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The genotype-phenotype map of seasonal plasticity in a butterfly

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I. Anna B. Shoshan, Ugo Pindeler, Christopher W. Wheat & Karl Gotthard. Repeated evolution of photoperiodic plasticity by different genetic architectures during range expansions in a butterfly. *Manuscript*.

II. Anna B. Shoshan, Kalle Tunström, Christopher W. Wheat & Karl Gotthard. CRISPR/Cas9-mediated high-efficiency knock-out of *yellow-y* gene and germline mutations unveils promising applications in the speckled wood butterfly *Pararge aegeria*. *Manuscript*.

Candidate contributions to thesis articles*

	I	**II**
Conceived the study	**Minor**	**Significant**
Designed the study	**Significant**	**Significant**
Collected the data	**Substantial**	**Substantial**
Analyzed the data	**Substantial**	**Substantial**
Manuscript preparation	**Substantial**	**Substantial**

*** Contribution Explanation**

Minor: contributed in some way, but contribution was limited.

Significant: provided a significant contribution to the work.

Substantial: took the lead role and performed the majority of the work.

Contents

Summary

Many species have evolved strategies to overcome seasonal fluctuations in resources. That includes winter diapause that is a type of hibernation found in a wide variety of insects in the temperate zone. The decision to induce diapause is made in advance of winter, as a response to a seasonal que, often the daylength. The daylength inducing diapause varies adaptively between species and populations. Understanding the genotype-phenotype map of this variation in the seasonal "timekeeping" is of importance as it can give insight into the evolution of seasonal timing as well as the processes driving adaptation of seasonal timing to changing environments. However, despite numerous studies identifying genomic regions associated with adaptive phenotypic variation in the timing of diapause induction, the relationship between genetic and phenotypic variation is still not well understood. For the speckled wood butterfly (*Pararge aegeria*), two regions highly associated with interpopulation differences in the timing of diapause induction have been identified, one on the Z-chromosome and another on an autosome. In **Paper I**, we examined if the impact of the Z-chromosome on the timing of diapause induction varies between populations from a latitudinal cline of *P. aegeria* in Sweden. Creating F1 reciprocal hybrid crosses between two closely related populations, we found no evidence of Z-linked effect on the difference in diapause timing. This contrasts with a study by Pruisscher et al. 2018, which identified a significant Z-linked effect on the timing of diapause induction between populations that are more distantly related. Together, these findings show that variation in the timing of diapause is, at least partly, due to variation within different genes across the butterfly's Swedish range. That the genotype-phenotype map can vary suggests that the trait may have a degree of genetic flexibility when adapting to new environments. In **Paper II** we aimed to improve the toolbox for exploring genetic regions and their functional effect on the phenotype in this species. Therefore, we evaluated the efficiency of the CRISPR/Cas9 technology in inducing targeted gene editing in *P. aegeria*'*s* germline. By targeting the melanin-promoting *yellow-y* gene, we observed color changes in first-instar larval head capsules and adult background coloration. Our results demonstrated high efficiency in *yellow-y* knock-out, with almost 80% of adults displaying mosaic or complete color transformation. Importantly, mating highly transformed individuals resulted in knock-out offspring, affirming successful germline transmission. Our studies advance the research of the genotype-phenotype in the timing of diapause induction in *P. aegeria*. First, we reveal that the genotype-phenotype map varies across the species range in Sweden suggesting some degree of flexibility in adaptation to new environments and second, we uncover that CRISPR/Cas9 has great potential in further studying the genotype-phenotype map of variation in the timing of diapause induction in *P. aegeria*.

Sammanfattning

Många arter har utvecklat strategier för att överleva säsongsvariationer i resurser. Det inkluderar diapaus som är en typ av vinterdvala som uppvisas av en stor andel av insekter i den tempererade zonen. Beslutet att inducera diapaus tas i förväg inför vintern, som svar på en säsongsmässig signal, oftast dagslängden. Dagslängden som inducerar diapaus varierar adaptivt mellan arter och populationer. Förståelsen av genotyp-fenotypkartan för denna variation i säsongsmässig "tidshållning" är av betydelse eftersom den kan ge insikter om evolutionen av säsongsmässig timing samt processerna som driver anpassningen av säsongsmässig timing till föränderliga miljöer. Trots ett flertal studier som identifierat genomiska regioner som är associerade med adaptiv fenotypisk variation i timingen av diapaus induktion är förhållandet mellan genetisk och fenotypisk variation fortfarande relativt outforskat. Hos kvickgräsfjärilen (*Pararge aegeria*) har två genomiska regioner som är starkt associerade med variation mellan populationer i timingen av diapaus induktion identifierats, en på Z-kromosomen och en annan på en autosom. I Artikel I undersökte vi om effekten av Z-kromosomen på timingen av diapause induktion varierar mellan populationer längs en latitudinell gradient av *P. aegeria* i Sverige. Genom att skapa F1-reciproka hybridkorsningar mellan två närliggande populationer fann vi inga bevis för en Z-länkad effekt på skillnaden i diapaus timing. Detta kontrasterar med en studie av Pruisscher et al. 2018, som identifierade en signifikant Z-länkad effekt på timingen av diapaus induktion mellan populationer som är mer avlägset besläktade. Tillsammans visar dessa fynd att variationen i timingen av diapaus, åtminstone delvis, beror på variation inom olika gener över fjärilens svenska utbredningsområde. Att genotyp-fenotypkartan kan variera antyder att egenskapen kan ha en grad av genetisk flexibilitet när den anpassar sig till nya miljöer. I Artikel II syftade vi till att förbättra verktygslådan för att utforska genetiska regioner och deras funktionella effekt på fenotypen hos denna art. Därför utvärderade vi effektiviteten av CRISPR/Cas9-tekniken för att inducera riktade genförändringar i *P. aegerias* könsceller. Genom att inrikta oss på *yellow-y* genen som är involverad i melaninproduktion, observerade vi färgförändringar i huvudkapslarna på första-stadie-larverna och de vuxnas fjärilarnas bakgrundsfärg. Resultaten visade att vi med hög effektivitet kunne generera *yellow-y* knock-outs, då nästan 80% av vuxna upp visar delvis- eller komplett färgtransformation. Dessutom visade vi att genom att para starkt transformerade individer producerades avkomma som bar på knock-out fenotypen, vilket bekräftar framgångsrik genetisk transformering av könsceller. Våra studier har flyttat fram forskningen om hur genotyp-fenotypkartan i timingen av diapaus induktion ser ut hos *P. aegeria*. För det första visar vi att genotyp-fenotypkartan varierar över artens utbredningsområde i Sverige vilket antyder en viss grad av flexibilitet i anpassningen till nya miljöer, och för det andra avslöjar vi att CRISPR/Cas9 har stor potential att ytterligare studera genotyp-fenotypkartan för variation i timingen av diapaus induktion hos *P. aegeria*.

Introduction

Seasonal adaptation – Winter diapause

Throughout the year, fluctuations in temperature and daylength results in seasonal changes influencing the availability of essential resources like food and mating opportunities of most temperate organisms. The fluctuations follow a pattern of annual predictability (Hut et al., 2013), enabling animals to develop adaptive strategies to these seasonal variations. Among seasonal strategies are color camouflage, changes in coat size, hibernation, migration and diapause (Dolnik & Gavrilov, 1980; Gwinner, 1996; Saunders, 2010; Williams et al., 2014; Mills et al., 2018). Significantly, these strategies are often initiated in advance of a specific season and require adaptations that enable seasonal timing. Understanding the genetic mechanisms underlying "timekeeping adaptations" can reveal the evolution of seasonal timing.

