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

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Highlights

- The morphology of the reintroduced butterflies is more stable than the non-translocated ones.
- The habitat connectivity decreased in the source and increased in the reintroduced population.
- The reintroduction reduced the genetic richness of the translocated population.

- The genetic structure of the reintroduced population changed over 30 generations.

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 morphological and genetic changes that occurred after 30 generations since the reintroduction. We also assessed the climatic and 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 climatic conditions and 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 have the potential to adapt to new habitats and respond to climatic 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.

Previous Next

Keywords

Dispersal; Geometric morphometry; Microsatellite; Bottleneck; Metapopulation; *Maculinea*

1. Introduction

Reintroductions are used in conservation biology as a tool to restore species loss after local extinction (Seddon et al., 2014). 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 offer the potential to study phenotypic plasticity and/or adaptations following reintroductions, providing an opportunity to understand whether and how organisms can cope 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 and 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 and Hall, 1980, Deredec and 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 (Andrews, 2015, Thomas et al., 2009) and *P. teleius* in the Netherlands from a Polish population (Wynhoff, 1998). *Phengaris* butterflies 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 and 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).

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), the current Polish and the reintroduced current Dutch metapopulations of *P. teleius* by investigating (i) population genetics, and (ii) morphology of adult butterflies. We also assessed climatic changes and metapopulation connectivity in our study systems over time to investigate their impact on changes observed in both metapopulations. 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, climate, 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 climatic conditions and landscape connectivity may be important factors leading to selection pressure on morphological traits and dispersal (Bonte et al., 2012).

2. Material and methods

2.1. 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 (50º01’N, 19º54’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.

