

CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Environmental Sciences

BACHELOR THESIS

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CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Environmental Sciences

Environmental Engineering



Reintroduction and conservation genetics of butterflies

BACHELOR THESIS

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Faculty of Environmental Sciences

BACHELOR THESIS ASSIGNMENT

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Environmental Engineering

Thesis title

Reintroduction and conservation genetics of butterflies

Objectives of thesis

Translocations of individuals of endangered species are important management to reinforce unstable or dying out populations. But success of these processes is usually very uncertain (Morris et al., 2021). Although reintroduction programs are frequently used as management for endangered species of butterflies (e.g. Andersen et al. 2014), our knowledge about their translocation techniques, strategies and results is still very poor (La Haye et al.2017, White et al. 2018).

Methodology

The main goal of the bachelor thesis will be to summarize information from studies that used molecular techniques for the evaluation of the reintroductions of butterflies. The thesis also tries to identify factors that influence these reintroduction programs' success. The thesis will make suggestions for monitoring of reintroduction of *Chazara briseis* in the Czech Central Highlands (Baranovská et al. 2020, Kadlec et al.2010).

The proposed extent of the thesis

max. 30 pages

Keywords

non-invasive DNA, Lepidoptera, population genetics, reintroduction

Recommended information sources

- Andersen A. et al. 2014. Assessing reintroduction schemes by comparing genetic diversity of reintroduced and source populations: A case study of the globally threatened large blue butterfly (*Maculinea arion*). *Biological conservation* 175: 34–41.
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- Kadlec T, Vrba P, Kepka P, Schmitt T, Konvicka M 2010. Tracking the decline of the once-common butterfly: Delayed oviposition, demography and population genetics in the hermit *Chazara briseis*. *Animal Conservation* 13: 172–183.
- La Haye, MJJ, Reiners, TE, Raedts, R et al. 2017. Genetic monitoring to evaluate reintroduction attempts of a highly endangered rodent. *Conserv Genet* 18: 877–892.
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- White LC, Moseby KE, Thomson VA, Donnellan SC, Austin JJ 2018. Long-term genetic consequences of mammal reintroductions into an Australian conservation reserve. *Biological Conservation* 219: 1–11.
-

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Prague on 26. 03. 2023

BACHELOR THESIS DECLARATION

I hereby declare that I have independently elaborated the bachelor/final thesis with the topic of: Reintroduction and conservation genetics of butterflies and that I have cited all of the information sources that I used in the thesis as listed at the end of the thesis in the list of used information sources.

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In Prague
27.03.2023



Camila Morfin Medina

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Abstract

This thesis provides an overview of Lepidoptera conservation genetics and techniques, with a focus on reintroductions. Lepidopterans play a crucial role in pollination and can serve as excellent bioindicators, making them essential for nature conservation. As a result of their continuous decrease, they are increasingly being used in conservation efforts. Strategies like reintroduction programs are regularly implemented and it is, therefore, necessary to have a deeper understanding of them, to ensure they have a positive effect on the species. This research seeks to identify the elements that impact the success or failure of Lepidoptera reintroductions. The review shows a strong correlation between reintroduction success and habitat quality and management. It also reveals a higher record of successful reintroductions for the projects that released individuals as caterpillars and eggs. Europe appears to be the leader in Lepidopteran reintroductions, accounting for over half of the reintroductions in this research. Additionally, most of the studied reintroductions were carried out in non-forest habitats, particularly grasslands. Reintroductions that established techniques to monitor the success before releasing the individuals were found to be successful, even in their initial stages, suggesting a positive influence on the overall outcome of reintroductions. If these influencing elements are considered, future reintroduction efforts, including those for the focus species, could be positively impacted. By examining and comparing previous reintroduction attempts, this work evaluates the implementation of this strategy for the conservation of the focus species, *Chazara briseis*.

Keywords: non-invasive DNA, Lepidoptera, population genetics, reintroduction

Abstrakt

Tato práce poskytuje přehled konzervační genetiky a technik Lepidoptera se zaměřením na reintrodukce. Lepidopterani hrají klíčovou roli při opylování a mohou sloužit jako vynikající bioindikátory, což je činí nezbytnými pro ochranu přírody. V důsledku jejich neustálého úbytku jsou stále více využívány v ochrannářských snahách. Strategie jako reintrodukční programy jsou pravidelně zaváděny, a proto je nutné jim hlouběji porozumět, aby bylo zajištěno, že budou mít na daný druh pozitivní vliv. Tento výzkum se snaží identifikovat prvky, které ovlivňují úspěch nebo neúspěch reintrodukce Lepidoptera. Přehled ukazuje silnou korelaci mezi úspěšností reintrodukce a kvalitou stanovišť a managementem. Odhaluje také vyšší rekord úspěšných reintrodukcí u projektů, které vypustily jedince jako housenky a vajíčka. Evropa se zdá být lídrem v reintrodukcích Lepidoptera, což představuje více než polovinu reintrodukcí v tomto výzkumu. Většina studovaných reintrodukcí byla navíc provedena na nelesních stanovištích, zejména travních porostech. Bylo zjištěno, že reintrodukce, které zavedly techniky pro sledování úspěchu před propuštěním jedinců, byly úspěšné, a to i v jejich počátečních fázích, což naznačuje pozitivní vliv na celkový výsledek reintrodukcí. Pokud budou tyto ovlivňující prvky zváženy, mohly by být pozitivně ovlivněny budoucí snahy o reintrodukci, včetně těch pro ohniskové druhy. Zkoumáním a porovnáním předchozích pokusů o reintrodukci tato práce hodnotí implementaci této strategie pro zachování ohniska druhu *Chazara briseis*.

Klíčová slova: neinvazivní DNA, Lepidoptera, populační genetiky, reintrodukce

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1. Introduction

Our planet is changing, particularly as a result of the rising demands of a continuously expanding population, and this is not news. Human activities have taken a toll on our ecosystems in several ways, from habitat fragmentation to pollution and overexploitation of biological resources; all of which has resulted in a vast loss of biodiversity. In consequence, there has been an increase in the number of endangered species all over the world. For instance, more than 40% of insect species are facing extinction, with Lepidoptera being one of the most affected orders, as reported by Sánchez-Bayo & Wyckhus (2019). Butterflies are crucial for nature conservation not only because of their role as pollinators, but because they can be great bioindicators as they are extremely susceptible to environmental conditions and climate change (Legal et al., 2020). As a response to these declines and to reduce these impacts, conservation efforts like captive breeding and reintroduction of species have gained popularity (Beck, 2001).

Reintroduction programs can be key to the conservation and protection of species, as well as to strengthen the current populations (Converse et al., 2013). However, even though they are frequently relied upon for the conservation of Lepidopterans, there are no clearly determined factors that will impact their results, nor a clear establishment of the techniques and strategies used (Seddon, 2015). Not only this but there is a lack of long-term monitoring of the reintroduced populations (but see Andersen et al., 2014), which could provide valuable insights to help perfect this conservation strategy.

Hence, this thesis will compare and further analyse previous Lepidoptera reintroduction projects with the aim of discovering the factors responsible for their failure or success, as they could be valuable for the conservation of the focus species in the Czech Republic, the endangered *Chazara briseis* (Linnaeus, 1764) (Lepidoptera: *Nymphalidae*). This research begins with an introduction to conservation genetics and related techniques, as well as an overview of the characteristics and risks associated with Lepidopteran reintroductions. Then, I present a review of different Lepidoptera reintroduction attempts, finalizing with an application of the obtained results to the focus species, *Chazara briseis*, assessing its current conservation status and how future efforts can benefit from this research.

2. Objectives

The overall goal of this thesis is to gather and analyze information related to reintroductions for conservation and reintroduction studies, particularly those performed on Lepidoptera. By studying previous Lepidoptera reintroductions, I seek to identify what influenced their success or failure.

In addition, it aims to provide a deeper understanding of conservation genetics of butterfly species. This thesis provides an overview on genetic markers used for the aforementioned studies, ideal non-invasive techniques for DNA extraction, and the differences between generations of a species before and after its captivity.

Moreover, it focuses on the characteristics of the species *Chazara briseis*, its current status, and conservation efforts. Similarly, I will assess general risks that can be present in reintroductions, as well as the parameters to evaluate the success rate of reintroductions. Together, these approaches to the topic at hand seek to assess whether this would be a viable solution for the studied species, enhance the results of upcoming conservation reintroductions, and provide threatened species a better chance of surviving in a world that is changing quickly.

3. Conservation genetics

The Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES) informed in their 2019 report that ecosystems have declined by around 50% when compared to historical earlier conditions, and about 25% of species face extinction, as they are currently threatened (Brondízio et al., 2019; Kardos, 2021). With these numbers in mind, conservation strategies have become increasingly popular, in search of protection of threatened species, and stopping or slowing this rapid decline as much as possible.

As better defined by Wan et al. (2004), conservation genetics refers to the “use of genetics to preserve species as dynamic entities capable of coping with environmental change”, with the goal of using genetic diversity to evaluate the population’s health and risk of extinction (Woodruff, 2001). Conservation and genetics have a strong, well-studied correlation, and the importance of genetic diversity is frequently highlighted (Allendorf et al., 2012). Genetic diversity needs to be considered for the long-term survival of any species, as populations with a higher genetic diversity are expected to have a better adaptation to rapid environmental change when compared to those with low genetic variation (Turlure et al., 2014; Kardos, 2021; Teixeira & Huber, 2021). The main mechanisms that are found to influence genetic diversity are inbreeding, genetic drift, mutation, gene flow, and natural selection (Andrews, 2010; Turlure et al., 2014).

There are two categories into which butterfly conservation activities can be divided, according to New (1997). The focus of the first of these categories is the protection of the butterfly fauna, following the theory that a highly diverse butterfly fauna is connected with high biodiversity and a generally stable ecosystem. The second group has the goal of restoring threatened and endangered species and their ecosystems and focuses on the preservation and overall protection of these species (New, 1997). I will now describe a couple of examples where genetics was applied with a conservation aim.

To demonstrate both the value of using molecular techniques when choosing conservation measures and the close relationship between molecular methods and habitat management, a study (Álvarez Hincapié et al., 2005) carried out in Antioquia, Colombia, aimed to improve the planning of future conservation strategies, particularly in fragmented ecosystems by using mitochondrial DNA from two butterfly species (*Hypoleria vanilia* and *Euptychia Hermes*) with different dispersion patterns. They determined the gene flow between the five forest terrains under study by looking at the genetic structure of the species, and their findings showed how the size, distance, and distribution of patch sizes have an impact on species with various dispersal capacities.

Similarly, with climate and land use changes having such a big impact on habitats, it is important to assess the effects on the species, the populations, and their dynamics, so that specific and effective conservation strategies are implemented to help with the long-term persistence of the species. Such is the case of the study carried out by Sherpa

et al. (2021), who aimed to evaluate the relationship between land use and climate changes and the population dynamics in a threatened butterfly species, *Coenonympha hero*. Using SNPs, they were able to look at the genetic diversity and structure of the populations in the French Jura massif and, from the three main regions studied in the area, they found three corresponding major genetic clusters, which showed genetic richness in the northern sites and rather low genetic diversity in the southern ones. Despite these variations, the majority of locations were in a demographic equilibrium, and therefore they determined the metapopulation to be dynamic and functional. Additionally, they used genetic inferences and species distribution modeling (SDM) to obtain a better understanding of population dynamics at the species distribution margin and predict future species ranges, as this can be crucial for conservation management. They compared demographic changes to changes in climatically suitable habitats, in this case, grasslands, by selecting environmental predictors that are best able to account for population size fluctuations across time and space (e.g., climatic, topographic, and habitat) (Sherpa et al., 2021).

