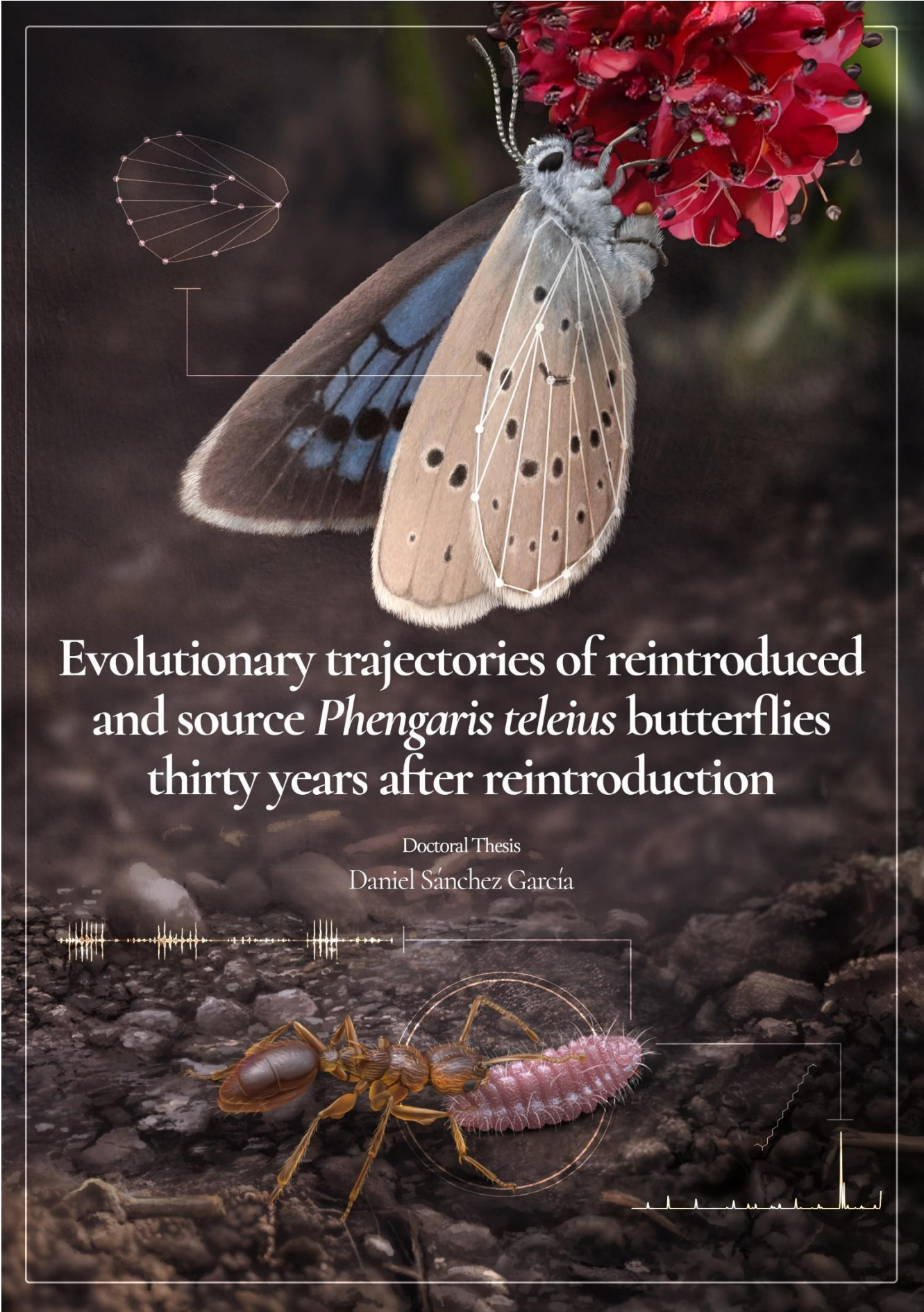

Evolutionary trajectories of reintroduced and source *Phengaris teleius* butterflies thirty years after reintroduction

Doctoral Thesis

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Manuscript 1. Sánchez-García, D., Wynhoff, I., d'Etorre, P., Leroy, C., Kajzer-Bonk, J., Maák, I., Barbero, F., Casacci, L.P. & Witek, M. Ongoing coevolution between reintroduced *Phengaris teleius* butterflies and their *Myrmica* host ants. In preparation for submission.

Manuscript 2. Sánchez-García, D., Wynhoff, I., Kajzer-Bonk, J., Sztencel-Jabłonka, A., Nowicki, P., Casacci, L.P. & Witek, M. Temporal and spatial variation of morphological traits and genetic structure in *Phengaris teleius* myrmecophilous butterflies following habitat changes three decades after reintroduction. Under review in *Global Ecology and Conservation*.

Manuscript 3. Sánchez-García, D., Casacci, L.P., Wynhoff, I., Chiara, V. & Witek, M. Changes in the wing spot pattern of the endangered butterfly *Phengaris teleius* thirty years after its reintroduction. Under review in *Insect Conservation and Diversity*.

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Abstract

Populations respond to environmental changes through biological adaptations. Specialists, which rely heavily on specific ecological niches or interaction partners, face a rapid decline in their populations due to misadaptation to changing conditions when they are unable to adapt quickly enough. Thus, host-parasite systems are particularly vulnerable to extinction, due to parasite host dependency and low resilience to replace lost interactions. Reintroductions serve as a conservation tool to restore species loss and ecological processes after extinction. Additionally, successful reintroductions offer insights into evolutionary changes in new habitats, as a tool to learn whether and how organisms can deal with new environmental conditions. Despite the complexity of butterfly reintroductions, it has been one of the more popular taxa being reintroduced in the last decades, notably successful with *Phengaris* species reintroduced in the UK and Netherlands. *Phengaris* species, serving as biodiversity indicators, exhibit a specialized lifecycle as social parasites of ants, with adaptations facilitating integration within host colonies. Their lifecycle complexities underscore the importance of considering both the parasite and host species in conservation strategies. They rely on specific host plants and *Myrmica* ants. Mimicking chemical and vibroacoustic signals of ants, they infiltrate and integrate into the host colonies. *Myrmica* species differ in cuticular hydrocarbon profiles, which is the main nestmate recognition mechanism. Thus, *Phengaris* caterpillars need to mimic specific host ant chemical profiles to deceive ant workers of their *Myrmica* host species and facilitate adoption into the colony. Geographical variations in host specificity demonstrate a mosaic of coevolution, highlighting the spatially diverse nature of species interactions and adaptations. While evidence supports this pattern in cuckoo species, such as *Phengaris alcon*, it remains unknown for the most generalist predatory species, namely *Phengaris teleius*.

After the extinction of *P. teleius* in the Netherlands in 1976, a successful reintroduction occurred in 1990, involving the translocation of 86 butterflies from Poland to the Moerputten nature reserve in the Netherlands. The reintroduction effort resulted in the establishment of a metapopulation with thousands of individuals. Three decades later, this study aimed to assess potential changes in adult butterflies and caterpillars between the source and reintroduced metapopulations. The study encompasses chemical, vibroacoustic, behavioral analysis in caterpillars and ants; and morphological and genetic analysis in adult butterflies. In addition, the morphological changes in adult butterfly hindwings were evaluated spatially and temporarily by integrating data from current butterflies with historical data of individuals from the source and reintroduced metapopulations. The study provides insights into ongoing coevolution processes, adaptations to new conditions, and genetic impacts of reintroduction. We hypothesized that chemical profiles and vibroacoustic signals in caterpillars differ between metapopulations, resembling those of their sympatric *Myrmica*

scabrinodis host ants. We also predicted more successful adoption and increased survival for caterpillars exposed to local host ants. Biotic and abiotic conditions likely influenced morphological traits differently between metapopulations, with the reintroduced metapopulation possibly exhibiting lower genetic variability. Moreover, metapopulation connectivity likely impacts selection pressure on butterfly morphology and dispersal. Additionally, factors like sexual selection, predation, and developmental stress may affect the hindwing spot pattern differently between metapopulations.

Our findings indicate that the reintroduced caterpillars differ in their chemical and vibroacoustic signals compared to their source metapopulation after 30 generations since the reintroduction. Notably, the reintroduced metapopulation emitted vibroacoustic signals more akin to those of their sympatric ant hosts, suggesting potential for local adaptation. However, our analysis did not uncover any evidence of improved performance in chemical mimicry. The adult butterflies also present differences among metapopulations. The Polish butterflies from the current source metapopulation exhibited greater body weight and thorax size compared to the reintroduced ones. They also had the largest hindwings among all studied metapopulations (current and historical). The wing shape and spot pattern variation also differed between metapopulations. Metapopulation connectivity changed over time, decreasing slowly in Poland, but sharply increasing in the Netherlands after habitat restoration. Moreover, the genetic analysis revealed differences in allelic richness, indicating a founder effect and bottleneck in the reintroduced metapopulation, with clear genetic structure differentiation among metapopulations and lower effective population size in the reintroduced metapopulation compared to the source one.

The study highlights the adaptability of *P. teleius* across its life stages, showcasing the capacity of the caterpillars for coevolution and adaptation to new ant host metapopulations. Furthermore, morphological changes in adult butterflies were observed in response to environmental pressures. Moreover, as a consequence of the reintroduction process the reintroduced metapopulation exhibited a distinct genetic structure and showed resilience to genetic variability loss, facilitating successful colonization and increasing the metapopulation size. Evidence suggests that *P. teleius* is a promising candidate for reintroduction efforts capable of thriving and adapting in newly reintroduced habitats.

Streszczenie (Abstract)

Osobniki danego gatunku reagują na zmiany środowiskowe poprzez adaptacje biologiczne. Gatunki specjalistów stoją często w obliczu szybkiego spadku populacji z powodu nieprawidłowego przystosowania się do nowych warunków (maladaptacji). Szczególnie podatne na wyginięcie są gatunki pasożytnicze ze względu na dużą zależność pasożyta od obecności i liczebności gospodarza. Reintrodukcja służy jako narzędzie ochrony, mające na celu przywrócenie utraconych populacji danego gatunku i procesów ekologicznych po ich wyginięciu. Dodatkowo, reintrodukcje pozwalają obserwować zmiany ewolucyjne w nowych siedliskach, co pozwala dowiedzieć się czy i w jaki sposób organizmy mogą radzić sobie w nowych warunkach środowiskowych. W ostatnich dziesięcioleciach przeprowadzono wiele procesów reintrodukcji motyli, w tym również motyli z rodzaju *Phengaris* reintrodukowanych w Wielkiej Brytanii i Holandii. Motyle te są dobrymi wskaźnikami różnorodności biologicznej, wykazują wyspecjalizowany cykl życiowy jako pasożyty społeczne mrówek i posiadają specyficzne adaptacje ułatwiające integrację gąsienic w koloniach mrówek gospodarzy. Złożoność ich cyklu życiowego podkreśla konieczność uwzględnienia w strategiach ochrony zarówno gatunku pasożyta (motyla), żywiciela (mrówek z rodzaju *Myrmica*) jak i obecności roślin żywicielskich. Gąsienice motyli, naśladując sygnały chemiczne i wibroakustyczne mrówek, infiltrują kolonie gospodarza a następnie integrują się w celu dalszego przeżycia w kolonii mrówek. Poszczególne gatunki mrówek *Myrmica* różnią się profilami węglowodorów kutykularnych, co jest głównym mechanizmem rozpoznawania się u mrówek. Zatem gąsienice motyli *Phengaris* muszą naśladować specyficzny profil mrówek gospodarzy, aby oszukać robotnice mrówek danego gatunku i ułatwić adopcję oraz zabranie ich do kolonii. Geograficzne różnice w specyficzności względem mrówek gospodarzy ukazują tzw. mozaikę koewolucji, podkreślając przestrzennie zróżnicowany charakter interakcji i wzajemnej adaptacji gatunków. Istnieją dowody potwierdzające ten wzór u niektórych gatunków *Phengaris*, takich jak *Phengaris alcon*, natomiast nie udało się tego wykazać dla najmniej specyficznego względem mrówek gospodarzy gatunku, a mianowicie *Phengaris teleius*.

Po wyginięciu ostatniej populacji *P. teleius* w Holandii w 1976 r., w 1990 r. nastąpiła udana reintrodukcja, obejmująca translokację 86 motyli z Polski do rezerwatu przyrody Moerputten w Holandii. Wysiłki związane z reintrodukcją zaowocowały utworzeniem metapopulacji składającej się obecnie z kilku tysięcy osobników. Trzy dekady później przedstawione w rozprawie doktorskiej badania miały na celu ocenę potencjalnych zmian jakie mogły zajść zarówno u dorosłych motyli jak i u gąsienic pochodzących z metapopulacji źródłowej (polskiej) i metapopulacji reintodukowanej (holenderskiej). Badania objęły analizy chemiczne (profile węglowodorów kutykularnych), wibroakustyczne i behawioralne gąsienic i mrówek oraz analizy morfologiczne i genetyczne dorosłych motyli. Ponadto ocenione zostały zmiany morfologiczne tylnych skrzydeł motyli w skali

przestrzennej i czasowej dzięki integracji danych dotyczących osobników z obecnych metapopulacji z danymi historycznymi osobników z metapopulacji źródłowej i reintodukowanej. Badania te zapewniają wgląd w trwający proces koewolucji, powstawanie adaptacji do nowych warunków oraz genetyczne skutki reintrodukcji. Postawiono hipotezę, że profile węglowodorów kutykularnych i sygnały wibroakustyczne u gąsienic różnią się w zależności od metapopulacji, przypominając profile ich lokalnych mrówek gospodarzy *Myrmica scabrinodis*. Przewidywano również skuteczniejszą adopcję gąsienic i zwiększoną ich przeżywalność w koloniach mrówek z lokalnych populacji. Założono, że warunki biotyczne i abiotyczne w różny sposób wpływały na cechy morfologiczne motyli z poszczególnych metapopulacji oraz że metapopulacja reintodukowana wykazuje się niższą zmiennością genetyczną. Założono również że łączność metapopulacji wpływa na dobór cech morfologicznych i zdolności do dyspersji motyli. Ponadto czynniki takie jak dobór płciowy, drapieżnictwo i stres w fazie rozwojowej mogą w różny sposób wpływać na wzorce kropek na skrzydłach motyli w metapopulacji źródłowej i reintrodukowanej.

Otrzymane wyniki wskazują, że gąsienice z metapopulacji reintrodukowanej różnią się sygnałami chemicznymi i wibroakustycznymi w porównaniu z metapopulacją źródłową po 30 pokoleniach od reintrodukcji. Gąsienice z reintrodukowanej metapopulacji emitują sygnały wibroakustyczne bardziej podobne do sygnałów emitowanych przez ich sympatycznych gospodarzy, co sugeruje potencjał adaptacyjny do lokalnych warunków. Nasze analizy nie wykazały natomiast dowodów na poprawę wydajności w zakresie mimikry chemicznej (profili węglowodorów kutykularnych). Dorosłe motyle również wykazują różnice morfologiczne pomiędzy metapopulacjami. Motyle ze współczesnej, polskiej metapopulacji źródłowej charakteryzują się wyższą masą ciała i szerokością tułowia w porównaniu z motylami z holenderskiej metapopulacji. Mają one też największe skrzydła spośród wszystkich badanych metapopulacji (współczesnych i historycznych). Kształt skrzydeł i zmienność wzoru kropek również różnią się w zależności od metapopulacji. Łączność metapopulacji zmieniała się w czasie; w polskiej metapopulacji powoli spadała zaś w holenderskiej istotnie wzrosła, zwłaszcza po dodatkowym odtworzeniu siedlisk. Sugeruje to, że łączność metapopulacji może być jednym z ważniejszych czynników wpływających na zmiany w cechach morfologicznych dorosłych motyli w obu metapopulacjach. Analiza genetyczna ujawniła różnice w bogactwie alleli, wskazując na efekt założyciela oraz wąskiego gardła w reintrodukowanej metapopulacji z wyraźnym zróżnicowaniem struktury genetycznej pomiędzy metapopulacjami i mniejszą efektywną wielkością populacji holenderskiej w porównaniu ze źródłową.

Otrzymane wyniki podkreślają zdolność przystosowawczą motyla *P. teleius* na wszystkich etapach cyklu życiowego, wskazując zdolność gąsienic do ewolucji adaptacji do nowych mrówek gospodarzy. Również zaobserwowano zmiany morfologiczne u dorosłych motyli w odpowiedzi na presję środowiskową. Co więcej, w wyniku procesu reintrodukcji holenderska metapopulacja

wykazuje odrębną strukturę genetyczną ale także odporność na utratę zmienności genetycznej, o czym świadczy pomyślna kolonizacja i wzrost liczebności metapopulacji. Otrzymane wyniki sugerują, że *P. teleius* jest obiecującym gatunkiem motyla do działań w zakresie reintrodukcji, zdolnym do rozwoju i adaptacji w nowych siedliskach.

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I wish to express my sincere gratitude to my supervisors, Magda and Luca, for their faith in me, their guidance, support, and feedback during the last five years. Their expertise and dedication have been instrumental in shaping this thesis and in my academic and personal growth.

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Abbreviations

ANOSIM - Analysis of similarities

ANOVA - Analysis of variance

CHC - Cuticular hydrocarbon

CVA - Canonical variates analysis

FA - Fluctuating asymmetry

GLM - Generalized linear model

GLMM - Generalized linear mixed model

NMDS - Non-metric multidimensional scaling

NL - the Netherlands

PERMANOVA - Permutational multivariate analysis of variance

PL - Poland

Table of Contents

Summary

Introduction

Biological adaptations play a crucial role in evolutionary processes, as organisms respond to environmental pressures. These adaptations are crucial for species survival and reproduction. Unraveling these phenomena not only enhances our understanding of evolutionary dynamics, but also provides critical insights for biodiversity conservation and addresses critical ecological issues.

The lack of adaptations to new conditions can result in decline of species. Insects are one of the most rapidly declining groups of animals, with specialists being particularly threatened (e.g., Zayed *et al.* 2005; Hallmann *et al.* 2017; Raven & Wagner 2021). In addition, interacting species are even more vulnerable to environmental changes and the risk of coextinction increases (e.g., Koh *et al.* 2004). While mutualistic networks can restore lost species interactions by creating new opportunistic ones, host-parasite systems are less resilient (Grass *et al.* 2018; Gawecka *et al.* 2022). In this perspective, when reintroducing a parasitic species, not only the parasite itself, but also the host species must be taken into account to increase the probability of success (Wynhoff *et al.* 2011).

Reintroductions are used in conservation biology as a tool to recover species loss or restore ecological processes after local extinction in a specific ecosystem (Seddon *et al.* 2014). This applied science provides management strategies for translocations (Taylor *et al.* 2017) and offers opportunities to study evolutionary changes in populations introduced to new habitats. For instance, it was demonstrated that *Gasterosteus aculeatus* only needed one generation to show changes in morphology (Wund *et al.* 2016) and *Martes americana* showed morphological variation 45 years after the translocation (Howell *et al.* 2016). Such changes can provide the potential for studying adaptations after reintroductions and give us an opportunity to learn whether and how organisms can deal with new environmental conditions.

Butterfly reintroductions have had a great importance during the last decades, because of their great rate of extinction and number of translocations (Thomas *et al.* 2004; Bellis *et al.* 2019). However, it is a complicated process and many populations become extinct in the first five years after reintroduction (Oates & Warren 1990). Despite that, two of the most successful reintroductions in insect conservation history have been implemented for the butterflies of the genus *Phengaris*; *P. arion* was reintroduced in the United Kingdom from a Swedish population (Thomas *et al.* 2009; Andrews 2015) and *P. teleius* in the Netherlands from a Polish population (Wynhoff 1998).

Phengaris butterflies serve as indicators and flagship species for biodiversity conservation (Thomas *et al.* 2004). They are univoltine butterflies with a very specialized lifecycle. They evolved as social parasites of ants, possessing adaptations to disrupt their host communication to enter and integrate within host colonies (Thomas *et al.* 2005). Their caterpillar requires two resources: species-specific host plants and *Myrmica* host ants (Elmes & Thomas 1992). Inside the ant nest, some species can be directly fed by ants via trophallaxis, commonly called cuckoo feeders, while others such as *P. teleius* can actively prey on ant brood, being called predatory

feeders (Thomas & Elmes 1998). In the case of *P. teleius*, females lay eggs on *Sanguisorba officinalis* flowerheads where the caterpillars live for about three weeks. After reaching the fourth instar, they abandon the host plant and need to be taken by a *Myrmica* ant to the nest for further development (Thomas 1984). *P. teleius* is a generalist social parasite that can be adopted by several *Myrmica* species, but in many populations *Myrmica scabrinodis* appears to be its primary host (Tartally *et al.* 2019).

Throughout the adoption process, the main communication mechanisms between butterfly caterpillars and ants involve those employed by the ants themselves, i.e. chemical signals (Akino *et al.* 1999; Schönrogge *et al.* 2004; Nash *et al.* 2008) and vibroacoustic signals (Barbero *et al.* 2009; Sala *et al.* 2014). Nestmate recognition in social insects is mainly mediated by cuticular hydrocarbons (CHCs) (e.g., D'Ettorre & Lenoir 2010). *Myrmica* ant species possess species-specific CHC profiles, which can vary both qualitatively and quantitatively (Elmes *et al.* 2002; Guillem *et al.* 2016). Intraspecific variation among populations of the same *Myrmica* species can be found, so that colonies exhibit the same CHCs that differ in relative abundance (Elmes *et al.* 2002; Guillem *et al.* 2016). *Phengaris* caterpillars mimic these chemical signals to parasitize *Myrmica* colonies. The host specificity pattern observed in *Phengaris* butterflies can be attributed to the significant differences in CHC profiles among *Myrmica* species (Witek *et al.* 2013). Additionally, *Phengaris* caterpillars not only mimic chemical signals but also produce vibroacoustic signals imitating those of ants (Barbero *et al.* 2009). These signals deceive ant workers into treating the caterpillars as their own larvae or even as their queen. The local adaptations that some *Phengaris* populations can exhibit to different *Myrmica* species show a geographical mosaic of coevolution (Tartally *et al.* 2019). This evolutionary mechanism denotes the spatially heterogeneous nature of species interactions and adaptations in different geographical regions. It is mainly proven for *Phengaris* cuckoo species (Nash *et al.* 2008; Thomas *et al.* 2013) whereas until now there is no evidence for the more generalist predatory *Phengaris teleius* (Tartally *et al.* 2019).

Following the extinction of *P. teleius* in the Netherlands in 1976, a significant reintroduction effort took place in 1990 (Wynhoff 1998). Eighty-six butterflies were translocated from a Polish metapopulation to the Dutch nature reserve of Moerputten. The success of the reintroduction has resulted in the establishment of a metapopulation, now comprising several thousand individuals (Irma Wynhoff, unpublished data). After 30 generations since the separation of the source and reintroduced metapopulations, we took a unique opportunity to study the possible changes that could appear in adult butterflies and caterpillars in the reintroduced and source metapopulations. The aim of the study was to test whether 1) *P. teleius* caterpillars could have evolved adaptations to their local hosts detecting potential ongoing coevolution processes between a social parasite and its host *Manuscript 1*; 2) the adult descendants of the individuals from the translocated and native metapopulations have retained the characteristics of the source metapopulation or they have changed and adapted to the new current conditions (*Manuscript 2* and *3*); and 3) the reintroduction process has produced any effect on the genetic structure of the translocated metapopulation (*Manuscript 2*). We carried out a multilevel comparison among the current Polish and current reintroduced Dutch metapopulations by

performing chemical, vibroacoustic, behavioral, morphological and genetic analyses. Moreover, the morphological changes in adult butterfly hindwings were studied including additional historical data from the source metapopulation from 1990 (year of the reintroduction) and 2003, and from the reintroduced metapopulation from 1996.

Aims of the study

The aim of this research was to study the adaptability of *P. teleius* caterpillars to new host populations (*Manuscript 1*) and assess potential changes in adult butterflies under different selective pressures (*Manuscript 2* and *3*) in the source and reintroduced metapopulations. We also studied the effect in the reintroduction on the genetics of the different metapopulations (*Manuscript 2*).

The caterpillar adaptability was studied by analyzing the chemical and vibroacoustical cues used by the caterpillar to mimic the ones of the ants and integrate into the colony. We compared cuticular hydrocarbon profiles of *P. teleius* caterpillars before and after adoption, as well as the chemical profiles of their host ants. Moreover, we analyzed the vibroacoustic signals emitted by pre-adoption caterpillars, ant workers and queens, along with the corresponding behavioral responses of ants to playbacked vibroacoustic stimuli. Additionally, we investigated the behavioral responses of ants towards the caterpillars in cross-metapopulation adoption experiments (*Manuscript 1*). We hypothesized that CHC profiles and vibroacoustic signals emitted by the caterpillars differ between metapopulations, being more similar to those of their sympatric *M. scabrinodis* host ants. Furthermore, we hypothesized that caterpillars exposed to host ants from the same metapopulation undergo a more favorable and successful adoption process, and have an increased survival within the colony.

The potential changes in adult butterflies were assessed by studying the hindwing morphology and different body measurements (*Manuscript 2*). Furthermore, we also studied the morphology and variability of the hindwing spot pattern among the reintroduced and source metapopulations (*Manuscript 3*). Moreover, we performed population genetics analysis to test a possible bottleneck and founder effect in the reintroduced metapopulation, and evaluate possible differences in the genetic structure of both metapopulations (*Manuscript 2*). Additionally, we assessed the metapopulation connectivity of our study systems over time (*Manuscript 2*). We hypothesized that different biotic and abiotic conditions, such as population size, habitat structure, and availability of host plants and host ants, have influenced the two current metapopulations differently, thus affecting the morphology of adult butterflies (*Manuscript 2*). And factors such as sexual selection, predation, and developmental stress, could have influenced their hindwing spot pattern characteristics (*Manuscript 3*). Additionally, we hypothesized that the reintroduced metapopulation is characterized by lower genetic variability compared to the source metapopulation, and after 30 years of separation a genetic differentiation has occurred between metapopulations (*Manuscript 2*). We also expected metapopulation connectivity to be one of the most important factors leading to selection pressure on morphological traits of butterflies and their dispersal.

Material and Methods

Study sites and data

P. teleius adult butterflies were collected to study their morphology and genetic structure of metapopulations. Butterfly caterpillars and ant colonies were collected to study their cuticular hydrocarbons profiles (CHCs), vibroacoustical signals, behavioral responses and butterfly caterpillar adoption and survival. The sampling was carried out in two sites: in the source Polish and in the Dutch reintroduced metapopulations. The source metapopulation occurs in the Vistula River Valley in the outskirts of Kraków city in Southern Poland (50°01'N, 19°54'E). The habitat of the focal butterfly species is a part of a large meadow complex with an area exceeding 200 ha and consisting of several dozens of nutrient-poor to mesotrophic meadows with varying densities of butterfly foodplant (Kajzer-Bonk *et al.* 2016). The sampling was performed in the three populations (K10, K1 and K25; Fig. 1) from where the individuals were originally captured in 1990 for the reintroduction. The reintroduced metapopulation is located in the nature reserve of Moerputten, located in the south of the city of 's-Hertogenbosch (the Netherlands) and covers the central part of the Natura 2000 area "Vlijmens Ven, Moerputten and Bossche Broek" (51°41'N, 5°15'E). Recently, *P. teleius* is restricted to one core population and two to three populations on other meadows within the nature reserve. The sampling was performed in three of those populations (BW, PHZ and KBW; Fig. 1).

Butterfly caterpillars and ants were collected in August 2019 for analysis of CHCs and vibroacoustic signal recordings, representing the pre-adoption samples. In July 2020, additional caterpillars and ants were collected to conduct a cross-metapopulation behavioral experiment. Furthermore, post-adoption caterpillars and ant samples for CHC analysis were obtained from selected colonies used in the behavioral experiment after adoption.

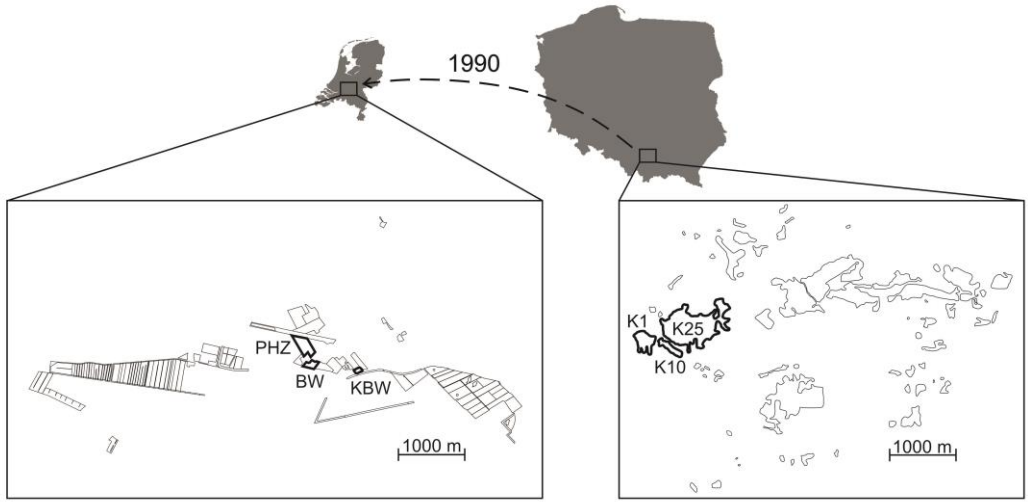

Fig. 1. Sites of the two studied metapopulations of *Phengaris teleius*. On the left: habitat patches of the Dutch reintroduced metapopulation; on the right: habitat patches of the Polish source metapopulation (habitat patches are considered to be sites where the butterfly food plant is present).

Additionally, ant workers were collected in August and October 2021 to perform a playback experiment with the previously recorded vibroacoustic signals (*Manuscript 1*).

Adult butterflies were collected in Poland in July 2019 and the Netherlands in July 2020 to perform morphological and genetic analysis (*Manuscript 2* and *3*). The historical samples of butterflies from the Polish metapopulation from 1990 and 2003, and from the Dutch metapopulation from 1996 were obtained from the entomological collections of the authors and used for morphological analysis (*Manuscript 2* and *3*).

CHC analysis

The chemical adaptations of *P. teleius* caterpillars were studied through analysis of CHCs profiles of pre-adopted and post-adopted butterfly caterpillars and *M. scabrinodis* ants. Pre-adopted caterpillars were obtained from the flowers of its foodplant (*Sanguisorba officinallis*) and CHCs were extracted within a few hours after leaving the foodplant. The CHCs profile of ants used for comparison with pre-adopted caterpillars were extracted within the first 24 hours after ant collection in the field. Pre-adoption samples were extracted from a total of 25 caterpillar and 21 ant samples from Poland and 19 caterpillar and 24 ant samples from the Netherlands. Post-adopted caterpillars were obtained from the colonies that successfully adopted a caterpillar during the behavioral experiment (see below). The caterpillars and ants were collected three days after adoption and stored at -20°C until CHC extraction. Post-adoption samples were extracted from a total of six caterpillars from the Polish source metapopulation, four caterpillars from Dutch reintroduced metapopulation and from their ant host from both Poland and the Netherlands. CHCs were extracted from a single caterpillar or five ant workers by placing them in a glass vial with 200 µl of hexane for 10 minutes. After extraction, all vials were stored at -20°C until gas-chromatograph analysis. Detailed information about gas-chromatography analysis can be found in *Manuscript 1*.

Vibroacoustic signal recordings

We used a custom-made device designed for recording the sounds emitted by *P. teleius* caterpillars and *M. scabrinodis* ants to study the vibroacoustic signal adaptations. We recorded a total of eight caterpillars, five ant queens and 10 ant workers (gathered from five colonies) from Poland, and six caterpillars, three ant queens and 10 ant workers (gathered from five colonies) from the Netherlands. The recording equipment included a recording chamber with a miniature moving-coil microphone attached at its center and one more microphone used to record ambient noise. Signals were combined after preamplification. The setup was placed in an anechoic chamber to minimize background noise and interference. Caterpillars and ants were individually placed on the microphone's surface and recorded for 10 minutes during the morning at room temperature. Analysis was done using Praat version 6.2.14 and the measurements included peak frequency, the third quartile of the energy spectrum, unit duration, mean intensity, energy of the peak frequency, and the ratio of the peak frequency energy to the total call energy. More details can be found in *Manuscript 1*.

Playback experiment

To test whether the sounds emitted by the caterpillars were able to produce a greater behavioral response in their sympatric host ants, we conducted

playback experiments in four ant colonies from Poland and five ant colonies from the Netherlands. Playback experiments were carried out in artificial arenas in which a speaker was glued to the bottom and covered with a thin layer of slightly damp soil. In each arena, we introduced five ant workers and allowed them to settle for 10 minutes before exposing them for 30 minutes to one of five vibroacoustic signals previously recorded: 1) *M. scabrinodis* queen, 2) *M. scabrinodis* worker, 3) sympatric larvae of *P. teleius*, and 4) allopatric larvae of *P. teleius*; 5) white noise (used as a control). We replicated the playback experiment per each colony from two to three times using new ants.

Behavioral essay

Pre-adoption caterpillar performance during interaction with ants was tested with a cross-metapopulation behavioral experiment. After collection, ant colonies were divided into two sub-colonies consisting of 100 workers (50 foragers and 50 intra-nidal workers) and 30 ant larvae and established in plastic boxes. Altogether, 15 colonies from the Polish metapopulation and 11 colonies from the Dutch metapopulation were used in the experiment. One sub-colony from each colony was used for the adoption of butterfly caterpillars from the sympatric metapopulation and the another one for the adoption of the allopatric caterpillars. A butterfly caterpillar was introduced in the plastic box at the furthest distance from the entrance of the ant nest. The observation started from the first contact of the caterpillar with ants and lasted 60 min. All behavioral events displayed by *M. scabrinodis* workers were registered. We also noted whether adoption occurred during the observation. If not, the boxes were checked every hour during the following five hours and after 24 hours since the observation was finished. The survival of the caterpillar was checked every day starting 24 hours after the adoption observation until its death.

Adult butterfly data collection

The butterflies were collected at different moments: from the source metapopulation in Poland in 1990 (the year of the reintroduction); from the current Polish metapopulation in 2019; from the reintroduced Dutch metapopulation in 2020 (***Manuscript 2*** and ***3***). Additionally, butterflies were collected from two other temporal moments: in 2003 from the Polish metapopulation and in 1996 from the Dutch metapopulation, specifically for studying hindwing morphometry (***Manuscript 2***). The butterflies were photographed on the right and left sides to study their hindwing morphology and spot pattern (see below). Then, the thorax width was measured with a caliper and the butterfly was weighed with a balance. We also removed 2–3 mm2 of the left hindwing to obtain material for the genetic analysis. Finally, the butterflies were marked to prevent re-sampling of the same individual and they were released (see ***Manuscript 2*** or ***3*** for more detailed information). The hindwings from historical previously collected individuals were also photographed.

Hindwing morphometry assessment

We used a total of 354 butterflies to study the morphology of the hindwing by applying geometric morphometric techniques. A combination of landmarks and sliding semilandmarks (Bookstein 1997) was applied to study the vein intersections (5 landmarks) and the outline of the wings (9 landmarks and 17 semilandmarks). Landmarks and

semilandmarks were used to estimate both wing shape and centroid size. Detailed information about landmark data procedures prior morphological analysis can be found in *Manuscript 2*.

Hindwing spot pattern morphometric approach

A total of 267 butterflies were used for studying the hindwing spot pattern. The spot pattern refers to the motif created by the combination of the spots found on the wings. Eleven landmarks were digitized in every picture to estimate both wing spot pattern shape and centroid size. Additionally, the landmarks previously digitized to study hindwing morphometry were used in further analysis to normalize the data. The raw data were subsetted and only the data from the left wing were used in the analysis to avoid bias based on directional asymmetry between left and right wings. Detailed information about landmark data procedures prior morphological analysis can be found in *Manuscript 3*.

Computer assisted spot detection

We developed a program in Python (3.9) to measure black spots area, the exact center coordinates from the hindwing images and whether spots were detected in the wing areas in which facultative points were expected (see *Manuscript 3* for more detailed information). A Graphical User Interface was also programmed to allow manual corrections of the errors made by the automatic detection program. After correction, the area and centroid (arithmetic mean) of each of the spots were calculated. Area was calculated in pixels and then converted to international units (mm2).

Metapopulation connectivity

In order to evaluate the changes in the spatial structure of both *P. teleius* metapopulations over the investigated period, we used Hanski's connectivity index I4 (Hanski 1994). A more detailed description of the calculation is presented in *Manuscript 2*: Methods A.1.

Genetic structure of the metapopulations

The genetic study was performed by using only butterflies from the two current metapopulations (Poland 2019 and the Netherlands 2020). We analyzed 118 butterflies from the Polish metapopulation and 134 from the Dutch metapopulation by using a small fragment of the hindwing. The butterflies were assayed at 17 microsatellite markers. Details about DNA extraction and microsatellite amplification are presented in *Manuscript 2*: Methods S1.

Statistical analysis

The CHC profile distances were calculated based on Bray-Curtis dissimilarity and used to assess differences between groups with PERMANOVA. The distance between different groups were fitted to GLMs. The changes in the CHC profile after caterpillar adoption were studied with a multi-level pattern analysis comparing the compounds of the different groups with a higher abundance respecting the pre-adopted caterpillars as a reference group. In order to assess disparities in vibroacoustic signals between the ants and pre-adoption caterpillars we performed analysis of similarities (ANOSIM). Additionally, vibroacoustic signal distances were calculated based on Euclidean distances and fitted to GLMs. Differences in the vibroacoustic parameters were tested by fitting the data to GLMMs. The ant

responses to the vibroacoustic stimuli during the playback experiment were also tested fitting the data to GLMMs. The different behaviors registered and the adoption success of the caterpillars during the behavioral experiment were analyzed with GLMMs. Caterpillar survival (days after adoption) was fitted to a Cox proportional-hazards model and the survival curves were estimated with the survfit() function (Therneau 2015).

Butterfly weight, thorax width, hindwing size and the ratios body weight/centroid size and thorax width/centroid size were analyzed with GLMs. Hindwing shape variation and allometry were tested by performing a Procrustes ANOVA with permutation. The morphological disparity variation was tested using residuals of a linear model fit to estimate the Procrustes variance. All the analyses were separately performed for females and males.