For insects inhabiting higher latitudes winter poses a significant challenge, as temperatures gets lower than what is necessary for growth and reproduction (Moore & Lee, 1991; Bale & Hayward, 2010; Hut et al., 2013). Consequently, while certain insects have evolved the ability to undertake extensive migrations to regions with milder climates (Chowdhury et al., 2021), the majority enter a state akin to hibernation known as winter diapause (Tauber et al., 1986). To survive the cold season, temperate insects suspend their development, decrease their metabolic activity (thus conserving energy), and rely on previously accumulated energy reserves until the cold period ends (Tauber et al., 1986). The life stage where insect diapause can occur is typically species-specific and can be either during egg, larval, pupal or adult stage (Gill et al., 2017).

For facultative diapausers, the "decision" to induce diapause occurs prior to the onset of the cold season through the use of environmental cues, like daylength and temperature (Clark & Platt, 1969; Tauber et al., 1986; Nylin et al., 1995). The decision-making process is significant as it profoundly impacts the organism's life history. For instance, winter diapause is often accompanied by an increase in development time before diapause is initiated (termed diapause induction) (Wang et al., 2007; Välimäki et al., 2013; Lindestad et al., 2021), presumably to allow physiological preparation and location selection (Wang et al., 2007). Furthermore, once diapause has been initiated, most species are unable to terminate it until a specific time, often spanning several months, has past, regardless if the temperature increases (Dong et al., 2013; Lehmann et al., 2017). Therefore, a correct assessment of the time of the year is essential.

Winter diapause is a plastic trait but the sensitivity to the environmental cue causing diapause induction is genetically determined and varies adaptively (Tauber et al., 1986; Gomi, 1996; Aalberg haugen & Gotthard, 2015; Ittonen et al., 2022). For species where daylength is one of the primary environmental cues, the

daylength (or photoperiod) causing diapause induction is often measured as a photoperiodic reaction norm or the critical photoperiod of a population (Danilevskii, 1965; Hut et al., 2013; Joschinski & Bonte, 2021; Lankinen et al., 2021). The critical photoperiod is defined as the daylength resulting in 50% of a population entering diapause under constant temperature (Koštál, 2011). Due to annual variation in daylength and length of the cold season across latitudes (Hut et al., 2013) several species have evolved latitudinal clines where the critical photoperiod increases with increasing latitude (Bradshaw, 1976; Nylin, 1995; Kato, 2005; Aalberg Haugen & Gotthard, 2015; Siemers et al., 2024). Studying the genetic variation underlying this variation in the timing of diapause induction can lead to insights into the genotype-phenotype map and the evolutionary processes that have driven the adaptation to the contemporary environments.

What we know to date

The ability to enter diapause appears to have evolved several times as insect species have expanded their distribution from the southern tropical regions into the northern temperate zone (Ragland & Keep, 2017). Therefore, it is not surprising that there are differences in the genotype-phenotype map between species (Ragland et al., 2019). The sensitivity to the cue causing diapause induction is in some species controlled by several genes with varying effect size (Gomi 1997; Pruisscher et al, 2018) and in others by a small set of genes (Suwa & Gotoh, 2006; Han & Denlinger, 2009). While some studies suggest inheritance to be primarily additive others show a combination of additivity and dominance (Mathias et al., 2007; Bradshaw et al., 2012). Additionally, the chromosomal position of the genes varies. In some species the trait is partly sex-linked (King et al., 1974; Pruisscher et al., 2017, 2018) while in others it may be exclusively regulated by genes on the autosomes (Tauber et al., 1977; Han & Denlinger, 2009; Söderlind & Nylin, 2011). Some studies have detected rapid adaptation in the timing of diapause induction to environmental changes (Bradshaw & Holzapfel 2001; Sadakiyo & Ishihara, 2011, Urbanski et al., 2012, Ittonen et al., 2022; Nielsen et al., 2023).

In many species, loci associated with the timing of diapause induction are within genomic regions containing circadian clock genes (Han & Denlinger, 2009; Yamada & Yamamoto, 2011; Ikeno et al., 2013 Gotthard & Wheat, 2019; Pruisscher et al., 2018, 2021; Lindestad et al., 2022). The specific clock genes vary between species but are sometimes core genes of the circadian clock. The circadian clock is involved with daily timing and may provide information about time in seasonal timing (Bünning, 1936; Bünsow, 1953). However, it is still not known how the circadian clock is involved with photoperiodism (Goto, 2022).

While the genotype-phenotype map of photoperiodism in insects is starting to be explored the genetic variants causing adaptive differences in the timing of diapause induction is still largely unknown (Ragland et

al., 2019). Knowledge of the genotype-phenotype map can provide insight into how well species may respond to environmental changes.

How to study the genotype-phenotype map

The often-used methods to explore the genetic variation behind adaptive phenotypic variation are candidate gene, linkage mapping and association mapping (Phillips, 2005; Courtier-Orgogozo, 2023). These methods find genomic regions associated with variation in phenotypes across environments and suggests whether a trait is regulated by a few or several genes, as well as the effect sizes of the regions (Phillips, 2005; Courtier-Orgogozo, 2023). However, often the effects of the identified regions on the phenotype are not further explored (Rockman, 2012; Courtier-Orgogozo, 2023). This is likely due to the difficulty in creating functional analysis of specific genomic regions.

Analyzing the functional effect of a specific genomic region on a phenotype can be done directly and indirectly. If there is a suspicion of genetic variation on a sex-chromosome contributing to phenotypic differences between populations, the region can be investigated indirectly by considering the entire sex chromosome as a region. A phenotypic effect would confirm that the sex chromosome contains allelic differences between populations causing a difference in their photoperiodic reaction norms. In practice, this is done by estimating photoperiodic reaction norms of F1 reciprocal hybrid crosses across relevant daylengths (King et al., 1974; Pruisscher et al., 2017, 2018). In butterflies and moths the males are the homogametic sex (ZZ) and the females are the heterogametic sex (ZW) (Traut & Marec, 1996). If the phenotype is additive the reciprocal hybrid males, having a chromosome from each population in each chromosome pair, is expected to have a reaction norm intermediate to the two pure populations (figure 1). The reciprocal hybrid females will as well have a chromosome from each population in each autosome pair, but only one Z-chromosome inherited from their father (Traut & Marec, 1996). Therefore, if the females of the two reciprocal crosses differ, while there is no such difference between the males, it is expected to be due to variation on the Z-chromosome effecting the reaction norm (figure 1) (King et al., 1974; Pruisscher et al., 2017, 2018). A limitation to this methodology is that it is only practically possible for species that can be bread in the laboratory.