4. Non-invasive and non-destructive techniques for DNA extraction in butterfly species

Non-invasive techniques have become a key part of molecular studies, particularly those involving threatened or rare species (Hamm et al., 2009). There is a difference between non-invasive and non-destructive (or non-lethal) techniques (Fig. 1): non-destructive sampling often involves capturing the individuals and clipping or sampling

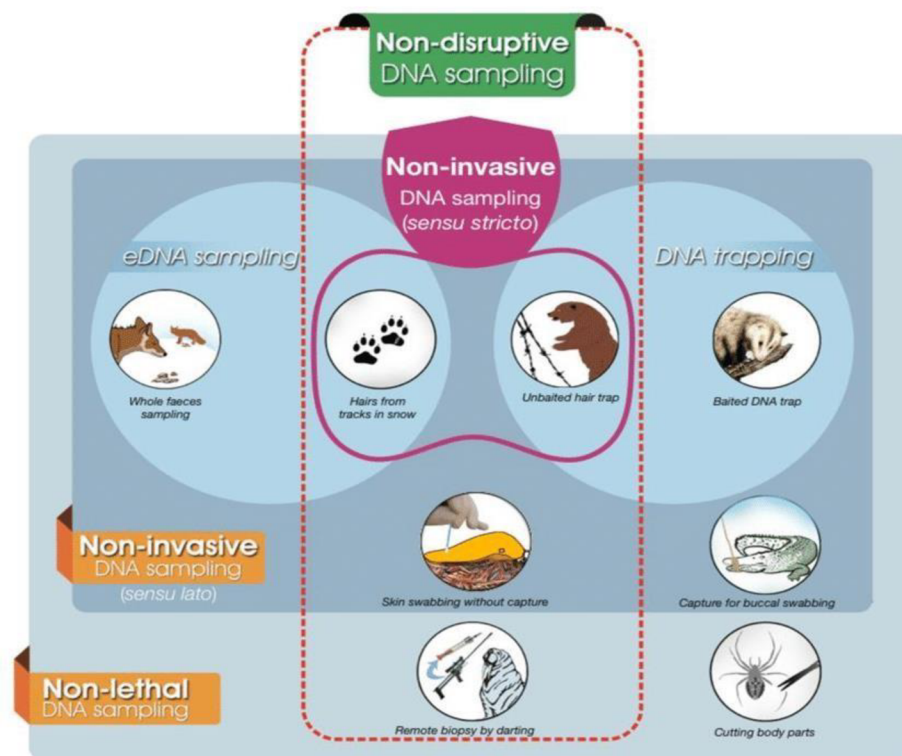


Fig. 1. Diagram showing the differences between non-lethal, non-disruptive, and non-invasive DNA sampling (Lefort et al., 2022).

invasively, without killing them (Lefort et al., 2022); meanwhile, on the other hand, non-invasive sampling refers to situations where the DNA is left behind by the individual and there is no need to capture or disrupt the species (Taberlet et al., 1999; Segelbacher, 2002; Lefort et al., 2022). The overall aim is to obtain the sample for DNA testing without harming, putting at risk or even capturing the individuals (Taberlet et al., 1999).

While it is possible to obtain the required information from feathers, droppings, or skin for larger vertebrate species, it is much harder when it comes to invertebrates (Storer et al., 2019). Particularly for Lepidoptera, several non-lethal techniques have been described as successful for the genetic sampling of endangered species, with the most common being wing clipping, tarsal clipping, and hemolymph sampling (Hamm et al., 2009). However, while some studies and sampling cases have resulted in no significant impact on the behavior of the species (Hamm et al., 2009), others have no record of the impacts or evaluation post-sampling, leading to uncertainty for future studies. As an alternative, non-invasive sampling has risen in popularity, obtaining the sample DNA for example from chorion (hard outer shell) from residual butterfly egg debris (Fig. 2), frass (feces), or exuviae (shed exoskeleton) (Fig. 3) (Ali et al., 2019; Storer et al., 2019).

The main disadvantages associated with non-invasive sampling are: a) it can be a time-consuming sampling process, and b) not obtaining enough information after the DNA amplification processes (Storer et al., 2019). However, recent studies performed on different species have proven that this is not always the case, turning non-invasive sampling into an advantageous alternative, particularly when it comes to studying endangered invertebrates, as will be explained in more detail ahead.

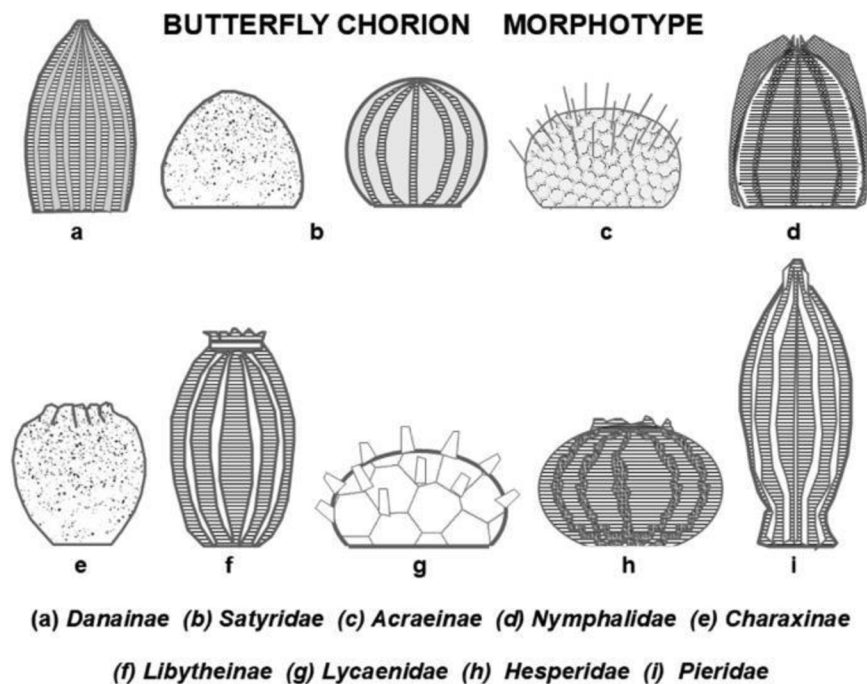


Fig. 2. Examples of different types of butterfly chorion from residual butterfly egg debris, that could be used for non-invasive DNA sampling (Lumini, 2022).

A study aimed to determine how effective it would be to use the chorion from hatched ovae of the Miami Blue Butterfly (*Cyclargus thomasi bethunebakeri*) for DNA extraction and analysis. After collecting and storing the egg debris samples, it was demonstrated that it is in fact possible to extract DNA of a high-enough quality, and can provide sufficient information for gene sequencing and further genetic studies, without compromising the individuals (Storer et al., 2019).

Another example of successful non-invasive sampling can be found in the study of the Oriental Armyworm (*Mythimna separata*), showing the effectiveness of frass and exuviae to obtain DNA. This approach has been proven more useful when obtained during the larval stage of the insects' development, or with the shedding of exoskeletons from instars, since there are larger quantities available for sampling, and due to the fragility of the exuviae itself. Despite the reduced DNA concentration in frass samples, they were able to raise the detection threshold by using Multiple Displacement Amplification (MDA) technologies. By employing this practical technique, they were able to use non-invasive sampling and carry on with further DNA extraction, pre-amplifying DNA by MDA, and eventually leading to effective PCR amplification of both exuviae and frass samples (Ali et al., 2019).



Fig. 3. Small Tortoiseshell butterfly (*Aglais urticae*), newly hatched and close to its exuvia (Alamy Stock Photo).

5. Genetic markers

Genetic markers have become key for obtaining information for conservation studies. As described by Hedrich et al. (2012), they can generally be defined as a specific DNA sequence with a known location on a chromosome. They are particularly useful in

conservation studies due to their ability to provide further details on the genetic variability of endangered species, as well as evolutionary and population biology (Sunnucks, 2000). Additionally, they are very convenient when dealing with non-invasive DNA samples, as they have the ability to still supply the required information, even if they are small in quantity or quality. Furthermore, they provide the possibility to be tested by polymerase chain reaction (PCR), together with the potential to provide comparability and aid in reaching conclusions from previous studies (Sunnucks, 2000).

It is important to select the correct genetic marker for the question at hand, understanding the highlights and challenges of each of them to obtain the desired results. Amongst the most widely used genetic markers for conservation and population studies of endangered species are microsatellites, SNPs, and mtDNA (Fig. 4). I will now describe each of them in more detail, as well as discuss their main advantages and disadvantages in terms of conservation of species.

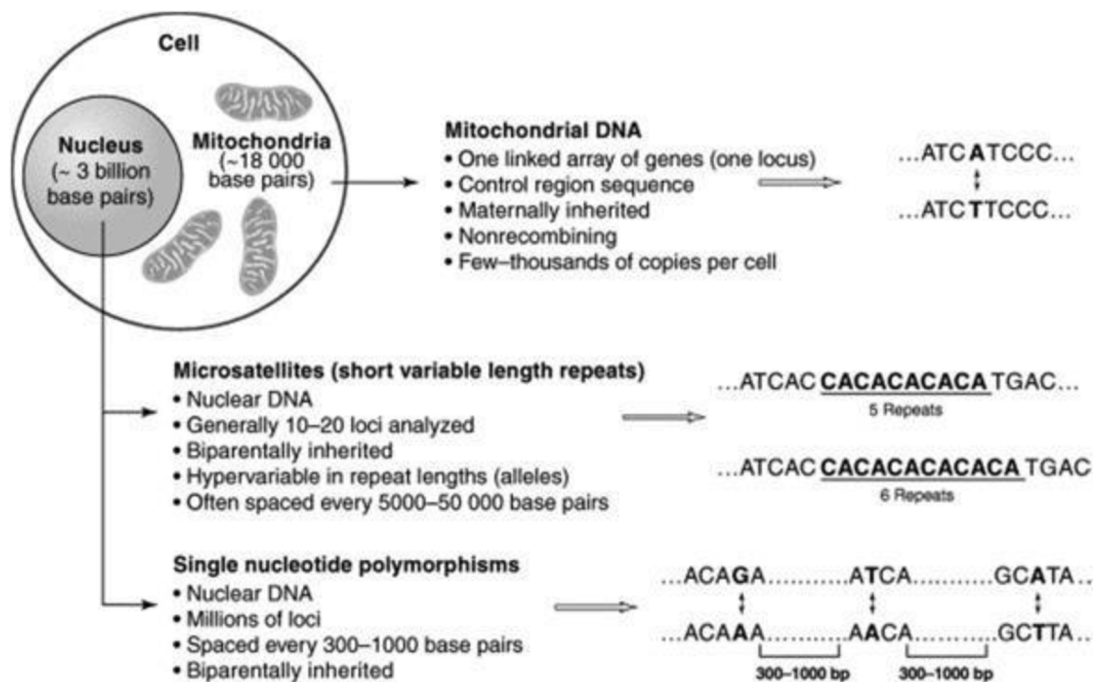


Fig. 4. Comparison between mitochondrial DNA (mtDNA), microsatellites, and single nucleotide polymorphisms (SNPs) (Sodhi et al., 2022).

5.1 Microsatellites

Microsatellites are part of the genetic markers also known as co-dominant markers, in which all the alleles present in a particular locus can be identified. They are among the most used for population genetics and conservation studies (Turlure et al., 2014). Microsatellites owe their popularity to their versatility, as they are neutral, variable, and can be reproduced (Selkoe & Toonen, 2006). They are short tandem repeats, also referred to as Simple Sequence Repeats (SSRs), and they have a high degree of length polymorphism, are easy to amplify by the use of PCR, and can provide a lot of

information (Wan et al., 2004). Besides this, since microsatellites are specific to the species, cross-contamination is not a big problem compared with other markers (Selkoe & Toonen, 2006). Therefore, they are very useful in phylogenetic studies and the study of gene flow, population size, and population genetics in general (Selkoe & Toonen, 2006).

However, it is still possible for genotyping errors to appear, as well as some difficulty in the isolation of the marker (Turlure et al., 2014). Besides, it is not always possible to compare data when genotyping from isolated individuals due to inconsistencies in allele size calling (Vignal et al., 2002). Data analysis is complicated and can be ambiguous because of null alleles, which are not amplified by PCR (Morin et al., 2004).