The spot pattern centroid size was tested for correlation with the wing centroid size to test if the spot pattern size can be used as a good estimator of wing size in further analysis. After testing for correlation, all the analyses were separately performed for females and males. The differences in the hindwing spot pattern shape and allometry among metapopulations were tested by performing a Procrustes ANOVA with permutation. Spot pattern centroid size was used as an estimator of wing size and used to remove the effect of allometry from shape pairwise comparisons. Spot presence, individual spot area, total spot area and spot fusion were fitted to GLMs. Fluctuating asymmetry was also tested for spot presence with a GLM. Both hindwings (left and right) were considered for this analysis. The wing spot pattern distance between individuals based on spot presence, area and fusion was calculated based on Bray-Curtis dissimilarity and the disparity among metapopulations was tested with PERMANOVA. Intra-metapopulation distances were compared to assess the spot pattern metapopulation variability and the distances were fitted to a GLM.

All GLM and GLMM predictors were tested for significance with ANOVA and the different groups of each model were pairwise compared with estimated marginal means (EMMs) tests.

The microsatellite loci were tested for Hardy-Weinberg Equilibrium (HWE) and Linkage disequilibrium. FSTAT was also used to assess population parameters: number of alleles, allelic richness (AR), observed heterozygosity (H_O), expected heterozygosity (H_S), inbreeding coefficient (F_{IS}) and fixation index (F_{ST}). Differences between the current Polish and Dutch metapopulations were assessed by a two-sided permutation test with 1000 permutations in FSTAT. The number of genetic clusters was inferred by implementing Bayesian clustering in Structure v2.3.4 (Pritchard *et al.* 2000; Falush *et al.* 2003; Hubisz *et al.* 2009). The effective population size was assessed for each metapopulation in LDNE (Waples & Do 2008) and the hypothesis of a bottleneck was tested with a two-phase model (TPM) with 30% of infinite alleles model (IAM) and 70% of stepwise mutation model (SM).

Results

Cuticular hydrocarbon adaptations

We identified a total of 31 cuticular hydrocarbon compounds. The composition of CHC profiles and the abundance of CHCs differed according to the pre- and post-adoption phase, metapopulation and between butterfly caterpillars and ants. The reintroduced Dutch caterpillars had a less similar CHC profile to the host ants, both before and after

their adoption compared to the Polish caterpillars. They also showed the smallest change in their CHC profile after the adoption and they did not show differences when compared to Dutch and Polish host ants. On the other hand, the Polish caterpillars had the biggest change in their CHC profile after adoption and presented the closest CHC profile when they were adopted by their sympatric host ants. In detail, nine CHC compounds increased their abundance in *P. teleius* caterpillars after their adoption. Those compounds also presented a higher abundance in the ant CHC profile compared to pre-adoption *P. teleius*.

Vibroacoustic adaptations

The vibroacoustic signals produced by ants and caterpillars differed among metapopulations. The caterpillars from each metapopulation produced sounds more similar to the ones of their sympatric host ants. Moreover, the caterpillars from Poland had the closest vibroacoustic signal overall when compared to their Polish sympatric host ants, but no difference was found between Polish and Dutch caterpillars when they were compared to Dutch host ants. When we tested the ant behavioral responses to the vibroacoustic signals emitted by the caterpillars, each ant metapopulation reacted more intensively to the sound produced by their sympatric caterpillars, reaching the level of response to the sounds produced by ants.

Behavioral interactions

The behavioral experiment tested the efficiency of the CHC profile and vibroacoustic signal adaptations from *P. teleius*. The number of antennations performed by ants did not show any significant difference in the presence of caterpillars from different metapopulations. However, the caterpillars from Poland in the presence of their sympatric host ants received more positive and less negative behaviors in comparison with any other combination. Additionally, the Dutch caterpillars were adopted in a lower proportion and survived for a shorter time after adoption.

Morphological differences in adult butterflies

Polish *P. teleius* females had a greater body weight while both females and males from Poland had a bigger thorax compared with the reintroduced Dutch butterflies. Butterflies from the current Polish metapopulation also had the biggest wings in comparison with any other metapopulations. We could not find differences in wing size when it was corrected with body weight or thorax width. The butterflies from the different metapopulations also differed in wing shape and those differences were partially explained by allometry. The Polish and the reintroduced Dutch metapopulations presented differences in hindwing shape between them and also in comparison with the source metapopulation from 1990. Additionally, the butterflies from the current Polish metapopulation showed the highest shape variability.

Hindwing spot pattern analysis

Spot pattern centroid size and wing centroid size showed a strong correlation proving that both can be used as an estimator of wing size. The butterflies from the different metapopulations differed in spot pattern shape and those differences were partially explained by allometry. The focal spot (spot identity) was the main factor determining its presence in the butterfly wings. There were two highly variable spots and we considered them as facultative spots, meaning

they were not present in many cases. One of them showed to be significantly more present in the Dutch metapopulation compared with the source and current Polish ones. The spot area was influenced by the metapopulation and the focal spot, but any clear trend was demonstrated. However, when taking into account the total melanized spot area, the Polish butterflies presented a higher value in both sexes. The focal spot was also the main factor determining the proportion of asymmetrical individuals. Only the spot number 10 showed a clear trend with a significantly higher proportion of asymmetrical individuals in the reintroduced Dutch metapopulation. Spot fusion was found in a significantly higher proportion in males from the Polish source metapopulation (from 1990) compared to males from the current Polish metapopulation. A similar but not statistically significant pattern was found for females. The spot pattern was analyzed in a general way combining the different studied parameters. We found to be affected by the metapopulation in both sexes. Additionally, the metapopulation also influenced the spot pattern intra-metapopulation variability. The butterflies from the reintroduced Dutch metapopulation showed a significantly lower spot pattern intra-metapopulation variability for both sexes.

Metapopulation connectivity

The results of metapopulation connectivity changes over the years in the two investigated metapopulations indicated a relatively slow decrease in connectivity in the Polish metapopulation and a sharp increase in the Dutch metapopulation after the habitat restoration program in the mid-2010s. The connectivity of the Dutch metapopulation was already substantially greater when the reintroduction occurred in 1990 than the highest ever registered value in the source Polish metapopulation.

Genetic structure of the current Polish and Dutch metapopulations

Nine out of the seventeen studied microsatellites were suitable for analysis. Only allelic richness showed a nearly significant difference among both metapopulations. The Dutch metapopulations showed to lose more than half of the pool of alleles found in the Polish metapopulation. We found evidence of founder effect and bottleneck in the reintroduced metapopulation and a clear genetic structure differentiation among metapopulations. Additionally, the effective population size of the reintroduced metapopulation was estimated much lower compared to the native Polish one.

Conclusions

The study reveals the adaptability of *P. teleius* during both its caterpillar and adult phases. Our findings demonstrate the capacity of the caterpillars for coevolution and adaptation to new ant host populations (***Manuscript 1***). Moreover, our research shows morphological changes in adult butterflies after being exposed to different environmental pressures (***Manuscript 2*** and ***3***). Additionally, the reintroduced metapopulation of *P. teleius* exhibited a different genetic structure than the source metapopulation, and resilience in the face of a loss of genetic variability, enabling a successful colonization of the new habitat (***Manuscript 2***).

We observed notable differences in the CHC profiles and vibroacoustic signals of the reintroduced caterpillars in the Netherlands compared to caterpillars from the source metapopulation in Poland after 30 generations since their

reintroduction. Despite the divergence, the reintroduced Dutch caterpillars did not exhibit a higher similarity of the chemical profile to their sympatric Dutch host ants when compared to their allopatric Polish hosts. However, their vibroacoustic signals showed to mimic better the ones of their sympatric host ants. Although our observations indicated changes in the Dutch caterpillars to make them more similar to the new host metapopulation, the reintroduced caterpillars have not fully adapted and achieved the level of mimicry as caterpillars from the source metapopulation with their local host ants in Poland. Nonetheless, these changes were enough to facilitate butterfly adoption, integration within the ant colony and the completeness of the vital cycle of the butterfly. As a consequence, the population size has increased since the reintroduction, indicating a successful reintroduction process. Our study provides evidence that *P. teleius*, being the more generalist *Phengaris* butterfly (Stankiewicz & Sielezniew 2002; Woyciechowski *et al.* 2006; Witek *et al.* 2010), possesses the capacity to locally adapt to new host populations (*Manuscript 1*).

The adult butterflies also differed in their morphology in both metapopulations in the last 30 generations. The reintroduced metapopulation due to the new habitat conditions for the translocated butterflies was expected to show greater changes compared to the source metapopulation. Meantime, the Polish butterflies were the ones showing the greater morphological changes, as a possible consequence of the habitat perturbation occurring in their metapopulation system during the last decades (*Manuscript 2*) and changes in wing melanization explained as an adaptation to different climatic conditions (*Manuscript 3*). Such changes demonstrate that *P. teleius* butterflies are good indicators of habitat changes and they are able to adapt to environmental alterations in a relatively short period of time. Moreover, the reduction of traits variability (e.g., spot pattern variability) and the increase in asymmetrical individuals in the Dutch metapopulation could be a sign of the loss of genetic variability after the reintroduction (*Manuscript 2* and *3*). In any case, the reintroduction of 86 individuals was enough for the population to survive, adapt, grow and expand the distribution area to new patches over 30 generations, despite the loss of genetic variability after the reintroduction *Manuscript 2*.

We offer evidence that *P. teleius*, despite its peculiar life cycle, is a good candidate species with enough adaptability to be successfully reintroduced in new habitats where it was previously lost. A source population containing host ants with a CHC profile closely similar to that of the host ants in the reintroduced area might improve the success of caterpillar adoption, especially considering their limited chemical adaptability (*Manuscript 1*). Moreover, employing a larger number of butterflies during reintroduction could help to prevent the loss of trait variability and the appearance of asymmetrical individuals as consequences of genetic variability loss after translocations (*Manuscript 2* and *3*).

References

Akino, T., Knapp, J.J., Thomas, J.A. & Elmes, G.W. (1999). Chemical mimicry and host specificity in the butterfly *Maculinea rebeli*, a social parasite of *Myrmica* ant colonies. *Proceedings of the Royal Society B: Biological Sciences*, 266, 1419–1426.

Andrews, P. (2015). A History of the Large Blue *Maculinea arion* subspecies eutyphron (Fruhstorfer, 1915) in Somerset. *Dispar*, 1–6.

Barbero, F., Bonelli, S., Thomas, J.A., Balletto, E. & Schönrogge, K. (2009). Acoustical mimicry in a predatory social parasite of ants. *Journal of Experimental Biology*, 212, 4084–4090.

Bellis, J., Bourke, D., Williams, C. & Dalrymple, S. (2019). Identifying factors associated with the success and failure of terrestrial insect translocations. *Biological Conservation*, 236, 29–36.

Bookstein, F.L. (1997). Landmark methods for forms without landmarks: localizing group differences in outline shape. *Proceedings of the Workshop on Mathematical Methods in Biomedical Image Analysis*, 1, 225–243.

D'Ettorre, P. & Lenoir, A. (2010). Nestmate Recognition. In: *Ant ecology* (eds. Lach, L., Parr, C.L. & Abbott, K.L.). Oxford University Press, Oxford, UK, pp. 194–209.

Elmes, G.W., Akino, T., Thomas, J.A., Clarke, R.T. & Knapp, J.J. (2002). Interspecific differences in cuticular hydrocarbon profiles of *Myrmica* ants are sufficiently consistent to explain host specificity by *Maculinea* (large blue) butterflies. *Oecologia*, 130, 525–535.

Elmes, G.W. & Thomas, J.A. (1992). Complexity of species conservation in managed habitats: interaction between *Maculinea* butterflies and their ant hosts. *Biodiversity and Conservation*, 1, 155–169.

Falush, D., Stephens, M. & Pritchard, J.K. (2003). Inference of population structure using multilocus genotype data: Linked loci and correlated allele frequencies. *Genetics*, 164, 1567–1587.

Gawecka, K.A., Pedraza, F. & Bascompte, J. (2022). Effects of habitat destruction on coevolving metacommunities. *Ecology Letters*, 25, 2597–2610.

Grass, I., Jauker, B., Steffan-Dewenter, I., Tscharntke, T. & Jauker, F. (2018). Past and potential future effects of habitat fragmentation on structure and stability of plant–pollinator and host–parasitoid networks. *Nature Ecology and Evolution*, 2, 1408–1417.

Guillem, R.M., Drijfhout, F.P. & Martin, S.J. (2016). Species-Specific Cuticular Hydrocarbon Stability within European *Myrmica* Ants. *Journal of Chemical Ecology*, 42, 1052–1062.

Hallmann, C.A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., *et al.* (2017). More than 75 percent decline over 27 years in total flying insect biomass in protected areas. *PLoS ONE*, 12.

Hanski, I. (1994). A practical model of metapopulation dynamics. *The Journal of Animal Ecology*, 63, 151–162.

Howell, P.E., Lundrigan, B. & Scribner, K.T. (2016). Environmental and genealogical effects on emergence of cranial morphometric variability in reintroduced American martens. *Journal of Mammalogy*, 97, 761–773.

Hubisz, M.J., Falush, D., Stephens, M. & Pritchard, J.K. (2009). Inferring weak population structure with the assistance of sample group information. *Molecular Ecology Resources*, 9, 1322–1332.

Kajzer-Bonk, J., Skorka, P., Nowicki, P., Bonk, M., Krol, W., Szpilyk, D., *et al.* (2016). Relative contribution of matrix structure, patch resources and management to the local densities of two large blue butterfly species. *PLoS ONE*, 11, 1–19.

Koh, L.P., Dunn, R.R., Sodhi, N.S., Colwell, R.K., Proctor, H.C. & Smith, V.S. (2004). Species coextinctions and the biodiversity crisis. *Science*, 305, 1632–1634.

Nash, D.R., Als, T.D., Maile, R., Jones, G.R. & Boomsma, J.J. (2008). A mosaic of Chemical Coevolution in a Large Blue Butterfly. *Science*, 319, 88–90.

Oates, M.R. & Warren, M.S. (1990). *A review of butterfly introductions in Britain and Ireland.* World Wide Fund for Nature, Godalming.

Pritchard, J.K., Stephens, M. & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, 155, 945–959.

Raven, P.H. & Wagner, D.L. (2021). Agricultural intensification and climate change are rapidly decreasing insect biodiversity. *Proceedings of the*

National Academy of Sciences of the United States of America, 118, 1–6.

Sala, M., Casacci, L.P., Balletto, E., Bonelli, S. & Barbero, F. (2014). Variation in butterfly larval acoustics as a strategy to infiltrate and exploit host ant colony resources. *PLoS ONE*, 9, 20–23.

Schönrogge, K., Wardlaw, J.C., Peters, A.J., Everett, S., Thomas, J.A. & Elmes, G.W. (2004). Changes in chemical signature and host specificity from larval retrieval to full social integration in the myrmecophilous butterfly *Maculinea rebeli*. *Journal of Chemical Ecology*, 30.

Seddon, P.J., Griffiths, C.J., Soorae, P.S. & Armstrong, D.P. (2014). Reversing defaunation: restoring species in a changing world. *Science*, 345, 406–412.

Stankiewicz, A. & Sielezniew, M. (2002). Host specificity of *Maculinea teleius* Bgstr. and M. nausithous Bgstr. (Lepidoptera: Lycaenidae) the new insight. *Annales Zoologici*, 52, 403–408.

Tartally, A., Thomas, J.A., Anton, C., Balletto, E., Barbero, F., Bonelli, S., *et al.* (2019). Patterns of host use by brood parasitic *Maculinea* butterflies across Europe. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 374.

Taylor, G., Canessa, S., Clarke, R.H., Ingwersen, D., Armstrong, D.P., Seddon, P.J., *et al.* (2017). Is Reintroduction Biology an Effective Applied Science? *Trends in Ecology and Evolution*, 32, 873–880.

Therneau, T. (2015). survival: A Package for Survival Analysis in R. (R package version 2.37-7).

Thomas, J.A. (1984). The behaviour and habitat requirements of *Maculinea nausithous* (the dusky large blue butterfly) and M. teleius (the scarce large blue) in France. *Biological Conservation*, 28, 325–347.

Thomas, J.A. & Elmes, G.W. (1998). Higher productivity at the cost of increased host-specificity when *Maculinea* butterfly larvae exploit ant colonies through trophallaxis rather than by predation. *Ecological Entomology*, 23, 457–464.

Thomas, J.A., Elmes, G.W., Sielezniew, M., Stankiewicz-Fiedurek, A., Simcox, D.J., Settele, J., *et al.* (2013). Mimetic host shifts in an endangered social parasite of ants. *Proceedings of the Royal Society B: Biological Sciences*, 280.

Thomas, J.A., Schönrogge, K. & Elmes, G.W. (2005). Specialization and host associations of social parasites of ants. In: *Insect evolutionary ecology* (eds. Fellowes, M.D.E., Holloway, G.J. & Rolff, J.). CABI Publishing, Wallingford, pp. 475–514.

Thomas, J.A., Simcox, D.J. & Clarke, R.T. (2009). Successful conservation of a threatened Maculinea butterfly. *Science*, 325, 80–83.

Thomas, J.A., Telfer, M.G., Roy, D.B., Preston, C.D., Greenwood, J.J.D., Asher, J., *et al.* (2004). Comparative Losses of British Butterflies, Birds, and Plants and the Global Extinction Crisis. *Science*, 303, 1879–1881.

Waples, R.S. & Do, C. (2008). LDNE: A program for estimating effective population size from data on linkage disequilibrium. *Molecular Ecology Resources*, 8, 753–756.

Witek, M., Casacci, L.P., Barbero, F., Patricelli, D., Sala, M., Bossi, S., *et al.* (2013). Interspecific relationships in co-occurring populations of social parasites and their host ants. *Biological Journal of the Linnean Society*, 109, 699–709.

Witek, M., Nowicki, P., Śliwińska, E.B., Skórka, P., Settele, J., Schönrogge, K., *et al.* (2010). Local host ant specificity of *Phengaris* (*Maculinea*) *teleius* butterfly, an obligatory social parasite of Myrmica ants. *Ecological Entomology*, 35, 557–564.

Woyciechowski, M., Slowik, J. & Muehlenberg, M. (2006). Hosts of the butterfly, *Maculinea teleius*, among Myrmica ants in northern Mongolia (Lepidoptera: Lycaenidae; Hymenoptera: Formicidae). *Sociobiology*, 48, 493–502.

Wund, M.A., Singh, O.D., Geiselman, A. & Bell, M.A. (2016). Morphological evolution of an anadromous threespine stickleback population

within one generation after reintroduction to Cheney Lake, Alaska. *Evolutionary Ecology Research*, 17, 203–224.

Wynhoff, I. (1998). Lessons from the reintroduction of *Maculinea teleius* and M. nausithous in the Netherlands. *Journal of Insect Conservation*, 2, 47–57.

Wynhoff, I., Gestel, R. van, Swaay, C. van & Langevelde, F. van. (2011). Not only the butterflies: Managing ants on road verges to benefit *Phengaris* (*Maculinea*) butterflies. *Journal of Insect Conservation*, 15, 189–206.

Zayed, A., Packer, L., Grixti, J.C., Ruz, L., Owen, R.E. & Toro, H. (2005). Increased genetic differentiation in a specialist versus a generalist bee: implications for conservation. *Conservation Genetics*, 6, 1017–1026.

Manuscript 1

Ongoing coevolution between reintroduced *Phengaris teleius* butterflies and their *Myrmica* host ants

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Abstract

Evolutionary dynamics between parasites and their hosts are crucial to understand how certain species adapt, thus shedding light on ecosystem resilience. Herein, we explore the potential for adaptive responses in the social parasitic butterfly, *Phengaris teleius*, towards its ant host. The past reintroduction of *P. teleius* in the Netherlands offered a unique opportunity to delve into ongoing coevolution processes in this host-parasite system. We compared parasites' chemical and acoustical adaptations to those of the main and most abundant host ant, *Myrmica scabrinodis*, by analyzing samples from the reintroduced and paired source populations. We performed cuticular hydrocarbon analysis, vibroacoustic signals comparison, playback experiments, and a behavioral essay to study the potential changes in the caterpillars during their interaction with the ant hosts. We found that the reintroduced caterpillars exhibit distinct chemical and vibroacoustic signals compared to their source population after 30 generations since their reintroduction. Interestingly, the reintroduced population emitted vibroacoustic signals that were more similar to those of their sympatric ant hosts, suggesting potential for local adaptation. However, our analysis did not reveal any evidence of better performance in chemical mimicry. We offer evidence that *P. teleius*, the most generalist among all *Phengaris* butterflies, is able to respond to habitat changes and modify chemical and vibroacoustic signals to adapt to a new host population.

Keywords: cuticular hydrocarbon, vibroacoustics, host-parasite interaction, geographical mosaic of coevolution

Introduction

Interactions between species, including coevolutionary dynamics between parasites and hosts, represent critical forces that drive evolution and speciation (Thompson 1999; (Summers *et al.* 2003). A peculiar type of antagonistic interaction, where the parasite exploits a whole society instead of a single organism, is called social parasitism. Social parasitism occurs in diverse taxa, including birds,

fish, and, particularly, social insects like bees or ants (Wisenden 1999; Davies 2000; (Nash & Boomsma 2008). Numerous organisms have developed adaptations to live within ant societies, reflecting a wide spectrum of interactions from mutualism to parasitism (Hölldobler & Wilson 1990). Several strategies evolved by social parasites of ants allow them to infiltrate and integrate within host colonies by disrupting their communication system (Thomas *et al.* 2005). Among the most intriguing adaptations are those employed by caterpillars of *Phengaris* butterflies to exploit resources within *Myrmica* ant colonies. The caterpillar of these obligate social parasites do not actively enter their host nests; they are retrieved by *Myrmica* foraging ants and carried inside the colony, where they are cared for and fed until adult emergence (Thomas & Settele 2004). *Phengaris* immature instars "cheat" their hosts, making ants firstly adopt and then tolerate them, by corrupting ants' primary communication channels based on chemical (Akino *et al.* 1999; Schönrogge *et al.* 2004; Nash *et al.* 2008) and vibroacoustic signals (Barbero *et al.* 2009a; Sala *et al.* 2014). Nestmate recognition in social insects is mainly mediated by cuticular hydrocarbons (CHCs) (e.g., D'Ettorre & Lenoir 2010). *Myrmica* ants possess species-specific CHC profiles that can vary both qualitatively and quantitatively, with intraspecific variation observed among populations from distinct geographical localities (Elmes *et al.* 2002; Guillem *et al.* 2016; Casacci *et al.* 2019b). Sharing a similar chemical profile enables ants to discriminate between kin and intruders (Vander Meer & Morel 1998). However, early stages of *Phengaris* caterpillars (pre-adoption) possess a simple blend of CHCs that mimics those of *Myrmica* ants, thus deceiving the foragers and promoting the parasite's adoption inside host colonies (Akino *et al.* 1999). Soon after entering the nest (post-adoption), the chemical similarity between the caterpillars and the workers increases, allowing the parasite to live in the host society until pupation (Schönrogge *et al.* 2004). Besides imitating chemical recognition cues, *Phengaris* caterpillars can also produce "calls" that closely resemble the vibroacoustic signals emitted by *Myrmica* ants, especially queens (Barbero *et al.* 2009b; Casacci *et al.* 2013; Schönrogge *et al.* 2017). Since vibrations are used for inter- and intra-cast communication in ants, the queen-like signals produced by the parasite effectively deceive the workers that treat the caterpillars as valuable items in the colony hierarchy, as their own larvae or even the queen.

Different *Phengaris* species and populations use various *Myrmica* ants as host, and these complex patterns represent a geographical mosaic of coevolution, at least for some species (Nash *et al.* 2008; Tartally *et al.* 2019). Moreover, the existence of local adaptations between these butterflies and their host ants was found in some populations of 'cuckoo'-feeding *Phengaris* group (Nash *et al.* 2008; Thomas *et al.* 2013; Casacci *et al.* 2019b; Tartally *et al.* 2019). Cuckoo species are fed directly by nurse ants via trophallaxis; thus, they require a spotless integration and full acceptance as kin in the host society, possibly leading to a high host-specific populations (Thomas & Elmes 1998). In contrast, predatory species, like *Phengaris arion* or *Phengaris teleius*, actively prey on ant broods and, once they are gathered from outside into the nest, they only need not to be discarded as intruders, having the possibility to parasitize a wider range of hosts.

To test whether generalist social parasites like *P. teleius* could evolve adaptations to their local hosts and detect potential ongoing coevolution processes in

this host-parasite system, we surveyed a reintroduced and its paired source metapopulation. A detailed description of the reintroduction is provided in (Wynhoff 1998). Briefly, 86 *P. teleius* adult butterflies were moved from the Polish metapopulation occurring in Krakow to the Dutch reserve of Moerputten in 1990. The successful reintroduction has led to the establishment of a metapopulation in the Netherlands, currently consisting of several thousand individuals (Irma Wynhoff, unpublished data).

A gap of almost 30 butterfly generations since the reintroduction offered a unique opportunity to study the chemical and acoustical adaptations of *P. teleius* butterflies to their main and most abundant host ant, *Myrmica scabrinodis*. We hypothesized that: 1) the CHC profile of the butterfly caterpillars differs between metapopulations and is more similar to the profile of local *M. scabrinodis* host ants; 2) the vibroacoustic signals emitted by pre-adoption *P. teleius* caterpillars differ among metapopulations and are more similar to cues emitted by local host ants; 3) the caterpillars exposed to host ants from their sympatric metapopulations pass through a more benevolent and successful adoption process, and present a higher survival within the ant colony.

Material and Methods

Collection of Phengaris teleius caterpillars and Myrmica scabrinodis ants

P. teleius caterpillars and *M. scabrinodis* ant colonies were collected from the source metapopulation (PL) located in the Vistula River Valley in Krakow, Southern Poland (50°01'N, 19°54'E) and from the reintroduced metapopulation (NL) in the nature reserve of Moerputten located in the south of the city of 's-Hertogenbosch in the Netherlands (51°41'N, 5°15'E).

The samples to study the pre-adoption CHC profile and vibroacoustic signals were collected in August 2019; the samples to perform the behavioral essay and study the post-adoption CHC profile were collected in July and August 2020; and the samples to perform the playback experiment were collected in August and October 2021.

P. teleius caterpillars were obtained from the flowers of its foodplant, *S. officinalis*. These samples are hereafter labeled as "pre-adoption". The selection of flowers with the presence of butterfly caterpillars was done in the field and single stems of *S. officinalis* were collected and taken to the laboratory. These plants were gathered into bunches of a few stems, placing the base of the stems in water. Each bunch was kept in a plastic container, in which the walls were covered by fluon to prevent *P. teleius* caterpillars from escaping. The containers were checked every morning and late afternoon to obtain butterfly caterpillars. Only the fourth-instar caterpillars were used in the study. *M scabrinodis* ant workers were collected and kept alive in plastic containers until used for different purposes. Before adopting a *P. teleius* caterpillar, it was called laboratory nest; when they host a butterfly caterpillar, they are named "post-adoption" colonies. In the same way, once inside the nests, *P. teleius* caterpillars are referred to as "post-adoption" caterpillars.

CHC extraction and GC-MS analysis

Pre-adoption *P. teleius* caterpillars' CHC compounds were extracted within a few hours after they left their host plant. Pre-adoption *M. scabrinodis* workers were processed one day after collecting in the field. Ant samples consisted of a group of five

ants pooled together into the same vial. Three replicates were taken from each sampling colony. Pre-adoption samples were extracted from a total of 21 *M. scabrinodis* and 25 *P. teleius* caterpillar samples from Poland, and 24 *M. scabrinodis* and 19 *P. teleius* caterpillar samples from the Netherlands.

The samples of post-adopted *P. teleius* caterpillars and their host *M. scabrinodis* were collected during the behavioral experiment on caterpillar adoption, whose detailed protocol is described below. The CHC profiles of the caterpillars and host ants were extracted three days after caterpillar adoption. For CHC extraction, we collected a total of three *P. teleius* caterpillars from the Polish source population adopted by their sympatric host ants and three adopted by the Dutch host ants; two *P. teleius* caterpillars from the Dutch reintroduced metapopulation adopted by their sympatric host ants and two adopted by Polish ants. Moreover, we also extracted the CHC profile from the host ants.

CHCs were extracted either from individual caterpillars or from pools of five ant workers by placing them in a glass vial with 200 µl of hexane for 10 minutes. After extraction, all vials (from pre- and post-adopted experiments) were stored at -20°C until analysis. For shipping, samples were evaporated. Prior to chemical analyses, *P. teleius* caterpillar samples were suspended in a final volume of 20 µL of pentane (HPLC grade; Sigma Aldrich) with n-heptadecane (n-C17: at 5 ng/µL in 100 ng) as internal standard. The *M. scabrinodis* ant extracts were suspended in 60 µL of pentane, with n-eicosane (n-C20: at 5 ng/µL in 300 ng) as internal standard. For caterpillar samples, 3 µL were added in manual injection, and for ant worker samples, an aliquot of 2 µL of the solution was injected using an Agilent G4513A Automatic Liquid Sampler into an Agilent Technologies 7890A gas chromatograph coupled with an Agilent 5975 C mass spectrometer (Agilent Technologies, Les Ulis, France). The GC column (30 m × 0.25 mm × 0.25 µm film) was coated with HP5MS (Agilent Technologies), and helium was used as a carrier gas (1 mL/min). Injection was splitless, and the oven temperature was set at 60 °C for 1 min, then it was raised from 60 °C to 220 °C at 20 °C.min−1, then to 250 °C at 3 °C.min−1 and then to 320°C at 5 °C.min−1 and held for 5 min. Mass spectra were recorded with electron impact ionization at 70 eV.

CHC raw data processing

The chromatograms were analyzed in MSD ChemStation E.02.01.1177 (Agilent Technologies) with the RTE integrator to calculate the area of each peak of interest using the proportion of the sum over the area of all peaks applying the following parameters. Data point sampling: 1, Detection filtering: 7 points, Start threshold: 0.020, Stop threshold: 0.000, Baseline reset > 50, If leading or trailing edge < 100, Minimum peak area: 0.1 % of largest Peak, Peak location: Centroid, Maximum number of peaks: 250 and Baseline Preference: Baseline drop else tangent. Hydrocarbons were identified on the basis of their mass spectra and retention times, and compared with known standards.

CHC peaks retention time was used to perform an automatic aligning of the compounds by applying an alignment algorithm implemented in the align_chromatograms() function (Ottensmann *et al.* 2018) involving three sequential steps: 1) each sample is aligned to a reference sample while maximizing overall similarity through linear shifts of retention times, 2) individual peaks are sorted into rows based

on close similarity of their retention times and 3) rows representing putatively homologous substances are merged. After aligning, all peaks were double-checked to ensure a correct identification. Only peaks with a retention time from 8 to 26.5 were selected.

CHC intensity values after peak integration were transformed to absolute abundance data by correcting with the internal standard. Data were filtered by selecting the compounds with an absolute abundance higher than or equal to 0.01% per sample. Only the compounds present in at least 70% of the samples per group (species+population) were taken. CHC absolute abundance was then divided by its sample dry mass weight, and log10 was applied to avoid over-representation of very abundant compounds. Samples were dry at 50ºC for a week, and subsequently, they were weighted in a Radwag microbalance MYA 5.4Y (± 1 µg) to estimate their dry mass.

Alkanes were removed from the dataset because of sample contamination to reduce noise during the analysis.

CHC statistical analysis

CHC profile distances between groups of individuals characterized by species (*P. teleius* caterpillar and *M. scabrinodis* ant), adoption state (pre and post-adoption) and population (Poland and the Netherlands) were calculated by the vegdist() function (Oksanen *et al.* 2022) with the Bray-Curtis dissimilarity index. A Permutational analysis of variance (PERMANOVA) by the adonis2() function (Oksanen *et al.* 2022) was applied to assess the significance of species (*P. teleius* caterpillar and *M. scabrinodis* ant) + adoption state (pre and post-adoption) and population (Poland and the Netherlands). The matrix of Bray-Curtis distances was subsetted in three different ways: 1) pre-adoption *P. teleius* and pre-adoption *M. scabrinodis*, 2) post-adoption *P teleius* and post-adoption *M. scabrinodis* and 3) pre-adoption *P. teleius* and post-adoption *P. teleius*. From each of these different subsets, distances were extracted and fitted to a generalized linear model with gaussian distribution, applying the ant and caterpillar populations as predictor variables (for instance, distance ~ population1 + population2) by using the glm() function (R Core Team 2023). Variable significance was tested with ANOVA with the Anova() function (Fox & Weisberg 2019), and the groups were pairwise-compared by applying estimated marginal means (EMMs) with Bonferroni correction by using the emmeans() function (Lenth 2023).

The CHC compounds of each group with a significantly higher abundance respecting pre-adoption *P. teleius* caterpillars were estimated by the multipatt() function (De Cáceres & Legendre 2009). The main data frame was subseted into three different ones (pre and post-adoption *M. scabrinodis* and post-adoption *P. teleius*) and pairwise compared with pre-adoption *P. teleius*.

All statistical analyses were performed in R (R Core Team 2023).

Vibroacoustics signal recordings

We utilized a custom-made equipment designed for recording the sounds produced by undisturbed, unstressed insects (see Riva *et al.* 2017 for a detailed description). We recorded a total of eight *P. teleius* caterpillars, five ant queens and 10 ant workers from Poland, and six *P. teleius* caterpillars, three ant queens and 10 ant workers from the Netherlands. The recording equipment included a recording chamber measuring 12.5 × 8 × 2 cm, housing a

miniature moving-coil microphone (with a sensitivity of 2.5 mV/Pa/1.0 kHz) attached at its center. The sampling rate was set to 44.10 kHz. Another identical moving-coil microphone was employed to record ambient noise, combining the signals from both microphones after passing through preamplifiers. The overall frequency range covered from 20 Hz to 20 kHz, with a gain of approximately 83 dB. A 12 V gel cell battery powered the recording equipment. The recording chamber and the microphone were situated within an anechoic chamber to further minimize background noise and interference. *P. teleius* caterpillars and ants were individually positioned on the microphone surface inside the recording chamber and recorded in the morning under room temperature conditions (23–25°C). Recording sessions lasted 10 minutes, starting 5 minutes after introducing the specimens into the recording chamber. We carefully examined segments containing acoustic recordings and digitally saved them in WAV format with a 16-bit amplitude resolution using Audacity version 3.0.3. Temporal and spectral features of the signals were subsequently measured using Praat version 6.2.14. We then selected three sequences of vibroacoustic signals per individual. In the case of ant stridulations, each sequence was made of alternate five units a and five units b, while in the case of caterpillars, each sequence was made of five units of the same type. For each unit, we measured six temporal, spectral and intensity vibroacoustic variables, namely: the peak frequency (Fpeak, Hz), the third quartile of the energy spectrum (Q75%, Hz; representing 75% of the call's energy), the unit duration (Δt, s), the mean intensity of the entire call, quantified by the root-mean-square signal level (RMS, dB), the energy of the peak frequency (EnFpeak, $Pa^2 \cdot s$) and the ratio of the peak frequency energy to the total call energy, expressed as a percentage (%EFpeak).

Playback experiments

To test whether the sounds emitted by the fourth instar caterpillars were able to produce a greater behavioral response in their sympatric host ants, we conducted playback experiments using four colonies from Poland and five colonies from the Netherlands in August and October 2021. Playback experiments were carried out in artificial arenas made of plastic cylinders (7 x 7 x 5 cm). A speaker was glued to the bottom of each arena. We covered the speaker with a thin layer of slightly damp soil to simulate natural conditions. In each arena, we introduced five *M. scabrinodis* ant workers and allowed them to settle for 10 minutes before exposing them to one of the five vibroacoustic signals previously recorded and emitted by 1) *M. scabrinodis* queens, 2) *M. scabrinodis* workers, 3) sympatric caterpillars of *P. teleius* and 4) allopatric caterpillars of *P. teleius*; a 5) white noise was used as a control. We employed MP3 devices to play continuous loops of the original recordings, adjusting the volume to match the natural level (for detailed volume adjustments, refer to Sala *et al.* 2014). Each experimental trial spanned 30 minutes, with behavior observations conducted in one-minute intervals for each arena, in sequential order for the six signal types, totaling six minutes of signal exposure per trial. Five different benevolent behaviors were registered during the observations: 1) walking – worker ants were attracted to the speaker but continued walking without stopping on it; 2) staying – workers rested on the speaker for at least 5 seconds; 3) antennating – worker ants approached the speaker and engaged with it by antennating it for at least 3 seconds; 4) guarding – workers stayed on the speaker

and raised the thorax and head keeping the mandibles opened; 5) digging - in a few instances, worker ants dug into the soil surrounding the speaker. We replicated the playback experiment per each colony from two to three times, using new *Myrmica* ants for each arena. The signal source in each arena was randomized to account for potential positional effects. Prior to each trial, new soil was introduced, and all equipment was thoroughly cleaned with absolute alcohol.

Vibroacoustics data analysis

We performed a non-metric multidimensional scaling ordination (NMDS) based on Euclidean distances, using the metaMDS() function (Oksanen *et al.* 2022). The euclidean distances were calculated with the normalized vibroacoustic parameters derived from the signal units emitted by the recorded specimens. In order to assess disparities in vibroacoustic signals between the host (queens and workers) and pre-adoption parasitic caterpillars from the two populations, we carried out analysis of similarities (ANOSIM), conducting 9999 permutations. This analysis was conducted using the anosim() function (Oksanen *et al.* 2022). The vibroacoustic signal Euclidean distances were also analyzed fitting the data to a general linear model with ant and caterpillar metapopulations as predictor variables (for instance, distance ~ ant + caterpillar metapopulation) by using the glm() function (R Core Team 2023). Differences in the average values of vibroacoustic parameters calculated on the signal units emitted by ant caste individuals and *M. teleius* pre-adoption caterpillars from the two populations were tested by applying linear mixed-effects models with individuals as a random factor (for instance, parameter ~ group + (1|individual)) by using the glmer() function (Bates *et al.* 2015). Predictor significance was tested with ANOVA using the Anova() function (Fox & Weisberg 2019). Groups were pairwise-compared by performing estimated marginal means (EMMs) tests with Bonferroni correction by using the emmeans() function (Lenth 2023).

We also compared the impact of the vibroacoustic signals on the total instances of behaviors exhibited by the worker ants of the two populations. To do so, we employed a negative binomial generalized linear mixed-effects model with "colony" as a random factor (for instance, behaviors ~ sounds + (1|colony)) by using the glmer.nb() function (Bates *et al.* 2015). Predictor significance was tested with ANOVA using the Anova() function (Fox & Weisberg 2019). Subsequently, we conducted post-hoc pairwise comparisons among the different factor levels by performing estimated marginal means (EMMs) tests with Bonferroni correction by using the emmeans() function (Lenth 2023).