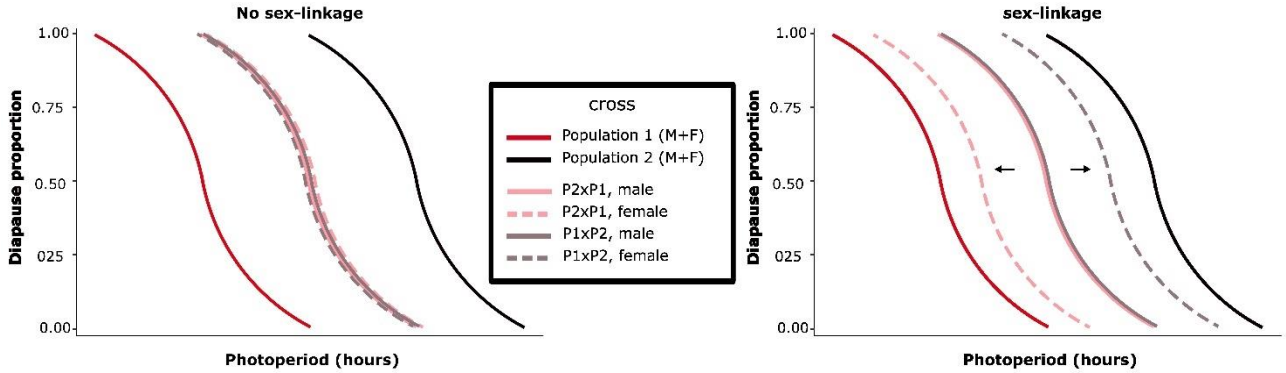

Figure 1. Illustration of expected results of F1 reciprocal hybrid crosses between two populations showing a difference in their reaction norms of daylengths inducing diapause. To the left the F1 reciprocal hybrid males and females have reaction norms in between the two pure populations indicating no Z-linked effect in the diapause response. To the right the F1 reciprocal hybrid female crosses deviate significantly from each other indicating a Z-linked effect explaining part of the difference in the timing of the diapause response between the two pure populations.

In case of repeated adaptation, where the same species has adapted to a similar environment more than once, the adaptation can be a result of allelic variation at the same genes or allelic variation at different genes (Conte et al., 2012; Bohutínská & Peichel, 2023). For instance, if the F1 reciprocal hybrid crosses of two populations gave the results to the left in figure 1, it would indicate that the difference in these two pure populations reaction norms is mainly due to variation on the autosomes. However, if two other populations show the results to the right in figure 1, where the F1 reciprocal hybrid female crosses deviate significantly from each other, the difference between those two pure populations is additionally partly due to variation on the Z-chromosome. Thereby, the difference between the four populations in the timing of diapause induction is caused by variation in different genes, in other words, the genotype-phenotype map varies between the populations. While gene-reuse is often expected within a species range (Conte et al., 2012), several factors can influence the chance of gene reuse (Gompel & Prud'homme, 2009) and therefore, the generality of the genotype-phenotype map should be investigated.

Analyzing the functional effect of specific genomic regions on a phenotype directly can be challenging. The classic method is introgression testing, where a genomic region is backcrossed from one population into another and thereby into a different genetic background (Laurie et al., 1997). However, single region tracking can be difficult (Laurie et al., 1997). Additionally, backcrossing can result in genetic hitchhiking and inbreeding rendering it challenging to separate the effect of the region from other genetic factors. Another option is to use the relatively new gene editing tool CRISPR/Cas9 (Cong et al., 2013). CRISPR/Cas9 enables the creation of targeted double stranded DNA cuts (Gasiunas et al., 2012; Jinek et al., 2012). However, after the DNA is cut the cell will mainly repair by non-homologue (NHEJ) end joining resulting in minor indels

(insertions or deletions) (Cong et al., 2013; Sander et al., 2014). While NHEJ is ideal for knocking out a gene it is not suitable for creating specific mutations. Homologue directed repair (HDR) is another cell mechanism where a donor plate is used to repair a break (Cong et al., 2013; Sander et al., 2014). By providing a donor plate with a predesigned sequence it is possible to insert an ecologically relevant mutation. The second option is still not common practice but several examples exist for Drosophila melanogaster (Douris et al., 2020) and the first examples in other insect species have been published (Wang et al., 2020; Heryanto et al., 2022). When CRISPR/Cas9 with DNA repair though HDR becomes common practice, it can greatly assist functional testing of genomic regions on the phenotype and building the genotype-phenotype map of ecologically important traits like the ability to time diapause induction. However, until now the efficiency of CRISPR/Cas9 in inducing germline mutations has only been tested in a limited number of lepidopteran ecological model species (Shirai et al., 2021; Connahs et al. 2022; Li et al., 2022; Okamura et al., 2022; Wang et al., 2023). As CRISPR/Cas9 often results in mosaic individuals (Perry et al., 2016; Bi et al., 2019), germline mutations are essential to secure mutations in the targeted tissue. Additionally, germline mutations are necessary, if the mutation has a small effect on the phenotype and a large number of individuals are needed to statistically test the genotype-phenotype relationship.

Pararge aegeria as a study species

The speckled wood butterfly (*Pararge aegeria*) is an ideal species for investigating the genetic variation underlying local variation in the timing of diapause induction as it is an ecological model species in seasonal adaptation, life cycle regulation and voltinism (Wiklund et al., 1983; Shreeve, 1986; Merckx & Van Dyck, 2006; Aalberg Haugen & Gotthard, 2015; Pruisscher et al., 2018; Lindestad et al., 2021). It is a facultative diapauser with a well-studied latitudinal cline in the critical photoperiod of diapause induction in Sweden (Aalberg Haugen & Gotthard, 2015) where it primarily diapauses as a pupa (Wiklund et al., 1983). The latitudinal cline in the critical photoperiod is accompanied with a change in voltinism (the number of generations/year) from two generations in the south to one generation in the northern distribution of the species in Sweden (Lindestad et al., 2019). For *P. aegeria* the main cue for diapause induction is daylength (in combination with temperature) (Lindestad et al., 2019).

A recent study performed a genome-wide scan and a candidate loci association analysis in F2 hybrids between two Swedish populations of *P. aegeria*, from the most northern and most southern part of the Swedish distribution, respectively. These populations differ strongly in their critical photoperiod (Sundsvall in the north: critical photoperiod 19h; Skåne in the south: critical photoperiod 16.3h) (Aalberg Haugen & Gotthard, 2015) (figure 2a). The analyses found that the timing of diapause induction is regulated by a combination of a few genes with large effect size and several genes with low effect size (Pruisscher et al.,

2018). Additionally, two loci were highly associated with the difference in diapause timing between these two populations, the *period* gene located on the Z-chromosome and a locus containing the *timeless* gene on an autosomal chromosome (Pruisscher et al., 2018). Both *period* and *timeless* are core genes of the circadian clock (Goto, 2013). As the *period* gene is located on the Z-chromosome, Pruisscher et al. (2018) additionally created F1 reciprocal hybrid crosses and found that the Z-chromosome does have genetic variation explaining part of the difference in the timing of diapause induction between these two populations (Pruisscher et al., 2018).

Interestingly, the two populations compared in the study by Pruisscher et al. 2018 originates from two separate range expansions of *P. aegeria* into Sweden (Nordström, 1955; Tison et al., 2014). While most of the Swedish range (including Sundsvall) expanded into Sweden before we have any records of the species, the most southern range (including Skåne) originates from an expansion into Sweden less than 100 years ago (~ year 1930) (figure 2a) (Nordström, 1955; Tison et al., 2014). These two distributions appear to still be genetically separated as limited gene flow appear between them (figure 2b) (Lindestad et al., 2021). As the species has expanded into the south of Sweden twice this can be considered an example of repeated adaptation and the genotype-phenotype map may differ between the expansions in the timing of diapause induction.