A study by Turlure et al. (2014) showed the efficiency of microsatellites for the analysis of genetic diversity and population structures of the threatened butterfly *Boloria aquilonaris*. Even with Lepidoptera being one of the groups that encounter difficulties when isolating microsatellites, linked to the considerable similarity in flanking areas between several microsatellites within the same species and/or the absence of conserved flanking regions resulting in unpredictable banding patterns, they were able to successfully isolate the microsatellite loci using next-generation sequencing. When compared with RAPDs (Random Amplified Polymorphic DNA), microsatellites provided a higher estimate resolution on the population structures. (Turlure et al., 2014).

5.2 SNPs

Single nucleotide polymorphisms (SNPs) are variations in a DNA sequence, arising from the difference of a single nucleotide in the genome (Brody, 2006). They increased their popularity in the 2000s and, typically, an extremely high density of SNPs can be found in genomes, which could perhaps increase cost-effectiveness (Vignal et al., 2002). When it comes to estimating genetic variation, a large number of SNPs is required since it has 2 alleles per loci (Morin et al., 2014). Additionally, it is recommended to do a simulation study to understand SNPs numbers and how to apply them according to the characteristics of the population genetics study at hand (Morin et al., 2004).

SNPs can be helpful and provide valuable information on population and genome dynamics, as well as connections between certain genes or other DNA structures and phenotypes (Vignal et al., 2002). Furthermore, they frequently produce comparable statistical data while offering greater genome coverage, higher-quality data, and greater analytical ease compared to other markers, e.g. microsatellites (Morin et al., 2004). However, it is possible to encounter a lack of information due to their bi-allelic nature, which reduces the power to detect the loss of allelic richness, particularly in relatedness studies (Vignal et al., 2002; Morin et al., 2004). Another limitation that is important to keep in mind, is the ascertainment bias that can arise in some applications (Morin et al., 2004).

5.3 *mtDNA*

Mitochondrial DNA (mtDNA) is best applied for the resolution of taxonomic problems and uncertainties since they contain historic genetic mutations (Wan et al., 2004). It has a relatively fast rate of base substitution, is effectively haploid, and has maternal inheritance, increasing its sensitivity to genetic drift and facilitating its isolation and manipulation (Moritz, 1994). However, this maternal inheritance can limit its use only from the maternal perspective, therefore forcing it to be more of an auxiliary marker (Wan et al., 2004). It is not generally considered effective for determining any individual-level events like identification, dispersal, and mating systems, nor for current changes in genetic structure, such as the recent loss of genetic variation (Wan et al., 2004). However, it is a great tool for the identification of species and the general, current status of population structure (Wan et al., 2004). Some of its other main uses include the establishment of interspecific hybridization and the detection of illegal hunting of threatened species, as explained by Arif et al. (2011).

Since the majority of cells contain several copies of the mtDNA molecule, these sequences can usually be obtained from small tissue samples with degraded DNA (Arif et al., 2011). Such is the case of the study performed by Lushai et al. in the year 2000, in which they used tissue from the wing tips of the endangered Apollo butterfly (*Parnassius apollo L.*). By using universal mtDNA primers, they were able to amplify the DNA in the sample and show the possibility of gene flow between the separate populations (Lushai et al., 2000).

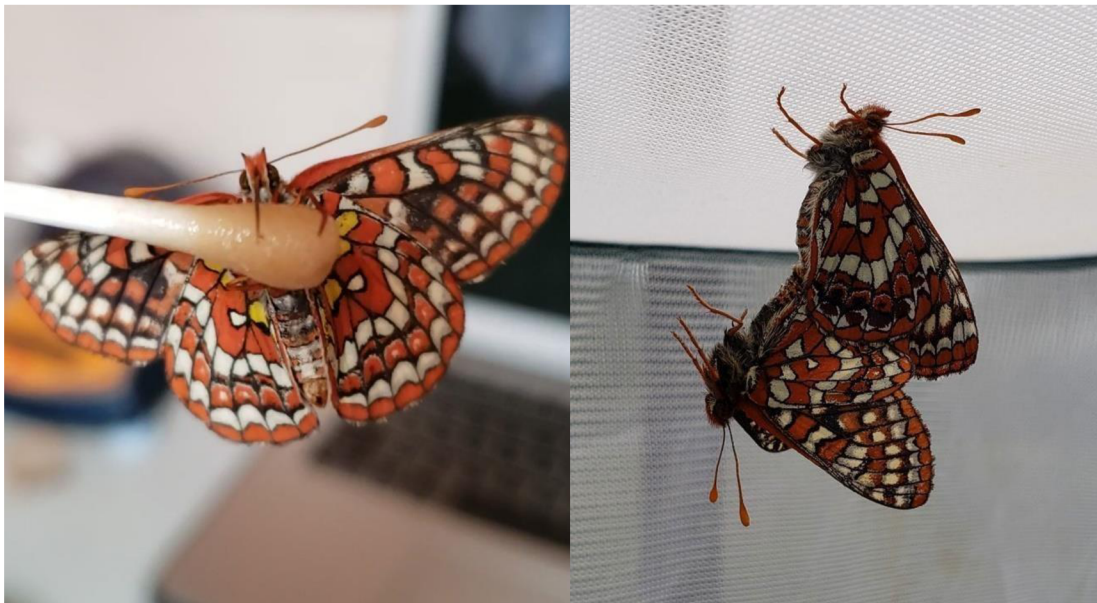
6. Reintroduction

6.1 *Definition & characteristics*

The reintroduction of species is one of many conservation strategies used in efforts to slow down species extinction and conserve biological diversity (Seddon et al., 2007). It consists of the planned release of a living organism into an area that was previously occupied by that species (indigenous range), with the main goal of its conservation and the re-establishment of a viable population (Seddon, 2010; IUCN, 2013; Bellis et al., 2019). While sometimes it is interchangeable with the term repatriation, the main difference between the two can be found in the way that reintroductions are only released in locations where the species is no longer available, while in repatriations, individuals are released into an area that is currently or was previously inhabited by the species (Kingsbury & Attum, 2009). There are tools available for conservationists to conduct reintroductions following general principles, such as the IUCN's Guidelines for Reintroductions and Other Conservation Translocations, as well as the guidelines proposed by Daniels et al. (2018), which are more specific to Lepidoptera.

A successful reintroduction needs careful planning, a pre-release health-risk assessment, strong local community support, and the use of corporate and media backing, as well as post-monitoring examinations (Beck, 2001; IUCN, 2013). It is also necessary to have basic biological knowledge of the focus species, habitat

requirements, and population biology (IUCN, 2013; Daniels et al., 2018). Some key components that could increase the chances of success of a butterfly reintroduction, according to Daniels et al. (2018), include a suitable habitat, access to nectar sources and host plants, and overall landscape features, together with microclimatic conditions. If the current habitat quality of the indigenous range is not suitable for reintroduction, it is possible to look for a different habitat that fulfills all the species' needs (Stephenson et al., 2019). The individuals selected to be the first to be reintroduced, known as source or founder individuals, can come from wild propagation, or captivity, and they should be from an appropriate population in terms of demography, genetics, health management, and behavior (IUCN, 2013). Captivity breeding and rearing takes place intending to then reintroduce a viable population into the wild, and it can be particularly valuable when the population is too fragile and cannot lose any more individuals (Lewis & Thomas, 2001; Crone et al., 2007) (Fig. 5). Post-release monitoring is also needed to evaluate the performance of the organism, the impact of the reintroduction program, and determine whether further management is needed (Daniels et al., 2018). Together with this, it is recommended for genetic monitoring to be performed, particularly in small release sites, to maximize the probability of persistence (Daniels et al., 2018).



*Fig. 5. Captive breeding of Taylor's checkerspot (*Euphydryas Editha taylori*) butterfly in Canada as part of their conservation breeding program. On the left is a female butterfly feeding from honey-water on a Q-tip. On the right two butterflies in a classic breeding pose (Polley, 2021).*

6.2 Parameters to evaluate the success rate

Although there are clear guidelines to follow when preparing a reintroduction, there is no universal definition or set of rules to determine whether a reintroduction has been successful or not (Robert et al., 2015; Seddon, 2015). Because of the unique qualities, population dynamics, and the ability of the reintroduced individuals—especially those sourced from captive populations—to acclimate to the reintroduction area, it can be

difficult to establish a definition for success that can be applied to all reintroductions (Seddon, 2015). However, it might be helpful to keep a certain goal in mind, with criteria that could potentially be restructured and tailored for the particular species.

With this in mind, the ultimate goal of a reintroduction can be considered to be biological success, in the form of self-sustaining populations, as best described by Pavlik (1996) who states there are four factors to take into account: abundance (population size and fertility), extent (population distribution and dispersal), resilience (genetic diversity), and persistence (self-sustainability). While it might be possible for these parameters not to apply to certain species that live for longer, they can be a good reference for Lepidoptera, as their lifespan tends to be shorter (Pavlik, 1996; Haskins, 2015). Similarly, in an attempt to define translocation success, research carried out by Bellis et al. (2019) focused on insect translocations, and established criteria that would help determine whether a translocation was successful or not, these being: a) the translocated population has survived for more than 10 years after the most recent release, and b) the results from the most recent monitoring show the population is still located at the release site. The goal of both these standards is to avoid misclassifying a reintroduction as successful in its early stage (Bellis et al., 2019). Comparably, the U. S. Fish and Wildlife Service defined reintroduction success as resilience, which is achieved if any decline in the number of occupied habitat patches is followed by increases of equal or greater magnitude over a period of 10 to 20 years (U.S. Fish and Wildlife Service, 2003).

An example of an initially successful reintroduction can be found in the study of Marttila et al. (1997), where they reintroduced the threatened Batton Blue butterfly (*Pseudophilotes baton schijfer muelleri*) to Finland, into a location where it had previously gone extinct. After following the correct management of the selected site, they relocated the butterflies and studied them following the mark-recapture method, with results the following year showing that the population had almost doubled in size. They also observed dispersal behavior from certain individuals. The reintroduction was considered successful at the time and even guaranteed the chances of long-term survival of the species. However, the population declined and eventually vanished in the years following the reintroduction, as a result of excessive rain during the flight period (K. Saarinen pers. comm., as cited in Bellis, 2021). Hence the importance of genetic monitoring, as it can be of great use when estimating the adaptability of reintroduced populations, and ultimately increase their chances of survival in the face of climate change or stochastic weather events.

In contrast, perhaps one of the most recognized, successful translocations is the reintroduction of the Large Blue butterfly (*Maculinea arion*), following its extinction in the United Kingdom in 1979. It is considered to be *Maculinea*'s longest-running, most significant project to date (Andersen et al., 2014). Not only has this reintroduction been successful in terms of population persistence over the years, but also in maintaining genetic diversity, as confirmed by a study by Andersen et al. (2014). By using microsatellites, they examined and compared the diversity of one of the source populations in Sweden with that of the reintroduced populations, and the results

showed a similar genetic diversity. They highlight and claim this success was a consequence of following the IUCN Reintroduction Guidelines and other protocols, the adequate restoration and continuous management of the reintroduction site, as well as the particular life-cycle of the species, making it an outstanding conservation project and an example for future reintroduction efforts (Andersen et al., 2014).

6.3 Captivity and release: changes between generations

Captive breeding has become a valuable conservation strategy for threatened species, with its popularity recently increasing in invertebrates (Lewis & Thomas, 2001). It can be a great conservation method for some species since these periods of captive breeding are necessary for their survival - while long-term recovery and conservation strategies are being developed, captivity breeding can take place to help in the preservation of endangered populations (Crone et al., 2007). Individuals are bred *ex-situ* - offsite from the natural location – with the aim for a suitable population to then be reintroduced to the wild (Lewis & Thomas, 2001). However, it is important to acknowledge and discuss possible genetic effects and plan accordingly for future reintroductions. The main concerns when it comes to the effect of captive-reared individuals being released into the wild include genetic consequences such as the loss of genetic diversity, potential behavioral and demographic changes, as well as the risk of parasites and diseases being reintroduced to wild populations (Lewis & Thomas, 2001; Crone et al., 2007).