Behavioral essay

Phengaris teleius caterpillars were collected in both source (PL) and reintroduced (NL) metapopulations from their host plant, *S. officinallis.* Just after field collection in the Netherlands, single stems bearing *P. teleius* caterpillars were shipped to Poland in 24 hours. Plants with caterpillars collected in the Polish metapopulation were also transported to Warsaw on the day of the collection. A behavioral experiment was performed in Warsaw in the laboratory of the Museum and Institute of Zoology in August 2020. *M. scabrinodis* ant colonies were collected in the field in both metapopulations and transported to Warsaw before the *P. teleius* caterpillars were collected. From each ant colony, two sub-colonies consisting of 100

workers (50 foragers and 50 intra-nidal workers) and 30 ant larvae were established in plastic boxes. Altogether, 15 colonies from the Polish metapopulation and 11 colonies from the Dutch metapopulation were used in the experiment. One sub-colony from each colony was used to observe the adoption of a butterfly caterpillar from the sympatric population, and another sub-colony for the adoption of an allopatric caterpillar. Only the fourth-instar caterpillars from the morning check were used for the adoption experiment. Sub-colonies of *M. scabrinodis* ants were kept in plastic boxes (23 x 15 x 6 cm) in which walls were covered with paraffin to prevent workers from escaping. A wet sponge covered by a plastic lid with an entrance notch to provide a suitable and dark place for ants was put on the side. Colonies were fed twice a week with a solution of sugar water and pieces of crickets placed on a circular metallic plate (Ø 3 cm). A butterfly caterpillar was put on a circular metallic plate (Ø 3 cm) at the furthest distance from the entrance of the ant nest to observe adoption. The time of caterpillar insertion and the time of the first contact with ants were noted and detailed observations were made from the first contact of the *P. teleius* caterpillar with workers, and lasted 60 min. All behavioral events displayed by *M. scabrinodis* workers were recorded and categorized as follows: 1) antennation, 2) grooming, 3) licking larval secretions, 4) picking up the caterpillar (caterpillar was taken by an ant and transported for some distance) and 5) aggression events like mandible gaping, biting and stinging. The number of sugary drops secreted by the caterpillars were also counted. The ant behaviors were divided into antennation, which was considered inspection behavior, positive behaviors (grooming, licking and picking up) and negative behaviors (aggression events). We also noted whether adoption occurred within 60 min after the first contact. If not, the boxes were checked during the following five hours, every hour after the end of the observation experiment. When the adoption did not occur even during this time, we checked the colony and the presence of the butterfly caterpillar after 24 hours. If the caterpillar was present inside the ant nest (together with workers and brood), we considered that the adoption happened within 24 hours. The survival of the caterpillar was checked every day, starting 24 hours after the adoption until its death. Every week, 10 ant larvae were added to each ant sub-colony to provide a food source for the *P. teleius* caterpillars.

Behavioral data analysis

The number of antennation events, positive behaviors, negative behaviors and number of drops were standardized so as not to underestimate the number of observations when the caterpillar was adopted or killed before the observation time finished. The values were divided by the number of minutes in which the observation ended and multiplied by 60. Antennation event counts were fitted to a generalized linear mixed-effects model with poisson distribution applying the ant, caterpillar population and their interaction as predictor variables and the ant colony as random factor (for instance, antennation ~ ant_population * caterpillar_population + (1|ant_colony)) by using the glmmTMB() function (Brooks *et al.* 2017). The positive and negative behaviors were fitted to a generalized linear mixed-effects model with negative binomial Type I and II distribution respectively, applying the ant, caterpillar metapopulation and their interaction as predictor variables, and the ant colony as random factor (for instance, positive ~

ant_population * caterpillar_population + (1|ant_colony)) by using the glmmTMB() function (Brooks *et al.* 2017). The number of drops produced by the caterpillars were also fitted to a generalized linear mixed-effects model with negative binomial Type II distribution. Predictor significance was tested with ANOVA using the Anova() function (Fox & Weisberg 2019) and the groups of each model were pairwise-compared by performing an estimated marginal means (EMMs) test with Bonferroni correction by using the emmeans() function (Lenth 2023). The number of positive and negative behaviors was correlated with the number of drops produced by the caterpillars by using the cor.test() function (R Core Team 2023).

Adoption was fitted to a generalized linear mixed-effects model with binomial distribution, applying the ant and caterpillar metapopulations as predictor variables and the ant colony as random factor (for instance, adoption ~ ant_population + caterpillar_population + (1|ant_colony)) by using the glmer() function (Bates *et al.* 2015). Predictor significance was tested with ANOVA using the Anova() function (Fox & Weisberg 2019), and groups were pairwise-compared by performing an estimated marginal means (EMMs) test with Bonferroni correction by using the emmeans() function (Lenth 2023).

Survival days were fitted to a Cox proportional-hazards model applying the ant and caterpillar metapopulation as predictor variables (for instance, Surv(survival_days + 1, adoption) ~ ant_population + caterpillar_population) by the coxph() function (Therneau 2023). Predictor significance was tested with ANOVA using the Anova() function (Fox & Weisberg 2019). Hazards ratios indicate a covariate level that is neutrally (= 1), positively (< 1) or negatively (> 1) associated with the length of survival respecting the reference level. Predicted survival proportions were estimated with the survfit() function (Therneau 2023). The survival curves were pairwise compared with an estimated marginal means (EMMs) test with Bonferroni correction by using the emmeans() function (Lenth 2023).

Results

Cuticular hydrocarbon adaptations

A total of 31 cuticular hydrocarbon compounds were identified both for *Myrmica scabrinodis* ants and *Phengaris teleius* caterpillars, and 23 of them were common compounds among all groups (Fig. S1). The list of analyzed compounds and their presence in the different groups can be found in Fig. S2. Additionally a graphical representation of the CHC profiles can be found in Fig. S3.

All *M. scabrinodis* and *P. teleius* groups (pre- and post-adoption) showed differences in their CHC profile (d.f. = 3, F = 408.67, p = 0.001). The CHC profile of the individuals was significantly influenced by the metapopulation from which they originated (d.f. = 1, F = 13.04, p = 0.001). However, not all groups displayed differences in their CHC profile among individuals of different metapopulations, as evidenced by the significance in the interaction between group and metapopulation (d.f. = 3, F = 9.9, p = 0.001). For instance, post-adoption host ants showed an overlap among the CHC profiles of individuals from Poland and the Netherlands (Fig. 1a).

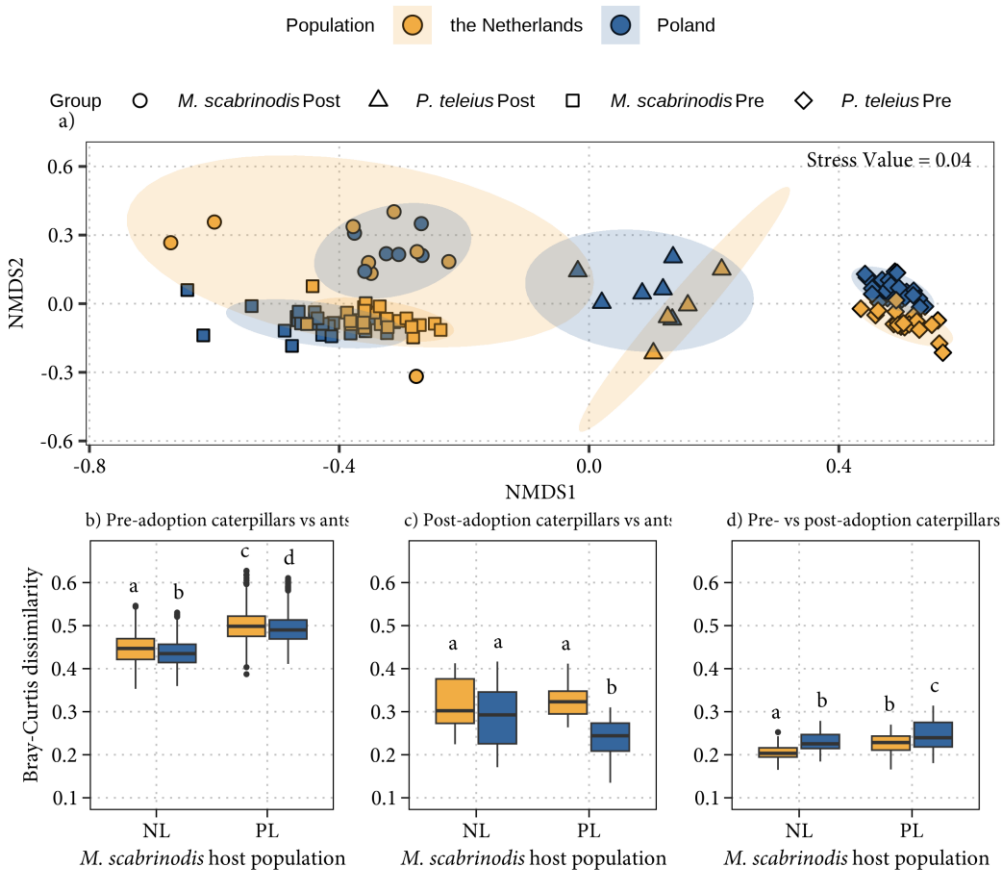

Fig. 1: CHC profiles comparison of the reintroduced and source metapopulation of *P. teleius* parasitic caterpillars and its host ant *M. scabrinodis*. (a) Non-Metric Multidimensional Scaling (NMDS) ordination graph representing the distribution of the different studied groups based on Bray-Curtis dissimilarity distances. Color indicates the metapopulation: yellow (the Netherlands) and blue (Poland). Shape indicates the species and pre- or post-adoption state of the individuals: circle (post-adoption *M. scabrinodis*), triangle (post-adoption *P. teleius*), rectangle (pre-adoption *M. scabrinodis*) and cross (pre-adoption *P. teleius*). Stress value = 0.04. On the bottom, Bray-Curtis dissimilarity distance comparisons between b) pre-adoption *P. teleius* and pre-adoption *M. scabrinodis*, c) post-adoption *P. teleius* and post-adoption *M. scabrinodis*, and d) post-adoption *P. teleius* and its correspondent pre-adoption *P. teleius* individuals. The X axis indicates the population of the *M. scabrinodis* host ants. The boxplot color indicates the metapopulation of origin for *P. teleius*: yellow (the Netherlands) and blue (Poland). Horizontal lines represent median values, the boxes the first and third quartiles and whiskers the maximum and minimum values. Dots represent outliers. Lower-case letters above boxplots indicate pairwise significant differences between groups based on an estimated marginal means (EMMs) test.

The CHC profile similarity between pre-adoption *P. teleius* parasite caterpillars and *M. scabrinodis* host ants was significantly affected by the metapopulation they belong (Table S1a). *P. teleius* pre-adoption caterpillars from Poland exhibited a closer CHC profile to any of the *M. scabrinodis* host ant metapopulations than *P. teleius* caterpillars from the Netherlands (Fig. 1b; Table S1b). Additionally, the

Polish *M. scabrinodis* host ants possessed a less similar CHC profile to pre-adopted *P. teleius* from any metapopulation than the Dutch host ants.

The CHC profile similarity between post-adoption *P. teleius* caterpillars and their host ants was also significantly affected by their metapopulations (Table S2a). The post-adopted reintroduced caterpillars did not show to be chemically closer to any of the host ants, while the Polish caterpillars showed to be more similar to their sympatric host (Fig. 1c; Table S2b). Additionally, the post-adopted caterpillars from Poland demonstrated the closest similarity to the ant CHC profile overall when parasitizing their sympatric host. However, no difference was found between Polish and Dutch caterpillars when they were compared to Dutch host ants (Table S2a).

We also analyzed how much the CHC profile of the caterpillars changed after the adoption by host ants from different metapopulations. The chemical signature was significantly affected by the metapopulation of both post-adoption caterpillars and host ants (Table S3a). The CHC profile of *P. teleius* caterpillars from the Netherlands changed less than that of the Polish caterpillars. The reintroduced Dutch caterpillars parasitizing their sympatric host ants showed the smallest changes in their CHC profile. In contrast, the Polish caterpillars reared by their sympatric host ants underwent the most significant changes in their CHC profile (Fig. 1d; Table S3b).

In order to test to what extent the post-adopted caterpillars changed their CHC profile to mimic their host ants, we compared between post-adoption caterpillars and host ants the compounds that were found in significantly higher amounts respecting the pre-adoption caterpillars. Post-adopted *P. teleius* and *M. scabrinodis* ants showed a high degree of similarity in the compounds whose abundance increased in the caterpillars when they were reared in the nest. In particular, nine out of the ten compounds found in pre-adopted *M. scabrinodis* were also found in post-adopted *P. teleius* (Fig. S4; Table S4). All the compounds that significantly increased in post-adopted *P. teleius* were also present in pre-adoption *M. scabrinodis*. Moreover, all compounds found in post-adoption *M. scabrinodis* were also found in the post-adoption caterpillars. However, post-adoption *P. teleius* had a higher abundance in two compounds not found in post-adoption *M. scabrinodis*, one of them (5,17-diMeC29) not detected in pre-adoption caterpillars.

Vibroacoustic adaptations

We recorded and analyzed the stridulations made by both worker and queen ants of *M. scabrinodis*, as well as the vibroacoustic signals produced by pre-adoption caterpillars of *P. teleius* collected in Poland and the Netherlands. Antand caterpillar vibroacoustic signals consisted of sequences (trains) of variable numbers of units (as shown in Fig. S5).

Non-metric multidimensional scaling ordination to analyze the vibroacoustic patterns of ants and caterpillars showed a separation among metapopulations and species (Fig. 2) confirmed by the analysis of similarities test (ANOSIM: $R = 0.537$, $p < 0.001$). The signals produced by *M. scabrinodis* queens were statistically distinct between metapopulations (ANOSIM: $R = 0.407$, $p < 0.001$) as well as those emitted by workers (ANOSIM: $R = 0.568$, $p < 0.001$). Also, the vibroacoustic pattern of *P. teleius* pre-adoption caterpillars differed between metapopulations (ANOSIM: $R = 0.536$, $p < 0.001$). Comparing the emissions of the parasitic caterpillars, they appeared significantly distinct from the host

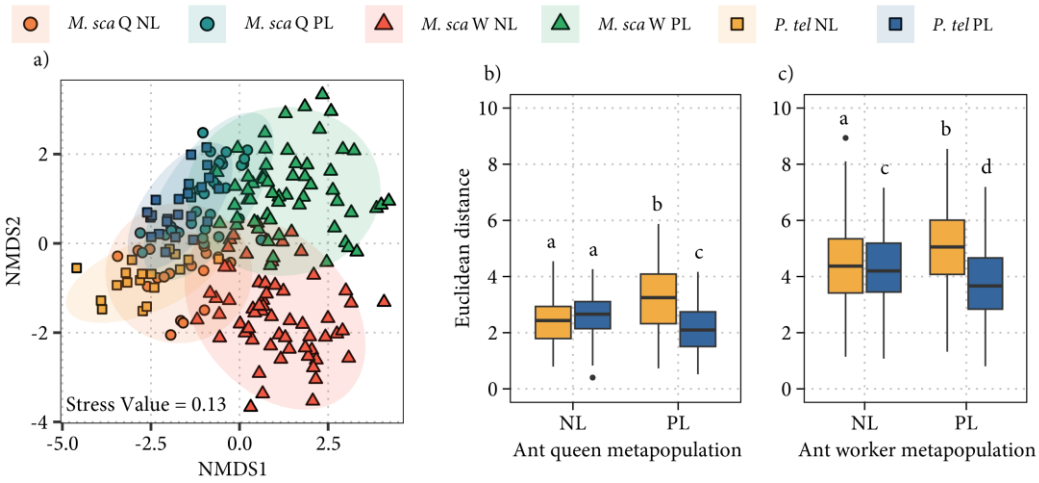

Fig. 2: Pattern of vibroacoustic signal emitted by *P. teleius* caterpillars, ant queens and workers. (a) Non-metric Multidimensional Scaling Ordination of vibracoustic signals emitted by *M. scabrinodis* workers and queens and *M. teleius* caterpillars based on normalized Euclidean distances calculated using single unit parameters. Each point represents 'average' values of pulse parameters calculated over a train of pulses (see Methods for more details). In the legend: *M. sca* refers to *M. scabrinodis*; *P. tel* refers to *P. teleius*; Q refers to queen; W refers to workers; NL refers to the Netherlands; and PL refers to Poland. b) Boxplots of Euclidean distances calculated on normalized vibroacoustic parameters of signals emitted by caterpillars and ant queens. c) Boxplots of Euclidean distances calculated on normalized vibroacoustic parameters of signals emitted by caterpillars and ant workers. Horizontal lines represent median values, the boxes the first and third quartiles and whiskers the maximum and minimum values. Dots represent outliers. Lower-case letters above boxplots indicate pairwise significant differences between castes based on an estimated marginal means (EMMs) test.

worker emissions, but *P. teleius* caterpillar signals were closer to those of queens. Nevertheless, in the source metapopulation, the signals of queens and *P. teleius* caterpillars showed the highest degree of similarity with an R value close to 0 (Poland, ANOSIM: $R = 0.082$, $p = 0.016$; the Netherlands, ANOSIM: $R = 0.231$, $p < 0.001$).

The vibroacoustic similarity was also analyzed fitting the euclidean distances among queen/ant workers and *P. teleius* caterpillars in a generalized linear model. The differences in vibroacoustic signals were significantly affected by the origin of the caterpillars, but not directly influenced by the origin of the host ants (Table S5a and 6a). The reintroduced caterpillars produced a vibroacoustic signal more similar to that of their sympatric host ants. Similarly, Polish caterpillars exhibited a signal profile comparable to that of their sympatric host ants. Overall, the caterpillars from Poland had the closest vibroacoustic signal when compared to their Polish sympatric host ants, but no difference was found between Polish and Dutch caterpillars when they were compared to Dutch host ants (Fig. 2b and 2c; Table S5b and 6b).

Our results revealed that the vibroacoustic signals emitted by the Polish *M. scabrinodis* queens and the Polish *P. teleius* caterpillars share similar values for all the estimated vibroacoustic parameters (Fig. S6; Table S7 and 8). Most of the vibroacoustic parameter values of the Dutch *P. teleius* caterpillars are similar

to those of their sympatric queens apart from the root-mean-square values (Fig. S6d; Table S8d). Moreover, the parameters related to sound amplitude (the root-mean-square and energy of the peak frequency) differed between *P. teleius* caterpillars from the Dutch and Polish metapopulation (Fig. S6d and 6e; Table S8d and 8e). The amplitude parameters not only differed in the caterpillars, but also showed a considerable divergence among ant metapopulations.

During the playback experiments, worker ants displayed no aggressive or alarmed behaviors. Instead, we observed five positive responses. Notably, the digging behavior, although observed infrequently, was never triggered by the white noise control stimulus. We analyzed the worker ants' reactions using generalized linear models, and the results indicated significant differences in their responses to the five vibroacoustic signals, when considering the total behaviors exhibited per minute of observation (Fig. 3 and Table S9). If we compare the behavioral reactions exhibited by the workers when we reproduced the sounds of *P. teleius* caterpillars, it is interesting to note that the workers of each metapopulation reacted significantly more to the sounds produced by their sympatric social parasite (Polish workers: $z = 5.079$, $p < 0.001$; Dutch workers: $z = -4.323$, $p < 0.001$).

Behavioral responses

During the adoption observations, the metapopulation of the caterpillars influenced the number of antennation events performed by the host ants (Table S10a). However, the number of antennation events did not differ among groups, representing all the combinations between adoptions in sympatric and allopatric hosts (Fig. 4a; Table S10b). The metapopulation of the ants and the interaction among the metapopulation of the caterpillars and ants did not show any significant effect (Table S10a).

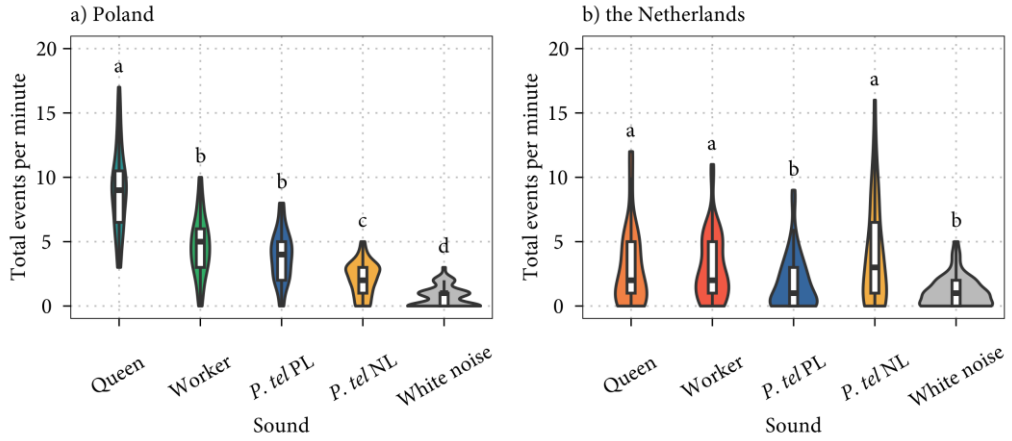

Fig. 3: Behavioral responses of *M. scabrinodis* workers from a) Poland and b) the Netherlands to vibroacoustic emissions of sympatric *M. scabrinodis* queens and workers, and the emission of sympatric and allopatric *P. teleius* caterpillars and to a control signal (white noise). Horizontal lines represent median values, the boxes the first and third quartiles and whiskers the maximum and minimum values. Lower-case letters above boxplots indicate pairwise significant differences between vibroacoustic stimuli based on an estimated marginal means (EMMs) test.

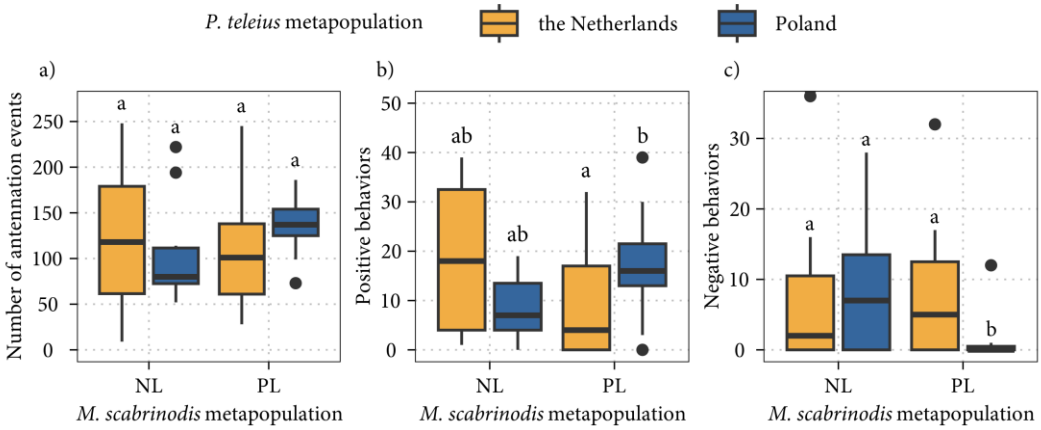

Fig. 4: *M. scabrinodis* ant and *P. teleius* caterpillar behavioral cross-metapopulation experiment results for a) number of antennation events, b) positive behaviors and c) negative behaviors. The color indicates the metapopulation of origin for *P. teleius*: yellow (the Netherlands) and blue (Poland). Horizontal lines represent median values, the boxes the first and third quartiles and whiskers the maximum and minimum values. Dots represent outliers. Lower-case letters indicate pairwise significant differences between groups based on an estimated marginal means (EMMs) test.

The number of positive behaviors from the Polish host ants to their sympatric *P. teleius* caterpillars was significantly higher compared to the positive reaction of the ants to the allopatric Dutch caterpillars, however the reaction of the Dutch ants did not differ between the caterpillars of the Dutch and Polish metapopulations (Fig. 4b; Table S11b). The metapopulation of the caterpillars and ants did not show to be a trigger for a change in the number of positive behaviors in any case (Table S11a). However, the interaction between both variables showed a cross-over effect with a higher median number of positive behaviors for the sympatric combinations.

The number of negative behaviors received by *P. teleius* caterpillars from the host ants was significantly lower for the sympatric Polish host-parasite combination compared to any other group (Fig. 4c; Table S12b). The caterpillar metapopulation did not show to be a general trigger for a change in the number of negative behaviors in any case, but the ant metapopulation and the interaction among caterpillar and ant metapopulation influenced the number of negative behaviors (Table S12a).

The number of drops produced by the caterpillars was also analyzed. None of the studied variables significantly affected the number of produced drops (Fig. S7a; Table S13). On the other hand, the number of drops were highly positively correlated with the number of positive behaviors performed by ants toward butterfly caterpillars ($r^2 = 0.49$, $p < 0.001$; Fig. S7b), but we did not find any correlation for the number of drops in relation to the number of ant negative behaviors ($r^2 = -0.16$, $p = 0.259$; Fig. S7c).

P. teleius caterpillars from Poland had a higher probability of being adopted (Fig. 5a). The metapopulation of the caterpillars influenced the adoption success, whereas the metapopulation of the host ants did not show any significant effect on the proportion of adopted individuals (Table S14).

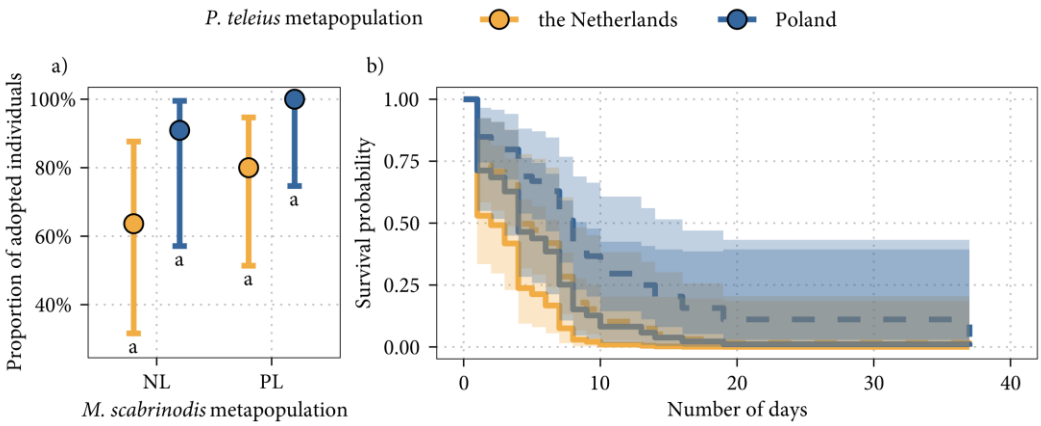

Fig. 5: *M. scabrinodis* ant and *P. teleius* caterpillar behavioral cross-metapopulation experiment results for a) adoption success and b) caterpillar survival probability. The color indicates the metapopulation of origin for *P. teleius*: yellow (the Netherlands) and blue (Poland). In plot a) lower-case letters indicate pairwise significant differences between groups based on an estimated marginal means (EMMs) test. In plot b) solid lines refers to the host ant Dutch metapopulation, while dotted ones refer to the Polish metapopulation.

The caterpillars' survival after adoption was significantly affected by the metapopulation of the host ants and marginally influenced by the metapopulation of the caterpillars (Fig. 5b; Table S15a). The hazard ratios showed a significant increase of the survival probability of the caterpillars after being adopted by the Polish host ants (HR = 0.49, p = 0.037) and a marginally significant increase of the survival when the caterpillars belonged to the Polish metapopulation (HR = 0.53, p = 0.052) (Table S16). The survival probability for the Polish caterpillars with their sympatric host ants showed significantly higher survival probability with respect to the Dutch caterpillar survival probability with their sympatric host ants (Table S15b).

Discussion

Chemical and acoustic signals play a vital role in communication within insect societies, and they are often mimicked by social parasites to deceive and exploit their host colonies. Mimicry strategies are frequently observed in myrmecophilous organisms closely interacting with ants. For instance, hoverflies of the genus *Microdon* mimic the chemical signature of their ant hosts' brood (Scarparo *et al.* 2019), while the parasitic beetles of the genus *Paussus* and the inquiline ant *Myrmica karavajevi* employ chemical and vibroacoustic deception to integrate into the colonies of their host ants (Di Giulio *et al.* 2009, 2015; Casacci *et al.* 2021). The subversion of these communication signals has been extensively studied in the butterflies of the genus *Phengaris*, which integrate into the colonies of *Myrmica* ants (reviewed by Barbero *et al.* 2012; Schönrogge *et al.* 2017; Casacci *et al.* 2019a). The degree to which larvae can successfully mimic such signals plays a fundamental role in their survival.

Cuticular hydrocarbon adaptations

Ants can detect CHCs at very low concentrations and discriminate methylated alkanes, thus small differences in CHCs relative proportions could be an

important factor during the integration of myrmecophilous parasites within ant colonies (Guerrieri *et al.* 2009; Ichinose & Lenoir 2010; Bos *et al.* 2012). Indeed, *Phengaris* cuckoo species, that require full acceptance as kin inside the colony structure, can chemically mimic their host ants more efficiently than predatory *Phengaris* butterflies at the cost of a high host-specificity (Thomas & Elmes 1998; Witek *et al.* 2013). They can acquire new chemical compounds from their host and synthesize their own molecules (Schönrogge *et al.* 2004). On the other hand, predatory species such as *P. teleius* are more host-generalist and their chemical mimicry is assumed to be based on the passive acquisition of compound and not on the active synthesis, but it has never been proved (Thomas *et al.* 1989).

In our study we tested whether and to what extent reintroduced caterpillars of *P. teleius* are able to change their chemical compounds to mimic their new, local host ants. Our findings revealed that the caterpillars of *P. teleius*, descendants of eighty-six individuals translocated from a Polish metapopulation to the Netherlands in the 1990, exhibit different chemical signals compared to caterpillars from the current source metapopulation after 30 generations since their reintroduction. On the other hand, the pre-adoption reintroduced caterpillars were not as chemically adapted to *M. scabrinodis* ants as the caterpillars from the source metapopulation. The different environmental conditions in the Netherlands could have influenced the capacity of the caterpillar to better mimic their host during the pre-adoption stage, as individuals can plastically modify their CHCs proportions according to the environmental conditions (Menzel *et al.* 2018). It is worth noting that the ants from the Netherlands presented the most similar CHC profile to any of the caterpillar metapopulations compared to the Polish ants. It could be explained as a consequence of the lack of parasitic pressure suffered by the Dutch ants during 14 years since the extinction of *P. teleius* in the Netherlands until their reintroduction (Wynhoff 1998). There is evidence of the existence of coevolutionary arms races between *Phengaris* butterflies and their *Myrmica* host ants, as in the case of *P. teleius* with *M. scabrinodis* as a host (Witek *et al.* 2016) or *P. alcon* with *M. rubra* (Nash *et al.* 2008).

After the adoption, the caterpillars changed their CHC profile to get a closer chemical match to their host ants. The reintroduced caterpillars showed a CHC profile that was equally similar to their sympatric Dutch and allopatric Polish hosts, whereas the native Polish caterpillars hosted by their sympatric ants were the ones that better mimic their hosts. Also, the CHC profile of the Dutch post-adopted caterpillars reared with their sympatric host ants changed from pre- to post-adoption stage in the lower proportion compared to Polish sympatric combination. It could be a consequence of the more recent colonization event of the butterflies in the Netherlands and a much longer coevolution process in the Polish system. *P. teleius* caterpillars after the adoption presented nine out of the 10 CHCs that were found in *M. scabrinodis* in a higher abundance than in the pre-adopted caterpillars, and five of those nine compounds were acquired during the integration of the caterpillar in the ant host colony. Interestingly, we found that 3-MeC23 was more abundant in caterpillars after adoption. This highlights the significance of 3-MeC23 in chemical recognition by *M. scabrinodis* workers, as demonstrated by Csata *et al.* (2017). We also detected 5,17-diMeC29 in wild (pre-adoption) *M. scabrinodis*

ants and in caterpillars three days after adoption, but not in the ants from the artificial nests (post-adoption) in which the caterpillars were reared, despite these ants being the only ones in physical contact with the caterpillars. The fact that *Myrmica* colonies under laboratory conditions presented slightly different CHC profiles than wild colonies (Sprenger & Menzel 2020), enabled us to identify 5,17-diMeC29 as a potential CHC synthesized by caterpillars. This synthesis may serve as an adaptive mechanism, with the caterpillars attempting to mimic the chemical signature of their ant hosts. It evinces the possible existence of active CHC synthesis in *P. teleius* to mimic their host CHC profile after adoption. The predatory strategy of *P. teleius* suggests that the passive adsorption of CHCs after the adoption could be produced not only from physical contact with their host ants, but also through feeding on ant larvae. It appears to be a widespread mechanism, as there is evidence that different taxa like ants, grasshoppers, and spiders present this type of CHC acquisition from the diet (Blomquist & Jackson 1973; Liang & Silverman 2000; Elgar & Allan 2004). For instance, the myrmecophilous spider *Cosmophasis bitaeniata* chemically mimics its host ants by feeding on their larvae and acquiring their CHCs (Elgar & Allan 2004).

We should mention that our CHC dataset was composed of methylated alkanes and alkenes. We do not expect the lack of linear alkanes in our dataset to be a mislead of the results. On the one hand, saturated alkanes are considered especially important for protection against desiccation, not being the aim of our study. On the other hand, methylated alkanes are the ones that mainly influence nestmate recognition, which perfectly defines our goal (D'Ettorre & Lenoir 2010).

Vibroacoustic signal adaptations

The effectiveness of *Phengaris* acoustic mimicry varies according to both caterpillar development (before and after adoption) and feeding strategies (cuckoo vs. predatory), as shown by Sala *et al.* (2014). In the post-adoption phase, the vibroacoustic signals produced by cuckoo species elicit higher responses in ant workers than those produced by predatory species, thus enabling the parasite to achieve high social status. In contrast, during the pre-adoption, the calls of the predatory species increased the attention of foragers more than the signals released by cuckoo species, supposedly complementing their less accurate chemical mimicry and eventually promoting their retrieval (Sala *et al.* 2014). Therefore, in our work, we compared the vibroacoustic signals produced by reintroduced and source caterpillars in the crucial pre-adoption phase when they should be subject to the highest evolutionary pressure.

We found that the reintroduced Dutch caterpillars showed divergence from their source metapopulation in their vibroacoustic signal. Moreover, the vibroacoustic signals emitted by caterpillars from the reintroduced metapopulation are more similar to the stridulations of Dutch workers than to those of Polish ants. No overall differences were detected when we compared the parasite's calls with Polish and Dutch queen stridulations, but, as already demonstrated (Barbero *et al.* 2009b; Sala *et al.* 2014), they are closer than the sounds of workers. However, not just the overall sound similarity, but the values of specific parameters of the vibroacoustic signals could be the key for sound mimicry in *Phengaris* butterflies. In the study conducted by Jang & Greenfield (1996), it was noted that the moth *Achroia grisella* demonstrated the ability to discern between sounds that differ by a

single component. Furthermore, these insects tend to show a preference for more regular and higher amplitude sounds. The amplitude parameters (i.e., energy of the peak frequency) diverged among *Phengaris* and *Myrmica* metapopulations, but the signals remained significantly similar between caterpillars and sympatric ant queens. Sound intensity was detected in the *Phengaris-Myrmica* systems as one of the key parameters in the similarity of the signals between caterpillars and queens (Sala *et al.* 2014). Our results provide support to the fact that intensity might be a pivotal component of the signals driving differentiation among populations and enhancing the vibroacoustic deception of the *Myrmica* hosts.

It is interesting to notice that the most distant vibroacoustic signals were detected between the reintroduced caterpillars and their past Polish host ants. This result matches with the fact that those caterpillars were never exposed to the source Polish ants since the reintroduction and their vibroacoustic signals evolved to become more similar to their current host in the Netherlands. The results of the playback experiments demonstrated that the sounds produced by the caterpillars triggered a higher response in their sympatric host ants, giving evidence of a finer vibroacoustic mimicry.

Behavioral responses

Ant and caterpillar behaviors observed during the adoption process can be used as proxy for the efficiency of chemical and acoustical strategies used by *P. teleius* to be retrieved into the ant colony. Our study showed a very clear pattern with a significantly lower number of negative behaviors in the sympatric group from Poland. Other types of behaviors such as antennations and positive behaviors were not significantly different between groups, but the same trend was demonstrated; more antennations and more positive behaviors were noted in sympatric host-parasite combinations. Although not statistically significant, also in the case of negative behaviors, ants from the Netherlands showed less negative behaviors toward sympatric caterpillars compared to allopatric ones. A similar experiment was previously performed by Witek *et al.* (2016), exposing *P. teleius* caterpillars to ant colonies from different populations (sympatric and allopatric). In this study, no difference was found in the number of antennations and negative behaviors, whereas the number of positive behaviors was higher for the sympatric group. Nevertheless, these kinds of results should be cautiously interpreted. The number of antennations is an exploratory behavior that serves to quantify the interest of the ants to gather information from the caterpillars. Its interpretation can be challenging due to its neutral nature and the absence of clear differences among groups in our data. On the other hand, the number of positive behaviors could be influenced by the amount of drops the caterpillar was able to produce during the interaction and trigger the ants to behave in a more benevolent way. We found a strong positive correlation between the number of drops and the number of ant positive behaviors. The number of drops produced by the caterpillars remained constant for all group combinations, so it had a similar effect in the final count of positive behaviors among groups due to correlation. While closely adapted caterpillars could naturally receive a benevolent treatment from the ants, the less adapted ones could benefit from the production of drops. It could create noise in the data and as a consequence, it could reduce the expected differences between sympatric and allopatric combinations. However, the

number of negative behaviors seems to be a more reliable variable to study. We did not find any correlation between the number of drops and number of negative behaviors, so the caterpillars could not avoid aggressiveness from the ants when they were recognized as intruders. We found no statistical differences in the proportion of adopted caterpillars from the reintroduced and source metapopulations, but we consider the almost 40% lower adoption probability of the Dutch sympatric group respecting the Polish one as a biologically relevant difference, providing evidence of a still ongoing adaptive process in the reintroduced metapopulation. Similar results in which the sympatric populations showed higher probability of adoption were previously found in *P. nausithous* and *P. teleius* (Solazzo *et al.* 2013; Witek *et al.* 2016). However, it was reported in *P. alcon* that a more successful adoption was produced in the presence of ant allopatric populations (Als *et al.* 2001). The low host-specificity of *P. teleius* might come at the cost of a more challenging adaptation to parasitism in new allopatric populations. Moreover, the Polish caterpillars in the presence of their sympatric host ants also showed a higher survival probability than the sympatric reintroduced Dutch group.

