigeron* and *Senecio* (Figure 4). The three lower dilutions of the positive control in the mixed floral background did not yield any sequences with Primer Set 2. Due to the uneven proportion of positive control species abundances in dilution series tests, it was determined that sequence abundance could not be quantified reliably, and remaining analyses were conducted by first converting taxonomic assignments to presence or absence.

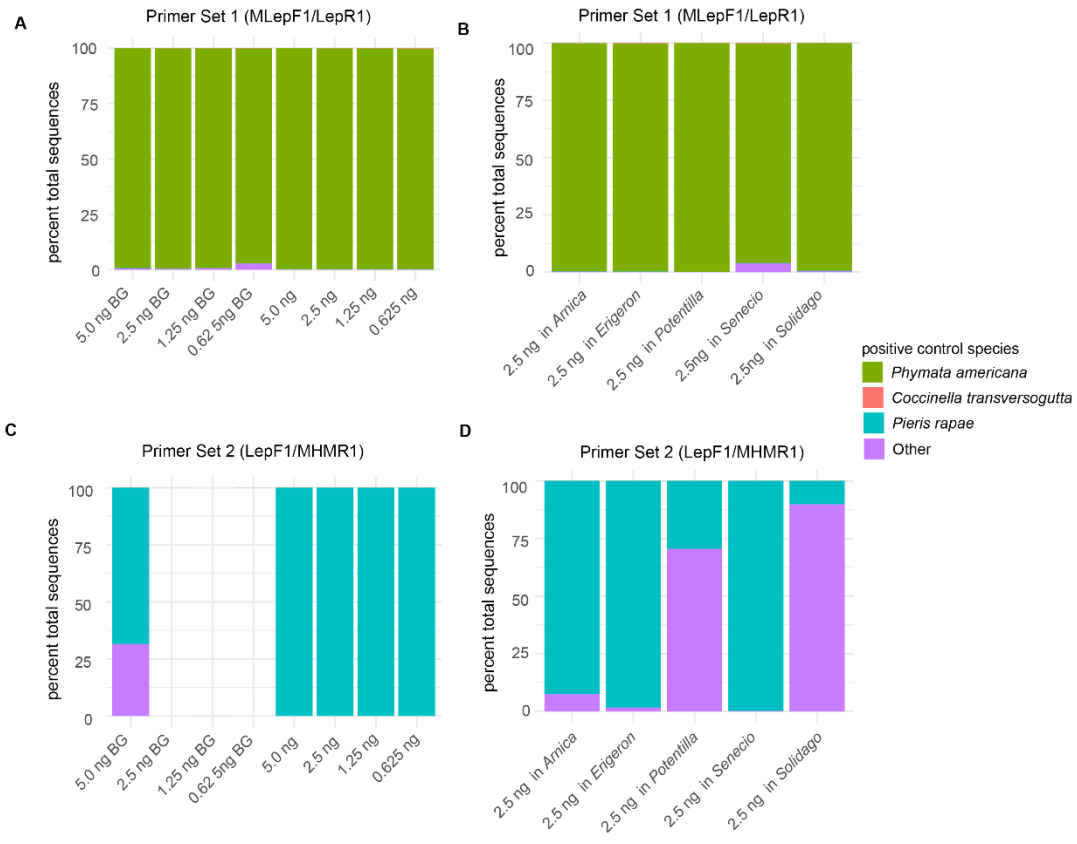


Figure 4. Sequence data from positive control dilution series indicate non-quantitative nature of sequencing data. Stacked bar plots indicate the percent of total sequences that were identified as each of five possible insect species included in mixed insect DNA positive controls (Figure 3). Positive control sample sequencing libraries were generated using both primer sets (Primer Set 1 = MLepF1/LepR1 and Primer Set 2 = LepF1/MHMR1). A) Positive control dilution series amplified with Primer Set 1 B) Positive control in genus specific floral background for Primer Set 1 C) Positive control dilution series amplified with Primer Set 2 D) Positive control in genus specific floral background for Primer Set 1. More than 90% of total sequences for each positive control sample generated with Primer Set 1 were identified to *Phymata americana*. *Pieris rapae* was the only positive control species identified with Primer Set 2 and the percent of total sequences identified to *Pieris rapae* varied among genus-specific floral backgrounds. Dilutions of 2.5 ng, 1.25 ng and 0.625 ng in the mixed floral background did not yield any sequences with Primer Set 2.

Positive control species were also identified in negative controls, though overall sequence abundance in negative controls was low. Three types of negative controls were included in analysis, a field negative control for each wildflower eDNA collection event, buffer only

negative controls for each DNA extraction, and PCR no-template controls (NTC) for each PCR plate. Information from negative controls was used to remove likely contaminants using the R package decontam (Davis et al., 2018) though the sequences of several taxa were still reported after this step (Supplemental Figure 2, Supplemental Table 9). Field negative controls amplified using Primer Set 1 had a mean sequence abundance of 2,225 with a range from 0 to 14,650 sequences. The field negative originating from the site *Big Prairie* had higher abundance (i.e. 14,650 sequences) while all other field negatives had a total sequence count below 3,500. Similarly, field negatives using Primer Set 2 had a mean abundance of 2,223 sequences with a range between 0 and 19,488 sequences. The field negative originating from the site *Rocky Knob* had 19,488 sequences while the other eight field negatives had a total sequence count below 500. Analysis of these outlier samples determined that the sequences were taxonomically assigned to Arthropoda, but they were not assigned to any lower taxonomic levels. The number of sequences obtained from buffer negative controls was low for samples amplified using Primer Set 2 (mean = 11, range = 0-35, SD = 13), while the average sequence abundance obtained from libraries prepared using Primer Set 1 was higher (mean = 5,187, range = 0 – 20,858, SD = 7,821). Buffer negatives associated with extractions 7 and 9 had the highest abundance values, the majority of which were assigned to *Phymata americana*. No template controls (NTCs) were low for both primer pairs, though somewhat higher for Primer Set 2 (mean = 1,142, range 29 – 2,490, SD = 1,014) than Primer Set 1 (mean = 131, range = 2 – 249, SD = 131) (Table 1). The majority of the sequences associated with NTCs for Primer Set 2 were assigned to *Pieris rapae*. Because positive control species were identified in negative controls and we did not use positive control

species DNA that is foreign to the study area, ASVs identified to all positive control species were removed for the remainder of analysis.

3.3 Evaluation of Technical Replicates of eDNA Samples Supports Their Importance for Detecting Unique Taxa

To assess the consistency of results from technical replicates, the unique ASVs identified in sequence data from individual replicates, as well as combined data from either two or three replicates were plotted in accumulation curves (Figure 5). Taxonomic assignment of unique ASVs can result in the same taxonomic identification because ASVs only differ by at least one nucleotide, while more mutations accrue in different species. Therefore, accumulation curves for the unique taxa identified in samples with additional replicates were also plotted. Select samples (n=81) were amplified and sequenced in three technical replicates using each primer set and included all contrived visit samples (n=31), samples with an observed natural butterfly visit before collection (n=25), and pooled samples of two to four wildflowers of the same genus and site (n=25) (Figure 3). Replicates were combined by considering an ASV or unique taxa present if it was found in any of the two or three replicates and absent if it was not found in any of the replicates being combined. For both primer sets, the individual technical replicates resulted in a similar final count of ASVs and unique taxa. The first replicate (PL1) of Primer Set 1 resulted in a final count of 2,771 ASVs and 102 unique taxa, the second replicate (PL2) had 2,520 ASVs and 95 unique taxa, and the third replicate (PL3) had 2,357 ASVs and 107 unique taxa. For Primer Set 2 the first replicate (PL4) had a final count of 907 ASVs and 43 unique taxa, the second replicate (PL5) had 907 ASVs and 42 unique taxa, and the third replicate (PL6) had 976 ASVs and 47 unique taxa. Combining the first two replicates of each primer set increased the final counts of ASVs to 3,900 and 1,558 and final counts of taxa to 117 and 51 for Primer Set 1 and

Primer Set 2, respectively. Combining all three replicates of each primer set increased the final counts of Primer Set 1 to 4,768 ASVs of 142 unique taxa and Primer Set 2 to 2,075 ASVS of 63 unique taxa. The number of ASVs and unique taxa increased with each technical replicate for both primer sets, indicating technical replicates are needed to identify unique taxa.

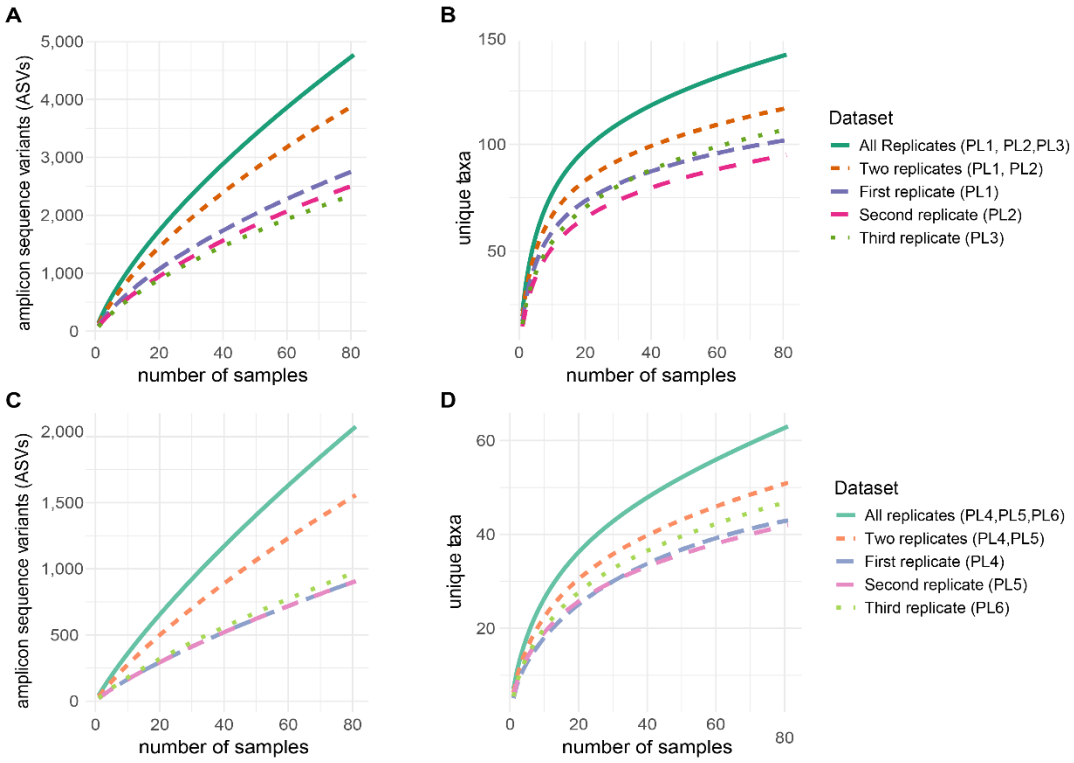


Figure 5. Unique ASVs and taxa accumulate with additional technical replicates. To assess consistency of taxonomic identifications, eighty-one wildflower eDNA samples were amplified and sequenced in three technical replicates with both primer sets. The total number of unique amplicon sequence variants (ASVs) and unique taxa identified from the ASVs increased with the inclusion of technical replicates. Abundance values were converted to presence (1) or absence (0) values and replicates were combined by considering the ASV or taxonomic ID as present if it was found in any of the two or three replicates being combined. Combining replicates increased the number of ASVs and unique taxa identified for both primer sets. The accumulation of unique amplicon sequence variants (ASVs) and unique taxa identified from the ASVs are plotted on the y-axes with each additional sample on the x-axes; A) Accumulation of ASVs with Primer Set 1 B) Accumulation of unique taxa with Primer Set 1 C) Accumulation of ASVs with Primer Set 2 D) Accumulation of unique taxa with Primer Set 2. Different colored and textured lines represent each replicate individually, the first two replicates combined, and all three replicates combined.

3.4 Assessment of Butterfly Associated eDNA on Wildflowers After Controlled Visitation Experiments

A controlled visitation field experiment was conducted at one site (St Mary) to assess whether butterfly eDNA could be identified with known visitation, and whether sequence abundance could be correlated with the number of contrived visits. Two butterfly species that were common to the study area and well-represented in the COI database (Supplemental Table 5), *Speyeria mormonia* and *Plebejus melissa*, were physically placed on flowers in either one, five, or ten "visits". While over 59 million sequences from 401 ASVs with Primer Set 2 and 800,000 sequences from 53 ASVs with Primer Set 1 were identified to Lepidoptera, no sequences were identified to either of the two target species. To investigate the sequences identified to Lepidoptera further, these ASV sequences from both primer sets were aligned directly with COI barcode sequences from regional records of the target species. Two *Speyeria mormonia* records in the DS-USCANLEP database that originated from Waterton, Alberta, Canada just north of Glacier National Park (BOLD record IDs: SSWLE2314-13, SSWLE2315-13), one *Plebejus melissa* record (EZBNA272-07) also from southern Alberta, as well as one record of *Plebejus idas* (LWUSA151-07), which is closely related and difficult to distinguish morphologically from *P. melissa*, from Madison Co., Montana were aligned to the Lepidopteran sequences. All four records shared an average pairwise-identity of 88% or less with all Lepidopteran identified sequences (Table 2), while all three species shared greater than 99% mean similarity with all other conspecifics in the database. These sequence alignments confirmed that the target butterfly species were not identified in any of the wildflower eDNA samples with contrived visitation.

Table 2. Mean pairwise similarity of alignments between regional COI records of contrived visit target species with sequences identified to Lepidoptera from contrived visit experiment. Sequences identified to Lepidoptera in contrived visit samples do not correspond to the butterfly species that were physically placed on the flowers. To ensure ASVs associated with Lepidoptera from contrived visit flowers did not correspond to target taxa, ASV sequences were directly aligned with target taxa COI records of *Speyeria mormonia* and *Plebejus melissa* in Geneious Prime. ASVs identified to Lepidoptera were aligned with two *Speyeria mormonia* records that originated from Waterton, Alberta, Canada directly north of Glacier National Park, one *Plebejus melissa* record also from southern Alberta, as well as one record of *Plebejus idas* from Madison Co., Montana were aligned to the Lepidopteran sequences and to all conspecific records in the COI database. All four records shared an average pairwise-identity of 88% or less with all Lepidopteran identified sequences with both primer sets while conspecific alignments shared greater than 96% similarity for all species.

	Records/ ASVs	[1]*S. mormonia* Waterton, AB	[2]*S. mormonia* Waterton, AB	[3]*P. melissa* Pincher Ck., AB	[4]*P. idas* Madison, MT
ASVs to Lepidoptera Primer Set 1	**53**	**81.54%**	**81.79%**	**68.42%**	**82.32%**
ASVs to Lepidoptera Primer Set 2	**401**	**88.06%**	**88.06%**	**87.95%**	**88.22%**
S. mormonia COI records	95	**99.23%**	**99.48%**	86.93%	87.03%
P. melissa COI records	41	86.57%	86.90%	**99.69%**	99.81%
P. idas COI records	122	86.48%	86.82%	96.62%	**99.44%**

[1] Record ID: SSWLE2315-13
[2] Record ID: SSWLE2314-13
[3] Record ID: EZBNA272-07
[4] Record ID: LWUSA151-07

3.5 Detections of Lepidoptera Across all Wildflower-Associated eDNA Samples

The two primer sets utilized in this study were established for their ability to differentiate species of Lepidoptera and to assess how these primer sets compare in this eDNA sample cohort, identifications of Lepidoptera across all wildflower eDNA samples were compared with both

primer sets. Of the nine insect orders detected with either primer set, Lepidoptera comprised 3.4% of total insect detections with Primer Set 1 (415 of 12,041 detections) and 29.5% of total insect detections with Primer Set 2 (995 of 3,368 detections) (Figure 6).

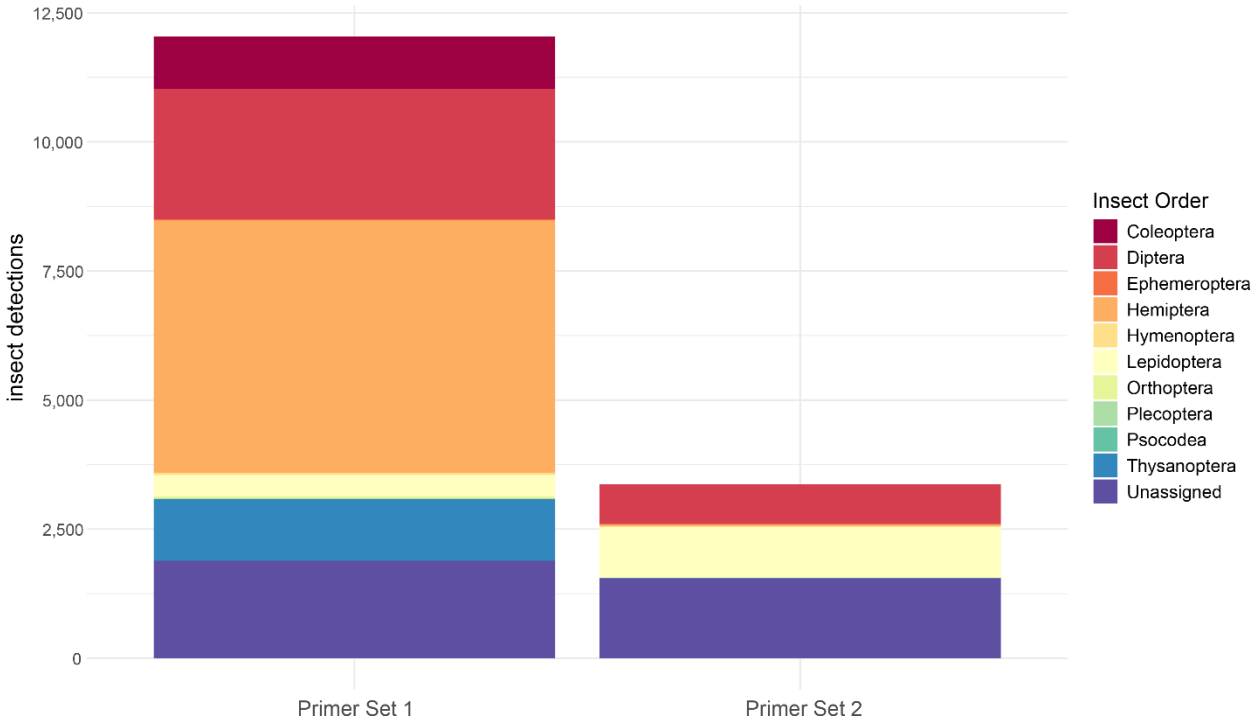


Figure 6. Detections of insect orders differ between two COI primer sets. The overall number of insect detections and the proportion of detections attributed to insect order differed between the two COI barcode primer sets. Nine insect orders were identified between both primer sets. Detections were defined as presence of an ASV identified to the taxonomic order across all samples, where samples with technical replicates were merged so that presence in any of three technical replicates were assigned a value of "1". The height of the stacked bars represents the sum of detections from all insect orders identified with each primer set, Primer Set 1 = MLepF1/LepR1 and Primer Set 2 = LepF1/MHMR1, while color and height represent the total number of detections from each insect order. Sequences obtained using Primer Set 1 included a total of 12,041 ASVs identified as Insecta; 40.6% (4,888) were Hemiptera, 30.0% (2,527) were Diptera, 9.9% (1,195) were Thysanoptera, 8.4% (1,014) were Coleoptera, 3.4% (415) were Lepidoptera, and less than 1% were identified as Hymenoptera, Orthoptera, Ephemeroptera, and Psocodea (45, 45, 20, and 3 detections, respectively). Sequences obtained using Primer Set 2 included a total of 3,368 ASVs identified to Insecta; 29.5% (995) were Lepidoptera, 22.8% were Diptera, 1.4% (48) were Hemiptera, and one detection each for Coleoptera, Hymenoptera, Plecoptera, and Thysanoptera. Primer Set 1 had 15.7% (1,889) of insect ASVs that were not identified to any order ('Unassigned') and Primer Set 2 had 46.1% of insect ASVs that were 'Unassigned'.

To compare the Lepidopteran identifications using the two primer sets, the number of ASVs identified to the order Lepidoptera was calculated for each wildflower-associated eDNA sample and primer set. The majority of samples (i.e., 101 of 145) amplified using Primer Set 1 included at least one identified Lepidoptera sequence (mean = 2.9, range 0 to 23, SD = 3.1) and 81 samples amplified using Primer Set 2 included at least one Lepidoptera detection (mean = 6.9, range 0 to 188, SD = 23.6) (Figure 7A). The median difference in the number of ASVs identified to Lepidoptera of paired samples between primer sets did not differ (paired Wilcoxon signed rank test with continuity correction, V = 3045.5, p-value = 0.73). A total of 19 Lepidopteran families were detected between the two primer sets, eight were shared, four were unique to Primer Set 1 and seven were unique to Primer Set 2 (Figure 7B). The most prevalently detected Lepidopteran families were Tortricidae (79 detections), Noctuidae (62 detections), and Coleophoridae (57 detections). Five butterfly families are expected in Glacier National Park and four butterfly families were detected between the two primer sets. One family, Hesperiidae was detected by both primer sets, eight detections with Primer Set 1 and one detection with Primer Set 2. Nymphalidae was only detected by Primer Set 1 (10 detections), and Lycaenidae (5 detections) and Papilionidae (1 detection) were only detected by Primer Set 2 (Figure 7C). No ASVs were identified to the family Pieridae after removal of *Pieris rapae* from the dataset.

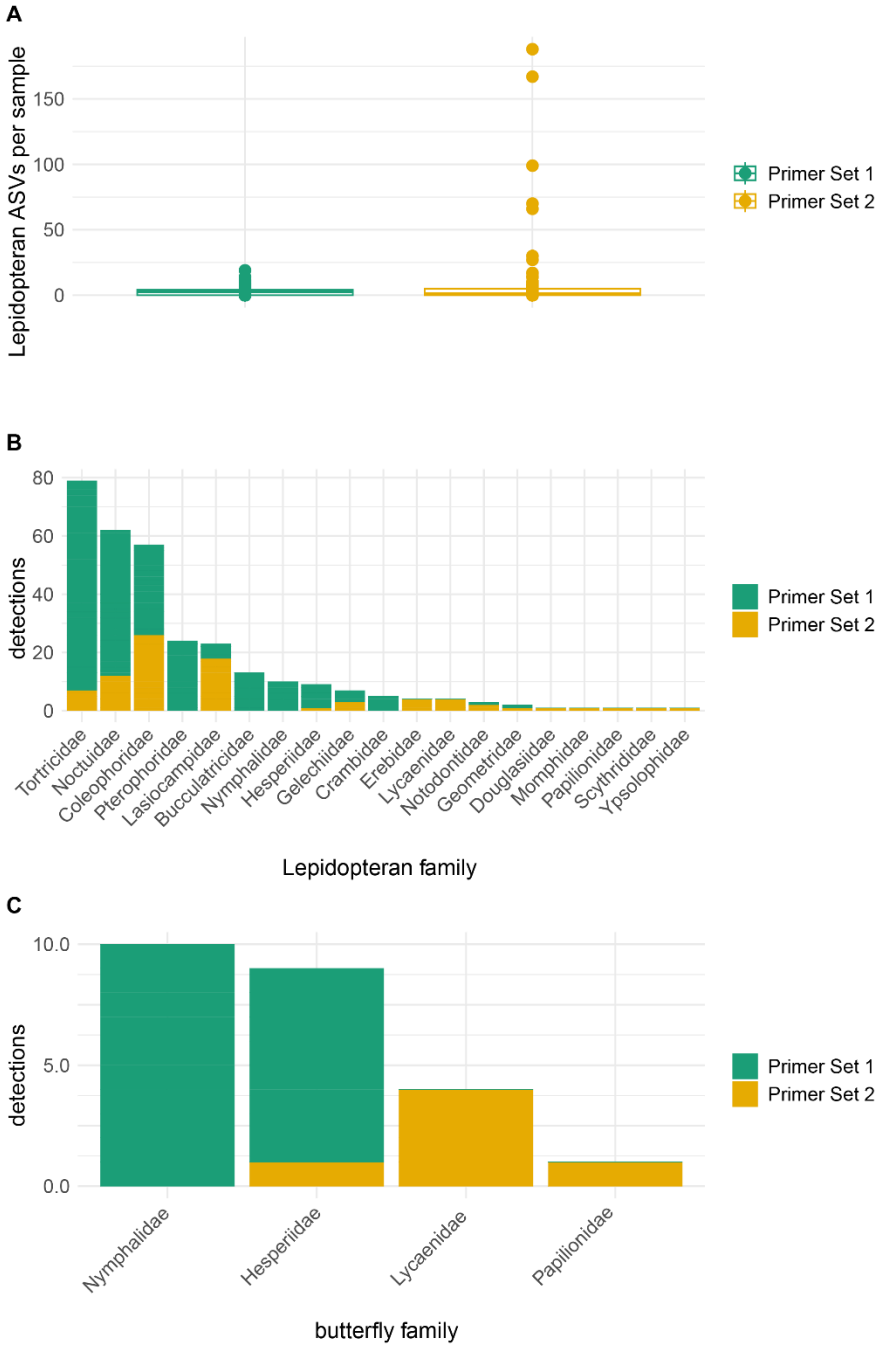


Figure 7. Detections of Lepidopteran taxa between two COI primer sets. The number of ASVs identified to Lepidopteran taxa did not differ at the order level but did differ at the family level when comparing the same wildflower-associated eDNA samples between the two primer sets. A) The number of ASVs identified to the order Lepidoptera per wildflower-associated eDNA sample for each primer set, Primer Set 1 (MLepF1/LepR1) and Primer Set 2 (LepF1/MHMR1), did not differ (Paired Wilcoxon signed rank test with continuity correction, V = 3045.5, p-value = 0.73). B) A total of nineteen Lepidopteran families were identified using both primer sets. The height of the stacked bars represents the number of detections of each family across all samples

while the colors represent the number of detections associated with each primer set (green = Primer Set 1 and yellow = Primer Set 2). The Lepidopteran families that were most detected included: Tortricidae (79 detections), Noctuidae (62 detections), and Coleophoridae (57 detections). Four families were only detected by Primer Set 1 (Pterophoridae, Bucculatricidae, Nymphalidae, and Crambidae) and seven families were only detected by Primer Set 2 (Erebidae, Lycaenidae, Douglasidae, Momphidae, Papilionidae, Scythrididae, and Ypsolophidae). C) A total of four butterfly families were detected using both primer sets. Nymphalidae (10 detections) was only identified by Primer Set 1, and eight of nine detections of Hesperiidae were detected by Primer Set 1. Lycaenidae (5 detections) and Papilionidae (1 detection) were only detected by Primer Set 2.

3.6 Comparison of Butterfly Detections by eDNA with Known Visitation and Traditional Survey Data

Butterflies that were identified from wildflower-associated eDNA samples were validated against observed butterfly visits before wildflower collection and with known butterfly site occupancy from traditional netting surveys. From the 74 individual wildflower-associated eDNA samples with contrived or observed butterfly visits and 71 pools representing 224 additional wildflower eDNA samples six unique butterfly identifications were made, four to species and two to genus. These six taxa were detected a total of 24 times (Primer Set 1 = 18 detections, Primer Set 2 = 6 detections) across 21 samples from four sites (Figure 8). *Oarisma garita* (Garita Skipperling, Hesperiidae) was the only butterfly species detected with both primer sets, six detections using Primer Set 1 and one detection using Primer Set 2.

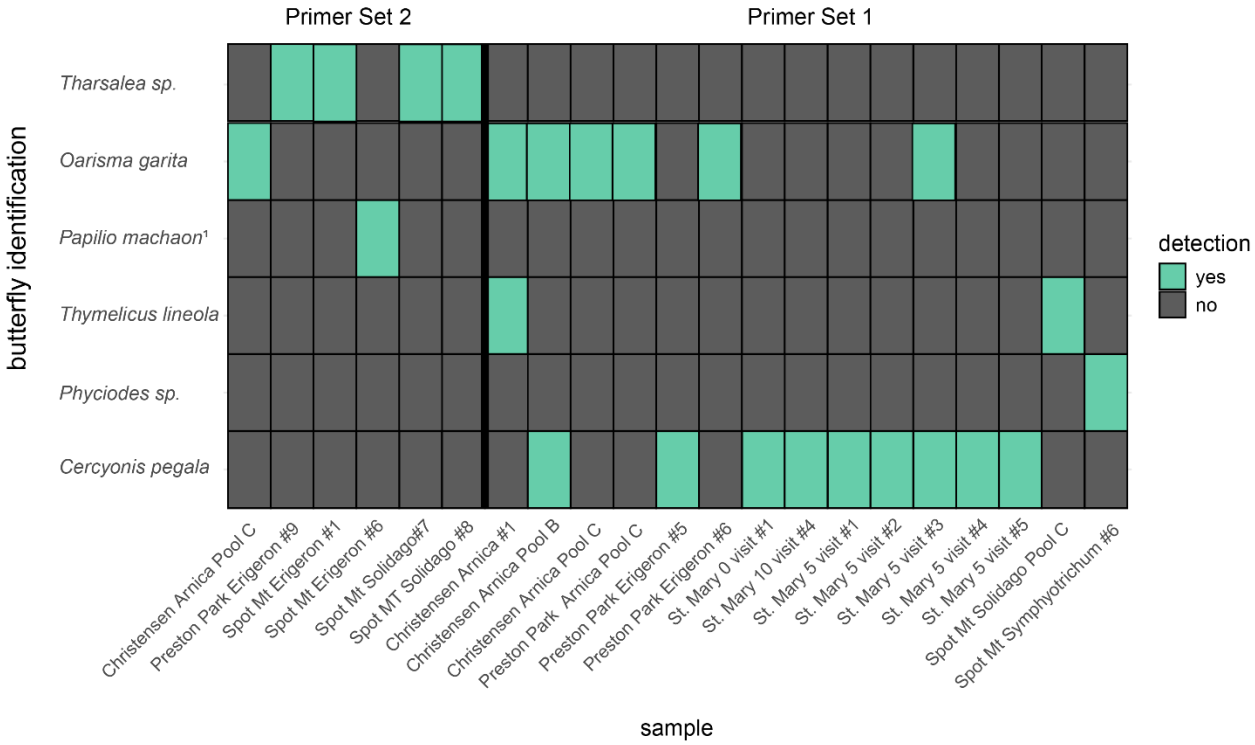


Figure 8: Few butterfly species were detected and identifications differed between the two primer sets. Examination of the butterflies detected in this study revealed that the sequences generated from 74 individual wildflower-associated eDNA samples with contrived or observed butterfly visits and 71 pools representing 224 additional wildflower eDNA samples (Figure 3) identified six unique butterflies, four identified to species, and two to genus. Only one species, *Oarisma garita,* was identified with both primer sets, Primer Set 1 (MLepF1/LepR1) and Primer Set 2 (LepR1/MHMR1). Green tiles indicate detections of a species while grey tiles indicate absence of a detection of the 21 wildflower-associated eDNA samples that had at least one detection. The vertical black bar divides detections from the two primer sets. [1]*Papilio machaon* was the only identification that differed between the bold-full-USCAN and the DSUSCAN-GNPSps reference databases. Results are shown using bold-full-USCAN as the reference database and when using the DSUSCAN-GNPSps reference database this ASV was identified to *Papilio zelicaon*.

Traditional netting surveys that were conducted at the ten field sites directly before wildflower collection in 2022 detected five butterfly families and 47 butterfly species, whereas eDNA data detected four families and six genera from all sites (Table 3). Five of the six eDNA butterfly identifications were in-line with known site-occupancy of that species from traditional netting surveys. Specifically, species that were identified with both netting surveys and eDNA

were: *Cercyonis pegala* (Common wood-nymph) at St Mary, *Tharsalea* (Coppers)*, Phyciodes* (Crescents) and *Thymelicus lineola* (European Skipper) at Spot Mt and Christensen, and *Oarisma garita* (Garita Skipperling) at Christensen (Supplemental Table 11). Identifications with eDNA that were not validated with netting surveys included *Tharsalea* at Christensen, *Oarisma garita* at Preston Park and St Mary, *Papilio* (Swallowtails) at Spot Mt, and *Cercyonis pegala* at Christensen. No butterfly detections were associated with expected species from the 43 samples that had a known natural butterfly visit before collection (Table 3).

All results are reported from taxonomic assignment using the bold-full-USCAN reference database which includes records of animal species from the United States and Canada. To test whether a more specific database would result in more butterfly identifications, taxonomic assignment was also conducted against a database specific to butterflies expected to be in Glacier National Park (DS-USCANLEP-GNPSps). No additional butterfly identifications were made and the only difference in results between the two reference databases was the identification of *Papilio machaon* (Papilionidae) from the Spot Mt site (Spot MT Erigeron #6). *Papilio machaon* (Old-world swallowtail) is not known to be present in Glacier National Park, and when ASVs were identified with the COI database specific to Glacier National Park butterflies (DSUSCANLEP-GNPSps) this ASV was assigned to *Papilio zelicaon* (Anise swallowtail). *Papilio zelicaon* was found near the Spot Mt. site but not during the traditional butterfly survey. The other butterfly identifications were consistent whether the bold-full-USCAN or the DS-USCANLEP-GNPsps database was used as reference.

Table 3. Comparison of butterfly species detections from wildflower-associated eDNA samples to contrived visitation, observed natural visitation, and known butterfly presence from traditional netting surveys.

Contrived Butterfly Visits			
	1	5	10
Speyeria	0/5	0/5	0/3
Plebejus	0/5	0/5	0/5
Observed Butterfly Visits			
flower genus	butterfly genus	flower - butterfly pair N	# detections eDNA
Arnica	*Thymelicus*	1	0
Erigeron	*Thymelicus*	2	0
Symphyotrichum	*Tharsalea*	10	0
Erigeron	*Tharsalea*	1	0
Solidago	*Tharsalea*	2	0
Arnica	*Tharsalea*	1	0
Arnica	*Speyeria*	1	0
Erigeron	*Speyeria*	7	0
Erigeron	*Pontia*	2	0
Senecio	*Pontia*	3	0
Arnica	*Phyciodes*	1	0
Erigeron	*Phyciodes*	3	0
Geranium	*Plebejus*	1	0
Erigeron	*Cercyonis*	1	0
Erigeron	*Colias*	2	0
Senecio	*Colias*	1	0
Erigeron	*Hesperia*	1	0
Known Presence by Traditional Netting			
site	# species netted	# taxa by eDNA	shared detections
Belly River	7	0	0
Big Prairie	7	0	0
Christensen	14	2	2
Preston Park	11	1	0
Rocky Knob	5	0	0
Spot Mt	11	4	3
St Mary	7	2	1
Sullivan	12	0	0
Two Dog	4	0	0
Baring Creek	6	0	0

Table 3 Continued

Overall Summary			
	netted detection	eDNA detection	shared detections
total taxa	47	6	5
Nymphalidae	17	2	2
Hesperidae	6	2	1
Lycaenidae	14	1	1
Papilionidae	2	1	0
Pieridae	8	0	0

3.7 Non-target Insect Species Identifications

Insects other than Lepidoptera were identified with both primer sets (Figure 6). Of the 12,041 insect ASV detections from Primer Set 1 and the bold-full-USCAN database, 40.6% (4,888) were Hemiptera (true bugs) and 30.0% (2,527) were Diptera (flies). Thysanoptera (thrips) and Coleoptera (beetles) comprised 9.9% (1,195) and 8.4% (1,014) of detections, respectively. Lepidoptera (butterflies and moths) comprised 3.4% (415) of detections and Hymenoptera (bees, wasps, ants, etc.: 45 detections), Orthoptera (grasshoppers, crickets, etc.: 45 detections) Ephemeroptera (mayflies: 20 detections) and Psocodea (booklice, barklice: 3 detections) were all less than 1% of total detections. Data generated from Primer set 2 resulted in a total of 3,368 insect detections: 29.5% (995 detections) to Lepidoptera, 22.8% to Diptera, 1.4% (48 detections) to Hemiptera, and one detection each for Coleoptera, Hymenoptera, Plecoptera (stoneflies), and Thysanoptera. There were 1,889 (15.7%) of insect ASVs produced using Primer Set 1 and 1,552 (46.1%) using Primer Set 2 that were not assigned to any order (‘Unassigned’).

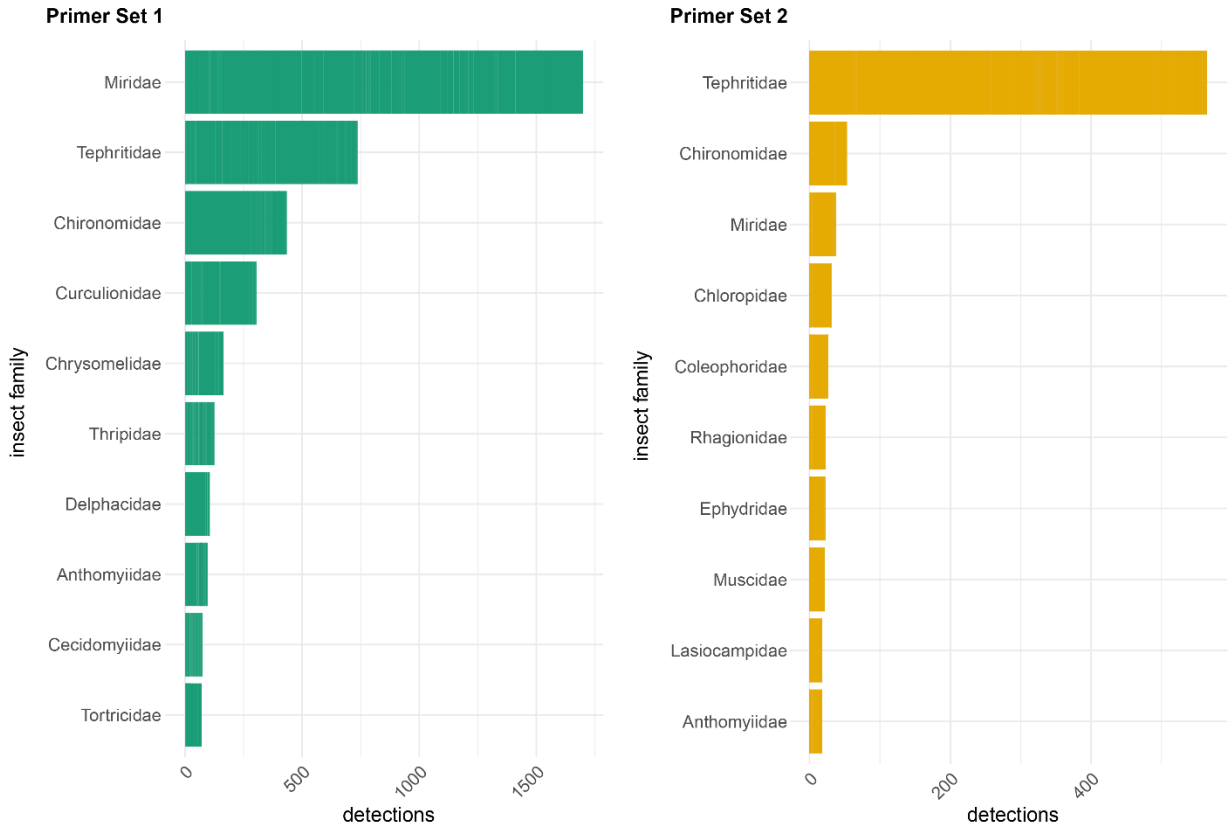


Figure 9. The ten most-detected families from all wildflower-associated eDNA samples with each primer set. Total detections were obtained by summing presence values of taxonomic assignments at the family level from all wildflower-associated eDNA samples. The top 10 insect families detected for A) Primer Set 1 and B) Primer Set 2 are plotted where the length of the bar represents the total detections of the insect family. Miridae, Tephritidae, and Chironomidae were three insect families with the most detections for both primer sets: Miridae:1,700 detections with Primer Set 1 and 38 detections with Primer Set 2; Tephritidae: 738 detections with Primer Set 1 and 565 detections with Primer Set 2; Chironomidae: 434 detections with Primer Set 1 and 53 detections with Primer Set 2. The only other shared family in the top 10 detections with both primer sets was Anthomyiidae (96 detections with Primer Set 1 and 18 detections with Primer Set 2).