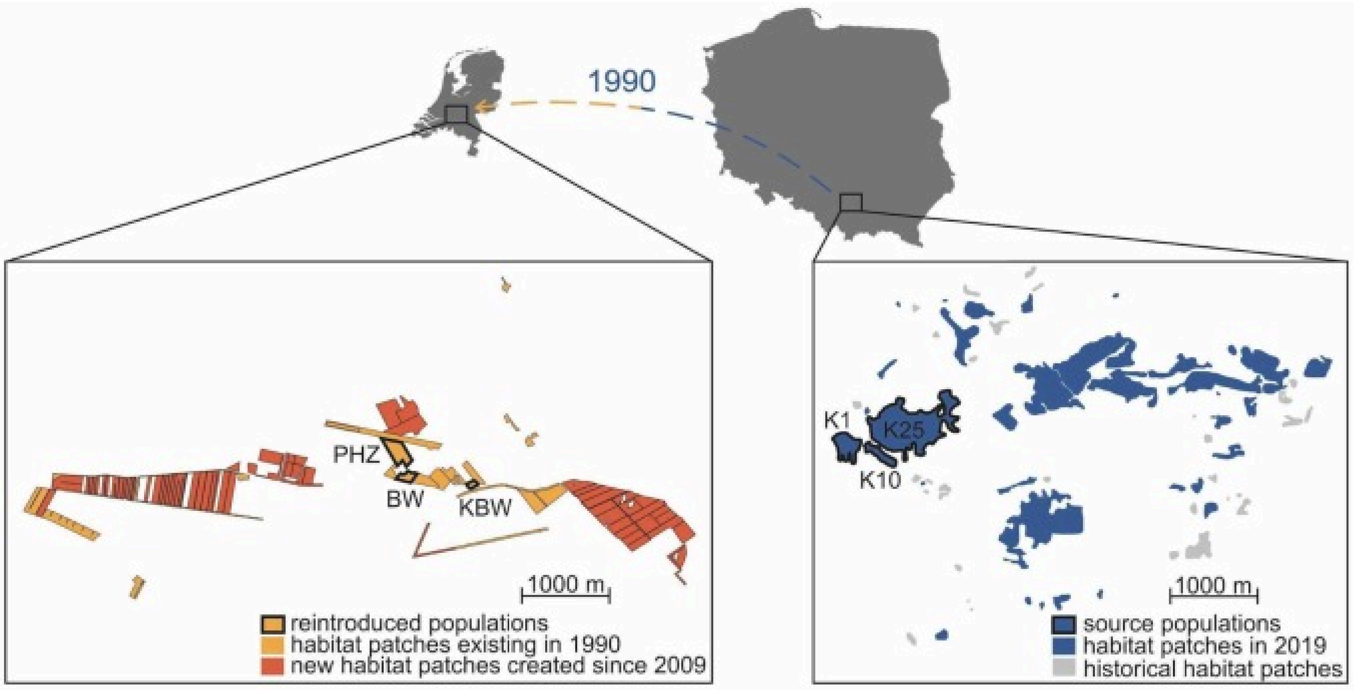

Fig. 1. Sites of the two studied metapopulations of *Phengaris teleius*. Habitat patches of the Dutch reintroduced metapopulation (on the left) and of the Polish source metapopulation (on the right) are shown. Habitat patches are considered to be sites where the butterfly food plant is present, mapped using GPS devices with 1-m accuracy (Nowicki et al., 2007). The blue patches are the ones existing in Poland in 2019, the gray patches are ones recently lost in Poland (data gathered from Kajzer-Bonk and Nowicki, 2023), 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 (data gathered from Sevilleja et al., 2022). 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. The maps were visualized in QGIS v. 3.4.15-Madeira software and then compiled in CorelDRAW v. 22.1.1.523.

2.2. 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 (Sevilleja et al., 2022, Wynhoff et al., 2017).

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).

2.3. 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 randomly collected in the field and obtained from the personal collection of PN and IW. The flow chart in Fig. 2 outlines the crucial steps of the study and specifies the times at which data were collected for the two populations (for additional information, refer to Table A.1).

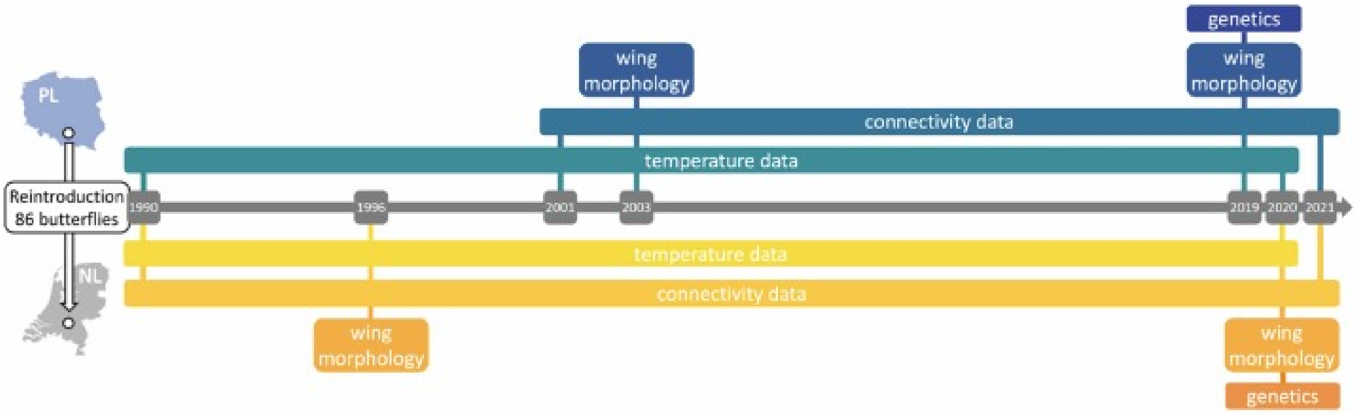

Fig. 2. Flow chart summarizing the data collected at the various time points for both metapopulations.

During the fieldwork in 2019 and 2020, 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 forewing 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.

2.4. 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. A.1). 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 A.1.

2.5. Temperature change over time

Temperature data were obtained from CRU-TS 4.06 (Harris et al., 2020) downscaled with WorldClim 2.1 (Fick and Hijmans, 2017) at a spatial resolution of 2.5 minutes (~21 km^2 at the equator). The mean temperature was calculated as the average of maximum and minimum temperatures per each month and year. Specifically, we considered the mean temperatures from March to August, as this period encompasses critical stages in the butterfly life cycle. From March to June caterpillars experience rapid development and undergo pupation inside *Myrmica* nests (Witek et al., 2011), whereas July-August represents the time when adult butterflies are active.