To summarize, thirty generations after the reintroduction of *P. teleius* in the Netherlands were just adequate to showcase differences in the adaptations of reintroduced butterfly caterpillars. Both chemical and acoustical signals were various from those used by Polish caterpillars. In the source system, caterpillars exhibited higher degree of mimicry toward their sympatric host ants, a phenomenon easily explained by a much longer period of coevolution in this host-parasite system. Nevertheless, the degree of mimicry in the reintroduced caterpillars sufficed to facilitate the adoption, integration into ant colonies, and the subsequent population growth after the reintroduction. The varying degrees of affinity in chemical and vibroacoustic communication signals between parasite and host in the Dutch site, where their coexistence is more recent, underscore the intricacies of host-parasite interactions and the multimodal adaptations required to achieve a heightened level of mimicry (Casacci *et al.* 2019a). Interestingly, our results suggest different coevolution in two communication systems used by butterflies to cheat their host ants. As suggested by Sala *et al.* (2014), cuckoo and predatory *Phengaris* caterpillars can invest differently in their chemical and acoustical signals considering also pre- and post-adoption stages. During the long adoption rituals, typical for the predatory species, pre-adoption caterpillars can rely more on acoustical emission to complement its chemical mimicry, thus they may have evolved more efficient vibroacoustic signals to compensate for the chemical deficiency. In fact, our result supports this hypothesis, as in the reintroduced *P. teleius* caterpillars the degree of acoustical mimicry towards their local host ants surpasses that of chemical cues, which is still much lower than in the sympatric Polish host-parasite system. Such results can be due to possible differences in the evolutionary costs of the two communication channels, as we expected acoustic mimicry to be easier to be adjusted instead of chemical cues, especially in light of our new results suggesting that *P. teleius* caterpillars could actively synthesize chemical compounds. Finally, we offer evidence that *P. teleius*, considered as the most generalist among all *Phengaris* butterflies (Stankiewicz & Sielezniew 2002; Woyciechowski *et al.* 2006; Witek *et al.* 2010), is able to evolve

adaptations to parasites and adapts to new populations of their host ants. *P. teleius* caterpillars were able to respond to habitat changes and modify their chemical and vibroacoustic signals to adapt to their new host population after reintroduction. It suggests that a geographical mosaic of coevolution can also occur in *P. teleius*.

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CRediT authorship contribution statement

Daniel Sánchez-García: Conceptualization, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization. Irma Wynhoff: Conceptualization, Methodology, Investigation. Patricia d'Ettorre: Investigation. Chloé Leroy: Investigation. Joanna Kajzer-Bonk: Investigation. István Maák: Investigation. Francesca Barbero: Investigation, Writing - Review & Editing. Luca Pietro Casacci: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Review & Editing. Magdalena Witek: Conceptualization, Methodology, Investigation, Writing - Original Draft, Supervision, Project administration, Funding acquisition.

Conflict of Interest Statement

The authors declare no conflict of interests.

References

Akino, T., Knapp, J.J., Thomas, J.A. & Elmes, G.W. (1999). Chemical mimicry and host specificity in the butterfly *Maculinea rebeli*, a social parasite of Myrmica ant colonies. *Proceedings of the Royal Society B: Biological Sciences*, 266, 1419–1426.

Als, T.D., Nash, D.R. & Boomsma, J.J. (2001). Adoption of parasitic *Maculinea alcon* caterpillars (Lepidoptera: Lycaenidae) by three Myrmica ant species. *Animal Behaviour*, 62, 99–106.

Barbero, F., Bonelli, S., Thomas, J.A., Balletto, E. & Schönrogge, K. (2009a). Acoustical mimicry in a predatory social parasite of ants. *Journal of Experimental Biology*, 212, 4084–4090.

Barbero, F., Patricelli, D., Witek, M., Balletto, E., Casacci, L.P., Sala, M., *et al.* (2012). *Myrmica* ants and their butterfly parasites with special focus on the acoustic communication. *Psyche*, 2012.

Barbero, F., Thomas, J.A., Bonelli, S., Balletto, E. & Schönrogge, K. (2009b). Queen Ants Make Distinctive Sounds That Are Mimicked by a Butterfly Social Parasite. *Science*, 323, 782–785.

Bates, D., Mächler, M., Bolker, B. & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, 67, 1–48.

Blomquist, G.J. & Jackson, L.L. (1973). Incorporation of labelled dietary n-alkanes into cuticular lipids of the grasshopper *Melanoplus sanguinipes*. *Journal of Insect Physiology*, 19, 1639–1647.

Bos, N., Dreier, S., Jørgensen, C.G., Nielsen, J., Guerrieri, F.J. & D'Ettorre, P. (2012). Learning and perceptual similarity among cuticular hydrocarbons in ants. *Journal of Insect Physiology*, 58, 138–146.

Brooks, M.E., Kristensen, K., van Benthem, K.J., Magnusson, A., Berg, C.W., Nielsen, A., *et al.*

(2017). glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *The R Journal*, 9, 378–400.

Casacci, L.P., Barbero, F., Ślipiński, P. & Witek, M. (2021). The inquiline ant *Myrmica karavajevi* uses both chemical and vibroacoustic deception mechanisms to integrate into its host colonies. *Biology*, 10.

Casacci, L.P., Bonelli, S., Balletto, E. & Barbero, F. (2019a). Multimodal Signaling in Myrmecophilous Butterflies. *Frontiers in Ecology and Evolution*, 7.

Casacci, L.P., Schönrogge, K., Thomas, J.A., Balletto, E., Bonelli, S. & Barbero, F. (2019b). Host specificity pattern and chemical deception in a social parasite of ants. *Scientific Reports*, 9, 1–10.

Casacci, L.P., Thomas, J.A., Sala, M., Treanor, D., Bonelli, S., Balletto, E., *et al.* (2013). Ant pupae employ acoustics to communicate social status in their colony's hierarchy. *Current Biology*, 23, 323–327.

Csata, E., Timuş, N., Witek, M., Casacci, L.P., Lucas, C., Bagnères, A.G., *et al.* (2017). Lock-picks: Fungal infection facilitates the intrusion of strangers into ant colonies. *Scientific Reports*, 7, 1–14.

D'Ettorre, P. & Lenoir, A. (2010). Nestmate Recognition. In: *Ant ecology* (eds. Lach, L., Parr, C.L. & Abbott, K.L.). Oxford University Press, Oxford, UK, pp. 194–209.

De Cáceres, M. & Legendre, P. (2009). Associations between species and groups of sites: Indices and statistical inference. *Ecology*, 90, 3566–3574.

Di Giulio, A., Maurizi, E., Barbero, F., Sala, M., Fattorini, S., Balletto, E., *et al.* (2015). The pied piper: A parasitic Beetle's melodies modulate ant behaviours. *PLoS ONE*, 10, 1–15.

Di Giulio, A., Rossi Stacconi, M.V. & Romani, R. (2009). Fine structure of the antennal glands of the ant nest beetle *Paussus favieri* (Coleoptera, Carabidae, Paussini). *Arthropod Structure and Development*, 38, 293–302.

Elgar, M.A. & Allan, R.A. (2004). Predatory spider mimics acquire colony-specific cuticular hydrocarbons from their ant model prey. *Naturwissenschaften*, 91, 143–147.

Elmes, G.W., Akino, T., Thomas, J.A., Clarke, R.T. & Knapp, J.J. (2002). Interspecific differences in cuticular hydrocarbon profiles of *Myrmica* ants are sufficiently consistent to explain host specificity by *Maculinea* (large blue) butterflies. *Oecologia*, 130, 525–535.

Fox, J. & Weisberg, S. (2019). *An R companion to applied regression*. Third. Sage, Thousand Oaks CA.

Guerrieri, F.J., Nehring, V., Jørgensen, C.G., Nielsen, J., Galizia, C.G. & D'Ettorre, P. (2009). Ants recognize foes and not friends. *Proceedings of the Royal Society B: Biological Sciences*, 276, 2461–2468.

Guillem, R.M., Drijfhout, F.P. & Martin, S.J. (2016). Species-Specific Cuticular Hydrocarbon Stability within European *Myrmica* Ants. *Journal of Chemical Ecology*, 42, 1052–1062.

Hölldobler, B. & Wilson, E.O. (1990). *The ants*. Harvard University Press, Cambridge.

Ichinose, K. & Lenoir, A. (2010). Hydrocarbons detection levels in ants. *Insectes Sociaux*, 57, 453–455.

Jang, Y. & Greenfield, M.D. (1996). Ultrasonic communication and sexual selection in wax moths: Female choice based on energy and asynchrony of male signals. *Animal Behaviour*, 51, 1095–1106.

Lenth, R.V. (2023). emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.8.4-1.

Liang, D. & Silverman, J. (2000). 'You are what you eat': Diet modifies cuticular hydrocarbons and nestmate recognition in the Argentine ant, *Linepithema humile*. *Naturwissenschaften*, 87, 412–416.

Menzel, F., Zumbusch, M. & Feldmeyer, B. (2018). How ants acclimate: Impact of climatic conditions

on the cuticular hydrocarbon profile. *Functional Ecology*, 32, 657–666.

Nash, D.R., Als, T.D., Maile, R., Jones, G.R. & Boomsma, J.J. (2008). A mosaic of Chemical Coevolution in a Large Blue Butterfly. *Science*, 319, 88–90.

Nash, D.R. & Boomsma, J.J. (2008). Communication between hosts and social parasites. In: *Sociobiology of communication : An interdisciplinary perspective* (eds. D'Etorre, P. & Hughes, D.P.). Oxford University Press, Oxford, UK, pp. 55–79.

Oksanen, J., Simpson, G.L., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., *et al.* (2022). vegan: Community ecology package. R package version 2.6-4.

Ottensmann, M., Stoffel, M.A., Nichols, H.J. & Hoffman, J.I. (2018). GCalignR: An r package for aligning gas-chromatography data for ecological and evolutionary studies. *PLOS ONE*.

R Core Team. (2023). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria.

Riva, F., Barbero, F., Bonelli, S., Balletto, E. & Casacci, L.P. (2017). The acoustic repertoire of lycaenid butterfly larvae. *Bioacoustics*, 26, 77–90.

Sala, M., Casacci, L.P., Balletto, E., Bonelli, S. & Barbero, F. (2014). Variation in butterfly larval acoustics as a strategy to infiltrate and exploit host ant colony resources. *PLoS ONE*, 9, 20–23.

Scarparo, G., D'Ettorre, P. & Di Giulio, A. (2019). Chemical Deception and Structural Adaptation in *Microdon* (Diptera, Syrphidae, Microdontinae), a Genus of Hoverflies Parasitic on Social Insects. *Journal of Chemical Ecology*, 45, 959–971.

Schönrogge, K., Barbero, F., Casacci, L.P., Settele, J. & Thomas, J.A. (2017). Acoustic communication within ant societies and its mimicry by mutualistic and socially parasitic myrmecophiles. *Animal Behaviour*, 134, 249–256.

Schönrogge, K., Wardlaw, J.C., Peters, A.J., Everett, S., Thomas, J.A. & Elmes, G.W. (2004). Changes in chemical signature and host specificity from larval retrieval to full social integration in the myrmecophilous butterfly *Maculinea rebeli*. *Journal of Chemical Ecology*, 30.

Solazzo, G., Moritz, R.F.A. & Settele, J. (2013). Choice behaviour of Myrmica rubra workers between ant larvae and larvae of their *Phengaris* (*Maculinea*) *nausithous* nest parasites. *Insectes Sociaux*, 60, 57–64.

Sprenger, P.P. & Menzel, F. (2020). Cuticular hydrocarbons in ants (Hymenoptera: Formicidae) and other insects: how and why they differ among individuals, colonies, and species. *Myrmecological News*, 30, 1–26.

Stankiewicz, A. & Sielezniew, M. (2002). Host specificity of *Maculinea teleius* Bgstr. and M. nausithous Bgstr. (Lepidoptera: Lycaenidae) the new insight. *Annales Zoologici*, 52, 403–408.

Summers, K., McKeon, S., Sellars, J., Keusenkothen, M., Morris, J., Gloeckner, D., *et al.* (2003). Parasitic exploitation as an engine of diversity. *Biological Reviews*, 78, 639–675.

Tartally, A., Thomas, J.A., Anton, C., Balletto, E., Barbero, F., Bonelli, S., *et al.* (2019). Patterns of host use by brood parasitic *Maculinea* butterflies across Europe. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 374.

Therneau, T.M. (2023). survival: A Package for Survival Analysis in R. R package version 3.5-7.

Thomas, A.J.A., Elmes, G.W., Wardlaw, J.C., Woyciechowski, M., Thomas, J.A., Elmes, G.W., *et al.* (1989). Host Specificity among *Maculinea* butterflies in *Myrmica* ant nests. *Oecologia*, 79, 452–457.

Thomas, J.A. & Elmes, G.W. (1998). Higher productivity at the cost of increased host-specificity when *Maculinea* butterfly larvae exploit ant colonies through trophallaxis rather than by predation. *Ecological Entomology*, 23, 457–464.

Thomas, J.A., Elmes, G.W., Sielezniew, M., Stankiewicz-Fiedurek, A., Simcox, D.J., Settele, J.,

et al. (2013). Mimetic host shifts in an endangered social parasite of ants. *Proceedings of the Royal Society B: Biological Sciences*, 280.

Thomas, J.A., Schönrogge, K. & Elmes, G.W. (2005). Specialization and host associations of social parasites of ants. In: *Insect evolutionary ecology* (eds. Fellowes, M.D.E., Holloway, G.J. & Rolff, J.). CABI Publishing, Wallingford, pp. 475–514.

Thomas, J.A. & Settele, J. (2004). Butterfly mimics of ants. *Nature*, 432, 283–284.

Vander Meer, R.K. & Morel, L. (1998). Nestmate Recognition in Ants. In: *Pheromone communication in social insects* (eds. Vander Meer, R.K., Breed, M.D., Winston, M. & Espelie, K.E.). Westview Press, Oxford, UK, pp. 79–103.

Witek, M., Casacci, L.P., Barbero, F., Patricelli, D., Sala, M., Bossi, S., *et al.* (2013). Interspecific relationships in co-occurring populations of social parasites and their host ants. *Biological Journal of the Linnean Society*, 109, 699–709.

Witek, M., Nowicki, P., Śliwińska, E.B., Skórka, P., Settele, J., Schönrogge, K., *et al.* (2010). Local host ant specificity of *Phengaris* (*Maculinea*) *teleius* butterfly, an obligatory social parasite of Myrmica ants. *Ecological Entomology*, 35, 557–564.

Witek, M., Ślipiński, P., Trigos Peral, G. & Csata, E. (2016). Consequences of the arms race between *Maculinea teleius* social parasite and *Myrmica* host ants for myrmecophilous butterfly conservation. *Journal of Insect Conservation*, 20, 887–893.

Woyciechowski, M., Slowik, J. & Muehlenberg, M. (2006). Hosts of the butterfly, *Maculinea teleius*, among *Myrmica* ants in northern Mongolia (Lepidoptera: Lycaenidae; Hymenoptera: Formicidae). *Sociobiology*, 48, 493–502.

Wynhoff, I. (1998). Lessons from the reintroduction of *Maculinea teleius* and *M. nausithous* in the Netherlands. *Journal of Insect Conservation*, 2, 47–57.

Supporting information

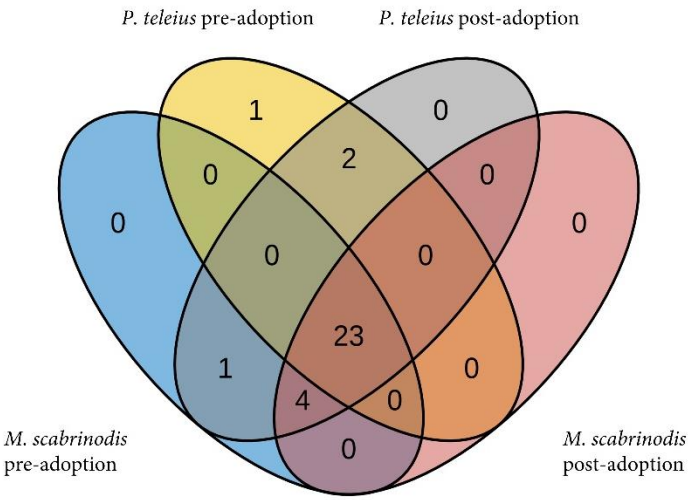


Fig. S1: Venn diagram representing the number of CHC compounds from each of the study groups.

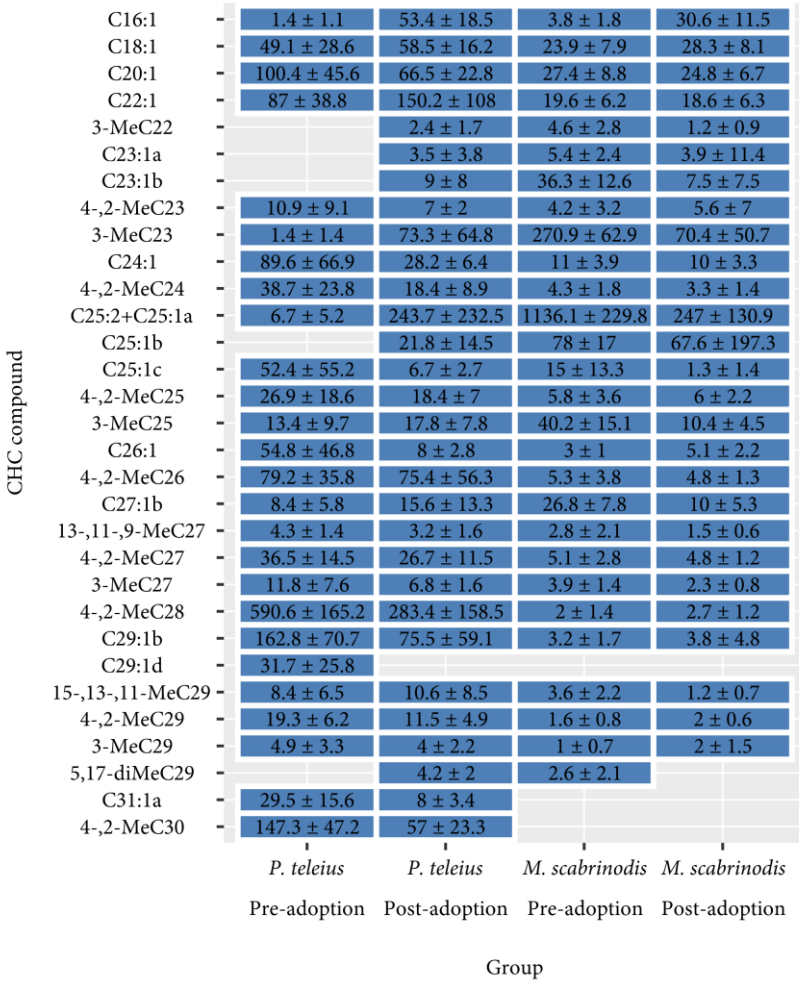


Fig. S2: Graphical representation of the CHC compounds analyzed in the study. Blue tiles indicate the presence of the CHC in the different groups. The values inside the tiles indicate the absolute abundance of the CHCs (ng) per mg of sample dry mass and their standard deviation.

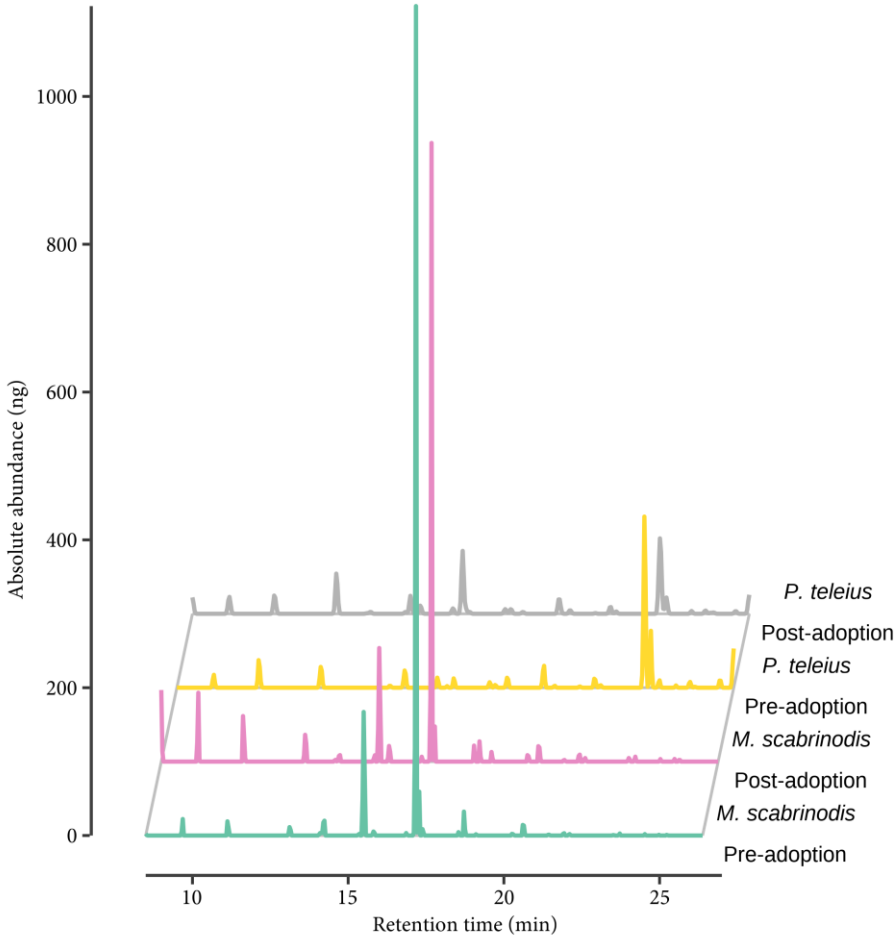


Fig. S3: Chromatogram representation of the CHC profile from the different studied groups. The X axis indicates the retention time in which the compound was detected during the GC-MS analysis. The Y axis indicates the absolute abundance (ng) of the CHC compounds per mg of sample dry mass.

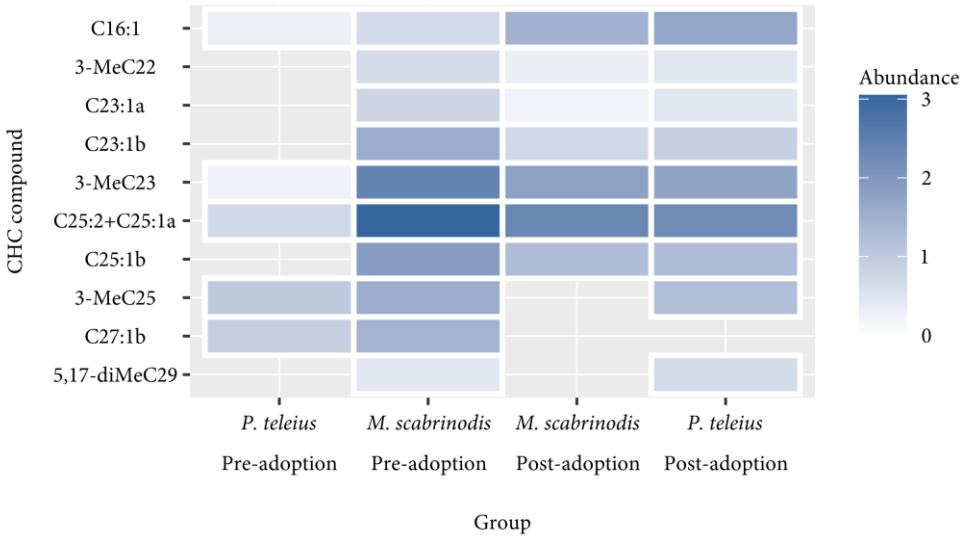


Fig. S4: CHC compounds of *M. scabrinodis* ants (pre- and post-adoption) and post-adoption *P. teleius* caterpillars with a significantly higher abundance than pre-adoption *P. teleius*. Color intensity indicates the log10 of compound abundance (ng) per mg of dry mass sample. Pre-adoption *P. teleius* tiles color intensity just indicate the abundance of the compounds as a reference level to compare the rest of the groups.

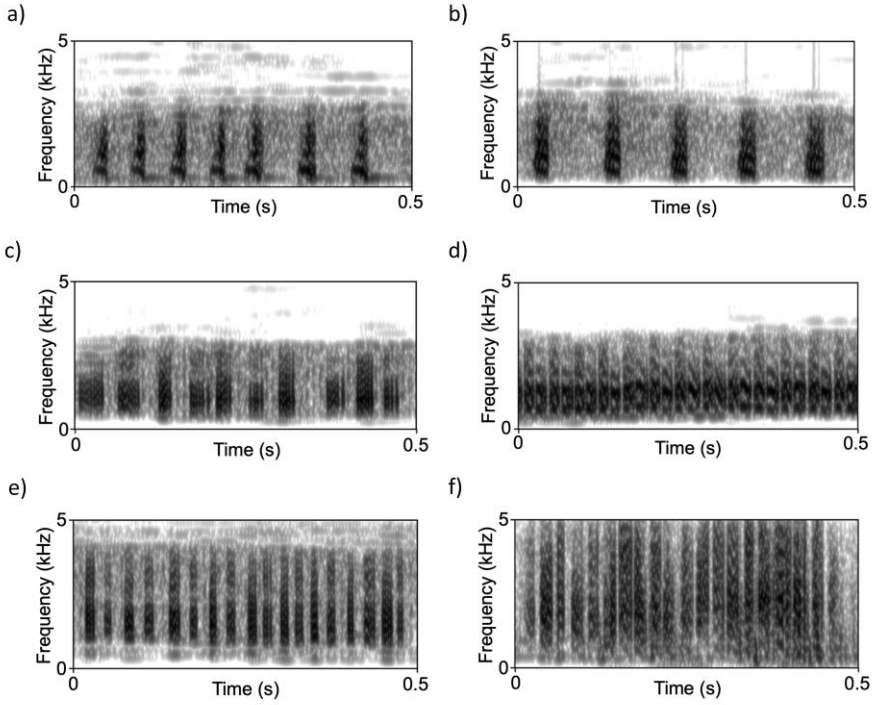


Fig. S5: Oscillograms and spectrograms of the stridulations emitted by pre-adoption caterpillars of *P. teleius*, *M. scabrinodis* queens and workers from a, c, e) Poland and b, d, f) the Netherlands. Spectrograms were generated in Praat using the following parameters: window shape = Gaussian, window length = 0.025 s, number of time steps = 1000, number of frequency steps = 500, dynamic range = 70 dB.

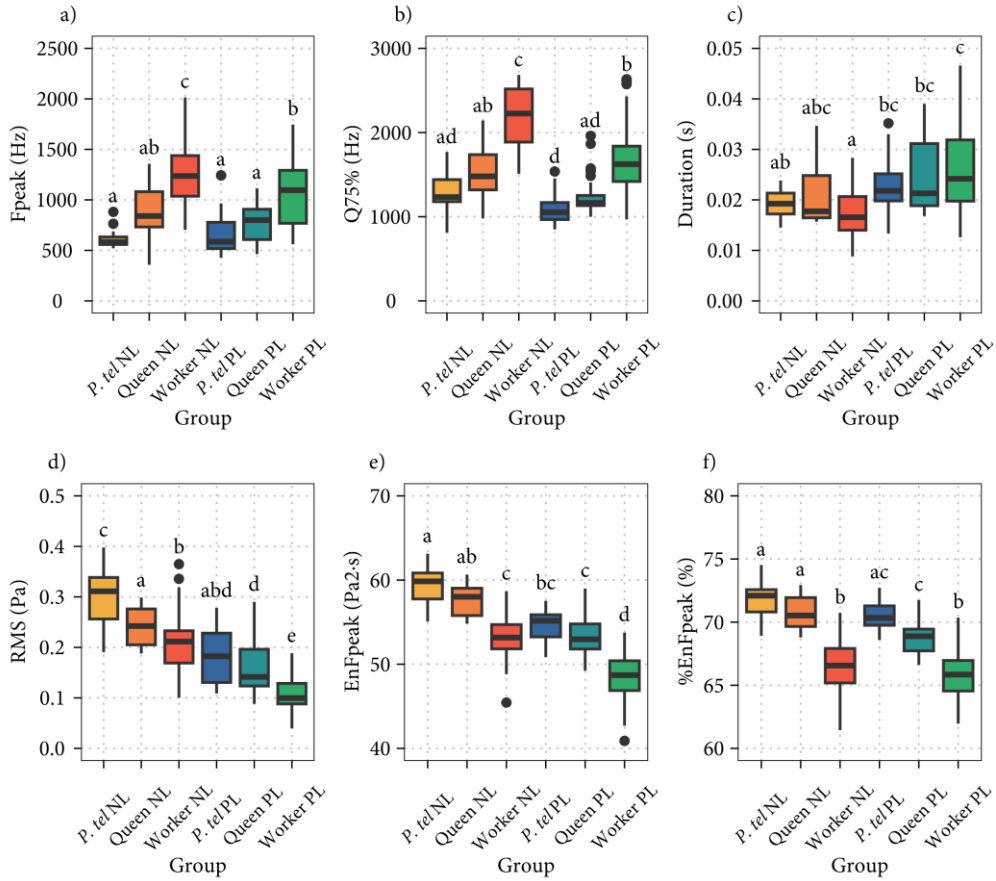


Fig. S6: Boxplots of vibroacoustic parameters, i.e., a) frequency peak (Fpeak, Hz), b) third frequency quartile (Q75%, Hz), c) unit duration (Duration, s), d) root-mean-square (RMS, Pa), e) energy of the frequency peak (EnFpeak, $Pa^2 \cdot s$), f) percentage of the energy of the frequency peak over the total energy (%EnFpeak, %), of stridulation units of the signals emitted by *M. scabrinodis* queens, workers and pre-adoption caterpillars of *P. teleius* from the Polish and Dutch metapopulations. Horizontal lines represent median values, the boxes the first and third quartiles and whiskers the maximum and minimum values. Dots represent outliers. Lower-case letters above boxplots indicate pairwise significant differences between castes based on an estimated marginal means (EMMs) test.

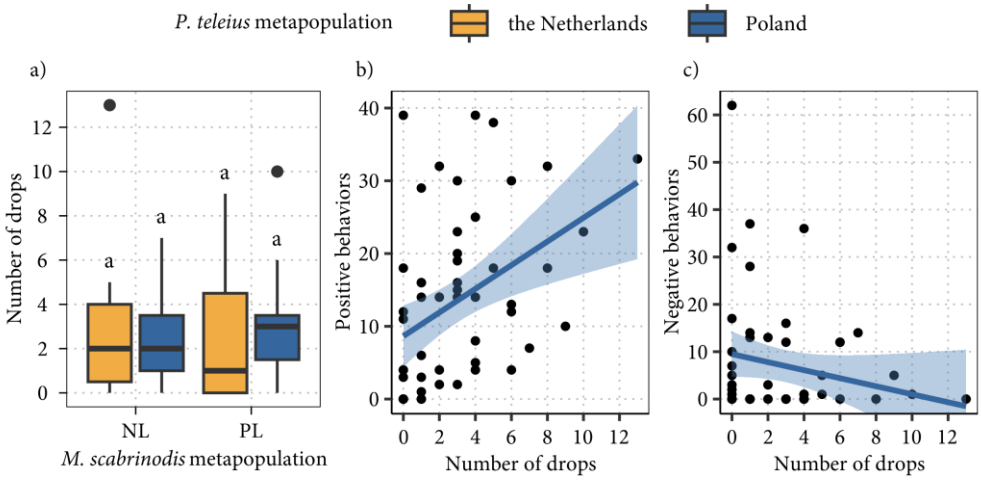


Fig. S7: *M. scabrinodis* ant and *P. teleius* caterpillar behavioral cross-metapopulation experiment results: a) number of drops produced by *P. teleius* caterpillars in the different host-parasite combinations. The color of the boxplots indicates the population of origin for *P. teleius*: yellow (the Netherlands) and blue (Poland). Horizontal lines represent median values, the boxes the first and third quartiles and whiskers the maximum and minimum values. Dots represent outliers. Lower-case letters above boxplots indicate pairwise significant differences between groups based on an estimated marginal means (EMMs) test; b) correlation between the number of drops and ant positive behaviors; and c) correlation between the number of drops and ant negative behaviors. The light blue surface represents the 95% interval of confidence.

Table S1: Statistical results from the analysis of pre-adoption cuticular hydrocarbon profile Bray-Curtis distances between *P. teleius* caterpillars and *M. scabrinodis* ants from different metapopulations. a) Generalized linear model (GLM) variable significance. b) Estimated marginal means (EMMs) pairwise comparisons among groups. The first term of each group in the contrast indicates the ant host metapopulation; the second term indicates the metapopulation of *P. teleius*. NL refers to the Netherlands and PL refers to Poland.

a)

Variable	d.f.	LR Chisq	p
Ant population	1	1,162.57	0.001***
Caterpillar population	1	31.53	0.001***
Ant population x caterpillar population	1	0.84	0.359

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

b)

Contrast	T	p
NL-NL vs PL-NL	-21.71	0.001***
NL-NL vs NL-PL	4.73	0.001***
NL-NL vs PL-PL	-19.97	0.001***
PL-NL vs NL-PL	27.59	0.001***
PL-NL vs PL-PL	3.17	0.009**
NL-PL vs PL-PL	-26.30	0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

Table S2: Statistical results from the analysis of post-adoption cuticular hydrocarbon profile Bray-Curtis distances between *P. teleius* caterpillars and *M. scabrinodis* ants from different metapopulations. a) Generalized linear model (GLM) variable significance. b) Estimated marginal means (EMMs) pairwise comparisons among groups. The first term of each group in the contrast indicates the ant host metapopulation; the second term indicates the metapopulation of *P. teleius*. NL refers to the Netherlands and PL refers to Poland.

a)

Variable	d.f.	LR Chisq	p
Ant population	1	4.16	0.041*
Caterpillar population	1	16.00	0.001***
Ant population x caterpillar population	1	3.99	0.046*

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

b)

Contrast	T	p
NL-NL vs PL-NL	-0.26	0.994
NL-NL vs NL-PL	1.84	0.266
NL-NL vs PL-PL	4.27	0.001***
PL-NL vs NL-PL	1.89	0.244
PL-NL vs PL-PL	4.08	0.001***
NL-PL vs PL-PL	2.84	0.029*

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

Table S3: Statistical results from the analysis of pre- and post-adoption cuticular hydrocarbon profile Bray-Curtis distances between *P. teleius* caterpillars and *M. scabrinodis* ants from different metapopulations. a) Generalized linear model (GLM) variable significance. b) Estimated marginal means (EMMs) pairwise comparisons among groups. The first term of each group in the contrast indicates the ant host metapopulation; the second term indicates the metapopulation of *P. teleius*. NL refers to the Netherlands and PL refers to Poland.

a)

Variable	d.f.	LR Chisq	p
Ant population	1	19.36	0.001***
Caterpillar population	1	36.13	0.001***
Ant population x caterpillar population	1	0.61	0.434

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

b)

Contrast	T	p
NL-NL vs PL-NL	-3.19	0.009**
NL-NL vs NL-PL	-4.80	0.001***
NL-NL vs PL-PL	-7.37	0.001***
PL-NL vs NL-PL	-1.13	0.672
PL-NL vs PL-PL	-3.70	0.002**
NL-PL vs PL-PL	-3.13	0.011*

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

Table S4: Multi-level pattern analysis results of the cuticular hydrocarbon (CHC) compounds with a significantly higher abundance in a) pre-adoption *M. scabrinodis* ant workers, b) post-adoption *M. scabrinodis* ant workers and c) post-adoption *P. teleius* caterpillars with the CHC profile of pre-adoption *P. teleius* caterpillars as a reference level. 'rpb' refers to the 'point biserial correlation coefficient.

a)

Compound	rpb	p
C16:1	0.65	0.003**
3-MeC22	0.94	0.003**
C23:1a	0.95	0.003**
C23:1b	0.99	0.003**
3-MeC23	0.99	0.003**
C25:2+C25:1a	0.98	0.003**
C25:1b	1.00	0.003**
3-MeC25	0.76	0.003**
C27:1b	0.76	0.003**
5,17-diMeC29	0.76	0.003**

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

b)

Compound	rpb	p
C16:1	0.96	0.001***
3-MeC22	0.83	0.001***
C23:1a	0.53	0.001***
C23:1b	0.89	0.001***
3-MeC23	0.95	0.001***
C25:2+C25:1a	0.82	0.001***
C25:1b	0.89	0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

c)

Compound	rpb	p
C16:1	0.98	0.001***
3-MeC22	0.88	0.001***
C23:1a	0.74	0.001***
C23:1b	0.90	0.001***
3-MeC23	0.93	0.001***
C25:2+C25:1a	0.87	0.001***
C25:1b	0.95	0.001***
3-MeC25	0.38	0.039*
5,17-diMeC29	0.79	0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

Table S5: Statistical results from the analysis of vibroacoustic signal Bray-Curtis distances between pre-adoption *P. teleius* caterpillars and *M. scabrinodis* ant queens from different metapopulations. a) Generalized linear model (GLM) variable significance. b) Estimated marginal means (EMMs) pairwise comparisons among groups. The first term of each group in the contrast indicates the ant host metapopulation; the second term indicates the metapopulation of *P. teleius*. NL refers to the Netherlands and PL refers to Poland.

a)

Variable	d.f.	LR Chisq	p
Queen population	1	2.74	0.098
Caterpillar population	1	237.54	0.001***
Queen population x caterpillar population	1	229.54	0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

b)

Contrast	T	p
NL-NL vs PL-NL	-12.54	0.001***
NL-NL vs NL-PL	-2.54	0.067
NL-NL vs PL-PL	5.10	0.001***
PL-NL vs NL-PL	10.76	0.001***
PL-NL vs PL-PL	21.46	0.001***
NL-PL vs PL-PL	8.67	0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

Table S6: Statistical results from the analysis of vibroacoustic signal Bray-Curtis distances between pre-adoption *P. teleius* caterpillars and *M. scabrinodis* ant workers from different metapopulations. a) Generalized linear model (GLM) variable significance. b) Estimated marginal means (EMMs) pairwise comparisons among groups. The first term of each group in the contrast indicates the ant host metapopulation; the second term indicates the metapopulation of *P. teleius*. NL refers to the Netherlands and PL refers to Poland.

a)

Variable	d.f.	LR Chisq	p
Worker population	1	0.08	0.781
Caterpillar population	1	413.67	0.001***
Worker population x caterpillar population	1	259.46	0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

b)

Contrast	T	p
NL-NL vs PL-NL	-11.99	0.001***
NL-NL vs NL-PL	2.99	0.017*
NL-NL vs PL-PL	12.95	0.001***
PL-NL vs NL-PL	15.81	0.001***
PL-NL vs PL-PL	25.77	0.001***
NL-PL vs PL-PL	10.76	0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