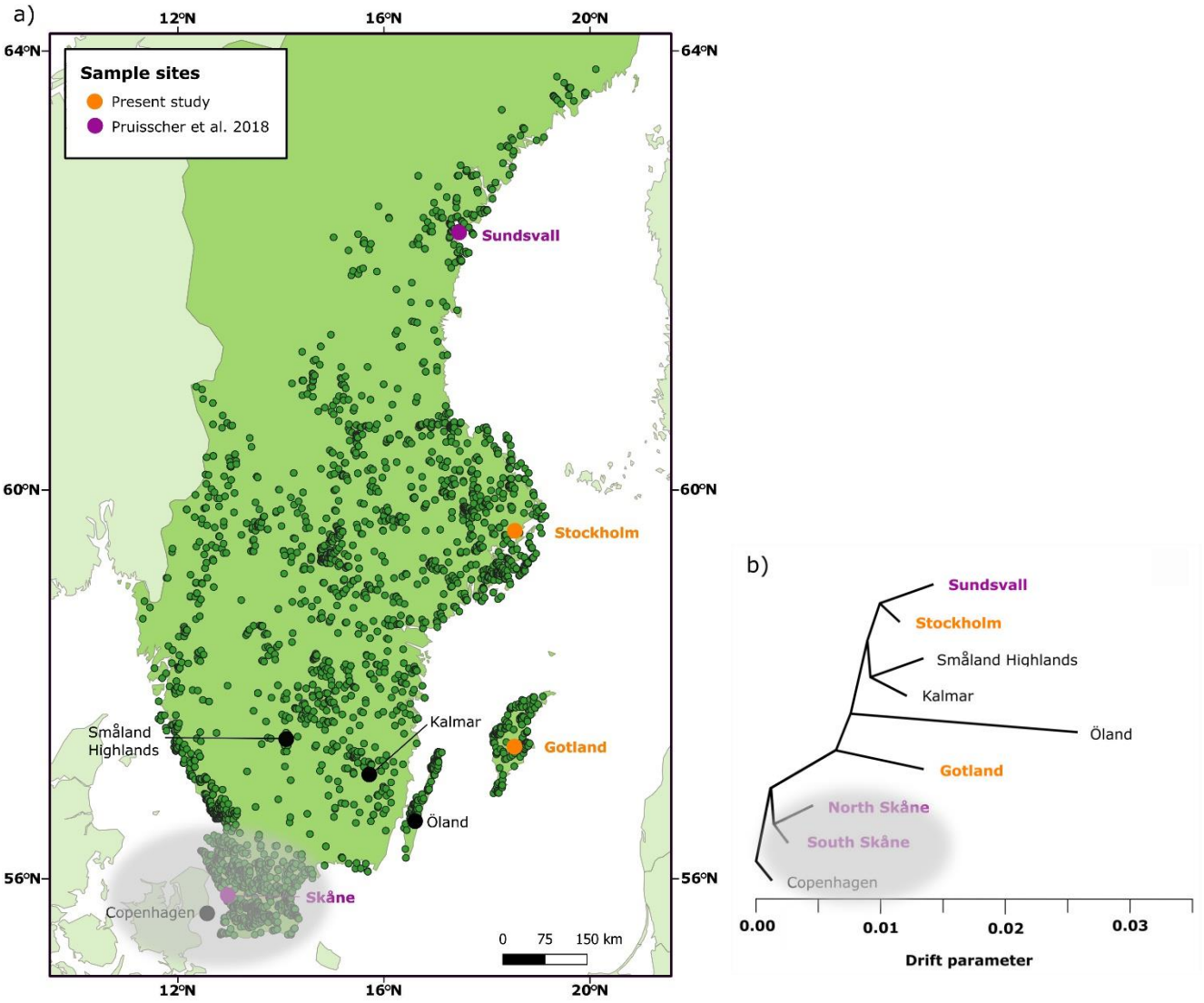

Figure 2. The current range of P. aegeria *in Sweden and the position of specific populations and their relations. a) Map of Sweden. The green dots indicate locations where adult* P. aegeria *were observed from January 2017 to May 2023 according to artportalen.se. The orange and purple dots represent sample sites; orange dots denote sites from Paper I (Stockholm and Gotland), while the purple dots denote sites from Pruisscher et al., 2018 (Sundsvall and Skåne). The grey bubble over southern Sweden and Denmark indicates the current distribution of populations from the latest expansion. b) Phylogenetic relationship among seven* P. aegeria *populations in Sweden, adapted from figure 1b in Lindestad et al., 2022. The phylogeography is based on whole-genome sequences, with branch length reflecting genetic drift levels. Orange markers denote populations sampled in Paper I, while purple markers represent populations sampled in Pruisscher et al. (2018). The grey bubble signifies the populations originating from the most recent expansion.*

The application of CRISPR/Cas9 gene editing has not yet been conducted in *P. aegeria*. Although the technology presents substantial advantages for exploring variation in the timing of diapause induction, CRISPR/Cas9 requires modification and testing before implementation to determine its efficiency, potential side-effects and capacity to induce germline mutations (Pattanayak et al., 2013; Mohr et al., 2016).

P. aegeria registers the "seasonal timing" at least twice during its development (figure 3). The first instance occurs during larval development, where short daylengths results in an extended larval development time (Nylin et al., 1989; Pruisscher et al., 2018; Lindestad et al., 2019). The second registration occurs in the last larval instar, at a critical stage where the final decision between entering diapause or proceed with direct development in the pupal stage is made (Nylin et al. 1989, Lindestad et al. 2019; 2021). Each of these decisions have their own photoperiodic reaction norm (Lindestad et al. 2019; 2021). Under laboratory conditions, they can be experimentally separated by exposing individuals to a constant daylength. Recent studies have revealed that these two decisions are made independently (Lindestad et al., 2021) and may also evolve independently over time (Nielsen et al., 2023), raising questions about the extent to which their genetic architecture overlaps.

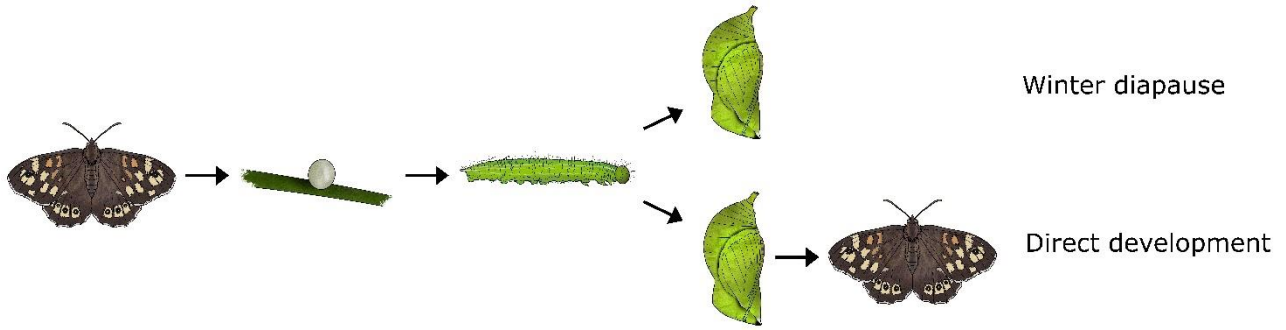

Figure 3. P. aegeria *in Sweden can diapause as a pupa or develop directly into an adult. The development path depends on the length of the days during larval development. Short days indicate that winter is approaching and will result in an induction of diapause. In* P. aegeria *the length of the day is registered during larval development (larval decision) and in the last larval instar, just before pupation (pupal decision).*