Several studies have been carried out to assess the possible consequences of captivity programs on butterflies, reporting different results. Morphological and reproductive traits of the Large White butterfly (*Pieris brassicae*) were investigated by Lewis and Thomas (2001), who chose this species as a model system since they had access to a population that had been kept in captivity for at least 100 generations. The obtained data showed that the reproductive behavior of the butterflies had adapted to the captive environment. Additionally, they reported that captive individuals were heavier and had smaller wings. It is important to register these changes, as these adaptations could potentially have an impact on demography and dispersal behavior when released back into the wild (Lewis & Thomas, 2001).

Another example of morphological and genetic changes in captivity-bred butterflies can be found in *Heliconius Melpomene*, a species characterized by its distinctive red or black wing patterns found in wild populations. However, a strange wing pattern variation that is not seen in the wild can be found in captivity-reared populations, with white or yellow elements along the hindwing, and even some individuals showing all their scales in these colors, with no trace of the original patterns. This mutation was named “ivory” for the purposes of the study (Fig. 6). While butterflies with small pattern variations showed typical behavior, the ivory butterflies showed that they are unable to fly, nor successfully mate or lay eggs (Hanly et al., 2021).

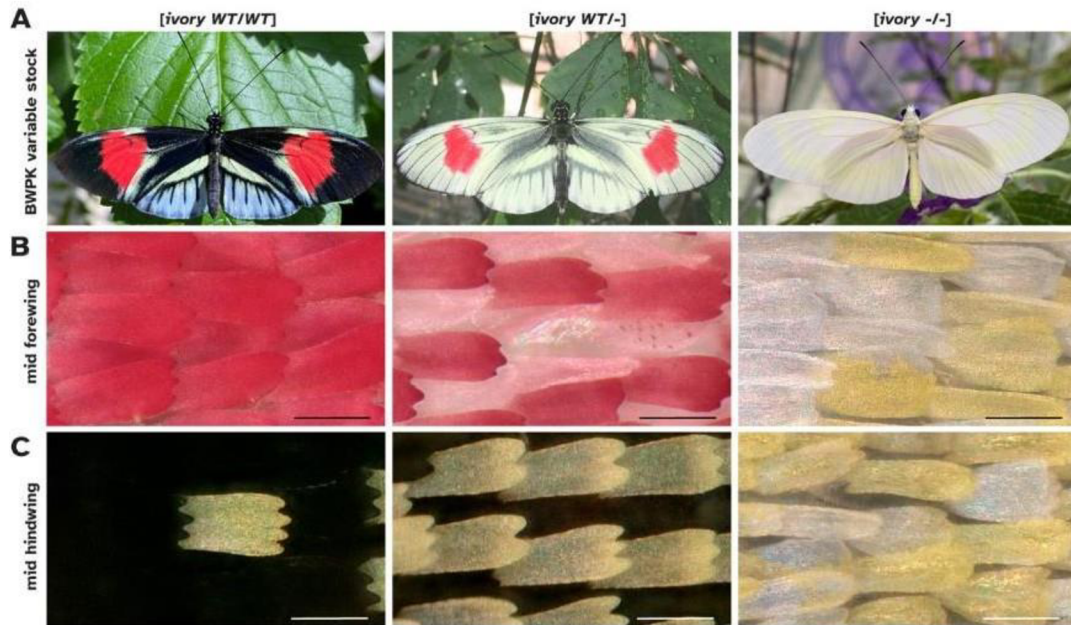


Fig. 6. Changes between generations of the captivity-bred *Heliconius Melpomene* butterfly. (A) Color variations depending on the allelic dosage of a co-dominant mutation. (B) Magnified view of the forewing red band region where abnormal scales formed and even changed color. (C) Magnified view of a central hindwing with usually black scales, which showed a color alteration into yellow or white. Adapted from Hanly et al., 2021.

Seeking to determine a change in genetic diversity, Miller et al. (2014) carried out a study comparing populations of the *Euphydryas editha quino* butterfly in captivity and those in the wild. They compared the data from microsatellites from the captive populations, with that from previous studies that included samples from wild populations. Even though their results did not show any substantial differences in genetic diversity, there was a clear tendency in the captive populations to have a much lower allelic richness when compared to the wild ones. As mentioned, captivity breeding can have impacts on the genetic diversity of the species, and it is then crucial to maintain enough variation to prevent inbreeding. This study suggests a periodic introduction of wild individuals into the captive population, as to help maintain allelic variety (Miller et al., 2014).

6.4 Risks

Threatened species can reach a critical level where the only viable strategy for their conservation is to be reintroduced, as to avoid them being restricted to captivity (Converse et al., 2013). However, there are risks involved, as better described by Anderson et al. (2014), who group the main risks associated with reintroduction into 4 broad categories: a) evolutionary, b) demographic, c) ecological, and d) disease. Similarly, the IUCN Reintroduction Guidelines (2013) describe 7 main risk categories, including financial and socio-economic risks (IUCN, 2013; Anderson et al., 2014). I will now present different risk categories that could arise in reintroductions if they are not approached adequately.

6.4.1 Evolutionary risks

Reintroductions frequently rely on offsite captivity breeding of the individuals (Ren et al., 2014), however, even in a controlled environment there is a possibility that poor sampling could lead to the protected species not having enough genetic diversity and representation, affecting the population with genetic homogenization or inbreeding and outbreeding depression (Anderson et al., 2014; Ren et al., 2014). It is also common for captivity-bred individuals to show a lower fitness than those in the wild as they may adapt to captivity conditions (Kang et al., 2015, as cited in Ren et al., 2014). Therefore, it is a possibility that the release of captive-reared individuals might endanger and limit the long-term viability of the species (Anderson et al., 2014).

6.4.2 Demographic

Demographic risks are those that have the potential to affect the population, such as when individuals from a wild population are removed for reintroduction purposes, leading to a short-term decline in viability and negative effects on the source population and other species in the ecosystem (IUCN, 2013). Population models, which can be used to predict how the population would react to management measures, have the potential to better control these risks (Converse & Armstrong, 2016).

6.4.3 Ecological

Another potential risk can be found in situations where there is an introduction of additional species along with the translocated organisms due to insufficient biosecurity standards, which could lead to the reintroduced individuals becoming invasive in the destination region, potentially causing irreversible damage (IUCN, 2013). For instance, the Cabbage White butterfly (*Pieris rapae*) has been introduced to different parts of the world over the past 160 years and has become an extremely abundant and highly destructive pest affecting crops in the *Brassicaceae* family across the planet (Ryan et al., 2019).

Furthermore, as stated by Banks et al. (2002) ecological risks might also be present in captivity-bred individuals in the form of predation risk, due to their unfamiliarity with the wild surroundings, making them more susceptible to predators, and increasing their short-term vulnerability (Banks et al., 2002).

6.4.4 Disease

Whether the released individuals come from captivity or the wild, there is still a possibility that they carry/suffer from a disease - the worst case scenario in this situation would be for the reintroduced species to fail to establish, and for other species at the release location to suffer declines due to the introduction of new parasites, or even an increase in the existing ones (Ewen et al., 2015). Because captive breeding and rearing systems present a high-density monoculture, they provide a high-risk environment for diseases, commonly caused by bacteria, viruses, fungi, microsporidia, and nematodes (Tanada & Kaya, 1993; Mattoni et al., 2003). Such was the case of the attempt to produce a large amount of Palos Verdes blue butterfly (*Glaucopsyche lygdamus palosverdesensis*) individuals for reintroduction to areas where the species

had been eradicated, as well as insurance against extinction, in which substantial losses were suffered due to a microsporidian infection (Mattoni et al., 2003).

Specific pathogens tend to be highlighted, like the intracellular bacterium *Wolbachia*, an endosymbiont that is transmitted from mother to offspring, present in invertebrates, and mostly known for its manipulations of the cellular and reproductive activities in hosts (Fig. 7) (Salunkhe et al., 2014; Daniels et al., 2018). Other inherited bacteria can be present in Lepidoptera, such as *Arsenophonus*, *Cardinium*, and *Spiroplasma*, with similar effects (Daniels et al., 2018).

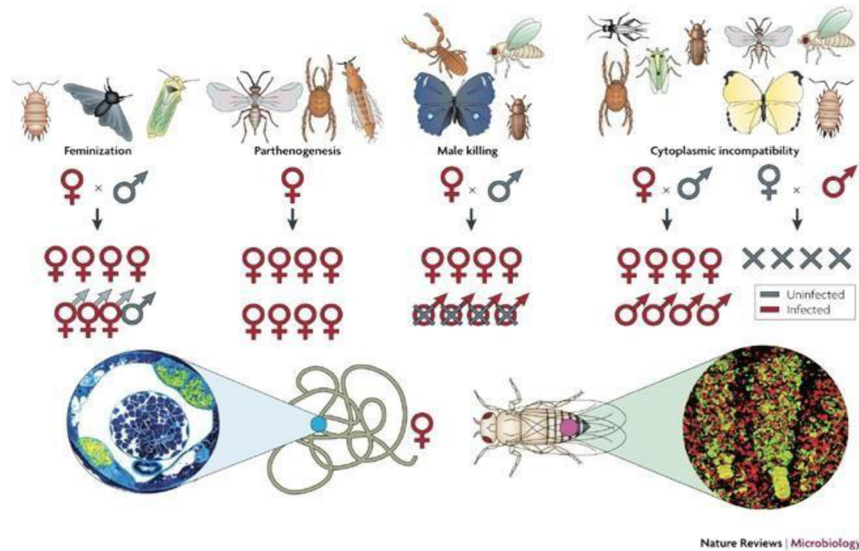


Fig. 7. Effects of *Wolbachia* in invertebrates: feminization affects males developing them into females, parthenogenesis stops males from reproducing, male killing removes males who are infected, and cytoplasmic incompatibility stops infected males from mating with females who are not infected (Werren et al., 2008).

6.4.5 Socio-economical and financial

A reintroduction could affect not only the habitat and species in it, but it could also impact human interests, such as perceived dangers from released plants, animals, and fungi, as well as negative publicity, and the endangerment of ecosystem services and/or food supplies (IUCN, 2013). If a reintroduction were to go wrong and have significant consequences, such as pests, the cost to solve it could be too high and not possible to cover, damaging the reputation of reintroductions, and reducing the probability of future conservation projects (IUCN, 2013).

7. Methodology

Two different types of research have been carried out as part of the process of this bachelor's thesis, in order to produce a complex and comprehensive overview from diverse angles. These methods of research include extensive research on the topics at hand, as well as collecting data from previous reintroduction attempts to analyze the factors that influence their success or failure.

7.1 Literature review

To provide further information on this topic, it was necessary to obtain a clearer understanding of it. To do this, I conducted separate keyword-based searches on different databases, but primarily Google Scholar. Firstly, by using keywords I deemed appropriate such as *conservation genetics*, *non-invasive sampling*, *reintroduction of butterflies*, and *Chazara briseis*, I started a broad search to confirm the availability of relevant information. Once I had a clearer picture of the amount of information I could have access to, I started deeper and more cautious research. I selected and opened the prompted articles and screened them based on how relevant they were to the selected research topic. Following this initial filter, I proceeded to read through the abstracts and/or introductions to determine whether they would provide valuable and relevant information, as well as to gain potential research points that could be valuable for my thesis. Once I had confirmed whether the article could provide suitable information, I then wrote down the details or copied the corresponding link and saved it on a separate document. Finally, I started researching for more detailed information by using the additional information obtained from the read literature, following the same procedure as before. I then created a structure for my thesis, dividing it into chapters and taking notes of relevant information.