Table S7: Generalized linear mixed model (GLMM) variable significance of different vibroacoustic parameters, i.e., a) peak frequency (Fpeak, Hz), b) third frequency quartile (Q75%, Hz), c) unit duration (Duration, s), d) root-mean-square (RMS, Pa), e) energy of the peak frequency (EnFpeak, Pa2·s), f) percentage of the energy of the peak frequency over the total energy (%EnFpeak, %) of stridulation units of the signals emitted by pre-adopted *P. teleius* caterpillars, *M. scabrinodis* ant queens and *M. scabrinodis* ant workers from the Netherlands and Poland. NL and PL refers to the Netherlands and Poland. it indicates the metapopulation of the ant queens, workers and caterpillars.

a) Peak frequency (Fpeak, Hz)

Contrast	T	p
Queen NL vs Worker NL	-4.17	0.001***
Queen NL vs P. tel NL	2.60	0.168
Queen NL vs Queen PL	1.60	1.000
Queen NL vs Worker PL	-2.02	0.686
Queen NL vs P. tel PL	2.15	0.534
Worker NL vs P. tel NL	8.09	0.001***
Worker NL vs Queen PL	6.55	0.001***
Worker NL vs Worker PL	3.56	0.007**
Worker NL vs P. tel PL	8.08	0.001***
P. tel NL vs Queen PL	-1.44	1.000
P. tel NL vs Worker PL	-5.74	0.001***
P. tel NL vs P. tel PL	-0.66	1.000
Queen PL vs Worker PL	-4.04	0.002**
Queen PL vs P. tel PL	0.89	1.000
Worker PL vs P. tel PL	5.50	0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

b) Third frequency quartile (Q75%, Hz)

Contrast	T	p
Queen NL vs Worker NL	-6.19	0.001***
Queen NL vs P. tel NL	2.58	0.174
Queen NL vs Queen PL	2.86	0.071
Queen NL vs Worker PL	-0.92	1.000
Queen NL vs P. tel PL	4.46	0.001***
Worker NL vs P. tel NL	8.60	0.001***
Worker NL vs Queen PL	10.75	0.001***
Worker NL vs Worker PL	8.22	0.001***
Worker NL vs P. tel PL	11.25	0.001***
P. tel NL vs Queen PL	-0.42	1.000
P. tel NL vs Worker PL	-3.94	0.003**
P. tel NL vs P. tel PL	2.13	0.510
Queen PL vs Worker PL	-4.54	0.001***
Queen PL vs P. tel PL	2.40	0.304
Worker PL vs P. tel PL	6.35	0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

c) Unit duration (Duration, s)

Contrast	T	p
Queen NL vs Worker NL	1.75	1.000
Queen NL vs P. tel NL	0.80	1.000
Queen NL vs Queen PL	-1.62	1.000
Queen NL vs Worker PL	-2.43	0.271
Queen NL vs P. tel PL	-0.63	1.000
Worker NL vs P. tel NL	-0.94	1.000
Worker NL vs Queen PL	-3.70	0.010**
Worker NL vs Worker PL	-7.38	0.001***
Worker NL vs P. tel PL	-3.20	0.030*
P. tel NL vs Queen PL	-2.27	0.421
P. tel NL vs Worker PL	-4.02	0.002**
P. tel NL vs P. tel PL	-1.70	1.000
Queen PL vs Worker PL	-1.06	1.000
Queen PL vs P. tel PL	0.79	1.000
Worker PL vs P. tel PL	2.30	0.365

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

d) Root-mean-square (RMS, Pa)

Contrast	T	p
Queen NL vs Worker NL	3.01	0.044*
Queen NL vs P. tel NL	-3.12	0.032*
Queen NL vs Queen PL	5.71	0.001***
Queen NL vs Worker PL	10.73	0.001***
Queen NL vs P. tel PL	2.83	0.080
Worker NL vs P. tel NL	-6.15	0.001***
Worker NL vs Queen PL	3.54	0.007**
Worker NL vs Worker PL	12.05	0.001***
Worker NL vs P. tel PL	0.84	1.000
P. tel NL vs Queen PL	7.85	0.001***
P. tel NL vs Worker PL	12.53	0.001***
P. tel NL vs P. tel PL	7.39	0.001***
Queen PL vs Worker PL	5.57	0.001***
Queen PL vs P. tel PL	-1.53	1.000
Worker PL vs P. tel PL	-5.68	0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

e) Energy of the peak frequency (EnFpeak, Pa2·s)

Contrast	T	p
Queen NL vs Worker NL	7.36	0.001***
Queen NL vs P. tel NL	-2.23	0.412
Queen NL vs Queen PL	5.98	0.001***
Queen NL vs Worker PL	14.21	0.001***
Queen NL vs P. tel PL	2.96	0.057
Worker NL vs P. tel NL	-8.76	0.001***
Worker NL vs Queen PL	-1.25	1.000
Worker NL vs Worker PL	10.77	0.001***
Worker NL vs P. tel PL	-2.76	0.115
P. tel NL vs Queen PL	7.16	0.001***
P. tel NL vs Worker PL	14.54	0.001***
P. tel NL vs P. tel PL	6.40	0.001***
Queen PL vs Worker PL	9.34	0.001***
Queen PL vs P. tel PL	-1.67	1.000
Worker PL vs P. tel PL	-8.71	0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

f) Percentage of the energy of the peak frequency over the total energy (%EnFpeak, %)

Contrast	T	p
Queen NL vs Worker NL	9.63	0.001***
Queen NL vs P. tel NL	-1.59	1.000
Queen NL vs Queen PL	3.93	0.002**
Queen NL vs Worker PL	11.31	0.001***
Queen NL vs P. tel PL	0.46	1.000
Worker NL vs P. tel NL	-10.40	0.001***
Worker NL vs Queen PL	-6.47	0.001***
Worker NL vs Worker PL	2.76	0.094
Worker NL vs P. tel PL	-8.33	0.001***
P. tel NL vs Queen PL	4.96	0.001***
P. tel NL vs Worker PL	11.96	0.001***
P. tel NL vs P. tel PL	2.49	0.202
Queen PL vs Worker PL	8.46	0.001***
Queen PL vs P. tel PL	-2.87	0.092
Worker PL vs P. tel PL	-9.97	0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

Table S8: EMMs (estimated marginal means) test results of the pairwise comparisons of different vibroacoustic parameters, i.e., a) peak frequency (Fpeak, Hz), b) third frequency quartile (Q75%, Hz), c) unit duration (Duration, s), d) root-mean-square (RMS, Pa), e) energy of the peak frequency (EnFpeak, Pa2·s), f) percentage of the energy of the peak frequency over the total energy (%EnFpeak, %) of stridulation units of the signals emitted by pre-adopted *P. teleius* caterpillars, *M. scabrinodis* ant queens and *M. scabrinodis* ant workers from the Netherlands and Poland. NL and PL refers to the Netherlands and Poland, respectly. It indicates the metapopulation of the ant queens, workers and caterpillars.

a) Peak frequency (Fpeak, Hz)

Contrast	T	p
Queen NL vs Worker NL	-4.17	0.001***
Queen NL vs P. tel NL	2.60	0.168
Queen NL vs Queen PL	1.60	1.000
Queen NL vs Worker PL	-2.02	0.686
Queen NL vs P. tel PL	2.15	0.534
Worker NL vs P. tel NL	8.09	0.001***
Worker NL vs Queen PL	6.55	0.001***
Worker NL vs Worker PL	3.56	0.007**
Worker NL vs P. tel PL	8.08	0.001***
P. tel NL vs Queen PL	-1.44	1.000
P. tel NL vs Worker PL	-5.74	0.001***
P. tel NL vs P. tel PL	-0.66	1.000
Queen PL vs Worker PL	-4.04	0.002**
Queen PL vs P. tel PL	0.89	1.000
Worker PL vs P. tel PL	5.50	0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

b) Third frequency quartile (Q75%, Hz)

Contrast	T	p
Queen NL vs Worker NL	-6.19	0.001***
Queen NL vs P. tel NL	2.58	0.174
Queen NL vs Queen PL	2.86	0.071
Queen NL vs Worker PL	-0.92	1.000
Queen NL vs P. tel PL	4.46	0.001***
Worker NL vs P. tel NL	8.60	0.001***
Worker NL vs Queen PL	10.75	0.001***
Worker NL vs Worker PL	8.22	0.001***
Worker NL vs P. tel PL	11.25	0.001***
P. tel NL vs Queen PL	-0.42	1.000
P. tel NL vs Worker PL	-3.94	0.003**
P. tel NL vs P. tel PL	2.13	0.510
Queen PL vs Worker PL	-4.54	0.001***
Queen PL vs P. tel PL	2.40	0.304
Worker PL vs P. tel PL	6.35	0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

c) Unit duration (Duration, s)

Contrast	T	p
Queen NL vs Worker NL	1.75	1.000
Queen NL vs P. tel NL	0.80	1.000
Queen NL vs Queen PL	-1.62	1.000
Queen NL vs Worker PL	-2.43	0.271
Queen NL vs P. tel PL	-0.63	1.000
Worker NL vs P. tel NL	-0.94	1.000
Worker NL vs Queen PL	-3.70	0.010**
Worker NL vs Worker PL	-7.38	0.001***
Worker NL vs P. tel PL	-3.20	0.030*
P. tel NL vs Queen PL	-2.27	0.421
P. tel NL vs Worker PL	-4.02	0.002**
P. tel NL vs P. tel PL	-1.70	1.000
Queen PL vs Worker PL	-1.06	1.000
Queen PL vs P. tel PL	0.79	1.000
Worker PL vs P. tel PL	2.30	0.365

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

d) Root-mean-square (RMS, Pa)

Contrast	T	p
Queen NL vs Worker NL	3.01	0.044*
Queen NL vs P. tel NL	-3.12	0.032*
Queen NL vs Queen PL	5.71	0.001***
Queen NL vs Worker PL	10.73	0.001***
Queen NL vs P. tel PL	2.83	0.080
Worker NL vs P. tel NL	-6.15	0.001***
Worker NL vs Queen PL	3.54	0.007**
Worker NL vs Worker PL	12.05	0.001***
Worker NL vs P. tel PL	0.84	1.000
P. tel NL vs Queen PL	7.85	0.001***
P. tel NL vs Worker PL	12.53	0.001***
P. tel NL vs P. tel PL	7.39	0.001***
Queen PL vs Worker PL	5.57	0.001***
Queen PL vs P. tel PL	-1.53	1.000
Worker PL vs P. tel PL	-5.68	0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

e) Energy of the peak frequency (EnFpeak, Pa2·s)

Contrast	T	p
Queen NL vs Worker NL	7.36	0.001***
Queen NL vs P. tel NL	-2.23	0.412
Queen NL vs Queen PL	5.98	0.001***
Queen NL vs Worker PL	14.21	0.001***
Queen NL vs P. tel PL	2.96	0.057
Worker NL vs P. tel NL	-8.76	0.001***
Worker NL vs Queen PL	-1.25	1.000
Worker NL vs Worker PL	10.77	0.001***
Worker NL vs P. tel PL	-2.76	0.115
P. tel NL vs Queen PL	7.16	0.001***
P. tel NL vs Worker PL	14.54	0.001***
P. tel NL vs P. tel PL	6.40	0.001***
Queen PL vs Worker PL	9.34	0.001***
Queen PL vs P. tel PL	-1.67	1.000
Worker PL vs P. tel PL	-8.71	0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

f) Percentage of the energy of the peak frequency over the total energy (%EnFpeak, %)

Contrast	T	p
Queen NL vs Worker NL	9.63	0.001***
Queen NL vs P. tel NL	-1.59	1.000
Queen NL vs Queen PL	3.93	0.002**
Queen NL vs Worker PL	11.31	0.001***
Queen NL vs P. tel PL	0.46	1.000
Worker NL vs P. tel NL	-10.40	0.001***
Worker NL vs Queen PL	-6.47	0.001***
Worker NL vs Worker PL	2.76	0.094
Worker NL vs P. tel PL	-8.33	0.001***
P. tel NL vs Queen PL	4.96	0.001***
P. tel NL vs Worker PL	11.96	0.001***
P. tel NL vs P. tel PL	2.49	0.202
Queen PL vs Worker PL	8.46	0.001***
Queen PL vs P. tel PL	-2.87	0.092
Worker PL vs P. tel PL	-9.97	0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

Table S9: Statistical results from the analysis of ant responses to different vibroacoustic signals during playback experiments. a) Generalized linear model (GLM) variable significance of ant worker data from Poland. b) Estimated marginal means (EMMs) pairwise comparisons among responses of ants from Poland to different vibroacoustic signals. c) Generalized linear model (GLM) variable significance of ant worker data from the Netherlands. d) Estimated marginal means (EMMs) pairwise comparisons among responses of ants from the Netherlands to different vibroacoustic signals. Queen refers to the ant queen signal, worker refers to the ant worker signal and P. tel refers to the signal produced by *P. teleius* caterpillars. NL and PL refers to the Netherlands and Poland, respectly. It indicates the metapopulation of origin.

a) GLM variable significance of ant workers from Poland

Variable	d.f.	Chisq	p
Vibroacoustic signal	4	404.66	0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

b) EMMs pairwise comparison of ant workers from Poland

Contrast	Z	p
Queen vs P. tel PL	10.09	0.001***
Queen vs P. tel NL	13.81	0.001***
Queen vs White noise	15.18	0.001***
Queen vs Worker	8.38	0.001***
P. tel PL vs P. tel NL	5.08	0.001***
P. tel PL vs White noise	9.73	0.001***
P. tel PL vs Worker	-1.94	0.520
P. tel NL vs White noise	6.04	0.001***
P. tel NL vs Worker	-6.86	0.001***
White noise vs Worker	-10.91	0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

c) GLM variable significance of ant workers from the Netherlands

Variable	d.f.	Chisq	p
Vibroacoustic signal	4	49.52	0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

d) EMMs pairwise comparison of ant workers from the Netherlands

Contrast	Z	p
Queen vs P. tel PL	2.98	0.028*
Queen vs P. tel NL	-1.35	1.000
Queen vs White noise	4.81	0.001***
Queen vs Worker	-0.38	1.000
P. tel PL vs P. tel NL	-4.32	0.001***
P. tel PL vs White noise	2.01	0.448
P. tel PL vs Worker	-3.33	0.009**
P. tel NL vs White noise	6.05	0.001***
P. tel NL vs Worker	0.96	1.000
White noise vs Worker	-5.13	0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

Table S10: Statistical results from the analysis of the number of antennations produced by *M. scabrinodis* ant workers in presence of *P. teleius* caterpillars from the Netherlands and Poland during the behavioral experiment. a) Generalized linear model (GLM) variable significance. b) Estimated marginal means (EMMs) pairwise comparisons. The first term of each group in the contrast indicates the ant host metapopulation; the second term indicates the metapopulation of *P. teleius*. NL refers to the Netherlands and PL refers to Poland.

a) Antennation

Variable	d.f.	Chisq	p
Ant metapopulation	1	2.97	0.085
Caterpillar metapopulation	1	4.74	0.030*
Ant metapopulation x Caterpillar metapopulation	1	0.69	0.405

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

b) Antennation

Contrast	Z	p
NL-NL vs PL-NL	-0.50	1.000
NL-NL vs NL-PL	-0.70	1.000
NL-NL vs PL-PL	-2.23	0.153
PL-NL vs NL-PL	-0.29	1.000
PL-NL vs PL-PL	-2.29	0.132
NL-PL vs PL-PL	-1.87	0.371

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

Table S11: Statistical results from the analysis of the number of positive behaviors produced by *M. scabrinodis* ant workers in presence of *P. teleius* caterpillars from the Netherlands and Poland during the behavioral experiment. a) Generalized linear model (GLM) variable significance. b) Estimated marginal means (EMMs) pairwise comparisons. The first term of each group in the contrast indicates the ant host metapopulation; the second term indicates the metapopulation of *P. teleius*. NL refers to the Netherlands and PL refers to Poland.

a) Positive behaviors

Variable	d.f.	Chisq	p
Ant metapopulation	1	0.38	0.540
Caterpillar metapopulation	1	0.97	0.325
Ant metapopulation x Caterpillar metapopulation	1	8.18	0.004**

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

b) Positive behaviors

Contrast	Z	p
NL-NL vs PL-NL	2.56	0.063
NL-NL vs NL-PL	1.30	1.000
NL-NL vs PL-PL	-0.05	1.000
PL-NL vs NL-PL	-1.27	1.000
PL-NL vs PL-PL	-2.74	0.037*
NL-PL vs PL-PL	-1.43	0.918

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

Table S12: Statistical results from the analysis of the number of negative behaviors produced by *M. scabrinodis* ant workers in presence of *P. teleius* caterpillars from the Netherlands and Poland during the behavioral experiment. a) Generalized linear model (GLM) variable significance. b) Estimated marginal means (EMMs) pairwise comparisons. The first term of each group in the contrast indicates the ant host metapopulation; the second term indicates the metapopulation of *P. teleius*. NL refers to the Netherlands and PL refers to Poland.

a) Negative behaviors

Variable	d.f.	Chisq	p
Ant metapopulation	1	5.62	0.018*
Caterpillar metapopulation	1	3.03	0.082
Ant metapopulation x Caterpillar metapopulation	1	4.51	0.034*

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

b) Negative behaviors

Contrast	Z	p
NL-NL vs PL-NL	0.25	1.000
NL-NL vs NL-PL	-0.40	1.000
NL-NL vs PL-PL	2.76	0.035*
PL-NL vs NL-PL	-0.68	1.000
PL-NL vs PL-PL	2.72	0.039*
NL-PL vs PL-PL	3.17	0.009**

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

Table S13: Statistical results from the analysis of the number of drops produced by *P. teleius* caterpillars from different metapopulations and exposed to different host ants. a) Generalized linear model (GLM) variable significance. b) Estimated marginal means (EMMs) pairwise comparison. The first term of each group in the contrast indicates the ant host metapopulation; the second term indicates the metapopulation of *P. teleius*. NL refers to the Netherlands and PL refers to Poland.

a) GLM variable significance

Variable	d.f.	Chisq	p
Ant metapopulation	1	0.04	0.848
Caterpillar metapopulation	1	0.00	0.944
Ant metapopulation x Caterpillar metapopulation	1	0.77	0.379

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

b) EMMs pairwise comparisons

Contrast	Z	p
NL-NL vs PL-NL	0.48	0.963
NL-NL vs NL-PL	0.63	0.924
NL-NL vs PL-PL	-0.09	1.000
PL-NL vs NL-PL	0.19	0.998
PL-NL vs PL-PL	-0.62	0.925
NL-PL vs PL-PL	-0.76	0.873

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

Table S14: Statistical results from the analysis of the adoption proportion of *P. teleius* caterpillars in presence of *M. scabrinodis* ants from the Netherlands and Poland. a) Generalized linear model (GLM) variable significance. b) Estimated marginal means (EMMs) pairwise comparison. The first term of each group in the contrast indicates the ant host metapopulation; the second term indicates the metapopulation of *P. teleius*. NL refers to the Netherlands and PL refers to Poland.

a) GLM variable significance

Variable	d.f.	Chisq	p
Ant metapopulation	1	1.68	0.195
Caterpillar metapopulation	1	4.13	0.042*

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

b) EMMs pairwise comparisons

Contrast	Z	p
NL-NL vs PL-NL	-1.30	1.000
NL-NL vs NL-PL	-2.03	0.253
NL-NL vs PL-PL	-2.30	0.128
PL-NL vs NL-PL	-0.89	1.000
PL-NL vs PL-PL	-2.03	0.253
NL-PL vs PL-PL	-1.30	1.000

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

Table S15: Statistical results from the analysis of the survival probability of *P. teleius* caterpillars in presence of *M. scabrinodis* ants from the Netherlands and Poland. a) Generalized linear model (GLM) variable significance. b) Estimated marginal means (EMMs) pairwise comparison.The first term of each group in the contrast indicates the ant host metapopulation; the second term indicates the metapopulation of *P. teleius*. NL refers to the Netherlands and PL refers to Poland.

a) GLM variable significance

Variable	d.f.	LR Chisq	p
Ant metapopulation	1	4.20	0.040*
Caterpillar metapopulation	1	3.72	0.054

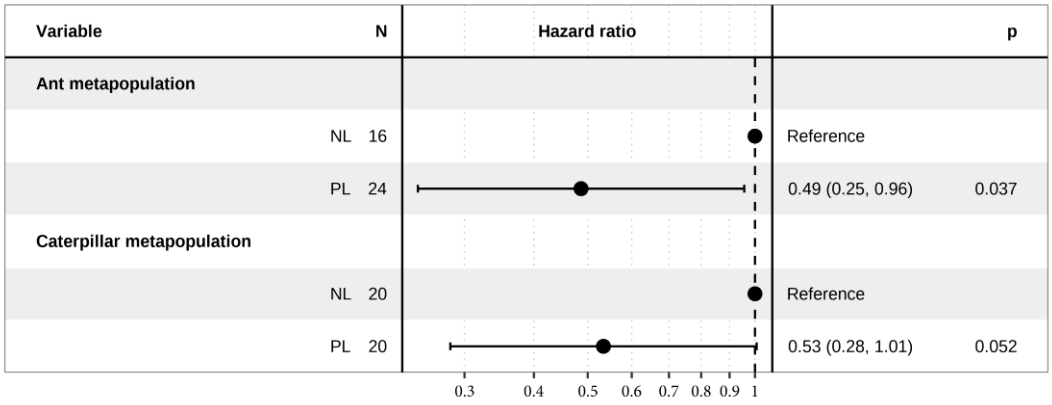
*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

b) EMMs pairwise comparisons

Contrast	Z	p
NL-NL vs PL-NL	2.09	0.220
NL-NL vs NL-PL	1.94	0.315
NL-NL vs PL-PL	2.82	0.028*
PL-NL vs NL-PL	-0.20	1.000
PL-NL vs PL-PL	1.94	0.315
NL-PL vs PL-PL	2.09	0.220

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

Table S16: Cox proportional-hazard ratios. On the left, the predictor variables (ant metapopulation and caterpillar metapopulation) and levels (NL: the Netherlands, PL: Poland). N refers to the sample size of each level. In the middle, hazard ratios. The dotted vertical line represents the reference level. NL was taken as a reference level for the two variables. On the right, the value and interval of confidence of the hazard ratio of each variable. p indicates the significance for each variable.



Manuscript 2

Temporal and spatial variation of morphological traits and genetic structure in *Phengaris teleius* myrmecophilous butterflies following habitat changes three decades after reintroduction

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Abstract

A successful reintroduction of *Phengaris teleius* performed in the Netherlands by translocating 86 individuals from a Polish metapopulation in 1990 represents a unique opportunity to study changes in butterflies from a source and reintroduced metapopulation after such a common conservation practice. Using multilevel comparisons, we tested the morphological and genetic changes that occurred after 30 generations since the reintroduction. We also assessed the connectivity changes that occurred over time in both metapopulation networks. Unexpectedly, we found more significant morphological changes in the current individuals from the source metapopulation, where both sexes had bigger hindwings with different shapes in comparison to the individuals from the original metapopulation in the year of the reintroduction and the ones from the current reintroduced metapopulation. The butterflies from the Dutch metapopulation also had smaller thorax width compared to the ones from the current source metapopulation. The observed morphological changes can be shaped by various factors like changes in habitat connectivity. Additionally, the genetic analysis revealed a differentiation between the source and reintroduced metapopulation. We found a loss of half of the allelic richness and a bottleneck effect in the reintroduced metapopulation compared to the current Polish one. Our results show that *Phengaris* butterflies are good indicators of environmental changes, including habitat fragmentation, but they also have the potential to adapt to new habitats and face global changes despite their complex life cycle. A proper long-term habitat management in reintroduced butterfly metapopulations and habitat restoration are key factors influencing the success of reintroduction.

Keywords: dispersal, geometric morphometry, microsatellite, bottleneck, metapopulation, Maculinea

Introduction

Currently, one of the most rapidly declining groups of animals are insects (e.g. Hallmann *et al.* 2017; Raven & Wagner 2021). This trend can be even stronger for specialist species with narrow environmental requirements (e.g. Zayed *et al.* 2005). Closely interacting species are more vulnerable to environmental changes, because their survival depends on the persistence of other organisms and is maintained by complex adaptations; thus, coextinction processes are more likely to occur in such cases (e.g. Koh *et al.* 2004). While coevolution can mitigate the negative effects of habitat loss and fragmentation in mutualistic networks by creating new opportunistic interactions (Gawecka *et al.* 2022), host-parasite systems are more vulnerable to habitat changes (Grass *et al.* 2018). In this perspective, when reintroducing a parasitic species, not only the parasite itself, but also the host species must be taken into account to increase the probability of success (Wynhoff *et al.* 2011).

Reintroduction is used in conservation biology as a tool to recover species loss after local extinction in a specific ecosystem (Seddon *et al.* 2014), also aiming at restoring ecological processes. Reintroduction biology is considered an applied science, with the aim of offering management strategies to implement animal and plant translocations (Taylor *et al.* 2017), but it can also represent a great opportunity to study evolutionary changes in populations translocated to new habitats. For instance, it was demonstrated that the anadromous threespine stickleback (*Gasterosteus aculeatus*) reintroduced in Alaska needed only one generation to show changes in morphology (Wund *et al.* 2016) and in the reintroduced American marten (*Martes americana*) morphological variation was detected 45 years after the translocation (Howell *et al.* 2016). Such changes can provide the potential for studying phenotypic plasticity and/or adaptations after reintroductions and give us an opportunity to learn whether and how organisms can deal with new habitat conditions.

During the last decades, in certain European countries butterflies experienced greater losses than vascular plants and terrestrial vertebrates (Thomas *et al.* 2004), and among insects, they were the most frequently translocated species with about 50 documented translocations involving this taxon (Bellis *et al.* 2019). Butterfly reintroduction appears to be a complicated process and many populations become extinct during the first five years (Oates & Warren 1990), mostly because of the poor knowledge of factors contributing to population decrease, the lack of specific ecological requirements in the new habitat or the small number of translocated individuals and the resulting consequences of the Allee effect (Dempster & Hall 1980; Deredec & Courchamp 2007; Thomas *et al.* 2009). Nevertheless, two of the most spectacular and successful reintroductions in insect conservation history have been implemented for the myrmecophilous butterflies of the genus *Phengaris* (=*Maculinea*); *P. arion* was reintroduced in the United Kingdom from a Swedish population (Thomas *et al.* 2009; Andrews 2015) and *P. teleius* in the Netherlands from a Polish population (Wynhoff 1998). *Phengaris* butterflies are indicators and flagship species for biodiversity conservation (Thomas & Settele 2004). They are univoltine butterflies having a very specialized lifecycle as they are social parasites of ants and their larvae require two resources: species-specific host plants and *Myrmica* host ants (Elmes & Thomas 1992). In the case of *P. teleius*, females lay eggs on

Sanguisorba officinalis flowerheads where caterpillars remain for about three weeks. After reaching the fourth instar, they abandon the host plant and must be taken by a *Myrmica* ant to the nest for further development (Thomas 1984). *P. teleius* has the widest host range among all *Phengaris* species, but *Myrmica scabrinodis* seems to be its main host (Tartally *et al.* 2019).

The successful reintroduction of *P. teleius* was the consequence of the translocation of eighty-six butterflies taken from a Polish metapopulation to the Dutch nature reserve of Moerputten in 1990. Nowadays, the Dutch metapopulation consists of about a few thousand butterflies (IW, unpublished data). A difference of almost 30 butterfly generations between the original and the reintroduced metapopulation offers a unique opportunity to study various changes which have occurred in both metapopulations. The main objective of our study was to evaluate whether the descendants of the translocated individuals have retained the characteristics of the source metapopulation or whether they have changed and adapted to the new conditions. Moreover, we could also investigate temporal changes that have occurred in the source metapopulation. We performed multilevel comparisons among the source (from 1990), current Polish and reintroduced current Dutch metapopulations of *P. teleius* by investigating (i) population genetics, and (ii) morphology of adult butterflies. We also assessed the metapopulation connectivity of our study systems over time. We hypothesize that the reintroduced metapopulation is characterized by lower genetic variation compared to the source population, and that after 30 years of separation a genetic differentiation has occurred. We also hypothesize that different biotic and abiotic conditions (e.g. population size, habitat structure, availability of host plants and host ants) have influenced the two current metapopulations in a different way affecting the morphology of adult butterflies. We expected that landscape connectivity may be one of the most important factors leading to selection pressure on morphological traits and dispersal (Bonte *et al.* 2012).

Material and methods

Study site of the source metapopulation

The studied *Phengaris teleius* butterfly metapopulation occurs in the Vistula River Valley in the outskirts of Kraków city in Southern Poland (50°01'N, 19°54'E). The area is mostly composed of abandoned or rarely extensively managed grasslands, arable fields, forests, and settlements (Kajzer-Bonk *et al.* 2016a). The habitats of the focal butterfly species are a part of a large meadow complex with an area exceeding 200 ha and consisting of several dozens of nutrient-poor to mesotrophic meadows with varying densities of *S. officinalis* (Fig. 1). The three investigated populations (K10, K1 and K25) are characterized by relatively large areas (2.4, 6.2 and 33.3 ha, respectively; Fig. 1). In these meadows the adult butterflies were collected for reintroduction in 1990. The estimated yearly population size reaches several dozens of thousands of individuals in each of the considered habitats.

Study site of the reintroduced metapopulation

The nature reserve Moerputten (115 ha) is located south of the city of 's-Hertogenbosch (the Netherlands) and covers the central part of the Natura 2000 area "Vlijmens Ven, Moerputten and Bossche Broek" (931 ha), (51°41'N, 5°15'E). The nature reserve consists of a central lake, surrounded

by willow forests and tall beds of *Phragmites*, *Typha* and tall *Carex* species. On the outer borders, different types of grasslands are found, where *P. teleius* finds its habitat. For a detailed description of the meadows see Wynhoff (1998). Nowadays, the area of wet meadows in Moerputten nature reserve has been enlarged with 250 ha of restored fen meadows in neighboring nature reserves with the aim of enlarging both the rare vegetation as well as the habitat of the butterfly (Wynhoff *et al.* 2017; Sevilleja *et al.* 2022).

The reintroduced metapopulation consisted of 33 males and 53 females that were translocated in 1990 from Poland to the moist meadows of Moerputten nature reserve (Wynhoff 1998). Recently, *P. teleius* is restricted to one core population on the meadows at the southern border of the core reserve and two to three populations on other meadows within the nature reserve (Fig. 1). We conducted the study at three closely located meadows (BW, PHZ, KBW) characterized by areas of 1.2, 1.9 and 0.4 ha, respectively (Fig. 1).

Data collection

Data were collected from the Polish and Dutch metapopulations in different moments in time: from the source metapopulation in Poland in 1990 (the year of the reintroduction; PL1990), from the Polish metapopulation in 2003 (PL2003), from the current metapopulation in Poland in 2019 (PL2019), from the reintroduced Dutch metapopulation in 1996 (NL1996) and from the reintroduced metapopulation in the Netherlands in 2020 (NL2020). The individuals which did not survive the trip for the reintroduction in 1990 were dried and preserved (n = 65, IW personal collection). The individuals from Poland 2003 (n = 63) and the Netherlands 1996 (n = 14) were

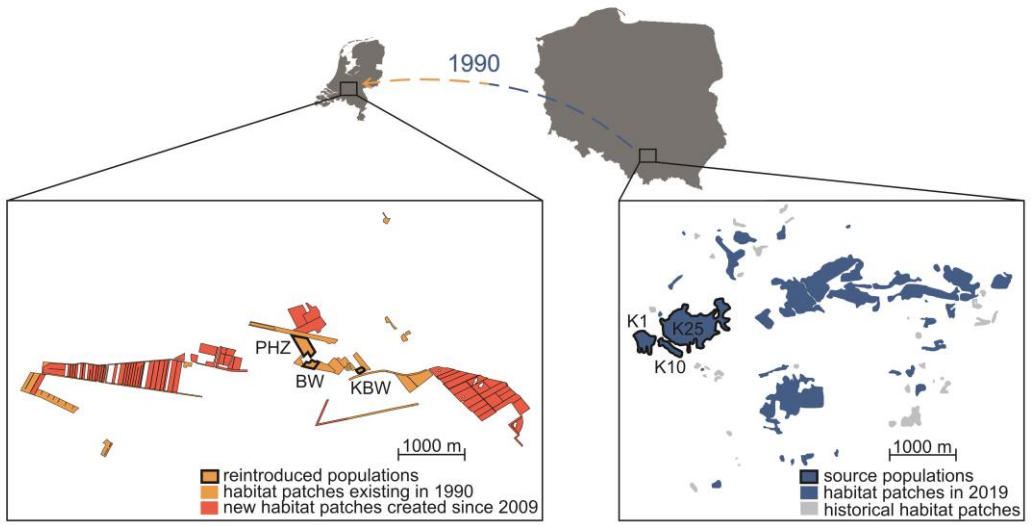

Fig. 1. Sites of the two studied metapopulations of *Phengaris teleius*. On the left: habitat patches of the Dutch reintroduced metapopulation; on the right: habitat patches of the Polish source metapopulation (habitat patches are considered to be sites where the butterfly food plant is present). The blue patches are the ones existing in Poland in 2019, the gray patches are ones recently lost in Poland, the yellow patches are the ones existing in 1990 during the reintroduction of *P. teleius* and the red patches are the sites where the restoration took place in the Netherlands. Patches with a black border are the collection sites from the source metapopulation in Poland and the ones where butterflies were reintroduced in the Netherlands.

randomly collected in the field and obtained from the personal collection of PN and IW. As not all types of data were collected in all studied metapopulations, therefore, a table summarizing the type of data collected in a given metapopulation at a given time is presented (Table S1).

During the fieldwork, 121 butterflies were captured in Kraków and 134 in Moerputten. Each butterfly was placed into a small jar and treated with carbon dioxide for ten seconds to anesthetize it. Then, the butterfly was gently laid on a millimetric paper and photographs of the left and right sides were taken using a Nikon D7200 camera and a Laowa 100 mm f/2.8 2x Ultra Macro APO lens. Photos were used later for morphological analyses (see below). Next, the thorax width was measured with a caliper (error: ± 0.01 mm). Then, the butterfly was placed into a small paper bag and weighed with an Ohaus Scout (SKX123) balance (error: ± 1 mg). Next, we removed 2–3 mm^2 of the left hindwing to obtain material for further genetic analysis. Finally, the butterflies were marked with a fine-tipped waterproof Stabilo pen on the ventral part of the right fore wing to prevent re-sampling of the same individual. All butterflies were released at the place of capture when they were fully awake again. The wings from all individuals were digitally photographed and used for morphological analysis (see below). The photos with low quality not allowing a correct visualization of the wing structure were excluded from the analysis.

Wing morphometry assessment

A total of 354 butterflies (PL1990: n = 65, PL2003: n = 63, PL2019: n = 119, NL1996: n = 14, NL2020: n = 93) were used for studying the morphology of the hindwing. Landmarks were digitized with the software tpsDig v.2.32 (Rohlf 2018). A combination of landmarks and sliding semilandmarks (Bookstein 1997) was applied to study the vein intersections (5 landmarks) and the outline of the wings (9 landmarks and 17 semilandmarks) (Fig. S1). As landmarks we considered points that could be precisely identified, while the semilandmarks were allowed to slide equidistantly along the outline trajectory. The landmarks and semilandmarks were used to estimate both wing shape and centroid size, as the square root of the sum of squared distances of all the coordinates, being the most appropriate measure for overall size (Bookstein 1997). Detailed information about landmark data procedures prior morphological analysis can be found in Methods S1.

Metapopulation connectivity

In order to evaluate the changes in the spatial structure of both *P. teleius* metapopulations over the investigated period, we used Hanski's connectivity index I_4 (Hanski 1994). A more detailed description of the calculation is presented in Methods S1.

Genetic structure of the metapopulations

The genetic study was performed by using only butterflies from two current metapopulations: PL2019 and NL2020. The total number of butterfly samples used for genetic analysis was n = 118 for the current Polish metapopulation (PL2019) and n = 134 for the Dutch metapopulation (NL2020). Unfortunately, the quality of DNA obtained from 30 tested samples from PL1990 did not allow us to amplify a satisfactory number of loci for the means of our analyses. The material was collected in the above-described populations in Poland (n_{K1} = 30, n_{K10} = 30 and n_{K25} = 58) and in the Netherlands (n_{KBW} = 54, n_{PHZ} = 31 and n_{BW} = 49). Details about DNA extraction and microsatellite amplification are

presented in Methods S1. Butterflies were assayed at 17 microsatellite markers: Macu: 1, 3, 8, 9, 11, 15, 16, 26, 31, 44 and Macari: 2, 5, 16, 18, 19, 22, 23 (Zeisset *et al.* 2005; Ugelvig *et al.* 2011; Ugelvig *et al.* 2012; Andersen *et al.* 2014). PCR products were run on an ABI 3500 xL automated sequencer with the GeneScan™ 600 LIZ® Size Standard and analyzed using GENEMAPPER 4.1 (Applied Biosystems).

Morphometric statistical analysis

Butterfly weight, thorax width and hindwing size were examined in the two current metapopulations with a generalized linear model including the metapopulation (i.e., weight ~ metapopulation) as a predictor variable by using the glm() function (R Core Team 2022). Wing size, estimated as centroid size, was also pairwise-compared by performing estimated marginal means (EMMs) test by using the emmeans() function (Lenth 2023).

The ratios of body weight/centroid size and thorax width/centroid size were analyzed for the two current metapopulations with a generalized linear model, using the metapopulation as a predictor variable (e.g., weight/centroid size ~ metapopulation) by using the glm() function (R Core Team 2022).

The differences in the wing shape among metapopulations and allometry were tested by using the ProcD.lm() function (Baken *et al.* 2021; Adams *et al.* 2023), which performs a Procrustes ANOVA with permutation for describing patterns of shape variation and covariation for a set of Procrustes shape variables. The model was built using the logarithm of the centroid size and the metapopulation as predictor variables. A pairwise comparison was also performed between metapopulations by applying an estimated marginal means (EMMs) test by using the function pairwise() (Collyer & Adams 2018; Collyer & Adams 2023). The effect of allometry was removed from the pairwise comparison by using shape ~ log(centroid size) as the null model. The morphological disparity between groups was also studied by using the morphol.disparity() function (Baken *et al.* 2021; Adams *et al.* 2023) which performs a pairwise comparison among groups using residuals of a linear model fit to estimate the Procrustes variance. The morphological disparity test was performed only using the data from the source and two current metapopulations due to the size equality required for the test and the small sample sizes available for the rest of the metapopulations (see Methods S1).