Paper I

In **Paper I** we elaborated on the results found by Pruisscher et al. 2018 and investigated whether the Z-linked effect present between the two populations that originated from the two different expansions (from Sundsvall and Skåne) is also present between two populations within the oldest expansion (from Stockholm and Gotland) (indicating gene-reuse) (Figure 2a). We recorded both the larval and pupal decisions to investigate if they showed the same pattern. We created F1 reciprocal hybrid crosses between these two more closely related populations and found that in none of the decisions the reciprocal hybrid female crosses deviated significantly from each other, as expected in case of a Z-linked effect (figure 4). Instead, the females generally entered diapause at a shorter daylength than the males, which is often seen in natural populations (Wiklund et al., 1992). It has been argued that this might be a consequence of selection

for protandry, a strategy in which males increase their mating potential by emerging earlier than the females (Wiklund et al., 1992).

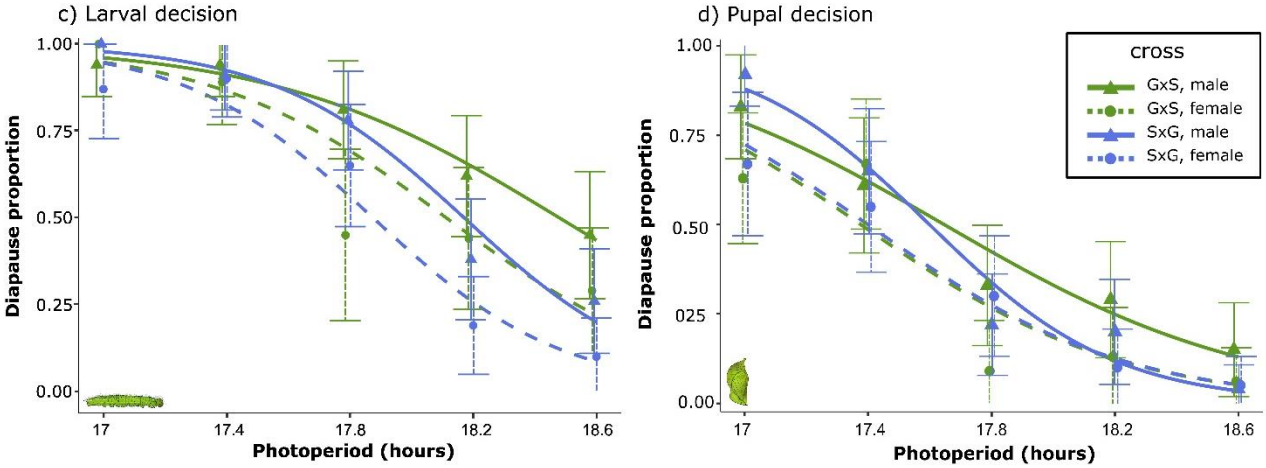

Figure 4. We found no Z-linked effect on the reaction norm of the two populations from the oldest expansion (Stockholm and Gotland) in either the larval or pupal reaction norms for the timing of diapause induction.

To confirm that the larval decision really did rely on different genetic variation between the two F1 reciprocal hybrid population crosses we compared our results to the raw data of Pruisscher et al. 2018. However, they only measured one daylength, so we pooled our data across all daylength treatments (figure 5a). Indeed, we found that there was a significant difference between the two studies in the larval decision indicating that the variation between populations is at least partly due to variation in different genes across the expansions of *P. aegeria* into Sweden. In other words, the genotype-phenotype map differs across the species range. This indicates that the trait is to some degree flexible and in some cases more than one "genetic solution" can solve a similar evolutionary challenge.

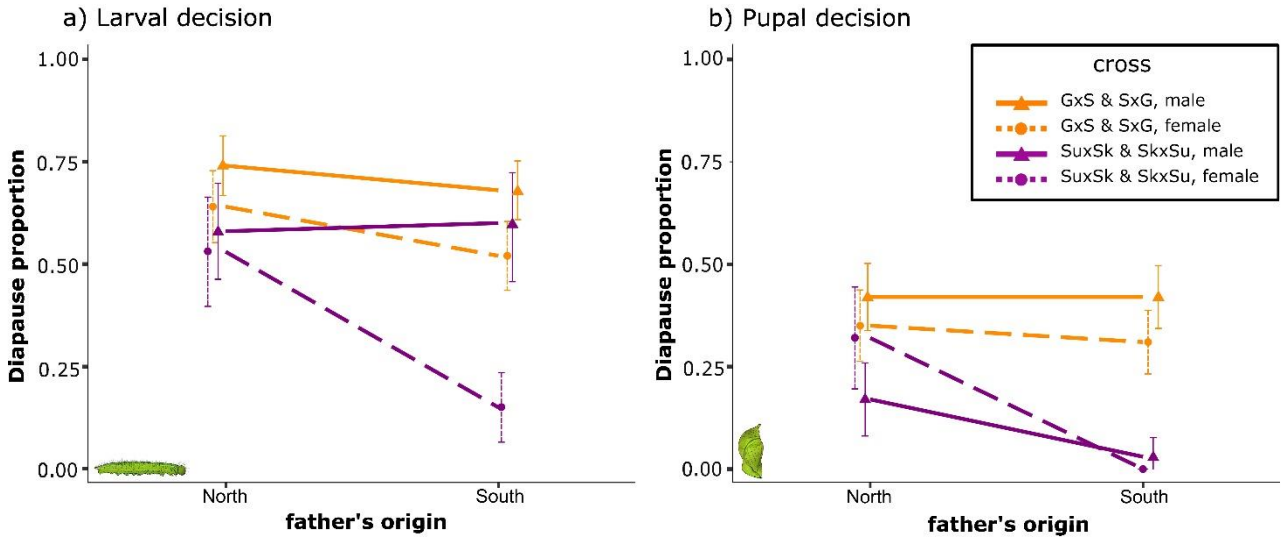

Figure 5. Proportion of diapausing individuals in reciprocal hybrid crosses conducted in Paper I (orange) and Pruisscher et al. (2018) (purple). Within each study, individuals are categorized based on the origin of their father (Stockholm=North, Gotland=South; Sundsvall=North, Skåne=South) and by sex (Females represented by stippled lines and dots; Males represented by solid lines and triangles). The bars represent the 95% confidence intervals of actual proportions. a) depicts larval decision, while b) depicts pupal decision.

We additionally compared the results of the pupal decision between the two studies (figure 5b). In both population crosses, no Z-linked effect was detected. This further corroborates that the larval and pupal decisions rely at least partly on different genetic architectures.

Gene-reuse is common in species experiencing repeated adaptation (Conte et al., 2012). The factors leading to the evolution of distinct genotype-phenotype maps in the timing of diapause induction of *P. aegeria* in Sweden remains unknown. One conceivable explanation could be that there was a difference in the standing genetic variation at the time of the two expansions. However, it is important to note that the genotype-phenotype map of seasonal timing may not be constant across the entire range of a species.