As I advanced, additional filters and screenings were put in place, such as prioritizing literature with a focus on conservation studies and/or invertebrates, filtering out papers without a reliable source, and focusing more on those published by science journals and universities. Lastly, and particularly when researching for chapter 6. Reintroduction, I selected and focused on recently published articles or case studies, as to use information as current as possible, considering the fast-paced climate and anthropogenic changes, and to avoid outdated studies (i.e. a reintroduction that was initially declared successful, but the current reintroduced population has disappeared from the location). In the case of chapter 9.1 *Chazara briseis*, some of the research papers and publications with useful information were written in Czech. Therefore, I used online translators to access the information and use it for my research. The last step of the literary research consisted of working directly on the chapters, reading through the articles pertinent to the topic of the chapter, checking notes, and writing. Mainly, I structured my research to provide a general explanation and understanding of the topic and then proceeded to provide a more specific approach. Throughout the writing process, I would still look up relevant articles from which my thesis could benefit, adding more relevant information to provide a complete research.

7.2 Collection of data

After gathering most of my literary research, I began looking for studies describing previous Lepidoptera translocations and reintroductions. Similar to the process I followed in my first research, I conducted separate keyword-based searches on different databases, this time expanding into other databases, such as the "Conservation Evidence Individual Studies repository", and the "Directory of Open Access Journals", and still using Google Scholar if necessary. Using keywords such as

reintroduction, Lepidoptera, and butterflies, I read the titles and descriptions of the suggested articles, reviewed their abstracts and/or introductions, and skimmed the rest of the article if the one I chose seemed like a good match. I then began looking for more in-depth information and gathering the data that I would need for my analysis. The information I looked for was mainly the place where it took place, the type of genetic markers that had been used (if any), the years that passed between the last reintroduction and monitoring, and the result of the reintroduction – in case it had already been determined. Together with this, I added information on the corresponding habitat, the number of released individuals, whether they had been sourced from the wild or were bred in captivity, and the techniques used to determine success before releasing the individuals. I tried to focus on articles, papers and reports with a relatively recent release date – mostly this century - so that the information would be as current as possible. Then, I created a spreadsheet to fill in the information. With the aid of my previous research, I had access to a review of terrestrial invertebrate translocations (Bellis, 2021), which provided me with several Lepidoptera projects that I had not found in my initial search. These projects were also screened in a similar way as initially described, and replaced with more recent studies if it was deemed necessary. If the reintroduction was declared unsuccessful, I took note of the reasons given by the researchers. If there was no information on post-release monitoring being carried out, or there were no clear results on the outcome of the project, I classified the reintroduction as Inconclusive (I). Once I had gathered the desired number of papers, I began the data analysis that would be part of my results.

8. Results

For the purpose of this thesis and to assess the success of reintroduction attempts for the conservation of Lepidoptera, I gathered literature documenting the reintroduction

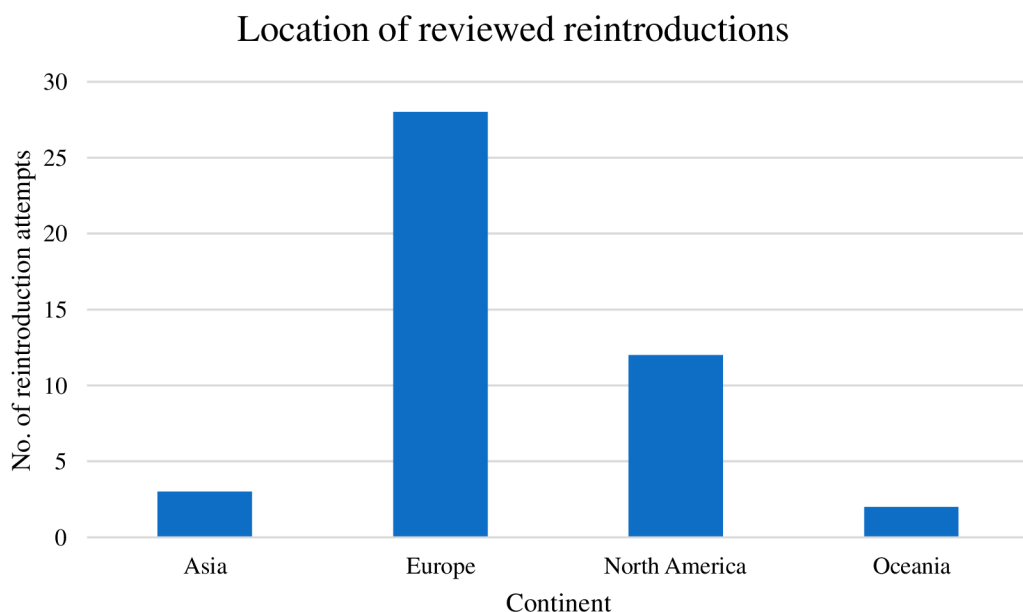


Fig. 8. Number of *Lepidoptera* reintroductions based on the location where they took place (n=45).

of 45 different species (Appendix 1). I compared the location where the reintroductions took place, the species' habitat, the use of genetic markers, the years between reintroductions and monitoring, the habitat, and information on the individuals, such as the number of released individuals, whether they came from wild or captive populations, as well as the result of the reintroduction according to the researchers, and the techniques used to determine success. Since reintroductions should be tailored to the species, my research did not take into account species-specific needs such as interspecific relationships (like myrmecophily) or migration.

The European continent was the site with the greatest number of Lepidoptera reintroductions (n=28), followed by North America (n=12). In third and fourth place are Asia (n=3) and Oceania (n=2) respectively (Fig. 8). Unfortunately, I was unable to find any information on reintroductions carried out in Africa or South America. It is important to take note of the great amount of conservation reintroductions carried out in the United Kingdom, as it accounted for over half (57%) of the reintroductions in Europe. Similarly, all of the reintroductions recorded for North America took place in the United States of America, where it seems to be rising in popularity as all of these reintroductions were carried out after the year 2000. While researching, I also found several action plans from the last decade where reintroductions were being designed for endangered Lepidopterans, which will possibly be carried out in the future.

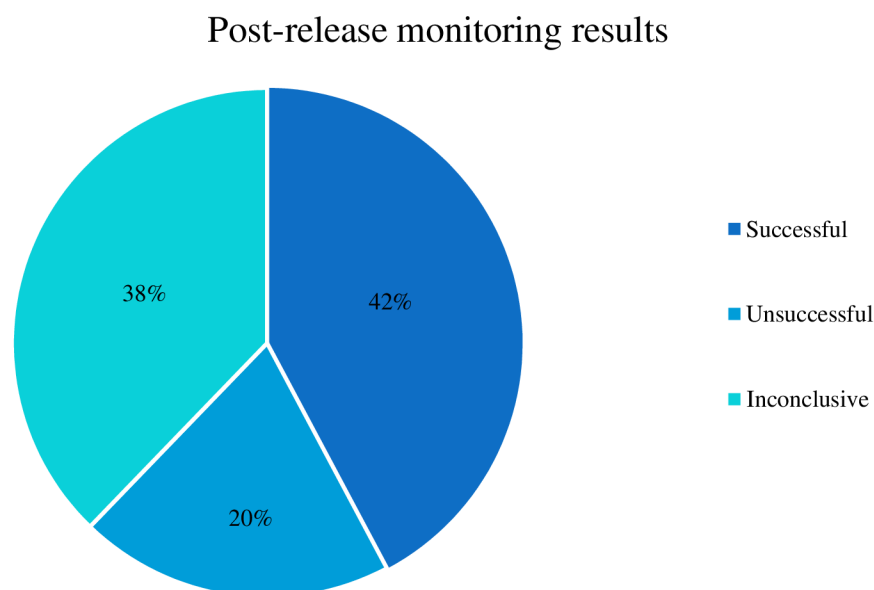


Fig. 9. Results of the reintroductions based on post-monitoring assessment.

The results obtained from the comparison of reintroduction studies and the collected data were very diverse, strengthening the statement that all reintroductions are species-specific, and therefore, the preparation and steps should be tailored to suit particular

requirements. For instance, 58% of the reintroduction attempts that were determined as successful had a period of approximately 10 years between the release and the most recent monitoring, thus reinforcing the previous parameters and definition of reintroduction success provided. From the remaining successful studies, 26% were monitored in a period from 5-9 years, and would therefore require further assessment to determine whether the long-term establishment of the population was successful or not. It is possible that they were considered successful by the researchers seeing that some of the particular goals of the study were achieved.

Reasons behind inconclusive results

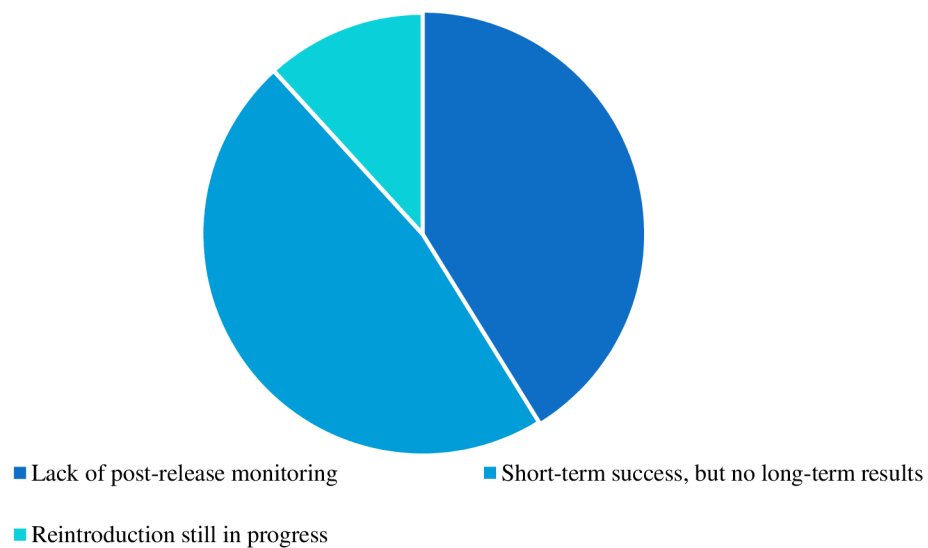


Fig. 10. Reasons why reintroductions were categorized as inconclusive based on the results seen post-release (n=17).

Evaluating the results obtained for each study after the release of the species, 19 of the reviewed studies were determined to be successful (42%), 9 were unsuccessful (20%), and 17 were classified as inconclusive (38%) (Fig. 9). The main cause of reintroductions being classified as inconclusive was the lack of post-release monitoring to evaluate the long-term establishment of the population adequately (Fig. 10). Out of the 17 reintroductions that fall under this category, 47% of them were considered as short-term successes (n=8) in the immediate years after the release, but lacked long-term monitoring to confirm the overall success. Additionally, some reintroductions were still in progress or had just been completed recently (n=2), and it was considered too soon to determine their success. The remaining reintroductions (n=7) were also missing the necessary monitoring after the release, but are different from the previous categories as they showed no indications of success early on, constituting 41% of the total studies in this category.

In the case of unsuccessful reintroductions, the reasons behind the lack of success were diverse, varying from inappropriate management of the location (n=3) leading to unsuitable conditions for the new population to establish, to bad weather conditions

(n=1), and death of the introduced eggs due to a parasitoid (n=1). The remaining studies were unable to determine why the reintroduction had failed, with unknown or unspecified reasons accounting for 44% of the cases in this category (Fig. 11).