2.6. 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 A.1.

2.7. 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 PL2019 and n = 134 for 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 A.1. 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 (Andersen et al., 2014, Ugelvig et al., 2012, Ugelvig et al., 2011, Zeisset et al., 2005). 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).

2.8. Morphometric statistical analysis

Butterfly weight, thorax width were examined in PL2019 and NL2020, while hindwing size was assessed in both metapopulations at all study times 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 PL2019 and NL2020 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 impact of temperature on wing size was also assessed. Hindwing centroid size values for females and males were fitted to a linear model with mean temperature and metapopulation as predictors (i.e., centroid size ~ temperature * metapopulation) by using the lm() function (R Core Team, 2022). The butterfly data from 1990 were considered as part of both metapopulation systems. Linear regression slopes for each metapopulation and sex were obtained from the emtrends() function (Lenth, 2023) and statistically compared by using the pairs() function.

The differences in the wing shape among metapopulations and allometry were tested by using the ProcD.lm() function (Adams et al., 2023, Baken et al., 2021), 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 and Adams, 2023, Collyer and Adams, 2018). 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 (Adams et al., 2023, Baken et al., 2021) 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 PL1990, PL2019 and NL2020 due to the size equality required for the test and the small sample sizes available for the rest of the metapopulations (see Methods A.1).

2.9. 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, 1000 iterations per batch), with Bonferroni correction, implemented in GENEPOP on the Web version 4.7 (Raymond and 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, 2001, Goudet, 1995). 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 regarding the above mentioned parameters between PL2019 and NL2020 were assessed by a two-sided permutation test with 1000 permutations in FSTAT (using the option: “Comparison among group of samples”). 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 and Smouse, 2012). To infer about a possible number of genetic clusters a Bayesian clustering approach implemented in Structure 2.3.4 (Falush et al., 2003, Hubisz et al., 2009, Pritchard et al., 2000) was used with the ancestry admixture model with correlated frequencies, with 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 and VonHoldt, 2012). The effective population size was assessed for each metapopulation in LDNE (Waples and 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 the infinite alleles model (IAM) and 70 % of the stepwise mutation model (SM), as an intermediate solution fitting to the reality best (see also discussion in Piry et al., 1999).

2.10. 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.

3. Results

3.1. 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, $\chi^2 = 6.85$, p = 0.009; males: d.f. = 1, $\chi^2 = 2.9$, p = 0.089; Fig. 3a). The thorax width was statistically bigger in PL2019 than in NL2020 in both sexes (GLM, females: d.f. = 1, $\chi^2 = 7.23$, p = 0.007; males: d.f. = 1, $\chi^2 = 20.38$, $p < 0.001$; Fig. 3b).