Table 4. Information on the ten most detected insect families of each primer set. Family level descriptions of the ten most detected families with both primer sets are given, including typical size and pertinent life history information. The number of detections with each primer set are included and the table is ordered by the total number of detections from both primer sets.

family	**common names**	**usual size**	**where typically found**	**life history / ecology notes**	**det. Set 1**	**det. Set 2**	**total det.**
Miridae	Leaf or Plant Bugs	< 10 mm	vegetation	phytophagous	1700	38	1738
Tephritidae	Fruit Flies	2-25 mm	flowers, vegetation	phytophagous, many oviposit on flower heads of *Asteraceae*	738	565	1303
Chironomidae	Midges	1-10 mm	in or near freshwater	adults congregate in large swarms near freshwater	434	53	487
Curculionidae	Snout Beetles	1-35 mm	vegetation	phytophagous	305	1	306
Chrysomelidae	Leaf Beetles	< 12 mm	flowers, vegetation	phytophagous	164	0	164
Thripidae	Common Thrips	< 1.5 mm	flowers, vegetation	phytophagous	124	1	125
Anthomyiidae	Anthomyid Flies	3-12 mm	flowers, dung	adults are nectar and pollen feeders	96	18	114
Delphacidae	Delphacid Plant Hoppers	1.5 - 10 mm	mainly grass feeders	common, mostly grass feeders	106	0	106
Rhagionidae	Snipe Flies	8-15 mm	wooded areas, dense vegetation	common	69	23	92
Tortricidae	Tortricid / Leafroller Moths	8-35 mm	vegetation	common, larvae are leaf rollers/leaf tiers	72	7	79
Cecidomyiidae	Gall Gnats	< 3 mm	vegetation	most larvae are gall makers, some plant feeders	73	1	74
Coleophoridae	Casebearer Moths	5-26 mm	vegetation	larvae are leaf miners	31	26	57
Chloropidae	Frit Flies	Small to very small	vegetation	common, larvae typically grass feeders	16	31	47

Table 4 Continued

Ephydridae	Shore Flies	< 6mm	in or near freshwater	--	1	23	24
Lasiocampidae	Tent Caterpillar Moths	22-105 mm	vegetation	larvae gregarious and form silken tents in vegetation	5	18	23
Muscidae	Muscid Flies/House Flies	3-12 mm	decaying material	--	0	21	21

The three most detected insect families were the same for both primer sets (Figure 9), Miridae (leaf or plant bugs), Tephritidae (fruit flies) and Chironomidae (midges). Specifically, ASVs assigned to the family Miridae were detected 1,700 times with Primer Set 1 and 38 with Primer Set 2, Tephritidae was detected 738 times with Primer Set 1 and 565 times with Primer Set 2, and Chironomidae had 434 detections with Primer Set 1 and 53 detections with Primer Set 2. The only other shared family in the top 10 detections with both primer sets was Anthomyiidae (Anthomyid flies) which had 96 detections with Primer Set 1 and 18 detections with Primer Set 2. Curculionidae (snout beetles: 305 detections), Chrysomelidae (leaf beetles: 164 detections), Thripidae (common thrips:124 detections) and Delphacidae (plant hoppers: 106 detections) were also commonly detected with Primer Set 1 but not with Primer Set 2 (0 to 1 detection). Family level descriptions of the top ten most commonly detected families for both primer sets are in Table 4.

4 Discussion

Wildflowers- can serve as a source of eDNA for insects; of nearly 300 million sequences obtained from eDNA samples in this study, over 278 million or 93.75% were identified as insect sequences, and 23% were identified as Lepidoptera. However, our results could not be used to accurately or precisely assess relative abundance of insect species. Butterflies were not detected

in accordance with contrived or known visitation to the flower or at equal or greater resolution to traditional surveys, meaning more methods development and benchmarking will be needed before wildflower-associated eDNA sampling can be an accurate and cost-effective method of surveying butterfly species.

4.1 Detection but Not Quantification

Wildflower-associated eDNA shows promise for detecting insect DNA. In this study we obtained hundreds of millions of high-quality sequences that were identified to insects and the nested metabarcoding strategy utilized allowed the sequencing of over 700 PCR reactions in parallel (Figure 2). While the combinatorial indexing strategy for tagging products from a PCR reaction has been the subject of concern due to the potential for high rates of index hopping (Schnell et al., 2015), estimates of index hopping in this sample cohort were low; less than 0.9% of sequences had unexpected tag combinations for all sub-libraries that included wildflower-associated samples (PL1 through PL8) (Supplemental Table 8). Although sequence abundance per sample was variable (Table 1), it is unlikely that low abundance samples were the result of the nested metabarcoding strategy. More than half of wildflower-associated eDNA samples with 0-10 sequences could be attributed to a sequencing sub-library (PL8) that had low yield after the final cleaning step during the sequencing library preparation (Supplemental Table 4). Overall, the nested metabarcoding strategy to sequence portions of the COI gene from wildflower-associated eDNA samples provided high quantity and quality of sequences for analysis of insect taxa.

Data from this study did not suggest that sequence abundance could be correlated with insect abundance. Specifically, of the five species expected to amplify of similar abundance in the insect DNA positive control samples, only three species were identified and one species,

Phymata americana, consistently comprised over 90% of total sequences from positive control samples, indicating potential taxonomic bias using Primer Set 1 (Figure 4). Only one of the five species was expected to amplify with Primer Set 2, *Pieris rapae,* as the only butterfly in the five-species positive control (Supplemental Figure 1). Although *Pieris rapae* was identified in nearly all positive control samples, the three lower dilutions (2.5 ng, 1.25 ng, and 0.625 ng) in the mixed floral background DNA did not yield any sequences (Supplemental Table 10). No apparent coding issues with the demultiplexing step, where sequences were binned back to samples using tag combinations associated with the sample origin, were found and the tag combinations associated with these samples were successfully demultiplexed on other PCR plates. It is possible that user error during library preparation resulted in these three samples not being added correctly to the sample cohort. For the remaining positive control dilutions, the proportion of *Pieris rapae* sequences, or relative abundance, varied from 10% to 90% of total sequences depending on what flower genera made up the background DNA. The floral background DNA was added as the average volume of wildflower eDNA template used across the study to approximate the background DNA in true samples. Additional tests with dilution series of both positive controls and different concentrations of background floral DNA could be more informative and advance the understanding of how sequence quantification varies from different sample origins for future studies working towards quantitative eDNA metabarcoding data.

Positive control tests suggested potential taxonomic bias, and there are several reasons why taxonomic bias may be observed in metabarcoding studies. Bias introduced by variable priming efficiency among taxa, where taxa that have fewer mismatches in the primer region may

amplify more efficiently, has been reported as a strong factor complicating abundance estimates (Piñol et al., 2015; Krehenwinkel et al., 2017). However, this does not explain the overabundance of *Phymata americana* for Primer Set 1 as *P. americana* and *Pieris rapae* both had one mismatch on the forward primer (MLepF1) and two mismatches on the reverse primer (LepR1) (Supplemental Figure 1). There is also inherent variation in mitochondrial gene copy numbers among taxa (Krehenwinkel et al., 2017; Garrido-Sanz et al., 2022) and even within taxa in cells originating from different organs (Wiesner et al., 1992) or from local adaptations to the environment, where high elevation and hypoxic-adapted individuals can have fewer mitochondrial DNA copies than lower elevation individuals of the same species (Liu et al., 2018). When inherent taxon-specific factors introducing biases are known, like primer binding efficiency and mitochondrial gene copy number, species correction factors can be incorporated into sequence abundance estimates to improve estimates of realized species abundance (Garrido-Sanz et al., 2022; Sickel et al., 2023), though in studies of diverse systems this requires a scope of phylogenetic knowledge that may not be available currently.

Insect eDNA detection, rather than abundance, was considered based on results from the positive control dilution tests and by assessing three technical replicates of high priority samples, where we found that additional technical replicates aided in the identification of rare taxa eDNA. Adding information from two technical replicates increased the number of ASVs detected by 1.5x on average for Primer Set 1 and an average of 1.7x for Primer Set 2 compared to individual replicates, while adding information from all three replicates increased ASVs detected by 1.9x and 2.2x, respectively. For both primer sets this translated to 1.2x the unique taxa with two replicates and 1.4x with three replicates compared to one replicate alone. Several studies have

found that consistency between replicates is more variable for low abundance than high abundance samples (Leray & Knowlton, 2017; Shirazi et al., 2021). Many wildflower-associated eDNA samples in this study were of low abundance, particularly for Primer Set 2, so it is perhaps unsurprising that PCR replicates were inconsistent (Supplemental Table 9). Rare DNA in mixed samples is also more likely to be amplified in a chance-like nature during PCR leading to inconsistency in the presence of rare DNA sequences between replicates (Shelton et al., 2023). Indeed, the accumulation curves for ASVs and unique taxa for both primer sets did not reach a visible asymptote indicating more unique taxa would be found with additional replicates (Figure 5). Because butterflies were of particular interest to this study and capturing both rare and abundant species in sequencing data would be critical to comparing detections to traditional surveys, low abundance samples were retained and detections from all three technical replicates were considered. While this approach may be less stringent than those that only consider taxa that are present in at least two or even three technical replicates (Alberdi et al., 2018), tandem observational and experimental data of butterfly presence and known contact with specific flowers allowed for butterfly detections from wildflower-associated eDNA sequencing to be validated.

4.2 Unique Lepidopteran Taxa Identified Between Two COI Primer Sets

Lepidopteran taxa were identified with both COI primer sets utilized in this study (Figure 6) though several Lepidopteran families were only identified with one primer set. The two primer sets did not significantly differ in the number, or richness of unique identifications at the order level but did differ at the family level for both moths and butterflies (Figure 7). For butterflies, Nymphalidae (brushfoot butterflies) were only detected with Primer Set 1 and Lycaenidae (blues

and coppers) and Papilionidae (swallowtails) were only detected with Primer Set 2. Hesperiidae (skippers) were detected with both primer sets but eight of nine detections were with Primer Set 1 (Figure 7C). The two sub regions amplified with both primer sets cover the full 658-bp COI barcode region with ~60-bp of overlap but yield very different results between the forward half (Primer Set 2: LepF1/MHMR1 – 310bp) and the latter half (Primer Set 1: MLepF1/LepR1 – 407bp) of the barcode region. These results suggest that further development of methods to characterize butterfly communities with eDNA metabarcoding will likely require multiple primer sets.

4.3 Few, but Credible, Detections of Butterfly eDNA from Wildflower-associated eDNA

Butterfly species eDNA were detected from wildflower-associated eDNA samples, though fewer than expected, and correspond to species we expect to see in Glacier National Park. There were 47 butterfly species that were captured during traditional netting surveys directly before wildflower collection and six butterfly genera (four to species) were identified from the COI analysis of wildflower-associated eDNA. The six unique butterfly taxa that were identified from eDNA were detected across 21 wildflower-associated eDNA samples. All but one sample were included as priority samples with three technical replicates, further supporting the importance of technical replicates for detecting rare taxa (Figure 8, Supplemental Table 9). Five out of the six species identified from eDNA were observed during traditional netting surveys. One species identified form eDNA alone, *Papilio machaon,* was alternatively identified to *Papilio zelicaon* when a database specific to Glacier National Park butterfly species was used as the reference. *Papilio zelicaon* is a species that was observed incidentally near the site but not during the traditional survey (Figure 8). For consistency between results the database of all North

American COI records (bold-full-USCAN) was reported for all results and all other butterfly identifications remained the same between both reference databases. From the ten sites that were sampled with both traditional netting surveys and wildflower-associated eDNA, the six butterfly taxa that were identified from eDNA were detected across four sites for a total of nine butterfly detections at the site level from eDNA. Six of those were detections shared by traditional sampling on the same day as wildflower collection (Table 3). However, an additional 42 butterfly species were found with traditional netting surveys across the ten sites. No species of the family Pieridae were identified by eDNA, after removing *Pieris rapae* sequences as likely spillover from positive controls, whereas eight Pierid species were detected with netting surveys. Given that there were so few butterfly species detections via eDNA overall, it could be chance that we did not identify any of the eight Pierid species known to be present in the sites via eDNA. Pierids are frequent flower visitors (Brock & Kaufmann, 2003) and identifications of *Pieris rapae* in positive controls indicated a member of the Pierid family could be identified with both primer sets. More investigation would be needed to speculate fully on why Pierid species were not detected by eDNA.

Detection of butterflies from wildflower-associated eDNA was also tested against known butterfly contact with individual flowers. No butterflies were identified from any of the 31 contrived visit samples or from the 43 wildflowers that had an observed natural visit before collection (Table 3). It is unlikely that the lack of butterfly detections is due to the resolution of the reference database. Butterflies are well represented in the COI barcode database; from a list of 126 species either observed by this study or by citizen scientists (iNaturalist) in Glacier National Park, only six species have fewer than 10 records and none have fewer than five records

(Supplemental Table 5). The primers used in this study were established in their ability to distinguish Lepidopteran species, and the flanking primers for each primer set (LepF1 and LepR1) are often used to create COI barcode references (Hajibabaei et al., 2006). Therefore, additional factors such as mechanisms for eDNA deposition and persistence on wildflowers, and the ability of these primers to distinguish butterflies in complex environmental samples, may be affecting the capability of detecting butterflies from wildflower-associated eDNA. These issues provide a wealth of opportunity for future research.

Predicting whether a butterfly would be expected to leave eDNA behind on a wildflower could be a function of flower morphology, the way that the butterfly interacts with the flower (e.g., nectaring vs. resting), or the time spent in contact with a flower. The probability of eDNA sloughing off of a butterfly from saliva, skin cells, or excrement while it interacts with the flower could also be affected by butterfly age, sex, and morphology (James & Lohman, 2023). Though butterflies frequently visit flowers to nectar or rest, their visits are often short and may not allow time for eDNA deposition. One study found that skipper butterflies (Hesperiidae) with medium to long proboscises spent an average of 6.5 seconds nectaring from short tubed flowers and 48 seconds from long tubed flowers (Bauder et al., 2015). In the second field season of the study described herein, which has yet to be sequenced or analyzed, butterfly handling time on the flower was quantified before collection. Butterfly handling time averaged 32 seconds (range: 3 seconds to 5 minutes, SD = 0.03) from 140 flowers, though nectaring time was not differentiated from resting. Contrived visits of *Speyeria mormonia* and *Plebejus melissa* in the contrived visit experiment totaled 50 seconds of resting time for the highest visit category (10 visits of 5 seconds each) and were not detected, so it could be that the average visit from a butterfly is not

long enough to facilitate detection using the eDNA metabarcoding protocol described herein. Additional testing with controlled experiments and longer handling times will be needed to determine the limits of detection for butterflies using wildflower eDNA.

The number of wildflower samples from each site was limited in this study and increasing the number of samples could result in more butterfly detections, especially if an average butterfly visit to one flower is below the limit of detection. However, analysis of technical replicates suggested more replicates of the same flower samples may also be needed to detect rare butterfly taxa, so additional wildflower samples are not the only factor to be considered to improve future study. Sequencing of individual flowers with multiple technical replicates and primers can quickly become cost prohibitive, so increasing the sample size of wildflowers collected and the number of technical replicates will require more pooling of eDNA in future studies. Careful examination and additional controlled experiments will be needed to assess how increasing the complexity of the sample by pooling multiple flowers will affect species richness and abundance estimates.

Detection of insect DNA may also be limited by eDNA collection and extraction methods, where even if there was enough DNA from butterflies deposited on flowers, it is possible that inefficiency at these steps in the workflow could limit the availability of target DNA in the amplified sample. Flower morphology may affect how much DNA is collected on the flower, with more complex surfaces potentially retaining more DNA (Harper et al., 2023). However, these qualities may also make it more difficult to efficiently extract all eDNA deposited on flowers with complex morphology. Storing flowers in cell lysis buffer for a week to 30 days has been employed as a way to maximize yield of eDNA, although increasing the length

of storage also increases the potential of PCR inhibitors releasing from the plant material (Johnson et al., 2023). Utilizing water to rinse eDNA off of flowers and filtering rinse water is another potential collection method (Yoneya et al., 2023; Avalos et al., 2024) which can have the added value of increasing the number of flower samples included in analysis without using additional costly reagents like cell lysis buffer. All of these methods will need to be tested and optimized further with controlled experiments to illuminate potential impacts of eDNA collection and extraction workflow decisions on sequencing results.

4.4 Wildflower associated eDNA Analysis Revealed Better Detection of Small, Flower-associated Insects

The insect families outside of Lepidoptera that were frequently detected from this eDNA metabarcoding cohort are small-bodied (less than 35 mm) and many are phytophagous, or plant eating, which pierce or chew plant material (Table 4). Two of the most detected families from both primer sets fall into these life history categories. Miridae, or plant bugs, was the first most detected family with Primer Set 1 and third with Primer Set 2, are less than 10 mm in size and are known to feed on flowers (Pan et al., 2015; Etl et al., 2022). The second most detected family with Primer Set 1 and first with Primer Set 2 was Tephritidae. This family includes fruit flies that are less than 25 mm in size and the subfamily Tephritinae predominately specialize on *Asteraceae* flowers for larval development (Mazzon et al., 2010). More than half of the wildflower eDNA samples collected were of *Asteraceae* (168 of 298 flowers). The third most detected insect family with Primer Set 1 and second most with Primer Set 2 was Chironomidae, or midges, which are primarily associated with freshwater, and may indicate detections originating from laboratory processing or from airborne eDNA landing on the flower before collection rather than insect DNA originating from flower contact. The remaining insect families

among the 10 most detected with either primer set follow similar life history patterns to the first two described, being small bodied or phytophagous. The two Lepidopteran families that were detected frequently with either primer set have larval stages associated with manipulating plant tissue, Tortricidae are commonly called leaf roller moths and Coleophoridae are commonly called casebearer moths or leaf miners. Physical specimens of only one of the most-detected families, thrips (Thysanoptera), were observed on the flowers during sample preparation; they were found in the sample envelope after the flower had been frozen. However, it is possible that egg or larval stages were present but not visible to the naked eye which would result in more DNA material associated with the flower than trace DNA from flower contact alone. While more study will be needed, the life history characteristics of the most detected families suggest that wildflower-associated eDNA may be biased towards detecting species that are small but likely numerous on the flowers and have life stages residing in flower heads or feeding on flower tissue, perhaps leaving behind more physical material or even entire bodies for DNA to be isolated than flower visitors feeding on nectar like butterflies.

4.5 Conclusion

Characterizing communities from terrestrial eDNA metabarcoding is a nascent but burgeoning field. While using wildflowers as a source of eDNA for insects shows potential, results from this study demonstrate that much more concerted study will be needed. Understanding of the deposition and persistence of DNA originating from different insect species and the taxonomic bias of the COI barcode in floral samples, will be needed before making inferences on whole floral associated insect communities from eDNA metabarcoding. Mesocosm and controlled field experiments are needed that account for varying visitation times and

behaviors of one insect taxon while limiting background DNA on the flower (excluding other insects) to determine the limit of detection for butterflies and other taxonomic groups. While particular attention is turned towards pollinators such as butterflies and bees due to their ecological impact and charismatic status, more tandem observational studies that also estimate diversity and abundance of smaller bodied insects associated with flowers will benefit inferences of taxonomic abundance from sequencing in future eDNA metabarcoding studies.

References

Alberdi, A., Aizpurua, O., Gilbert, M. T. P., & Bohmann, K. (2018). Scrutinizing key steps for reliable metabarcoding of environmental samples. *Methods in Ecology and Evolution*, *9*(1), 134–147. https://doi.org/10.1111/2041-210X.12849

Alvanou, M. V., Tokamani, M., Toros, A., Sandaltzopoulos, R., Zampakas, K., Tananaki, C., Melfou, K., & Giantsis, I. A. (2024). Using Pollen DNA Metabarcoding to Assess the Foraging Preferences of Honeybees in Kastoria Region, Greece. *Proceedings*, *94*(1), Article 1. https://doi.org/10.3390/proceedings2024094044

Ariza, M., Fouks, B., Mauvisseau, Q., Halvorsen, R., Alsos, I. G., & de Boer, H. J. (2023). Plant biodiversity assessment through soil eDNA reflects temporal and local diversity. *Methods in Ecology and Evolution*, *14*(2), 415–430. https://doi.org/10.1111/2041-210X.13865

Avalos, G., Trott, R., Ballas, J., Lin, C. H., Raines, C., Iwanowicz, D., Goodell, K., & Richardson, R. T. (2024). Prospects of pollinator community surveillance using terrestrial environmental DNA metagenetics. *Environmental DNA*, *6*(1), e492. https://doi.org/10.1002/edn3.492

Bale, J. S., Lenteren, J. C. van, & Bigler, F. (2008). Biological control and sustainable food production. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*(1492), 761. https://doi.org/10.1098/rstb.2007.2182

Barnes, M. A., & Turner, C. R. (2016). The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics*, *17*(1), 1–17. https://doi.org/10.1007/s10592-015-0775-4

Bauder, J. a. S., Morawetz, L., Warren, A. D., & Krenn, H. W. (2015). Functional constraints on the evolution of long butterfly proboscides: Lessons from Neotropical skippers (Lepidoptera: Hesperiidae). *Journal of Evolutionary Biology*, *28*(3), 678–687. https://doi.org/10.1111/jeb.12601

Beng, K. C., & Corlett, R. T. (2020). Applications of environmental DNA (eDNA) in ecology and conservation: Opportunities, challenges and prospects. *Biodiversity and Conservation*, *29*(7), 2089–2121. https://doi.org/10.1007/s10531-020-01980-0

Bokulich, N. A., Kaehler, B. D., Rideout, J. R., Dillon, M., Bolyen, E., Knight, R., Huttley, G. A., & Caporaso, J. G. (2018). Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome*, *6*(1), 90. https://doi.org/10.1186/s40168-018-0470-z

BOLD, I. D. B. (2023). *BOLD DNA Barcode Reference Library 31-MAR-2023*. https://doi.org/10.5883/DP-BOLD_Public.31-Mar-2023

BOLD Systems. (2024). *Public BIN Search*. https://www.boldsystems.org/index.php/Public_BINSearch?searchtype=records

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*(8), 852–857. https://doi.org/10.1038/s41587-019-0209-9

Brock, J., P., & Kaufmann, K. (2003). *Field Guide to Butterflies of North America*. Hillstar Editions, L.C.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), Article 7. https://doi.org/10.1038/nmeth.3869

Carson, R. (1962). *Silent Spring* (1st ed.). Houghton Mifflin.

Clarke, L. J., Soubrier, J., Weyrich, L. S., & Cooper, A. (2014). Environmental metabarcodes for insects: In silico PCR reveals potential for taxonomic bias. *Molecular Ecology Resources*, *14*(6), 1160–1170. https://doi.org/10.1111/1755-0998.12265

Clarke, L. J., Beard, J. M., Swadling, K. M., & Deagle, B. E. (2017). Effect of marker choice and thermal cycling protocol on zooplankton DNA metabarcoding studies. *Ecology and Evolution*, *7*(3), 873–883. https://doi.org/10.1002/ece3.2667

Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A., & Callahan, B. J. (2018). Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome*, *6*(1), 226. https://doi.org/10.1186/s40168-018-0605-2

Debinski, D. M., Moranz, R. A., Delaney, J. T., Miller, J. R., Engle, D. M., Winkler, L. B., McGranahan, D. A., Barney, R. J., Trager, J. C., Stephenson, A. L., & Gillespie, M. K. (2011). A cross-taxonomic comparison of insect responses to grassland management and land-use legacies. *Ecosphere*, *2*(12), art131. https://doi.org/10.1890/ES11-00226.1

Deiner, K., Bik, H. M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., Creer, S., Bista, I., Lodge, D. M., de Vere, N., Pfrender, M. E., & Bernatchez, L. (2017). Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. *Molecular Ecology*, *26*(21), 5872–5895. https://doi.org/10.1111/mec.14350

D'Ercole, J., Dincă, V., Opler, P. A., Kondla, N., Schmidt, C., Phillips, J. D., Robbins, R., Burns, J. M., Miller, S. E., Grishin, N., Zakharov, E. V., DeWaard, J. R., Ratnasingham, S., & Hebert, P. D. N. (2021). A DNA barcode library for the butterflies of North America.

PeerJ, *9*, e11157. https://doi.org/10.7717/peerj.11157

Dirzo, R., Young, H. S., Galetti, M., Ceballos, G., Isaac, N. J. B., & Collen, B. (2014). Defaunation in the Anthropocene. *Science*, *345*(6195), 401–406. https://doi.org/10.1126/science.1251817

Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32*(5), 1792–1797. https://doi.org/10.1093/nar/gkh340

Elbrecht, V., & Leese, F. (2015). Can DNA-Based Ecosystem Assessments Quantify Species Abundance? Testing Primer Bias and Biomass—Sequence Relationships with an Innovative Metabarcoding Protocol. *PLOS ONE*, *10*(7), e0130324. https://doi.org/10.1371/journal.pone.0130324

Etl, F., Kaiser, C., Reiser, O., Schubert, M., Dötterl, S., & Schönenberger, J. (2022). Evidence for the recruitment of florivorous plant bugs as pollinators. *Current Biology*, *32*(21), 4688-4698.e6. https://doi.org/10.1016/j.cub.2022.09.013

Ficetola, G. F., Miaud, C., Pompanon, F., & Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biology Letters*, *4*(4), 423–425. https://doi.org/10.1098/rsbl.2008.0118

Forister, M. L., McCall, A. C., Sanders, N. J., Fordyce, J. A., Thorne, J. H., O'Brien, J., Waetjen, D. P., & Shapiro, A. M. (2010). Compounded effects of climate change and habitat alteration shift patterns of butterfly diversity. *Proceedings of the National Academy of Sciences*, *107*(5), 2088–2092. https://doi.org/10.1073/pnas.0909686107

Gamonal Gomez, N., Sørensen, D. H., Chua, P. Y. S., & Sigsgaard, L. (2023). Assessing flower-visiting arthropod diversity in apple orchards through metabarcoding of environmental DNA from flowers and visual census. *Environmental DNA*, *5*(1), 117–131. https://doi.org/10.1002/edn3.362

Garrido-Sanz, L., Senar, M. À., & Piñol, J. (2022). Relative species abundance estimation in artificial mixtures of insects using mito-metagenomics and a correction factor for the mitochondrial DNA copy number. *Molecular Ecology Resources*, *22*(1), 153–167. https://doi.org/10.1111/1755-0998.13464

Gorelick, N., Hancher, M., Dixon, M., Ilyushchenko, S., Thau, D., & Moore, R. (2017). Google Earth Engine: Planetary-scale geospatial analysis for everyone. *Remote Sensing of Environment*, *202*, 18–27. https://doi.org/10.1016/j.rse.2017.06.031

Gregorič, M., Kutnjak, D., Bačnik, K., Gostinčar, C., Pecman, A., Ravnikar, M., & Kuntner, M. (2022). Spider webs as eDNA samplers: Biodiversity assessment across the tree of life.

Molecular Ecology Resources, *22*(7), 2534–2545. https://doi.org/10.1111/1755-0998.13629

Guenay-Greunke, Y., Bohan, D. A., Traugott, M., & Wallinger, C. (2021). Handling of targeted amplicon sequencing data focusing on index hopping and demultiplexing using a nested metabarcoding approach in ecology. *Scientific Reports*, *11*(1), Article 1. https://doi.org/10.1038/s41598-021-98018-4

Hajibabaei, M., Janzen, D. H., Burns, J. M., Hallwachs, W., & Hebert, P. D. N. (2006). DNA barcodes distinguish species of tropical Lepidoptera. *Proceedings of the National Academy of Sciences*, *103*(4), 968–971. https://doi.org/10.1073/pnas.0510466103

Hakimzadeh, A., Asbun, A. A., Albanese, D., Bernard, M., Buchner, D., Callahan, B., Caporaso, G., Curd, E., Djemiel, C., Durling, M. B., Elbrecht, V., Gold, Z., Gweon, H., Hajibabaei, M., Hildebrand, F., Mikryukov, V., Normandeau, E., Ozkurt, E., Palmer, J. M., … Anslan, S. (2023). *A pile of pipelines: An overview of the bioinformatics software for metabarcoding data analyses* [Preprint]. Preprints. https://doi.org/10.22541/au.167610608.87815963/v1

Harper, L. R., Niemiller, M. L., Benito, J. B., Paddock, L. E., Knittle, E., Molano-Flores, B., & Davis, M. A. (2023). BeeDNA: Microfluidic environmental DNA metabarcoding as a tool for connecting plant and pollinator communities. *Environmental DNA*, *5*(1), 191–211. https://doi.org/10.1002/edn3.370

Hassan, S., Sabreena, Poczai, P., Ganai, B. A., Almalki, W. H., Gafur, A., & Sayyed, R. Z. (2022). Environmental DNA Metabarcoding: A Novel Contrivance for Documenting Terrestrial Biodiversity. *Biology*, *11*(9), Article 9. https://doi.org/10.3390/biology11091297

Hebert, P. D. N., Cywinska, A., Ball, S. L., & deWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings. Biological Sciences*, *270*(1512), 313–321. https://doi.org/10.1098/rspb.2002.2218

Hebert, P. D. N., Ratnasingham, S., & deWaard, J. R. (2003). Barcoding animal life: Cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings. Biological Sciences*, *270 Suppl 1*(Suppl 1), S96-99. https://doi.org/10.1098/rsbl.2003.0025

Hughes, J. B., Daily, G. C., & Ehrlich, P. R. (1997). Population Diversity: Its Extent and Extinction. *Science*, *278*(5338), 689–692. https://doi.org/10.1126/science.278.5338.689

Ii, M. S. R., O'Rourke, D. R., Kaehler, B. D., Ziemski, M., Dillon, M. R., Foster, J. T., & Bokulich, N. A. (2021). RESCRIPt: Reproducible sequence taxonomy reference database management. *PLOS Computational Biology*, *17*(11), e1009581.

https://doi.org/10.1371/journal.pcbi.1009581

James, D. G., & Lohman, D. J. (2023). *The Lives of Butterflies: A Natural History of Our Planet's Butterfly Life*. Princeton University Press.

Johnson, M. D., Katz, A. D., Davis, M. A., Tetzlaff, S., Edlund, D., Tomczyk, S., Molano-Flores, B., Wilder, T., & Sperry, J. H. (2023). Environmental DNA metabarcoding from flowers reveals arthropod pollinators, plant pests, parasites, and potential predator–prey interactions while revealing more arthropod diversity than camera traps. *Environmental DNA*, *5*(3), 551–569. https://doi.org/10.1002/edn3.411

Kaunisto, K. M., Roslin, T., Sääksjärvi, I. E., & Vesterinen, E. J. (2017). Pellets of proof: First glimpse of the dietary composition of adult odonates as revealed by metabarcoding of feces. *Ecology and Evolution*, *7*(20), 8588–8598. https://doi.org/10.1002/ece3.3404

Kirse, A., Bourlat, S. J., Langen, K., & Fonseca, V. G. (2021). Metabarcoding Malaise traps and soil eDNA reveals seasonal and local arthropod diversity shifts. *Scientific Reports*, *11*(1), Article 1. https://doi.org/10.1038/s41598-021-89950-6

Kirtane, A., Kleyer, H., & Deiner, K. (2023). Sorting states of environmental DNA: Effects of isolation method and water matrix on the recovery of membrane-bound, dissolved, and adsorbed states of eDNA. *Environmental DNA*, *5*(3), 582–596. https://doi.org/10.1002/edn3.417

Kitson, J. J. N., Hahn, C., Sands, R. J., Straw, N. A., Evans, D. M., & Lunt, D. H. (2019). Detecting host–parasitoid interactions in an invasive Lepidopteran using nested tagging DNA metabarcoding. *Molecular Ecology*, *28*(2), 471–483. https://doi.org/10.1111/mec.14518

Krehenwinkel, H., Wolf, M., Lim, J. Y., Rominger, A. J., Simison, W. B., & Gillespie, R. G. (2017). Estimating and mitigating amplification bias in qualitative and quantitative arthropod metabarcoding. *Scientific Reports*, *7*(1), 17668. https://doi.org/10.1038/s41598-017-17333-x

Lear, G., Dickie, I., Banks, J., Boyer, S., Buckley, H., Buckley, T., Cruickshank, R., Dopheide, A., Handley, K., Hermans, S., Kamke, J., Lee, C., MacDiarmid, R., Morales, S., Orlovich, D., Smissen, R., Wood, J., & Holdaway, R. (2018). Methods for the extraction, storage, amplification and sequencing of DNA from environmental samples. *New Zealand Journal of Ecology*. https://doi.org/10.20417/nzjecol.42.9

Leempoel, K., Hebert, T., & Hadly, E. A. (2020). A comparison of eDNA to camera trapping for assessment of terrestrial mammal diversity. *Proceedings of the Royal Society B: Biological Sciences*, *287*(1918), 20192353. https://doi.org/10.1098/rspb.2019.2353

Lennard, K., Fields, C., Botha, G., Holmes, J., Di Tommaso, P., & Clark, L. (2020). *TADA: Beta*

v0.5 release (Version 0.5-beta) [Computer software]. https://doi.org/10.5281/zenodo.4208836

Leray, M., & Knowlton, N. (2017). Random sampling causes the low reproducibility of rare eukaryotic OTUs in Illumina COI metabarcoding. *PeerJ*, *5*, e3006. https://doi.org/10.7717/peerj.3006

Lister, B. C., & Garcia, A. (2018). Climate-driven declines in arthropod abundance restructure a rainforest food web. *Proceedings of the National Academy of Sciences*, *115*(44), E10397–E10406. https://doi.org/10.1073/pnas.1722477115

Liu, R., Jin, L., Long, K., Tang, Q., Ma, J., Wang, X., Zhu, L., Jiang, A., Tang, G., Jiang, Y., Li, X., & Li, M. (2018). Analysis of mitochondrial DNA sequence and copy number variation across five high-altitude species and their low-altitude relatives. *Mitochondrial DNA Part B*, *3*(2), 847–851. https://doi.org/10.1080/23802359.2018.1501285

Losey, J. E., & Vaughan, M. (2006). The Economic Value of Ecological Services Provided by Insects. *BioScience*, *56*(4), 311–323. https://doi.org/10.1641/0006-3568(2006)56[311:TEVOES]2.0.CO;2

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal*, *17*(1), Article 1. https://doi.org/10.14806/ej.17.1.200

Mazzon, L., Martinez-Sañudo, I., Simonato, M., Squartini, A., Savio, C., & Girolami, V. (2010). Phylogenetic relationships between flies of the Tephritinae subfamily (Diptera, Tephritidae) and their symbiotic bacteria. *Molecular Phylogenetics and Evolution*, *56*(1), 312–326. https://doi.org/10.1016/j.ympev.2010.02.016

McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLOS ONE*, *8*(4), e61217. https://doi.org/10.1371/journal.pone.0061217

Nørgaard, L., Olesen, C. R., Trøjelsgaard, K., Pertoldi, C., Nielsen, J. L., Taberlet, P., Ruiz-González, A., De Barba, M., & Iacolina, L. (2021). eDNA metabarcoding for biodiversity assessment, generalist predators as sampling assistants. *Scientific Reports*, *11*(1), Article 1. https://doi.org/10.1038/s41598-021-85488-9

Ollerton, J., Winfree, R., & Tarrant, S. (2011). How many flowering plants are pollinated by animals? *Oikos*, *120*(3), 321–326. https://doi.org/10.1111/j.1600-0706.2010.18644.x

Pan, H., Lu, Y., Xiu, C., Geng, H., Cai, X., Sun, X., Zhang, Y., Williams III, L., Wyckhuys, K. A. G., & Wu, K. (2015). Volatile fragrances associated with flowers mediate host plant alternation of a polyphagous mirid bug. *Scientific Reports*, *5*(1), 14805. https://doi.org/10.1038/srep14805

Patch, E.M. (1938). Without Benefit of Insects *Bulletin of the Brooklyn Entomological Society 33*(1): 1-9

Pentinsaari, M., Salmela, H., Mutanen, M., & Roslin, T. (2016). Molecular evolution of a widely-adopted taxonomic marker (COI) across the animal tree of life. *Scientific Reports*, *6*(1), 35275. https://doi.org/10.1038/srep35275

Piñol, J., Mir, G., Gomez-Polo, P., & Agustí, N. (2015). Universal and blocking primer mismatches limit the use of high-throughput DNA sequencing for the quantitative metabarcoding of arthropods. *Molecular Ecology Resources*, *15*(4), 819–830. https://doi.org/10.1111/1755-0998.12355

Pornon, A., Andalo, C., Burrus, M., & Escaravage, N. (2017). DNA metabarcoding data unveils invisible pollination networks. *Scientific Reports*, *7*(1), Article 1. https://doi.org/10.1038/s41598-017-16785-5

R Core Team. (2023). *R: A Language and Environment for Statistical Computing* [Computer software]. R Foundation for Statistical Computing. https://www.R-project.org/

Rader, R., Bartomeus, I., Garibaldi, L. A., Garratt, M. P. D., Howlett, B. G., Winfree, R., Cunningham, S. A., Mayfield, M. M., Arthur, A. D., Andersson, G. K. S., Bommarco, R., Brittain, C., Carvalheiro, L. G., Chacoff, N. P., Entling, M. H., Foully, B., Freitas, B. M., Gemmill-Herren, B., Ghazoul, J., … Woyciechowski, M. (2016). Non-bee insects are important contributors to global crop pollination. *Proceedings of the National Academy of Sciences*, *113*(1), 146–151. https://doi.org/10.1073/pnas.1517092112

Ratnasingham, S., & Hebert, P. D. N. (2007). bold: The Barcode of Life Data System (http://www.barcodinglife.org). *Molecular Ecology Notes*, *7*(3), 355–364. https://doi.org/10.1111/j.1471-8286.2007.01678.x

Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ*, *4*, e2584. https://doi.org/10.7717/peerj.2584

Ronca, S., Ford, C. S., Allanguillame, J., Szabo, C., Kipling, R., & Wilkinson, M. J. (2023). The value of twinned pollinator-pollen metabarcoding: Bumblebee pollination service is weakly partitioned within a UK grassland community. *Scientific Reports*, *13*(1), Article 1. https://doi.org/10.1038/s41598-023-44822-z

Ruppert, K. M., Kline, R. J., & Rahman, M. S. (2019). Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. *Global Ecology and Conservation*, *17*, e00547. https://doi.org/10.1016/j.gecco.2019.e00547

Schnell, I. B., Bohmann, K., & Gilbert, M. T. P. (2015). Tag jumps illuminated – reducing

sequence-to-sample misidentifications in metabarcoding studies. *Molecular Ecology Resources*, *15*(6), 1289–1303. https://doi.org/10.1111/1755-0998.12402

Seibold, S., Rammer, W., Hothorn, T., Seidl, R., Ulyshen, M. D., Lorz, J., Cadotte, M. W., Lindenmayer, D. B., Adhikari, Y. P., Aragón, R., Bae, S., Baldrian, P., Barimani Varandi, H., Barlow, J., Bässler, C., Beauchêne, J., Berenguer, E., Bergamin, R. S., Birkemoe, T., … Müller, J. (2021). The contribution of insects to global forest deadwood decomposition. *Nature*, *597*(7874), 77–81. https://doi.org/10.1038/s41586-021-03740-8

Shelton, A. O., Gold, Z. J., Jensen, A. J., D′Agnese, E., Andruszkiewicz Allan, E., Van Cise, A., Gallego, R., Ramón-Laca, A., Garber-Yonts, M., Parsons, K., & Kelly, R. P. (2023). Toward quantitative metabarcoding. *Ecology*, *104*(2), e3906. https://doi.org/10.1002/ecy.3906

Shen, W., Le, S., Li, Y., & Hu, F. (2016). SeqKit: A Cross-Platform and Ultrafast Toolkit for FASTA/Q File Manipulation. *PLOS ONE*, *11*(10), e0163962. https://doi.org/10.1371/journal.pone.0163962

Shirazi, S., Meyer, R. S., & Shapiro, B. (2021). Revisiting the effect of PCR replication and sequencing depth on biodiversity metrics in environmental DNA metabarcoding. *Ecology and Evolution, 11*(22), 15766–15779. https://doi.org/10.1002/ece3.8239

Sickel, W., Zizka, V., Scherges, A., Bourlat, S. J., & Dieker, P. (2023). Abundance estimation with DNA metabarcoding – recent advancements for terrestrial arthropods. *Metabarcoding and Metagenomics, 7*, e112290. https://doi.org/10.3897/mbmg.7.112290

Soulé, M. E. (1991). Conservation: Tactics for a Constant Crisis. *Science*, *253*(5021), 744–750. https://doi.org/10.1126/science.253.5021.744

Taberlet, P., Coissac, E., Hajibabaei, M., & Rieseberg, L. H. (2012). Environmental DNA. *Molecular Ecology*, *21*(8), 1789–1793. https://doi.org/10.1111/j.1365-294X.2012.05542.x

Thomsen, P. F., & Sigsgaard, E. E. (2019). Environmental DNA metabarcoding of wild flowers reveals diverse communities of terrestrial arthropods. *Ecology and Evolution*, *9*(4), 1665–1679. https://doi.org/10.1002/ece3.4809

Thornton, M. M., Shrestha, R., Wei, Y., Thornton, P. E., Kao, S.-C., & Wilson, B. E. (2022). *Daymet: Daily Surface Weather Data on a 1-km Grid for North America, Version 4 R1*. ORNL Distributed Active Archive Center. https://doi.org/10.3334/ORNLDAAC/2129

Toju, H., & Baba, Y. G. (2018). DNA metabarcoding of spiders, insects, and springtails for exploring potential linkage between above- and below-ground food webs. *Zoological Letters*, *4*(1), 4. https://doi.org/10.1186/s40851-018-0088-9

van der Heyde, M., Bunce, M., & Nevill, P. (2022). Key factors to consider in the use of environmental DNA metabarcoding to monitor terrestrial ecological restoration. *Science of The Total Environment*, *848*, 157617. https://doi.org/10.1016/j.scitotenv.2022.157617

Valentin, R. E., Kyle, K. E., Allen, M. C., Welbourne, D. J., & Lockwood, J. L. (2021). The state, transport, and fate of aboveground terrestrial arthropod eDNA. *Environmental DNA*, *3*(6), 1081–1092. https://doi.org/10.1002/edn3.229

Walker, F. M., Williamson, C. H. D., Sanchez, D. E., Sobek, C. J., & Chambers, C. L. (2016). Species From Feces: Order-Wide Identification of Chiroptera From Guano and Other Non-Invasive Genetic Samples. *PLoS ONE*, *11*(9), e0162342. https://doi.org/10.1371/journal.pone.0162342

Wiesner, R. J., Rüegg, J. C., & Morano, I. (1992). Counting target molecules by exponential polymerase chain reaction: Copy number of mitochondrial DNA in rat tissues. *Biochemical and Biophysical Research Communications*, *183*(2), 553–559. https://doi.org/10.1016/0006-291X(92)90517-O

Wilson, E. O., & Peter, F. M. (Eds.). (1988). *Biodiversity*. National Academies Press (US). http://www.ncbi.nlm.nih.gov/books/NBK219272/

Yoneya, K., Ushio, M., & Miki, T. (2023). Non-destructive collection and metabarcoding of arthropod environmental DNA remained on a terrestrial plant. *Scientific Reports*, *13*(1), 7125. https://doi.org/10.1038/s41598-023-32862-4

Zhang, J., Cong, Q., Shen, J., Opler, P. A., & Grishin, N. V. (2020). Genomic evidence suggests further changes of butterfly names. *The Taxonomic Report of the International Lepidoptera Survey*, *8*, 7.