Reasons behind unsuccessful results

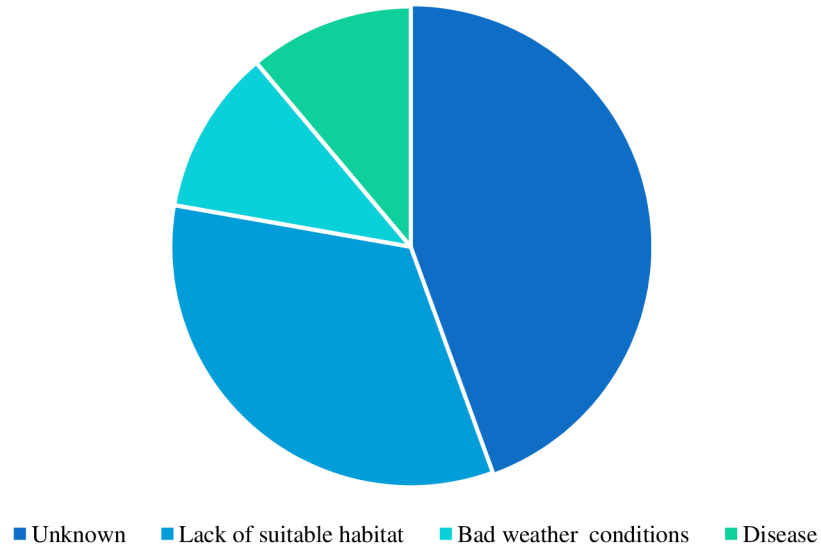


Fig. 11. Reasons why reintroductions were categorized as unsuccessful based on the results seen post-release (n=9).

Additionally, I recorded the number of reintroduction attempts that used genetic markers, if any, and which ones if it was the case (Fig. 12). Out of the reintroductions that were reviewed, it was only the minority, 9%, that did use genetic markers in the process (n=4). Out of these, all of them used microsatellites, and one used a combination of microsatellites and mtDNA. None of the studies recorded used SNPs.

Use of genetic markers

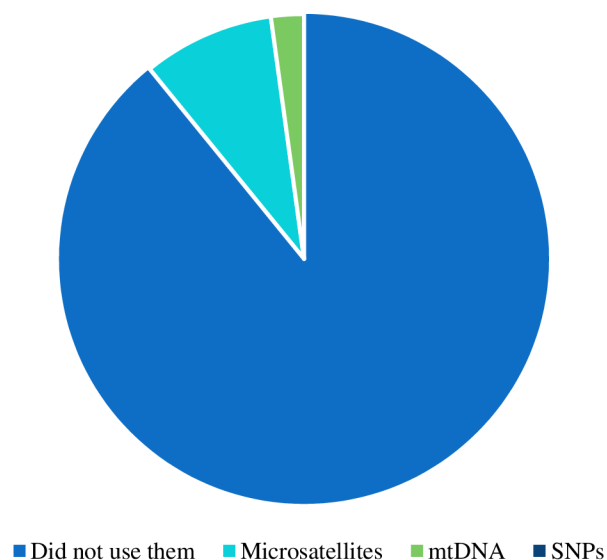


Fig. 12. Use of genetic markers in reviewed reintroduction studies (n=4).

Likewise, I collected information on the different habitats corresponding to the different species and where the reintroductions took place. The majority of species belong to different types of grasslands (n=29), ranging from coastal grasslands, to dry grasslands and sand grasslands, adding up to 64% of the total studies. Other habitats recorded include bedrock (n=2), woodlands and forests (n=14), wetlands and bogs (n=4), savanna and sandhills (n=2), and scrublands (n=2). Furthermore, I recorded information on the source individuals – whether they came from captivity or wild populations, together with the number of individuals released and the translocation techniques (pupae, caterpillars, or adults), if available. In terms of source individuals, the results showed an almost equal division between those coming from captivity (42%) and those from the wild (38%), with only one study using both of them. The rest of the studies did not specify where the source individuals were taken from (n=8), as shown in Figure 13.

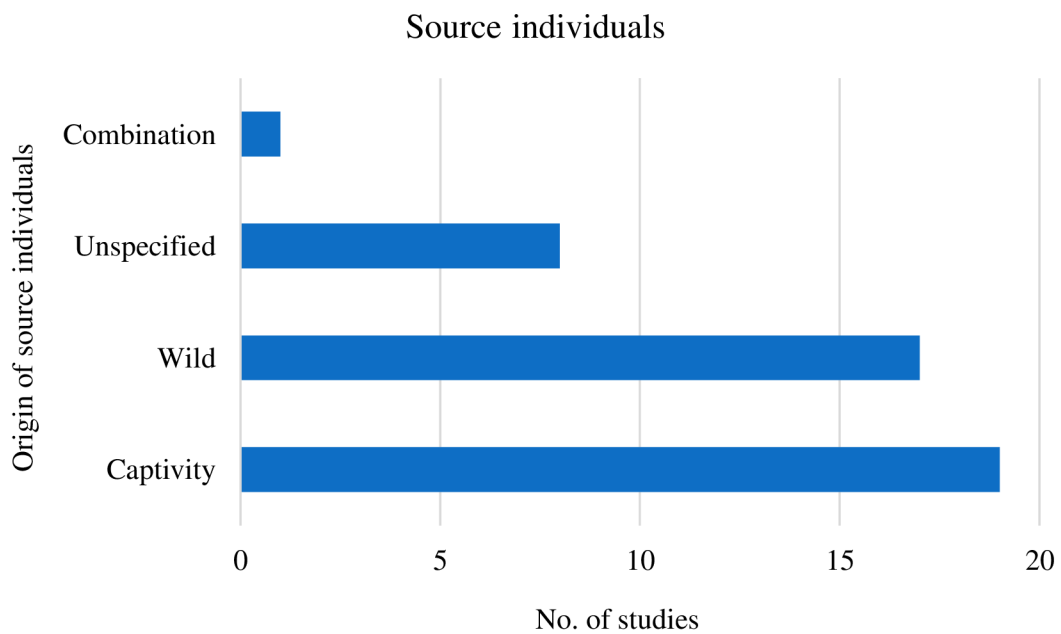


Fig. 13. Chart showing the count of chosen origin of source individuals for the different reintroduction studies.

As for the released individuals, I obtained a wide variety of results, both in the number of individuals released in each reintroduction and in the techniques used (Fig. 14). The individuals released ranged from eggs (n=1) to larval nests (n=2), caterpillars (n=13), pupae (n=2), and adults (n=8). Besides these categories, some studies did not specify the type of individuals released, but only the number (n=4), and some did not provide any information at all (n=9). Lastly, there were also studies in which there was a combination of individuals released (n=6). The number of individuals released recorded in the reviewed studies was also very diverse, with the lowest amount being a total of 4 adults released, and the highest going up to 42,000 caterpillars. Almost half of the studies released less than a thousand individuals (48%), while a quarter of the studies did not indicate the number of individuals released (24%). The remaining

studies released individuals on ranges going from 1,000-4,999 (n=9) to 5,000-9,999 (n=1), and above 10,000 individuals (n=2).

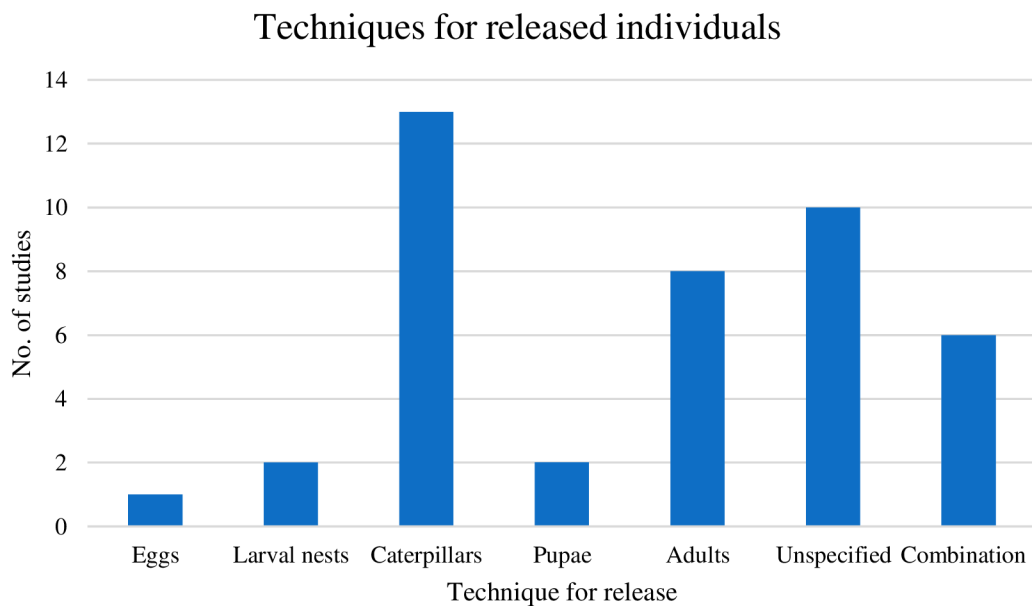


Fig. 14. Chart displaying the techniques for released individuals and the number of studies that recorded these categories.

9. Discussion

With several Lepidoptera species often being considered to be ideal umbrella species, they are a popular focus group for conservation strategies (Legal et al., 2020), and it was therefore not difficult to find relevant reintroduction studies for this order. However, even with the amount of literature available, there were still certain obstacles present while carrying out this research, one of them being the lack of details present in said studies. Over a third of the reviewed reintroductions were determined to be successful by their researchers, which I found quite surprising, considering the risks behind them and how sensitive some species can be to changes in their habitat. Regardless of the number of successful reintroductions, there is still a lot of work to be done in the design and execution of these programs, as the amount of unsuccessful (20%) and inconclusive (38%) reintroduction attempts accounted for over half of the total in my research. It is important to take into consideration that these results only represent the available literature and therefore there is a possibility for the success ratio not to be reflective of all Lepidoptera translocations.

The main reason behind reintroductions being declared unsuccessful was the lack of suitable habitat for the new population to settle, due to inappropriate location management, adding up to a third (33%) of the studies in this category. Understanding the target species' environmental needs such as the overall landscape features, and the distribution of host plants and nectar sources is therefore essential to increase the chances of reintroduction success (IUCN, 2013; Daniels et al., 2018). Adding to these requirements, climate conditions have to be suitable, as they can lead to the failure of

reintroductions too (e.g. Marttila et al., 1997). This is a sensitive requirement nowadays, considering the effects of climate change on the ecosystems and species, particularly habitat specialist Lepidopterans. However, the most striking result from this analysis was the lack of reasons given for the failure of the majority of the reviewed reintroductions (44%). Perhaps the number of released individuals was not high enough, or the conditions of the captivity-bred populations did not prove to be of sufficient quality to establish in the new location, due to inbreeding. There is a possibility that the ecological risks were not evaluated, and the reintroduction failed as a result of competition with other pollinators, or predation. Assessing the risks before reintroducing the individuals can improve the chances of success, and it should be documented as an important part of planning for the reintroduction. Furthermore, knowing why previous reintroductions were not successful can be as important as knowing the steps to proceed with a reintroduction, since it gives examples of what not to do, and narrows down the possibility of failure. In the same way, monitoring the reintroduced population years after the release is key, because it is necessary to assess the individual's current state and performance, whether any additional management decisions are required, and ultimately determine the results of the reintroduction program (Daniels et al., 2018). This research's results show that a third (33%) of the total reintroductions did not provide further post-release monitoring. The reasons for the lack of monitoring are still unknown. There is a possibility that this information was not available due to a shortage of funds, or that the researchers chose not to disclose this information because the reintroduction was ultimately deemed a failure. Regardless, there is a lot to learn from the past and mistakes, and therefore, making this type of data available is not only suggested but strongly encouraged.