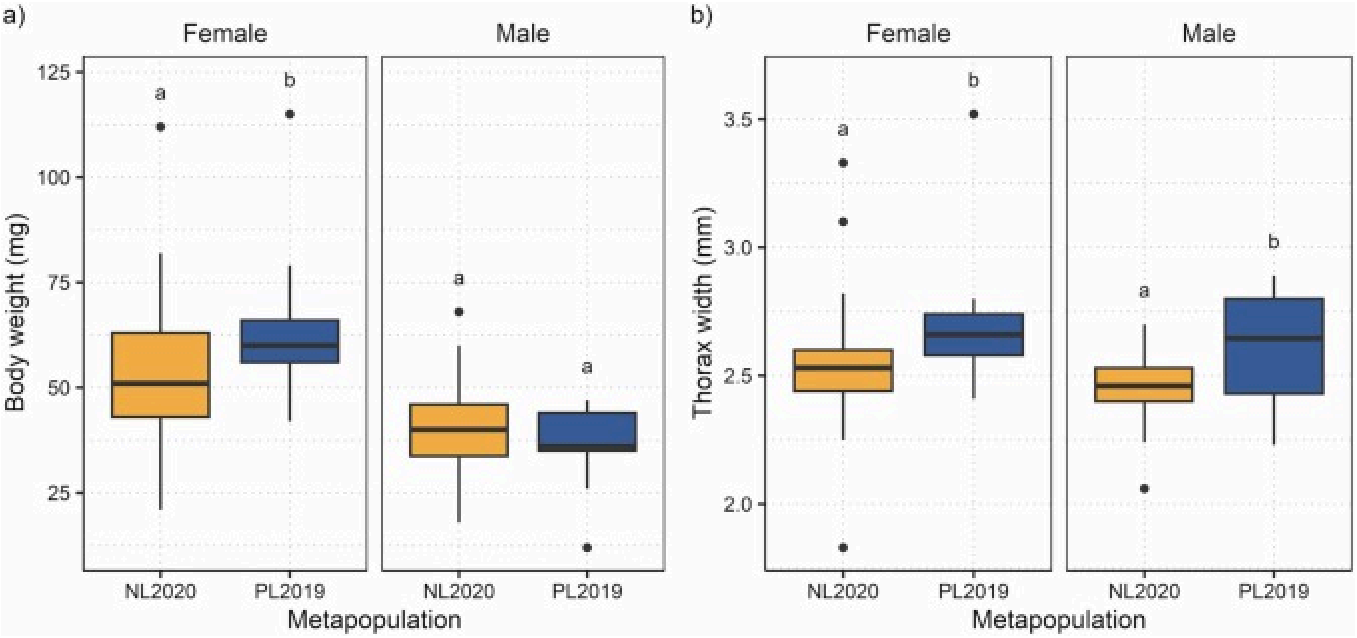

Fig. 3. *P. teleius* body weight (a) and thorax width (b) in PL2019 (blue) and NL2020 (yellow) metapopulations for females and males. The boxes depict the interquartile range, and horizontal black lines indicate median values. Vertical black lines extend from the minimum to the maximum non-outlier values and black dots indicate the outliers. Different letters at the top of the boxplots indicate statistically significant differences between groups.

3.2. 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 = 89.43, $p < 0.001$; males: d.f. = 4, χ^2 = 75.17, $p < 0.001$). 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. 4a and b; Table A.2).