Genetic structure of the metapopulations - statistical analysis

The analyses were carried out on two levels: 1) large regional scale between metapopulations and 2) local scale comparing the populations in three meadows in each region separately. To check if loci and groups were in Hardy-Weinberg Equilibrium (HWE), we used an exact probability test (Markov chain parameters: 10,000 dememorizations, 100 batches, 1,000 iterations per batch), with Bonferroni correction, implemented in GENEPOP on the Web version 4.7 (Raymond & Rousset 1995; Rousset 2008). Genotyping data were checked for amplification errors (large allele dropout, stuttering, and null alleles) using MICRO-CHECKER version 2.2.3 (Van Oosterhout *et al.* 2004). Linkage disequilibrium for all loci pairs was checked in FSTAT (Goudet 1995; Goudet 2001). FSTAT was also used to assess basic population parameters: number of alleles, allelic richness (AR), observed heterozygosity (H_O), expected heterozygosity (H_S), inbreeding coefficient (F_{IS}) and fixation index (F_{ST}). Allelic richness was calculated using the refraction

method for n = 28. Differences between the current Polish and Dutch metapopulations were assessed by a two-sided permutation test with 1,000 permutations in FSTAT. Allelic patterns, mean values with standard error of allele number, number of alleles with frequency over 5% and number of private alleles were calculated in GenAlEx6.5 (Peakall & Smouse 2012). To infer about a possible number of genetic clusters a Bayesian clustering approach implemented in Structure 2.3.4 (Pritchard *et al.* 2000; Falush *et al.* 2003; Hubisz *et al.* 2009) was used with the ancestry admixture model with correlated frequencies, with and without sampling location as prior information, with 100,000 Markov chain Monte Carlo (MCMC) replicates in each run, 50,000 MCMC after burn-in, and 10 replicate runs for each K (from 1 to 8). To determine the final number of clusters the Evanno method was used (Evanno *et al.* 2005) as implemented in Structure Harvester (Earl & VonHoldt 2012). The effective population size was assessed for each metapopulation in LDNE (Waples & Do 2008). This software facilitates calculations with different thresholds for the lowest allele frequencies considered. We selected a threshold of 0.02 as an intermediate solution. To test the hypothesis of a bottleneck we used Bottleneck 1.2.02 (Piry *et al.* 1999). We used the two-phase model (TPM) with 30% of infinite alleles model (IAM) and 70% of stepwise mutation model (SM), as an intermediate solution fitting to the reality best (see also discussion: Piry *et al.* (1999)).

Ethics approval statement

The butterfly study protocol was approved by the Regional Directorate for Environmental Protection from Kraków (decisions OP-I.6401.156.2019.KW) to perform the fieldwork in Poland. National State Forestry, Natuurmonumenten and the Province of Northern Brabant gave us permission to access the Moerputten nature reserve and carry out the survey in the Netherlands.

Results

Body weight and thorax width analysis

Adult females of *P. teleius* had a greater body weight in PL2019 than in NL2020, but males did not show any difference between the metapopulations (GLM, females: d.f. = 1, χ^2 = 6.85, p = 0.009; males: d.f. = 1, χ^2 = 2.9, p = 0.089; Fig. 2a). The thorax width was statistically bigger in PL2019 than in NL2020 in both sexes (GLM, females: d.f. = 1, χ^2 = 7.23, p = 0.007; males: d.f. = 1, χ^2 = 20.38, p < 0.001; Fig. 2b).

Hindwing size analysis

Differences in hindwing size were found among the different metapopulations (PL1990, PL2003, PL2019, NL1996, NL2020) for both females and males (GLM, females: d.f. = 4, χ^2 = 91.39, p < 0.001; males: d.f. = 4, χ^2 = 77.74, p < 0.001). Females from PL2019 had the largest wings, while females from the rest of the metapopulations did not significantly differ. However, females from NL2020 and PL2003 showed a higher mean value in comparison with PL1990. Similarly, males from PL2019 had the largest wings, while no statistically significant difference was found in hindwing size among the other studied metapopulations (Fig. 3a and b; Table S2).

The ratio between centroid size and body weight did not show any difference between the two current *P. teleius* metapopulations for both sexes (GLM, females: d.f. = 1, χ^2 = 3.21, p = 0.073; males: d.f. = 1, χ^2 = 4.83, p = 0.028; Fig. S2a). Likewise, the ratio

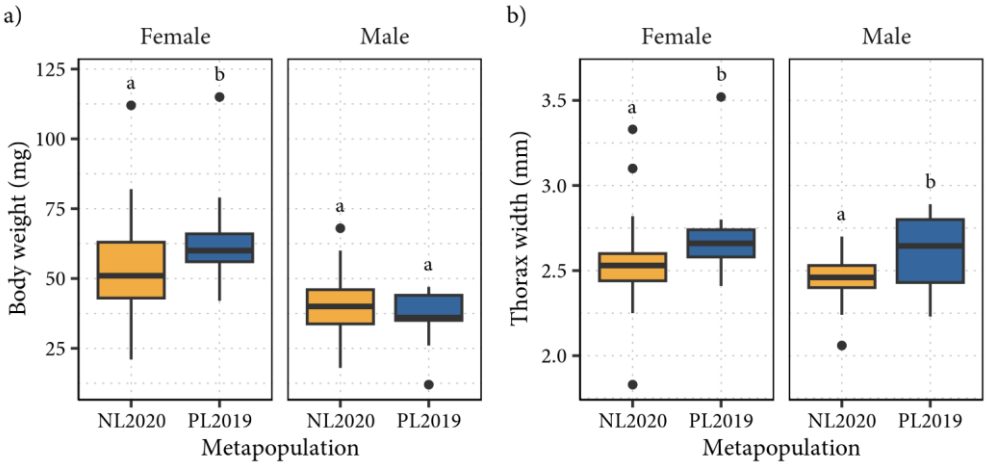

Fig. 2. *P. teleius* body weight (a) and thorax width (b) in the current metapopulation from Poland (PL2019: blue) and the Netherlands (NL2020: yellow) for females and males. The boxes depict the interquartile range, and horizontal black lines indicate median values. Vertical black lines extend from the minimum to the maximum non-outlier values and black dots indicate the outliers. Different letters at the top of the boxplots indicate statistically significant differences between groups.

between centroid size and thorax width also did not differ between the two current metapopulations (GLM, females: d.f. = 1, χ^2 = 0, p = 0.952; males: d.f. = 1, χ^2 = 0, p = 0.978; Fig. S2b).

Hindwing shape analysis

Both females and males showed differences in hindwing shape among metapopulations (Procrustes ANOVA, females: d.f. = 4, Z = 6.665, p = 0.001; males: d.f. = 4, Z = 5.541, p = 0.001; Fig. 3c and d). Differences in shape are partially explained by the change in centroid size, showing an allometric effect (Procrustes ANOVA, females: d.f. = 1, Z = 4.014, p = 0.001; males: d.f. = 1, Z = 3.775, p = 0.001; Fig. S3). The two current Polish and Dutch metapopulations showed statistically significant differences in shape when compared with the rest of the metapopulations apart from NL1996 for both females and males (Table S3). The other metapopulations did not show statistically significant differences between each other. Additionally, females and males from PL2019 also showed a higher shape variability than the rest of the metapopulations (Table S4).

Metapopulation connectivity

The results of connectivity changes over the years in both investigated metapopulations clearly indicated a gradual, although relatively slow, decrease in the connectivity in the Polish metapopulation from 0.39 to 0.3, apparently due to the loss of habitat patches through direct destruction (conversion to build-up areas, or less frequently afforestation) or through natural succession leading to meadow vegetation overgrowth and disappearance of *S. officinalis* host plants (cf. Kajzer-Bonk & Nowicki 2023). In turn, the connectivity in the Dutch metapopulation remained relatively stable for a long period till mid 2010s, but then sharply increased, from 0.99 to 2.75, thanks to the successful habitat restoration program (Wynhoff *et al.* 2017; Sevilleja *et al.* 2022). Most importantly, the results revealed that in mid 1990s when the reintroduction of *P. teleius* in the

Netherlands occurred, the connectivity of its habitat patch system was already substantially greater than that of the Polish metapopulation from which the reintroduced individuals originated. A graphical representation of the connectivity changes can be found in Fig. S4.

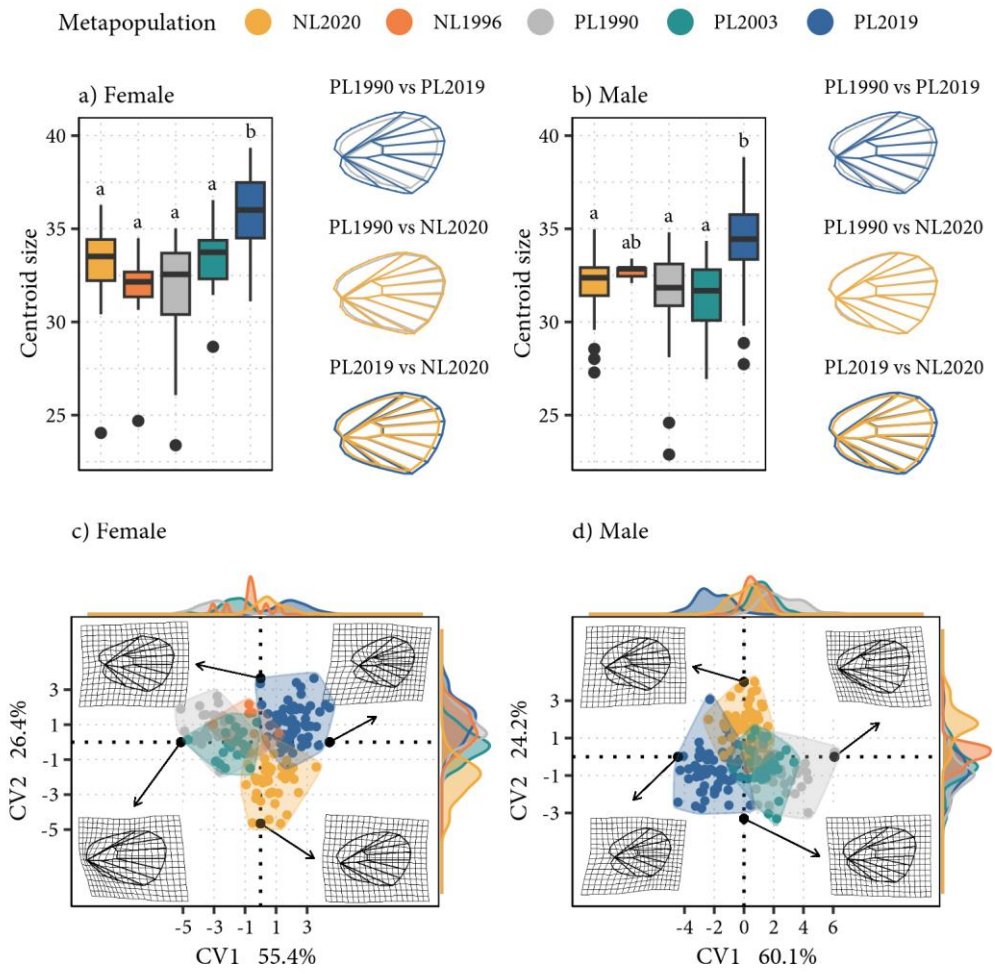

Fig. 3. Hindwing morphological comparison of *P. teleius* from the source (PL1990: grey), current and past Polish (PL2019: blue and PL2003: marine blue) and current and past reintroduced Dutch (NL2020: yellow and NL1996: orange) metapopulations. Size comparison for females (a) and males (b). The boxes depict the interquartile range, and horizontal black lines indicate median values. Vertical black lines extend from the minimum to the maximum non-outlier values and black dots indicate the outliers. Different letters at the top of the boxplots indicate statistically significant differences between groups (see Table S5). Hindwing graphical representation shows differences in size between each pairwise comparison. Procrustes CVA shape representation for females (c) and males (d). The axis densigrams represent the distribution of each canonical variate. The black dots and hindwing graphical representation indicate the most extreme values of each canonical variate and its corresponding estimated wing shape.

Genetic structure of the current Polish and Dutch metapopulations

Five out of 17 studied microsatellites proved to be monophyletic in both metapopulations: Macari5 (148 bp), Macari16 (168 bp), Macari18 (107 bp), Macu26 (90 bp) and Macu31 (104 bp). Loci Macu1 and Macari44 were not in HWE ($p < 0.001$). Loci Macari44 had null alleles (with frequencies 0.11 for the Polish and 0.05 for the Dutch metapopulation). Linkage disequilibrium was found only for one loci pair, i.e. Macari19 and Macari44. Some signs of null alleles were also detected in Macu15, but with low frequency (0.08) and only for one Polish population (K25) when groups were analyzed separately. Some discrepancy from HWE (heterozygosity excess) was noted for Macari3, but on the verge of statistical significance and only in two populations. Therefore, these two loci were not excluded from the analyses. Finally, nine loci (Macu3, Macu8, Macu9, Macu11, Macu15, Macu16, Macari02, Macari22, Macari23) were chosen for further analyses. There were no significant differences between Polish and Dutch metapopulations regarding allelic richness, observed heterozygosity H_o, expected heterozygosity H_e, inbreeding coefficient F_{IS} and genetic diversity F_{ST} (Table S6).

However, the difference in allelic richness (AR) was nearly significant ($p = 0.06$), and the Polish and Dutch metapopulations differed more than two-folds in number of alleles (62 vs. 30, respectively). Mean value of allele number was 6.89 ± 2.02 (SE) in Poland and 3.33 ± 0.67 in the Netherlands, number of private alleles 3.56 ± 1.43 vs. 0 ± 0.0, whereas number of alleles with frequencies over 5% was 2.78 ± 0.70 vs. 2.67 ± 0.47. Many of the alleles occurring with low frequencies in the Polish metapopulation were not found in the Dutch metapopulation. Also a founder effect can be seen, as some alleles of low frequencies in Poland increased their frequency in the Netherlands. More detailed changes in the allelic pattern are presented in Fig. 4a (for instance loci Macari16 with 21 alleles in Poland and 7 in Netherlands).

Genetic distances F_{ST} between all populations were low to moderate (from 0.003 to 0.126; Table S7), but significantly different except for the F_{ST} between the population K1 and K10. All genetic distances were low within the Polish and Dutch metapopulations and moderate in cross metapopulation comparisons.

No structuring was found in either of the metapopulations; the only well-grounded divide found (based on the rate of change in the log probability) was between the Polish and Dutch metapopulation with all individuals showing levels of admixture lower than 10% (Fig. 4b). No signs of population size reduction (bottleneck) were found in the Polish metapopulation, but it was found in the Dutch metapopulation (Wilcoxon test for heterozygosity excess, Poland: $p = 1$; the Netherlands: $p = 0.005$). The effective population size was estimated at 1165 individuals (Confidence interval: 177-∞) for the Polish metapopulation and 167 (Confidence interval: 69-4301) for the Dutch one.

Discussion

Our results demonstrated that after almost three decades of separation between the source and reintroduced metapopulations of *P. teleius*, morphological and genetic changes are observed in both metapopulations. Differences between the studied metapopulations are expected under independent development in different habitat types and climatological conditions. Since the translocated

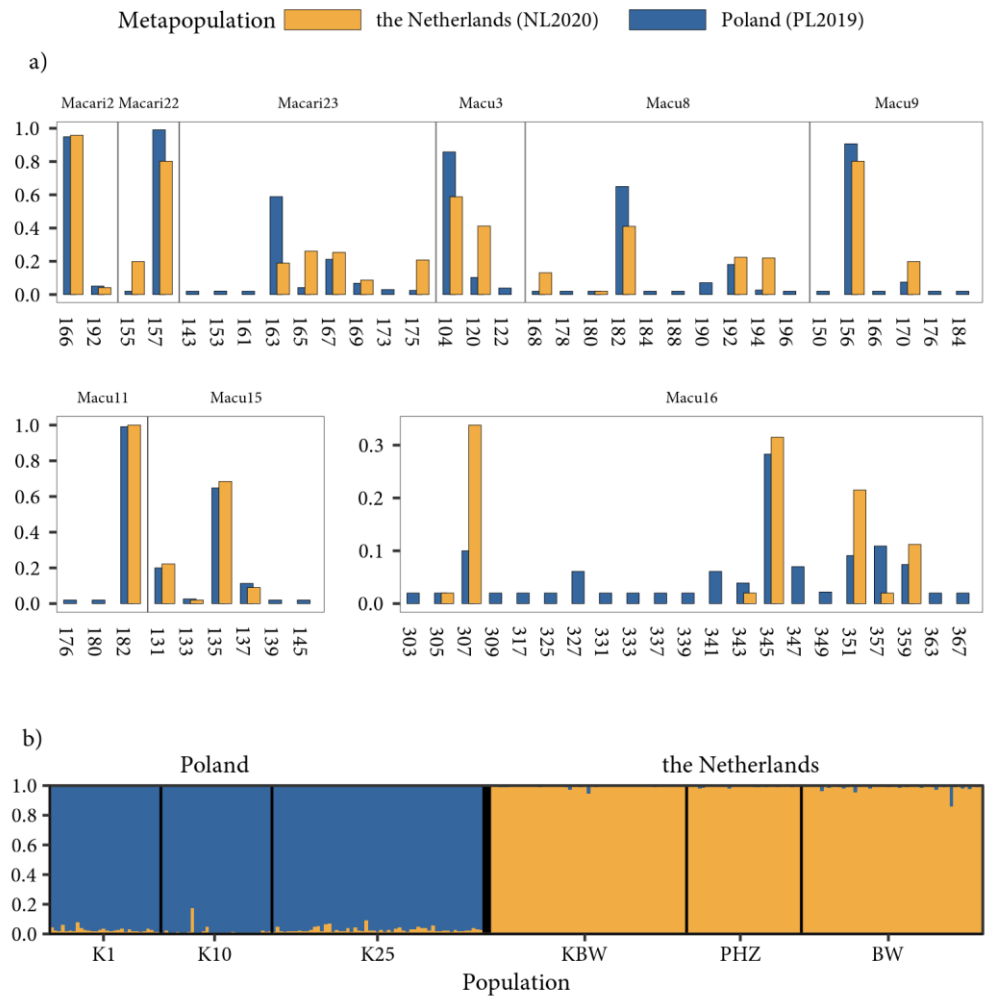

Fig. 4. Analysis of the nine studied microsatellite markers for the Polish and Dutch *P. teleius* metapopulation: (a) Microsatellite allele frequencies. Microsatellite names are given at the top of each box. Blue (Polish) and yellow (Dutch) bars indicate the frequency of each allele. Allele frequencies lower than 0.02 are represented as 0.02 to improve the graphical representation. Numbers in the x axis indicate allele size; (b) Cluster membership for *P. teleius* individuals from each sampling site identified using Structure analysis with location as prior information. Individuals are represented by vertical bars, with colors showing the probability of assignment to different genetic clusters. Cluster membership is based on K = 2 divisions, which had the highest likelihood, and the average of 10 iterations. Names of sampling sites are given above the membership diagram.

butterflies have undergone mostly environmental changes, the reintroduced metapopulation was expected to show greater changes since the moment of separation with respect to the source metapopulation. Meantime, the Polish butterflies were the ones showing the greater morphological changes. Genetic changes are in line with our assumptions, with the reintroduced and current

metapopulations being genetically different after 30 years of separation.

Habitat connectivity and differences in morphological traits among the studied metapopulations

Our results revealed that butterflies of the two current metapopulations (source and reintroduced) are different in their morphological traits. Individuals of both sexes from the Polish metapopulation have wider thoraxes and females are heavier compared to Dutch butterflies. They also have bigger wings that vary in their shape. Such differences in morphological traits among populations of the same butterfly species were also found in other studies. Butterflies of *Erebia medusa* from different populations have been found to have different wing size and shape, mostly due to the climate conditions (Mikitová *et al.* 2022). Interestingly, temporal morphological differences in the wing size and shape were also found in the Polish source metapopulation and these differences have been mostly observed in the last 16 years, which is halfway between the reintroduction time and current situation. The wing size increase in time is more directional and visible in females but currently bigger wings are found in both sexes. Various factors and/or their synergistic effect could be responsible for observed changes. Habitat connectivity can be one of the most important factors affecting dispersal, mostly distances covered by butterflies. Dispersal distances depend, among others, on the butterfly flying capacity, which in turn is related to morphological traits (e.g. wing size and thorax mass). This relationship was demonstrated both at inter-species (Sekar 2012) and intra-species level (Berwaerts *et al.* 2002; Merckx & Van Dyck 2006). Different trends in habitat management, and in turn habitat connectivity, can be found in the source and reintroduced metapopulations. In the Netherlands the Life+ project "Blues in the Marshes" started in 2012 and led to the restoration of fen meadows in the Natura 2000 reserve (Wynhoff *et al.* 2017; Sevilleja *et al.* 2022), which increased the potential habitat of the butterflies. At the Polish site, part of the suitable *P. teleius* habitats was included in the Natura 2000 network in 2011, but nevertheless half of the patches occupied by *P. teleius* have disappeared in the last two decades (Kajzer-Bonk & Nowicki 2022). Such environmental changes have influenced the connectivity among *P. teleius* habitat patches that decreased in the Polish metapopulation and increased in the Dutch one (Fig. 1). Some empirical studies and theoretical models demonstrated that habitat fragmentation can lead to lower butterfly dispersal propensity (e.g., Heino & Hanski 2001; Schtickzelle *et al.* 2006) and also influence morphological traits connected with dispersal ability. However, it is also possible that a fragmented landscape in some environmental conditions can favor higher mobility. A study on *Pararge aegeria* butterflies demonstrated that females developing in fragmented agricultural habitats allocated more mass to flight muscles than individuals developed in woodland landscapes (Merckx & Van Dyck 2006). Also, the theoretical model by Heino & Hanski (2001) predicts that under certain conditions, dispersal can increase with habitat fragmentation due to deterioration of patch quality manifested by changes in carrying capacity. In the Polish metapopulation not only habitat patch number decreased but habitat quality also changed due to prolonged meadow abandonment and goldenrod invasion (Kajzer-Bonk *et al.* 2016b), which led to a strong decrease of two main resources

of *P. teleius*; the abundance of host plants and the number of *Myrmica* nests (Kajzer-Bonk *et al.* 2016a, b). Despite these changes, the meadow complex in Poland is still supporting the largest metapopulation of *P. teleius* in Europe (Nowicki 2017), and local populations (patches) typically consist of several hundreds to a few thousand individuals (Nowicki *et al.* 2007). Recently, a positive density-dependent emigration was proven for *P. teleius*, indicating that once the carrying capacity is exceeded, the emigration propensity is doubling in males and rising threefold in females (Nowicki *et al.* 2014; Plazio *et al.* 2020). It is known that dispersal can be subject to strong, opposite selection pressures (Schtickzelle *et al.* 2006). Some factors select against dispersal behavior, like the costs of crossing unsuitable habitats and high mortality rate during dispersal, both connected with high habitat fragmentation, whereas other factors can promote dispersal (e.g., avoidance of kin competition or temporal variation of reproductive success in local populations). Thus, it is possible that in the current Polish metapopulation, despite the increase of the habitat fragmentation, individuals are affected by other selective pressures favoring higher dispersal abilities. Following Schtickzelle *et al.* (2006), we argue that in a fragmented landscape the individuals with bigger thoraxes and bigger wings have higher survival probabilities during dispersal and as a consequence an increased fitness. Our results demonstrated changes not only in the wing size but also in the wing shape. Giving an explanation related to shape variation remains more complicated, as such changes can be associated to different factors, like predation risk, mimicry, mating strategy and larval diet quality (see in Le Roy *et al.* 2019). Additionally, we also found slightly different results in the strength of the allometric effect in the shape of females and males, which could be explained by the differential effect of natural selection between sexes (DeVries *et al.* 2010). Moreover, the lower morphological variability found in the reintroduced metapopulation compared with the current Polish metapopulation might be explained by the effect of a lower genetic diversity, population size and isolation. A similar pattern was found in *Parnassius apollo*, for which the highest morphological variability was found in an Alpine metapopulation with the highest level of genetic diversity compared to other smaller and isolated populations (Habel *et al.* 2012).

Genetic structure of metapopulations

Our results demonstrated much lower allelic richness in the reintroduced Dutch metapopulation, which harbors only half of alleles present in the current Polish metapopulation. Although there were no statistically significant differences in the rest of the genetic parameters analyzed, it is worth noting that samples from the Netherlands were mainly collected in the core population and two smaller ones, which host most butterflies in this metapopulation system, whereas in Poland the study was restricted only to three populations out of the existing 33. Therefore, we can assume the whole genetic variation in the Polish metapopulation is much higher than the one detected in our study. No changes in heterozygosity were observed, the values of heterozygosity themselves being not very high in both metapopulations, comparable to those of endangered *Phengaris arion* in the Danish and Swedish populations (Ugelvig *et al.* 2011). This result is not surprising as allelic richness is known to be more sensitive to number reduction than heterozygosity (Frankham 1995). The same phenomenon has been

observed in a bottlenecked population of *P. arion* (Ugelvig *et al.* 2011). The 30-year period of separate history of the source and reintroduced metapopulation are apparent in the results of the admixture analysis, where the division between both metapopulations is clearly visible. There was no pronounced structure in any of the metapopulations at the population level as can be seen in Bayesian clustering and low pairwise genetic distances, which is in accordance with ecological data showing *P. teleius* dispersal among meadow patches (e.g., Plazio *et al.* 2020). The estimated effective population size is substantially bigger for the Polish metapopulation, however these results must be treated with some caution due to quite broad confidence intervals of the estimates. The estimate of effective population size for the Polish metapopulation is likely to be underestimated due to sampling of only three sites of a big metapopulation system. The Dutch metapopulation bears evident and strongly supported signs of a bottleneck (highly significant results regardless of the mutation model assumed), which are not seen in the Polish metapopulation. The evident bottleneck can be assigned to the reintroduction event being by its nature bound with genetic pool reduction. However, our estimate of the Dutch effective population size highly surpasses the number of translocated butterflies, thus pointing to the success of the reintroduction. This estimate is also very high compared with the effective population size of *P. arion* in England, estimated to be 25 individuals, 19 years after translocation of 281 caterpillars from Sweden (Andersen *et al.* 2014).

Study limitations

While the most comprehensive study designs demand repeats and complex methodology (i.e., pre- and post-impact sampling in both: control and experimental environments (Christie *et al.* 2019)), our study fulfills this approach only partially, as we had no replications and limited access to the historical data. Hence, there is a potential risk that wing samples from 1990 may be biased towards weaker individuals, which died during the trip, and potentially bearing different trait values. However, we argue that our historical data of butterfly wings reflects an accurate representation, as the wing size of the Dutch butterflies from 1996 and the Polish butterflies from 2003, that were randomly collected, are not different from those from 1990 (see Fig. 3a and Table S2). It should be stressed that the main changes in landscape composition and connectivity started in both considered locations after this period (see Fig. S4). Moreover, there was no significant correlation observed between the survival of *Pontia occidentalis* butterflies and their wing size or body mass (Kingsolver 1999), which could be an additional argument to support that our samples from 1990 were collected without methodological bias. Additionally, our efforts to analyze historical genetic data were unsuccessful, mostly due to problems with microsatellite amplification. Thus, in future studies, more sensitive methods as SNPs should be considered for application (Puckett & Eggert 2016).

Conclusions for conservation and management implications

Together with earlier research, our study implies that *Phengaris* butterflies are good indicators of habitat changes. Despite their peculiar life cycle, they are able to adapt to environmental alterations in a relatively short period of time. This can give us an optimistic expectation that as long as their food

plants and host ant species are present, they may persist both global and local changes, facing climate change and habitat fragmentation. The reintroduction of less than 100 individuals of *P. teleius* butterflies was enough for the metapopulation to survive over 30 generations, grow and expand to the new patches, showing the effectiveness of the sample size of translocated butterflies. Our results bear significance for a proper habitat management in reintroduced butterfly populations as it has been carried out in the Dutch nature reserve for the last 30 years starting from the reintroduction event. It shows how habitat restoration can lead to the increase of population size after reintroduction and it can be an important indication for potential future reintroductions of *Phengaris* butterflies. In many cases, more effort and attention are devoted to monitoring introduced populations, meanwhile our results emphasize the importance of also following the changes taking place in the source populations. It can be essential as usually such populations are the more resilient ones and could serve as a source for future reintroduction efforts. Therefore, maintaining such populations in healthy condition should be a priority in species conservation practices. We also would like to draw attention to the importance of conducting regular monitoring of morphological traits of butterflies using non-lethal methods, both in the source and reintroduced populations, as they may be characterized by high phenotypic plasticity and serve as a good indicator of environmental changes.

CRediT authorship contribution statement

Daniel Sánchez-García: Conceptualization, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization. Irma Wynhoff: Conceptualization, Methodology, Investigation, Resources, Writing - Review & Editing. Joanna Kajzer-Bonk: Investigation, Writing - Review & Editing, Visualization. Anna Sztencel-Jabłonka: Formal analysis, Writing - Review & Editing. Piotr Nowicki: Formal analysis, Writing - Review & Editing. Luca Pietro Casacci: Conceptualization, Methodology, Investigation, Writing - Review & Editing. Magdalena Witek: Conceptualization, Methodology, Investigation, Writing - Original Draft, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Adams, D.C., Collyer, M.L., Kaliontzopoulou, A. & Baken, E.K. (2023). Geomorph: Software for geometric morphometric analyses. R package version 4.0.6.

Andersen, A., Simcox, D.J., Thomas, J.A. & Nash, D.R. (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.

Andrews, P. (2015). A History of the Large Blue *Maculinea arion* subspecies eutyphron (Fruhstorfer, 1915) in Somerset. *Dispar*, 1–6.

Baken, E.K., Collyer, M.L., Kaliontzopoulou, A. & Adams, D.C. (2021). Geomorph v4.0 and gmShiny: Enhanced analytics and a new graphical interface for a comprehensive morphometric experience. *Methods in Ecology and Evolution*, 12, 2355–2363.

Bellis, J., Bourke, D., Williams, C. & Dalrymple, S. (2019). Identifying factors associated with the success and failure of terrestrial insect translocations. *Biological Conservation*, 236, 29–36.

Berwaerts, K., Dyck, H.V.A.N. & Aerts, P. (2002). Does flight morphology relate to flight performance? An experimental test with the butterfly Pararge aegeria. *Functional Ecology*, 16, 484–491.

Bonte, D., Van Dyck, H., Bullock, J.M., Coulon, A., Delgado, M., Gibbs, M., *et al.* (2012). Costs of dispersal. *Biological Reviews*, 87, 290–312.

Bookstein, F.L. (1997). Landmark methods for forms without landmarks: localizing group differences in outline shape. *Proceedings of the Workshop on Mathematical Methods in Biomedical Image Analysis*, 1, 225–243.

Christie, A.P., Amano, T., Martin, P.A., Shackelford, G.E., Simmons, B.I. & Sutherland, W.J. (2019). Simple study designs in ecology produce inaccurate estimates of biodiversity responses. *Journal of Applied Ecology*, 56, 2742–2754.

Collyer, M. & Adams, D. (2023). *RRPP: Linear model evaluation with randomized residuals in a permutation procedure*.

Collyer, M.L. & Adams, D.C. (2018). RRPP: An r package for fitting linear models to high-dimensional data using residual randomization. *Methods in Ecology and Evolution*, 9, 1772–1779.

Dempster, J.P. & Hall. (1980). An attempt at re-establishing the swallowtail butterfly at Wicken Fen. *Ecological Entomology*, 5, 327–334.

Deredec, A. & Courchamp, F. (2007). Importance of the Allee effect for reintroductions. *Ecoscience*, 14, 440–451.

DeVries, P.J., Penz, C.M. & Hill, R.I. (2010). Vertical distribution, flight behaviour and evolution of wing morphology in Morpho butterflies. *Journal of Animal Ecology*, 79, 1077–1085.

Earl, D.A. & VonHoldt, B.M. (2012). STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources*, 4, 359–361.

Elmes, G.W. & Thomas, J.A. (1992). Complexity of species conservation in managed habitats: interaction between *Maculinea* butterflies and their ant hosts. *Biodiversity and Conservation*, 1, 155–169.

Evanno, G., Regnaut, S. & Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Molecular Ecology*, 14, 2611–2620.

Falush, D., Stephens, M. & Pritchard, J.K. (2003). Inference of population structure using multilocus genotype data: Linked loci and correlated allele frequencies. *Genetics*, 164, 1567–1587.

Frankham, R. (1995). Effective population size/adult population size ratios in wildlife: A review. *Genetics Research*, 66, 95–107.

Gawecka, K.A., Pedraza, F. & Bascompte, J. (2022). Effects of habitat destruction on coevolving metacommunities. *Ecology Letters*, 25, 2597–2610.

Goudet, J. (1995). FSTAT (Version 1.2): A Computer Program to Calculate F-Statistics. *Journal of Heredity*, 86, 485–486.

Goudet, J. (2001). FSTAT, a program to estimate and test gene diversities and fixation indices, version 2.9.3.

Grass, I., Jauker, B., Steffan-Dewenter, I., Tscharntke, T. & Jauker, F. (2018). Past and potential future effects of habitat fragmentation on structure and

stability of plant–pollinator and host–parasitoid networks. *Nature Ecology and Evolution*, 2, 1408–1417.

Habel, J.C., Reuter, M., Drees, C. & Pfaender, J. (2012). Does isolation affect phenotypic variability and fluctuating asymmetry in the endangered Red *Apollo*? *Journal of Insect Conservation*, 16, 571–579.

Hallmann, C.A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., *et al.* (2017). More than 75 percent decline over 27 years in total flying insect biomass in protected areas. *PLoS ONE*, 12.

Hanski, I. (1994). A practical model of metapopulation dynamics. *The Journal of Animal Ecology*, 63, 151–162.

Heino, M. & Hanski, I. (2001). Evolution of migration rate in a spatially realistic metapopulation model. *American Naturalist*, 157, 495–511.

Howell, P.E., Lundrigan, B. & Scribner, K.T. (2016). Environmental and genealogical effects on emergence of cranial morphometric variability in reintroduced *American martens*. *Journal of Mammalogy*, 97, 761–773.

Hubisz, M.J., Falush, D., Stephens, M. & Pritchard, J.K. (2009). Inferring weak population structure with the assistance of sample group information. *Molecular Ecology Resources*, 9, 1322–1332.

Kajzer-Bonk, J. & Nowicki, P. (2022). Butterflies in trouble: The effectiveness of Natura 2000 network in preventing habitat loss and population declines of endangered species in urban area. *Ecological Indicators*, 135, 108518.

Kajzer-Bonk, J. & Nowicki, P. (2023). Vanishing meadows — Quantitative analysis of factors driving population declines of endangered butterflies. *Biological Conservation*, 282, 110050.

Kajzer-Bonk, J., Skorka, P., Nowicki, P., Bonk, M., Krol, W., Szpilyk, D., *et al.* (2016a). Relative contribution of matrix structure, patch resources and management to the local densities of two large blue butterfly species. *PLoS ONE*, 11, 1–19.

Kajzer-Bonk, J., Szpiłyk, D. & Woyciechowski, M. (2016b). Invasive goldenrods affect abundance and diversity of grassland ant communities (Hymenoptera: Formicidae). *Journal of Insect Conservation*, 20, 99–105.

Kingsolver, J.G. (1999). Experimental analyses of wing size, flight, and survival in the western white butterfly. *Evolution*, 53, 1479–1490.

Koh, L.P., Dunn, R.R., Sodhi, N.S., Colwell, R.K., Proctor, H.C. & Smith, V.S. (2004). Species coextinctions and the biodiversity crisis. *Science*, 305, 1632–1634.

Le Roy, C., Debat, V. & Llaurens, V. (2019). Adaptive evolution of butterfly wing shape: from morphology to behaviour. *Biological Reviews*, 94, 1261–1281.

Lenth, R.V. (2023). emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.8.4-1.

Merckx, T. & Van Dyck, H. (2006). Landscape structure and phenotypic plasticity in flight morphology in the butterfly *Pararge aegeria*. *Oikos*, 113, 226–232.

Mikitová, B., Šemeláková, M. & Panigaj, Ľ. (2022). Wing morphology and eyespot pattern of Erebia medusa (Lepidoptera, Nymphalidae) vary along an elevation gradient in the Carpathian Mountains. *Nota Lepidopterologica*, 45, 233–250.

Nowicki, P. (2017). Survey precision moderates the relationship between population size and stability. *Biological Conservation*, 212, 310–315.

Nowicki, P., Pepkowska, A., Kudlek, J., Skórka, P., Witek, M., Settele, J., *et al.* (2007). From metapopulation theory to conservation recommendations: Lessons from spatial occurrence and abundance patterns of *Maculinea* butterflies. *Biological Conservation*, 140, 119–129.

Nowicki, P., Vrabec, V., Binzenhöfer, B., Feil, J., Zakšek, B., Hovestadt, T., *et al.* (2014). Butterfly dispersal in inhospitable matrix: Rare, risky, but long-distance. *Landscape Ecology*, 29, 401–412.

Oates, M.R. & Warren, M.S. (1990). *A review of butterfly introductions in Britain and Ireland.* World Wide Fund for Nature, Godalming.

Peakall, R. & Smouse, P.E. (2012). GenALEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics*, 28, 2537–2539.

Piry, S., Luikart, G. & Cornuet, J.M. (1999). BOTTLENECK: A computer program for detecting recent reductions in the effective population size using allele frequency data. *Journal of Heredity*, 90, 502–503.

Plazio, E., Margol, T. & Nowicki, P. (2020). Intersexual differences in density-dependent dispersal and their evolutionary drivers. *Journal of Evolutionary Biology*, 33, 1495–1506.

Pritchard, J.K., Stephens, M. & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, 155, 945–959.

Puckett, E.E. & Eggert, L.S. (2016). Comparison of SNP and microsatellite genotyping panels for spatial assignment of individuals to natal range: A case study using the American black bear (*Ursus americanus*). *Biological Conservation*, 193, 86–93.

R Core Team. (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. R version 4.2.1.

Raven, P.H. & Wagner, D.L. (2021). Agricultural intensification and climate change are rapidly decreasing insect biodiversity. *Proceedings of the National Academy of Sciences of the United States of America*, 118, 1–6.

Raymond, M. & Rousset, F. (1995). GENEPOP (Version 1.2): Population Genetics Software for Exact Tests and Ecumenicism. *Journal of Heredity*, 86, 248–249.

Rohlf, J.F. (2018). tpsDig version 2.32.

Rousset, F. (2008). GENEPOP'007: A complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources*, 8, 103–106.

Schtickzelle, N., Mennechez, G.G. & Baguette, M. (2006). Dispersal Depression With Habitat Fragmentation. *Ecology*, 87, 1057–1065.

Seddon, P.J., Griffiths, C.J., Soorae, P.S. & Armstrong, D.P. (2014). Reversing defaunation: restoring species in a changing world. *Science*, 345, 406–412.