Paper II

In **Paper II** we explored the efficiency and ability to induce germline mutations using the gene editing tool CRISPR/Cas9 in *P. aegeria*. If the efficiency is high it opens for the possibility of using CRISPR/Cas9 for further functional analysis of genetic regions in this species. We targeted the *yellow-y* gene that appears to have a conserved function as a dark melanin promoting factor across lepidopteran taxa (Shirai et al., 2021). Knocking-out the *yellow-y* gene has previously been successfully done in several lepidopterans (Perry et al., 2016; Liu et al., 2020; Shirai et al., 2021) and makes it easy to phenotype successful mutations. In *P. aegeria* the first instar larva carries a dark brown head capsule and adults have a brown background color on the wings. Therefore, the identification of successful knock-outs was done as first larval instar, based on

whether the head capsule was dark brown as the wildtype or light brown, and as adults based on whether the background color of the wings was brown like the wildtype or <50%, >50% or 100% yellow (figure 6).

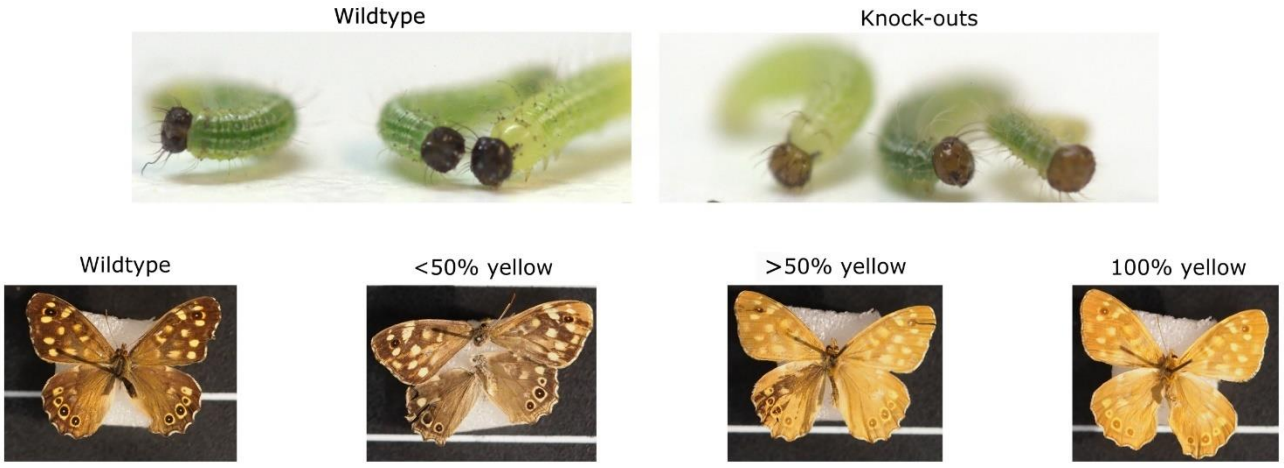

Figure 6. Phenotyping first instar larva and adults for knockout of the yellow-y *gene. Larvae were categorized as "Wildtype" or "Knock-outs" depending on if they had a dark brown or light brown head capsule. Adults were categorized into one of four categories: Wildtype (100% brown background color as the wildtype), <50% yellow (less than 50% of cells had a yellow color indicating a knockout of the* yellow-y *gene), >50% yellow or 100% yellow.*

To determine any difference in efficiency across factors we had four treatments where the construct varied in the number of sgRNAs (single stranded RNAs that guide the Cas9 endonuclease to the cutting site) and the concentration of Cas9-sgRNA. A total of 555 eggs were injected with a CRISPR/Cas9 construct and out of them 146 (26%) survived to egg hatching and 90 (16%) survived to adult eclosion. In total, 57% of the larvae were scored as knock-outs and out of 87 phenotyped adults almost 80% displayed at least partial transformation. The different treatments did not deviate significantly in the number of individuals transformed suggesting that the results are less sensitive to the factors varied in this study in the *yellow-y* gene in *P. aegeria*.

Matings between adults from the categories '>50% yellow' and '100% yellow' from three of the four treatments resulted in fully knock-out offspring (table 1), indicating a high degree of germline mutations in the mated adults.

Table 1. Categorization of offspring from two observed matings and two net cages with unobserved matings.

Parents (F x M)	Larvae	Knock-out larvae	Survival to adult eclosion	100% yellow
Mating 1 (y1+y3 x y1+y3)	15	15	14	14
Mating 2 (y1 x y1+y3)	15	15	13	13
Cage Matings*	45	45	32	32

***Cages with unobserved matings between adults from treatment y1+y3, y1+s4 and y1.**

Overall, the high efficiency of CRISPR/Cas9 and the ability to induce germline mutations suggests that *P. aegeria* could serve as a valuable model species for further investigating the genotype-phenotype map of variation in the timing of diapause induction, especially as homology-directed repair-mediated knock-in becomes more feasible.

Future directions

Since knock-in mediated by homology-directed repair using CRISPR/Cas9 is not easily performed yet, it will take a while before we can directly test the effect of minor loci on the variation in the timing of diapause induction. However, other steps can be taken toward building the genotype-phenotype map of the timing of diapause induction in this species. For instance, a relatively large deletion within the *timeless* gene is fixed in the Gotland population and is not present in any other populations studied across the range in Sweden (Lindestad et al., 2022). The *timeless* gene is part of the genomic region having a large effect on the difference in the timing of diapause induction between the two populations from each their expansion, from Sundsvall and Skåne (Pruisscher et al., 2018). Additionally, the *timeless* gene is a clock gene and therefore may influence the "sense of timing". Therefore, it can be hypothesized that the deletion may explain part of the difference in the photoperiodic reaction norm between the Gotland population and the rest of the populations in Sweden and thereby may be an additional example of a different genotype-phenotype map within *P. aegeria's* Swedish range.

To investigate if this deletion has an influence on the timing of diapause induction it may be possible to combine introgression testing and CRISPR/Cas9. The size of the deletion allows phenotyping with a simple PCR and makes it possible to track in a breeding design. Therefore, it is possible to investigate if the deletion has an effect on the timing of diapause induction using introgression testing. However, this will still not solve the problems associated with hitchhiking and inbreeding making the evaluation of the effect less stringent. By using CRISPR/Cas9 the deletion can be created in individuals from another population by targeting each side of the deletion using two separate sgRNAs. Unfortunately, the deletion expands exon

15 and part of exon 14 (out of 16 exons), and a double-stranded DNA cut followed by non-homologue end joining will most likely result in an early stop codon that may lead to a deletion that is even larger than the natural deletion. Therefore, at present the exact natural deletion is difficult to create using CRISPR/Cas9. However, by combining the two methods it may be possible to entangle if this deletion is an additional example of a difference in the genotype-phenotype map across the range of *P. aegeria* affecting the timing of diapause induction.

Our studies demonstrate differing genotype-phenotype maps of the timing of diapause induction among populations of *P. aegeria* in Sweden. Moreover, we highlight the promising potential of CRISPR/Cas9 for future investigation of variation in diapause timing in this species. Advancing the knowledge of the genotype-phenotype map of the timing of diapause induction in this and other insects can give insight to the evolution of photoperiodism for seasonal timing - an adaptation enabling organisms to anticipate seasonal fluctuations in resources and develop adaptive strategies.