CHAPTER FOUR

CONCLUSION

Butterflies are diverse, sensitive to environmental conditions, and relatively easy to capture and identify, making them useful both as indicators of ecological change and as test subjects for validating novel molecular tools of characterizing insect communities. Temporal changes to a high-elevation, high-latitude butterfly community over 35 years were examined in chapter two and data from the traditional resurvey effort of butterflies was utilized to test new methods of identifying insects from wildflower-associated eDNA in chapter three. Together, this work examined a butterfly community documented from the past, provided a new baseline of assessment in the present, and identified areas for improvement of future monitoring with molecular techniques.

Comparison of the butterfly community in Glacier National Park from the past survey (1988-1989) to the present (2022-2023) revealed that, contrary to predictions, butterfly species richness and community composition did not change overall compared to 35 years ago. While it is encouraging that we did not see widespread change, this positivity is cautious, as results from the two present study years were very different from each other and thus their comparisons to the past. Butterfly species richness was lower in 2022 than all other years and similar in 2023 compared to the past study years, although the number of butterfly individuals caught was higher in 2023 than all years. With two variable years in the present study, it is impossible to state a trend in the butterfly community with confidence from the current data. Distinct butterfly communities were found in different habitat types across the park during each of the four sampling years, with mesic meadows consistently supporting the highest number of species.

Additional years of monitoring may be useful in identifying an overall trend with more confidence while also tracking distinct communities that are likely to shift with increasing stress from climate change (Debinski et al., 2013). Future monitoring would benefit from estimates of abundance in addition to species richness, as declines in abundance may be evident before loss of species. The present study included monitoring of skippers (Hesperiidae) as well as floral resources, which were both not assessed in the past. Skippers are important to monitor because they may be at higher risk due to climate change (Forister et al., 2023). The floral resources assessment showed that plant generic richness was correlated with butterfly species richness, providing additional baseline information for future monitoring of this alpine butterfly community in the context of climate change.

Future monitoring efforts will also benefit from the ability to reliably characterize terrestrial insect communities from environmental DNA, which holds potential for rapid, cost-effective, and non-invasive assessment of biodiversity. To aid the development of these methods, the traditional resurvey effort of butterflies was utilized as tandem observational data to test detection of insects from wildflower-associated eDNA. High-quality and abundant insect sequences were obtained from wildflower-associated eDNA samples, and six unique butterfly taxa were identified, five of which were verified as present at the survey sites using traditional netting surveys on the same day as wildflower collection. Although detection of butterflies from wildflower-associated eDNA shows promise, traditional survey methods captured 42 additional butterfly species that were not identified by eDNA, and we did not detect butterflies that we had physically placed or observed on the flower before collection. Further research to understand the origin, deposition, and persistence of DNA in relation to different flower species and other

smaller-bodied and lesser-known insect species will be needed before inferences on the whole community of wildflower-associated insects can be drawn.

In a world characterized by change where loss of insect biodiversity holds implications for the welfare of both wild and human landscapes, butterflies can provide expediency in the monitoring of ecological change and testing novel molecular methods of characterizing insect communities. Together this work indicates the importance of continued traditional butterfly monitoring efforts while working in parallel with controlled experiments to further develop the protocols to reliably detect butterflies and other insects with flower-associated eDNA.

References

Debinski, D. M., Caruthers, J. C., Cook, D., Crowley, J., & Wickham, H. (2013). Gradient-based habitat affinities predict species vulnerability to drought. *Ecology, 94*(5), 1036–1045. https://doi.org/10.1890/12-0359.1

Forister, M. L., Grames, E. M., Halsch, C. A., Burls, K. J., Carroll, C. F., Bell, K. L., Jahner, J. P., Bradford, T. A., Zhang, J., Cong, Q., Grishin, N. V., Glassberg, J., Shapiro, A. M., & Riecke, T. V. (2023). Assessing risk for butterflies in the context of climate change, demographic uncertainty, and heterogeneous data sources. *Ecological Monographs, 93*(3), e1584. https://doi.org/10.1002/ecm.1584

CUMULATIVE REFERENCES CITED

Alberdi, A., Aizpurua, O., Gilbert, M. T. P., & Bohmann, K. (2018). Scrutinizing key steps for reliable metabarcoding of environmental samples. *Methods in Ecology and Evolution*, *9*(1), 134–147. https://doi.org/10.1111/2041-210X.12849

Alvanou, M. V., Tokamani, M., Toros, A., Sandaltzopoulos, R., Zampakas, K., Tananaki, C., Melfou, K., & Giantsis, I. A. (2024). Using Pollen DNA Metabarcoding to Assess the Foraging Preferences of Honeybees in Kastoria Region, Greece. *Proceedings*, *94*(1), Article 1. https://doi.org/10.3390/proceedings2024094044

Ariza, M., Fouks, B., Mauvisseau, Q., Halvorsen, R., Alsos, I. G., & de Boer, H. J. (2023). Plant biodiversity assessment through soil eDNA reflects temporal and local diversity. *Methods in Ecology and Evolution*, *14*(2), 415–430. https://doi.org/10.1111/2041-210X.13865

Avalos, G., Trott, R., Ballas, J., Lin, C. H., Raines, C., Iwanowicz, D., Goodell, K., & Richardson, R. T. (2024). Prospects of pollinator community surveillance using terrestrial environmental DNA metagenetics. *Environmental DNA*, *6*(1), e492. https://doi.org/10.1002/edn3.492

Bale, J. S., Lenteren, J. C. van, & Bigler, F. (2008). Biological control and sustainable food production. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*(1492), 761. https://doi.org/10.1098/rstb.2007.2182

Barnes, M. A., & Turner, C. R. (2016). The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics*, *17*(1), 1–17. https://doi.org/10.1007/s10592-015-0775-4

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*, *67*(1), 1–48. https://doi.org/10.18637/jss.v067.i01

Bauder, J. a. S., Morawetz, L., Warren, A. D., & Krenn, H. W. (2015). Functional constraints on the evolution of long butterfly proboscides: Lessons from Neotropical skippers (Lepidoptera: Hesperiidae). *Journal of Evolutionary Biology*, *28*(3), 678–687. https://doi.org/10.1111/jeb.12601

Beng, K. C., & Corlett, R. T. (2020). Applications of environmental DNA (eDNA) in ecology and conservation: Opportunities, challenges and prospects. *Biodiversity and Conservation*, *29*(7), 2089–2121. https://doi.org/10.1007/s10531-020-01980-0

Bokulich, N. A., Kaehler, B. D., Rideout, J. R., Dillon, M., Bolyen, E., Knight, R., Huttley, G. A., & Caporaso, J. G. (2018). Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome*, *6*(1), 90. https://doi.org/10.1186/s40168-018-0470-z

BOLD, I. D. B. (2023). *BOLD DNA Barcode Reference Library 31-MAR-2023*. https://doi.org/10.5883/DP-BOLD_Public.31-Mar-2023

BOLD Systems. (2024). *Public BIN Search*. https://www.boldsystems.org/index.php/Public_BINSearch?searchtype=records

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*(8), 852–857. https://doi.org/10.1038/s41587-019-0209-9

Borcard, D., Gillet, F., & Legendre, P. (2011). *Numerical Ecology with R*. Springer. https://doi.org/10.1007/978-1-4419-7976-6

Brock, J., P., & Kaufmann, K. (2003). *Field Guide to Butterflies of North America*. Hillstar Editions, L.C.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), Article 7. https://doi.org/10.1038/nmeth.3869

Carson, R. (1962). *Silent Spring* (1st ed.). Houghton Mifflin.

Cayton, H. L., Haddad, N. M., Gross, K., Diamond, S. E., & Ries, L. (2015). Do growing degree days predict phenology across butterfly species? *Ecology*, *96*(6), 1473–1479. https://doi.org/10.1890/15-0131.1

Chinn, B. (2014). *Lepidoptera of Glacier National Park* (Third). Project 4 Publishing.

Clarke, L. J., Soubrier, J., Weyrich, L. S., & Cooper, A. (2014). Environmental metabarcodes for insects: In silico PCR reveals potential for taxonomic bias. *Molecular Ecology Resources*, *14*(6), 1160–1170. https://doi.org/10.1111/1755-0998.12265

Clarke, L. J., Beard, J. M., Swadling, K. M., & Deagle, B. E. (2017). Effect of marker choice and thermal cycling protocol on zooplankton DNA metabarcoding studies. *Ecology and Evolution*, *7*(3), 873–883. https://doi.org/10.1002/ece3.2667

Conrad, K. F., Warren, M. S., Fox, R., Parsons, M. S., & Woiwod, I. P. (2006). Rapid declines of common, widespread British moths provide evidence of an insect biodiversity crisis. *Biological Conservation*, *132*(3), 279–291. https://doi.org/10.1016/j.biocon.2006.04.020

Crossley, M. S., Smith, O. M., Berry, L. L., Phillips-Cosio, R., Glassberg, J., Holman, K. M.,

Holmquest, J. G., Meier, A. R., Varriano, S. A., McClung, M. R., Moran, M. D., & Snyder, W. E. (2021). Recent climate change is creating hotspots of butterfly increase and decline across North America. *Global Change Biology*, *27*(12), 2702–2714. https://doi.org/10.1111/gcb.15582

Dalton, R. M., Underwood, N. C., Inouye, D. W., Soulé, M. E., & Inouye, B. D. (2023). Long-term declines in insect abundance and biomass in a subalpine habitat. *Ecosphere*, *14*(8), e4620. https://doi.org/10.1002/ecs2.4620

Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A., & Callahan, B. J. (2018). Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome*, *6*(1), 226. https://doi.org/10.1186/s40168-018-0605-2

Debinski, D. M., & Brussard, P. F. (1994). Using Biodiversity Data to Assess Species-Habitat Relationships in Glacier National Park, Montana. *Ecological Applications*, *4*(4), 833–843. https://doi.org/10.2307/1942012

Debinski, D. M., Jakubauskas, M. E., & Kindscher, K. (2000). Montane Meadows as Indicators of Environmental Change. In S. S. Sandhu, B. D. Melzian, E. R. Long, W. G. Whitford, & B. T. Walton (Eds.), *Monitoring Ecological Condition in the Western United States: Proceedings of the Fourth Symposium on the Environmental Monitoring and Assessment Program (EMAP), San Franciso, CA, April 6–8, 1999* (pp. 213–225). Springer Netherlands. https://doi.org/10.1007/978-94-011-4343-1_18

Debinski, D. M., Moranz, R. A., Delaney, J. T., Miller, J. R., Engle, D. M., Winkler, L. B., McGranahan, D. A., Barney, R. J., Trager, J. C., Stephenson, A. L., & Gillespie, M. K. (2011). A cross-taxonomic comparison of insect responses to grassland management and land-use legacies. *Ecosphere*, *2*(12), art131. https://doi.org/10.1890/ES11-00226.1

Debinski, D. M., Caruthers, J. C., Cook, D., Crowley, J., & Wickham, H. (2013). Gradient-based habitat affinities predict species vulnerability to drought. *Ecology*, *94*(5), 1036–1045. https://doi.org/10.1890/12-0359.1

Deiner, K., Bik, H. M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., Creer, S., Bista, I., Lodge, D. M., de Vere, N., Pfrender, M. E., & Bernatchez, L. (2017). Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. *Molecular Ecology*, *26*(21), 5872–5895. https://doi.org/10.1111/mec.14350

Deiner, K., Yamanaka, H., & Bernatchez, L. (2021). The future of biodiversity monitoring and conservation utilizing environmental DNA. *Environmental DNA*, *3*(1), 3–7. https://doi.org/10.1002/edn3.178

D'Ercole, J., Dincă, V., Opler, P. A., Kondla, N., Schmidt, C., Phillips, J. D., Robbins, R., Burns, J. M., Miller, S. E., Grishin, N., Zakharov, E. V., DeWaard, J. R., Ratnasingham, S., &

Hebert, P. D. N. (2021). A DNA barcode library for the butterflies of North America. *PeerJ*, *9*, e11157. https://doi.org/10.7717/peerj.11157

Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C., & Martin, P. R. (2008). Impacts of climate warming on terrestrial ectotherms across latitude. *Proceedings of the National Academy of Sciences*, *105*(18), 6668–6672. https://doi.org/10.1073/pnas.0709472105

Diamond, S. E., Frame, A. M., Martin, R. A., & Buckley, L. B. (2011). Species' traits predict phenological responses to climate change in butterflies. *Ecology*, *92*(5), 1005–1012. https://doi.org/10.1890/10-1594.1

Dirzo, R., Young, H. S., Galetti, M., Ceballos, G., Isaac, N. J. B., & Collen, B. (2014). Defaunation in the Anthropocene. *Science*, *345*(6195), 401–406. https://doi.org/10.1126/science.1251817

Dray, S., Bauman, D., Blanchet, G., Borcard, D., Clappe, S., Guénard, G., Jombart, T., Larocque, G., Legendre, P., Madi, N., & Wagner, H. H. (2023). *adespatial: Multivariate Multiscale Spatial Analysis*. https://CRAN.R-project.org/package=adespatial

Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32*(5), 1792–1797. https://doi.org/10.1093/nar/gkh340

Elbrecht, V., & Leese, F. (2015). Can DNA-Based Ecosystem Assessments Quantify Species Abundance? Testing Primer Bias and Biomass—Sequence Relationships with an Innovative Metabarcoding Protocol. *PLOS ONE*, *10*(7), e0130324. https://doi.org/10.1371/journal.pone.0130324

Etl, F., Kaiser, C., Reiser, O., Schubert, M., Dötterl, S., & Schönenberger, J. (2022). Evidence for the recruitment of florivorous plant bugs as pollinators. *Current Biology*, *32*(21), 4688-4698.e6. https://doi.org/10.1016/j.cub.2022.09.013

Ficetola, G. F., Miaud, C., Pompanon, F., & Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biology Letters*, *4*(4), 423–425. https://doi.org/10.1098/rsbl.2008.0118

Forister, M., & Shapiro, A. (2003). Climatic trends and advancing spring flight of butterflies in lowland California. *Global Change Biology*, *9*, 1130–1135. https://doi.org/10.1046/j.1365-2486.2003.00643.x

Forister, M. L., McCall, A. C., Sanders, N. J., Fordyce, J. A., Thorne, J. H., O'Brien, J., Waetjen, D. P., & Shapiro, A. M. (2010). Compounded effects of climate change and habitat alteration shift patterns of butterfly diversity. *Proceedings of the National Academy of*

Sciences,107(5), 2088–2092. https://doi.org/10.1073/pnas.0909686107

Forister, M. L., Pelton, E. M., & Black, S. H. (2019). Declines in insect abundance and diversity: We know enough to act now. *Conservation Science and Practice, 1*(8), e80. https://doi.org/10.1111/csp2.80

Forister, M. L., Halsch, C. A., Nice, C. C., Fordyce, J. A., Dilts, T. E., Oliver, J. C., Prudic, K. L., Shapiro, A. M., Wilson, J. K., & Glassberg, J. (2021). Fewer butterflies seen by community scientists across the warming and drying landscapes of the American West. *Science, 371*(6533), 1042–1045. https://doi.org/10.1126/science.abe5585

Forister, M. L., Grames, E. M., Halsch, C. A., Burls, K. J., Carroll, C. F., Bell, K. L., Jahner, J. P., Bradford, T. A., Zhang, J., Cong, Q., Grishin, N. V., Glassberg, J., Shapiro, A. M., & Riecke, T. V. (2023). Assessing risk for butterflies in the context of climate change, demographic uncertainty, and heterogeneous data sources. *Ecological Monographs*, *93*(3), e1584. https://doi.org/10.1002/ecm.1584

Gamonal Gomez, N., Sørensen, D. H., Chua, P. Y. S., & Sigsgaard, L. (2023). Assessing flower-visiting arthropod diversity in apple orchards through metabarcoding of environmental DNA from flowers and visual census. *Environmental DNA, 5*(1), 117–131. https://doi.org/10.1002/edn3.362

Garrido-Sanz, L., Senar, M. À., & Piñol, J. (2022). Relative species abundance estimation in artificial mixtures of insects using mito-metagenomics and a correction factor for the mitochondrial DNA copy number. *Molecular Ecology Resources*, *22*(1), 153–167. https://doi.org/10.1111/1755-0998.13464

Garzella, C., & Debinski, D. (2020). *Using Biodiversity Data to Assess Species-Habitat Relationships in Glacier National Park, Montana* (Version 8). Environmental Data Initiative. https://doi.org/10.6073/pasta/222532f38cb2e996244a60be7d048261

Gerlach, J., Samways, M., & Pryke, J. (2013). Terrestrial invertebrates as bioindicators: An overview of available taxonomic groups. *Journal of Insect Conservation, 17*(4), 831–850. https://doi.org/10.1007/s10841-013-9565-9

Gorelick, N., Hancher, M., Dixon, M., Ilyushchenko, S., Thau, D., & Moore, R. (2017). Google Earth Engine: Planetary-scale geospatial analysis for everyone. *Remote Sensing of Environment, 202*, 18–27. https://doi.org/10.1016/j.rse.2017.06.031

Gregorič, M., Kutnjak, D., Bačnik, K., Gostinčar, C., Pecman, A., Ravnikar, M., & Kuntner, M. (2022). Spider webs as eDNA samplers: Biodiversity assessment across the tree of life. *Molecular Ecology Resources*, *22*(7), 2534–2545. https://doi.org/10.1111/1755-0998.13629

Guenay-Greunke, Y., Bohan, D. A., Traugott, M., & Wallinger, C. (2021). Handling of targeted amplicon sequencing data focusing on index hopping and demultiplexing using a nested metabarcoding approach in ecology. *Scientific Reports*, *11*(1), Article 1. https://doi.org/10.1038/s41598-021-98018-4

Habel, J. C., Ulrich, W., Gros, P., Teucher, M., & Schmitt, T. (2023). Butterfly species respond differently to climate warming and land use change in the northern Alps. *Science of The Total Environment*, *890*, 164268. https://doi.org/10.1016/j.scitotenv.2023.164268

Hajibabaei, M., Janzen, D. H., Burns, J. M., Hallwachs, W., & Hebert, P. D. N. (2006). DNA barcodes distinguish species of tropical Lepidoptera. *Proceedings of the National Academy of Sciences*, *103*(4), 968–971. https://doi.org/10.1073/pnas.0510466103

Hakimzadeh, A., Asbun, A. A., Albanese, D., Bernard, M., Buchner, D., Callahan, B., Caporaso, G., Curd, E., Djemiel, C., Durling, M. B., Elbrecht, V., Gold, Z., Gweon, H., Hajibabaei, M., Hildebrand, F., Mikryukov, V., Normandeau, E., Ozkurt, E., Palmer, J. M., … Anslan, S. (2023). *A pile of pipelines: An overview of the bioinformatics software for metabarcoding data analyses* [Preprint]. Preprints. https://doi.org/10.22541/au.167610608.87815963/v1

Hall, M. H. P., & Fagre, D. B. (2003). Modeled Climate-Induced Glacier Change in Glacier National Park, 1850–2100. *BioScience*, *53*(2), 131–140. https://doi.org/10.1641/0006-3568(2003)053[0131:MCIGCI]2.0.CO;2

Hallmann, C. A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., Stenmans, W., Müller, A., Sumser, H., Hörren, T., Goulson, D., & Kroon, H. de. (2017). More than 75 percent decline over 27 years in total flying insect biomass in protected areas. *PLOS ONE*, *12*(10), e0185809. https://doi.org/10.1371/journal.pone.0185809

Halsch, C. A., Shapiro, A. M., Fordyce, J. A., Nice, C. C., Thorne, J. H., Waetjen, D. P., & Forister, M. L. (2021). Insects and recent climate change. *Proceedings of the National Academy of Sciences*, *118*(2), e2002543117. https://doi.org/10.1073/pnas.2002543117

Han, D., Zhang, C., Wang, C., She, J., Sun, Z., Zhao, D., Bian, Q., Han, W., Yin, L., Sun, R., Wang, X., & Cheng, H. (2021). Differences in Response of Butterfly Diversity and Species Composition in Urban Parks to Land Cover and Local Habitat Variables. *Forests*, *12*(2), Article 2. https://doi.org/10.3390/f12020140

Harper, L. R., Niemiller, M. L., Benito, J. B., Paddock, L. E., Knittle, E., Molano-Flores, B., & Davis, M. A. (2023). BeeDNA: Microfluidic environmental DNA metabarcoding as a tool for connecting plant and pollinator communities. *Environmental DNA*, *5*(1), 191–211. https://doi.org/10.1002/edn3.370

Hassan, S., Sabreena, Poczai, P., Ganai, B. A., Almalki, W. H., Gafur, A., & Sayyed, R. Z.

(2022). Environmental DNA Metabarcoding: A Novel Contrivance for Documenting Terrestrial Biodiversity. *Biology*, *11*(9), Article 9. https://doi.org/10.3390/biology11091297

Hebert, P. D. N., Cywinska, A., Ball, S. L., & deWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings. Biological Sciences*, *270*(1512), 313–321. https://doi.org/10.1098/rspb.2002.2218

Hebert, P. D. N., Ratnasingham, S., & deWaard, J. R. (2003). Barcoding animal life: Cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings. Biological Sciences*, *270 Suppl 1*(Suppl 1), S96-99. https://doi.org/10.1098/rsbl.2003.0025

Hill, G. M., Kawahara, A. Y., Daniels, J. C., Bateman, C. C., & Scheffers, B. R. (2021). Climate change effects on animal ecology: Butterflies and moths as a case study. *Biological Reviews*, *96*(5), 2113–2126. https://doi.org/10.1111/brv.12746

Hughes, J. B., Daily, G. C., & Ehrlich, P. R. (1997). Population Diversity: Its Extent and Extinction. *Science*, *278*(5338), 689–692. https://doi.org/10.1126/science.278.5338.689

Ii, M. S. R., O'Rourke, D. R., Kaehler, B. D., Ziemski, M., Dillon, M. R., Foster, J. T., & Bokulich, N. A. (2021). RESCRIPt: Reproducible sequence taxonomy reference database management. *PLOS Computational Biology*, *17*(11), e1009581. https://doi.org/10.1371/journal.pcbi.1009581

IPCC. (2023). *Climate Change 2023: Synthesis Report. Contribution of Working Groups I, II and III to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change* (H. Lee, J. Skea, P. Shukla, P. Zhai, V. Masson-Delmotte, A. Pirani, W. Moufouma-Okia, C. Péan, R. Pidcock, S. Connors, J. B. R. Matthews, M. Chen, Y. Sokona, M. I. Aguilar, C. Mintenbeck, A. Okem, B. Rama, J. Wilcock, W. Maycock, & M. Tignor, Eds.). IPCC. https://doi.org/10.59327/IPCC/AR6-9789291691647

James, D. G., & Lohman, D. J. (2023). *The Lives of Butterflies: A Natural History of Our Planet's Butterfly Life*. Princeton University Press.

Jarvis, B. (2018, November 27). The Insect Apocalypse Is Here. *The New York Times*. https://www.nytimes.com/2018/11/27/magazine/insect-apocalypse.html

Johnson, M. D., Katz, A. D., Davis, M. A., Tetzlaff, S., Edlund, D., Tomczyk, S., Molano-Flores, B., Wilder, T., & Sperry, J. H. (2023). Environmental DNA metabarcoding from flowers reveals arthropod pollinators, plant pests, parasites, and potential predator–prey interactions while revealing more arthropod diversity than camera traps. *Environmental DNA*, *5*(3), 551–569. https://doi.org/10.1002/edn3.411

Kassambara, A. (2023). *rstatix: Pipe-Friendly Framework for Basic Statistical Tests*. https://CRAN.R-project.org/package=rstatix

Kaunisto, K. M., Roslin, T., Sääksjärvi, I. E., & Vesterinen, E. J. (2017). Pellets of proof: First glimpse of the dietary composition of adult odonates as revealed by metabarcoding of feces. *Ecology and Evolution, 7*(20), 8588–8598. https://doi.org/10.1002/ece3.3404

Kerner, J. M., Krauss, J., Maihoff, F., Bofinger, L., & Classen, A. (2023). Alpine butterflies want to fly high: Species and communities shift upwards faster than their host plants. *Ecology, 104*(1), e3848. https://doi.org/10.1002/ecy.3848

Kirse, A., Bourlat, S. J., Langen, K., & Fonseca, V. G. (2021). Metabarcoding Malaise traps and soil eDNA reveals seasonal and local arthropod diversity shifts. *Scientific Reports, 11*(1), Article 1. https://doi.org/10.1038/s41598-021-89950-6

Kirtane, A., Kleyer, H., & Deiner, K. (2023). Sorting states of environmental DNA: Effects of isolation method and water matrix on the recovery of membrane-bound, dissolved, and adsorbed states of eDNA. *Environmental DNA, 5*(3), 582–596. https://doi.org/10.1002/edn3.417

Kitahara, M., Yumoto, M., & Kobayashi, T. (2008). Relationship of butterfly diversity with nectar plant species richness in and around the Aokigahara primary woodland of Mount Fuji, central Japan. *Biodiversity and Conservation, 17*(11), 2713–2734. https://doi.org/10.1007/s10531-007-9265-4

Kitson, J. J. N., Hahn, C., Sands, R. J., Straw, N. A., Evans, D. M., & Lunt, D. H. (2019). Detecting host–parasitoid interactions in an invasive Lepidopteran using nested tagging DNA metabarcoding. *Molecular Ecology, 28*(2), 471–483. https://doi.org/10.1111/mec.14518

Kral-O'Brien, K. C., Karasch, B. M., Hovick, T. J., Moranz, R. A., & Harmon, J. P. (2020). Morphological traits determine detectability bias in North American grassland butterflies. *Ecosphere, 11*(12), e03304. https://doi.org/10.1002/ecs2.3304

Krehenwinkel, H., Wolf, M., Lim, J. Y., Rominger, A. J., Simison, W. B., & Gillespie, R. G. (2017). Estimating and mitigating amplification bias in qualitative and quantitative arthropod metabarcoding. *Scientific Reports, 7*(1), 17668. https://doi.org/10.1038/s41598-017-17333-x

Layberry, R., Hall, P., & Lafontaine, D. (1998). *The Butterflies of Canada*. University of Toronto Press. https://doi.org/10.3138/9781442623163

Lear, G., Dickie, I., Banks, J., Boyer, S., Buckley, H., Buckley, T., Cruickshank, R., Dopheide, A., Handley, K., Hermans, S., Kamke, J., Lee, C., MacDiarmid, R., Morales, S.,

Orlovich, D., Smissen, R., Wood, J., & Holdaway, R. (2018). Methods for the extraction, storage, amplification and sequencing of DNA from environmental samples. *New Zealand Journal of Ecology*. https://doi.org/10.20417/nzjecol.42.9

Leempoel, K., Hebert, T., & Hadly, E. A. (2020). A comparison of eDNA to camera trapping for assessment of terrestrial mammal diversity. *Proceedings of the Royal Society B: Biological Sciences*, *287*(1918), 20192353. https://doi.org/10.1098/rspb.2019.2353

Legendre, P., & Condit, R. (2019). Spatial and temporal analysis of beta diversity in the Barro Colorado Island forest dynamics plot, Panama. *Forest Ecosystems*, *6*(1), 7. https://doi.org/10.1186/s40663-019-0164-4

Lennard, K., Fields, C., Botha, G., Holmes, J., Di Tommaso, P., & Clark, L. (2020). *TADA: Beta v0.5 release* (Version 0.5-beta) [Computer software]. https://doi.org/10.5281/zenodo.4208836

Leray, M., & Knowlton, N. (2017). Random sampling causes the low reproducibility of rare eukaryotic OTUs in Illumina COI metabarcoding. *PeerJ*, *5*, e3006. https://doi.org/10.7717/peerj.3006

Lister, B. C., & Garcia, A. (2018). Climate-driven declines in arthropod abundance restructure a rainforest food web. *Proceedings of the National Academy of Sciences*, *115*(44), E10397–E10406. https://doi.org/10.1073/pnas.1722477115

Liu, R., Jin, L., Long, K., Tang, Q., Ma, J., Wang, X., Zhu, L., Jiang, A., Tang, G., Jiang, Y., Li, X., & Li, M. (2018). Analysis of mitochondrial DNA sequence and copy number variation across five high-altitude species and their low-altitude relatives. *Mitochondrial DNA Part B*, *3*(2), 847–851. https://doi.org/10.1080/23802359.2018.1501285

Losey, J. E., & Vaughan, M. (2006). The Economic Value of Ecological Services Provided by Insects. *BioScience*, *56*(4), 311–323. https://doi.org/10.1641/0006-3568(2006)56[311:TEVOES]2.0.CO;2

Lüdecke, D. (2018). ggeffects: Tidy Data Frames of Marginal Effects from Regression Models. *Journal of Open Source Software*, *3*(26), 772. https://doi.org/10.21105/joss.00772

Lyon, N. J., Stein, D. S., Debinski, D. M., Miller, J. R., & Schacht, W. H. (2021). Responses of Flowering Plant and Butterfly Communities to Experimental Herbicide and Seeding Treatments for Native Grassland Restoration. *Ecological Restoration*, *39*(3), 168–181. https://doi.org/10.3368/er.39.3.168

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal*, *17*(1), Article 1. https://doi.org/10.14806/ej.17.1.200

Mazzon, L., Martinez-Sañudo, I., Simonato, M., Squartini, A., Savio, C., & Girolami, V. (2010). Phylogenetic relationships between flies of the Tephritinae subfamily (Diptera, Tephritidae) and their symbiotic bacteria. *Molecular Phylogenetics and Evolution*, *56*(1), 312–326. https://doi.org/10.1016/j.ympev.2010.02.016

McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLOS ONE*, *8*(4), e61217. https://doi.org/10.1371/journal.pone.0061217

Mi, C., Ma, L., Yang, M., Li, X., Meiri, S., Roll, U., Oskyrko, O., Pincheira-Donoso, D., Harvey, L. P., Jablonski, D., Safaei-Mahroo, B., Ghaffari, H., Smid, J., Jarvie, S., Kimani, R. M., Masroor, R., Kazemi, S. M., Nneji, L. M., Fokoua, A. M. T., … Du, W. (2023). Global Protected Areas as refuges for amphibians and reptiles under climate change. *Nature Communications*, *14*(1), 1389. https://doi.org/10.1038/s41467-023-36987-y

Molina-Martínez, A., León-Cortés, J. L., Regan, H. M., Lewis, O. T., Navarrete, D., Caballero, U., & Luis-Martínez, A. (2016). Changes in butterfly distributions and species assemblages on a Neotropical mountain range in response to global warming and anthropogenic land use. *Diversity and Distributions*, *22*(11), 1085–1098. https://doi.org/10.1111/ddi.12473

Mora, C., Tittensor, D. P., Adl, S., Simpson, A. G. B., & Worm, B. (2011). How Many Species Are There on Earth and in the Ocean? *PLoS Biology*, *9*(8), e1001127. https://doi.org/10.1371/journal.pbio.1001127

Nørgaard, L., Olesen, C. R., Trøjelsgaard, K., Pertoldi, C., Nielsen, J. L., Taberlet, P., Ruiz-González, A., De Barba, M., & Iacolina, L. (2021). eDNA metabarcoding for biodiversity assessment, generalist predators as sampling assistants. *Scientific Reports*, *11*(1), Article 1. https://doi.org/10.1038/s41598-021-85488-9

Ogram, A., Sayler, G. S., & Barkay, T. (1987). The extraction and purification of microbial DNA from sediments. *Journal of Microbiological Methods*, *7*(2), 57–66. https://doi.org/10.1016/0167-7012(87)90025-X

Oksanen, J., Simpson, G. L., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., Solymos, P., Stevens, M. H. H., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., Caceres, M. D., Durand, S., … Weedon, J. (2022). *vegan: Community Ecology Package*. https://CRAN.R-project.org/package=vegan

Ollerton, J., Winfree, R., & Tarrant, S. (2011). How many flowering plants are pollinated by animals? *Oikos*, *120*(3), 321–326. https://doi.org/10.1111/j.1600-0706.2010.18644.x

Owens, A. C. S., Cochard, P., Durrant, J., Farnworth, B., Perkin, E. K., & Seymoure, B. (2020).

Light pollution is a driver of insect declines. *Biological Conservation*, *241*, 108259. https://doi.org/10.1016/j.biocon.2019.108259

Pan, H., Lu, Y., Xiu, C., Geng, H., Cai, X., Sun, X., Zhang, Y., Williams III, L., Wyckhuys, K. A. G., & Wu, K. (2015). Volatile fragrances associated with flowers mediate host plant alternation of a polyphagous mirid bug. *Scientific Reports*, *5*(1), 14805. https://doi.org/10.1038/srep14805

Patch, E.M. (1938). Without Benefit of Insects *Bulletin of the Brooklyn Entomological Society 33*(1): 1-9

Pederson, G. T., Graumlich, L. J., Fagre, D. B., Kipfer, T., & Muhlfeld, C. C. (2010). A century of climate and ecosystem change in Western Montana: What do temperature trends portend? *Climatic Change*, *98*(1), 133–154. https://doi.org/10.1007/s10584-009-9642-y

Pentinsaari, M., Salmela, H., Mutanen, M., & Roslin, T. (2016). Molecular evolution of a widely-adopted taxonomic marker (COI) across the animal tree of life. *Scientific Reports*, *6*(1), 35275. https://doi.org/10.1038/srep35275

Pepin, N., Bradley, R. S., Diaz, H. F., Baraer, M., Caceres, E. B., Forsythe, N., Fowler, H., Greenwood, G., Hashmi, M. Z., Liu, X. D., Miller, J. R., Ning, L., Ohmura, A., Palazzi, E., Rangwala, I., Schöner, W., Severskiy, I., Shahgedanova, M., Wang, M. B., … Mountain Research Initiative EDW Working Group. (2015). Elevation-dependent warming in mountain regions of the world. *Nature Climate Change*, *5*(5), Article 5. https://doi.org/10.1038/nclimate2563

Pepin, N. C., Arnone, E., Gobiet, A., Haslinger, K., Kotlarski, S., Notarnicola, C., Palazzi, E., Seibert, P., Serafin, S., Schöner, W., Terzago, S., Thornton, J. M., Vuille, M., & Adler, C. (2022). Climate Changes and Their Elevational Patterns in the Mountains of the World. *Reviews of Geophysics*, *60*(1), e2020RG000730. https://doi.org/10.1029/2020RG000730

Piñol, J., Mir, G., Gomez-Polo, P., & Agustí, N. (2015). Universal and blocking primer mismatches limit the use of high-throughput DNA sequencing for the quantitative metabarcoding of arthropods. *Molecular Ecology Resources*, *15*(4), 819–830. https://doi.org/10.1111/1755-0998.12355

Pornon, A., Andalo, C., Burrus, M., & Escaravage, N. (2017). DNA metabarcoding data unveils invisible pollination networks. *Scientific Reports*, *7*(1), Article 1. https://doi.org/10.1038/s41598-017-16785-5

R Core Team. (2023). *R: A Language and Environment for Statistical Computing* [Computer software]. R Foundation for Statistical Computing. https://www.R-project.org/

Rader, R., Bartomeus, I., Garibaldi, L. A., Garratt, M. P. D., Howlett, B. G., Winfree, R.,

Cunningham, S. A., Mayfield, M. M., Arthur, A. D., Andersson, G. K. S., Bommarco, R., Brittain, C., Carvalheiro, L. G., Chacoff, N. P., Entling, M. H., Foully, B., Freitas, B. M., Gemmill-Herren, B., Ghazoul, J., … Woyciechowski, M. (2016). Non-bee insects are important contributors to global crop pollination. *Proceedings of the National Academy of Sciences*, *113*(1), 146–151. https://doi.org/10.1073/pnas.1517092112

Rantanen, M., Karpechko, A. Y., Lipponen, A., Nordling, K., Hyvärinen, O., Ruosteenoja, K., Vihma, T., & Laaksonen, A. (2022). The Arctic has warmed nearly four times faster than the globe since 1979. *Communications Earth & Environment*, *3*(1), 1–10. https://doi.org/10.1038/s43247-022-00498-3

Ratnasingham, S., & Hebert, P. D. N. (2007). bold: The Barcode of Life Data System (http://www.barcodinglife.org). *Molecular Ecology Notes*, *7*(3), 355–364. https://doi.org/10.1111/j.1471-8286.2007.01678.x

Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ*, *4*, e2584. https://doi.org/10.7717/peerj.2584

Ronca, S., Ford, C. S., Allanguillame, J., Szabo, C., Kipling, R., & Wilkinson, M. J. (2023). The value of twinned pollinator-pollen metabarcoding: Bumblebee pollination service is weakly partitioned within a UK grassland community. *Scientific Reports*, *13*(1), Article 1. https://doi.org/10.1038/s41598-023-44822-z

Ruppert, K. M., Kline, R. J., & Rahman, M. S. (2019). Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. *Global Ecology and Conservation*, *17*, e00547. https://doi.org/10.1016/j.gecco.2019.e00547

Schnell, I. B., Bohmann, K., & Gilbert, M. T. P. (2015). Tag jumps illuminated – reducing sequence-to-sample misidentifications in metabarcoding studies. *Molecular Ecology Resources*, *15*(6), 1289–1303. https://doi.org/10.1111/1755-0998.12402

Scott, J., A. (1986). *The Butterflies of North America A Natural History and Field Guide*. Stanford University Press.