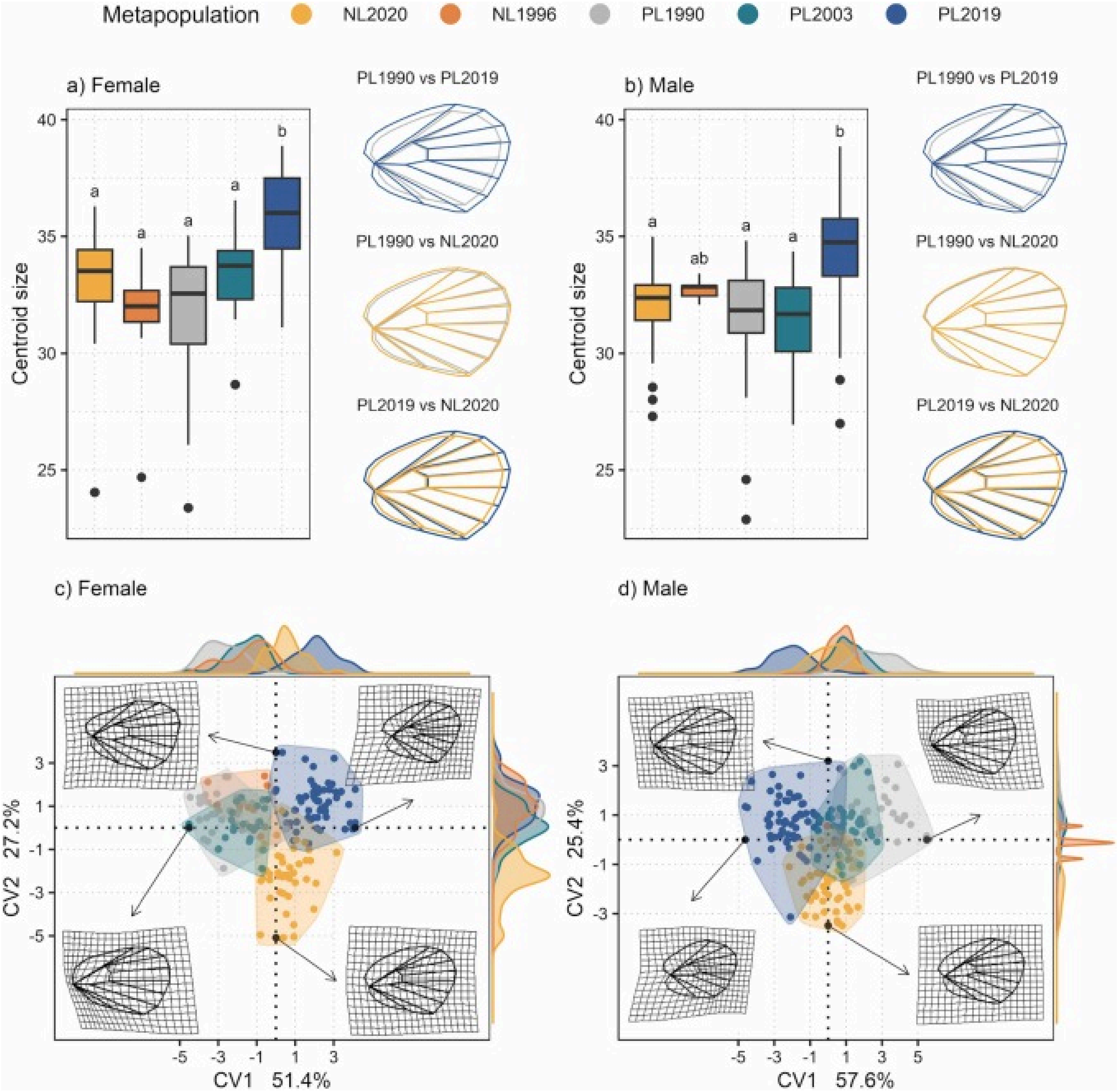

Fig. 4. Hindwing morphological comparison of *P. teleius* from PL1990 (grey), PL2019 (blue), PL2003 (marine blue), NL2020 (yellow) and NL1996 (orange) metapopulations. Size comparison for females (a) and males (b). The boxes depict the interquartile range, and horizontal black lines indicate median values. 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 A.5). 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.

The ratio between centroid size and body weight did not show any difference between PL2019 and NL2020 for both sexes (GLM, females: d.f. = 1, χ^2 = 2.19, p = 0.139; males: d.f. = 1, χ^2 = 1.96, p = 0.162; Fig. A.2a). Likewise, the ratio between centroid size and thorax width also did not differ between PL2019 and NL2020 (GLM, females: d.f. = 1, χ^2 = 0.08, p = 0.777; males: d.f. = 1, χ^2 = 0.12, p = 0.728; Fig. A.2b).

3.3. Hindwing shape analysis

Both females and males showed differences in hindwing shape among metapopulations (Procrustes ANOVA, females: d.f. = 4, Z = 6.542, p = 0.001; males: d.f. = 4, Z = 5.938, p = 0.001; Fig. 4c 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 = 3.889, p = 0.001; males: d.f. = 1, Z = 3.379, p = 0.001; Fig. A.3). PL2019 and NL2020 showed statistically significant differences in shape when compared with the rest of the metapopulations apart from NL1996 for both females and males (Table A.3). 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 A.4).

3.4. Temperature change and its relation to hindwing size

An increase in mean temperature was detected at both metapopulation sites from 1990 to 2020. Interestingly, the mean temperature in the Netherlands showed lower values compared to Poland for the same period (Fig. 5a). The current temperature values in the Netherlands are equal to those observed in Poland in 2000. Our analysis revealed a significant effect of mean temperature on hindwing size for both females (d.f. = 1, F = 51.84, $p < 0.001$; Fig. 5b) and males (d.f. = 1, F = 16.65, $p < 0.001$). Our data indicate that hindwing centroid size has increased over time in the Polish metapopulation following mean temperature increase, while in the reintroduced metapopulation, mean temperature had minimal or no effect on centroid size for both females (slope NL = 0.72, slope PL = 2.65, d.f. = 195, T = −4.32, $p < 0.001$) and males (slope NL = −0.04, slope PL = 1.94, d.f. = 213, T = −4.03, $p < 0.001$).