Sekar, S. (2012). A meta-analysis of the traits affecting dispersal ability in butterflies: Can wingspan be used as a proxy? *Journal of Animal Ecology*, 81, 174–184.

Sevilleja, C.G., Van Langevelde, F., Gallego-Zamorano, J., Bassignana, C.F. & Wynhoff, I. (2022). Sod translocation to restore habitats of the myrmecophilous butterfly *Phengaris* (*Maculinea*) *teleius* on former agricultural fields. *Ecology and Evolution*, 12, 1–14.

Tartally, A., Thomas, J.A., Anton, C., Balletto, E., Barbero, F., Bonelli, S., *et al.* (2019). Patterns of host use by brood parasitic *Maculinea* butterflies across Europe. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 374.

Taylor, G., Canessa, S., Clarke, R.H., Ingwersen, D., Armstrong, D.P., Seddon, P.J., *et al.* (2017). Is Reintroduction Biology an Effective Applied Science? *Trends in Ecology and Evolution*, 32, 873–880.

Thomas, J.A. (1984). The behaviour and habitat requirements of Maculinea nausithous (the dusky large blue butterfly) and *M. teleius* (the scarce large blue) in France. *Biological Conservation*, 28, 325–347.

Thomas, J.A. & Settele, J. (2004). Butterfly mimics of ants. *Nature*, 432, 283–284.

Thomas, J.A., Simcox, D.J. & Clarke, R.T. (2009). Successful conservation of a threatened *Maculinea* butterfly. *Science*, 325, 80–83.

Thomas, J.A., Telfer, M.G., Roy, D.B., Preston, C.D., Greenwood, J.J.D., Asher, J., *et al.* (2004). Comparative Losses of British Butterflies, Birds, and Plants and the Global Extinction Crisis. *Science*, 303, 1879–1881.

Ugelvig, L.V., Andersen, A., Boomsma, J.J. & Nash, D.R. (2012). Dispersal and gene flow in the rare, parasitic Large Blue butterfly *Maculinea arion*. *Molecular Ecology*, 21, 3224–3236.

Ugelvig, L.V., Nielsen, P.S., Boomsma, J.J. & Nash, D.R. (2011). Reconstructing eight decades of genetic variation in an isolated Danish population of the large blue butterfly *Maculinea arion*. *BMC Evolutionary Biology*, 11, 201.

Van Oosterhout, C., Hutchinson, W.F., Wills, D.P.M. & Shipley, P. (2004). MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, 4, 535–538.

Waples, R.S. & Do, C. (2008). LDNE: A program for estimating effective population size from data on linkage disequilibrium. *Molecular Ecology Resources*, 8, 753–756.

Wund, M.A., Singh, O.D., Geiselman, A. & Bell, M.A. (2016). Morphological evolution of an anadromous threespine stickleback population within one generation after reintroduction to Cheney Lake, Alaska. *Evolutionary Ecology Research*, 17, 203–224.

Wynhoff, I. (1998). Lessons from the reintroduction of *Maculinea teleius* and *M. nausithous* in the Netherlands. *Journal of Insect Conservation*, 2, 47–57.

Wynhoff, I., Gestel, R. van, Swaay, C. van & Langevelde, F. van. (2011). Not only the butterflies: Managing ants on road verges to benefit *Phengaris* (*Maculinea*) butterflies. *Journal of Insect Conservation*, 15, 189–206.

Wynhoff, I., Kolvoort, A.M., Bassignana, C.F., Berg, M.P. & Van Langevelde, F. (2017). Fen meadows on the move for the conservation of *Maculinea* (*Phengaris*) *teleius* butterflies. *Journal of Insect Conservation*, 21, 379–392.

Zayed, A., Packer, L., Grixti, J.C., Ruz, L., Owen, R.E. & Toro, H. (2005). Increased genetic differentiation in a specialist versus a generalist bee: implications for conservation. *Conservation Genetics*, 6, 1017–1026.

Zeisset, I., Als, T.D., Settele, J. & Boomsma, J.J. (2005). Microsatellite markers for the large blue butterflies *Maculinea nausithous* and *Maculinea alcon* (Lepidoptera: Lycaenidae) and their amplification in other *Maculinea* species. *Molecular Ecology Notes*, 5, 165–168.

Supporting information 1

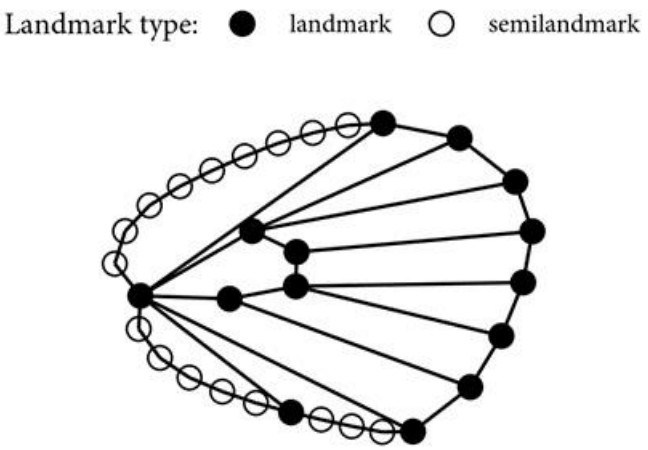

Fig. S1. Landmark and semilandmark location in the general consensus hindwing of *P. teleius*.

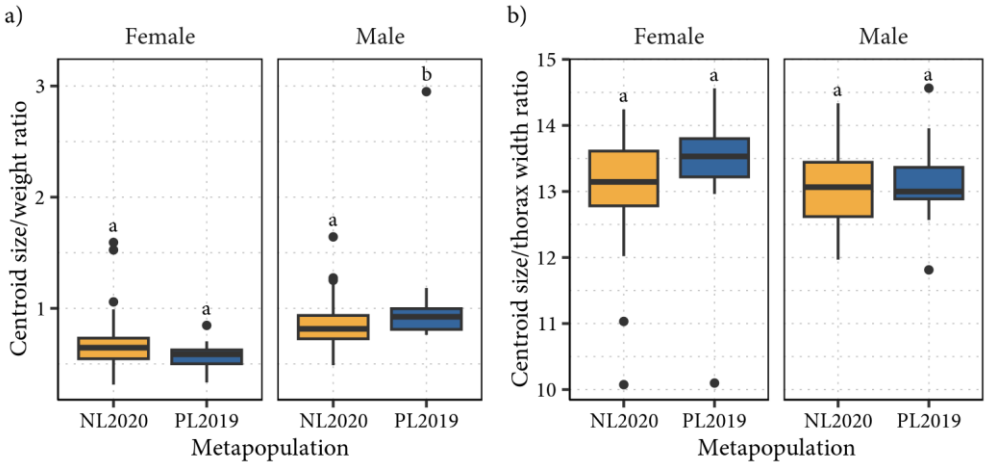

Fig. S2. *P. teleius* butterfly ratio of hindwing centroid size/weight (a) and centroid size/thorax width (b) comparison between the current metapopulation from Poland (PL2019) and the reintroduced metapopulation from the Netherlands (NL2020) for females and males.

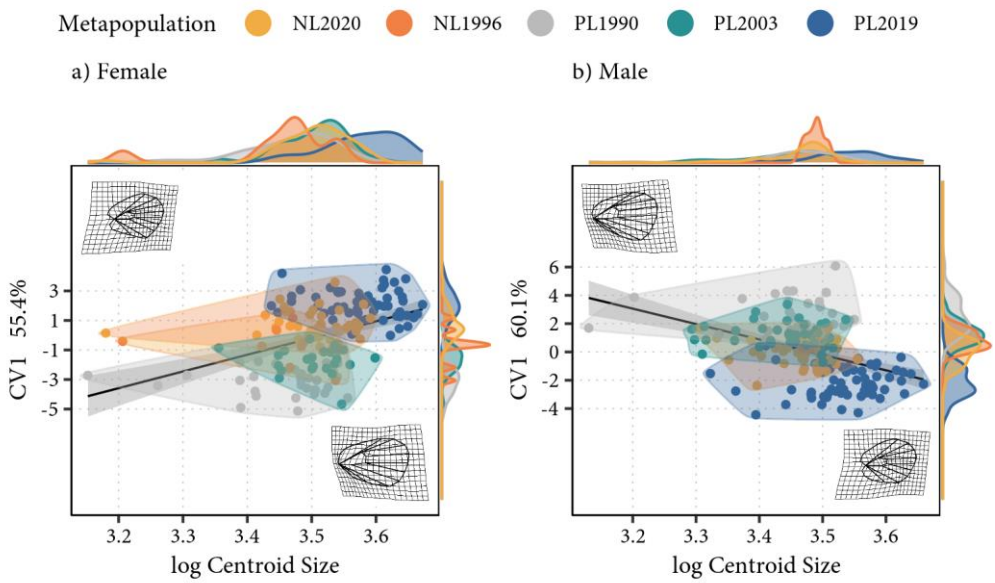

Fig. S3. Representation of *P. teleius* females (a) and males (b) butterfly hindwings shape allometry from the source (PL1990: grey), current and past Polish (PL2019: blue and PL2003: marine blue) and current and past reintroduced Dutch (NL2020: yellow and NL1996: orange) metapopulations.

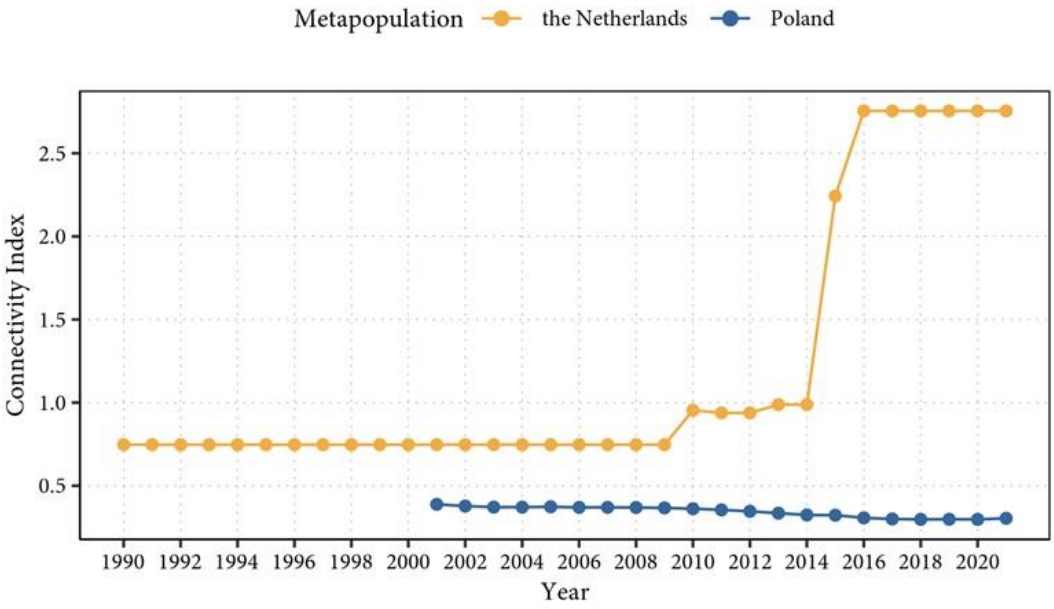

Fig. S4. *P. teleius* metapopulation connectivity for the Polish (blue line) and Dutch (yellow line) metapopulations calculated from 2001 for the Polish and from 1990 for the Dutch metapopulation.

Table S1. Summary of data collected in the different studied metapopulations. "+" means presence of data, "-" means absence of data for a specific metapopulation.

Metapopulation	Body weight/thorax size	Hindwing size and shape	Hindwing morphological disparity	Genetics
PL1990	-	+	+	-
PL2003	-	+	-	-
PL2019	+	+	+	+
NL1996	-	+	-	-
NL2020	+	+	+	+

Table S2. EMMs (estimated marginal means) test results for the female and male hindwing centroid size pairwise comparison between the source (PL1990), current and past Polish (PL2019 and PL2003) and current and past reintroduced Dutch (NL2020 and NL1996) metapopulations.

		NL2020	PL1990	PL2003	PL2019
Female	NL1996	t = -1.97 p = 0.286	t = -0.24 p = 0.999	t = -1.94 p = 0.301	t = -5.41 p < 0.001***
	NL2020	-	t = 2.75 p = 0.052	t = -0.16 p = 1	t = -6.02 p < 0.001***
	PL1990	-	-	t = -2.49 p = 0.099	t = -8.52 p < 0.001***
	PL2003	-	-	-	t = -4.77 p < 0.001***
Male	NL1996	t = 0.75 p = 0.943	t = 1.22 p = 0.737	t = 1.52 p = 0.549	t = -1.75 p = 0.405
	NL2020	-	t = 1.03 p = 0.841	t = 1.74 p = 0.413	t = -6.15 p < 0.001***
	PL1990	-	-	t = 0.54 p = 0.983	t = -6.32 p < 0.001***
	PL2003	-	-	-	t = -7.58 p < 0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

Table S3. LSMs (Least Square Means) test results for the female and male hindwing shape pairwise comparison between the source (PL1990), current and past Polish (PL2019 and PL2003) and current and past reintroduced Dutch (NL2020 and NL1996) metapopulations.

		NL2020	PL1990	PL2003	PL2019
Females	NL1996	Z = 0.62 p = 0.265	Z = 1.04 p = 0.156	Z = -0.27 p = 0.604	Z = 1.61 p = 0.051
	NL2020	-	Z = 4.53 p < 0.001***	Z = 2.39 p = 0.008**	Z = 4.4 p < 0.001***
	PL1990	-	-	Z = 0.93 p = 0.163	Z = 5.56 p < 0.001***
	PL2003	-	-	-	Z = 3.27 p = 0.003**
Males	NL1996	Z = -0.25 p = 0.593	Z = 0.66 p = 0.255	Z = -0.03 p = 0.513	Z = -0.15 p = 0.559
	NL2020	-	Z = 3.93 p < 0.001***	Z = 1.74 p = 0.048*	Z = 2.29 p = 0.012*
	PL1990	-	-	Z = 2.47 p = 0.008**	Z = 5.15 p < 0.001***
	PL2003	-	-	-	Z = 3.48 p < 0.001***

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

Table S4. Morphological disparity significance values for female and male hindwing shape pairwise comparison between the source (PL1990), current Polish (PL2019) and current reintroduced Dutch (NL2020) metapopulations.

		NL1990	PL2019
Female	NL2020	0.699	0.225
	PL1990	-	0.107
Male	NL2020	0.755	0.034*
	PL1990	-	0.114

Female Procrustes variances:
NL2020 = 0.0013; PL1990 = 0.0012; PL2019 = 0.0015

Male Procrustes variances:
NL2020 = 0.0011; PL1990 = 0.0011; PL2019 = 0.0014

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

Table S6. Comparison of genetic parameters between the Polish and Dutch metapopulations.

	PL	NL	p
AR	4.58	2.92	0.062
H_O	0.33	0.44	0.112
H_S	0.34	0.43	0.083
F_{IS}	0.03	-0.02	0.138
F_{ST}	0.01	0.02	0.378

PL = Poland, NL = the Netherlands

AR = allelic richness
H_O = observed heterozygosity
H_S = expected heterozygosity
F_{IS} = inbreeding coefficient
F_{ST} = fixation index

Table S7. Pairwise genetic distances F_{ST} between all studied populations from the current Polish metapopulation (K1, K10, K25) and the reintroduced Dutch metapopulation (KBW, PHZ, BW).

	K1	K10	K25	KBW	PHZ	BW
K1	-	0.003 p = 0.140	0.005 p = 0.003	0.083 p = 0.003	0.096 p = 0.003	0.076 p = 0.003
K10		-	0.014 p = 0.007	0.122 p = 0.003	0.126 p = 0.003	0.105 p = 0.003
K25			-	0.101 p = 0.003	0.115 p = 0.003	0.091 p = 0.003
KBW				-	0.029 p = 0.003	0.019 p = 0.003
PHZ					-	0.024 p = 0.003

Metapopulation connectivity

Hanski's connectivity index calculates the connectivity of a given habitat patch (i) based on the distances d_{ij} (in km, measured between patch centers) separating it from other patches in the system (j = 1 to k; $j \neq i$) and their areas S_j (in ha):

$$I_4 = \sum \exp(-\alpha d_{ij}) S_j^a \qquad (1)$$

where α and a are species specific scaling parameters. Habitat patches are considered to be sites where *Sanguisorba officinalis* (the butterfly foodplant) is present. The parameter α (distance dependence) reflects the chances of individuals to reach particular distances, while a (immigration scaling) describes how the chances of immigration to other patches depend on their sizes. The values of α = 6.6 and a = 0.54 were adopted after Nowicki *et al.* (2014), where they were derived through dispersal analysis for *P. teleius* in the Polish metapopulation, but also proved typical for other metapopulations of this species in predominantly grassland landscapes. Hanski's connectivity index was first calculated separately for each patch. Subsequently, in order to assess the average connectivity level within each metapopulation in a given year, we calculated its mean value for all the existing patches as well as the weighted mean, with weights proportional to the patch area-based carrying capacity (and thus also to potential population size) for *P. teleius* and defined as S_i^b, where b = 0.67 after Nowicki *et al.* (2007). The calculations were conducted for each year between 1990 and 2021 for the Dutch metapopulation, but in the Polish metapopulation they were restricted to the period starting from 2001, where the habitat patches were mapped for the first time. Nevertheless, we should stress that prior to this year the spatial structure remained mostly unchanged for at least 10-20 years. It is worth noting that whenever some patches are completely lost and/or partly reduced in size between years, then the connectivity within the metapopulation should normally decrease, but occasional slight increases are possible in the cases when only very isolated patches (thus of little value for the connectivity of other patches, but at the same time negatively affecting the mean value) are lost. Similarly, the emergence of new habitat patches in a metapopulation system should normally lead to increased connectivity, although exceptions from this general rule are possible.

Wing morphometry assessment

Landmark oversampling for centroid size and shape analysis was tested by performing the Landmark Sampling Evaluation Curve (LaSEC) with 99 iterations using the lasec() function (Watanabe 2017). This performs an ordinary Procrustes alignment to superimpose the distribution of specimens in the subsampled data to that of the full dataset and calculate the degree of congruence between subsampled and full data sets by performing a Procrustes Sum of Squares (PSS) as a measure of fit. The minimum number of landmarks to reach a fit $\geq$ 0.95 were 11 for the centroid size and 24 for shape, so oversampling was not considered.

To obtain shape variables from landmark data, a Generalized Procrustes Analysis (GPA) was performed using the gpagen() function (Baken *et al.* 2021; Adams *et al.* 2023). Then, directional asymmetry was tested for shape and centroid size differences between the left and right wings using the bilat.symmetry() function (Baken

et al. 2021; Adams *et al.* 2023). No differences were found, allowing us to use either the left wing, or if absent, the right one for the statistical analysis (Supporting information 2: Table S1 and 2).

The outliers were selected and removed using the plotOutliers() function in which the individuals are ordered by their Procrustes distances from the mean shape (Baken *et al.* 2021; Adams *et al.* 2023). All specimens above the upper quartile were considered outliers and consequently removed from the dataset (n = 2). Additionally, to account for the impact of uneven sample size for wing shape morphological disparity analysis a different data subset was created for this specific test. The number of individuals of each group was randomly constrained by the group with the smallest sample size (females: n = 34, males: n = 30). Because of this limitation, just the three main metapopulations with a big enough sample size (PL1990, PL2019 and NL2020) were taken into account for this analysis.

Genetic structure of the metapopulations

Genomic DNA was extracted by homogenizing the wing fragment in a solution of 100 µl 5% chelex and 1 µl of proteinase K. The samples were firstly incubated at 56°C for 3 hours, and secondly at 95°C for 15 min., then centrifuged at 13000 rpm for 10 min. Then, 40µl of supernatant was stored at -20 °C. Four sets of multiplex reactions were used with the forward primers labeled. The PCRs were performed in a total volume of 15 µl composed of 1 µl of DNA template, Multiplex PCR Master Mix (Qiagen), water and primers. For PCR amplification, a thermal cycler (Applied Biosystems) was used with the following PCR profile: 95 °C for 15 min., then 35 cycles of: 94 °C for 30 s, 52 °C or 56 °C or 58 °C (depending on the multiplex) for 90 s, 72 °C for 90 s, with the final elongation of 72 °C for 30 min.

References

Adams, D.C., Collyer, M.L., Kaliontzopoulou, A. & Baken, E.K. (2023). Geomorph: Software for geometric morphometric analyses. R package version 4.0.6.

Baken, E.K., Collyer, M.L., Kaliontzopoulou, A. & Adams, D.C. (2021). Geomorph v4.0 and gmShiny: Enhanced analytics and a new graphical interface for a comprehensive morphometric experience. *Methods in Ecology and Evolution*, 12, 2355–2363.

Nowicki, P., Pepkowska, A., Kudlek, J., Skórka, P., Witek, M., Settele, J., *et al.* (2007). From metapopulation theory to conservation recommendations: Lessons from spatial occurrence and abundance patterns of Maculinea butterflies. *Biological Conservation*, 140, 119–129.

Nowicki, P., Vrabec, V., Binzenhöfer, B., Feil, J., Zakšek, B., Hovestadt, T., *et al.* (2014). Butterfly dispersal in inhospitable matrix: Rare, risky, but long-distance. *Landscape Ecology*, 29, 401–412.

Watanabe, A. (2017). LaMBDA: LandMark-based data assessment. R package version 0.1.1.0000.

Supporting information 2

Table S1. Bilateral asymmetry ANOVA test results for *P. teleius* female and male hindwing centroid size analysis.

		Df	SS	MS	Rsq	F	Z	p[a]
Female	Individual	147	2,127.84	14.47	0.98	56.78	10.57	0.001***
	Side	1	0.06	0.06	0.00	0.23	-0.24	0.604
Male	Individual	160	1,778.95	11.12	0.98	64.49	17.55	0.001***
	Side	1	0.04	0.04	0.00	0.22	-0.35	0.631

[a] *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

Table S2. Bilateral asymmetry ANOVA test results for *P. teleius* female and male hindwing shape analysis.

		Df	SS	MS	Rsq	F	Z	p[a]
Female	Individual	147	0.31	0.00	0.72	2.66	11.72	0.001***
	Side	1	0.00	0.00	0.00	1.14	0.50	0.305
Male	Individual	160	0.30	0.00	0.69	2.25	14.47	0.001***
	Side	1	0.00	0.00	0.00	1.45	1.11	0.149

[a] *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

Manuscript 3

Changes in the wing spot pattern of the endangered butterfly *Phengaris teleius* thirty years after its reintroduction.

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Abstract

The spot pattern of butterfly wings plays an important role in species and mate recognition, predation avoidance and thermoregulation. We studied the hindwing spot pattern of 267 individuals of *Phengaris teleius*, reintroduced from Poland to the Netherlands in 1990. Our samples included butterflies collected during the reintroduction in the source population and 30 years later in the Netherlands and Poland. The presence, size, fluctuating asymmetry and fusion of the spots, and the overall spot pattern shape and variability were analyzed. We found differences among metapopulations for all tested variables, however when considering variables at spot level the significant differences were only found for two out of eleven spots. The presence of those two spots was highly variable among metapopulations. Additionally, two spots were sometimes merged, and this fusion was found in a lower proportion for the two current metapopulations compared to the source one. While the spot size did not show any clear pattern, the significant increase of the overall spot size for the current Polish metapopulation was interpreted as a change in wing melanization and explained as an adaptation to climatic conditions. Moreover, a higher proportion of asymmetrical individuals and a lower spot pattern variability were observed in the reintroduced metapopulation. The results revealed differences among metapopulations, possibly explained by differences in various factors like avoiding predation, mating strategy or caterpillar development. Additionally, the spot pattern variability and FA differences could be a direct consequence of the loss of genetic variability due to the reintroduction.

Keywords: Lycaenidae, Maculinea, melanization, translocation

Introduction

The research of wing pattern and wing morphology in butterflies and its ecological and evolutionary significance has a long tradition (Parchem *et al.* 2007). The Lycaenidae family is one of the most species-rich groups of butterflies (Robbins 1982), but the significance of wing color and wing pattern is much less studied than in other butterfly families. Color and spot pattern of wings can have important functions like predation avoidance (Wourms & Wasserman 1985), mate recognition (Rutowski & Rajyaguru 2013) and thermoregulation (Taylor-Cox *et al.* 2020). It has been demonstrated that in Lycaenidae, hindwing

spots can play an important role as species and partner recognition signals (Fordyce *et al.* 2002). Studying the wing spot pattern can also provide important knowledge about a population status through comparing fluctuating asymmetry (FA) and trait variability (i.e., wing spot pattern variability) among various populations. Thus, FA and general trait variability can be sensitive to environmental and genetic stress (Parsons 1992). In *Phengaris arion* (Lycaenidae), variation in the wing melanization level and in the number of black spots on wings have been found among different populations both in Finland as well as in Poland (Väisänen *et al.* 1994; Sielezniew & Dziekańska 2011), probably due to different climatic conditions.

Phengaris butterflies are obligate social parasites of *Myrmica* ants and they require two different resources for the larval development; namely, specific food-plant species and *Myrmica* ant hosts (Thomas 1980). Such complex adaptations pertaining to closely interacting species make them much more vulnerable to environmental changes. Nowadays, many butterfly populations are declining (Swaay *et al.* 2011) and myrmecophilous butterflies including *Phengaris* spp. are those that suffer greatly (Settele & Kühn 2009). In response to environmental changes and local population extinctions, actions including the translocation or reintroduction of threatened species toward new or former sites are performed. *Phengaris* butterflies, despite their complicated life cycle, were successfully reintroduced in two cases: *P. arion* from Sweden to the United Kingdom (Thomas *et al.* 2009) and *P. teleius* from Poland to the Netherlands in 1990 (Wynhoff 1998). The latter reintroduction was performed by introducing 86 butterflies from the metapopulation system from the Kraków region (Wynhoff 1998). Nowadays, the difference of thirty butterfly generations since the reintroduction gives the opportunity to study possible changes and adaptations that could have occurred. As the reintroduction process is connected with changes of many habitat parameters and very often leads to the decrease of genetic variation in reintroduced populations, studying its potential effect on the butterfly wing pattern could be enlightening. The aim of our study was to test whether and how the number of black spots, their size, symmetry and the shape and variability of the spot pattern have changed between the source and reintroduced metapopulation of *P. teleius* after thirty years since the reintroduction. As we also possess the wings of butterflies from the Polish source metapopulation from 1990 (the year of reintroduction), we also analyzed how the wing spot patterns diverged through time between the source original metapopulation and the current reintroduced and source ones.

Material and methods

Study site of the source metapopulation

The studied *Phengaris* (=*Maculinea*) *teleius* butterfly metapopulation occurs in the outskirts of Kraków city in South Poland (50°01'N, 19°54'E). The landscape of this valley is composed mostly of abandoned or rarely managed grasslands, arable fields, forests, and settlements (Kajzer-Bonk *et al.* 2016). The habitats of the focal butterfly species are a part of a large meadow complex with an area exceeding 200 ha and consisting of several dozens of nutrient-poor to mesotrophic meadows with varying densities of *Sanguisorba officinalis*, the only foodplant of caterpillars of *P. teleius*. The three investigated meadow patches, where butterflies were collected, are

characterized by relatively large areas (2.4, 6.2 and 33.3 ha, respectively). Currently, the whole meadow complex faces increasing urbanization pressure, due to growing human settlement and infrastructure (Kajzer-Bonk & Nowicki 2022).

Study site of the reintroduced metapopulation

The nature reserve Moerputten (115 ha) is located south of the city of 's-Hertogenbosch (The Netherlands) and covers the central part of the Natura 2000 nature reserve "Vlijmens Ven, Moerputten en Bossche Broek" (931 ha; 51°41'N, 5°15'E). On the outer borders of Moerputten nature reserve, partially within the forest, different types of grasslands are found, of which the hay meadows with a high abundance of *S. officinalis* are the most important habitat of *P. teleius*. The historical metapopulation of *P. teleius* was extinct in 1976, then *P. teleius* specimens were reintroduced in 1990 on the moist meadows (Wynhoff 1998), at that time the only suitable site of this butterfly species in the Netherlands. The reintroduced metapopulation consisted of 33 males and 53 females of *P. teleius*. Nowadays, *P. teleius* is restricted to two core populations on the meadows at the southern border of the core reserve and two to three small populations on other meadows within the nature reserve.

Experimental design and sampling

Data were collected from two metapopulations (Polish and Dutch) and from temporally different moments: in 1990 (the year of reintroduction) from the source metapopulation from Poland (=PL1990), in 2019 from the current metapopulation from Poland (=PL2019) and in 2020 from the reintroduced metapopulation in the Netherlands (=NL2020). Wings of individuals from PL1990 used for the analysis were coming from butterflies, which did not survive the trip from Poland to the Netherlands for the reintroduction. They were dried and preserved (n = 63) in an entomological box. Wings from these individuals were digitally photographed and used for morphological and spot pattern analysis (see below).

Butterflies were collected in 2019 in three closely located meadows in the Kraków region, which are the areas where in 1990 *P. teleius* adults were collected for reintroduction. In 2020, butterflies were also collected on three closely located meadows in Moerputten nature reserve. Butterflies were captured with entomological nets. After capture, each butterfly was put into a small jar and treated with carbon dioxide for ten seconds to anesthetize it. Then the butterfly was laid on millimetric graph paper and photographs were taken from the left and right side. The hindwings were photographed using a Nikon D7200 camera and a Laowa 100 mm macro lens. Finally, the butterflies were marked with fine-tipped waterproof Stabilo pen on the ventral part of the right forewing to prevent re-sampling of the same individual. All butterflies were released at the place of capture when they were fully awake again.

Geometric morphometric approach

A total of 267 butterflies (PL1990 n = 63, PL2019 n = 112, NL2020 n = 92) were used for studying the wing spot pattern morphology of the hindwings. The spot pattern refers to the motif created by the combination of the different spots found on the wing. Eleven landmarks were digitized in every picture with the software tpsDig v.2.32 (Bookstein 1997; Rohlf 2018). We considered as landmarks the points that could be precisely identified (i.e. spot location; Fig. 1). The landmarks were used to estimate both wing spot pattern shape and centroid size, as the square

root of the sum of squared distances of all the coordinates, being the most appropriate measure for overall size (Bookstein 1997). Additionally, thirty-one landsmarks were digitized for calculating the wing centroid size to use in further analysis to normalize the data.

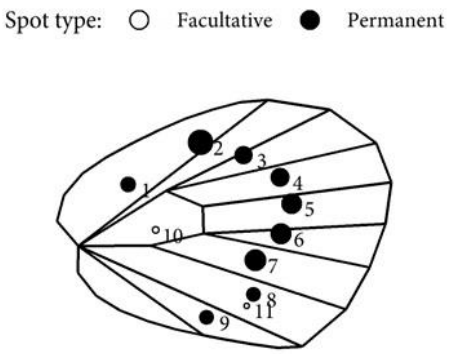

Fig. 1. Spot pattern landmark locations in the general consensus hindwing of *P. teleius*. Spot size represents their average area and the numbers indicate the spot identification code.

Landmark oversampling for centroid size and shape analysis was tested by performing the Landmark Sampling Evaluation Curve (LaSEC) with 100 iterations by using the lasec() function (Watanabe 2017). This performs an ordinary Procrustes alignment to superimpose the distribution of specimens in the subsampled data to that of the full dataset and calculate the degree of congruence between subsampled and full data sets by performing a Procrustes Sum of Squares (PSS) as a measure of fit. The minimum number of landmarks to reach a fit ≥ 0.95 were 6 for the centroid size and 9 for shape, so oversampling was not considered.

The raw data were subsetted and only the data from the left wing were used in the analysis to avoid bias based on directional asymmetry between left and right wings. To obtain shape variables from landmark data, a Generalized Procrustes Analysis (GPA) was performed using the gpagen() function (Baken *et al.* 2021; Adams *et al.* 2023). The outliers were selected and removed by using the plotOutliers() function in which the individuals are ordered by their Procrustes distances from the mean shape (Baken *et al.* 2021; Adams *et al.* 2023). All specimens above the upper quartile were considered outliers and consequently removed from the dataset (n = 1).

Computer assisted spot detection

We developed a program in Python (3.9) to measure black spots area and the exact center coordinates from the hindwing images. The image management and treatment were based on the opencv library (Bradski 2000). All images were first rotated and resized so that the scale and orientation were the same for all pictures. Images were converted to grayscale and blurred to avoid that scales on butterfly wings bias the circularity values. The lightning (average value) of the images were corrected so that each butterfly wing would have the same average level of brightness. Black spots were identified from these images using an adaptive thresholding by applying the adaptiveThreshold() function from the opencv package (Bradski 2000). Those spots were also filtered by size to keep only spots whose size was matching the expected ones. Finally, we associated these automatically detected spots with the manually recorded spot positions using the Hungarian algorithm provided by the linear_sum_assignment() function from the scipy package (Virtanen *et al.* 2020). We also determined whether spots were detected in the wing areas in which facultative points were expected (i.e. spot number 10 and 11).

A Graphical User Interface was also programmed to allow manual corrections of the errors made by the automatic detection program. At this stage, users could redraw the detected spot's areas, remove or add facultative spots, or reassign them. All images were verified and corrected if necessary. After this correction, the area and centroid (arithmetic mean) of each of the spots were calculated. Area was calculated in pixels and then converted to international units (mm^2).

All python scripts are available to download from a GitHub public repository: (https://github.com/VioletteChiara/Wing_spots)

Morphometric statistical analysis

The spot pattern centroid size was tested for correlation with the wing centroid size by applying the cor.test() function (R Core Team 2023) to test if the spot pattern size can be used as a good estimator of wing size in further analysis. After testing for centroid size correlation the data frame was subsetted and all the analyses were separately performed for females and males.

The differences in the hindwing spot pattern shape among metapopulations and allometry were tested by using the ProcD.lm() function (Baken *et al.* 2021; Adams *et al.* 2023). It performs a Procrustes ANOVA with permutation for describing patterns of shape variation and covariation for a set of Procrustes shape variables. The model was built using as predictor variables the logarithm of the spot pattern centroid size and metapopulation. Spot pattern centroid size was used as an estimator of wing size because of its high correlation with wing centroid size, as reported in the results (Supplementary Material, Fig. S1). A pairwise comparison was also performed between metapopulations by applying an estimated marginal means (EMMs) test by using the function pairwise() (Collyer & Adams 2018; Collyer & Adams 2023). The effect of allometry was removed from the pairwise comparison by using shape ~ log(centroid size) as the null model.

Spot presence and area/centroid size ratio were analyzed with a generalized linear model with binomial distribution using the metapopulation and spot location as predictor variables (for instance, presence ~ metapopulation * spot location) by using the glm() function (R Core Team 2023). The total surface of the melanized black area was calculated as the sum of the area of all spots and divided by the spot pattern centroid size to normalize. It was analyzed with a generalized linear model with binomial distribution as the ratio between the total melanized area/centroid size, using the metapopulation as a predictor variable by using the glm() function (R Core Team 2023). The spot fusion (between the spots number 8 and 11) were also analyzed with a generalized linear model with binomial distribution using the metapopulation as a predictor variable (for instance, fusion ~ metapopulation * spot) by using the glm() function (R Core Team 2023).

Fluctuating asymmetry was tested for spot presence. Both hindwings (left and right) were considered for this analysis. A new binomial variable indicating asymmetry of each of the spots was created with 0 value for asymmetry (when in one of the wings from the same individual the spot was present and in the another one was absent) and 1 value for symmetry (when in both wings the spot was present or absent). Spot fluctuating asymmetry was analyzed with a generalized linear model with binomial distribution using the metapopulation and spot location as predictor variables (for instance,

asymmetry ~ metapopulation * spot) by using the glm() function (R Core Team 2023).

The wing spot pattern distance between individuals based on spot presence, area and fusion (between the 8 and 11 spot) was calculated by using the vegdist() function (Oksanen *et al.* 2022) with the Bray-Curtis dissimilarity index. A Permutational analysis of variance (PERMANOVA) was applied to assess the significance of the metapopulation by the adonis2() function (Oksanen *et al.* 2022). Intra-metapopulation distances were compared to assess the spot pattern metapopulation variability. The distances were fitted to a generalized linear model with Gaussian distribution using the metapopulation as a predictor variable (for instance, distance ~ metapopulation) by using the glm() function (R Core Team 2023).

Variable significance for all generalized linear models was tested with ANOVA with the Anova() function (Fox *et al.* 2023) and the different metapopulations were pairwise-compared by performing estimated marginal means (EMMs) tests by using the emmeans() function (Lenth 2023).

Results

Morphometric analyses

Correlation between wing and spot pattern centroid size

Spot pattern centroid size and wing centroid size showed a significantly strong correlation ($r^2 = 0.94$, $P < 0.001$; Fig. S1), proving that both can be used as an estimator of wing size.

Wing spot pattern shape

The analysis of the wing spot pattern shape showed differences among the individuals from the source (PL1990), current Polish (PL2019) and reintroduced Dutch (NL2020) metapopulations for both females and males (Procrustes ANOVA, females: d.f. = 2, Z = 3.574, p = 0.001; males: d.f. = 2, Z = 3.535, p = 0.001; Fig. 2). Part of the differences in the spot pattern shape is explained by the change in centroid size, showing an allometric effect (Procrustes ANOVA, females: d.f. = 1, Z = 2.738, p = 0.003; males: d.f. = 1, Z = 2.796, p = 0.002; Fig. S2). All spot pattern shape pairwise comparisons show statistically significant differences (Table S1).

Spot pattern analysis

The proportion of spot presence in female wings differed when considering different metapopulations and varied among spots (metapopulation: d.f. = 2, χ^2 = 7.09, p = 0.029; spot identity: d.f. = 10, χ^2 = 259.32, p < 0.001; Fig. 3a). However, the interaction between metapopulation and spot identity did not show any significant effect on the proportion of spot presence (d.f. = 20, χ^2 = 26.33, p = 0.155). In the case of males, the proportion of spot presence only differed when considering different spots (spot identity: d.f. = 10, χ^2 = 224.2, p < 0.001; Fig. 3b). The butterfly metapopulation and the interaction between metapopulation and spot identity did not show any significant effect on the proportion of spot presence (metapopulation: d.f. = 2, χ^2 = 0.1, p = 0.95; interaction: d.f. = 20, χ^2 = 18.78, p = 0.536).

We considered the two highly variable spots number 10 and 11 as facultative spots. The spot number 10 did not show a very clear trend in terms of presence among the different metapopulations, but the spot number 11 is significantly more present in the Dutch metapopulation (NL2020) compared to the current Polish metapopulation (PL2019) for both sexes.