Seibold, S., Rammer, W., Hothorn, T., Seidl, R., Ulyshen, M. D., Lorz, J., Cadotte, M. W., Lindenmayer, D. B., Adhikari, Y. P., Aragón, R., Bae, S., Baldrian, P., Barimani Varandi, H., Barlow, J., Bässler, C., Beauchêne, J., Berenguer, E., Bergamin, R. S., Birkemoe, T., … Müller, J. (2021). The contribution of insects to global forest deadwood decomposition. *Nature*, *597*(7874), 77–81. https://doi.org/10.1038/s41586-021-03740-8

Selwood, K. E., & Zimmer, H. C. (2020). Refuges for biodiversity conservation: A review of the evidence. *Biological Conservation*, *245*, 108502.

https://doi.org/10.1016/j.biocon.2020.108502

Shelton, A. O., Gold, Z. J., Jensen, A. J., D′Agnese, E., Andruszkiewicz Allan, E., Van Cise, A., Gallego, R., Ramón-Laca, A., Garber-Yonts, M., Parsons, K., & Kelly, R. P. (2023). Toward quantitative metabarcoding. *Ecology*, *104*(2), e3906. https://doi.org/10.1002/ecy.3906

Shen, W., Le, S., Li, Y., & Hu, F. (2016). SeqKit: A Cross-Platform and Ultrafast Toolkit for FASTA/Q File Manipulation. *PLOS ONE*, *11*(10), e0163962. https://doi.org/10.1371/journal.pone.0163962

Shirazi, S., Meyer, R. S., & Shapiro, B. (2021). Revisiting the effect of PCR replication and sequencing depth on biodiversity metrics in environmental DNA metabarcoding. *Ecology and Evolution*, *11*(22), 15766–15779. https://doi.org/10.1002/ece3.8239

Sickel, W., Zizka, V., Scherges, A., Bourlat, S. J., & Dieker, P. (2023). Abundance estimation with DNA metabarcoding – recent advancements for terrestrial arthropods. *Metabarcoding and Metagenomics*, *7*, e112290. https://doi.org/10.3897/mbmg.7.112290

Siddig, A. A. H., Ellison, A. M., Ochs, A., Villar-Leeman, C., & Lau, M. K. (2016). How do ecologists select and use indicator species to monitor ecological change? Insights from 14 years of publication in Ecological Indicators. *Ecological Indicators*, *60*, 223–230. https://doi.org/10.1016/j.ecolind.2015.06.036

Soulé, M. E. (1991). Conservation: Tactics for a Constant Crisis. *Science*, *253*(5021), 744–750. https://doi.org/10.1126/science.253.5021.744

Stork, N. E. (2018). How Many Species of Insects and Other Terrestrial Arthropods Are There on Earth? *Annual Review of Entomology*, *63*(Volume 63, 2018), 31–45. https://doi.org/10.1146/annurev-ento-020117-043348

Taberlet, P., Coissac, E., Hajibabaei, M., & Rieseberg, L. H. (2012). Environmental DNA. *Molecular Ecology*, *21*(8), 1789–1793. https://doi.org/10.1111/j.1365-294X.2012.05542.x

Thomas, J. A. (2005). Monitoring change in the abundance and distribution of insects using butterflies and other indicator groups. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *360*(1454), 339–357. https://doi.org/10.1098/rstb.2004.1585

Thomsen, P. F., & Sigsgaard, E. E. (2019). Environmental DNA metabarcoding of wild flowers reveals diverse communities of terrestrial arthropods. *Ecology and Evolution*, *9*(4), 1665–1679. https://doi.org/10.1002/ece3.4809

Thornton, M. M., Shrestha, R., Wei, Y., Thornton, P. E., Kao, S.-C., & Wilson, B. E. (2022). *Daymet: Daily Surface Weather Data on a 1-km Grid for North America, Version 4 R1*. ORNL Distributed Active Archive Center. https://doi.org/10.3334/ORNLDAAC/2129

Thornton, P. E., Shrestha, R., Thornton, M., Kao, S.-C., Wei, Y., & Wilson, B. E. (2021). Gridded daily weather data for North America with comprehensive uncertainty quantification. *Scientific Data*, *8*(1), Article 1. https://doi.org/10.1038/s41597-021-00973-0

Toju, H., & Baba, Y. G. (2018). DNA metabarcoding of spiders, insects, and springtails for exploring potential linkage between above- and below-ground food webs. *Zoological Letters*, *4*(1), 4. https://doi.org/10.1186/s40851-018-0088-9

Uhler, J., Redlich, S., Zhang, J., Hothorn, T., Tobisch, C., Ewald, J., Thorn, S., Seibold, S., Mitesser, O., Morinière, J., Bozicevic, V., Benjamin, C. S., Englmeier, J., Fricke, U., Ganuza, C., Haensel, M., Riebl, R., Rojas-Botero, S., Rummler, T., … Müller, J. (2021). Relationship of insect biomass and richness with land use along a climate gradient. *Nature Communications*, *12*(1), 5946. https://doi.org/10.1038/s41467-021-26181-3

Valentin, R. E., Kyle, K. E., Allen, M. C., Welbourne, D. J., & Lockwood, J. L. (2021). The state, transport, and fate of aboveground terrestrial arthropod eDNA. *Environmental DNA*, *3*(6), 1081–1092. https://doi.org/10.1002/edn3.229

van der Heyde, M., Bunce, M., & Nevill, P. (2022). Key factors to consider in the use of environmental DNA metabarcoding to monitor terrestrial ecological restoration. *Science of The Total Environment*, *848*, 157617. https://doi.org/10.1016/j.scitotenv.2022.157617

van der Sluijs, J. P., & Vaage, N. S. (2016). Pollinators and Global Food Security: The Need for Holistic Global Stewardship. *Food Ethics*, *1*(1), 75–91. https://doi.org/10.1007/s41055-016-0003-z

van Klink, R., Bowler, D. E., Gongalsky, K. B., Swengel, A. B., Gentile, A., & Chase, J. M. (2020). Meta-analysis reveals declines in terrestrial but increases in freshwater insect abundances. *Science*, *368*(6489), 417–420. https://doi.org/10.1126/science.aax9931

Wagner, D. L. (2020). Insect Declines in the Anthropocene. *Annual Review of Entomology*, *65*(Volume 65, 2020), 457–480. https://doi.org/10.1146/annurev-ento-011019-025151

Walker, F. M., Williamson, C. H. D., Sanchez, D. E., Sobek, C. J., & Chambers, C. L. (2016). Species From Feces: Order-Wide Identification of Chiroptera From Guano and Other Non-Invasive Genetic Samples. *PLoS ONE*, *11*(9), e0162342. https://doi.org/10.1371/journal.pone.0162342

Wallisdevries, M. F., Van Swaay, C. A. M., & Plate, C. L. (2012). Changes in nectar supply: A

possible cause of widespread butterfly decline. *Current Zoology*, *58*(3), 384–391. https://doi.org/10.1093/czoolo/58.3.384

Warren, A. D., Davis, K. J., Stangeland, E. M., Pelham, J. P., Willmott, K. R., & Grishin, N. V. (2024, September 3). *Illustrated Lists of American Butterflies*. http://butterfliesofamerica.com

Warren, M. S., Maes, D., van Swaay, C. A. M., Goffart, P., Van Dyck, H., Bourn, N. A. D., Wynhoff, I., Hoare, D., & Ellis, S. (2021). The decline of butterflies in Europe: Problems, significance, and possible solutions. *Proceedings of the National Academy of Sciences*, *118*(2), e2002551117. https://doi.org/10.1073/pnas.2002551117

Welti, E. A. R., Joern, A., Ellison, A. M., Lightfoot, D. C., Record, S., Rodenhouse, N., Stanley, E. H., & Kaspari, M. (2021). Studies of insect temporal trends must account for the complex sampling histories inherent to many long-term monitoring efforts. *Nature Ecology & Evolution*, *5*(5), 589–591. https://doi.org/10.1038/s41559-021-01424-0

Wepprich, T., Adrion, J. R., Ries, L., Wiedmann, J., & Haddad, N. M. (2019). Butterfly abundance declines over 20 years of systematic monitoring in Ohio, USA. *PLOS ONE*, *14*(7), e0216270. https://doi.org/10.1371/journal.pone.0216270

Whitlock, C., Cross, W., Maxwell, B., Silverman, N., & Wade, A. A. (2017). *2017 Montana Climate Assessment*. Montana State University ScholarWorks. https://doi.org/10.15788/M2WW8W

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York. https://ggplot2.tidyverse.org

Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L. D., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J., Kuhn, M., Pedersen, T. L., Miller, E., Bache, S. M., Müller, K., Ooms, J., Robinson, D., Seidel, D. P., Spinu, V., … Yutani, H. (2019). Welcome to the tidyverse. *Journal of Open Source Software*, *4*(43), 1686. https://doi.org/10.21105/joss.01686

Wiesner, R. J., Rüegg, J. C., & Morano, I. (1992). Counting target molecules by exponential polymerase chain reaction: Copy number of mitochondrial DNA in rat tissues. *Biochemical and Biophysical Research Communications*, *183*(2), 553–559. https://doi.org/10.1016/0006-291X(92)90517-O

Wilson, E. O. (1987). The Little Things That Run the World (The Importance and Conservation of Invertebrates). *Conservation Biology*, *1*(4), 344–346.

Wilson, E. O., & Peter, F. M. (Eds.). (1988). *Biodiversity*. National Academies Press (US). http://www.ncbi.nlm.nih.gov/books/NBK219272/

Winkler, K., Fuchs, R., Rounsevell, M., & Herold, M. (2021). Global land use changes are four times greater than previously estimated. *Nature Communications*, *12*(1), 2501. https://doi.org/10.1038/s41467-021-22702-2

Wix, N., Reich, M., & Schaarschmidt, F. (2019). Butterfly richness and abundance in flower strips and field margins: The role of local habitat quality and landscape context. *Heliyon*, *5*(5), e01636. https://doi.org/10.1016/j.heliyon.2019.e01636

Yoneya, K., Ushio, M., & Miki, T. (2023). Non-destructive collection and metabarcoding of arthropod environmental DNA remained on a terrestrial plant. *Scientific Reports*, *13*(1), 7125. https://doi.org/10.1038/s41598-023-32862-4

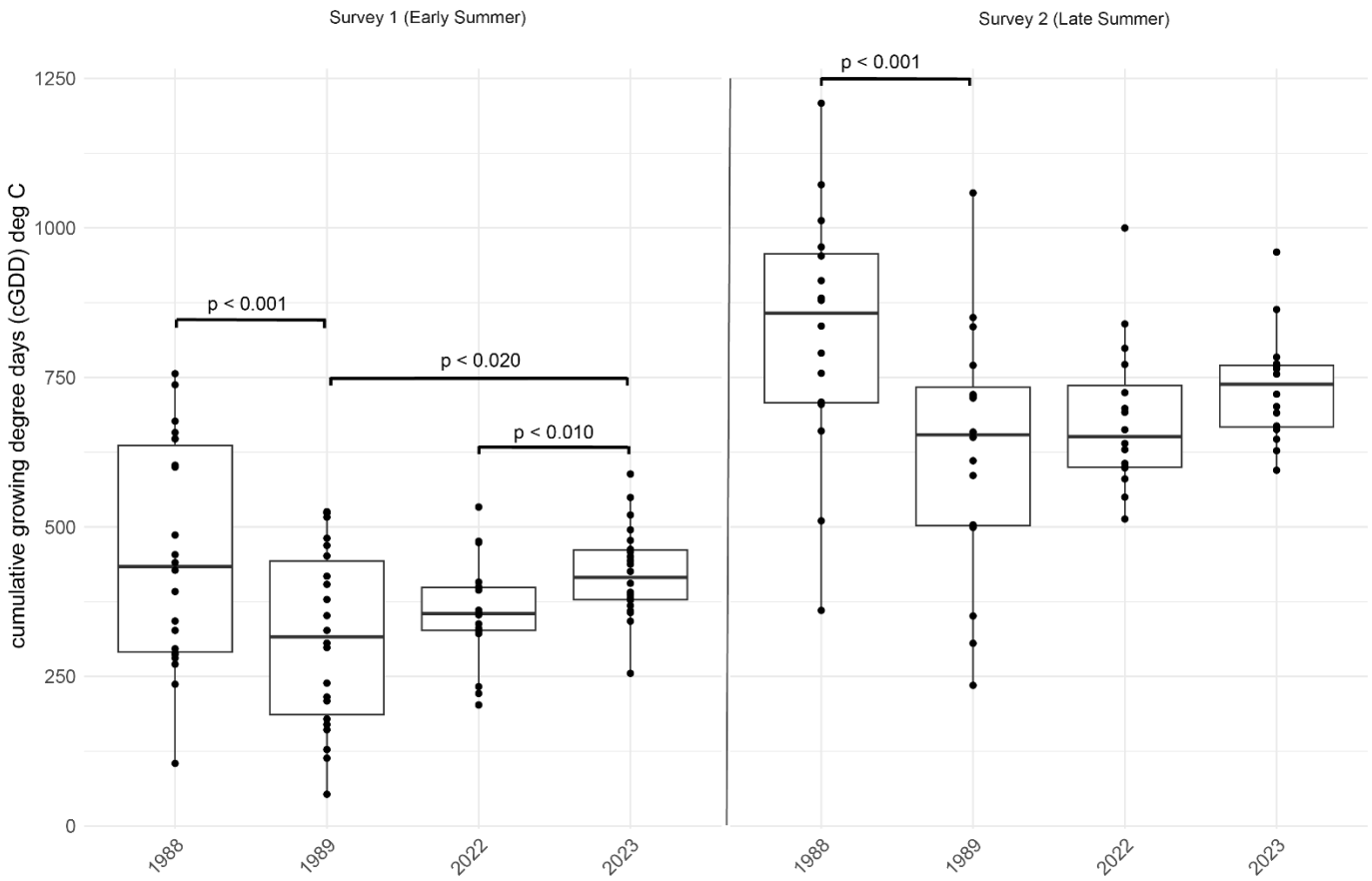
Zhang, J., Cong, Q., Shen, J., Opler, P. A., & Grishin, N. V. (2020). Genomic evidence suggests further changes of butterfly names. *The Taxonomic Report of the International Lepidoptera Survey*, *8*, 7.

Zhang, J., Cong, Q., Shen, J., Song, L., Gott, R. J., Boyer, P., Guppy, C. S., Kohler, S., Lamas, G., Opler, P. A., & Grishin, N. V. (2022). Taxonomic discoveries enabled by genomic analysis of butterflies. *The Taxonomic Report of the International Lepidoptera Survey*, *10*(7), 1–59. https://doi.org/10.5281/zenodo.7160429

APPENDICES

APPENDIX A

SUPPLEMENTARY INFORMATION FOR CHAPTER TWO



Supplemental Figure 1. Cumulative growing degree days associated with survey dates vary between consecutive years more than between past and present studies. To assess similarity in conditions for butterflies between surveys in each year of study, the cumulative growing degree days (cGDD) associated with each site were calculated between March 15th of the survey year and each survey date included in analysis. Paired t-tests were used to assess the mean of differences in cGDD between survey dates at the same sites between each pairwise year comparison; Survey 1 (early summer: ~June 1st – July 15th) and Survey 2 (late summer: ~July 1st – August 31st) were analyzed separately. All 22 sites were surveyed in early summer in all four years but not all sites were surveyed for a second time in late summer; Survey 2 was only included in analysis for sites that had a second survey in all four years (n=16). P-values were adjusted (p.adj) for multiple testing using a Bonferonni correction.

Supplemental Table 1. Butterfly monitoring sites in Glacier National Park. Coordinates and site descriptions for each of the 23 butterfly monitoring sites that were resurveyed in 2022 and 2023. Each site was broken into three (named A, B, or C) 50-meter by 50-meter subplots, except for McGee Meadow and MFF West Glacier which were surveyed as three 60-meter transects. The coordinates, elevation, and bearings of the 50-meter arms (or 60-meter transect) of the subplot from the point are listed. The coordinates of plot or transect B (bolded) for each site were used as the site coordinates for site related climate data.

Site - Plot	Habitat Type	Type	Lat.	Long.	Elevation (m)	Plot bearings from waypoint	Veg transect bearing from waypoint
HiddenLakeA	high alpine	plot	48.6880	-113.7434	2170	130, 220	175
HiddenLakeB	high alpine	plot	48.6868	-113.7436	2141	130, 220	175
HiddenLakeC	high alpine	plot	48.6863	-113.7419	2147	130, 220	175
PrestonParkA	high alpine	plot	48.7149	-113.6420	2189	70, 160	115
PrestonParkB	high alpine	plot	48.7140	-113.6427	2183	120, 210	165
PrestonParkC	high alpine	plot	48.7126	-113.6460	2165	120, 210	165
StonyIndianA	high alpine	plot	48.8819	-113.8637	2107	150, 240	195
StonyIndianB	high alpine	plot	48.8869	-113.8657	1930	20, 110	65
StonyIndianC	high alpine	plot	48.8893	-113.8713	1916	40, 130	85
GraniteParkA	high alpine	plot	48.7725	-113.7716	2064	230, 320	275
GraniteParkB	high alpine	plot	48.7718	-113.7727	2040	200, 290	245
GraniteParkC	high alpine	plot	48.7725	-113.7736	2034	200, 290	245
ScenicPointA	high alpine	plot	48.4802	-113.3267	2251	150, 240	195
ScenicPointB	high alpine	plot	48.4813	-113.3252	2242	150, 240	195
ScenicPointC	high alpine	plot	48.4823	-113.3238	2245	150, 240	195
FiftyMtA	high alpine	plot	48.8540	-113.8586	2072	20, 290	335
FiftyMtB	high alpine	plot	48.8538	-113.8560	2092	20, 290	335
FiftyMtC	high alpine	plot	48.8550	-113.8569	2095	20, 290	335
SpotMtA	alpine	plot	48.5242	-113.3321	1817	30, 120	75

Supplemental Table 1 Continued

SpotMtB	alpine	plot	48.5254	-113.3323	1828	30, 120	75
SpotMtC	alpine	plot	48.5265	-113.3317	1845	30, 120	75
BaringCreekA	alpine	plot	48.6917	-113.6064	1790	30,120	75
BaringCreekB	alpine	plot	48.6927	-113.6071	1796	30,120	75
BaringCreekC	alpine	plot	48.6940	-113.6077	1817	30,120	75
WilburCreekA	alpine	plot	48.8050	-113.6796	1632	90,0	45
WilburCreekB	alpine	plot	48.8065	-113.6785	1689	90,0	45
WilburCreekC	alpine	plot	48.8071	-113.6767	1742	90,0	45
FlattopA	alpine	plot	48.8085	-113.8579	1909	330,60	15
FlattopB	alpine	plot	48.8037	-113.8579	1904	200,290	245
FlattopC	alpine	plot	48.7901	-113.8477	1822	150,240	195
ChristensenA	mesic	plot	48.6286	-114.0225	1149	0,90	45
ChristensenB	mesic	plot	48.6288	-114.0248	1149	0,90	45
ChristensenC	mesic	plot	48.6302	-114.0244	1152	0,90	45
BellyRiverA	mesic	plot	48.9580	-113.6928	1422	0,270	315
BellyRiverB	mesic	plot	48.9590	-113.6943	1411	0,270	315
BellyRiverC	mesic	plot	48.9578	-113.6955	1415	0,270	315
SullivanA	mesic	plot	48.6880	-114.1578	1084	0,90	45
SullivanB	mesic	plot	48.6874	-114.1559	1084	0,90	45
SullivanC	mesic	plot	48.6868	-114.1541	1086	0,90	45
LonePineA	mesic	plot	48.7477	-114.2511	1092	90,180	135
LonePineB	mesic	plot	48.7465	-114.2503	1089	30,120	75
LonePineC	mesic	plot	48.7454	-114.2505	1084	110,220	155
HiddenMeadowA	mesic	plot	48.7590	-114.2321	1169	90,180	135
HiddenMeadowB	mesic	plot	48.7577	-114.2321	1169	90,180	135
HiddenMEadowC	mesic	plot	48.7564	-114.2320	1165	90,180	135
DesantosA	mesic	plot	49.0569	-113.5137	1384	220,310	265
DesantosB	mesic	plot	49.0559	-113.5131	1384	220,310	265
DesantosC	mesic	plot	49.0549	-113.5126	1397	220,310	265

Supplemental Table 1 Continued

SaintMaryA	mesic	plot	48.7431	-113.4406	1377	140,250	185
SaintMaryB	mesic	plot	48.7444	-113.4411	1372	140,250	185
SaintMaryC	mesic	plot	48.7456	-113.4416	1371	250,340	295
TwoDogA	xeric	plot	48.7145	-113.4790	1388	330,60	15
TwoDogB	xeric	plot	48.7138	-113.4808	1386	330,60	15
TwoDogC	xeric	plot	48.7131	-113.4825	1385	330,60	15
BigPrairieA	xeric	plot	48.8387	-114.3448	1108	20,110	65
BigPrairieB	xeric	plot	48.8382	-114.3435	1109	20,110	65
BigPrairieC	xeric	plot	48.8378	-114.3419	1106	20,110	65
RockyKnobA	xeric	plot	48.4833	-114.0453	1003	160,250	205
RockyKnobB	xeric	transect	48.4840	-114.0451	1014	300	300
RockyKnobC	xeric	plot	48.4839	-114.0467	1025	270,360	315
RoundPrairieA	xeric	plot	48.8619	-114.3572	1125	30,120	75
RoundPrairieB	xeric	plot	48.8628	-114.3560	1124	30,120	75
RoundPrairieC	xeric	plot	48.8625	-114.3552	1125	30,120	75
RoundPrairieC	xeric	plot	48.8619	-114.3552	1125	30,120	75
McGeeA	hydric	transect	48.5941	-114.0360	1178	60	60
McGeeB	hydric	transect	48.5958	-114.0350	1175	170	170
McGeeC	hydric	transect	48.5973	-114.0347	1179	170	170
MFFWestGlacierA	riparian	transect	48.5002	-113.9698	982	70	70
MFFWestGlacierB	riparian	transect	48.5004	-113.9731	981	70	70
MFFWestGlacierC	riparian	transect	48.5006	-113.9756	981	70	70

Supplemental Table 2: Survey dates and associated growing degree days for all analyzed butterfly surveys. The cumulative growing degree days (cGDD) were calculated for each of 22 sites between March 15th and the survey date of each year. Plot B from each site was used as the central coordinates. Each site was surveyed at least once for each survey year, so all 22 sites have an early summer survey (Survey 1) but not all sites were surveyed for a second late summer survey (Survey2); only sites that had Survey 2 for all four years were included in analysis.

			Survey 1 (Early Summer)							
			1988		1989		2022		2023	
LOCALITY	Lat	Long	Date	cGDD	Date	cGDD	Date	cGDD	Date	cGDD
Big Prairie	48.83838	-114.3435	6/24/1988	486	7/4/1989	516	7/1/2022	361	6/7/2023	381
Rocky Knob	48.48403	-114.0451	6/30/1988	600	6/30/1989	481	7/2/2022	396	6/6/2023	391
Spot Mt	48.52539	-113.3323	8/3/1988	658	7/8/1989	306	7/14/2022	330	7/9/2023	478
Baring Creek	48.69273	-113.6071	7/10/1988	428	6/13/1989	128	7/27/2022	474	6/29/2023	387
Stony Indian	48.88686	-113.8657	7/21/1988	270	7/27/1989	170	8/2/2022	322	7/22/2023	368
Flattop	48.80374	-113.8579	7/25/1988	440	7/14/1989	209	8/4/2022	476	7/24/2023	549
Hidden Lake	48.68681	-113.7436	7/19/1988	286	7/25/1989	216	8/18/2022	533	7/16/2023	359
Granite Park	48.77175	-113.7727	7/20/1988	327	7/19/1989	179	7/28/2022	330	7/20/2023	426
Scenic Point	48.48125	-113.3252	6/15/1988	105	7/5/1989	161	7/13/2022	202	7/10/2023	342
50 Mt	48.85375	-113.856	7/26/1988	343	7/15/1989	113	8/3/2022	353	7/23/2023	406
McGee	48.59577	-114.035	6/6/1988	281	6/24/1989	379	7/12/2022	476	6/23/2023	520
Christensen	48.62882	-114.0248	7/18/1988	756	7/6/1989	526	6/28/2022	325	6/12/2023	443
Belly River	48.95901	-113.6943	6/22/1988	392	6/27/1989	327	7/7/2022	358	6/27/2023	495
Sullivan	48.68737	-114.1559	7/7/1988	647	6/24/1989	418	6/29/2022	338	6/8/2023	390
Lone Pine	48.74651	-114.2503	7/8/1988	648	6/22/1989	404	6/30/2022	355	6/13/2023	458
Hidden Meadow	48.75774	-114.2321	7/8/1988	603	7/3/1989	469	6/30/2022	326	6/13/2023	438
Desantos	49.05594	-113.5131	6/10/1988	296	6/17/1989	298	7/8/2022	400	7/2/2023	588
St Mary	48.74444	-113.4411	7/20/1988	738	7/12/1989	523	7/9/2022	408	6/22/2023	463
Preston Park	48.71402	-113.6427	7/22/1988	237	7/7/1989	53	7/26/2022	222	7/15/2023	255

Supplemental Table 2 Continued

Wilbur Creek	48.80647	-113.6785	8/5/1988	677	7/19/1989	352	7/6/2022	233	6/26/2023	357
Two Dog	48.71381	-113.4808	6/10/1988	289	6/12/1989	239	7/9/2022	394	6/22/2023	450
Round Prairie	48.86278	-114.356	6/22/1988	454	6/29/1989	452	7/1/2022	356	6/7/2023	378
			Survey 2 (Late Summer)							
			1988		**1989**		**2022**		**2023**	
LOCALITY	**Lat**	**Long**	**Date**	**cGDD**	**Date**	**cGDD**	**Date**	**cGDD**	**Date**	**cGDD**
Big Prairie	48.83838	-114.3435	7/28/1988	883	7/20/1989	716	7/21/2022	606	7/1/2023	627
Rocky Knob	48.48403	-114.0451	7/29/1988	953	7/14/1989	650	7/29/2022	772	6/30/2023	647
Spot Mt	48.52539	-113.3323	8/17/1988	791	8/7/1989	611	8/12/2022	698	8/3/2023	773
Baring Creek	48.69273	-113.6071	8/3/1988	660	7/12/1989	305	8/15/2022	725	8/2/2023	756
Stony Indian	48.88686	-113.8657	-	-	-	-	-	-	-	-
Flattop	48.80374	-113.8579	-	-	-	-	-	-	-	-
Hidden Lake	48.68681	-113.7436	-	-	-	-	-	-	-	-
Granite Park	48.77175	-113.7727	8/11/1988	510	7/25/1989	235	8/17/2022	550	8/18/2023	722
Scenic Point	48.48125	-113.3252	7/24/1988	360	7/28/1989	351	8/11/2022	513	8/4/2023	595
50 Mt	48.85375	-113.856	-	-	-	-	-	-	-	-
McGee	48.59577	-114.035	8/4/1988	968	7/26/1989	770	8/17/2022	1000	7/13/2023	764
Christensen	48.62882	-114.0248	8/22/1988	1208	8/14/1989	1058	7/25/2022	663	7/14/2023	784
Belly River	48.95901	-113.6943	7/22/1988	709	7/15/1989	499	8/1/2022	692	7/21/2023	769
Sullivan	48.68737	-114.1559	7/28/1988	912	7/27/1989	835	7/19/2022	580	7/8/2023	701
Lone Pine	48.74651	-114.2503	8/11/1988	1072	7/28/1989	850	7/23/2022	640	7/13/2023	769
Hidden Meadow	48.75774	-114.2321	8/11/1988	1012	7/23/1989	719	7/23/2022	601	7/7/2023	663
Desantos	49.05594	-113.5131	7/22/1988	757	7/19/1989	586	8/6/2022	799	8/1/2023	959
St Mary	48.74444	-113.4411	-	-	-	-	-	-	-	-
Preston Park	48.71402	-113.6427	-	-	-	-	-	-	-	-
Wilbur Creek	48.80647	-113.6785	8/23/1988	836	8/18/1989	659	8/9/2022	629	7/26/2023	669
Two Dog	48.71381	-113.4808	7/19/1988	705	7/12/1989	503	8/10/2022	839	7/27/2023	864
Round Prairie	48.86278	-114.356	7/28/1988	879	7/21/1989	721	7/21/2022	599	7/7/2023	691

Supplemental Table 3. Units of reproductive ramets counted in floral resource surveys for each plant genera. Floral resources were quantitively assessed in each plot for each site visit in 2022 and 2023. Reproductive ramets were counted along a 1x 60-meter belt transect situated diagonally from the plot waypoint (Supplemental Table 2). The units counted as a reproductive ramet for each plant genus that was identified is listed. All but thirteen plant genera were counted as stalks originating from the soil as the reproductive unit and those that were instead counted as flowering heads are listed at the top of the table.

Genus	**Ramet Unit**	**Genus**	**Ramet Unit**	**Genus**	**Ramet Unit**	**Genus**	**Ramet Unit**
Cerastium	head	*Campanula*	stalk	*Lactuca*	stalk	*Rumex*	stalk
Cherleria	head	*Castilleja*	stalk	*Leucanthemum*	stalk	*Sambucus*	stalk
Cornus	head	*Ceanothus*	stalk	*Linaria*	stalk	*Sandbergia*	stalk
Dasiphora	head	*Centaurea*	stalk	*Linum*	stalk	*Scutellaria*	stalk
Dryas	head	*Chamerion*	stalk	*Lithospermum*	stalk	*Sedum*	stalk
Eremogone	head	*Cicuta*	stalk	*Lomatium*	stalk	*Senecio*	stalk
Erythranthe	head	*Cirsium*	stalk	*Lupinus*	stalk	*Silene*	stalk
Fragaria	head	*Claytonia*	stalk	*Maianthemum*	stalk	*Sisyrinchium*	stalk
Geranium	head	*Collinsia*	stalk	*Melilotus*	stalk	*Smelowskia*	stalk
Medicago	head	*Collomia*	stalk	*Micranthes*	stalk	*Solidago*	stalk
Rosa	head	*Comandra*	stalk	*Microsteris*	stalk	*Spiraea*	stalk
Saxifraga	head	*Comarum*	stalk	*Moerhingia*	stalk	*Spiranthes*	stalk
Stellaria	head	*Crepis*	stalk	*Monarda*	stalk	*Symphoricarpos*	stalk
Achillea	stalk	*Delphinium*	stalk	*Montia*	stalk	*Symphyotrichum*	stalk
Agoseris	stalk	*Drymocallis*	stalk	*Myosotis*	stalk	*Taraxacum*	stalk
Allium	stalk	*Epilobium*	stalk	*Nothocalis*	stalk	*Thlaspi*	stalk
Anaphalis	stalk	*Erigeron*	stalk	*Orthocarpos*	stalk	*Tonestus*	stalk
Anemone	stalk	*Eriognum*	stalk	*Oxytropis*	stalk	*Toxicoscordion*	stalk
Antennaria	stalk	*Eriogonum*	stalk	*Packera*	stalk	*Tragopogon*	stalk
Anticlea	stalk	*Erysismum*	stalk	*Parnassia*	stalk	*Triantha*	stalk
Apocynum	stalk	*Erythronium*	stalk	*Pedicularis*	stalk	*Trifolium*	stalk
Aquilegia	stalk	*Euphorbia*	stalk	*Penstemon*	stalk	*Turritis*	stalk

Supplemental Table 3 Continued

Arabis	stalk	*Gaillardia*	stalk	*Perideridia*	stalk	*Valeriana*	Stalk
Arctostaphylus	stalk	*Galium*	stalk	*Phacelia*	stalk	*Varbascum*	stalk
Arnica	stalk	*Gentiana*	stalk	*Pilosella*	stalk	*Verbascum*	stalk
Astragalus	stalk	*Geum*	stalk	*Pinguicula*	stalk	*Veronica*	stalk
Balsamorhiza	stalk	*Hedysarum*	stalk	*Potentilla*	stalk	*Vicia*	stalk
Barbarea	stalk	*Heracleum*	stalk	*Primula*	stalk	*Xerophyllum*	stalk
Bistorta	stalk	*Heterotheca*	stalk	*Prunella*	stalk		
Boechera	stalk	*Heuchera*	stalk	*Prunus*	stalk		
Bupluerum	stalk	*Hieracium*	stalk	*Pulsatella*	stalk		
Calochortus	stalk	*Holodiscus*	stalk	*Ranunculus*	stalk		
Cammassia	stalk	*Hypericum*	stalk	*Rubus*	stalk		

Supplemental Table 4. Results of detrended correspondence analysis (DCA) indicated use of canonical correspondence analysis (CCA). A detrended correspondence analysis (DCA) was conducted on species data for each survey year to inform the choice of canonical correspondence analysis (CCA) over redundancy analysis (RDA) to assess species-habitat relationships (Figure 6). DCA1 < 3 indicated RDA would be more appropriate, DCA1 > 4 indicated CCA would be more appropriate, and DCA1 between 3 and 4 indicated either analysis would be sufficient. For all years except 1989, DCA1 was greater than 3. For consistency and because CCA does not assume a linear relationship of species data to environmental gradients, CCA was used for analysis of all four years.

1988	DCA1	DCA2	DCA3	DCA4
Eigenvalues	0.4059	0.2345	0.2168	0.154
Additive Eigenvalues	0	0	0	0
Decorana values	0.4217	0.2711	0.1685	0.0857
Axis lengths	3.3041	2.7463	1.9649	1.8441
1989	DCA1	DCA2	DCA3	DCA4
Eigenvalues	0.3844	0.2347	0.1456	0.14708
Additive Eigenvalues	0	0	0	0
Decorana values	0.3922	0.2437	0.1406	0.08337
Axis lengths	2.5249	2.4409	1.7462	1.4031
2022	DCA1	DCA2	DCA3	DCA4
Eigenvalues	0.6159	0.3763	0.3113	0.18135
Additive Eigenvalues	0	0	0	0
Decorana values	0.6229	0.3608	0.2217	0.09221
Axis lengths	4.5982	4.0307	2.8012	1.6239
2023	DCA1	DCA2	DCA3	DCA4
Eigenvalues	0.4437	0.2589	0.2085	0.1832
Additive Eigenvalues	0	0	0	0
Decorana values	0.4537	0.2748	0.1848	0.1102
Axis lengths	3.3447	2.565	1.9573	2.3827

Supplemental Table 5. Species list of Hesperiidae (skippers) identified in the present study. The butterfly family Hesperiidae (skippers) was not included in identifications to species in the past study but were identified in the present study surveys. Thirteen species of skippers were identified in the present study and the number of individuals found in each present study year, 2022 and 2023, are listed in decreasing order of those found in 2022.

	Species	**2022**	**2023**
1	*Thymelicus lineola*	36	48
2	*Hesperia colorado*	7	2
3	*Gesta persius*	6	1
4	*Oarisma garita*	4	10
5	*Amblyscirtes vialis*	2	4
6	*Carterocephalus skada*	2	1
7	*Polites mystic*	2	4
8	*Hesperia assiniboia*	1	0
9	*Ochlodes sylvanoides*	0	1
10	*Polites draco*	0	2
11	*Polites peckius*	0	1
12	*Pyrgus centaureae*	0	1
13	*Burnsius communis*	0	1

Supplemental Table 6. Results of paired t-tests comparing cumulative growing degree days (cGDD) of early and late survey dates in all years of study. The cGDD associated with each site were calculated between March 15th of the survey year and each survey date included in analysis. Paired t-tests were used to assess the mean of differences in cGDD between survey dates at the same sites between each pairwise year comparison; Survey 1 (early summer: ~June 1st – July 15th) and Survey 2 (late summer: ~July 1st – August 31st) were analyzed separately. All 22 sites were surveyed in early summer in all four years but not all sites were surveyed for a second time in late summer; Survey 2 was only included in analysis for sites that had a second survey in all four years (n=16). P-values were adjusted (p.adj) for multiple testing using a Bonferonni correction.

Survey 1								
Response	**group1**	**group2**	**n1**	**n2**	**statistic**	**df**	**p**	**p.adj**
cGDD	1988	1989	22	22	5.11	21	4.7E-05	0.000281
cGDD	1988	2022	22	22	2.08	21	0.05	0.302
cGDD	1988	2023	22	22	0.716	21	0.482	1
cGDD	1989	2022	22	22	-1.37	21	0.185	1
cGDD	1989	2023	22	22	-3.49	21	0.002	0.013
cGDD	2022	2023	22	22	-3.61	21	0.002	0.01
Survey 2								
Response	**group1**	**group2**	**n1**	**n2**	**statistic**	**df**	**p**	**p.adj**
cGDD	1988	1989	16	16	9.267147	15	1.35E-07	8.10E-07
cGDD	1988	2022	16	16	2.637318	15	0.019	0.112
cGDD	1988	2023	16	16	1.63837	15	0.122	0.732
cGDD	1989	2022	16	16	-0.88558	15	0.39	1
cGDD	1989	2023	16	16	-1.82129	15	0.089	0.532
cGDD	2022	2023	16	16	-2.04149	15	0.059	0.355

Supplemental Table 7: Results of paired t-tests comparing site-level species richness in each year of study. The mean difference in species richness between paired sites differed between 2022 and all other survey years. Paired t-tests for each pairwise year comparison from four years, two past study years (1988 and 1989) and two present study years (2022 and 2023), tested the mean difference of species richness between each of 22 sites between years. P-values were adjusted for multiple testing using a Bonferonni correction (p.adj).

Response	group1	group2	n1	n2	statistic	df	p	p.adj
species richness	1988	1989	22	22	0.098	21	0.923	1
species richness	1988	2022	22	22	4.28	21	3.36E-04	0.002
species richness	1988	2023	22	22	0.49	21	0.629	1
species richness	1989	2022	22	22	3.86	21	0.000898	0.005
species richness	1989	2023	22	22	0.401	21	0.693	1
species richness	2022	2023	22	22	-3.25	21	0.004	0.023

Supplemental Table 8: Temporal beta diversity indices for each site and year comparisons. The difference in community composition, using Jaccard dissimilarity coefficients (D), between each of 22 sites in different years (each pairwise year comparison of four study years, 1988 and 1989 of the past study and 2022 and 2023 of the present study) was calculated and decomposed into species gains (C) and species losses (B). The overall sign change for each site in each year comparison are depicted as '-' (loss), '+' (gain) or '0' (no change).