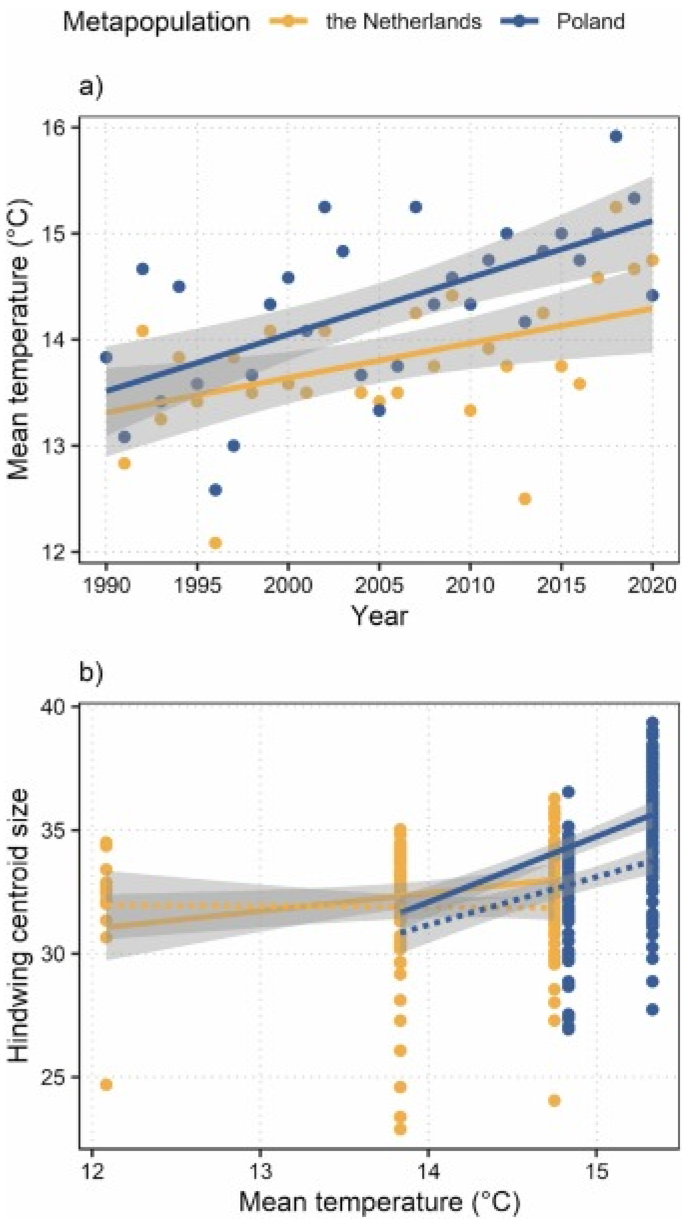

Fig. 5. Mean temperature conditions in the site of the studied metapopulations from Poland and the Netherlands from 1990 to 2020. The temperature values for each year are calculated as the mean value for the period from March to August. (a) Mean temperature per year; (b) effect of mean temperature on *P. teleius* hindwing centroid size. Solid lines represent females and dotted lines represent males.

3.5. 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 and 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 (Sevilleja et al., 2022, Wynhoff et al., 2017). 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. A.4.

3.6. 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 PL2019 and NL2020 regarding allelic richness, observed heterozygosity H_o, expected heterozygosity H_e, inbreeding coefficient F_{IS} and genetic diversity F_{ST} (Table A.6).

However, the difference in allelic richness (AR) was nearly significant ($p = 0.06$), and PL2019 and NL2020 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 PL2019 were not found in NL2020. 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. 6a (for instance loci Macari16 with 21 alleles in Poland and 7 in Netherlands).