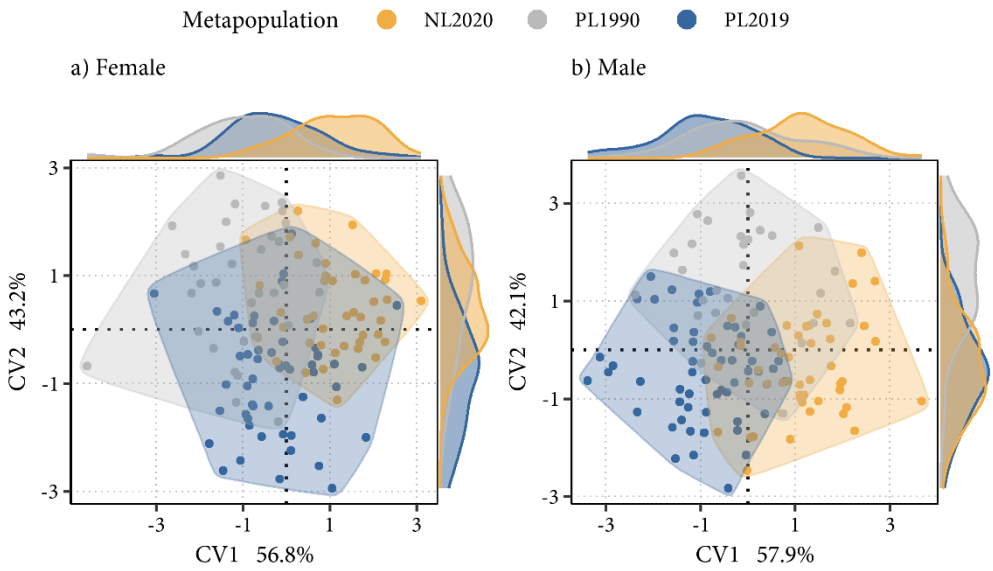

Fig. 2. Procrustes CVA shape representation of *P. teleius* wing spot pattern from the Polish source (PL1990), current Polish (PL2019) and reintroduced Dutch (NL2020) metapopulations. The axis densigrams represent the distribution of each canonical variate.

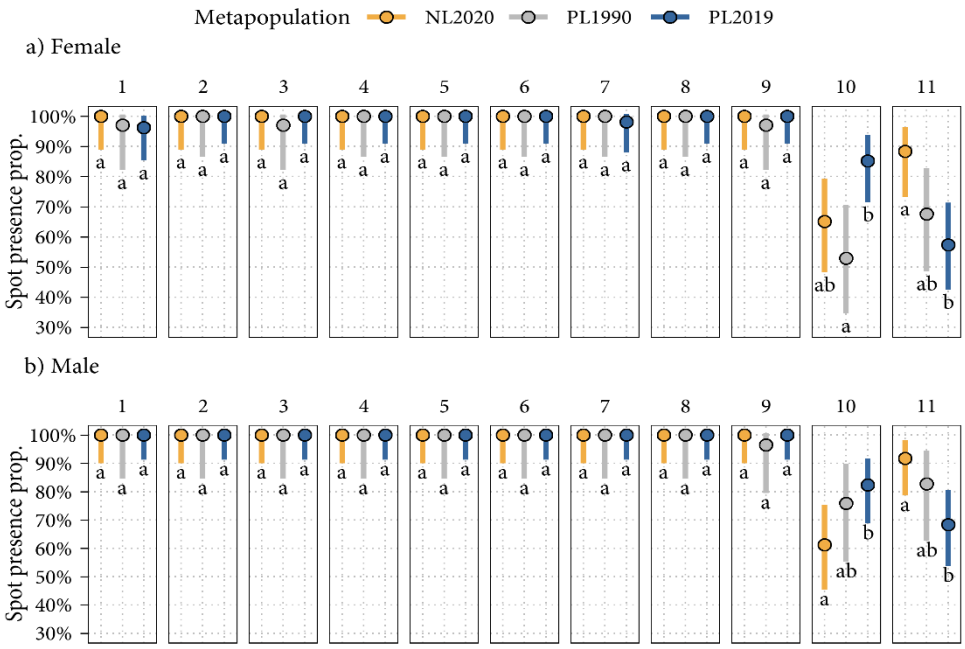

Fig. 3. Proportion of spot presence in *P. teleius* a) females and b) males from the source (PL1990), current Polish (PL2019) and reintroduced Dutch (NL2020) metapopulations. Boxes represent the results for the different wing spots. Different letters below dots indicate statistically significant differences between groups.

The spot area/centroid size ratio was significantly affected by the butterfly metapopulation and the different spots (females, metapopulation: d.f. = 2, χ^2 = 28.42, p < 0.001; spot identity: d.f. = 10, χ^2 = 830.23, p < 0.001; males, metapopulation: d.f. = 2, χ^2 = 34.15, p < 0.001; spot identity: d.f. = 10, χ^2 = 997.65, p < 0.001; Fig. S3). However, the interaction between both variables did not show any significant effect (females, interaction: d.f. = 20, χ^2 = 17.73, p = 0.605; males, interaction: d.f. = 20, χ^2 = 23.2, p = 0.279).

The total melanized spot black area/centroid size ratio was also significantly influenced by the metapopulation of the butterflies both for females and males (females: d.f. = 2, χ^2 = 6.99, p = 0.03; males: d.f. = 2, χ^2 = 8.15, p = 0.017). Polish butterflies from the current metapopulation (PL2019) presented a higher relative surface occupied by the spots with respect to the source metapopulation (PL1990), but this difference was only significant for females (Fig. 4). In any case, the current Polish butterflies always presented a higher mean value of melanized surface.

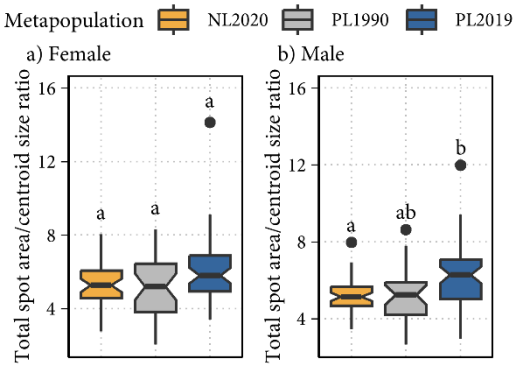

Fig. 4. Total wing spot area/centroid size ratio comparison of *P. teleius* a) females and b) males from the source (PL1990), current Polish (PL2019) and reintroduced Dutch (NL2020) metapopulations. Different letters at the top of the boxplots indicate statistically significant differences between groups.

The proportion of females with symmetrical spots presented differences based on the different spots and the interaction between butterfly metapopulation and spot identity (spot identity: d.f. = 10, χ^2 = 310.47, p < 0.001; interaction: d.f. = 20, χ^2 = 40.12, p = 0.005; Fig. 5a). However, the butterfly metapopulation did not show any significant effect on the proportion of symmetrical females (d.f. = 2, χ^2 = 5.22, p = 0.073). In the case of males, the proportion of symmetrical individuals was influenced by the butterfly metapopulation and the different spots (metapopulation: d.f. = 2, χ^2 = 13.68, p = 0.001; spot identity: d.f. = 10, χ^2 = 363.87, p < 0.001; Fig. 5b). However, the interaction between metapopulation and spot identity did not show any significant effect on the proportion of symmetrical males (d.f. = 20, χ^2 = 21.38, p = 0.375). Only the spot number 10 showed a clear trend with a significantly higher proportion of asymmetrical individuals in the reintroduced Dutch metapopulation.

The proportion of spot fusion (between spot number 8 and 11) in females did not differ among metapopulations (metapopulation: d.f. = 2, χ^2 = 5.22, p = 0.073; Fig. 6a). However, the proportion of spot fusion in males was significantly influenced by butterfly metapopulation (metapopulation: d.f. = 2, χ^2 = 8.18, p = 0.017; Fig. 6b). Spot fusion was found in a significantly higher proportion in males from the Polish source metapopulation (PL1990) compared to males from the current Polish metapopulation (PL2019). A similar but not statistically significant pattern was found for females.

The PERMANOVA analysis showed a significant effect of the butterfly metapopulation on the wing spot pattern for both sexes (female, d.f. = 2; F = 5.57; p = 0.001; male, d.f. = 2; F = 5.15; p = 0.002; Fig. S4). Additionally, the metapopulation also showed a

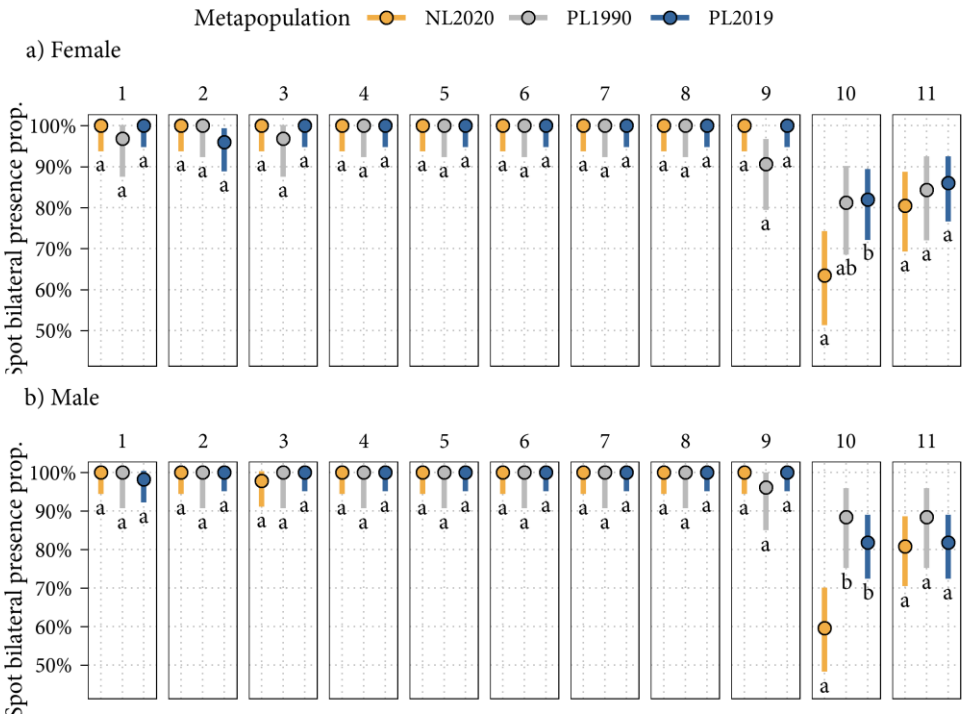

Fig. 5. Proportion of symmetrical *P. teleius* a) female and b) male spots from the source (PL1990), current Polish (PL2019) and reintroduced Dutch (NL2020) metapopulations. Boxes represent the results for the different wing spots. Different letters in the bottom of the dots indicate statistically significant differences between groups.

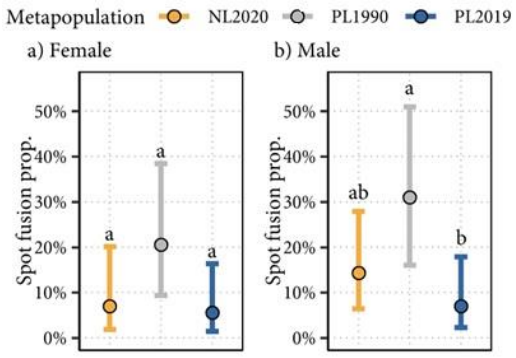

Fig. 6. Proportion of wing spot fusion (spot number 8 and 11) of *P. teleius* a) female and b) male butterflies from the source (PL1990), current Polish (PL2019) and reintroduced Dutch (NL2020) metapopulations. Different letters in the top of the dots indicate statistically significant differences between groups.

significant effect on the spot pattern intra-metapopulation variability (female, d.f. = 2; χ^2 = 303.54; p < 0.001; male, d.f. = 2; χ^2 = 178.08; p < 0.001; Fig. 7). The butterflies from the reintroduced Dutch metapopulation (NL2020) showed a significantly lower spot pattern intra-metapopulation variability for both sexes (Fig. 7). In the case of females, the highest variability was found among individuals coming from the source metapopulation from 1990 (PL1990).

Discussion

Our results revealed that the wing spot pattern in *P. teleius* butterflies can change along the time in a specific metapopulation, but it can also be affected by a reintroduction process and the consequent environ-

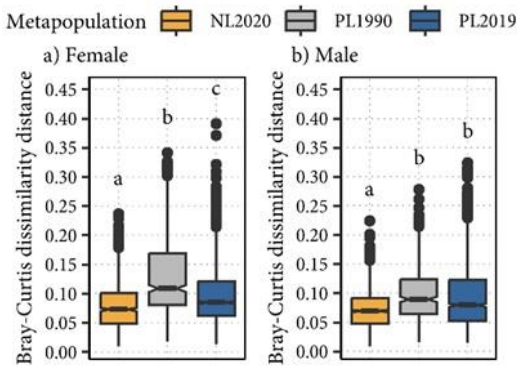

Fig. 7. Bray-Curtis dissimilarity distance of the wing spot pattern for a) females and b) males between the source (PL1990), current Polish (PL2019) and reintroduced Dutch (NL2020) metapopulation. Letters indicate statistically significant differences between boxplots.

mental changes. The differences we found in the spot pattern shape can be associated with wing shape changes. Other studies have already reported differences in wing shape between metapopulations of the same butterfly species (Mikitová *et al.* 2022). Factors like predation risk, mimicry, mating strategy and larval diet quality could indirectly be responsible for the observed changes in the spot pattern shape through direct changes in the wing shape (see in Le Roy *et al.* 2019). Furthermore, we observed slightly different results in the strength of the allometric effect in the spot pattern shape of females and males. This variation may be attributed to the distinct impact of natural selection on each gender, while males presented a specific wing shape according to the environmental needs, females were more constrained and strongly influenced by size, as expounded by DeVries *et al.* (2010).

The differences found in the presence of particular spots on wings between different populations of the same butterfly species have been already found in other studies on Lycaenidae. It has been reported that none of the hindwing spots were constant in Polish populations of *Phengaris arion* (Sielezniew & Dziekańska 2011) and wing spots were less numerous in southern populations compared to northern ones. Another study of the same butterfly species in Finland demonstrated that western populations of *P. arion* present fewer and smaller spots on wings (Väisänen *et al.* 1994). Our study showed that while most spots remain present in the wings of most individuals, regardless of the metapopulation they come from, some other spots (number 10 and 11) have a very high variation in their presence, thus they could be considered as facultative spots. The spot number 10 did not show a very clear trend among the studied metapopulations, but the spot number 11 shows a significantly lower presence in the wings of butterflies from the current Polish metapopulation compared to butterflies from the reintroduced Dutch metapopulation. This clear difference could be the effect of the genetic differentiation between these two metapopulations, already found in a parallel study (author's unpublished results). It is known that parent spot size is correlated with offspring spot presence in some species of butterflies, suggesting that the inheritance of the spot pattern could be described as a dosage model with different thresholds below which each spot is absent (Brakefield & Noordwijk 1985). We found the same trend in both females and males, however it has been observed that expression for particular spots is sex-dependent in some species of butterflies (i.e. hindwing spots in *Maniola jurtina* (Brakefield & Noordwijk 1985); or forewing spots in *Pieris rapae* (Stoehr *et al.* 2016)). In fact, the female wing spot pattern can work as an effective mate-recognition signal between individuals of different species, being hindwing spots one of the most

important traits in the recognition (Fordyce *et al.* 2002). Additionally, we also found evidence that spot size is influenced by metapopulation, but there was no clear common trend among spots. The spot size is directly determined at gene level (Holloway *et al.* 1993), but different spots from the same wing can be under different selective pressures (Obara & Rutowski 2023). There have been reported short-term changes in wing spot size during a 23 years period in *Papilio polytes* (Katoh *et al.* 2017). Nevertheless, the total spot black area indicates a clearer increase in melanization in butterflies from the current Polish metapopulation in comparison to the butterflies from 1990 of the same metapopulation and to the reintroduced Dutch metapopulation. The current Polish butterflies just present statistically significant differences in comparison with the source metapopulation for females and the Dutch reintroduced metapopulation for males, however, the higher mean value for the current Polish butterflies could be interpreted as a biologically relevant increase in melanization in any of the cases. Melanization contributes to absorbing solar radiation rapidly and it is suspected to be an adaptation to cooler environments (Dennis & Shreeve 1989). Butterfly wings tend to be darker moving northwards. However, this cannot be extrapolated and specific traits of the species can be crucial to determine if melanization plays an important role in the adaptations to cooler conditions (Nylin 2009). Blue lycenids as *P. teleius* are generally considered dorsal reflectance baskers and an exception for latitudinal increase in melanization from Britain (Dennis & Shreeve 1989). During dorsal reflectance basking, the overall area of wings acts as sunlight reflectors directing the radiation to the thorax to warm the muscles (Dennis 1993). It has been proposed by Nylin (2009) that dorsal reflectance baskers might benefit from an augmentation of iridescence in the central region of their wings to enhance body warming. Consequently, darker individuals have rather reduced abilities for dorsal reflectance basking and thermoregulation. It could be interpreted as an adaptation to global warming. Additionally, in *Colias* butterflies, it was demonstrated that an increase of melanization in female wings results in a notably higher egg maturation rate, potentially enhancing reproductive success (Ellers & Boggs 2004). This may hold particular significance for *Phengaris* butterflies, given their short-lived adult stage (Nowicki *et al.* 2005), in which females likely face even greater selection pressures than males.

The wing spot pattern can also offer potential to study FA in relation to the environmental conditions. It has been proved that the eyespot symmetry can be sensitive to stress (Brakefield 1997). Moreover, spot pattern FA do not generally show indication of heritable variation, however mutant individuals with additional spots seem to be more heritably influenced by exhibiting more non-directional asymmetry (Brakefield & Breuker 1996). In the case of our study, we presume the more probably explanation for the significantly higher degree of FA detected in the spot number 10 for the reintroduced Dutch metapopulation could be the increase of the stress level during the caterpillar development due to different environmental conditions and the loss of genetic variability due to the bottleneck that the reintroduced metapopulation suffered during the reintroduction (author's unpublished results). Furthermore, there is little known about the importance of the wing spot pattern in *P. teleius* in avoiding predation, but we might expect that a

different degree of predation could also affect FA values. Predators may select for symmetry in visual warnings, due to asymmetric signals being more difficult to remember (Forsman & Merilaita 1999). It is suggested that the eyespots under strong visual selection from predators may show reduced FA (Brakefield & Breuker 1996). On the other hand, predation can be also avoided from attracting the predator to attack non-vital body parts. The "false head" hypothesis states that the attack of a visual predator focuses on the hindwing anal angle of the butterflies presenting distinct markings, which decide the predator into attacking a "false head" (Robbins 1980). We found a lower proportion of individuals presenting fusion between the spot number 8 and 11 for the current Polish and Dutch metapopulations. We did not get a statistically significant difference in some of the pairwise comparisons, but the more than 10% lower proportion in the spot fusion found in both current metapopulations compared to the source metapopulation from 1990 could be biologically relevant. It could indicate a temporal change in biotic factor as predation risk. When both spots are present and not fusioned they could work as a "false head", attracting the attention of the predators and offering the butterflies a higher chance to escape. It has been empirically proven that the wings spots are the main signals influencing the bird point attack on butterflies (Wourms & Wasserman 1985) and several experiments found evidence that individuals with "false head" in their hindwings have a higher probability to escape from different kind of predators (Wourms & Wasserman 1985; Sourakov 2013).

Our results revealed the highest spot pattern variability in the source Polish metapopulation from 1990 and the lowest one in the Dutch reintroduced metapopulation. Such results can be attributed to the loss of genetic variability. The Dutch metapopulation suffered a bottleneck and a consequent reduction of genetic richness due to the reintroduction, while the current Polish metapopulation could be affected by the reduction of the metapopulation size (author's unpublished data).

To conclude, changes in the wing spot presence, size, FA, pattern shape and variability have been observed both in the current Polish and Dutch reintroduced metapopulations respecting the original source Polish metapopulation from 1990. Most of those changes could be probably explained by different factors such as predation risk, mimicry, mating strategy, global warming and caterpillar developmental conditions. The loss of the spot pattern metapopulation variability can be explained by the direct effect of the reintroduction and the loss of genetic variability. Moreover, the differences in FA could be also related to the reduction of genetic variability (Parsons 1992). However, FA as an indicator of population conservation status should be carefully taken into account as changes in FA could also be a response to differences in environmental conditions (Windig *et al.* 2000). For instance, neither significant rise in asymmetry nor decline of morphological variability were detected after several bottlenecks in a population of *Parnasius apollo* (Habel *et al.* 2012).

CRediT authorship contribution statement

Daniel Sánchez-García: Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization. Luca Pietro Casacci: Conceptualization, Methodology, Investigation, Writing - Review & Editing. Irma Wynhoff: Conceptualization,

Methodology, Investigation, Resources, Writing - Review & Editing. Violette Chiara: Methodology, Software, Writing - Review & Editing. Magdalena Witek: Conceptualization, Methodology, Investigation, Writing - Original Draft, Supervision, Project administration, Funding acquisition.

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References

Adams, D.C., Collyer, M.L., Kaliontzopoulou, A. & Baken, E.K. (2023). Geomorph: Software for geometric morphometric analyses. R package version 4.0.6.

Baken, E.K., Collyer, M.L., Kaliontzopoulou, A. & Adams, D.C. (2021). Geomorph v4.0 and gmShiny: Enhanced analytics and a new graphical interface for a comprehensive morphometric experience. *Methods in Ecology and Evolution*, 12, 2355–2363.

Bookstein, F.L. (1997). Landmark methods for forms without landmarks: localizing group differences in outline shape. *Proceedings of the Workship on Mathematical Methods in Biomedical Image Analysis*, 1, 225–243.

Bradski, G. (2000). The OpenCV Library. *Dr. Dobb's Journal of Software Tools.*

Brakefield, P.M. (1997). Phenotypic plasticity and fluctuating asymmetry as responses to environmental stress in the butterfly *Bicyclus anynana*. *Environmental Stress, Adaptation and Evolution*, 65–78.

Brakefield, P.M. & Breuker, C.J. (1996). The genetical basis of fluctuating asymmetry for developmentally integrated traits in a butterfly eyespot pattern. *Proceedings of the Royal Society B: Biological Sciences*, 263, 1557–1563.

Brakefield, P.M. & Noordwijk, A.J. van. (1985). The genetics of spot pattern characters in the meadow brown butterfly *Maniola jurtina* (Lepidoptera: Satyrinae). *Heredity*, 54, 275–284.

Collyer, M. & Adams, D. (2023). *RRPP: Linear model evaluation with randomized residuals in a permutation procedure*.

Collyer, M.L. & Adams, D.C. (2018). RRPP: An r package for fitting linear models to high-dimensional data using residual randomization. *Methods in Ecology and Evolution*, 9, 1772–1779.

Dennis, R.L.H. (1993). *Butterflies and climate change*. Manchester University Press, Manchester.

Dennis, R.L.H. & Shreeve, T.G. (1989). Butterfly wing morphology variation in the British Isles: the influence of climate, behavioural posture and the hostplant-habitat. *Biological Journal of the Linnean Society*, 38, 323–348.

DeVries, P.J., Penz, C.M. & Hill, R.I. (2010). Vertical distribution, flight behaviour and evolution of wing morphology in Morpho butterflies. *Journal of Animal Ecology*, 79, 1077–1085.

Ellers, J. & Boggs, C.L. (2004). Functional ecological implications of intraspecific differences in wing melanization in *Colias* butterflies. *Biological Journal of the Linnean Society*, 82, 79–87.

Fordyce, J.A., Nice, C.C., Forister, M.L. & Shapiro, A.M. (2002). The significance of wing pattern diversity in the Lycaenidae: Mate discrimination

by two recently diverged species. *Journal of Evolutionary Biology*, 15, 871–879.

Forsman, A. & Merilaita, S. (1999). Fearful symmetry: Pattern size and asymmetry affects aposematic signal efficacy. *Evolutionary Ecology*, 13, 131–140.

Fox, J., Weisberg, S. & Price, B. (2023). *Car: Companion to applied regression*.

Habel, J.C., Reuter, M., Drees, C. & Pfaender, J. (2012). Does isolation affect phenotypic variability and fluctuating asymmetry in the endangered Red *Apollo*? *Journal of Insect Conservation*, 16, 571–579.

Holloway, G.J., Brakefield, P.M. & Kofman, S. (1993). The genetics of wing pattern elements in the polyphenic butterfly, *Bicyclus anynana*. *Heredity*, 70, 179–186.

Kajzer-Bonk, J. & Nowicki, P. (2022). Butterflies in trouble: The effectiveness of Natura 2000 network in preventing habitat loss and population declines of endangered species in urban area. *Ecological Indicators*, 135, 108518.

Kajzer-Bonk, J., Skorka, P., Nowicki, P., Bonk, M., Krol, W., Szpilyk, D., *et al.* (2016). Relative contribution of matrix structure, patch resources and management to the local densities of two large blue butterfly species. *PLoS ONE*, 11, 1–19.

Katoh, M., Tatsuta, H. & Tsuji, K. (2017). Rapid evolution of a Batesian mimicry trait in a butterfly responding to arrival of a new model. *Scientific Reports*, 7, 1–7.

Le Roy, C., Debat, V. & Llaurens, V. (2019). Adaptive evolution of butterfly wing shape: from morphology to behaviour. *Biological Reviews*, 94, 1261–1281.

Lenth, R.V. (2023). *Emmeans: Estimated marginal means, aka least-squares means*.

Mikitová, B., Šemeláková, M. & Panigaj, Ľ. (2022). Wing morphology and eyespot pattern of Erebia medusa (Lepidoptera, Nymphalidae) vary along an elevation gradient in the Carpathian Mountains. *Nota Lepidopterologica*, 45, 233–250.

Nowicki, P., Witek, M., Skórka, P., Settele, J. & Woyciechowski, M. (2005). Population ecology of the endangered butterflies *Maculinea teleius* and M. nausithous and the implications for conservation. *Population Ecology*, 47, 193–202.

Nylin, S. (2009). Gradients in butterfly biology. In: *Ecology of butterflies in europe* (eds. Settele, J., Shreeve, T., Konvička, M. & Van Dyck, H.). Cambridge University Press, Cambridge, UK, pp. 198–216.

Obara, Y. & Rutowski, R. (2023). Contrasting Size Distributions Among the Wing Spots of a Pierid Butterfly Suggest Different Selective Histories. *Zoological Science*, 40, 219–223.

Oksanen, J., Simpson, G.L., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., *et al.* (2022). *Vegan: Community ecology package*.

Parchem, R.J., Perry, M.W. & Patel, N.H. (2007). Patterns on the insect wing. *Current Opinion in Genetics and Development*, 17, 300–308.

Parsons, P.A. (1992). Fluctuating asymmetry: A biological monitor of environmental and genomic stress. *Heredity*, 68, 361–364.

R Core Team. (2023). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria.

Robbins, R.K. (1980). The Lycaenid "False Head" hypothesis: historical review and quantitative analysis. *Journal of the Lepidopterists' Society*, 34, 194–208.

Robbins, R.K. (1982). How many butterflies species. *News of the Lepidopterists' Society*, 40–41.

Rohlf, J.F. (2018). tpsDig version 2.32.

Rutowski, R.L. & Rajyaguru, P.K. (2013). Male-specific Iridescent Coloration in the Pipevine Swallowtail (*Battus philenor*) is Used in Mate Choice by Females but not Sexual Discrimination by Males. *Journal of Insect Behavior*, 26, 200–211.

Settele, J. & Kühn, E. (2009). Understanding of ecosystem interactions and management has led to a major advance in the conservation of specialized insects. *Science*, 325, 41–42.

Sielezniew, M. & Dziekańska, I. (2011). Geographical variation in wing pattern in *Phengaris*

(=*Maculinea*) *arion* (L.) (Lepidoptera: Lycaenidae): Subspecific differentiation or clinal adaptation? *Annales Zoologici*, 61, 739–750.

Sourakov, A. (2013). Two heads are better than one: False head allows *Calycopis cecrops* (Lycaenidae) to escape predation by a Jumping Spider, *Phidippus pulcherrimus* (Salticidae). *Journal of Natural History*, 47, 1047–1054.

Stoehr, A.M., Hayes, K. & Wojan, E.M. (2016). Assessing the Role of Wing Spots in Intraspecific Communication in the Cabbage White Butterfly (*Pieris rapae* L.) Using a Simple Device to Increase Butterfly Responses. *Journal of Insect Behavior*, 29, 243–255.

Swaay, C. van, Maes, D., Collins, S., Munguira, M.L., Šašić, M., Settele, J., *et al.* (2011). Applying IUCN criteria to invertebrates: How red is the Red List of European butterflies? *Biological Conservation*, 144, 470–478.

Taylor-Cox, E.D., Macgregor, C.J., Corthine, A., Hill, J.K., Hodgson, J.A. & Saccheri, I.J. (2020). Wing morphological responses to latitude and colonisation in a range expanding butterfly. *PeerJ*, 8.

Thomas, J. (1980). Why did the large blue become extinct in britain? *Oryx*, 15, 243–247.

Thomas, J.A., Simcox, D.J. & Clarke, R.T. (2009). Successful conservation of a threatened *Maculinea* butterfly. *Science*, 325, 80–83.

Väisänen, R., Heliövaara, K. & Somerma, P. (1994). Wing variation of *Maculinea arion* (Linnaeus) in Finland (Lepidoptera, Lycaenidae). *Entomologica Fennica*, 5, 139–146.

Virtanen, P., Gommers, R., Oliphant, T.E., Haberland, M., Reddy, T., Cournapeau, D., *et al.* (2020). SciPy 1.0: fundamental algorithms for scientific computing in Python. *Nature Methods*, 17, 261–272.

Watanabe, A. (2017). LaMBDA: LandMark-based data assessment. R package version 0.1.1.0000.

Windig, J.J., Rintamäki, P.T., Cassel, A. & Nylin, S. (2000). How useful is fluctuating asymmetry in conservation biology: Asymmetry in rare and abundant Coenonympha butterflies. *Journal of Insect Conservation*, 4, 253–261.

Wourms, M.K. & Wasserman, F.E. (1985). Butterfly wing markings are more advantageous during handling than during the initial strike of an avian predator. *Evolution*, 39, 845–851.

Wynhoff, I. (1998). Lessons from the reintroduction of *Maculinea teleius* and *M. nausithous* in the Netherlands. *Journal of Insect Conservation*, 2, 47–57.

Supplementary material

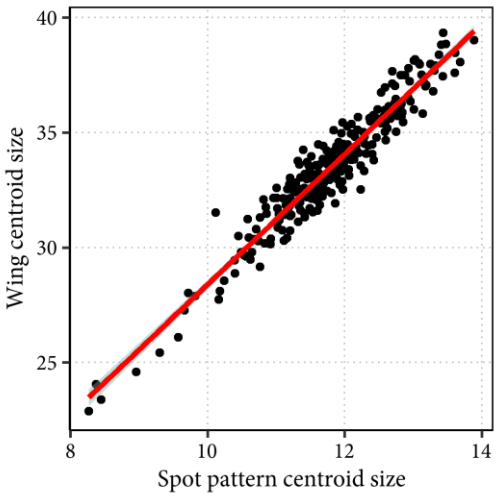

Fig. S1. Correlation plot of spot pattern centroid size and wing centroid size of the hindwings.

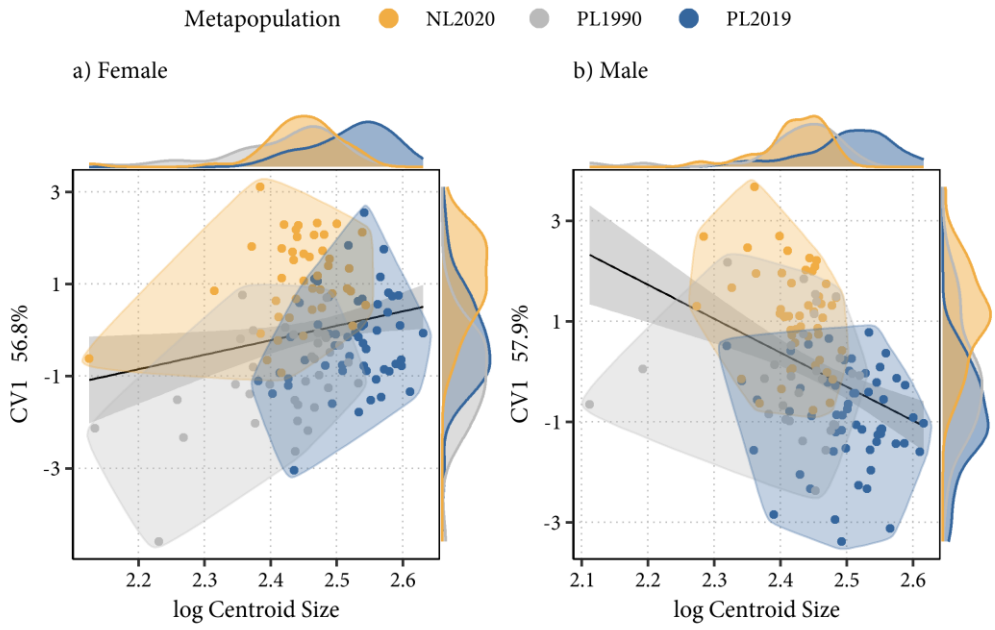

Fig. S2. *P. teleius* a) female and b) male butterflies wings spot pattern shape allometry from the Polish and Dutch metapopulations in different years: PL1990 (grey: year of the reintroduction), PL2019 (blue: original polish metapopulation) and NL2020 (yellow: reintroduced Dutch metapopulation).

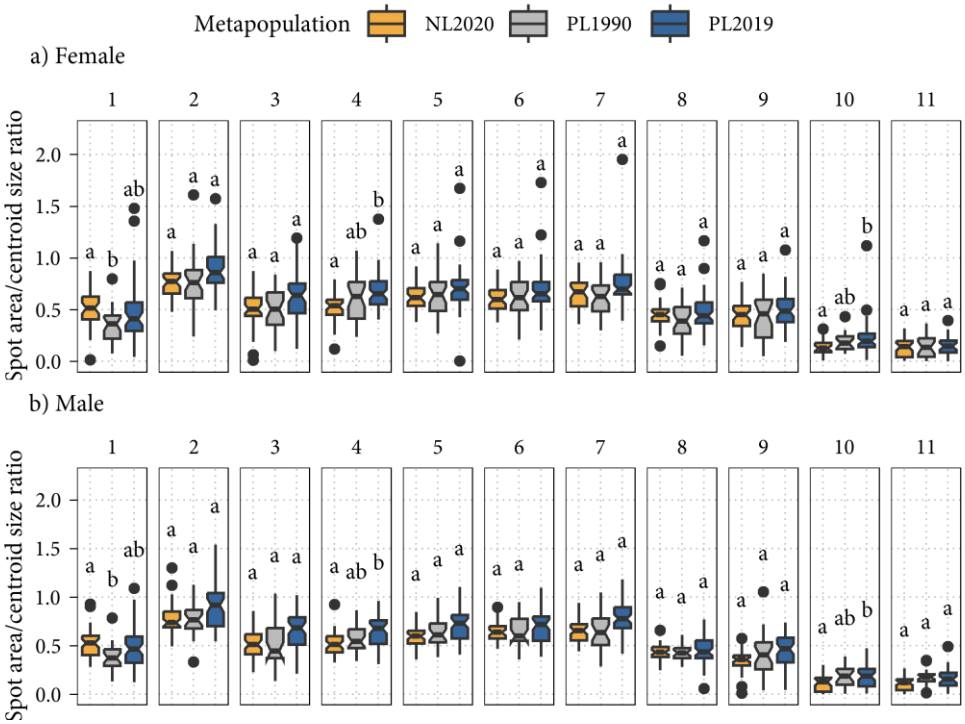

Fig. S3. Wing spot area/centroid size ratio comparison of *P. teleius* a) female and b) male from the source (PL1990) and current Polish (PL2019) and reintroduced Dutch (NL2020) metapopulations. Boxes represent the results for the different wing spots. Different letters in the top of the boxplots indicate statistically significant differences between groups.

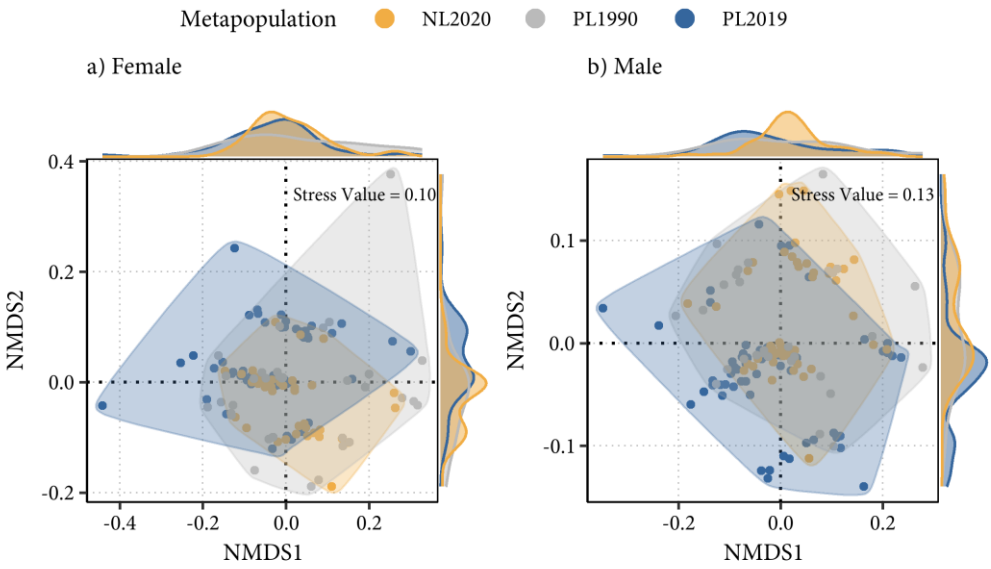

Fig. S4. Non-metric multidimensional scaling (NMDS) representation of *P. teleius* a) female and b) male butterfly wing spot pattern coming from the Polish source (PL1990), current Polish (PL2019) and Dutch (NL2020) metapopulations. The axis densigrams represent the distribution of each NMDS component.

Table S1. LSMs (Least Square Means) test results for the female and male wing spot pattern shape pairwise comparison between the source (PL1990), current Polish (PL2019) and reintroduced Dutch (NL2020) metapopulation.

		PL1990	PL2019
Females	NL2020	Z = 2.94 P = 0.002**	Z = 3.14 p < 0.001***
	PL1990	-	Z = 1.39 p = 0.083
Males	NL2020	Z = 2.25 P = 0.015*	Z = 3.49 p < 0.001***
	PL1990	-	Z = 1.93 p = 0.028*

*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001