	1988 v 1989				**1988 v 2022**				**1988 v 2023**			
LOCALITY	B/(A+B+C)	C/(A+B+C)	D=(B+C)/(A+B+C)	Sign	B/(A+B+C)	C/(A+B+C)	D=(B+C)/(A+B+C)	+/-	B/(A+B+C)	C/(A+B+C)	D=(B+C)/(A+B+C)	+/-
50 Mt	0.600	0.200	0.800	-	0.500	0.333	0.833	-	0.273	0.273	0.545	0
Baring Creek	**0.519**	**0.370**	**0.889**	**-**	**0.632**	**0.105**	**0.737**	**-**	**0.600**	**0.150**	**0.750**	**-**
Belly River	0.345	0.207	0.552	-	0.414	0.207	0.621	-	0.455	0.303	0.758	-
Big Prairie	0.524	0.286	0.810	-	0.526	0.211	0.737	-	0.400	0.250	0.650	-
Christensen	0.154	0.269	0.423	+	0.375	0.208	0.583	-	0.310	0.345	0.655	+
Desantos	0.053	0.474	0.526	+	0.357	0.286	0.643	-	0.294	0.412	0.706	+
Flattop	0.769	0.077	0.846	-	0.500	0.143	0.643	-	0.412	0.294	0.706	-
Granite Park	**0.391**	**0.261**	**0.652**	**-**	**0.450**	**0.150**	**0.600**	**-**	**0.684**	**0.105**	**0.789**	**-**
Hidden Lake	**0.333**	**0.400**	**0.733**	**+**	**0.368**	**0.526**	**0.895**	**+**	**0.235**	**0.471**	**0.706**	**+**
Hidden Meadow	0.440	0.320	0.760	-	0.727	0.227	0.955	-	0.385	0.346	0.731	-
Lone Pine	**0.417**	**0.417**	**0.833**	**0**	**0.364**	**0.364**	**0.727**	**0**	**0.269**	**0.462**	**0.731**	**+**
McGee	0.313	0.438	0.750	+	0.500	0.357	0.857	-	0.462	0.308	0.769	-
Preston Park	**0.500**	**0.100**	**0.600**	**-**	**0.600**	**0.100**	**0.700**	**-**	**0.176**	**0.471**	**0.647**	**+**
Rocky Knob	0.211	0.526	0.737	+	0.333	0.400	0.733	+	0.333	0.400	0.733	+
Round Prairie	0.118	0.588	0.706	+	0.500	0.300	0.800	-	0.231	0.462	0.692	+
Scenic Point	0.263	0.474	0.737	+	0.417	0.167	0.583	-	0.375	0.375	0.750	0
Spot Mt	0.333	0.296	0.630	-	0.423	0.269	0.692	-	0.500	0.208	0.708	-
St Mary	0.471	0.471	0.941	0	0.500	0.438	0.938	-	0.500	0.357	0.857	-
Stony Indian	**0.278**	**0.333**	**0.611**	**+**	**0.412**	**0.294**	**0.706**	**-**	**0.261**	**0.478**	**0.739**	**+**
Sullivan	**0.238**	**0.381**	**0.619**	**+**	**0.350**	**0.350**	**0.700**	**0**	**0.200**	**0.480**	**0.680**	**+**

Supplemental Table 8 Continued

Two Dog	0.368	0.263	0.632	-	0.474	0.263	0.737	-	0.526	0.263	0.789	-
Wilbur Creek	**0.263**	**0.316**	**0.579**	**+**	**0.300**	**0.350**	**0.650**	**+**	**0.304**	**0.435**	**0.739**	**+**
	1989 v 2022				**1989 v 2023**				**2022 v 2023**			
LOCALITY	B/(A+B+C)	C/(A+B+C)	D=(B+C)/(A+B+C)	+/-	B/(A+B+C)	C/(A+B+C)	D=(B+C)/(A+B+C)	+/-	B/(A+B+C)	C/(A+B+C)	D=(B+C)/(A+B+C)	+/-
50 Mt	0.333	0.556	0.889	+	0.200	0.600	0.800	+	0.273	0.455	0.727	+
Baring Creek	**0.611**	**0.278**	**0.889**	**-**	**0.556**	**0.278**	**0.833**	**-**	**0.333**	**0.417**	**0.750**	**+**
Belly River	0.370	0.296	0.667	-	0.419	0.387	0.806	-	0.217	0.261	0.478	+
Big Prairie	0.438	0.375	0.813	-	0.333	0.444	0.778	+	0.143	0.357	0.500	+
Christensen	0.400	0.120	0.520	-	0.286	0.214	0.500	-	0.200	0.400	0.600	+
Desantos	0.550	0.100	0.650	-	0.478	0.217	0.696	-	0.143	0.357	0.500	+
Flattop	0.222	0.667	0.889	+	0.167	0.750	0.917	+	0.231	0.462	0.692	+
Granite Park	**0.450**	**0.300**	**0.750**	**-**	**0.647**	**0.176**	**0.824**	**-**	**0.538**	**0.154**	**0.692**	**-**
Hidden Lake	**0.368**	**0.474**	**0.842**	**+**	**0.278**	**0.444**	**0.722**	**+**	**0.350**	**0.400**	**0.750**	**+**
Hidden Meadow	0.667	0.222	0.889	-	0.304	0.391	0.696	+	0.111	0.667	0.778	+
Lone Pine	0.333	0.333	0.667	0	0.240	0.440	0.680	+	0.174	0.391	0.565	+
McGee	0.533	0.267	0.800	-	0.462	0.154	0.615	-	0.300	0.300	0.600	0
Preston Park	**0.333**	**0.167**	**0.500**	**-**	**0.125**	**0.688**	**0.813**	**+**	**0.000**	**0.714**	**0.714**	**+**
Rocky Knob	0.474	0.211	0.684	-	0.444	0.167	0.611	-	0.333	0.333	0.667	0
Round Prairie	0.688	0.063	0.750	-	0.444	0.167	0.611	-	0.091	0.545	0.636	+
Scenic Point	0.588	0.176	0.765	-	0.500	0.300	0.800	-	0.167	0.417	0.583	+
Spot Mt	0.286	0.143	0.429	-	0.429	0.143	0.571	-	0.333	0.167	0.500	-
StMary	0.273	0.182	0.455	-	0.364	0.182	0.545	-	0.364	0.273	0.636	-
Stony Indian	**0.444**	**0.278**	**0.722**	**-**	**0.227**	**0.409**	**0.636**	**+**	**0.227**	**0.545**	**0.773**	**+**
Sullivan	**0.409**	**0.273**	**0.682**	**-**	**0.333**	**0.467**	**0.800**	**+**	**0.167**	**0.458**	**0.625**	**+**
Two Dog	0.412	0.294	0.706	-	0.500	0.333	0.833	-	0.308	0.231	0.538	-
Wilbur Creek	**0.222**	**0.222**	**0.444**	**0**	**0.238**	**0.333**	**0.571**	**+**	**0.158**	**0.263**	**0.421**	**+**

Supplemental Table 9: Comparison of generalized linear mixed effects models for model selection predicting butterfly species richness. Four separate generalized linear mixed effects models were used to test the effects of elevation, habitat and study year on observed species richness. Models including elevation and habitat were fit separately because alpine and high alpine designated sites are characterized based on elevation. Model 1 tested for an interaction of year and elevation, and Model 3 tested independent effects of year and elevation on species richness. Model 2 tested for an interaction of year and habitat, and Model 4 tested independent effects of year and habitat on species richness. All models were fit with a random effect for site (LOCALITY) and a Poisson distribution with a log-link function. One site was excluded from Models 2 and 4 (McGee) because habitat was characterized as hydric which was not replicated for any other sites. The incidence rate ratios (IRR), 95% confidence interavals (CI) and p-values for each predictor and model are given in the table. P-value below 0.05 are bolded.

	Model 1			**Model 3**			**Model 2**			**Model 4**		
Predictors	**IRR**	**95% CI**	**p**	**IRR**	**95% CI**	**p**	**IRR**	**95% CI**	**p**	**IRR**	**95% CI**	**p**
(Intercept)	12.64	10.89 - 14.66	**<0.001**	12.59	10.86 - 14.61	**<0.001**	11.17	8.01 - 15.57	**<0.001**	11.51	9.05 - 14.63	**<0.001**
Year [1989]	0.98	0.83 - 1.16	0.99	0.99	0.84 - 1.17	0.933	1.16	0.78 - 1.72	0.477	0.99	0.83 - 1.16	0.864
Year [2022]	0.76	0.63 - 0.90	**0.002**	0.76	0.64 - 0.90	**0.002**	0.76	0.48 - 1.18	0.217	0.76	0.63 - 0.91	**0.002**
Year [2023]	0.96	0.81 - 1.13	0.609	0.96	0.81 - 1.13	0.641	0.91	0.60 - 1.39	0.666	0.97	0.82 - 1.14	0.699
Elevation	0.96	0.82 - 1.11	0.579	0.91	0.81- 1.02	0.092						
Year [1989] X Elevation	0.89	0.75 - 1.05	0.16									
Year [2022] X Elevation	0.97	0.81 - 1.16	0.741									
Year [2023] X Elevation	0.95	0.80- 1.12	0.516									
Habitat [mesic]							1.32	0.88 - 1.97	0.175	1.35	1.04 - 1.77	**0.027**

Supplemental Table 9 Continued

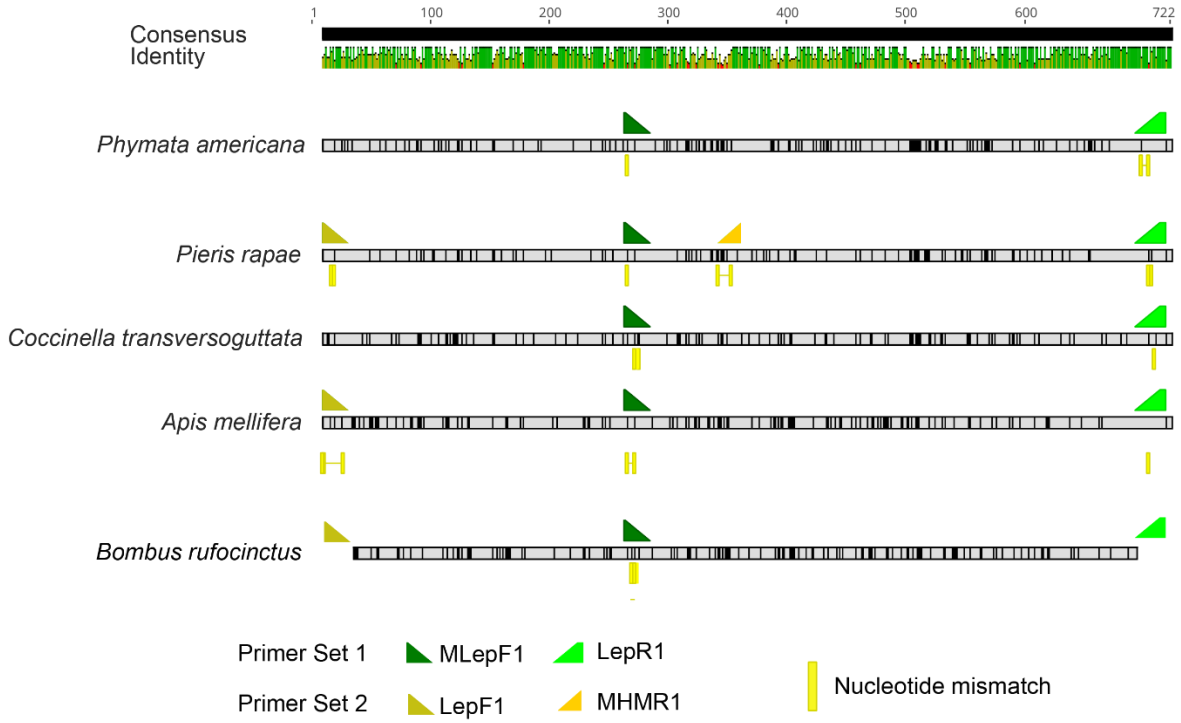
Habitat [alpine]			1.29	0.84 - 1.98	0.245	1.16	0.87 - 1.54	0.327
Habitat [high alpine]			0.94	0.60 - 1.47	0.779	0.88	0.66 - 1.19	0.415
Year [1989] X Habitat [mesic]			0.92	0.57 - 1.49	0.743			
Year [2022] X Habitat [mesic]			1.03	0.61 - 1.76	0.903			
Year [2023] X Habitat [mesic]			1.17	0.71 - 1.93	0.536			
Year [1989] X Habitat [alpine]			0.72	0.43 - 1.22	0.225			
Year [2022] X Habitat [alpine]			0.96	0.54 - 1.70	0.891			
Year [2023] X Habitat [alpine]			0.95	0.55 - 1.63	0.844			

Supplemental Table 9 Continued

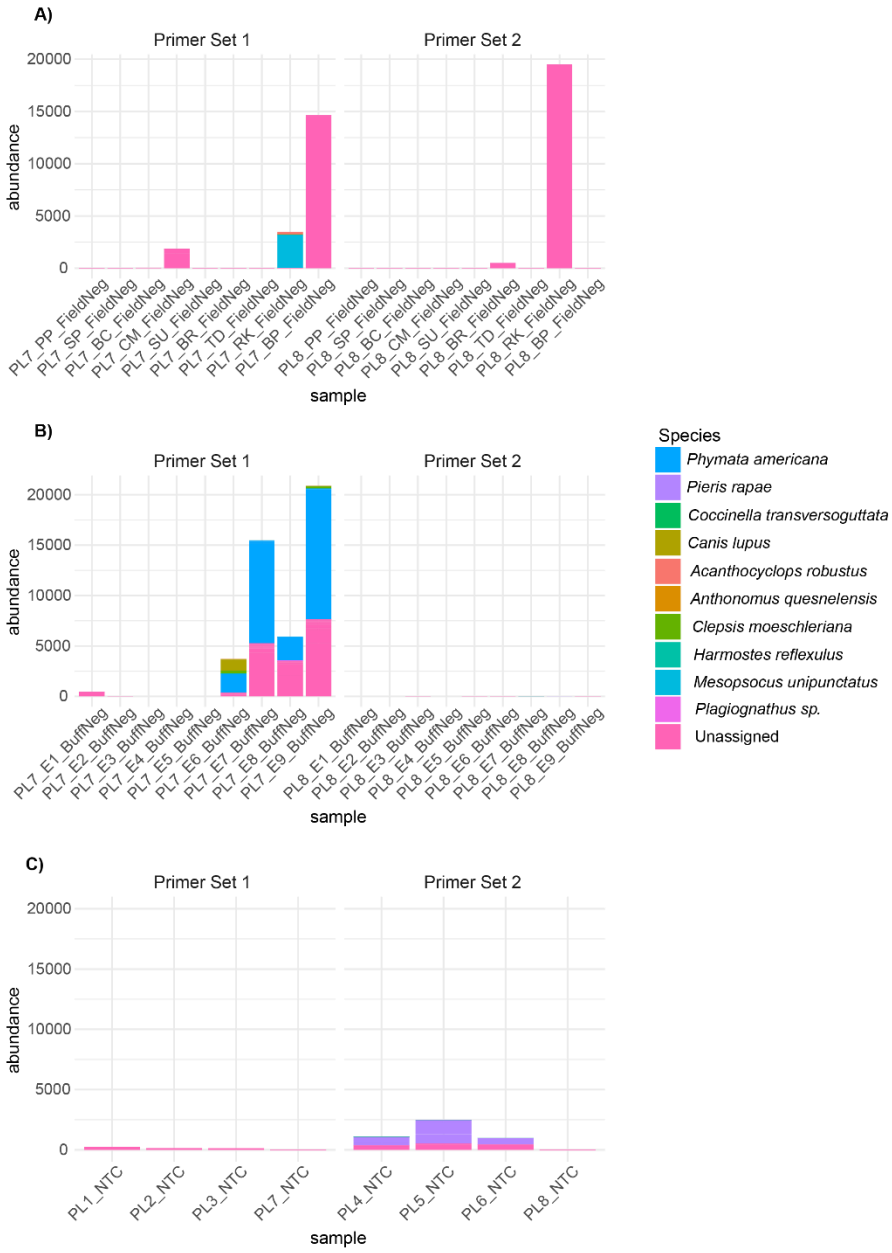
Year [1989] X Habitat [high alpine]			0.77	0.44 - 1.34	0.353
Year [2022] X Habitat [high alpine]			1	0.55 - 1.83	0.997
Year [2023] X Habitat [high alpine]			1.06	0.60 - 1.87	0.851
N	[22]LOCALITY	[22]LOCALITY	[21]LOCALITY		[21]LOCALITY
Observations	88	88	84		84
Marginal R2 / Conditional R2	0.158 / 0.473	0.149 / 0.468	0.294 / 0.469		0.280 / 0.459
AIC	490.004	486.099	478.854		464.18

APPENDIX B

SUPPLEMENTARY INFORMATION FOR CHAPTER THREE



Supplemental Figure 1. Expected primer binds of both COI primer sets on positive control species COI sequences. Two *cytochrome c oxidase I* (COI) gene regions were amplified for high throughput sequencing (Illumina NovaSeq). Positive control sample sequencing libraries were generated using both primer sets (Primer Set 1 = MLepF1/LepR1 and Primer Set 2 = LepF1/MHMR1) which amplified a mixed species positive control of equal amounts of DNA from five insect species: one plant bug (*Phymata americana*), one butterfly (*Pieris rapae*), one beetle (*Coccinella transversoguttata*), and two bees (*Bombus rufocinctus* and *Apis mellifera*). Expected primer binds are shown from alignments to COI reference sequences from each species. Reference sequences of the COI gene were accessed from NCBI, except for *Bombus rufocinctus* which did not have the full gene reference available and a COI barcode record from the Barcode of Life Database (BOLD) was accessed. All five species were expected to amplify with Primer Set 1; both primers have two or fewer mismatches on the target sequence. Only *Pieris rapae* was expected to amplify with Primer Set 2. The primers LepF1 and LepR1 were used to create the COI reference sequence of *Bombus rufocinctus.*so primer binding is inferred.



Supplemental Figure 2. Species identified in negative controls. Three types of negative controls were included in the study and throughout all steps of analysis. **A)** Each wildflower collection event included a field negative control where a sterile gauze pad was opened and handled with sampling equipment, **B)** a buffer only negative control for each DNA extraction and **C)** a no template controls (NTC) on each PCR plate. All negative controls were sequenced with both primer sets (Primer Set 1 = MLepF1/LepR1 and Primer Set 2 = LepF1/MHMR1). The height of the stacked bars indicates the total sequences for each control for with stacked colors indicating species that were identified. *Phymata americana* was the most abundant species detected in negative controls of Primer Set 1 and *Pieris* was identified in no template controls of plates 4-6, both species used in positive controls.

Supplemental Table 1. All wildflowers collected for eDNA metabarcoding and sequences. Wildflowers associated with butterfly occupancy or visitation were collected for eDNA metabarcoding and high throughput sequencing (Illumina NovSeq) of the *cytochrome c oxidase I* (COI) gene. A total of 298 flowers of 12 genera were collected from ten sites in Glacier National Park between July 1st and August 16th, 2022. All flowers were collected directly after traditional butterfly surveys and 43 flowers had an observed natural butterfly visit before collection. Wildflowers from Saint Mary are associated with a field experiment where two butterfly species were physically placed on flowers in a series of contrived visits.

Site	Date Collected	Sample ID	Butterfly Species	Number of Visits
BaringCreek	7/27/2022	BC_Eriogonum1	NA	NA
BaringCreek	7/27/2022	BC_Eriogonum2	NA	NA
BaringCreek	7/27/2022	BC_Eriogonum3	NA	NA
BaringCreek	7/27/2022	BC_Eriogonum4	NA	NA
BaringCreek	7/27/2022	BC_Eriogonum5	NA	NA
BaringCreek	7/27/2022	BC_Eriogonum6	NA	NA
BaringCreek	7/27/2022	BC_Eriogonum7	NA	NA
BaringCreek	7/27/2022	BC_Eriogonum8	NA	NA
BaringCreek	7/27/2022	BC_Eriogonum9	NA	NA
BaringCreek	7/27/2022	BC_Eriogonum10	NA	NA
BaringCreek	7/27/2022	BC_Packera1	NA	NA
BaringCreek	7/27/2022	BC_Packera2	NA	NA
BaringCreek	7/27/2022	BC_Packera3	NA	NA
BaringCreek	7/27/2022	BC_Packera4	NA	NA
BaringCreek	7/27/2022	BC_Packera5	NA	NA
BaringCreek	7/27/2022	BC_Packera6	NA	NA
BaringCreek	7/27/2022	BC_Packera7	NA	NA
BaringCreek	7/27/2022	BC_Packera8	NA	NA
BaringCreek	7/27/2022	BC_Packera9	NA	NA
BaringCreek	7/27/2022	BC_Packera10	NA	NA
BaringCreek	7/27/2022	BC_Dasiphora1	NA	NA
BaringCreek	7/27/2022	BC_Dasiphora2	NA	NA
BaringCreek	7/27/2022	BC_Dasiphora3	NA	NA
BaringCreek	7/27/2022	BC_Dasiphora4	NA	NA
BaringCreek	7/27/2022	BC_Dasiphora5	NA	NA
BaringCreek	7/27/2022	BC_Dasiphora6	NA	NA
BaringCreek	7/27/2022	BC_Dasiphora7	NA	NA
BaringCreek	7/27/2022	BC_Dasiphora8	NA	NA
BaringCreek	7/27/2022	BC_Dasiphora9	NA	NA

Supplemental Table 1 Continued

BaringCreek	7/27/2022	BC_Dasiphora10	NA	NA
BellyRiver	7/7/2022	BR_Geranium1	NA	NA
BellyRiver	7/7/2022	BR_Geranium2	NA	NA
BellyRiver	7/7/2022	BR_Geranium3	NA	NA
BellyRiver	7/7/2022	BR_Geranium4	NA	NA
BellyRiver	7/7/2022	BR_Geranium5	NA	NA
BellyRiver	7/7/2022	BR_Geranium6	NA	NA
BellyRiver	7/7/2022	BR_Geranium7	NA	NA
BellyRiver	7/7/2022	BR_Geranium8	NA	NA
BellyRiver	7/7/2022	BR_Geranium9	NA	NA
BellyRiver	7/7/2022	BR_Senecio1	NA	NA
BellyRiver	7/7/2022	BR_Senecio2	NA	NA
BellyRiver	7/7/2022	BR_Senecio3	NA	NA
BellyRiver	7/7/2022	BR_Senecio4	NA	NA
BellyRiver	7/7/2022	BR_Senecio5	NA	NA
BellyRiver	7/7/2022	BR_Senecio6	NA	NA
BellyRiver	7/7/2022	BR_Senecio7	NA	NA
BellyRiver	7/7/2022	BR_Senecio8	NA	NA
BellyRiver	7/7/2022	BR_Senecio9	NA	NA
BellyRiver	7/7/2022	BR_Senecio10	NA	NA
BellyRiver	7/7/2022	BR_Dasiphora1	NA	NA
BellyRiver	7/7/2022	BR_Dasiphora2	NA	NA
BellyRiver	7/7/2022	BR_Dasiphora3	NA	NA
BellyRiver	7/7/2022	BR_Dasiphora4	NA	NA
BellyRiver	7/7/2022	BR_Dasiphora5	NA	NA
BellyRiver	7/7/2022	BR_Dasiphora6	NA	NA
BellyRiver	7/7/2022	BR_Dasiphora7	NA	NA
BellyRiver	7/7/2022	BR_Dasiphora8	NA	NA
BigPrairie	7/1/2022	BP_Arnica1	NA	NA
BigPrairie	7/1/2022	BP_Arnica2	NA	NA
BigPrairie	7/1/2022	BP_Arnica3	NA	NA
BigPrairie	7/1/2022	BP_Arnica4	NA	NA
BigPrairie	7/1/2022	BP_Arnica5	NA	NA
BigPrairie	7/1/2022	BP_Arnica6	NA	NA
BigPrairie	7/1/2022	BP_Arnica7	NA	NA
BigPrairie	7/1/2022	BP_Arnica8	NA	NA
BigPrairie	7/1/2022	BP_Arnica9	NA	NA

Supplemental Table 1 Continued

BigPrairie	7/1/2022	BP_Arnica10	NA	NA
BigPrairie	7/1/2022	BP_Eriogonum1	NA	NA
BigPrairie	7/1/2022	BP_Eriogonum2	NA	NA
BigPrairie	7/1/2022	BP_Eriogonum3	NA	NA
BigPrairie	7/1/2022	BP_Eriogonum4	NA	NA
BigPrairie	7/1/2022	BP_Eriogonum5	NA	NA
BigPrairie	7/1/2022	BP_Eriogonum6	NA	NA
BigPrairie	7/1/2022	BP_Eriogonum7	NA	NA
BigPrairie	7/1/2022	BP_Eriogonum8	NA	NA
BigPrairie	7/1/2022	BP_Eriogonum9	NA	NA
BigPrairie	7/1/2022	BP_Eriogonum10	NA	NA
BigPrairie	7/1/2022	BP_Eriogonum11	NA	NA
BigPrairie	7/1/2022	BP_Eriogonum12	NA	NA
BigPrairie	7/1/2022	BP_Eriogonum13	NA	NA
BigPrairie	7/1/2022	BP_Eriogonum14	NA	NA
BigPrairie	7/1/2022	BP_Eriogonum15	NA	NA
BigPrairie	7/1/2022	BP_Eriogonum16	NA	NA
BigPrairie	7/1/2022	BP_Eriogonum17	NA	NA
BigPrairie	7/1/2022	BP_Eriogonum18	NA	NA
BigPrairie	7/1/2022	BP_Eriogonum19	NA	NA
BigPrairie	7/1/2022	BP_Eriogonum20	NA	NA
Christensen	7/25/2022	CM_Erigeron1	Phyciodes	1
Christensen	7/25/2022	CM_Erigeron6	Boloria	1
Christensen	7/25/2022	CM_Erigeron10	Thymelicus lineola	1
Christensen	7/25/2022	CM_Arnica1	Phyciodes	1
Christensen	7/25/2022	CM_Arnica6	Phyciodes	1
Christensen	7/25/2022	CM_Achillea1	NA	NA
Christensen	7/25/2022	CM_Achillea2	NA	NA
Christensen	7/25/2022	CM_Achillea3	NA	NA
Christensen	7/25/2022	CM_Achillea4	NA	NA
Christensen	7/25/2022	CM_Achillea5	NA	NA
Christensen	7/25/2022	CM_Achillea6	NA	NA
Christensen	7/25/2022	CM_Achillea7	NA	NA
Christensen	7/25/2022	CM_Achillea8	NA	NA
Christensen	7/25/2022	CM_Achillea9	NA	NA
Christensen	7/25/2022	CM_Achillea10	NA	NA
Christensen	7/25/2022	CM_Erigeron2	NA	NA

Supplemental Table 1 Continued

Christensen	7/25/2022	CM_Erigeron3	NA	NA
Christensen	7/25/2022	CM_Erigeron4	NA	NA
Christensen	7/25/2022	CM_Erigeron5	NA	NA
Christensen	7/25/2022	CM_Erigeron7	NA	NA
Christensen	7/25/2022	CM_Erigeron8	NA	NA
Christensen	7/25/2022	CM_Erigeron9	NA	NA
Christensen	7/25/2022	CM_Arnica2	NA	NA
Christensen	7/25/2022	CM_Arnica3	NA	NA
Christensen	7/25/2022	CM_Arnica4	NA	NA
Christensen	7/25/2022	CM_Arnica5	NA	NA
Christensen	7/25/2022	CM_Arnica7	NA	NA
Christensen	7/25/2022	CM_Arnica8	NA	NA
Christensen	7/25/2022	CM_Arnica9	NA	NA
Christensen	7/25/2022	CM_Arnica10	NA	NA
PrestonPark	8/16/2022	PP_Arnica1	*Tharsalea*	1
PrestonPark	8/16/2022	PP_Erigeron1	*Colias meadii*	1
PrestonPark	8/16/2022	PP_Erigeron3	*Colias meadii*	1
PrestonPark	8/16/2022	PP_Erigeron4	*Speyeria mormonia*	1
PrestonPark	8/16/2022	PP_Erigeron5	*Speyeria mormonia*	1
PrestonPark	8/16/2022	PP_Erigeron6	*Speyeria mormonia*	1
PrestonPark	8/16/2022	PP_Erigeron7	*Speyeria mormonia*	1
PrestonPark	8/16/2022	PP_Erigeron8	*Speyeria mormonia*	1
PrestonPark	8/16/2022	PP_Erigeron9	*Aglais milberti*	1
PrestonPark	8/16/2022	PP_Erigeron10	*Speyeria mormonia*	1
PrestonPark	8/16/2022	PP_Senecio6	*Pontia occidentalis*	1
PrestonPark	8/16/2022	PP_Senecio7	*Colias meadii*	1
PrestonPark	8/16/2022	PP_Senecio8	*Pontia occidentalis*	1
PrestonPark	8/16/2022	PP_Senecio9	*Pontia occidentalis*	1
PrestonPark	8/16/2022	PP_Senecio10	*Aglais milberti*	1
PrestonPark	8/16/2022	PP_Arnica2	NA	NA
PrestonPark	8/16/2022	PP_Arnica3	NA	NA
PrestonPark	8/16/2022	PP_Arnica4	NA	NA
PrestonPark	8/16/2022	PP_Arnica5	NA	NA
PrestonPark	8/16/2022	PP_Arnica6	NA	NA
PrestonPark	8/16/2022	PP_Arnica7	NA	NA
PrestonPark	8/16/2022	PP_Arnica8	NA	NA
PrestonPark	8/16/2022	PP_Arnica9	NA	NA

Supplemental Table 1 Continued

PrestonPark	8/16/2022	PP_Arnica10	NA	NA
PrestonPark	8/16/2022	PP_Erigeron2	NA	NA
PrestonPark	8/16/2022	PP_Senecio1	NA	NA
PrestonPark	8/16/2022	PP_Senecio2	NA	NA
PrestonPark	8/16/2022	PP_Senecio3	NA	NA
PrestonPark	8/16/2022	PP_Senecio4	NA	NA
PrestonPark	8/16/2022	PP_Senecio5	NA	NA
RockyKnob	7/2/2022	RK_Achillea1	NA	NA
RockyKnob	7/2/2022	RK_Achillea2	NA	NA
RockyKnob	7/2/2022	RK_Achillea3	NA	NA
RockyKnob	7/2/2022	RK_Achillea4	NA	NA
RockyKnob	7/2/2022	RK_Achillea5	NA	NA
RockyKnob	7/2/2022	RK_Achillea6	NA	NA
RockyKnob	7/2/2022	RK_Achillea7	NA	NA
RockyKnob	7/2/2022	RK_Achillea8	NA	NA
RockyKnob	7/2/2022	RK_Achillea9	NA	NA
RockyKnob	7/2/2022	RK_Achillea10	NA	NA
RockyKnob	7/2/2022	RK_Drymocallis1	NA	NA
RockyKnob	7/2/2022	RK_Drymocallis2	NA	NA
RockyKnob	7/2/2022	RK_Drymocallis3	NA	NA
RockyKnob	7/2/2022	RK_Drymocallis4	NA	NA
RockyKnob	7/2/2022	RK_Drymocallis5	NA	NA
RockyKnob	7/2/2022	RK_Drymocallis6	NA	NA
RockyKnob	7/2/2022	RK_Drymocallis7	NA	NA
RockyKnob	7/2/2022	RK_Drymocallis8	NA	NA
RockyKnob	7/2/2022	RK_Drymocallis9	NA	NA
RockyKnob	7/2/2022	RK_Drymocallis10	NA	NA
RockyKnob	7/2/2022	RK_Geranium1	NA	NA
RockyKnob	7/2/2022	RK_Geranium2	NA	NA
RockyKnob	7/2/2022	RK_Geranium3	NA	NA
RockyKnob	7/2/2022	RK_Geranium4	NA	NA
RockyKnob	7/2/2022	RK_Geranium5	NA	NA
RockyKnob	7/2/2022	RK_Geranium6	NA	NA
RockyKnob	7/2/2022	RK_Geranium7	NA	NA
RockyKnob	7/2/2022	RK_Geranium8	NA	NA
RockyKnob	7/2/2022	RK_Geranium9	NA	NA
RockyKnob	7/2/2022	RK_Geranium10	NA	NA

Supplemental Table 1 Continued

SpotMt	8/12/2022	SP_Symphyotrichum8	*Tharsalea*	1
SpotMt	8/12/2022	SP_Symphyotrichum1	*Tharsalea*	1
SpotMt	8/12/2022	SP_Symphyotrichum2	*Tharsalea*	1
SpotMt	8/12/2022	SP_Symphyotrichum3	*Tharsalea*	1
SpotMt	8/12/2022	SP_Symphyotrichum4	*Tharsalea*	1
SpotMt	8/12/2022	SP_Symphyotrichum5	*Tharsalea*	1
SpotMt	8/12/2022	SP_Symphyotrichum6	*Tharsalea*	1
SpotMt	8/12/2022	SP_Symphyotrichum7	*Tharsalea*	1
SpotMt	8/12/2022	SP_Symphyotrichum9	*Tharsalea*	1
SpotMt	8/12/2022	SP_Symphyotrichum10	*Tharsalea*	1
SpotMt	8/12/2022	SP_Solidago7	*Tharsalea*	1
SpotMt	8/12/2022	SP_Solidago8	*Tharsalea*	1
SpotMt	8/12/2022	SP_Erigeron1	*Cercyonis oetus*	1
SpotMt	8/12/2022	SP_Erigeron2	*Thymelicus lineola*	1
SpotMt	8/12/2022	SP_Erigeron3	*Pontia*	1
SpotMt	8/12/2022	SP_Erigeron4	*Pontia*	1
SpotMt	8/12/2022	SP_Erigeron5	*Phyciodies*	1
SpotMt	8/12/2022	SP_Erigeron6	*Hesperia*	1
SpotMt	8/12/2022	SP_Erigeron7	*Tharsalea*	1
SpotMt	8/12/2022	SP_Erigeron10	*Speyeria mormonia*	1
SpotMt	8/12/2022	SP_Solidago1	NA	NA
SpotMt	8/12/2022	SP_Solidago2	NA	NA
SpotMt	8/12/2022	SP_Solidago3	NA	NA
SpotMt	8/12/2022	SP_Solidago4	NA	NA
SpotMt	8/12/2022	SP_Solidago5	NA	NA
SpotMt	8/12/2022	SP_Solidago6	NA	NA
SpotMt	8/12/2022	SP_Solidago9	NA	NA
SpotMt	8/12/2022	SP_Solidago10	NA	NA
SpotMt	8/12/2022	SP_Erigeron8	NA	NA
SpotMt	8/12/2022	SP_Erigeron9	NA	NA
StMary	8/10/2022	SM_SPMO_1_1	*Speyeria mormonia*	1
StMary	8/10/2022	SM_SPMO_1_2	*Speyeria mormonia*	1
StMary	8/10/2022	SM_SPMO_1_3	*Speyeria mormonia*	1
StMary	8/10/2022	SM_SPMO_1_4	*Speyeria mormonia*	1
StMary	8/10/2022	SM_SPMO_1_5	*Speyeria mormonia*	1
StMary	8/10/2022	SM_SPMO_5_1	*Speyeria mormonia*	5
StMary	8/10/2022	SM_SPMO_5_2	*Speyeria mormonia*	5

Supplemental Table 1 Continued

StMary	8/10/2022	SM_SPMO_5_3	*Speyeria mormonia*	5
StMary	8/10/2022	SM_SPMO_5_4	*Speyeria mormonia*	5
StMary	8/10/2022	SM_SPMO_5_5	*Speyeria mormonia*	5
StMary	8/10/2022	SM_SPMO_10_1	*Speyeria mormonia*	10
StMary	8/10/2022	SM_SPMO_10_2	*Speyeria mormonia*	10
StMary	8/10/2022	SM_SPMO_10_3	*Speyeria mormonia*	10
StMary	8/10/2022	SM_PLML_1_1	*Plebejus melissa*	1
StMary	8/10/2022	SM_PLML_1_2	*Plebejus melissa*	1
StMary	8/10/2022	SM_PLML_1_3	*Plebejus melissa*	1
StMary	8/10/2022	SM_PLML_1_4	*Plebejus melissa*	1
StMary	8/10/2022	SM_PLML_1_5	*Plebejus melissa*	1
StMary	8/10/2022	SM_PLML_5_1	*Plebejus melissa*	5
StMary	8/10/2022	SM_PLML_5_2	*Plebejus melissa*	5
StMary	8/10/2022	SM_PLML_5_3	*Plebejus melissa*	5
StMary	8/10/2022	SM_PLML_5_4	*Plebejus melissa*	5
StMary	8/10/2022	SM_PLML_5_5	*Plebejus melissa*	5
StMary	8/10/2022	SM_PLML_10_1	*Plebejus melissa*	10
StMary	8/10/2022	SM_PLML_10_2	*Plebejus melissa*	10
StMary	8/10/2022	SM_PLML_10_3	*Plebejus melissa*	10
StMary	8/10/2022	SM_PLML_10_4	*Plebejus melissa*	10
StMary	8/10/2022	SM_PLML_10_5	*Plebejus melissa*	10
StMary	8/10/2022	SM_CTRL_0_1	NA	0
StMary	8/10/2022	SM_CTRL_0_2	NA	0
StMary	8/10/2022	SM_CTRL_0_3	NA	0
Sullivan	8/10/2022	SU_Arnica1	*Speyeria*	1
Sullivan	8/10/2022	SU_Arnica3	*Thymelicus lineola*	1
Sullivan	7/19/2022	SU_Arnica2	NA	NA
Sullivan	7/19/2022	SU_Arnica4	NA	NA
Sullivan	7/19/2022	SU_Arnica5	NA	NA
Sullivan	7/19/2022	SU_Arnica6	NA	NA
Sullivan	7/19/2022	SU_Arnica7	NA	NA
Sullivan	7/19/2022	SU_Arnica8	NA	NA
Sullivan	7/19/2022	SU_Arnica9	NA	NA
Sullivan	7/19/2022	SU_Arnica10	NA	NA
Sullivan	7/19/2022	SU_Erigeron1	NA	NA
Sullivan	7/19/2022	SU_Erigeron2	NA	NA
Sullivan	7/19/2022	SU_Erigeron3	NA	NA
Sullivan	7/19/2022	SU_Erigeron4	NA	NA

Supplemental Table 1 Continued

Sullivan	7/19/2022	SU_Erigeron5	NA	NA
Sullivan	7/19/2022	SU_Erigeron6	NA	NA
Sullivan	7/19/2022	SU_Erigeron7	NA	NA
Sullivan	7/19/2022	SU_Erigeron8	NA	NA
Sullivan	7/19/2022	SU_Erigeron9	NA	NA
Sullivan	7/19/2022	SU_Erigeron10	NA	NA
Sullivan	7/19/2022	SU_Potentilla1	NA	NA
Sullivan	7/19/2022	SU_Potentilla2	NA	NA
Sullivan	7/19/2022	SU_Potentilla3	NA	NA
Sullivan	7/19/2022	SU_Potentilla4	NA	NA
Sullivan	7/19/2022	SU_Potentilla5	NA	NA
Sullivan	7/19/2022	SU_Potentilla6	NA	NA
Sullivan	7/19/2022	SU_Potentilla7	NA	NA
Sullivan	7/19/2022	SU_Potentilla8	NA	NA
Sullivan	7/19/2022	SU_Potentilla9	NA	NA
Sullivan	7/19/2022	SU_Potentilla10	NA	NA
TwoDog	7/9/2022	TD_Geranium3	*Icaricia icariodies*	1
TwoDog	7/9/2022	TD_Potentilla1	NA	NA
TwoDog	7/9/2022	TD_Potentilla2	NA	NA
TwoDog	7/9/2022	TD_Potentilla3	NA	NA
TwoDog	7/9/2022	TD_Potentilla4	NA	NA
TwoDog	7/9/2022	TD_Potentilla5	NA	NA
TwoDog	7/9/2022	TD_Potentilla6	NA	NA
TwoDog	7/9/2022	TD_Potentilla7	NA	NA
TwoDog	7/9/2022	TD_Potentilla8	NA	NA
TwoDog	7/9/2022	TD_Potentilla9	NA	NA
TwoDog	7/9/2022	TD_Potentilla10	NA	NA
TwoDog	7/9/2022	TD_Geranium1	NA	NA
TwoDog	7/9/2022	TD_Geranium2	NA	NA
TwoDog	7/9/2022	TD_Geranium4	NA	NA
TwoDog	7/9/2022	TD_Geranium5	NA	NA
TwoDog	7/9/2022	TD_Geranium6	NA	NA
TwoDog	7/9/2022	TD_Geranium7	NA	NA
TwoDog	7/9/2022	TD_Geranium8	NA	NA
TwoDog	7/9/2022	TD_Geranium9	NA	NA
TwoDog	7/9/2022	TD_Geranium10	NA	NA
TwoDog	7/9/2022	TD_Arnica1	NA	NA

Supplemental Table 1 Continued

TwoDog	7/9/2022	TD_Arnica2	NA	NA
TwoDog	7/9/2022	TD_Arnica3	NA	NA
TwoDog	7/9/2022	TD_Arnica4	NA	NA
TwoDog	7/9/2022	TD_Arnica5	NA	NA
TwoDog	7/9/2022	TD_Arnica6	NA	NA
TwoDog	7/9/2022	TD_Arnica7	NA	NA
TwoDog	7/9/2022	TD_Arnica8	NA	NA
TwoDog	7/9/2022	TD_Arnica9	NA	NA
TwoDog	7/9/2022	TD_Arnica10	NA	NA

Supplemental Table 2. Pooling of wildflower eDNA from samples without known butterfly visits. Wildflower eDNA samples that were not associated with either an observed natural visit or a contrived visit of butterflies were pooled to increase the throughput of samples that could be sequenced. Wildflower eDNA from two to four individual flowers of the same genus and from the same site were pooled in equal amounts (10 ng) each and the DNA concentration of the resulting pool was calculated. The volume needed to add 5 ng of eDNA pool as template for a PCR reaction is also calculated. DNA concentrations were obtained using an Invitrogen Qubit 4.0 fluorometer and the 1x dsDNA High Sensitivity (HS) Assay Kit (Fisher Scientific).