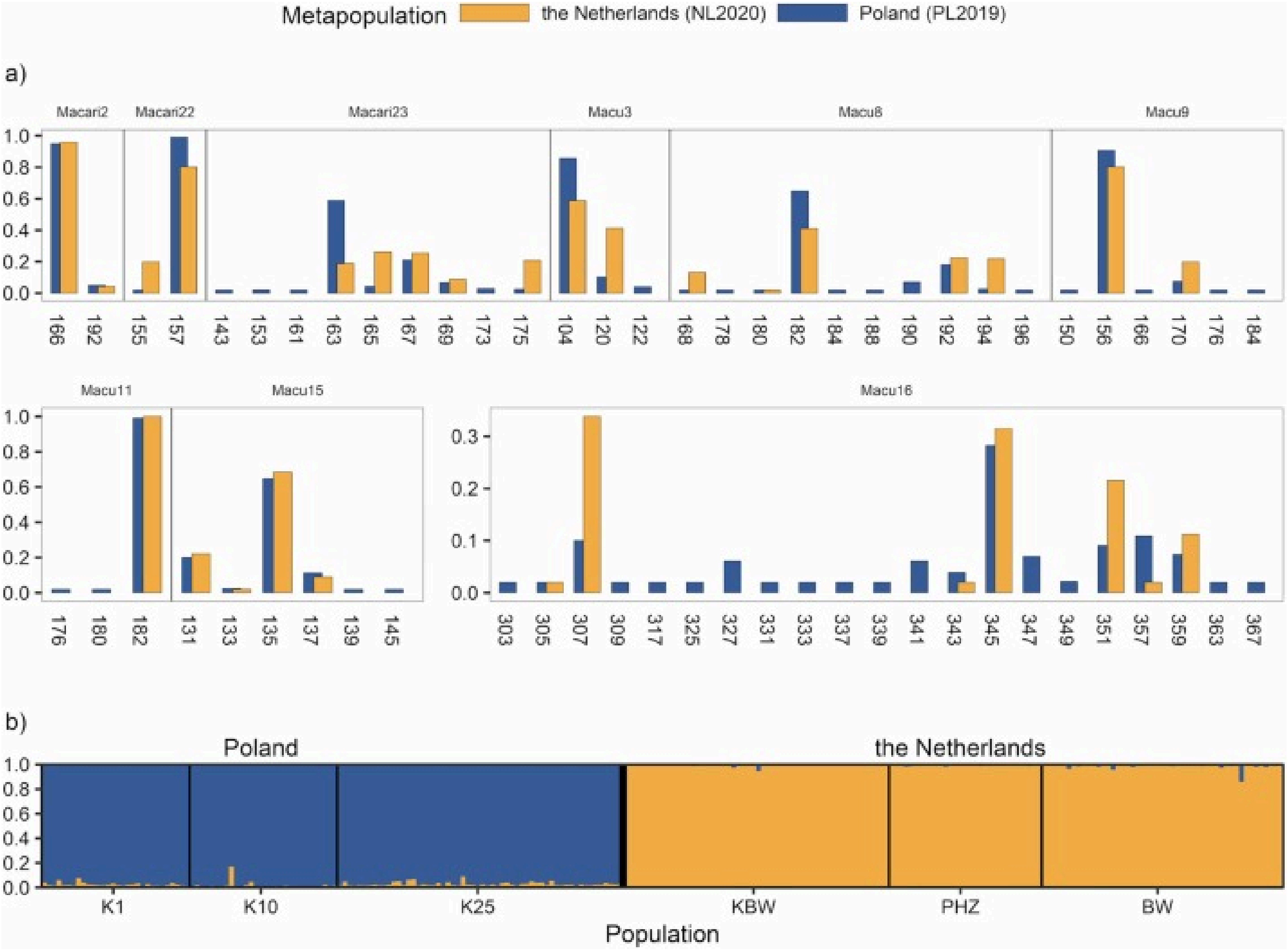

Fig. 6. Analysis of the studied nine microsatellite markers for PL2019 and NL2020 *P. teleius* metapopulations: (a) Microsatellite allele frequencies. Microsatellite names are given at the top of each box. Blue (Polish) and yellow (Dutch) bars indicate the frequency of each allele. Allele frequencies 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 chosen using the Evanno method (Evanno et al., 2005) and the average of 10 iterations. Names of sampling sites are given above the membership diagram.