Despite the majority of reintroductions reviewed using captivity-reared individuals as their source and contrary to my expectations, over half of the successful reintroductions (58%) released individuals from wild sources and not from captivity. According to these findings, it could be assumed that reintroductions with source individuals obtained from wild populations are more successful than those with source individuals from captivity. But, analysing the reintroductions that were determined to be inconclusive or unsuccessful, only one study was declared as failed because of complications with the captivity-rearing process, while the rest of them provided different reasons for their results (Figure 10 and Figure 11). This could raise the question of whether individuals being sourced from wild populations or captivity would in fact have any influence on the success of the reintroduction. Perhaps the majority of successful studies using wild-sourced individuals could be explained by the fact that these reintroductions first took place, on average, 17 years ago, and the donor populations could have been strong enough to provide sufficient individuals at the time. However, it is important to consider the recent and continuous decline in ecosystems and biodiversity loss (Brondízio et al., 2019; Kardos, 2021), and that populations nowadays might not be able to endure the loss of more individuals. Consequently, it is key for captivity-breeding programs to be improved and perfected,

as they could be the only alternative solution to strengthen current populations or aid in future translocation attempts (Lewis & Thomas, 2001; Crone et al., 2007).

The majority of the successful reintroductions released individuals in stages earlier than adulthood, mostly caterpillars and eggs, and showing only 38% of the studies released adults. With this in mind, one can assume that there is a higher possibility of success when releasing caterpillars or eggs. When assessing the reintroductions to establish the relationship between the number of individuals released and the chances of reintroduction success, the results were inconclusive. While it is true that the 2 reintroductions with the highest numbers of released individuals were successful (e.g. Boggs et al., 2006; Davis et al., 2021), the rest of the reintroductions do not show particularly large numbers of released individuals when compared with unsuccessful or inconclusive attempts. In fact, over half of the successful reintroductions released less than 1,000 individuals (58%), with as low as 10 butterflies released. It is commonly recognized that if the reintroduced population has a larger number of source individuals, then it will be less susceptible to the impacts of genetic diversity loss and inbreeding depression, as well as stochastic events, and therefore the chances of reintroduction success and long-term establishment will be higher (Turlure et al., 2014; Bellis, 2021). However, this review did not display a substantial difference in results. This could be clarified with a larger study sample, or even if the studies that did not specify this information were to make it available.

After proving the importance of genetic diversity for the long-term survival and establishment of Lepidoptera populations, I did not expect to find that such a low number of reintroduction studies used genetic markers. While microsatellites were used in all of them, giving reason to believe that they are the preferred marker for reintroductions, the sample size is too small to determine this, and would therefore need more extensive research before making conclusions. The results are varied, with a 50/50 division of the use of genetic markers between successful reintroductions and those that are inconclusive. Both of the inconclusive reintroductions that used them (e.g. Daniels, 2009; Nakahama & Isagi, 2017) lack further monitoring, which would be necessary to strengthen the relationship between reintroduction success and the use of genetic markers.

Finally, I looked into the techniques used to monitor the success of the reintroductions, before the release of the individuals. In other words, what parameters were determined by the researchers to declare the reintroduction a success? I was surprised to see that only 20% (n=9) of the studies provided this information, as in my eyes it is helpful to set goals to reach when carrying out a research or project. The reintroductions that set these techniques are almost equally distributed among the successful (55%) and inconclusive (45%) reintroductions, with none recorded for those that failed. Setting these kinds of goals for monitoring success can facilitate the research in its later stages, as it could indicate what to look for, what could be missing, or even work as an additional motivation for researchers not to omit this crucial step. Similarly, it could also aid in narrowing down the particular needs of the species to be reintroduced. Based on the obtained results, establishing these techniques pre-release could

influence the success of the reintroduction. From the inconclusive reintroductions with this parameter, all but one were declared successful in the immediate years after the release. Of course, long-term monitoring would be needed to determine the success of the reintroduction, but it can be seen as a positive start. The techniques described in the reintroductions were varied, with some specifying the presence of individuals over a certain period as a goal (e.g. Fred & Brommer, 2015; Kukkonen, 2021), and others focusing more on reaching a certain number of individuals (e.g. Soorae, 2018). Still, as expected, they all coincided in their ultimate goal: the persistence of individuals, whether at the release site or with evidence of dispersal.

9.1 *Chazara Briseis* – distribution, ecology, threat and conservation management

Chazara briseis, also known as The Hermit, is a relatively large butterfly part of the Lepidopteran family *Nymphalidae* (Fig. 15). It lives on warm grasslands with exposed bedrock, particularly short-stemmed grasslands, ideally steppe (Kadlec et al., 2010; John et al., 2018). It is a univoltine butterfly, meaning it only has one generation per year, with a relatively long life. Females lay individual, white eggs on few and sparse drying patches of short fescue grass (*Festuca ovina* L. agg.), or rocks and pebbles in the surrounding area of them (John et al., 2018). The caterpillars feed on various types of grasses but mainly fescue grass and occasionally meadow brome (*Bromus erectus*) (Vrba et al., 2021). Adults use patches with taller stands with flowers as a source of nectar, sucking on nectar-producing plants such as cream scabious (*Scabiosa ochroleuca*), thistles (*Carduus* spp.), and centaury (*Centaurea* spp.) (Vrba et al., 2021).

Chazara briseis individuals require extensive territories to form populations and to assure their survival, and will disperse if the location is smaller than 1 ha (Kadlec et al., 2010). Additionally, only about a quarter of the recorded individuals will successfully establish a new generation, decreasing the effective population by half (Kadlec et al., 2009; Vrba et al., 2009). It is considered a valuable bioindicator of short-

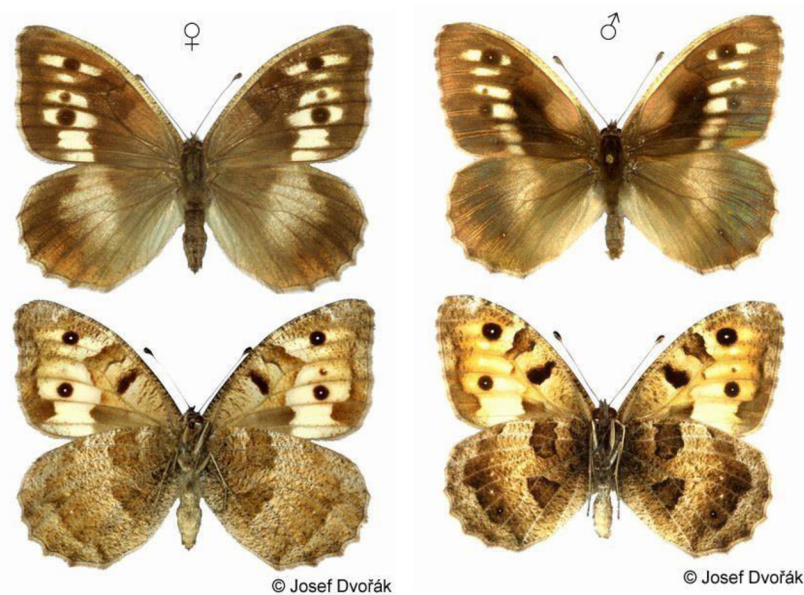


Fig. 15. Female and male specimens of *Chazara briseis* (Dvořák, n.d.; lepidoptera.cz).

stemmed steppes, and its protection will guarantee the efficient protection of other threatened steppe grassland plants and animals (John et al., 2018).

Chazara briseis is found in southern and central Europe, as well as North Africa, southern Russia, Turkey, and Iran, and through Asia Minor to northern China (Kadlec et al., 2010; John et al., 2018) (Fig. 16). It has suffered a fast decline from most of Central Europe, with only one metapopulation remaining in the Czech Republic, located in České středohoří (Central Bohemian Highlands) (Bartoňová et al., 2021) (Fig. 17). While the last assessment from the IUCN Red List of Threatened Species for *Chazara briseis* listed it as Near Threatened in Europe, with a decreasing population trend (Van Swaay et al., 2010), the local Red List for the Czech Republic listed it as Critically Endangered in their 2017 assessment (Hejda et al., 2017), only to confirm the fast decline that the species has suffered in the Czech Republic. In 2006, there were approximately 1800 individuals in the metapopulation, according to Vrba et al. (2009); but by 2017, the number was only determined to be around 400-500 (Baranovská & Moravec, 2020; Vrba et al., 2021). Additionally, previous records had registered 106 cells in the period from 1951-2000, while later on it was stated that in 2020 only a single metapopulation occupying 8 steppe patches and covering 2 grid cells was located in the Czech Republic, showing a decline of over 98% in the last 20 years (Bartoňová et al., 2021). The main cause of the decline is the loss of suitable habitat due to a decrease in grazing activities, as well as degradation from the invasion of non-native species and inappropriate care of the area (Vrba et al., 2009).

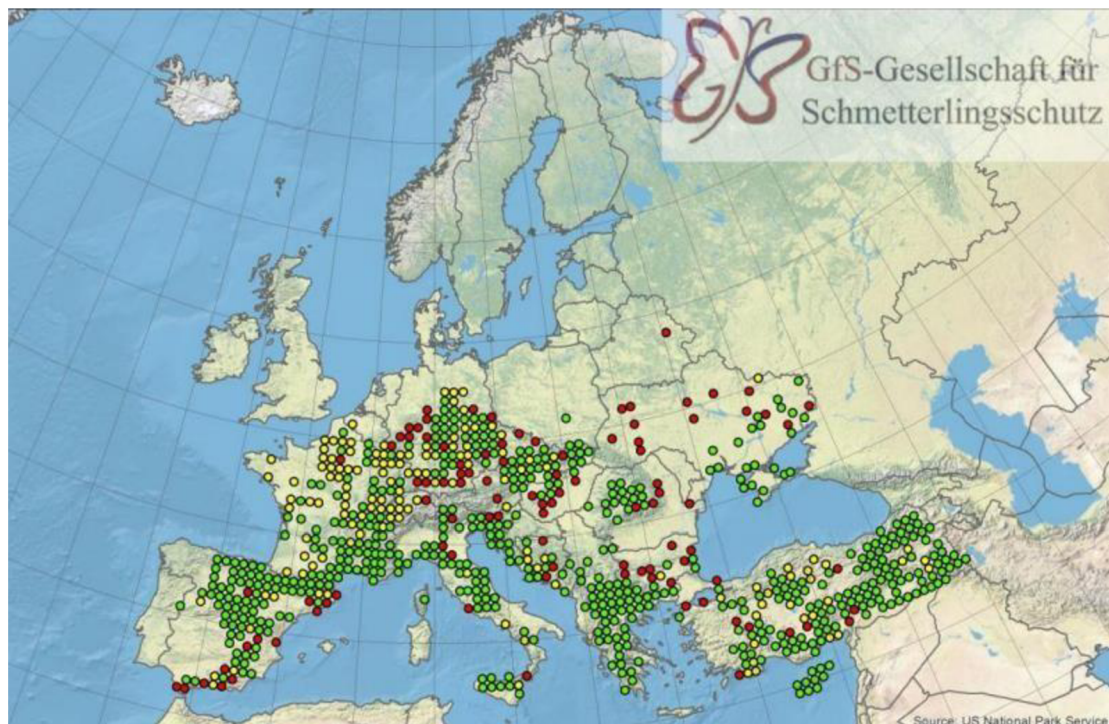


Fig. 16. Map showing the distribution of *Chazara briseis* in Europe. Adapted from Kudrna et al., 2011 (ufz.de).