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References

Aalberg Haugen, I. M., & Gotthard, K. (2015). Diapause induction and relaxed selection on alternative developmental pathways in a butterfly. *Journal of Animal Ecology*, *84*(2), 464-472.

Bale, J. S., & Hayward, S. A. L. (2010). Insect overwintering in a changing climate. *Journal of Experimental Biology*, *213*(6), 980-994.

Bi, H. L., Xu, J., He, L., Zhang, Y., Li, K., & Huang, Y. P. (2019). CRISPR/Cas9-mediated ebony knockout results in puparium melanism in Spodoptera litura. *Insect science*, *26*(6), 1011-1019.

Bohutínská, M., & Peichel, C. L. (2023). Divergence time shapes gene reuse during repeated adaptation. *Trends in ecology & evolution.*

Bradshaw, W. E. (1976). Geography of photoperiodic response in diapausing mosquito. *Nature*, *262*(5567), 384-386.

Bradshaw, W. E., Emerson, K. J., Catchen, J. M., Cresko, W. A., & Holzapfel, C. M. (2012). Footprints in time: comparative quantitative trait loci mapping of the pitcher-plant mosquito, Wyeomyia smithii. *Proceedings of the Royal Society B: Biological Sciences*, *279*(1747), 4551-4558.

Bradshaw, W. E., & Holzapfel, C. M. (2001). Genetic shift in photoperiodic response correlated with global warming. *Proceedings of the National Academy of Sciences*, *98*(25), 14509-14511.

Bünning, E. (1936). *Die Entstehung der Variationsbewegungen bei den Pflanzen* (pp. 235-347). Springer Berlin Heidelberg.

Bünsow, R. (1953). Endogene Tagesrhythmik und Photoperiodismus bei Kalanchoe blossfeldiana. *Planta*, *42*, 220-252.

Chowdhury, S., Fuller, R. A., Dingle, H., Chapman, J. W., & Zalucki, M. P. (2021). Migration in butterflies: a global overview. *Biological Reviews*, *96*(4), 1462-1483.

Clark, S. H., & Platt, A. P. (1969). Influence of photoperiod on development and larval diapause in the viceroy butterfly, Limenitis archippus. *Journal of Insect Physiology*, *15*(10), 1951-1957.

Connahs, H., Tan, E. J., Ter, Y. T., Dion, E., Matsuoka, Y., Bear, A., & Monteiro, A. (2022). The yellow gene regulates behavioural plasticity by repressing male courtship in Bicyclus anynana butterflies. *Proceedings of the Royal Society B*, *289*(1972), 20212665.

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W., Marraffini, L. A., & Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science*, *339*(6121), 819–823.

Conte, G. L., Arnegard, M. E., Peichel, C. L., & Schluter, D. (2012). The probability of genetic parallelism and convergence in natural populations. *Proceedings of the Royal Society B: Biological Sciences*, *279*(1749), 5039-5047.

Courtier-Orgogozo, V. (2023). The Loci of Insect Phenotypic Evolution. *Current Opinion in Insect Science*, 101134.

Danilevskii, A. S. (1965). Photoperiodism and seasonal development of insects. *Photoperiodism and seasonal development of insects.*

Dolnik, V. R., & Gavrilov, V. M. (1980). Photoperiodic control of the molt cycle in the Chaffinch (Fringilla coelebs). *The Auk*, *97*(1), 50-62.

Dong, Y. C., Wang, Z. J., Clarke, A. R., Pereira, R., Desneux, N., & Niu, C. Y. (2013). Pupal diapause development and termination is driven by low temperature chilling in Bactrocera minax. *Journal of pest science*, *86*, 429-436.

Douris, V., Denecke, S., Van Leeuwen, T., Bass, C., Nauen, R., & Vontas, J. (2020). Using CRISPR/Cas9 genome modification to understand the genetic basis of insecticide resistance: Drosophila and beyond. *Pesticide biochemistry and physiology*, *167*, 104595.

Gasiunas, G., Barrangou, R., Horvath, P., & Siksnys, V. (2012). Cas9–crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *National Acad SciencesG Gasiunas, R Barrangou, P Horvath, V SiksnysProceedings of the National Academy of Sciences, 2012•National Acad Sciences*, *109*(39). https://doi.org/10.1073/pnas.1208507109

Gill, H. K., Goyal, G., & Chahil, G. (2017). Insect diapause: a review. *J. Agric. Sci. Technol*, *7*, 454-473.

Gomi, T. (1997). Geographic variation in critical photoperiod for diapause induction and its temperature dependence in Hyphantria cunea Drury (Lepidoptera: Arctiidae). *Oecologia*, *111*, 160-165.

Gompel, N., & Prud'homme, B. (2009). The causes of repeated genetic evolution. *Developmental biology*, *332*(1), 36-47.

Goto, S. G. (2013). Roles of circadian clock genes in insect photoperiodism. *Entomological Science*, *16*(1), 1-16.

Goto, S. G. (2022). Photoperiodic time measurement, photoreception, and circadian clocks in insect photoperiodism. *Applied Entomology and Zoology*, *57*(3), 193-212.

Gotthard, K., & Wheat, C. W. (2019). Diapause: circadian clock genes are at it again. *Current Biology*, *29*, R1245–R1246

Gwinner, E. (1996). Circannual clocks in avian reproduction and migration. *Ibis*, *138*(1), 47-63.

Han, B., & Denlinger, D. L. (2009). Mendelian inheritance of pupal diapause in the flesh fly, Sarcophaga bullata. *Journal of Heredity*, *100*(2), 251-255.

Heryanto, C., Hanly, J. J., Mazo-Vargas, A., Tendolkar, A., & Martin, A. (2022). Mapping and CRISPR homology-directed repair of a recessive white eye mutation in Plodia moths. *Iscience*, *25*(3), 103885.

Hut, R. A., Paolucci, S., Dor, R., Kyriacou, C. P., & Daan, S. (2013). Latitudinal clines: an evolutionary view on biological rhythms. *Proceedings of the Royal Society B: Biological Sciences*, *280*(1765), 20130433.
Lander, E. S., & Schork, N. J. (2006). Genetic dissection of complex traits. *Focus*, *265*(3), 2037-458.

Ikeno, T., Ishikawa, K., Numata, H., & Goto, S. G. (2013). Circadian clock gene Clock is involved in the photoperiodic response of the bean bug Riptortus pedestris. *Physiological Entomology*, *38*(2), 157-162.

Ittonen, M., Hagelin, A., Wiklund, C., & Gotthard, K. (2022). Local adaptation to seasonal cues at the fronts of two parallel, climate-induced butterfly range expansions. *Ecology Letters*, *25*(9), 2022-2033.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, *337*(6096), 816–821. https://doi.org/10.1126/SCIENCE.1225829

Joschinski, J., & Bonte, D. (2021). Diapause and bet-hedging strategies in insects: a meta-analysis of reaction norm shapes. *Oikos*, *130*(8), 1240-1250.

Kato, Y. (2005). Geographic variation in photoperiodic response for the induction of pupal diapause in the Aristolochia-feeding butterfly Atrophaneura alcinous. *Applied entomology and zoology*, *40*(2), 347-350.