Genetic distances F_{ST} between all populations were low to moderate (from 0.003 to 0.126; Table A.7), but significantly different except for the F_{ST} between the population K1 and K10. All genetic distances were low within PL2019 and NL2020 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 PL2019 and NL2020 with all individuals showing levels of admixture lower than 10 % (Fig. 6b).

No signs of population size reduction (bottleneck) was found in PL2019, but it was found in NL2020 (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 PL2019 and 167 (Confidence interval: 69–4301) for NL2020.

4. 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 spatial structure of habitats and climatological conditions. Since the translocated 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.

4.1. Habitat connectivity and differences in morphological traits among the studied metapopulations

Our results revealed that butterflies of the two current metapopulations (PL2019 and NL2020) 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 metapopulation (PL1990-PL2019) 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 may 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 and 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 (Sevilleja et al., 2022, Wynhoff et al., 2017), 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 and Nowicki, 2022). Such environmental changes have influenced the connectivity among *P. teleius* habitat patches that slightly decreased in the Polish metapopulation from 0.39 to 0.3 and strongly increased in the Dutch one from 0.99 to 2.75 (Fig. 1). Some empirical studies and theoretical models demonstrated that habitat fragmentation can lead to lower butterfly dispersal propensity (e.g., Heino and 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 and Van Dyck, 2006). Also, the theoretical model by Heino and 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. Such a situation appears to be occurring in the Polish metapopulation of *P. teleius*. It is important to notice that in the Polish metapopulation not only habitat patch number decreased but habitat quality also negatively 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, Kajzer-Bonk et al., 2016b). 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 could be possible that in the current Polish metapopulation, despite the increase of the habitat fragmentation, individuals are affected by other selective pressures, primarily the deterioration of patch quality, and, in turn, the reduction of the carrying capacity, which could have favored individuals with higher dispersal abilities. As pointed out by Thomas et al. (2001) variation in the habitat quality is an important parameter in metapopulation dynamics beside habitat connectivity and patch areas.

Moreover, climatic conditions could potentially be among the factors contributing to differences in morphological changes between reintroduced and source metapopulations. While mean spring and summer temperatures have risen in both sites, these changes are more pronounced in Poland. Currently, mean temperatures in the Netherlands have reached levels comparable to those observed in the Polish site in the early 2000s. The results of studies investigating the effect of temperature on body and wing size in butterflies yield conflicting findings, with some indicating negative effects (e.g., Büyükyilmaz and Tseng, 2022), and others showing positive effects (e.g., Na et al., 2021). Generally, it has been demonstrated that in terrestrial arthropods, size tends to increase on average in univoltine species (Horne et al., 2015). Our data also reveal a positive effect of temperature on the wing size of *P. teleius*. Given that temperature changes were more pronounced and rapid in the Polish site, this could potentially lead to an increase of the wing size of Polish butterflies. In contrast, Dutch individuals have not exhibited significant morphological changes, possibly because climatic conditions have not shifted drastically since the reintroduction of butterflies from Poland to Netherlands in 1990.

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 (NL2020) compared with the Polish metapopulation (PL2019) 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).

4.2. Genetic structure of metapopulations

Our results demonstrated much lower allelic richness in NL2020, which harbors only half of alleles present in PL2019. 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).

4.3. 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. 4a and Table A.2). It should be stressed that the main changes in landscape composition and connectivity started in both considered locations after this period (see Fig. A.4). 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 such as SNPs should be considered for application (Puckett and Eggert, 2016).

4.4. Conclusions for conservation and management implications

Our study implies that *Phengaris* butterflies, despite their peculiar life cycle, 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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Appendix A. Supplementary material

Supplementary material.

Supplementary material.

Recommended articles

Data Availability

Raw data and R code are available for download from https://doi.org/10.6084/m9.figshare.25093298.v1 ↗.

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