Site	Pool Name	Sample	DNA conc (ng/ul)	ul for 10ng	DNA conc of Pool (Total ng/total ul)	ul of pool for 5ng in PCR
Preston Park	**PP_ArnicaA**	PP_Arnica2	4.46	2.2	**1.2**	**4.2**
		PP_Arnica3	1.19	8.4		
		PP_Arnica4	0.674	14.8		
		TOTAL		**25.5**		
	PP_ArnicaB	PP_Arnica5	0.594	16.8	**0.4**	**11.4**
		PP_Arnica6	0.258	38.8		
		PP_Arnica7	0.788	12.7		
		TOTAL		**68.3**		
	PP_ArnicaC	PP_Arnica8	0.648	15.4	**0.8**	**6.0**
		PP_Arnica9	2.3	4.3		
		PP_Arnica10	0.624	16.0		
		TOTAL		**35.8**		
	PP_SenecioA	PP_Senecio1	1.36	7.4	**0.6**	**8.1**
		PP_Senecio2	0.582	17.2		
		PP_Senecio3	0.412	24.3		
		TOTAL		**48.8**		
	PP_SenecioB	PP_Senecio4	2.26	4.4	**1.2**	**4.0**
		PP_Senecio5	0.862	11.6		
		TOTAL		**16.0**		
Spot Mt	**SP_SolidagoA**	SP_Solidago1	2.88	3.5	**3.3**	**1.5**
		SP_Solidago2	4.02	2.5		
		SP_Solidago3	3.28	3.0		
		TOTAL		**9.0**		
	SP_SolidagoB	SP_Solidago4	3.12	3.2	**4.1**	**1.2**
		SP_Solidago5	2.96	3.4		

Supplemental Table 2 Continued

		SP_Solidago6	12.8	0.8		
		TOTAL		**7.4**		
Spot Mt	**SP_SolidagoC**	SP_Solidago9	6.18	1.6	**4.3**	**1.2**
		SP_Solidago10	3.24	3.1		
		TOTAL		**4.7**		
	SP_ErigeronA	SP_Erigeron8	0.818	12.2	**0.8**	**6.5**
		SP_Erigeron9	0.724	13.8		
		TOTAL		26.0		
Christensen	**CM_AchilleaA**	CM_Achillea1	2.14	4.7	**4.0**	**1.2**
		CM_Achillea2	7.46	1.3		
		CM_Achillea3	7	1.4		
		TOTAL		**7.4**		
	CM_AchilleaB	CM_Achillea4	3.9	2.6	**3.8**	**1.3**
		CM_Achillea5	4.24	2.4		
		CM_Achillea6	3.48	2.9		
		TOTAL		**7.8**		
	CM_AchilleaC	CM_Achillea7	6.5	1.5	**2.0**	**2.5**
		CM_Achillea8	0.628	15.9		
		CM_Achillea9	5.52	1.8		
		CM_Achillea10	9.26	1.1		
		TOTAL		**20.4**		
	CM_ErigeronA	CM_Erigeron2	0.936	10.7	**1.0**	**4.9**
		CM_Erigeron3	0.684	14.6		
		CM_Erigeron4	2.4	4.2		
		TOTAL		**29.5**		
	CM_ErigeronB	CM_Erigeron5	0.878	11.4	**1.3**	**4.0**
		CM_Erigeron7	1.33	7.5		
		CM_Erigeron8	1.31	7.6		
		CM_Erigeron9	1.96	5.1		
		TOTAL		**31.6**		
	CM_ArnicaA	CM_Arnica2	6.38	1.6	**3.5**	**1.4**
		CM_Arnica3	3.66	2.7		
		CM_Arnica4	2.32	4.3		
		TOTAL		**8.6**		

Supplemental Table 2 Continued

Christensen	**CM_ArnicaB**	CM_Arnica5	3	3.3	**2.8**	**1.8**
		CM_Arnica7	2.5	4.0		
		CM_Arnica8	3.02	3.3		
		TOTAL		**10.6**		
	CM_ArnicaC	CM_Arnica9	0.986	10.1	**1.6**	**3.2**
		CM_Arnica10	1.12	8.9		
		TOTAL		**19.1**		
Sullivan	**SU_ArnicaA**	SU_Arnica2	2.72	3.7	**2.9**	**1.7**
		SU_Arnica4	3.16	3.2		
		SU_Arnica5	2.88	3.5		
		TOTAL		**10.3**		
	SU_ArnicaB	SU_Arnica6	2.68	3.7	**2.0**	**2.5**
		SU_Arnica7	1.89	5.3		
		SU_Arnica8	1.67	6.0		
		TOTAL		**15.0**		
	SU_ArnicaC	SU_Arnica9	2.18	4.6	**1.4**	**3.7**
		SU_Arnica10	0.996	10.0		
		TOTAL		**14.6**		
	SU_ErigeronA	SU_Erigeron1	1.18	8.5	**1.4**	**3.7**
		SU_Erigeron2	1.6	6.3		
		SU_Erigeron3	1.39	7.2		
		TOTAL		**21.9**		
	SU_ErigeronB	SU_Erigeron4	0.762	13.1	**1.2**	**4.0**
		SU_Erigeron5	1.69	5.9		
		SU_Erigeron6	1.98	5.1		
		TOTAL		**24.1**		
	SU_ErigeronC	SU_Erigeron7	0.686	14.6	**0.8**	**6.2**
		SU_Erigeron8	0.658	15.2		
		SU_Erigeron9	1.44	6.9		
		SU_Erigeron10	0.796	12.6		
		TOTAL		**49.3**		
	SU_PotentillaA	SU_Potentilla1	0.892	11.2	**0.4**	**13.2**
		SU_Potentilla2	0.322	31.1		
		SU_Potentilla3	0.27	37.0		
		TOTAL		**79.3**		

Supplemental Table 2 Continued

Belly River	**SU_PotentillaB**	SU_Potentilla4	0.262	38.2	**0.2**	**20.2**
		SU_Potentilla5	0.23	43.5		
		SU_Potentilla6	0.254	39.4		
		TOTAL		**121.0**		
	SU_PotentillaC	SU_Potentilla7	0.216	46.3	**0.4**	**12.6**
		SU_Potentilla8	0.458	21.8		
		SU_Potentilla9	0.5	20.0		
		SU_Potentilla10	0.782	12.8		
		TOTAL		**100.9**		
	BR_DasiphoraA	BR_Dasiphora1	0.26	38.5	**0.3**	**19.3**
		BR_Dasiphora2	0.302	33.1		
		BR_Dasiphora3	0.226	44.2		
		TOTAL		**115.8**		
	BR_DasiphoraB	BR_Dasiphora4	0.372	26.9	**0.2**	**21.5**
		BR_Dasiphora5	0.186	53.8		
		BR_Dasiphora6	0.206	48.5		
		TOTAL		**129.2**		
	BR_DasiphoraC	BR_Dasiphora7	0.194	51.5	**0.2**	**27.8**
		BR_Dasiphora8	0.168	59.5		
		TOTAL		**111.1**		
	BR_GeraniumA	BR_Geranium1	0.544	18.4	**0.8**	**6.4**
		BR_Geranium2	2.8	3.6		
		BR_Geranium3	0.612	16.3		
		TOTAL		**38.3**		
	BR_GeraniumB	BR_Geranium4	1.11	9.0	**0.9**	**5.6**
		BR_Geranium5	3.02	3.3		
		BR_Geranium6	0.474	21.1		
		TOTAL		**33.4**		
	BR_GeraniumC	BR_Geranium7	1.6	6.3	**0.7**	**7.6**
		BR_Geranium8	0.256	39.1		
		BR_Geranium9	0.454	22.0		
		TOTAL		**45.3**		
	BR_SenecioA	BR_Senecio1	3.92	2.6	**2.4**	**2.0**
		BR_Senecio2	2.36	4.2		
		BR_Senecio3	1.82	5.5		
		TOTAL		**12.3**		

Supplemental Table 2 Continued

Belly River	**BR_SenecioB**	BR_Senecio4	4.04	2.5	**3.1**	**1.6**
		BR_Senecio5	2.62	3.8		
		BR_Senecio6	3.06	3.3		
		TOTAL		**9.6**		
	BR_SenecioC	BR_Senecio7	3.46	2.9	**4.1**	**1.2**
		BR_Senecio8	2.2	4.5		
		BR_Senecio9	4.38	2.3		
		BR_Senecio10	6.7	1.5		
		TOTAL		**9.7**		
Baring Creek	**BC_DasiphoraA**	BC_Dasiphora1	0.116	86.2	**0.1**	**35.6**
		BC_Dasiphora2	0.144	69.4		
		BC_Dasiphora3	0.172	58.1		
		TOTAL		**213.8**		
	BC_DasiphoraB	BC_Dasiphora4	0.366	27.3	**0.3**	**18.8**
		BC_Dasiphora5	0.354	28.2		
		BC_Dasiphora6	0.174	57.5		
		TOTAL		**113.0**		
	BC_DasiphoraC	BC_Dasiphora7	0.854	11.7	**0.4**	**12.4**
		BC_Dasiphora8	0.114	87.7		
		BC_Dasiphora9	0.108	92.6		
		BC_Dasiphora10	0.636	15.7		
		TOTAL		**99.4**		
	BC_PackeraA	BC_Packera1	3.44	2.9	**3.1**	**1.6**
		BC_Packera2	3.44	2.9		
		BC_Packera3	2.5	4.0		
		TOTAL		**9.8**		
	BC_PackeraB	BC_Packera4	2.88	3.5	**3.3**	**1.5**
		BC_Packera5	3.52	2.8		
		BC_Packera6	3.68	2.7		
		TOTAL		**9.0**		
	BC_PackeraC	BC_Packera7	2.14	4.7	**6.5**	**0.8**
		BC_Packera8	6.92	1.4		
		BC_Packera9	2.78	3.6		
		BC_Packera10	4.38	2.3		
		TOTAL		**6.1**		

Supplemental Table 2 Continued

Baring Creek	**BC_EriogonumA**	BC_Eriogonum1	0.556	18.0	**0.5**	**11.0**
		BC_Eriogonum2	0.24	41.7		
		BC_Eriogonum3	1.56	6.4		
		TOTAL		**66.1**		
	BC_EriogonumB	BC_Eriogonum4	3.7	2.7	**1.9**	**2.7**
		BC_Eriogonum5	1.45	6.9		
		BC_Eriogonum6	1.56	6.4		
		TOTAL		**16.0**		
	BC_EriogonumC	BC_Eriogonum7	0.618	16.2	**1.2**	**4.3**
		BC_Eriogonum8	2.26	4.4		
		BC_Eriogonum9	0.716	14.0		
		BC_Eriogonum10	1.86	5.4		
		TOTAL		**34.6**		
Rocky Knob	**RK_DrymocallisA**	RK_Drymocallis1	0.264	37.9	**0.2**	**24.3**
		RK_Drymocallis2	0.28	35.7		
		RK_Drymocallis3	0.138	72.5		
		TOTAL		**146.1**		
	RK_DrymocallisB	RK_Drymocallis4	0.124	80.6	**0.2**	**24.9**
		RK_Drymocallis5	0.152	65.8		
		RK_Drymocallis6	3.46	2.9		
		TOTAL		**149.3**		
	RK_DrymocallisC	RK_Drymocallis7	0.258	38.8	**0.7**	**7.4**
		RK_Drymocallis8	0.496	20.2		
		RK_Drymocallis9	0.224	44.6		
		RK_Drymocallis10	0.334	29.9		
		TOTAL		**58.9**		
	RK_GeraniumA	RK_Geranium1	1.6	6.3	**1.9**	**2.6**
		RK_Geranium2	1.87	5.3		
		RK_Geranium3	2.44	4.1		
		TOTAL		**15.7**		
	RK_GeraniumB	RK_Geranium4	3.08	3.2	**2.0**	**2.5**
		RK_Geranium5	1.73	5.8		
		RK_Geranium6	1.69	5.9		
		TOTAL		**14.9**		

Supplemental Table 2 Continued

Rocky Knob	**RK_GeraniumC**	RK_Geranium7	2	5.0	**5.9**	**0.8**
		RK_Geranium8	5.78	1.7		
		RK_Geranium9	1.29	7.8		
		RK_Geranium10	2.1	4.8		
		TOTAL		**6.7**		
	RK_AchilleaA	RK_Achillea1	1.17	8.5	**2.1**	**2.4**
		RK_Achillea2	4.64	2.2		
		RK_Achillea3	2.72	3.7		
		TOTAL		**14.4**		
	RK_AchilleaB	RK_Achillea4	1.1	9.1	**1.6**	**3.1**
		RK_Achillea5	2.52	4.0		
		RK_Achillea6	1.87	5.3		
		TOTAL		**18.4**		
	RK_AchilleaC	RK_Achillea7	1.78	5.6	**1.1**	**4.4**
		RK_Achillea8	1.79	5.6		
		RK_Achillea9	0.414	24.2		
		RK_Achillea10	2.16	4.6		
		TOTAL		**35.4**		
Big Prairie	**BP_ArnicaA**	BP_Arnica1	5.84	1.7	**2.2**	**2.3**
		BP_Arnica2	2.68	3.7		
		BP_Arnica3	1.23	8.1		
		TOTAL		**13.6**		
	BP_ArnicaB	BP_Arnica4	2.06	4.9	**2.0**	**2.5**
		BP_Arnica5	2.3	4.3		
		BP_Arnica6	1.75	5.7		
		TOTAL		**14.9**		
	BP_ArnicaC	BP_Arnica7	1.93	5.2	**2.7**	**1.9**
		BP_Arnica8	1.02	9.8		
		BP_Arnica9	1.27	7.9		
		BP_Arnica10	1.86	5.4		
		TOTAL		**15.0**		
	BP_EriogonumA	BP_Eriogonum1	0.332	30.1	**0.6**	**9.0**
		BP_Eriogonum2	0.79	12.7		
		BP_Eriogonum3	0.908	11.0		
		TOTAL		**53.8**		

Supplemental Table 2 Continued

Big Prairie	**BP_EriogonumB**	BP_Eriogonum4	2.74	3.6	**0.6**	**8.9**
		BP_Eriogonum5	1.06	9.4		
		BP_Eriogonum6	0.248	40.3		
		TOTAL		**53.4**		
	BP_EriogonumC	BP_Eriogonum7	2.24	4.5	**0.4**	**13.0**
		BP_Eriogonum8	0.846	11.8		
		BP_Eriogonum9	0.162	61.7		
		TOTAL		**78.0**		
	BP_EriogonumD	BP_Eriogonum10	0.278	36.0	**0.3**	**18.0**
		BP_Eriogonum11	0.674	14.8		
		BP_Eriogonum12	0.174	57.5		
		TOTAL		**108.3**		
	BP_EriogonumE	BP_Eriogonum13	0.79	12.7	**0.4**	**11.9**
		BP_Eriogonum14	0.26	38.5		
		BP_Eriogonum15	0.492	20.3		
		TOTAL		**71.4**		
	BP_EriogonumF	BP_Eriogonum16	0.188	53.2	**0.6**	**9.1**
		BP_Eriogonum17	0.512	19.5		
		BP_Eriogonum18	0.136	73.5		
		BP_Eriogonum20	0.296	33.8		
		TOTAL		**72.7**		
Two Dog	**TD_GeraniumA**	TD_Geranium1	0.602	16.6	**0.6**	**8.4**
		TD_Geranium2	0.51	19.6		
		TD_Geranium4	0.708	14.1		
		TOTAL		**50.3**		
	TD_GeraniumB	TD_Geranium5	0.534	18.7	**0.7**	**6.9**
		TD_Geranium6	1.38	7.2		
		TD_Geranium7	0.638	15.7		
		TOTAL		**41.6**		
	TD_GeraniumC	TD_Geranium8	0.512	19.5	**0.5**	**10.0**
		TD_Geranium9	0.246	40.7		
		TD_Geranium10	2.36	4.2		
		TOTAL		**60.2**		

Supplemental Table 2 Continued

Two Dog	**TD_PotentillaA**	TD_Potentilla1	0.53	18.9	**0.7**	**7.6**
		TD_Potentilla2	0.898	11.1		
		TD_Potentilla3	0.63	15.9		
		TOTAL		**45.9**		
	TD_PotentillaB	TD_Potentilla4	0.364	27.5	**0.5**	**9.1**
		TD_Potentilla5	0.862	11.6		
		TD_Potentilla6	0.638	15.7		
		TOTAL		**54.7**		
	TD_PotentillaC	TD_Potentilla7	0.35	28.6	**0.6**	**7.9**
		TD_Potentilla8	0.292	34.2		
		TD_Potentilla9	0.396	25.3		
		TD_Potentilla10	0.456	21.9		
		TOTAL		**62.8**		
	TD_ArnicaA	TD_Arnica1	4.7	2.1	**2.1**	**2.4**
		TD_Arnica2	2.4	4.2		
		TD_Arnica3	1.2	8.3		
		TOTAL		**14.6**		
	TD_ArnicaB	TD_Arnica4	1.32	7.6	**1.4**	**3.5**
		TD_Arnica5	1.27	7.9		
		TD_Arnica6	1.71	5.8		
		TOTAL		**21.3**		
	TD_ArnicaC	TD_Arnica7	2.64	3.8	**2.0**	**2.5**
		TD_Arnica8	3.38	3.0		
		TD_Arnica9	0.74	13.5		
		TD_Arnica10	3.58	2.8		
		TOTAL		**20.3**		

Supplemental Table 3. PCR primers used in the study. Wildflower-associated eDNA samples were prepared for high throughput sequencing (Illumina NovSeq) of two *cytochrome c oxidase I* (COI) gene regions with a 2-step PCR nested metabarcoding method (Figure 2). In the first PCR (PCR1), fusion primers were used to add a unique 8-nucleotide sequence (internal index) for sample tagging, a 0-6-bp variable spacer to increase sequence heterogeneity and an extension sequence to bind sequencing adapters in the second PCR. Twelve forward and eight reverse primers were ordered with unique internal indices to tag a full 96-well PCR plate with unique combinations of indices on forward and reverse primers. A second set of nine forward and reverse primers were used in a second PCR reaction (PCR2) to add Illumina sequencing adapters, unique dual indexes (Illumina Index) and an extension sequence to bind products from PCR1. The same tagging scheme was used for both primer sets: Primer Set 1 (MLepF1/LepR1) and Primer Set 2 (LepF1/MHMR1).

PCR1 - Primer Set 1						
Primer Name	**For/ Rev**	**Extension sequence**	**Internal Index**	**Spacer**	**Amplicon primer**	Whole Primer sequence 5'-3'
MLepF1_F1	For	tcgtcggcagcgtcagatgt gtataagagacag	**tcgcctta**	**none**	gctttcccacgaataaataata	tcgtcggcagcgtcagatgtgtataagagacag**tcg** **cctta**gctttcccacgaataaataata
MLepF1_F2	For	tcgtcggcagcgtcagatgt gtataagagacag	**ctagtacg**	**t**	gctttcccacgaataaataata	tcgtcggcagcgtcagatgtgtataagagacag**cta** **gtacgt**gctttcccacgaataaataata
MLepF1_F3	For	tcgtcggcagcgtcagatgt gtataagagacag	**ttctgcct**	**gt**	gctttcccacgaataaataata	tcgtcggcagcgtcagatgtgtataagagacag**ttct** **gcctgt**gctttcccacgaataaataata

Supplemental Table 3 Continued

MLepF1_F4	For	tcgtcggcagcgtcagatgt gtataagagacag	**gctcagga**	**cga**	gctttcccacgaataaataata	Tcgtcggcagcgtcagatgtgtataagagacag**gct cagga**cgagctttcccacgaataaataata
MLepF1_F5	For	tcgtcggcagcgtcagatgt gtataagagacag	**aggagtcc**	**atga**	gctttcccacgaataaataata	tcgtcggcagcgtcagatgtgtataagagacag**agg agtcc**atgagctttcccacgaataaataata
MLepF1_F6	For	tcgtcggcagcgtcagatgt gtataagagacag	**catgccta**	**tgcga**	gctttcccacgaataaataata	tcgtcggcagcgtcagatgtgtataagagacag**cat gccta**tgcgagctttcccacgaataaataata
MLepF1_F7	For	tcgtcggcagcgtcagatgt gtataagagacag	**gtagagag**	**gagtgg**	gctttcccacgaataaataata	tcgtcggcagcgtcagatgtgtataagagacag**gta gagag**gagtggctttcccacgaataaataata
MLepF1_F8	For	tcgtcggcagcgtcagatgt gtataagagacag	**cctctctg**	**cctgtgg**	gctttcccacgaataaataata	tcgtcggcagcgtcagatgtgtataagagacag**cct ctctg**cctgtggctttcccacgaataaataata
MLepF1_F9	For	tcgtcggcagcgtcagatgt gtataagagacag	**agcgtagc**	**none**	gctttcccacgaataaataata	tcgtcggcagcgtcagatgtgtataagagacag**agc gtagc**gctttcccacgaataaataata
MLepF1_F10	For	tcgtcggcagcgtcagatgt gtataagagacag	**cagcctcg**	**gagtgg**	gctttcccacgaataaataata	tcgtcggcagcgtcagatgtgtataagagacag**cag cctcg**gagtggctttcccacgaataaataata

Supplemental Table 3 Continued

MLepF1_F11	For	tcgtcggcagcgtcagatgt gtataagagacag	**tgcctctt**	**tgcga**	gctttcccacgaataaataata	Tcgtcggcagcgtcagatgtgtataagagacag**tgc ctctttgcga**gctttcccacgaataaataata
MLepF1_F12	For	tcgtcggcagcgtcagatgt gtataagagacag	**tcctctac**	**atga**	gctttcccacgaataaataata	tcgtcggcagcgtcagatgtgtataagagacag**tcct ctacatga**gctttcccacgaataaataata
LepR1_R1	Rev	gtctcgtgggctcggagatgt gtataagagacag	**tagatcgc**	**none**	taaacttctggatgtccaaaaaatca	gtctcgtgggctcggagatgtgtataagagacag**ta gatcgc**taaacttctggatgtccaaaaaatca
LepR1_R2	Rev	gtctcgtgggctcggagatgt gtataagagacag	**ctctctat**	**t**	taaacttctggatgtccaaaaaatca	gtctcgtgggctcggagatgtgtataagagacag**ctc tctatt**taaacttctggatgtccaaaaaatca
LepR1_R3	Rev	gtctcgtgggctcggagatgt gtataagagacag	**tatcctct**	**gt**	taaacttctggatgtccaaaaaatca	gtctcgtgggctcggagatgtgtataagagacag**tat cctctgt**taaacttctggatgtccaaaaaatca
LepR1_R4	Rev	gtctcgtgggctcggagatgt gtataagagacag	**agagtaga**	**cga**	taaacttctggatgtccaaaaaatca	gtctcgtgggctcggagatgtgtataagagacag**ag agtagacga**taaacttctggatgtccaaaaaatca
LepR1_R5	Rev	gtctcgtgggctcggagatgt gtataagagacag	**gtaaggag**	**atga**	taaacttctggatgtccaaaaaatca	gtctcgtgggctcggagatgtgtataagagacag**gt aaggagatga**taaacttctggatgtccaaaaaatca

Supplemental Table 3 Continued

LepR1_R6	Rev	gtctcgtgggctcggagatgt gtataagagacag	**actgcata**	**tgcga**	taaacttctggatgtccaaaaaatca	Gtctcgtgggctcggagatgtgtataagagacag**ac tgcata**tgcgataaacttctggatgtccaaaaaatca
LepR1_R7	Rev	gtctcgtgggctcggagatgt gtataagagacag	**aaggagta**	**gagtgg**	taaacttctggatgtccaaaaaatca	gtctcgtgggctcggagatgtgtataagagacag**aa ggagta**gagtggtaaacttctggatgtccaaaaaat ca
LepR1_R8	Rev	gtctcgtgggctcggagatgt gtataagagacag	**ctaagcct**	**cctgtgg**	taaacttctggatgtccaaaaaatca	gtctcgtgggctcggagatgtgtataagagacag**ct aagcct**cctgtggtaaacttctggatgtccaaaaaat ca
PCR1 - Primer Set 2						
Primer Name	**For/ Rev**	**Extension sequence**	**Internal Index**	**Spacer**	**Amplicon primer**	**Whole primer sequence**
LepF1_F101	For	tcgtcggcagcgtcagatgt gtataagagacag	**tcgcctta**	**none**	attcaaccaatcataaagatat	tcgtcggcagcgtcagatgtgtataagagacag**tc gcctta**attcaaccaatcataaagatat

Supplemental Table 3 Continued

LepF1_F102	For	tcgtcggcagcgtcagatgt gtataagagacag	**ctagtacg**	**t**	attcaaccaatcataaagatat	Tcgtcggcagcgtcagatgtgtataagagacag**c** **tagtacg**tattcaaccaatcataaagatat
LepF1_F103	For	tcgtcggcagcgtcagatgt gtataagagacag	**ttctgcct**	**gt**	attcaaccaatcataaagatat	tcgtcggcagcgtcagatgtgtataagagacag**tt** **ctgcctgt**attcaaccaatcataaagatat
LepF1_F104	For	tcgtcggcagcgtcagatgt gtataagagacag	**gctcagga**	**cga**	attcaaccaatcataaagatat	tcgtcggcagcgtcagatgtgtataagagacag**g** **ctcaggacga**attcaaccaatcataaagatat
LepF1_F105	For	tcgtcggcagcgtcagatgt gtataagagacag	**aggagtcc**	**atga**	attcaaccaatcataaagatat	tcgtcggcagcgtcagatgtgtataagagacag**a** **ggagtccatga**attcaaccaatcataaagatat
LepF1_F106	For	tcgtcggcagcgtcagatgt gtataagagacag	**catgccta**	**tgcga**	attcaaccaatcataaagatat	tcgtcggcagcgtcagatgtgtataagagacag**c** **atgcctatgcga**attcaaccaatcataaagatat
LepF1_F107	For	tcgtcggcagcgtcagatgt gtataagagacag	**gtagagag**	**gagtgg**	attcaaccaatcataaagatat	tcgtcggcagcgtcagatgtgtataagagacag**gt** **agagaggagtgg**attcaaccaatcataaagatat
LepF1_F108	For	tcgtcggcagcgtcagatgt gtataagagacag	**cctctctg**	**cctgtgg**	attcaaccaatcataaagatat	tcgtcggcagcgtcagatgtgtataagagacag**c** **ctctctgcctgtgg**attcaaccaatcataaagatat

Supplemental Table 3 Continued

LepF1_F109	For	tcgtcggcagcgtcagatgt gtataagagacag	**agcgtagc**	**none**	attcaaccaatcataaagatat	Tcgtcggcagcgtcagatgtgtataagagacag**c** **agcctcg**attcaaccaatcataaagatat
LepF1_F110	For	tcgtcggcagcgtcagatgt gtataagagacag	**cagcctcg**	**gagtgg**	attcaaccaatcataaagatat	tcgtcggcagcgtcagatgtgtataagagacag**c** **agcctcggagtgg**attcaaccaatcataaagatat
LepF1_F111	For	tcgtcggcagcgtcagatgt gtataagagacag	**tgcctctt**	**tgcga**	attcaaccaatcataaagatat	tcgtcggcagcgtcagatgtgtataagagacag**tg** **cctctttgcga**attcaaccaatcataaagatat
LepF1_F112	For	tcgtcggcagcgtcagatgt gtataagagacag	**tcctctac**	**atga**	attcaaccaatcataaagatat	tcgtcggcagcgtcagatgtgtataagagacag**tc** **ctctacatga**attcaaccaatcataaagatat
MHMR1_R101	Rev	gtctcgtgggctcggagatgt gtataagagacag	**tagatcgc**	**none**	cctgttccagctccattttc	gtctcgtgggctcggagatgtgtataagagacag**t** **agatcgc**cctgttccagctccattttc
MHMR1_R102	Rev	gtctcgtgggctcggagatgt gtataagagacag	**ctctctat**	**t**	cctgttccagctccattttc	gtctcgtgggctcggagatgtgtataagagacag**c** **tctctatt**cctgttccagctccattttc
MHMR1_R103	Rev	gtctcgtgggctcggagatgt gtataagagacag	**tatcctct**	**gt**	cctgttccagctccattttc	gtctcgtgggctcggagatgtgtataagagacag**t** **atcctctgt**cctgttccagctccattttc

Supplemental Table 3 Continued

MHMR1_R104	Rev	gtctcgtgggctcggagatgt gtataagagacag	**agagtaga**	**cga**	cctgttccagctccattttc	Gtctcgtgggctcggagatgtgtataagagacag **agagtagacga**cctgttccagctccattttc
MHMR1_R105	Rev	gtctcgtgggctcggagatgt gtataagagacag	**gtaaggag**	**atga**	cctgttccagctccattttc	gtctcgtgggctcggagatgtgtataagagacag **gtaaggagatga**cctgttccagctccattttc
MHMR1_R106	Rev	gtctcgtgggctcggagatgt gtataagagacag	**actgcata**	**tgcga**	cctgttccagctccattttc	gtctcgtgggctcggagatgtgtataagagacag **actgcatatgcga**cctgttccagctccattttc
MHMR1_R107	Rev	gtctcgtgggctcggagatgt gtataagagacag	**aaggagta**	**gagtgg**	cctgttccagctccattttc	gtctcgtgggctcggagatgtgtataagagacag **aaggagtagagtgg**cctgttccagctccattttc
MHMR1_R108	Rev	gtctcgtgggctcggagatgt gtataagagacag	**ctaagcct**	**cctgtgg**	cctgttccagctccattttc	gtctcgtgggctcggagatgtgtataagagacag**c** **taagcctcctgtgg**cctgttccagctccattttc

Supplemental Table 3 Continued

PCR2 Primers					
Primer Name	**For/ Rev**	**Illumina Adapter**	**Illumina Index**	**Extension Sequence**	**Whole Primer Sequence 5' - 3'**
i5_F_200	For	aatgatacggcgaccaccga gatctacac	ctctctat	tcgtcggcagcgtc	Aatgatacggcgaccaccgagatctacacctctctattcgtcggcagcgtc
i5_F_201	For	aatgatacggcgaccaccga gatctacac	tatcctct	tcgtcggcagcgtc	aatgatacggcgaccaccgagatctacactatcctcttcgtcggcagcgtc
i5_F_202	For	aatgatacggcgaccaccga gatctacac	gtaaggag	tcgtcggcagcgtc	aatgatacggcgaccaccgagatctacacgtaaggagtcgtcggcagcgtc
i5_F_203	For	aatgatacggcgaccaccga gatctacac	actgcata	tcgtcggcagcgtc	aatgatacggcgaccaccgagatctacacactgcatatcgtcggcagcgtc
i5_F_204	For	aatgatacggcgaccaccga gatctacac	aaggagta	tcgtcggcagcgtc	aatgatacggcgaccaccgagatctacacaaggagtatcgtcggcagcgtc

Supplemental Table 3 Continued

i5_F_205	For	aatgatacggcgaccaccga gatctacac	ctaagcct	tcgtcggcagcgtc	Aatgatacggcgaccaccgagatctacacctaagccttcgtcggcagcgtc
i5_F_206	For	aatgatacggcgaccaccga gatctacac	cgtctaat	tcgtcggcagcgtc	aatgatacggcgaccaccgagatctacaccgtctaattcgtcggcagcgtc
i5_F_207	For	aatgatacggcgaccaccga gatctacac	tctctccg	tcgtcggcagcgtc	aatgatacggcgaccaccgagatctacactctctccgtcgtcggcagcgtc
i5_F_208	For	aatgatacggcgaccaccga gatctacac	tcgactag	tcgtcggcagcgtc	aatgatacggcgaccaccgagatctacactcgactagtcgtcggcagcgtc
i5_F_209	For	aatgatacggcgaccaccga gatctacac	ttctagct	tcgtcggcagcgtc	aatgatacggcgaccaccgagatctacacttctagcttcgtcggcagcgtc
i7_R_250	Rev	caagcagaagacggcatac gagat	taaggcga	gtctcgtgggctcgg	caagcagaagacggcatacgagattaaggcgagtctcgtgggctcgg
i7_R_251	Rev	caagcagaagacggcatac gagat	cgtactag	gtctcgtgggctcgg	caagcagaagacggcatacgagatcgtactaggtctcgtgggctcgg

Supplemental Table 3 Continued

i7_R_252	Rev	caagcagaagacggcatacgagat	aggcagaa	gtctcgtgggctcgg	Caagcagaagacggcatacgagataggcagaagtctcgtgggctcgg
i7_R_253	Rev	caagcagaagacggcatacgagat	tcctgagc	gtctcgtgggctcgg	caagcagaagacggcatacgagattcctgagcgtctcgtgggctcgg
i7_R_254	Rev	caagcagaagacggcatacgagat	ggactcct	gtctcgtgggctcgg	caagcagaagacggcatacgagatggactcctgtctcgtgggctcgg
i7_R_255	Rev	caagcagaagacggcatacgagat	taggcatg	gtctcgtgggctcgg	caagcagaagacggcatacgagattaggcatggtctcgtgggctcgg
i7_R_256	Rev	caagcagaagacggcatacgagat	ctctctac	gtctcgtgggctcgg	caagcagaagacggcatacgagatctctctacgtctcgtgggctcgg
i7_R_257	Rev	caagcagaagacggcatacgagat	cgaggctg	gtctcgtgggctcgg	caagcagaagacggcatacgagatcgaggctggtctcgtgggctcgg
i7_R_258	Rev	caagcagaagacggcatacgagat	aagaggca	gtctcgtgggctcgg	caagcagaagacggcatacgagataagaggcagtctcgtgggctcgg
i7_R_259	Rev	caagcagaagacggcatacgagat	gtagagga	gtctcgtgggctcgg	caagcagaagacggcatacgagatgtagaggagtctcgtgggctcgg

Supplemental Table 4. Description of final sequence library. Indexed pools of COI amplicons (PCR2 pools), each diluted to 10 µM, were pooled into a single sample for sequencing. The nine PCR2 sub-libraries (Figure 2) were diluted with molecular grade water (Invitrogen) to a concentration of 10 µM DNA, and 5 µl of each of those samples were combined prior to sequencing on an Illumina NovaSeq at the University of Illinois Roy J. Carver Biotechnology Center. The DNA concentration (DNA Conc.), average (avg) size in base pairs, and the volume of PCR2 product to reach 5 µL of 10 µM, as well as the final sequencing library concentration and size are given in the table.

PCR2 Library Name	**DNA Conc. (ng/ul)**	**Avg Size (bp)**	**nM DNA**	**Volume for 5µL of 10µM stock**	**Volume of dH20**
PL1_PS_MLepF1_LepR1_Rep1	13.30	641	31.97	1.6	3.4
PL2_PS_MLepF1_LepR1_Rep2	10.20	634	24.79	2.0	3.0
PL3_PS_MLepF1.LepR1_Rep3	11.30	631	27.59	1.8	3.2
PL4_PS_LepF1.MHMR1_Rep1	2.74	503	8.39	6.0	0.0
PL5_PS_LepF1.MHMR1_Rep2	5.12	507	15.56	3.2	1.8
PL6_PS_LepF1.MHMR1_Rep3	2.88	505	8.79	5.7	0
PL7_RS_MLepF1.LepR1	14.40	614	36.14	1.4	3.6
PL8_RS_LepF1.MHMR1	0.19	474	0.62	81.0	0.0
PL9_dilutions	18.40	607	46.71	1.1	3.9
CFD_COI_2023_FinalPool	**3.74**	**610**	**9.45**		

Supplemental Table 5. COI barcode records of Glacier National Park butterfly species. Butterflies are well represented in the COI or Barcode of Life Database (BOLD) including butterfly species expected to be found in Glacier National Park. Barcode records associated with 835 butterfly species of North America were accessed through the project name DS-USCANLEP in the BOLD database and filtered for a list of 126 butterfly species that could be expected in Glacier National Park. The number of records for each species was tallied and are ordered by decreasing number of records. None of the 126 species have fewer than five COI barcode records, only six have fewer than 10 and 25 have fewer 20 records.

#	Species Name	Number of Records	#	Species Name	Number of Records
1	*Boloria chariclea*	279	64	*Papilio zelicaon*	35
2	*Glaucopsyche lygdamus*	242	65	*Callophrys eryphon*	34
3	*Icaricia lupini*	211	66	*Celastrina echo*	34
4	*Burnsius communis*	210	67	*Polites peckius*	34
5	*Agriades glandon*	175	68	*Boloria selene*	33
6	*Thymelicus lineola*	155	69	*Colias alexandra*	33
7	*Hesperia colorado*	147	70	*Parnassius smintheus*	33
8	*Icaricia saepiolus*	138	71	*Vanessa cardui*	33
9	*Plebejus idas*	122	72	*Phyciodes mylitta*	31
10	*Cupido amyntula*	120	73	*Polites draco*	31
11	*Cercyonis pegala*	109	74	*Tharsalea heteronea*	31
12	*Cercyonis oetus*	103	75	*Celastrina ladon*	30
13	*Speyeria mormonia*	95	76	*Erynnis pacuvius*	30
14	*Limenitis arthemis*	94	77	*Lycaena phlaeas*	30
15	*Pieris rapae*	93	78	*Phyciodes pallida*	30
16	*Euphydryas anicia*	89	79	*Chlosyne palla*	29
17	*Pieris marginalis*	89	80	*Colias nastes*	29
18	*Phyciodes pulchella*	84	81	*Speyeria coronis*	29
19	*Euphydryas editha*	80	82	*Papilio rutulus*	28
20	*Ochlodes sylvanoides*	79	83	*Satyrium sylvinus*	28
21	*Speyeria hesperis*	79	84	*Amblyscirtes vialis*	27
22	*Hesperia assiniboia*	78	85	*Anthocharis sara*	27
23	*Speyeria cybele*	76	86	*Callophrys polios*	27
24	*Polygonia faunus*	74	87	*Colias eurytheme*	27
25	*Coenonympha tullia*	73	88	*Euphydryas colon*	27
26	*Callophrys gryneus*	72	89	*Speyeria callippe*	27
27	*Colias interior*	68	90	*Glaucopsyche piasus*	26
28	*Icaricia acmon*	67	91	*Colias eriphyle*	25
29	*Colias christina*	65	92	*Callophrys spinetorum*	24
30	*Erynnis persius*	63	93	*Euptoieta claudia*	24
31	*Thorybes pylades*	63	94	*Polygonia progne*	24
32	*Nymphalis antiopa*	59	95	*Satyrium saepium*	24
33	*Tharsalea dorcas*	59	96	*Oeneis uhleri*	23

Supplemental Table 5 Continued

34	*Boloria freija*	57	97	*Callophrys sheridanii*	22
35	*Colias gigantea*	56	98	*Limenitis archippus*	22
36	*Icaricia icarioides*	56	99	*Phyciodes batesii*	22
37	*Pontia occidentalis*	56	100	*Pterourus eurymedon*	22
38	*Papilio canadensis*	56	101	*Parnassius clodius*	21
39	*Speyeria zerene*	56	102	*Aglais milberti*	19
40	*Oeneis chryxus*	55	103	*Boloria epithore*	18
41	*Polites themistocles*	53	104	*Nymphalis californica*	18
42	*Tharsalea mariposa*	52	105	*Pontia sisymbrii*	17
43	*Tharsalea helloides*	51	106	*Speyeria edwardsii*	17
44	*Vanessa atalanta*	50	107	*Callophrys mossii*	16
45	*Erynnis icelus*	48	108	*Erebia discoidalis*	16
46	*Speyeria atlantis*	47	109	*Satyrium titus*	16
47	*Polygonia satyrus*	46	110	*Colias meadii*	15
48	*Pyrgus centaureae*	45	111	*Tharsalea hyllus*	14
49	*Callophrys augustinus*	44	112	*Boloria astarte*	13
50	*Polygonia gracilis*	42	113	*Pyrgus ruralis*	13
51	*Erebia epipsodea*	41	114	*Chlosyne damoetas*	11
52	*Euphilotes ancilla*	41	115	*Danaus plexippus*	11
53	*Plebejus melissa*	41	116	*Polygonia oreas*	11
54	*Oarisma garita*	40	117	*Speyeria egleis*	11
55	*Polites mystic*	40	118	*Tharsalea editha*	11
56	*Lycaena cupreus*	39	119	*Tharsalea nivalis*	11
57	*Phyciodes tharos*	39	120	*Vanessa annabella*	11
58	*Carterocephalus skada*	37	121	*Boloria alberta*	6
59	*Euchloe ausonides*	37	122	*Limenitis weidemeyerii*	6
60	*Boloria frigga*	36	123	*Colias scudderii*	5
61	*Hesperia juba*	36	124	*Erynnis afranius*	5
62	*Speyeria aphrodite*	36	125	*Euphydryas gillettii*	5
63	*Limenitis lorquini*	35	126	*Papilio multicaudata*	5
				total	**6345**

Supplemental Table 6. Amplicon sequence variants (ASVs) that were removed from analysis as contaminants. ASVs were identified as likely contaminants using the R package *decontam* with the frequency algorthim and a threshold set to 0.5. The ASVs that were identified as contaminants and removed from analysis with their associated taxonomy are in the table below. ASVs were identified using the VSEARCH global alignment tool in Qiime2 with a 95% similarity threshold and the bold_full_USCAN database.