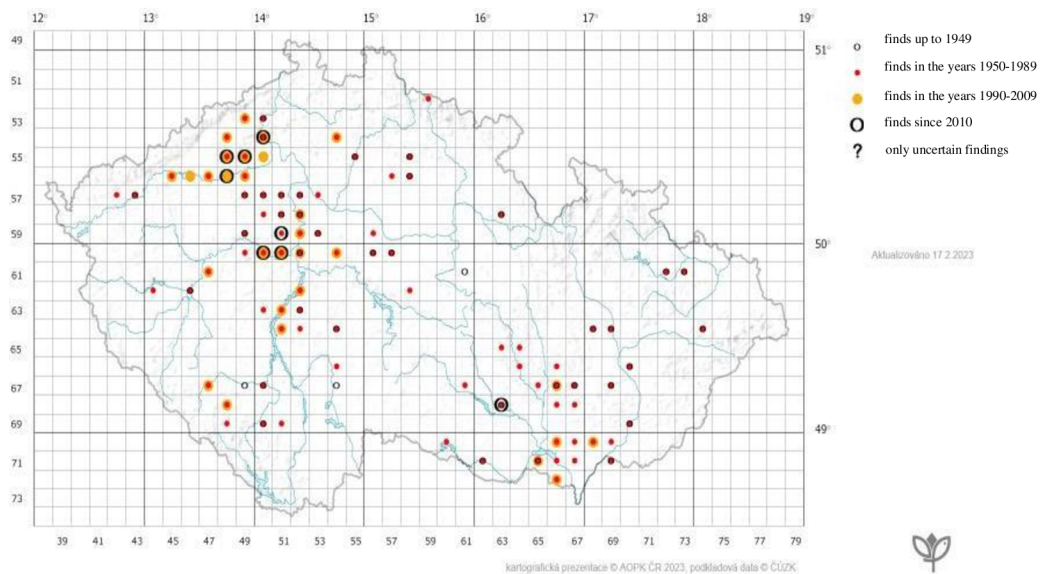


Fig. 17. Map showing the occurrence of *Chazara briseis* in the Czech Republic. Adapted from Nature Conservation Discovery Database of the Agentura ochrany přírody a krajiny České Republiky (AOPK, 2023).

Conservation efforts have been in place since 2006, and have been applied not only to the species itself but also to the habitat, in the form of habitat restoration with strategies such as sheep grazing, manual mowing of coarse patches, litter removal, and raking of the ground (John et al., 2018; Bartoňová et al., 2021). Great efforts such as that from the project LIFE+ Steppe Lounského středohoří are worth mentioning. This project was set in motion with the main goal being the restoration of steppe lawns and it was considered to be successful, covering a total area of 320 ha, by mowing and grazing with sheep and goats, as well as removing certain trees (John et al., 2018). Together with this, offsite breeding is in place, and extra eggs, larvae, and adults have been moved back yearly to the previously managed patches close to their original site, as explained in detail by Bartoňová et al. (2021). As part of the captivity-rearing process, since 2017, two wild-caught butterflies have been added yearly to the ex-situ population, to avoid genetic influences like inbreeding, leading to the mortality rate significantly decreasing. Additional releases have been successful in the historically occupied site of Český Kras (Bohemian Kras), in the following localities: Třesina (approximately 50-60 individuals), and Radotín (around 20-30 adults counted). Another attempt took place in Zlatý kůň, but unfortunately, it failed to establish (Bartoňová et al., 2021).

The Regional Action Plan (RAP) for *Chazara briseis* was published in 2018, with the latest report on its implementation released in 2022 (Fig. 18). The previously mentioned conservation measures both for habitat restoration and captivity breeding are still active, with no genetic anomalies detected at the time (Andres et al., 2022). Additionally, there is an ongoing genetic study, and while the complete results have not been published yet, preliminary results have shown the genetic variability has been relatively preserved (John et al., 2018; Andres et al., 2022). Reintroductions to the

locations Církvice and Radobýl have been carried out, both considered relatively successful and promising (Andres et al., 2022), but of course, further monitoring is necessary to determine the ultimate success of the reintroduction. Additionally, new potential locations have been determined, these being Kalvárie and Vraníky. With these efforts in mind, it is expected that the populations will grow stronger in the next year (Andres et al., 2022).

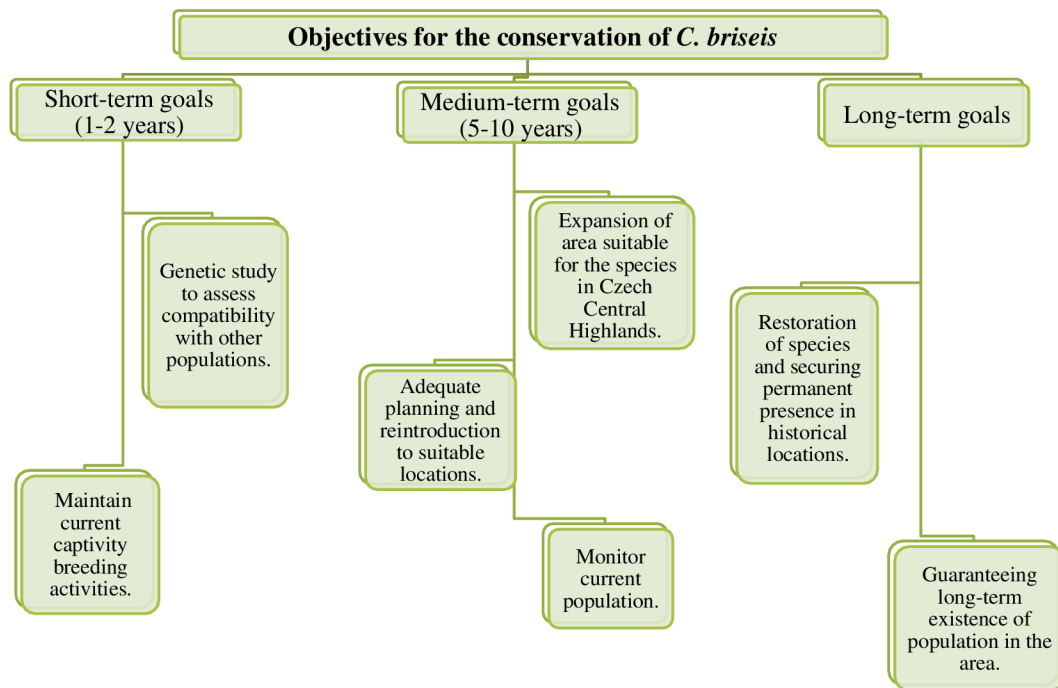


Fig. 18. Summary of the objectives for the conservation of *Chazara briseis* according to the Regional Action Plan created in 2018 (John et al., 2018).

9.2 Suggestions for future reintroductions and conservation efforts

According to the reviewed Lepidoptera reintroduction projects, it is possible to establish that habitat quality and continuous management, along with knowledge of the species requirements for the landscape features and climate conditions, and establishing clear techniques for success monitoring throughout the reintroduction, are the main factors influencing the outcome of these projects. Other important factors include the life stage of the released individuals, and quite possibly the number as well, even though a sample this size was unable to determine clear results. With this in mind, it is possible to suggest strategies that could have a positive impact on future reintroductions for *Chazara briseis*.

As stated in the RAP for *Chazara briseis*, habitat restoration is currently in place and has been for some time, always taking into consideration the species' needs as they are extensively researched. Similarly, and seeing how detailed and well-planned the RAP is, the techniques for success have been determined clearly (Fig. 18), which should

prove to benefit the outcome of future reintroductions. While it is still too early to determine the success of previously carried out reintroductions for this species, it could be recommended to release individuals as caterpillars or eggs, instead of adults. In addition, and perhaps on a personal note, involving local communities in the restoration and reintroduction efforts can also be beneficial as it can increase awareness and support for conservation efforts, leading to more effective management of the restored habitats. Without a doubt, continuous and long-term monitoring is strongly recommended, as it is crucial to track the success of the reintroduction as well as to determine any potential threats and to include them in the decision-making process. Together with this, it is highly advisable to carry on with the ongoing genetic study described in the RAP, to investigate the genetic diversity not only of the captive populations but of those who have been reintroduced. Maintaining a high genetic diversity is key to keeping highly resilient populations, and increasing the population's adaptability to changing conditions. The sustainability and resilience of *Chazara briseis* populations also depend on migration among specific populations, which must function as metapopulations. Techniques that can support these functional metapopulations include genetic monitoring and capture-recapture methods, which are currently in place (Baranovská & Moravec, 2020). Seeing their popularity in the reviewed reintroductions, and their great results in other butterfly genetic diversity studies (e.g. Andersen et al., 2014; Miller et al., 2014), microsatellites and SNPs could be seen as a promising choice for genetic markers. Perhaps for the individuals in captivity, it could be possible to follow the previously presented non-invasive DNA sampling techniques, by using frass, chorion from residual eggs, or exuviae, as it has been proven to be effective. Alternatively, and possibly more useful for field studies, non-destructive sampling is recommended, aiming to have as little disruption on the individuals as possible. For other Lepidopteran *Nymphalidae* family members (*Vanessa cardui* and *Satyrodes eurydice*), removing minor parts of the hind wing has been successful since it did not affect their behaviour or survival while still supplying enough DNA in field tests, as seen in the study by Hamm et al. (2009). However, this sampling method would undoubtedly need to be tested on *Chazara briseis* to guarantee that it has no harmful effects on the individuals. Lastly, I recommend maintaining the results of the ongoing monitoring programs and any further research as available to the public as possible. In this research, I encountered plenty of reintroductions that were not fully documented and left important details inconclusive, possibly because of the outcome of the reintroduction itself, or because the efforts stopped abruptly. Regardless of the reason, having this information available and in the open has the potential to be of great help to future reintroduction studies.

Overall, the combination of perfected captivity rearing, ongoing genetic research, involvement of local communities, and education and outreach programs can greatly increase the chances of successful restoration and reintroduction of *Chazara briseis*. However, it is important to carefully monitor and manage these efforts to ensure the long-term sustainability of the species and its habitat.

10. Conclusion

My goal was to determine the factors influencing the success of Lepidopteran reintroductions. By reviewing 45 previous Lepidoptera reintroduction attempts, I was able to achieve it, identifying not only the elements that have an impact on the outcome of said reintroductions but also the challenges faced and aspects that need to be improved. By learning from these experiences, the success and sustainability of future reintroduction efforts can be improved, thereby helping to restore populations of endangered, threatened, and extirpated species to their native habitats.

Through my research, I identified a strong relationship between the management and quality of the habitat and reintroduction success. Additionally, I found that projects that had clear techniques to monitor the success of the reintroduction throughout the process proved to have successful outcomes. I also found that reintroductions had a higher success rate when releasing individuals as caterpillars and eggs. Surprisingly, the use of genetic techniques was quite low in the reviewed reintroductions, with microsatellites being the common genetic marker, and further research would be needed to determine whether they have a direct influence on the outcome of the reintroduction itself. Lastly, I identified that the main complication with Lepidopteran reintroductions is the lack of information available to the public, which goes hand-in-hand with the lack of post-release and long-term monitoring. Sharing the results of reintroductions, regardless of the outcome, could facilitate future conservation efforts.

Concretely for *Chazara briseis*, there is plenty of information available on its basic biology, habitat requirements, and current state and conservation measures, which is a great advantage and starting point. The species' current Regional Action Plan has clear and well-defined goals, and it shows continuous monitoring, which can have a positive influence on the success of future reintroductions. The ongoing habitat restoration efforts in the Central Bohemian Highlands in the Czech Republic should also prove to be helpful and provide a suitable location for new *Chazara briseis* populations to establish.

I believe that the combination of the current action plan, the success levels of the reintroductions in progress, how the captive breeding process has been mastered, as well as the active habitat restoration, are all positive indicators for the future of *Chazara briseis*.

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Figure 1: Lefort, M., Cruickshank, R., Descovich, K., Adams, N., Barun, A., Emami-Khoyi, A., Ridden, J., Smith, V., Sprague, R., Waterhouse, B. & Boyer, S., 2022: Blood, sweat and tears: a review of non-invasive DNA sampling. Volume 2. doi:10.24072/pcjournal.98.

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Figure 3: New hatched Small tortoiseshell (*Aglais urticae*), a butterfly of the Nymphalidae family, close to its exuvia, Switzerland. GFC Collection, Alamy Stock Photo (online) [cit. 2023.02.17], available at: <https://www.alamy.com/new-hatched-small-tortoiseshell-aglais-urticae-a-butterfly-of-the-nymphalidae-family-close-to-its-exuvia-switzerland-image216145167.html>.

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11. Appendix

11.1 List of reviewed Lepidoptera reintroductions

CZECH UNIVERSITY OF LIFE SCIENCES

Faculty Of Environmental Sciences

Reintroduction and conservation genetics of butterflies

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2023