ASV_ID	Primer Set	Taxonomy
a587d8619fc3d01df9109567d46db847	1	tax=k__Animalia;p__Arthropoda;c__Insecta
b05cf0de9037ae07842fd2803e1d1523	1	tax=k__Animalia;p__Arthropoda;c__Insecta
bc535dbc6c589f1c0438c47df03dc358	1	tax=k__Animalia;p__Arthropoda;c__Insecta
cf9a8cec5ebc228b8391cb4b19e35275	1	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Diptera
93335ae5260a562b1a330ee40193be25	1	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Hemiptera
301d3cd71ddebce956b62a3a1fe5b663	1	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Hemiptera;f__Miridae;sf__Mirinae;g__Lygus
efa21d5397f726ce142eab56388c3066	1	tax=k__Animalia;p__Arthropoda;c__Insecta
0932eb88c7561956482b7b2a545886ae	1	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Coleoptera
e73f5c7ecb0b3b4b5b6c6ca73b621210	1	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Coleoptera
609d9707c950f9f3ee86cb4b1847921a	1	tax=k__Animalia;p__Arthropoda;c__Insecta
6bf69c20d15e8b5faf4a63966de50f64	1	Unassigned
1d479d84179bcda6cd4f4a0c303091eb	1	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Hemiptera
278c5740fad03d92a8dbb68386bc5344	1	Unassigned

Supplemental Table 6 Continued

6c527a13ebbcf9bada3dc5b4ee883661	1	Unassigned
6afa0b4cce12899a476239379dfcba15	1	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Hemiptera
21451988b4f2d0b16a3c1526d3a5e57b	1	tax=k__Animalia;p__Arthropoda;c__Insecta
ae01692ea2682d5e21c3933bf0ddd281	1	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Hemiptera
44d0b914468d4efd013bcb7ec3e52b5c	1	Unassigned
fcdb799c95178afd3c49784b5b58bd6e	1	tax=k__Animalia;p__Arthropoda;c__Insecta
268a81f697fb2719d3f745d2810de9e1	1	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Hemiptera;f__Miridae;sf__Mirinae;g__Lygus
deb0a465b982109e0b7ccb4f66ae1142	1	Unassigned
1b49a6319e9613d3181eb491a1e030e8	1	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Hemiptera
ab71dc66969a2939f3cbf2ffd4d1813f	1	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Lepidoptera
4c7d443e96816b48371569135a031d68	2	tax=k__Animalia;p__Arthropoda;c__Insecta
4cf0a71d3cefcf6240a9c78fb825fe61	2	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Lepidoptera
750364cf673a9c553746b43d0245ef41	2	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Diptera;f__Tephritidae;sf__Tephritinae;g__Campiglossa;s__None
7a229731611825b9ccd205a6f506e402	2	tax=k__Animalia;p__Arthropoda;c__Insecta
cdc99d0f19eb99a569c95c02eea2eabc	2	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Diptera;f__Tephritidae;sf__Tephritinae;g__Tephritis

Supplemental Table 6 Continued

f7fef312f0e230c8c7153dbbd7fc32af	2	tax=k__Animalia;p__Arthropoda;c__Insecta
e81911be7b8d79505c9e6ced8f7d135b	2	Unassigned
47621f68005c70a43d270a5f97d0aa81	2	tax=k__Animalia;p__Arthropoda;c__Insecta
605f12d4af30b3a43c1883d6ede23c1b	2	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Diptera;f__Tephritidae;sf__Tephritinae;g__Campiglossa

Supplemental Table 7. ASVs that were removed from analysis by an additional sequence length filter. Sequences that were less than 100 bp of the expected size for each amplicon, 310 bp less for MLepF1/LepR1 (Primer Set 1) and 210 bp or less for LepF1/MHMR1 (Primer Set 2), were removed. The ASV identifiers, length in base pairs (bp), abundance of the sequence in the raw data ('Seqs') are given in the table along with the associated taxonomy of each ASV identified with the VSEARCH global alignment tool in QIIME 2 with a 95% similarity threshold and the bold_full_USCAN database. A total of 176 ASVs were removed, 122 originating from MLepF1/LepR1 54 and from LepF1/MHMR1.

	ASV_ID	Primer Set	Length (bp)	Sequences	Taxonomy
1	00518d27a3511cac9502693260801983	1	156	741	Unassigned
2	0326d8a32c4886ba59da8e61217aa2d2	1	157	9976	Unassigned
3	033413ad684944f0d49943fc410ea075	1	152	137	Unassigned
4	0a5dd5e3c366a99a77c919e61986d2f3	1	142	3233	Unassigned
5	0e43f5c342d49e0ede0db7affc166f9f	1	111	20	Unassigned
6	101a223f95e589feef4b2f055a29edce	1	299	1170	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Lepidoptera;f__Erebidae;sf__Arctiinae;g__Crambidia;s__Crambidia cephalica
7	10312964e94783e688090be44e9aa2b0	1	281	23	Unassigned
8	1081c92e7c3e04de9e8f6487ae39a7c8	1	299	57	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Lepidoptera;f__Erebidae;sf__Arctiinae;g__Crambidia;s__Crambidia cephalica
9	109da7085439cc8a376b03a4e3cd0bf8	1	131	10935	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Lepidoptera;Unassigned
10	13d50ffdbc24026b15b8a0d4d7d21516	1	182	68783	Unassigned
11	16207a666ebd6f26bc266fc5dc712789	1	89	1676	Unassigned
12	16913b3397e1665734a7202949d10133	1	78	132	Unassigned
13	16bcacf9c442daf02a534549f9891367	1	182	147170	Unassigned
14	17a98a569bccdd34eef366a82424d876	1	299	3	Unassigned
15	191d145178879be75d937b89aa363b74	1	170	3782	Unassigned
16	257a447b530b0ce79ee6cdb2c8e5f063	1	237	126	Unassigned
17	26ed6856a8d4e69a87ee18cd534e1f9b	1	288	82	Unassigned
18	2729abb89c34e40f3c1c3a717fe88545	1	126	98	Unassigned

Supplemental Table 7 Continued

19	2782b400d565ef875bf27334df736c80	1	299	165	Unassigned
20	2a8201b395c7eb2853cba0908e9fd943	1	183	4	Unassigned
21	2bfb4b29143f74606009df42efc7fbbc	1	182	392	Unassigned
22	2e8f1539256f95732320c40f374fda52	1	182	563	Unassigned
23	2f4d110c467351ced34a2f3cd36fa5b5	1	182	173	Unassigned
24	30f9193f844ab638071a3fd42bda9c7f	1	94	994	Unassigned
25	31e5cb3fff8799847ae9b37bf0b96088	1	216	27	Unassigned
26	35d9fe4329473f3063013e1dc0eb8c4c	1	237	16	Unassigned
27	3663737132bedb43a7b6ebb86cf6a648	1	250	366	Unassigned
28	373457428af364e05550597895db45ab	1	134	8778	Unassigned
29	3a58651d73b70056b00e8ec687966a09	1	158	6	Unassigned
30	3af3b178c1d44919621b67f833da5ee3	1	79	173065	Unassigned
31	43fb8788291c69b823781f1b4e6372ad	1	95	13983	Unassigned
32	46a1dd1354c501091e9e0c8c9238ed39	1	281	214	Unassigned
33	4845a353aca597c063bcd26dc4c19023	1	134	221	Unassigned
34	49e618d2c7674093d762801e4894de5f	1	142	3	Unassigned
35	4cd1eccad07a60e1ed982a40e41de2b7	1	182	25	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Hymenoptera;Unassigned
36	4e68b97c55a11b1b4c18389cb0b9ca13	1	299	2	Unassigned
37	50aa53d962e1fd458f88747f576ee9f1	1	39	630	Unassigned
38	52d7d731819284717927727a29e6915a	1	190	18	Unassigned
39	5409480213faee0c3bbba7455662dc22	1	299	1756	Unassigned
40	54460865580d95f51ab6e1df76b5f73c	1	250	96	Unassigned
41	5a4f6cba4250836f3dca94d373e5a688	1	181	77	Unassigned
42	5bc69ea8587da9c5d269e97dc48a111c	1	275	34231	Unassigned

Supplemental Table 7 Continued

43	5f67e5e83f04507e15a2a919901ebd18	1	299	141	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Lepidoptera;f__Erebidae;sf__Arctiinae;g__Crambidia;s__Crambidia cephalica
44	60b9cdb77b5fb5411a9b700493b23d81	1	301	4	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Hymenoptera;f__Apidae;sf__Apinae;g__Bombus;Unassigned
45	640825dc875b6f00b9ee6bce06f0bb77	1	182	388	Unassigned
46	6a13d513e355820fa14d169d8e526a51	1	277	19	Unassigned
47	6a7653e32836f779bd0d62134dbec3f8	1	220	382	Unassigned
48	6bb80571bd1c6cc89d3a80540597827f	1	182	886	Unassigned
49	6ca725a46a99be431d2632380c853d8b	1	153	4	Unassigned
50	6d1234471267e5ed12fc66bcca4e15a1	1	123	261	Unassigned
51	73aa3355b6218fdbcd6e76b2d5c90e8a	1	134	1459	Unassigned
52	75ba970fd0c586bf70204b4f9b61867d	1	200	2	Unassigned
53	76cb8d9e2d77e76d3d648a60cde1f4ca	1	41	122	Unassigned
54	77077d77493419a917842744d8dda162	1	200	7	Unassigned
55	78e9d0055f111d09b245ed75f8e30b42	1	179	7	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Lepidoptera;Unassigned
56	7a1e96d97cf160795bbf2b43dbe73fd2	1	299	7	Unassigned
57	7a685c830c9ed835f91f61db4e3ee246	1	179	4	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Lepidoptera;Unassigned
58	7bf9ca1329a51734c03abf52d25b78c1	1	275	31	Unassigned
59	7d57f8a66ff115f2d954bcc332d57bca	1	179	4526	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Lepidoptera;Unassigned
60	7df8f0aeb6c6e49c4396f09d0433edb8	1	152	643	Unassigned
61	7fc4dbd8082d19f3e095be03a3a1a709	1	158	557	Unassigned
62	801a28c765755d4a1dfad69cb47cb4ca	1	159	90	Unassigned
63	83798e5538544a6699fdc423ce66b28a	1	191	24	Unassigned

Supplemental Table 7 Continued

64	8451f821f4a5f3f79e34f2675468da5f	1	182	13	Unassigned
65	858e311bf833472bb21cda90c275b703	1	79	106	Unassigned
66	8729bad4ae14329d226863bca1c76276	1	174	621	Unassigned
67	899db99482d7126a5a0b852559345163	1	286	153	Unassigned
68	8a146f6c135e11346481b62de0625b51	1	182	873	Unassigned
69	8b52d54da0f0d3e8ad5001bb6a332193	1	226	25	Unassigned
70	8ba7d3702523c5f5c7541a6c5c35ad1b	1	182	756	Unassigned
71	8c526002d6038557ae9be4884c9ebda4	1	153	59	Unassigned
72	8e7b8ef0cff3e2b2937e05d3c1a9530c	1	153	7	Unassigned
73	8ec6af9951c22cc751ca0a9bfd9fb59a	1	79	51	Unassigned
74	8f65dd03a10ecccb391bb039b44eed9d	1	203	1440	Unassigned
75	91b44452e91efb20e79fbb350522b537	1	299	526	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Lepidoptera;f__Erebidae;sf__Arctiinae;g__Crambidia;s__Crambidia n. sp. 1
76	93501c0da78501f3f10d947710e24388	1	176	24	Unassigned
77	939ed47900e7dfa3c6ded4937d900454	1	250	359	Unassigned
78	95fcea0e7a19cf9cbb868f197c557fd8	1	250	168	Unassigned
79	995e99d267497d3ff237a21ed351ef5a	1	134	40	Unassigned
80	9ee245e56bc4e73d9c2be107b1cceaae	1	179	811	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Lepidoptera;Unassigned
81	9f8273b1662085637159ec85743ae1e0	1	30	3302	Unassigned
82	a4ede907c31a363c4ead591957757f6c	1	179	1359	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Lepidoptera;f__Coleophoridae;sf__Coleophorinae;g__Coleophora;Unassigned
83	ab4e67dbded46536cc48994aca4daac5	1	250	400	Unassigned
84	ac57a918ed3a1a980cd24ff071529fc3	1	179	33980	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Lepidoptera;f__Coleophoridae;sf__Coleophorinae;g__Coleophora;Unassigned
85	af79c01f08f907545fe90f8b607187ec	1	182	339	Unassigned

Supplemental Table 7 Continued

86	b30334e2f25c2f2c5c3effc94919e42a	1	277	511	Unassigned
87	b3696712bae9c32bf3af85c4aabfa291	1	179	209	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Lepidoptera;f__Coleophoridae;sf__Coleophorinae;g__Coleophora;Unassigned
88	b541b8cad0bdb4a82fe47b5207f9a5bf	1	307	237	Unassigned
89	b6a4846d12397cdba36720e9fa27837c	1	182	968	Unassigned
90	b7277270f1365b69035bbd6cd0a1b3a8	1	241	7	Unassigned
91	baed8763488801c4ceadbe656d3dd292	1	250	95	Unassigned
92	bbf8755f66a55810a9a21ec427591182	1	250	237	Unassigned
93	bd69d8a07054d60c11f1596a7afc4390	1	190	241	Unassigned
94	bec84551665c82574515e68a8ecfe5b2	1	233	3	Unassigned
95	c12617ccd4ce4cc1001d2651c919ec33	1	36	9307	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Lepidoptera;Unassigned
96	c6b0b87d2598d3b5e0c2dca651cd6b3c	1	127	404	Unassigned
97	c6e4cc0c6eaa703065a36ea062fd5aa6	1	250	37	Unassigned
98	c9a95d8abce0df22716e9b1c816ddc7b	1	299	32555	Unassigned
99	cbf7134bcbfc1788a19dbf39592aee1b	1	134	15	Unassigned
100	cc35cd32f086abece4f54e92e1645fe9	1	123	3	Unassigned
101	ce88a3305b93e34faf8760901e1634d1	1	178	17	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Lepidoptera;f__Erebidae;sf__Arctiinae;g__Crambidia;Unassigned
102	cf19239d1779b7cc7f4d7af9c2736e70	1	179	512	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Lepidoptera;f__Coleophoridae;sf__Coleophorinae;g__Coleophora;Unassigned
103	d04ec9d1703b2f35da52d75ab9ee91a8	1	182	46	Unassigned
104	d125b1e126a864815f986aaf2d58d8de	1	179	28359	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Lepidoptera;Unassigned
105	d35aa69ae17c31398ab9237261101142	1	135	20	Unassigned

Supplemental Table 7 Continued

106	d56165a9cd6103b8cf981861868f46d8	1	131	13	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Lepidoptera;f__Coleophoridae;sf__Coleophorinae;g__Coleophora;s__Coleophora JFL150
107	da69de3f466d326855063af184bd9e3f	1	250	36686	Unassigned
108	dd1917a5a11e7e5e9acbecdec4ef62ca	1	157	5416	Unassigned
109	e14dac0ef324f4a15d6c99c44114fc04	1	130	66	tax=k__Animalia;Unassigned
110	e544fa2a7619956178cf068334680f65	1	182	978	Unassigned
111	e5e88e819d209f667faa4679f7ed3811	1	194	268	Unassigned
112	e8c5c5831abf049f972ffe72c9b86e2d	1	182	27	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Lepidoptera;f__Erebidae;sf__Arctiinae;g__Crambidia;Unassigned
113	e90245a7f120ea3a43c3c880ba9f5f0c	1	167	398	Unassigned
114	e93d74aae051d5ff24721e40618c8b3b	1	190	1064	Unassigned
115	f2103e6b52f78c71d76e8cb2eed16909	1	179	19687	tax=k__Animalia;Unassigned
116	f2a483a0ec858c94264652f03ee81620	1	142	33	Unassigned
117	f4be567b0229446ab0f2ee83bf36be6b	1	174	18656	tax=k__Animalia;p__Arthropoda;c__Insecta;o__Lepidoptera;f__Erebidae;sf__Arctiinae;g__Crambidia;Unassigned
118	f75abc176df04b6576810de4d6efa57e	1	194	483	Unassigned
119	f7b0aba8dedb667447518248179ed075	1	133	15	Unassigned
120	fc5929926b619fa9bc1f5ef11560773d	1	182	1	Unassigned
121	fd5a619a6ee81f4f3576510b9f62471b	1	30	1472	Unassigned
122	fda886eed725f1f6130755f00f2216bd	1	153	51	Unassigned
123	002d6553447adf318d37a19ef7311554	2	174	302	Unassigned
124	06f47b8b109eedccb927cc4d16ff8989	2	206	1	Unassigned
125	10af1b7269891d3b02330bb646a29dce	2	202	2	Unassigned
126	1367d1e9fd0b8faa1170c07eef70c0a5	2	188	42	Unassigned

Supplemental Table 7 Continued

127	16e5d104fc9a37871b0325c450ec65bd	2	185	533	Unassigned
128	18e2316bfa789e35f6fd93b89596bccb	2	170	71	Unassigned
129	19118f6f920a6792e424f1edb6d5d580	2	50	2359	Unassigned
130	19e55260308e96d2ed0a4f92790eb29a	2	139	79	Unassigned
131	1ff4ca6c5130cf1c5e09a98af25aecad	2	101	146	Unassigned
132	244f710ede1d622aaf3b749c35628a3b	2	40	1609	Unassigned
133	270ba46d535a46c663af71cdba1b193a	2	208	8	Unassigned
134	31b05fa035725c08cb8ab80cfe695c97	2	100	430	Unassigned
135	34c41ed0acd73b7559b9d9bc0d1d1ac3	2	138	4	Unassigned
136	366d421be77d0b370a0272f10a4271a6	2	182	11	Unassigned
137	3e08a527d294d749394b0b0b9bd16ad0	2	206	98	Unassigned
138	3f0857ebf4627e46bbb5799bbe15818b	2	208	546	Unassigned
139	4c55e8c53212a88a9bb667b26bb95dab	2	32	22	Unassigned
140	5097e656158effd134dde16d24986903	2	206	321	Unassigned
141	598475d72fe4ff538f8d06432dac21b0	2	43	169	Unassigned
142	59e0a4ddd4864db393801b674e555fbe	2	86	260	Unassigned
143	5a4b302d7959185a57561d71320a9cae	2	41	209	Unassigned
144	5f09e0f0455d331c400f72adf86bd839	2	126	941	Unassigned
145	693a2d0d7fdd13ce20195a06b2f06de0	2	50	43	Unassigned
146	6db9b42d0d84d6ae6bc034b71d22afb8	2	173	699	Unassigned
147	75f33aceaed2fb24a3576550b853edca	2	22	39	Unassigned
148	76c400b961b8d45cc8576b1abf08ee3e	2	52	142	Unassigned
149	783fa42075cd0dfa2856cb4dc1637a91	2	206	113217	Unassigned
150	8723788fcda35a16ef5097b4aeb3f508	2	206	1658	Unassigned
151	8c65a719eb2d51aa11d715beb1bf0d1f	2	206	274	Unassigned
152	8d930917ac6c705ff7cc130737fb4280	2	113	192	Unassigned
153	8fc814483811a3543d2c88ac0b35c1f6	2	22	96	Unassigned
154	9ab347fab5e68e1ddfca852c88b72c75	2	206	122	Unassigned

Supplemental Table 7 Continued

155	a4d592d668c4cab6bed097629a3f02e3	2	36	12475	Unassigned
156	a4d9a6a64386f2368e5c776699d34221	2	51	1523	Unassigned
157	a520361ecdbe34a61e6aab8672aa0b04	2	123	23	Unassigned
158	a5487b4161e0ac959ee86de59754ca7d	2	32	21	Unassigned
159	ad8ec1dd5074dff02fbc794b37a4d6d8	2	32	273	Unassigned
160	b4033ad6efdd124711b66ae422d368ad	2	22	12	tax=k__Animalia;p__Arthropoda:Unassigned
161	b8328437fab9aba690afa8939492fc3f	2	206	155	Unassigned
162	bd5d03297276bd575dc8428e3cc084a7	2	184	15	Unassigned
163	c6549a6fcb359ff1d60e9bada3f17d9f	2	206	62	Unassigned
164	c778a67e2f19c8e799a0f6d33c8234d6	2	53	110	Unassigned
165	c9fe0a2a64d99f89630d91d07b1dc087	2	43	1030	Unassigned
166	ce64dfb06d578d1c2ba08b3514aa307b	2	206	67	Unassigned
167	d147b26c0cae742e123016049b1df03a	2	206	60	Unassigned
168	d17439e6f81e04fd09d3d36dec6c4dcc	2	28	7523	Unassigned
169	d6ecb9fb01f8c2b3419a11a4050c5e93	2	73	269	Unassigned
170	dbce8076132a4d8655b9e03c4e4c752a	2	100	655	Unassigned
171	dd1346238c2a16118a4788321192802d	2	22	779	Unassigned
172	e886e9edbd2f3ebb4311cfdead56aae7	2	70	772	Unassigned
173	ea532d8dd1f4445f1521b37e3b00ec08	2	191	31	Unassigned
174	efd318edb5f265dee14b155694ecb7a1	2	206	1901	Unassigned
175	eff8f9926c4e88b5ebe5c2cccf2a732f	2	108	4637	Unassigned
176	f64463b8f970d6c8493be1250e1eca92	2	78	290	Unassigned

Supplemental Table 8. Raw sequences from Illumina NovaSeq and index hopping estimates. The final sequencing library that was sent for high throughput sequencing (Illumina NovaSeq) of two *cytochrome c oxidase I* (COI) gene regions contained nine sub-libraries which were associated with pooled PCR plates (PL) of tagged PCR reactions from wildflower-associated eDNA samples and all positive and negative controls. The raw number of reads for each forward (R1) and reverse (R2) read are listed for each sub-library. During demultiplexing of raw sequence reads by combinatorial indexes for samples within each library, the total reads that had unexpected tag combinations were divided by the total reads of expected tag combinations to derive an index hopping rate.

Raw Sequences					Demultiplexing		
Library / Plate (PL)	Primer Set	Description	Read #	Number of Reads	Total Reads Unexpected Tags	Total Reads Expected Tags	Index hopping rate
PL1	1	high priority samples rep 1	R1 R2	56,801,858 56,801,858	208,946	56,068,155	0.371
PL2	1	high priority samples rep 2	R1 R2	55,627,087 55,627,087	115,345	55,067,297	0.209
PL3	1	high priority samples rep 3	R1 R2	54,011,706 54,011,706	129,803	53,397,555	0.242
PL4	2	high priority samples rep 1	R1 R2	36,547,719 36,547,719	10,868	35,944,813	0.03
PL5	2	high priority samples rep 2	R1 R2	49,951,581 49,951,581	13,827	49,329,770	0.028
PL6	2	high priority samples rep 3	R1 R2	44,009,066 44,009,066	11,613	43,546,211	0.027
PL7	1	one rep remaining samples	R1 R2	44,673,198 44,673,198	364,445	43,960,730	0.822
PL8	1	one rep remaining samples	R1 R2	2,353,056 2,353,056	175	2,105,280	0.008

Supplemental Table 8 Continued

PL9	1 & 2	positive control dilutions	R1 R2	59,420,942 59,420,942	5,604,188	53,160,075	9.537
			total raw	806,792,426			

Supplemental Table 9. Sequence and quality control summary. Several quality control steps were applied to the sequences output from the TADA bioinformatic pipeline. An additional length filter was applied to filter out sequences that were 100 base-pairs (bp) less than the expected size for each primer pair, the R package decontam was used to remove likely contaminants and amplicon sequence variants (ASVs) that had fewer than 5 sequences associated with them across all samples were removed, and finally, assigned sequence variants (ASVs) that identified any of the five species used in the positive controls (*Pieris rapae, Bombus rufocinctus, Apis mellifera, Phymata americana, Coccinella transversoguttata*) were removed. The number of sequences associated with each sample output from the TADA pipeline and the number of sequences removed at each quality control step are listed in the table.

Priority Samples							
Plate	**Primer Set**	**SampleName2**	**Sequences Post TADA Pipeline**	**Seqs lost after length filter**	**Seqs lost after decontam**	**Seqs lost after ASV < 5**	**Seqs lost after pos ctrl species removed**
PL1	1	SM_CTRL_0_1	276076	0	34	8	0
PL2	1	SM_CTRL_0_1	481789	0	0	0	134
PL3	1	SM_CTRL_0_1	367346	0	0	9	62
PL4	2	SM_CTRL_0_1	229	0	0	0	0
PL5	2	SM_CTRL_0_1	3599	0	0	6	11
PL6	2	SM_CTRL_0_1	351	21	0	0	2
PL1	1	SM_CTRL_0_2	597676	0	0	23	0
PL2	1	SM_CTRL_0_2	387406	16	0	14	41
PL3	1	SM_CTRL_0_2	216764	45	0	14	57
PL4	2	SM_CTRL_0_2	4814	0	0	0	12
PL5	2	SM_CTRL_0_2	17462	0	0	0	34
PL6	2	SM_CTRL_0_2	550	0	0	0	13
PL1	1	SM_CTRL_0_3	4083	0	0	11	0
PL2	1	SM_CTRL_0_3	1289185	0	0	8	25
PL3	1	SM_CTRL_0_3	700426	6	0	11	15
PL4	2	SM_CTRL_0_3	13015	6	0	0	3
PL5	2	SM_CTRL_0_3	5460	0	0	0	3
PL6	2	SM_CTRL_0_3	205198	0	0	1	0
PL1	1	SM_PLML_1_1	62587	0	0	0	5
PL2	1	SM_PLML_1_1	133394	14	0	0	4

Supplemental Table 9 Continued

PL3	1	SM_PLML_1_1	104418	0	0	9	1
PL4	2	SM_PLML_1_1	562	0	0	6	0
PL5	2	SM_PLML_1_1	71	0	0	0	0
PL6	2	SM_PLML_1_1	29549	0	0	0	0
PL1	1	SM_PLML_1_2	75224	1918	0	4	0
PL2	1	SM_PLML_1_2	70872	149	0	3	4
PL3	1	SM_PLML_1_2	81576	313	0	1	3
PL4	2	SM_PLML_1_2	534163	0	0	0	0
PL5	2	SM_PLML_1_2	446102	0	0	0	0
PL6	2	SM_PLML_1_2	224735	0	0	0	0
PL1	1	SM_PLML_1_3	93554	0	0	6	0
PL2	1	SM_PLML_1_3	119981	0	0	2	2
PL3	1	SM_PLML_1_3	135205	0	0	6	262
PL4	2	SM_PLML_1_3	9909	0	0	3	16
PL5	2	SM_PLML_1_3	34973	0	0	0	30
PL6	2	SM_PLML_1_3	6349	0	0	0	6
PL1	1	SM_PLML_1_4	121775	8	0	0	0
PL2	1	SM_PLML_1_4	163982	2	0	8	0
PL3	1	SM_PLML_1_4	83731	0	0	12	219
PL4	2	SM_PLML_1_4	66180	0	0	0	12
PL5	2	SM_PLML_1_4	2233	0	0	4	23
PL6	2	SM_PLML_1_4	2781	142	0	0	17
PL1	1	SM_PLML_1_5	172268	0	0	8	0
PL2	1	SM_PLML_1_5	109787	0	0	0	0
PL3	1	SM_PLML_1_5	63647	0	0	2	0
PL4	2	SM_PLML_1_5	2911	0	0	0	0
PL5	2	SM_PLML_1_5	1531	0	0	0	0
PL6	2	SM_PLML_1_5	442	0	0	0	0
PL1	1	SM_PLML_10_1	224512	39	0	3	0
PL2	1	SM_PLML_10_1	143561	3236	0	7	0
PL3	1	SM_PLML_10_1	151227	0	0	13	1043

Supplemental Table 9 Continued

PL4	2	SM_PLML_10_1	1163	0	0	0	7
PL5	2	SM_PLML_10_1	4610	0	0	2	53
PL6	2	SM_PLML_10_1	6810	296	0	0	43
PL1	1	SM_PLML_10_2	825503	643	0	13	0
PL2	1	SM_PLML_10_2	919857	0	0	15	0
PL3	1	SM_PLML_10_2	528575	0	0	16	0
PL4	2	SM_PLML_10_2	9588	0	0	0	0
PL5	2	SM_PLML_10_2	24127	0	0	0	0
PL6	2	SM_PLML_10_2	4353	0	0	0	0
PL1	1	SM_PLML_10_3	3296681	17	0	31	5
PL2	1	SM_PLML_10_3	3047937	0	1043	12	2
PL3	1	SM_PLML_10_3	2552332	378	0	32	8
PL4	2	SM_PLML_10_3	2284	0	0	0	0
PL5	2	SM_PLML_10_3	12032	0	0	0	0
PL6	2	SM_PLML_10_3	433	22	0	0	0
PL1	1	SM_PLML_10_4	1152883	18	0	5	8
PL2	1	SM_PLML_10_4	515274	0	0	2	17
PL3	1	SM_PLML_10_4	436882	0	0	5	3
PL4	2	SM_PLML_10_4	73148	0	0	0	0
PL5	2	SM_PLML_10_4	18371	0	0	0	0
PL6	2	SM_PLML_10_4	25276	0	0	0	0
PL1	1	SM_PLML_10_5	143990	3796	0	15	12
PL2	1	SM_PLML_10_5	195969	477	0	0	3
PL3	1	SM_PLML_10_5	143791	8	0	12	7
PL4	2	SM_PLML_10_5	177	0	0	0	0
PL5	2	SM_PLML_10_5	374	0	0	3	0
PL6	2	SM_PLML_10_5	561	0	0	0	0
PL1	1	SM_PLML_5_1	359528	16412	5	9	11
PL2	1	SM_PLML_5_1	180978	10025	4	12	6
PL3	1	SM_PLML_5_1	216354	75	1	6	3
PL4	2	SM_PLML_5_1	1672909	0	0	3	0

Supplemental Table 9 Continued

PL5	2	SM_PLML_5_1	2582230	0	0	10	0
PL6	2	SM_PLML_5_1	1746149	0	0	8	0
PL1	1	SM_PLML_5_2	1866604	0	0	18	23
PL2	1	SM_PLML_5_2	2620385	0	0	18	40
PL3	1	SM_PLML_5_2	2085632	2	0	5	9
PL4	2	SM_PLML_5_2	525500	0	0	11	0
PL5	2	SM_PLML_5_2	565565	0	0	16	0
PL6	2	SM_PLML_5_2	668619	0	0	12	0
PL1	1	SM_PLML_5_3	2322085	5	0	3	13
PL2	1	SM_PLML_5_3	2746337	8	0	14	7
PL3	1	SM_PLML_5_3	2306879	14	0	8	13
PL4	2	SM_PLML_5_3	344900	0	0	1	0
PL5	2	SM_PLML_5_3	1086657	0	0	1	0
PL6	2	SM_PLML_5_3	481038	0	0	3	5
PL1	1	SM_PLML_5_4	877329	0	0	7	0
PL2	1	SM_PLML_5_4	920048	0	0	4	1
PL3	1	SM_PLML_5_4	652343	192	0	6	0
PL4	2	SM_PLML_5_4	280	0	0	0	2
PL5	2	SM_PLML_5_4	524	0	0	0	0
PL6	2	SM_PLML_5_4	1133	0	0	0	0
PL1	1	SM_PLML_5_5	22693	0	0	18	0
PL2	1	SM_PLML_5_5	9663	6	0	7	17
PL3	1	SM_PLML_5_5	33627	0	0	6	991
PL4	2	SM_PLML_5_5	1307	772	0	2	25
PL5	2	SM_PLML_5_5	2708	0	0	0	70
PL6	2	SM_PLML_5_5	4726	0	0	0	10
PL1	1	SM_SPMO_1_1	74032	71	0	3	0
PL2	1	SM_SPMO_1_1	79887	71	0	5	13057
PL3	1	SM_SPMO_1_1	75378	19526	0	3	21
PL4	2	SM_SPMO_1_1	134542	0	0	0	0
PL5	2	SM_SPMO_1_1	263844	0	0	0	6

Supplemental Table 9 Continued

PL6	2	SM_SPMO_1_1	62533	0	0	0	4
PL1	1	SM_SPMO_1_2	59360	0	0	11	0
PL2	1	SM_SPMO_1_2	113503	2	0	6	72622
PL3	1	SM_SPMO_1_2	124713	8	0	19	56535
PL4	2	SM_SPMO_1_2	56237	0	0	0	39803
PL5	2	SM_SPMO_1_2	126238	0	0	0	70807
PL6	2	SM_SPMO_1_2	80051	3	0	0	46196
PL1	1	SM_SPMO_1_3	3895847	17	0	24	0
PL2	1	SM_SPMO_1_3	4289423	5	0	17	25
PL3	1	SM_SPMO_1_3	3220408	0	0	21	2113
PL4	2	SM_SPMO_1_3	9640	0	0	0	19
PL5	2	SM_SPMO_1_3	8640	71	0	0	72
PL6	2	SM_SPMO_1_3	3062	0	0	0	31
PL1	1	SM_SPMO_1_4	1418381	0	2338	6	0
PL2	1	SM_SPMO_1_4	1717201	0	2745	16	30
PL3	1	SM_SPMO_1_4	1136325	0	1688	17	33
PL4	2	SM_SPMO_1_4	13908	0	92	0	7
PL5	2	SM_SPMO_1_4	5586	546	0	2	32
PL6	2	SM_SPMO_1_4	49490	0	0	4	17
PL1	1	SM_SPMO_1_5	427834	15	0	4	1
PL2	1	SM_SPMO_1_5	247861	0	0	9	0
PL3	1	SM_SPMO_1_5	161707	0	0	4	5
PL4	2	SM_SPMO_1_5	7676	0	0	2	13
PL5	2	SM_SPMO_1_5	13352	12	0	9	0
PL6	2	SM_SPMO_1_5	13265	0	0	1	18
PL1	1	SM_SPMO_10_1	64463	1325	0	2	0
PL2	1	SM_SPMO_10_1	50128	1384	0	11	0
PL3	1	SM_SPMO_10_1	62085	162	0	4	1
PL4	2	SM_SPMO_10_1	8665694	0	3879	2	20
PL5	2	SM_SPMO_10_1	9463623	0	3755	16	0
PL6	2	SM_SPMO_10_1	6585332	0	2470	10	22

Supplemental Table 9 Continued

PL1	1	SM_SPMO_10_4	148538	2	330	11	12
PL2	1	SM_SPMO_10_4	189760	90	0	13	5
PL3	1	SM_SPMO_10_4	127589	1660	0	9	6
PL4	2	SM_SPMO_10_4	261	0	0	4	0
PL5	2	SM_SPMO_10_4	1006	0	0	3	0
PL6	2	SM_SPMO_10_4	389	0	0	0	0
PL1	1	SM_SPMO_10_5	405874	0	0	2	6
PL2	1	SM_SPMO_10_5	575264	0	0	10	17
PL3	1	SM_SPMO_10_5	453154	0	14	7	3
PL4	2	SM_SPMO_10_5	157492	0	0	0	0
PL5	2	SM_SPMO_10_5	93528	0	0	0	0
PL6	2	SM_SPMO_10_5	7588	0	0	4	0
PL1	1	SM_SPMO_5_1	114582	11372	0	5	0
PL2	1	SM_SPMO_5_1	98508	9349	0	2	0
PL3	1	SM_SPMO_5_1	221185	27815	0	9	3475
PL4	2	SM_SPMO_5_1	5924475	0	0	9	88354
PL5	2	SM_SPMO_5_1	8513038	168	0	24	7650
PL6	2	SM_SPMO_5_1	9209724	0	7006	2	148062
PL1	1	SM_SPMO_5_2	611488	0	0	10	4
PL2	1	SM_SPMO_5_2	684448	5	0	0	4
PL3	1	SM_SPMO_5_2	408563	0	0	2	5
PL4	2	SM_SPMO_5_2	4471	0	0	0	0
PL5	2	SM_SPMO_5_2	787	0	0	0	0
PL6	2	SM_SPMO_5_2	61053	0	0	5	12
PL1	1	SM_SPMO_5_3	38825	1	71	1	0
PL2	1	SM_SPMO_5_3	59931	5	0	6	1
PL3	1	SM_SPMO_5_3	61959	7	0	5	7
PL4	2	SM_SPMO_5_3	2561	0	0	0	6
PL5	2	SM_SPMO_5_3	3333	0	0	0	0
PL6	2	SM_SPMO_5_3	2566	41	0	0	6
PL1	1	SM_SPMO_5_4	216319	1300	0	7	0

Supplemental Table 9 Continued

PL2	1	SM_SPMO_5_4	110998	780	0	0	6
PL3	1	SM_SPMO_5_4	114146	298	0	7	513
PL4	2	SM_SPMO_5_4	1280224	0	0	13	133
PL5	2	SM_SPMO_5_4	2217	0	0	5	306
PL6	2	SM_SPMO_5_4	1885921	4	0	10	78
PL1	1	SM_SPMO_5_5	208857	17	0	5	0
PL2	1	SM_SPMO_5_5	286673	0	0	1	0
PL3	1	SM_SPMO_5_5	156193	3	203	0	493
PL4	2	SM_SPMO_5_5	4891	0	0	3	97
PL5	2	SM_SPMO_5_5	2544	0	0	3	265
PL6	2	SM_SPMO_5_5	27336	0	0	0	149
PL1	1	PP_Erigeron1	118595	32437	0	2	0
PL2	1	PP_Erigeron1	54921	1243	0	0	0
PL3	1	PP_Erigeron1	43861	518	0	4	0
PL4	2	PP_Erigeron1	208	0	0	0	15
PL5	2	PP_Erigeron1	4556	678	0	0	37
PL6	2	PP_Erigeron1	14655	21	0	0	22
PL1	1	PP_Erigeron3	3700	6	0	0	0
PL2	1	PP_Erigeron3	11188	0	0	1	0
PL3	1	PP_Erigeron3	6617	0	0	0	0
PL4	2	PP_Erigeron3	10395	0	0	0	0
PL5	2	PP_Erigeron3	527	0	0	0	0
PL6	2	PP_Erigeron3	33367	0	0	0	0
PL1	1	PP_Erigeron4	4022660	0	6251	6	0
PL2	1	PP_Erigeron4	3267017	132	3698	1	0
PL3	1	PP_Erigeron4	2940906	0	3355	2	0
PL4	2	PP_Erigeron4	2680191	0	4488	23	0
PL5	2	PP_Erigeron4	4627832	0	7065	63	0
PL6	2	PP_Erigeron4	7624771	48	10609	14	0
PL1	1	PP_Erigeron5	1041339	195	26	3	0
PL2	1	PP_Erigeron5	1198992	2486	1785	8	18

Supplemental Table 9 Continued

PL3	1	PP_Erigeron5	555260	4149	333	13	0
PL4	2	PP_Erigeron5	3659025	0	0	32	0
PL5	2	PP_Erigeron5	3152055	590	0	21	0
PL6	2	PP_Erigeron5	2140376	177	0	4	0
PL1	1	PP_Erigeron6	430768	0	282	8	0
PL2	1	PP_Erigeron6	258484	0	142	14	0
PL3	1	PP_Erigeron6	252561	0	161	11	6
PL4	2	PP_Erigeron6	2804	0	0	3	8
PL5	2	PP_Erigeron6	139599	0	0	3	0
PL6	2	PP_Erigeron6	4291	10	0	5	12
PL7	1	PP_Erigeron7	3910385	0	205	2	0
PL8	2	PP_Erigeron7	583024	0	0	1	0
PL7	1	PP_Erigeron8	620233	0	0	2	1093
PL8	2	PP_Erigeron8	54	0	0	3	2
PL1	1	PP_Erigeron9	454708	0	0	18	0
PL2	1	PP_Erigeron9	684308	0	0	4	0
PL3	1	PP_Erigeron9	358148	2	0	2	0
PL4	2	PP_Erigeron9	21164	6489	0	0	0
PL5	2	PP_Erigeron9	14652	39	0	2	0
PL6	2	PP_Erigeron9	61368	423	0	3	0
PL1	1	PP_Senecio10	82673	23	0	3	0
PL2	1	PP_Senecio10	93297	994	0	2	5
PL3	1	PP_Senecio10	46906	0	0	7	0
PL4	2	PP_Senecio10	6633	0	0	0	0
PL5	2	PP_Senecio10	2105	0	0	5	0
PL6	2	PP_Senecio10	9567	0	0	2	0
PL1	1	PP_Senecio6	511	0	0	0	0
PL2	1	PP_Senecio6	216	0	0	3	0
PL3	1	PP_Senecio6	129	0	0	0	0
PL4	2	PP_Senecio6	637	0	0	0	2
PL5	2	PP_Senecio6	417	0	0	0	0

Supplemental Table 9 Continued

PL6	2	PP_Senecio6	666	8	0	4	0
PL1	1	PP_Senecio7	2087	0	0	2	0
PL2	1	PP_Senecio7	857	0	0	0	0
PL3	1	PP_Senecio7	72	0	0	1	0
PL4	2	PP_Senecio7	665	28	0	3	40
PL5	2	PP_Senecio7	39228	461	0	3	107
PL6	2	PP_Senecio7	117681	116617	0	4	30
PL1	1	PP_Senecio8	34602	0	0	0	0
PL2	1	PP_Senecio8	13449	0	0	4	0
PL3	1	PP_Senecio8	15340	0	0	3	0
PL4	2	PP_Senecio8	3256	533	0	2	0
PL5	2	PP_Senecio8	9223	8	0	1	0
PL6	2	PP_Senecio8	2600	15	0	1	0
PL1	1	PP_Senecio9	101545	15	0	15	0
PL2	1	PP_Senecio9	102548	0	0	5	0
PL3	1	PP_Senecio9	58323	221	0	16	0
PL4	2	PP_Senecio9	35016	0	0	3	0
PL5	2	PP_Senecio9	803	0	0	0	0
PL6	2	PP_Senecio9	63136	0	0	0	0
PL1	1	CM_Arnica1	797969	0	0	4	0
PL2	1	CM_Arnica1	587489	0	0	6	0
PL3	1	CM_Arnica1	740813	0	0	16	3
PL4	2	CM_Arnica1	753735	0	1572	0	0
PL5	2	CM_Arnica1	1357501	0	3657	9	0
PL6	2	CM_Arnica1	589376	0	1134	5	7
PL1	1	CM_Arnica6	137	0	0	2	0
PL2	1	CM_Arnica6	622	530	0	0	0
PL3	1	CM_Arnica6	184	0	0	1	0
PL4	2	CM_Arnica6	50967	2163	12	0	0
PL5	2	CM_Arnica6	173339	3303	560	0	0
PL6	2	CM_Arnica6	192	0	0	0	0

Supplemental Table 9 Continued

PL1	1	CM_Erigeron1	212	0	0	0	0
PL2	1	CM_Erigeron1	278	0	0	0	0
PL3	1	CM_Erigeron1	59	0	0	0	0
PL4	2	CM_Erigeron1	146633	0	0	0	2
PL5	2	CM_Erigeron1	228801	178	0	2	0
PL6	2	CM_Erigeron1	500515	0	0	0	0
PL1	1	CM_Erigeron6	1411	11	0	0	0
PL2	1	CM_Erigeron6	867	0	0	0	0
PL3	1	CM_Erigeron6	1339	0	0	0	0
PL4	2	CM_Erigeron6	17896	0	0	0	21
PL5	2	CM_Erigeron6	65975	0	0	0	47
PL6	2	CM_Erigeron6	1353	102	0	0	17
PL1	1	CM_Erigeron10	31278	8	0	0	0
PL2	1	CM_Erigeron10	6619	6	0	0	0
PL3	1	CM_Erigeron10	9355	125	0	0	0
PL4	2	CM_Erigeron10	93	0	0	0	18
PL5	2	CM_Erigeron10	2736	236	0	0	44
PL6	2	CM_Erigeron10	168	0	0	0	27
PL1	1	SP_Erigeron2	17690	12	0	6	0
PL2	1	SP_Erigeron2	15228	0	0	8	0
PL3	1	SP_Erigeron2	13135	0	0	6	3
PL4	2	SP_Erigeron2	236	0	0	0	1
PL5	2	SP_Erigeron2	21780	0	0	0	1
PL6	2	SP_Erigeron2	459	0	0	0	4
PL1	1	SP_Erigeron4	1758183	106	0	11	0
PL2	1	SP_Erigeron4	2499090	92	0	7	0
PL3	1	SP_Erigeron4	2062336	11	0	14	3
PL4	2	SP_Erigeron4	1144993	0	0	2	0
PL5	2	SP_Erigeron4	2273687	0	0	1	0
PL6	2	SP_Erigeron4	1316492	0	0	1	0
PL7	1	SP_Erigeron5	478193	0	0	4	11

Supplemental Table 9 Continued

PL8	2	SP_Erigeron5	6931	0	0	0	0
PL1	1	SP_Erigeron6	3211	0	0	1	0
PL2	1	SP_Erigeron6	3358	270	0	4	0
PL3	1	SP_Erigeron6	9289	0	0	12	2
PL4	2	SP_Erigeron6	13473	0	0	0	0
PL5	2	SP_Erigeron6	80097	21	0	1	0
PL6	2	SP_Erigeron6	15437	0	0	0	0
PL1	1	SP_Symphyotrichum1	2971	0	0	3	3
PL2	1	SP_Symphyotrichum1	28159	0	0	0	1
PL3	1	SP_Symphyotrichum1	4218	621	0	9	6
PL4	2	SP_Symphyotrichum1	33	0	0	0	0
PL5	2	SP_Symphyotrichum1	167	43	0	0	0
PL6	2	SP_Symphyotrichum1	68	0	0	0	0
PL1	1	SP_Symphyotrichum2	2893	0	0	0	1
PL2	1	SP_Symphyotrichum2	23147	7	0	0	6
PL3	1	SP_Symphyotrichum2	1425	1	0	5	397
PL4	2	SP_Symphyotrichum2	958	0	0	0	12
PL5	2	SP_Symphyotrichum2	1255	0	0	0	31
PL6	2	SP_Symphyotrichum2	3959	2359	0	0	7
PL1	1	SP_Symphyotrichum3	19411	14498	0	4	0
PL2	1	SP_Symphyotrichum3	5706	3749	0	0	0
PL3	1	SP_Symphyotrichum3	11500	9532	0	0	0
PL4	2	SP_Symphyotrichum3	219911	0	0	0	17
PL5	2	SP_Symphyotrichum3	597077	0	0	0	18
PL6	2	SP_Symphyotrichum3	541911	0	0	0	15
PL1	1	SP_Erigeron1	141134	15	401	8	8
PL2	1	SP_Erigeron1	156764	0	1723	3	14
PL3	1	SP_Erigeron1	65208	0	0	4	6
PL4	2	SP_Erigeron1	14129	0	0	4	0
PL5	2	SP_Erigeron1	5069	0	0	0	0
PL6	2	SP_Erigeron1	13072	0	0	1	0

Supplemental Table 9 Continued

PL1	1	SP_Solidago7	78353	7	0	4	0
PL2	1	SP_Solidago7	35404	4	0	0	0
PL3	1	SP_Solidago7	48038	10	0	1	0
PL4	2	SP_Solidago7	1742	0	0	0	0
PL5	2	SP_Solidago7	398	0	0	0	0
PL6	2	SP_Solidago7	15346	0	0	0	0
PL1	1	SP_Solidago8	117584	21	0	2	18
PL2	1	SP_Solidago8	60182	0	0	6	1
PL3	1	SP_Solidago8	80396	0	0	6	0
PL4	2	SP_Solidago8	222	0	0	7	0
PL5	2	SP_Solidago8	640	0	0	0	0
PL6	2	SP_Solidago8	716	0	0	5	0
PL1	1	SU_ArnicaA	266156	0	0	10	0
PL2	1	SU_ArnicaA	105672	0	0	9	15
PL3	1	SU_ArnicaA	190584	0	0	7	1
PL4	2	SU_ArnicaA	248	0	0	0	0
PL5	2	SU_ArnicaA	33131	0	0	0	0
PL6	2	SU_ArnicaA	3525	0	0	0	0
PL1	1	SU_ArnicaB	112	0	0	0	0
PL2	1	SU_ArnicaB	7	0	0	0	0
PL3	1	SU_ArnicaB	33	0	0	8	0
PL4	2	SU_ArnicaB	9023	0	0	0	25
PL5	2	SU_ArnicaB	63517	0	0	4	98
PL6	2	SU_ArnicaB	12296	0	0	0	11
PL1	1	SU_ArnicaC	4464	37	0	0	0
PL2	1	SU_ArnicaC	80	10	0	5	0
PL3	1	SU_ArnicaC	11058	9519	0	3	0
PL4	2	SU_ArnicaC	5871	0	0	0	14
PL5	2	SU_ArnicaC	11016	0	0	2	58
PL6	2	SU_ArnicaC	1105	0	0	4	41
PL1	1	SU_ErigeronA	738	0	0	3	0

Supplemental Table 9 Continued

PL2	1	SU_ErigeronA	313	0	0	0	0
PL3	1	SU_ErigeronA	190	0	0	4	0
PL4	2	SU_ErigeronA	6300	0	0	0	0
PL5	2	SU_ErigeronA	1293	0	0	2	0
PL6	2	SU_ErigeronA	1211	0	0	0	0
PL1	1	SU_ErigeronB	408920	1016	616	10	0
PL2	1	SU_ErigeronB	204906	224	0	6	0
PL3	1	SU_ErigeronB	237005	1451	261	13	10780
PL4	2	SU_ErigeronB	4729	0	0	0	189
PL5	2	SU_ErigeronB	40512	0	0	1	708
PL6	2	SU_ErigeronB	26909	0	0	4	245
PL1	1	SU_ErigeronC	580818	102	887	14	0
PL2	1	SU_ErigeronC	504625	80	1012	13	8
PL3	1	SU_ErigeronC	508487	5	354	6	10504
PL4	2	SU_ErigeronC	584	0	0	0	349
PL5	2	SU_ErigeronC	1206	0	0	6	802
PL6	2	SU_ErigeronC	7856	0	47	6	211
PL1	1	SU_PotentillaA	297070	52	563	6	0
PL2	1	SU_PotentillaA	526720	48	0	5	0
PL3	1	SU_PotentillaA	341572	3	0	1	23032
PL4	2	SU_PotentillaA	227292	0	0	0	443
PL5	2	SU_PotentillaA	266377	0	0	0	1666
PL6	2	SU_PotentillaA	396215	0	0	3	621
PL1	1	SU_PotentillaC	492608	12	0	2	0
PL2	1	SU_PotentillaC	569121	10	1677	5	3
PL3	1	SU_PotentillaC	330530	0	0	14	36215
PL4	2	SU_PotentillaC	351512	0	0	0	133
PL5	2	SU_PotentillaC	973936	0	16	5	235
PL6	2	SU_PotentillaC	824447	0	21	0	87
PL1	1	CM_ArnicaA	3458059	724	18312	30	0
PL2	1	CM_ArnicaA	2642968	6526	11639	8	94

Supplemental Table 9 Continued

PL3	1	CM_ArnicaA	2287053	471	10554	7	0
PL4	2	CM_ArnicaA	962454	0	2177	5	0
PL5	2	CM_ArnicaA	2779802	0	5889	9	0
PL6	2	CM_ArnicaA	1913075	0	2564	9	0
PL1	1	CM_ArnicaB	2898538	38821	14953	18	3
PL2	1	CM_ArnicaB	2464317	9984	11789	2	103731
PL3	1	CM_ArnicaB	2021764	124319	5769	6	0
PL4	2	CM_ArnicaB	1165206	0	3484	4	0
PL5	2	CM_ArnicaB	2466423	65	4946	11	0
PL6	2	CM_ArnicaB	1876593	788	6607	9	0
PL1	1	CM_ArnicaC	492492	27	0	18	0
PL2	1	CM_ArnicaC	184225	0	0	2	61
PL3	1	CM_ArnicaC	351392	3	0	10	1
PL4	2	CM_ArnicaC	396	0	0	0	0
PL5	2	CM_ArnicaC	1221	0	0	3	0
PL6	2	CM_ArnicaC	1031	0	0	0	10
PL1	1	CM_ErigeronA	2429	4	0	0	0
PL2	1	CM_ErigeronA	901	9	0	2	0
PL3	1	CM_ErigeronA	1585	261	0	0	0
PL4	2	CM_ErigeronA	24973	0	0	0	15
PL5	2	CM_ErigeronA	4450	553	0	0	18
PL6	2	CM_ErigeronA	8958	291	0	0	14
PL1	1	CM_ErigeronB	617	15	0	0	0
PL2	1	CM_ErigeronB	977	0	0	0	0
PL3	1	CM_ErigeronB	280	0	0	0	0
PL4	2	CM_ErigeronB	1367070	0	0	6	0
PL5	2	CM_ErigeronB	498304	0	0	0	0
PL6	2	CM_ErigeronB	220827	0	0	0	0
PL1	1	CM_AchilleaA	94785	0	0	1	0
PL2	1	CM_AchilleaA	56616	0	0	8	0
PL3	1	CM_AchilleaA	47638	0	0	7	16

Supplemental Table 9 Continued

PL4	2	CM_AchilleaA	386	0	0	0	0
PL5	2	CM_AchilleaA	279	0	0	0	0
PL6	2	CM_AchilleaA	3702	1030	0	4	0
PL1	1	CM_AchilleaB	337313	0	33	4	0
PL2	1	CM_AchilleaB	256981	0	160	7	0
PL3	1	CM_AchilleaB	261819	0	17	3	7
PL4	2	CM_AchilleaB	185	42	0	0	0
PL5	2	CM_AchilleaB	80	0	0	0	0
PL6	2	CM_AchilleaB	230	169	0	0	0
PL1	1	CM_AchilleaC	780	0	0	0	0
PL2	1	CM_AchilleaC	932	0	0	0	0
PL3	1	CM_AchilleaC	992	0	0	1	0
PL4	2	CM_AchilleaC	151	0	0	0	1
PL5	2	CM_AchilleaC	64	0	0	2	0
PL6	2	CM_AchilleaC	70	0	0	0	0
PL1	1	SP_ErigeronA	61390	25	0	9	0
PL2	1	SP_ErigeronA	27322	0	0	8	8
PL3	1	SP_ErigeronA	29662	0	0	2	19
PL4	2	SP_ErigeronA	3780	0	0	0	0
PL5	2	SP_ErigeronA	13810	473	0	0	0
PL6	2	SP_ErigeronA	22580	660	0	0	0
PL1	1	SP_SolidagoA	219748	0	46	3	0
PL2	1	SP_SolidagoA	121111	0	0	0	3
PL3	1	SP_SolidagoA	127085	0	0	11	263
PL4	2	SP_SolidagoA	0	0	0	0	0
PL5	2	SP_SolidagoA	0	0	0	0	0
PL6	2	SP_SolidagoA	0	0	0	0	0
PL1	1	SP_SolidagoB	847244	0	95	11	0
PL2	1	SP_SolidagoB	712347	0	7	11	0
PL3	1	SP_SolidagoB	890516	0	481	9	0
PL4	2	SP_SolidagoB	0	0	0	0	0

Supplemental Table 9 Continued

PL5	2	SP_SolidagoB	1	0	0	1	0
PL6	2	SP_SolidagoB	0	0	0	0	0
PL1	1	SP_SolidagoC	19262	0	0	6	0
PL2	1	SP_SolidagoC	14813	0	0	0	0
PL3	1	SP_SolidagoC	12455	0	0	5	38
PL4	2	SP_SolidagoC	1765	0	0	0	0
PL5	2	SP_SolidagoC	248	8	0	0	0
PL6	2	SP_SolidagoC	2569	0	0	2	0
PL1	1	PP_ArnicaA	3212341	0	0	1	0
PL2	1	PP_ArnicaA	3615913	0	0	5	0
PL3	1	PP_ArnicaA	2035280	0	0	6	1
PL4	2	PP_ArnicaA	1	0	0	0	0
PL5	2	PP_ArnicaA	1	0	0	1	0
PL6	2	PP_ArnicaA	0	0	0	0	0
PL1	1	PP_ArnicaB	3373001	0	0	19	0
PL2	1	PP_ArnicaB	3368858	0	0	14	7
PL3	1	PP_ArnicaB	2262255	0	0	12	0
PL4	2	PP_ArnicaB	0	0	0	0	0
PL5	2	PP_ArnicaB	0	0	0	0	0
PL6	2	PP_ArnicaB	0	0	0	0	0
PL1	1	PP_ArnicaC	604293	0	0	8	0
PL2	1	PP_ArnicaC	458499	0	0	7	0
PL3	1	PP_ArnicaC	402004	0	0	6	0
PL4	2	PP_ArnicaC	0	0	0	0	0
PL5	2	PP_ArnicaC	0	0	0	0	0
PL6	2	PP_ArnicaC	1	0	0	1	0
PL1	1	PP_SenecioA	28287	0	0	1	0
PL2	1	PP_SenecioA	50999	0	0	2	0
PL3	1	PP_SenecioA	32999	1459	0	7	0
PL4	2	PP_SenecioA	0	0	0	0	0
PL5	2	PP_SenecioA	1	0	0	1	0

Supplemental Table 9 Continued

PL6	2	PP_SenecioA	0	0	0	0	0
PL1	1	PP_SenecioB	39264	0	1	8	0
PL2	1	PP_SenecioB	29080	0	0	2	0
PL3	1	PP_SenecioB	68700	0	0	9	3
PL4	2	PP_SenecioB	0	0	0	0	0
PL5	2	PP_SenecioB	0	0	0	0	0
PL6	2	PP_SenecioB	0	0	0	0	0
Remaining Samples							
Plate	**Primer Set**	**SampleName2**	**Raw Sequences**	**Seqs lost after length filter**	**Seqs lost after decontam**	**Seqs lost after ASV < 5**	**Seqs lost after pos ctrl species removed**
PL7	1	BC_DasiphoraA	4758	0	0	0	0
PL8	2	BC_DasiphoraA	203	0	0	0	0
PL7	1	BC_DasiphoraB	88528	0	0	13	17
PL8	2	BC_DasiphoraB	16	0	0	0	0
PL7	1	BC_DasiphoraC	2753880	0	0	7	27
PL8	2	BC_DasiphoraC	374950	0	0	0	0
PL7	1	BC_EriogonumA	120140	7	0	0	0
PL8	2	BC_EriogonumA	11	0	0	0	3
PL7	1	BC_EriogonumB	2249	0	0	2	0
PL8	2	BC_EriogonumB	6	0	0	0	0
PL7	1	BC_EriogonumC	63820	0	0	3	12
PL8	2	BC_EriogonumC	8155	0	0	0	0
PL7	1	BC_PackeraA	2847493	0	0	4	25
PL8	2	BC_PackeraA	124	105	0	0	0
PL7	1	BC_PackeraB	980707	0	0	0	15
PL8	2	BC_PackeraB	14	0	0	0	0
PL7	1	BC_PackeraC	27921	1	0	6	1430
PL8	2	BC_PackeraC	3782	155	0	0	0
PL7	1	BP_ArnicaA	14804	0	0	1	0

Supplemental Table 9 Continued

PL8	2	BP_ArnicaA	153316	110	0	0	0
PL7	1	BP_ArnicaB	0	0	0	0	0
PL8	2	BP_ArnicaB	9	0	0	0	0
PL7	1	BP_ArnicaC	213	0	0	2	0
PL8	2	BP_ArnicaC	7	0	0	0	0
PL7	1	BP_EriogonumA	34	0	0	0	0
PL8	2	BP_EriogonumA	7	0	0	4	0
PL7	1	BP_EriogonumB	22501	0	0	5	0
PL8	2	BP_EriogonumB	82	0	0	0	0
PL7	1	BP_EriogonumC	8115	0	0	0	0
PL8	2	BP_EriogonumC	0	0	0	0	0
PL7	1	BP_EriogonumD	206	0	0	0	0
PL8	2	BP_EriogonumD	1	0	0	1	0
PL7	1	BP_EriogonumE	0	0	0	0	0
PL8	2	BP_EriogonumE	716	269	0	0	0
PL7	1	BP_EriogonumF	0	0	0	0	0
PL8	2	BP_EriogonumF	13	0	0	0	0
PL7	1	BR_DasiphoraA	1072841	0	0	2	1073
PL8	2	BR_DasiphoraA	2521	0	0	0	0
PL7	1	BR_DasiphoraB	1642568	12	0	1	1373
PL8	2	BR_DasiphoraB	118	88	0	0	0
PL7	1	BR_DasiphoraC	1694155	0	0	6	0
PL8	2	BR_DasiphoraC	2334	0	0	0	0
PL7	1	BR_GeraniumA	27628	0	0	2	12
PL8	2	BR_GeraniumA	54	50	0	0	0
PL7	1	BR_GeraniumB	175303	18	0	3	26
PL8	2	BR_GeraniumB	6	0	0	0	0
PL7	1	BR_GeraniumC	5323	0	0	2	19
PL8	2	BR_GeraniumC	23	23	0	0	0
PL7	1	BR_SenecioA	942562	0	0	9	27
PL8	2	BR_SenecioA	4	0	0	0	0

Supplemental Table 9 Continued

PL7	1	BR_SenecioB	1083109	1	1166	1	15977
PL8	2	BR_SenecioB	541	541	0	0	0
PL7	1	BR_SenecioC	1325895	16	881	2	1252
PL8	2	BR_SenecioC	736	729	0	0	7
PL7	1	PP_Arnica1	72909	0	0	2	17
PL8	2	PP_Arnica1	4575	0	0	0	0
PL7	1	PP_Erigeron10	729284	4	0	0	1465
PL8	2	PP_Erigeron10	20	0	0	0	2
PL7	1	RK_AchilleaA	119	0	0	0	0
PL8	2	RK_AchilleaA	7	0	0	0	0
PL7	1	RK_AchilleaB	25801	0	0	3	0
PL8	2	RK_AchilleaB	12	0	0	0	0
PL7	1	RK_AchilleaC	4124	0	0	0	0
PL8	2	RK_AchilleaC	726	4	0	0	0
PL7	1	RK_DrymocallisA	123968	0	0	9	12
PL8	2	RK_DrymocallisA	823	0	0	0	0
PL7	1	RK_DrymocallisB	869136	0	0	0	0
PL8	2	RK_DrymocallisB	0	0	0	0	0
PL7	1	RK_DrymocallisC	899539	0	0	0	0
PL8	2	RK_DrymocallisC	149	0	0	0	0
PL7	1	RK_GeraniumA	22675	0	0	0	2
PL8	2	RK_GeraniumA	9122	0	0	0	0
PL7	1	RK_GeraniumB	11235	0	0	0	0
PL8	2	RK_GeraniumB	0	0	0	0	0
PL7	1	RK_GeraniumC	0	0	0	0	0
PL8	2	RK_GeraniumC	0	0	0	0	0
PL7	1	SP_Erigeron10	86542	0	0	0	2
PL8	2	SP_Erigeron10	36	0	0	5	0
PL7	1	SP_Erigeron3	75063	0	0	1	1
PL8	2	SP_Erigeron3	22	0	0	0	0
PL7	1	SP_Erigeron7	108340	0	0	6	0

Supplemental Table 9 Continued

PL8	2	SP_Erigeron7	240	0	0	0	0
PL7	1	SP_Symphyotrichum10	1466	0	0	4	0
PL8	2	SP_Symphyotrichum10	0	0	0	0	0
PL7	1	SP_Symphyotrichum4	23287	105	0	2	1
PL8	2	SP_Symphyotrichum4	2032	0	0	0	0
PL7	1	SP_Symphyotrichum5	100783	0	0	0	0
PL8	2	SP_Symphyotrichum5	6	0	0	0	0
PL7	1	SP_Symphyotrichum6	930090	0	0	2	0
PL8	2	SP_Symphyotrichum6	2414	0	0	0	0
PL7	1	SP_Symphyotrichum7	79627	1403	0	3	0
PL8	2	SP_Symphyotrichum7	18811	0	0	0	0
PL7	1	SP_Symphyotrichum8	107440	0	0	0	331
PL8	2	SP_Symphyotrichum8	2	0	0	0	2
PL7	1	SP_Symphyotrichum9	346363	79	0	7	363
PL8	2	SP_Symphyotrichum9	10897	0	0	0	5
PL7	1	SU_Arnica1	48649	0	0	6	0
PL8	2	SU_Arnica1	16	0	0	0	0
PL7	1	SU_Arnica3	210850	0	59	0	15
PL8	2	SU_Arnica3	6020	112	0	0	0
PL7	1	TD_ArnicaA	432291	0	0	1	0
PL8	2	TD_ArnicaA	0	0	0	0	0
PL7	1	TD_ArnicaB	93830	0	68	2	785
PL8	2	TD_ArnicaB	0	0	0	0	0
PL7	1	TD_ArnicaC	95559	0	0	0	0
PL8	2	TD_ArnicaC	0	0	0	0	0
PL7	1	TD_Geranium3	786575	0	0	2	14
PL8	2	TD_Geranium3	191565	0	0	0	0
PL7	1	TD_GeraniumA	0	0	0	0	0
PL8	2	TD_GeraniumA	1523	1523	0	0	0
PL7	1	TD_GeraniumB	2629	82	0	0	0
PL8	2	TD_GeraniumB	11617	0	0	0	6

Supplemental Table 9 Continued

PL7	1	TD_GeraniumC	228778	0	0	1	0
PL8	2	TD_GeraniumC	0	0	0	0	0
PL7	1	TD_PotentillaA	21514	0	0	0	0
PL8	2	TD_PotentillaA	2	0	0	0	0
PL7	1	TD_PotentillaB	2637666	0	0	0	0
PL8	2	TD_PotentillaB	0	0	0	0	0
PL7	1	TD_PotentillaC	21510	0	0	3	0
PL8	2	TD_PotentillaC	0	0	0	0	0
Controls							
Plate	PrimerPair	SampleName2	Raw Sequences	Seqs lost after length filter	Seqs lost after decontam	Seqs lost after abundance thresh	Seqs lost after pos ctrl sps
PL2	1	PosCtrl_noBG	121846	121792	0	0	NA
PL3	1	PosCtrl_noBG	8821	8785	0	2	NA
PL4	2	PosCtrl_noBG	751170	0	0	2	NA
PL5	2	PosCtrl_noBG	1682106	0	0	3	NA
PL6	2	PosCtrl_noBG	1009840	0	0	0	NA
PL7	1	PosCtrl_noBG	17323	17204	0	0	NA
PL8	2	PosCtrl_noBG	353917	0	0	0	NA
PL1	1	PosCtrl_wBG	1136187	22850	2645	11	NA
PL2	1	PosCtrl_wBG	1036859	33310	409	9	NA
PL3	1	PosCtrl_wBG	5105382	8222	0	5	NA
PL4	2	PosCtrl_wBG	910949	192	0	0	NA
PL5	2	PosCtrl_wBG	2129647	64	0	2	NA
PL6	2	PosCtrl_wBG	463150	0	0	0	NA
PL7	1	PosCtrl_wBG	5093689	5379	0	6	NA
PL8	2	PosCtrl_wBG	217034	155	0	0	NA
PL7	1	BR_FieldNeg	2	0	0	0	0
PL8	2	BR_FieldNeg	480	0	0	0	5
PL7	1	BP_FieldNeg	14650	0	0	0	0

Supplemental Table 9 Continued

PL8	2	BP_FieldNeg	1	0	0	0	0
PL7	1	RK_FieldNeg	3480	0	0	0	0
PL8	2	RK_FieldNeg	19488	0	0	0	0
PL7	1	BC_FieldNeg	30	0	0	0	0
PL8	2	BC_FieldNeg	0	0	0	0	0
PL7	1	SP_FieldNeg	373	0	361	0	0
PL8	2	SP_FieldNeg	5	0	0	0	0
PL7	1	PP_FieldNeg	3	0	0	0	0
PL8	2	PP_FieldNeg	5	0	0	0	0
PL7	1	TD_FieldNeg	0	0	0	0	0
PL8	2	TD_FieldNeg	12	0	0	0	0
PL7	1	SU_FieldNeg	0	0	0	0	0
PL8	2	SU_FieldNeg	12	0	0	0	0
PL7	1	CM_FieldNeg	1844	0	0	0	0
PL8	2	CM_FieldNeg	0	0	0	0	0
PL7	1	E1_BuffNeg	443	0	0	0	0
PL8	2	E1_BuffNeg	0	0	0	0	0
PL7	1	E2_BuffNeg	14	0	0	3	0
PL8	2	E2_BuffNeg	2	0	0	0	0
PL7	1	E3_BuffNeg	0	0	0	0	0
PL8	2	E3_BuffNeg	2	0	0	0	0
PL7	1	E4_BuffNeg	0	0	0	0	0
PL8	2	E4_BuffNeg	3	0	0	3	0
PL7	1	E5_BuffNeg	0	0	0	0	0
PL8	2	E5_BuffNeg	3	0	0	0	0
PL7	1	E6_BuffNeg	3732	0	0	7	1915
PL8	2	E6_BuffNeg	12501	12475	0	0	24
PL7	1	E7_BuffNeg	15737	0	0	14	10207
PL8	2	E7_BuffNeg	34	0	0	0	20
PL7	1	E8_BuffNeg	5928	0	0	1	2352
PL8	2	E8_BuffNeg	18	0	0	0	18

Supplemental Table 9 Continued

PL7	1	E9_BuffNeg	20863	0	0	5	12971
PL8	2	E9_BuffNeg	12	0	0	0	8
PL1	1	NTC	299	50	0	0	0
PL2	1	NTC	171	32	0	0	0
PL3	1	NTC	140	0	0	7	0
PL4	2	NTC	1142	0	75	0	692
PL5	2	NTC	2563	0	71	2	1952
PL6	2	NTC	1008	0	27	0	529
PL7	1	NTC	2	0	0	0	0
PL8	2	NTC	29	0	0	0	22

Supplemental Table 10. Raw and percent of total sequences for positive control species in all positive control samples. Positive control samples with equal amounts of DNA from five insect species were included on each PCR plate and in a dilution series, and the raw sequences and percent of total sequences attributed to each of three control species that were identified, *Phymata americana, Coccinella transversoguttata,* and *Pieris rapae* are given. Two bee species were included in the five-species positive control, *Apis meliffera* and *Bombus rufocinctus*, and were not identified in any of the positive control samples. Positive control DNA was suspended in molecular grade water (Invitrogen) or water containing background DNA (BG) (i.e., DNA from 10 wildflowers each of five genera) on PCR plates three (PL3) through eight (PL8). On a ninth PCR plate, the mixed positive control was diluted to 5.0 ng, 2.5 ng, 1.25 ng and 0.625 ng per microliter in a series with background DNA (wBG) and in water alone (noBG). Floral backgrounds for each of the five plant genera were also created from the 10 wildflowers of each genus (*Arnica, Erigeron, Senecio, Solidago,* and *Potentilla*) that were included in the mixed wildflower DNA background and 2.5 ng of positive control was added to each floral background in separate reactions. The dilution series and genus-specific background DNA controls were run in one replicate with each primer set.

			Phymata americana		*Coccinella transversoguttata*		*Pieris rapae*		
Primer Set	PLATE	Sample	Raw	%	Raw	%	Raw	%	Total of Sample
Primer Set 1 (MLepF1/LepR1)	PLATE 3	PosCtrl wBG	5,005,932	98.05	10,682	0.02	4,015	0.08	5,105,382
		PosCtrl noBG	-	-	-	-	-	-	8,821
	PLATE 7	PosCtrl wBG	5,015,434	98.46	3,536	0.07	786	0.02	5,093,689
		PosCtrl noBG	-	-	-	-	-	-	17,323
	PLATE 9 (Dilutions)	5.0ng wBG	2,831,493	99.25	1,079	0.04	2,221	0.08	2,852,948
		2.5ng wBG	3,937,514	99.53	2,012	0.05	2,553	0.06	3,955,955
		1.25ng wBG	3,525,524	99.26	1,692	0.05	1,526	0.04	3,551,947
		0.625 wBG	2,786,579	97.11	2,566	0.09	2,555	0.09	2,869,599

Supplemental Table 10 Continued

Primer Set 1 (MLepF1/LepR1)		5.0ng noBG	4,472,750	99.76	1,561	0.03	4,879	0.11	4,483,604
		2.5ng noBG	4,296,525	99.76	2,053	0.05	4,454	0.10	4,307,062
		1.25ng noBG	2,536,532	99.74	1,937	0.08	2,977	0.12	2,543,248
		0.625ng noBG	4,227,092	99.51	9,371	0.22	6,938	0.16	4,248,019
		2.5ng in Arnica BG	4,815,542	99.65	3,055	0.06	1,449	0.03	4,832,526
		2.5ng in Erigeron BG	3,104,931	99.58	4,810	0.15	1,964	0.06	3,118,076
		2.5ng in Senecio BG	3,768,177	95.83	5,593	0.14	3,303	0.08	3,932,230
		2.5ng in SolidagoBG	3,061,078	99.36	1,060	0.03	2,475	0.08	3,080,668
		2.5ng in Potentilla BG	2,879,386	99.78	841	0.03	1,474	0.05	2,885,801
Primer Set 2 (LepF1/MHMR1)	PLATE 4	PosCtrl wBG	-	-	-	-	863,885	94.83	910,949
		PosCtrl noBG	-	-	-	-	751,122	99.99	751,170
	PLATE 5	PosCtrl wBG	-	-	-	-	1,949,547	91.54	2,129,647
		PosCtrl noBG	-	-	-	-	1,681,926	99.99	1,682,106
	PLATE 6	PosCtrl wBG	-	-	-	-	306,902	66.26	463,150
		PosCtrl noBG	-	-	-	-	1,009,706	99.99	1,009,840
	PLATE 8	PosCtrl wBG	-	-	-	-	152,910	70.45	217,034

Supplemental Table 10 Continued

Primer Set 2 (LepF1/MHMR1)		PosCtrl noBG	-	-	-	-	353,917	100.00	353,917
	PLATE 9 (Dilutions)	5.0ng wBG	-	-	-	-	3,398	68.47	4,963
		2.5ng wBG	-		-		-		-
		1.25ng wBG	-		-		-		-
		0.625 wBG	-		-		-		-
		5.0ng noBG	-	-	-	-	4,391	100.00	4,391
		2.5ng noBG	-	-	-	-	7,524	100.00	7,524
		1.25ng noBG	-	-	-	-	1,339	100.00	1,339
		0.625ng noBG	-	-	-	-	1,166	100.00	1,166
		2.5ng in Arnica BG	-	-	-	-	1,097	92.57	1,185
		2.5ng in Erigeron BG	-	-	-	-	1,793	98.30	1,824
		2.5ng in Senecio BG	-	-	-	-	1,644	99.82	1,647
		2.5ng in SolidagoBG	-	-	-	-	697	10.16	6,858
		2.5ng in Potentilla BG	-	-	-	-	3,584	29.47	12,163

Supplemental Table 11. Butterfly species netted from traditional surveys at wildflower eDNA sites. Traditional butterfly surveys were conducted at all nine sites on the same date as the wildflower associated eDNA samples were collected. The butterfly species name, its associated family, the number of individuals netted during the traditional survey, and whether the butterfly species was identified from wildflower associated eDNA samples collected at the site are listed in the table. The species that were identified by eDNA sequencing are bolded.

Site	Date	Species	Family	number netted	eDNA detection
BellyRiver	7/7/2022	*Cupido amyntula*	Lycaenidae	1	No
BellyRiver	7/7/2022	*Erebia epipsodea*	Nymphalidae	6	No
BellyRiver	7/7/2022	*Euchloe ausonides*	Pieridae	5	No
BellyRiver	7/7/2022	*Glaucopsyche lygdamus*	Lycaenidae	1	No
BellyRiver	7/7/2022	*Icaricia icarioides*	Lycaenidae	2	No
BellyRiver	7/7/2022	*Phyciodes cocyta*	Nymphalidae	1	No
BellyRiver	7/7/2022	*Phyciodes pulchella*	Nymphalidae	1	No
BellyRiver	7/7/2022	*Tharsalea helloides*	Lycaenidae	1	No
BigPrairie	7/1/2022	*Callophrys eryphon*	Lycaenidae	1	No
BigPrairie	7/1/2022	*Erebia epipsodea*	Nymphalidae	7	No
BigPrairie	7/1/2022	*Erynnis persius*	Hesperiidae	1	No
BigPrairie	7/1/2022	*Hesperia comma*	Hesperiidae	1	No
BigPrairie	7/1/2022	*Icaricia acmon*	Lycaenidae	3	No
BigPrairie	7/1/2022	*Phyciodes cocyta*	Nymphalidae	1	No
BigPrairie	7/1/2022	*Phyciodes pulchella*	Nymphalidae	1	No
Christensen	7/25/2022	*Argynnis hesperis*	Nymphalidae	1	No
Christensen	7/25/2022	*Boloria selene*	Nymphalidae	2	No
Christensen	7/25/2022	*Coenonympha inornata*	Nymphalidae	2	No
Christensen	7/25/2022	*Colias christina*	Pieridae	1	No
Christensen	7/25/2022	*Colias eriphyle*	Pieridae	2	No
Christensen	7/25/2022	*Colias eurythme*	Pieridae	3	No
Christensen	7/25/2022	*Colias interior*	Pieridae	2	No
Christensen	**7/25/2022**	***Oarisma garita***	**Hesperiidae**	**2**	**Yes**
Christensen	7/25/2022	*Phyciodes cocyta*	Nymphalidae	9	No
Christensen	7/25/2022	*Phyciodes pulchella*	Nymphalidae	1	No
Christensen	7/25/2022	*Polites themistocles*	Hesperiidae	1	No
Christensen	7/25/2022	*Pontia occidentalis*	Pieridae	1	No
Christensen	7/25/2022	*Tharsalea mariposa*	Lycaenidae	3	No
Christensen	**7/25/2022**	***Thymelicus lineola***	**Hesperiidae**	**4**	**Yes**
PrestonPark	8/16/2022	*Aglais milberti*	Nymphalidae	1	No
PrestonPark	8/16/2022	*Argynnis mormonia*	Nymphalidae	8	No
PrestonPark	8/16/2022	*Colias meadii*	Pieridae	9	No
PrestonPark	8/16/2022	*Colias nastes*	Pieridae	2	No
PrestonPark	8/16/2022	*Euphydryas chalcedona*	Nymphalidae	1	No
PrestonPark	8/16/2022	*Euptoieta claudia*	Nymphalidae	1	No

Supplemental Table 11 Continued

PrestonPark	8/16/2022	*Lycaena cuprea*	Lycaenidae	1	No
PrestonPark	8/16/2022	*Parnassius smintheus*	Papilionidae	8	No
PrestonPark	8/16/2022	*Plebejus idas*	Lycaenidae	1	No
PrestonPark	8/16/2022	*Plebejus melissa*	Lycaenidae	3	No
PrestonPark	8/16/2022	*Pontia occidentalis*	Pieridae	1	No
RockyKnob	7/2/2022	*Argynnis hesperis*	Nymphalidae	2	No
RockyKnob	7/2/2022	*Icaricia icarioides*	Lycaenidae	3	No
RockyKnob	7/2/2022	*Nymphalis antiopa*	Nymphalidae	1	No
RockyKnob	7/2/2022	*Papilio eurymedon*	Papilionidae	2	No
RockyKnob	7/2/2022	*Phyciodes cocyta*	Nymphalidae	2	No
SpotMt	8/12/2022	*Argynnis edwardsii*	Nymphalidae	1	No
SpotMt	8/12/2022	*Argynnis mormonia*	Nymphalidae	1	No
SpotMt	8/12/2022	*Cercyonis oetus*	Nymphalidae	5	No
SpotMt	8/12/2022	*Cercyonis oetus*	Nymphalidae	1	No
SpotMt	8/12/2022	*Colias eriphyle*	Pieridae	3	No
SpotMt	8/12/2022	*Icaricia icarioides*	Lycaenidae	1	No
SpotMt	8/12/2022	*Parnassius smintheus*	Papilionidae	2	No
SpotMt	**8/12/2022**	***Phyciodes pulchella***	**Nymphalidae**	**1**	**Yes (genus)**
SpotMt	8/12/2022	*Pontia occidentalis*	Pieridae	2	No
SpotMt	**8/12/2022**	***Tharsalea florus***	**Lycaenidae**	**3**	**Yes (genus)**
SpotMt	**8/12/2022**	***Tharsalea heteronea***	**Lycaenidae**	**2**	**Yes (genus)**
SpotMt	**8/12/2022**	***Thymelicus lineola***	**Hesperiidae**	**3**	**Yes**
StMary	8/10/2022	*Argynnis mormonia*	**Nymphalidae**	5	No
StMary	8/10/2022	*Cercyonis oetus*	**Nymphalidae**	1	No
StMary	**8/10/2022**	***Cercyonis pegala***	**Nymphalidae**	**4**	**Yes**
StMary	8/10/2022	*Colias eriphyle*	Pieridae	3	No
StMary	8/10/2022	*Hesperia assiniboia*	Hesperiidae	1	No
StMary	8/10/2022	*Plebejus melissa*	Lycaenidae	6	No
StMary	8/10/2022	*Thymelicus lineola*	Hesperiidae	2	No
Sullivan	7/19/2022	*Argynnis atlantis*	Nymphalidae	1	No
Sullivan	7/19/2022	*Argynnis mormonia*	Nymphalidae	1	No
Sullivan	7/19/2022	*Argynnis zerene*	Nymphalidae	1	No
Sullivan	7/19/2022	*Cercyonis pegala*	Nymphalidae	1	No
Sullivan	7/19/2022	*Chlosyne palla*	Nymphalidae	1	No
Sullivan	7/19/2022	*Coenonympha inornata*	Nymphalidae	1	No
Sullivan	7/19/2022	*Icaricia saepiolus*	Lycaenidae	1	No
Sullivan	7/19/2022	*Phyciodes cocyta*	Nymphalidae	8	No
Sullivan	7/19/2022	*Phyciodes tharos*	Nymphalidae	2	No
Sullivan	7/19/2022	*Tharsalea hyllus*	Lycaenidae	3	No
Sullivan	7/19/2022	*Tharsalea mariposa*	Lycaenidae	3	No
Sullivan	7/19/2022	*Thymelicus lineola*	Hesperiidae	3	No
TwoDog	7/9/2022	*Coenonympha inornata*	Nymphalidae	6	No

Supplemental Table 11 Continued

TwoDog	7/9/2022	*Erebia epipsodea*	Nymphalidae	1	No
TwoDog	7/9/2022	*Icaricia icarioides*	Lycaenidae	10	No
TwoDog	7/9/2022	*Phyciodes pulchella*	Nymphalidae	6	No