King, A. B. S. (1974). Photoperiodic induction and inheritance of diapause in Pionea forficalis (Lepidoptera: Pyralidae). *Entomologia Experimentalis et Applicata*, *17*(3), 397-409.

Koštál, V. (2011). Insect photoperiodic calendar and circadian clock: independence, cooperation, or unity?. *Journal of insect physiology*, *57*(5), 538-556.

Lankinen, P., Kastally, C., & Hoikkala, A. (2021). Nanda-Hamner curves show huge latitudinal variation but no circadian components in Drosophila montana photoperiodism. *Journal of Biological Rhythms*, *36*(3), 226-238.

Laurie, C. C., True, J. R., Liu, J., & Mercer, J. M. (1997). An introgression analysis of quantitative trait loci that contribute to a morphological difference between Drosophila simulans and D. mauritiana. *Genetics*, *145*(2), 339-348.

Lehmann, P., Van Der Bijl, W., Nylin, S., Wheat, C. W., & Gotthard, K. (2017). Timing of diapause termination in relation to variation in winter climate. *Physiological Entomology*, *42*(3), 232-238.

Li, Y., Liu, Z., Liu, C., Shi, Z., Pang, L., Chen, C., Chen, C., Chen, Y., Pan, R., Zhou, W., Chen, X. X., Rokas, A., Huang, J., & Shen, X. X. (2022). HGT is widespread in insects and contributes to male courtship in lepidopterans. *Cell*, *185*(16), 2975-2987.

Lindestad, O., Aalberg Haugen, I. M., & Gotthard, K. (2021). Watching the days go by: Asymmetric regulation of caterpillar development by changes in photoperiod. *Ecology and Evolution*, *11*(10), 5402-5412.

Lindestad, O., Nylin, S., Wheat, C. W., & Gotthard, K. (2022). Local adaptation of life cycles in a butterfly is associated with variation in several circadian clock genes. *Molecular Ecology*, *31*(5), 1461-1475.

Lindestad, O., Wheat, C. W., Nylin, S., & Gotthard, K. (2019). Local adaptation of photoperiodic plasticity maintains life cycle variation within latitudes in a butterfly. *Ecology*, *100*(1), e02550.

Livraghi, L., Vodă, R., Evans, L. C., Gibbs, M., Dincă, V., Holland, P. W., Shreeve, T. G., Vila, R., Dapporto, L., & Breuker, C. J (2018). Historical and current patterns of gene flow in the butterfly Pararge aegeria. *Journal of Biogeography*, *45*(7), 1628-1639.

Liu, X. L., Han, W. K., Ze, L. J., Peng, Y. C., Yang, Y. L., Zhang, J., Yan, Q., & Dong, S. L. (2020). Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 mediated knockout reveals functions of the yellow-y gene in Spodoptera litura. *Frontiers in Physiology*, *11*, 615391.

Mathias, D., Jacky, L., Bradshaw, W. E., & Holzapfel, C. M. (2007). Quantitative trait loci associated with photoperiodic response and stage of diapause in the pitcher-plant mosquito, Wyeomyia smithii. *Genetics*, *176*(1), 391-402.

Merckx, T., & Van Dyck, H. (2006). Landscape structure and phenotypic plasticity in flight morphology in the butterfly Pararge aegeria. *Oikos*, *113*(2), 226-232.

Mills, L. S., Bragina, E. V., Kumar, A. V., Zimova, M., Lafferty, D. J., Feltner, J., Davis, B. M., Hackländer, K., Alves, P. C., Good, J. M., Melo-Ferreira, J., Dietz, A., Abramov, A. V., Lopatina, N., & Fay, K. (2018). Winter color polymorphisms identify global hot spots for evolutionary rescue from climate change. *Science*, *359*(6379), 1033-1036.

Mohr, S. E., Hu, Y., Ewen-Campen, B., Housden, B. E., Viswanatha, R., & Perrimon, N. (2016). CRISPR guide RNA design for research applications. *The FEBS journal*, *283*(17), 3232-3238.

Moore, M. V., & Lee Jr, R. E. (1991). Surviving the big chill: overwintering strategies of aquatic and terrestrial insects. *American Entomologist*, *37*(2), 111-118.

Nielsen, M. E., Nylin, S., Wiklund, C., & Gotthard, K. (2023). Evolution of butterfly seasonal plasticity driven by climate change varies across life stages. *Ecology Letters*, *26*(9), 1548-1558.

Nordström, F. (1955). De fennoskandiska dagfjärilarnas utbredning. Lepidoptera, Diurna (Rhopalocera & Hesperioidea). *Kungliga Fysiografiska Sällskapets Handlingar (N.F.)*, 66, 1–157.

Nylin, S., Wickman, P. O., & Wiklund, C. (1989). Seasonal plasticity in growth and development of the speckled wood butterfly, Pararge aegeria (Satyrinae). *Biological Journal of the Linnean Society*, *38*(2), 155-171.

Nylin, S., Wickman, P. O., & Wiklund, C. (1995). Life-cycle regulation and life history plasticity in the speckled wood butterfly: are reaction norms predictable?. *Biological Journal of the Linnean Society*, *55*(2), 143-157.

Okamura, Y., Dort, H., Reichelt, M., Tunström, K., Wheat, C. W., & Vogel, H. (2022). Testing hypotheses of a coevolutionary key innovation reveals a complex suite of traits involved in defusing the mustard oil bomb. *Proceedings of the National Academy of Sciences*, *119*(51), e2208447119.

Pattanayak, V., Lin, S., Guilinger, J. P., Ma, E., Doudna, J. A., & Liu, D. R. (2013). High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nature biotechnology*, *31*(9), 839-843.

Perry, M., Kinoshita, M., Saldi, G., Huo, L., Arikawa, K., & Desplan, C. (2016). Molecular logic behind the three-way stochastic choices that expand butterfly colour vision. *Nature*, *535*(7611), 280-284.

Phillips, P. C. (2005). Testing hypotheses regarding the genetics of adaptation. *Genetica*, *123*, 15-24.

Pruisscher, P., Larsdotter-Mellström, H., Stefanescu, C., Nylin, S., Wheat, C. W., & Gotthard, K. (2017). Sex-linked inheritance of diapause induction in the butterfly Pieris napi. *Physiological Entomology*, *42*(3), 257-265.

Pruisscher, P., Nylin, S., Gotthard, K., & Wheat, C. W. (2018). Genetic variation underlying local adaptation of diapause induction along a cline in a butterfly. *Molecular ecology*, *27*(18), 3613-3626.

Pruisscher, P., Nylin, S., Wheat, C. W., & Gotthard, K. (2021). A region of the sex chromosome associated with population differences in diapause induction contains highly divergent alleles at clock genes. *Evolution*, *75*(2), 490-500.

Ragland, G. J., Armbruster, P. A., & Meuti, M. E. (2019). Evolutionary and functional genetics of insect diapause: a call for greater integration. *Current opinion in insect science*, *36*, 74-81.

Ragland, G. J., & Keep, E. (2017). Comparative transcriptomics support evolutionary convergence of diapause responses across I nsecta. *Physiological Entomology*, *42*(3), 246-256.

Sadakiyo, S., & Ishihara, M. (2011). Rapid seasonal adaptation of an alien bruchid after introduction: geographic variation in life cycle synchronization and critical photoperiod for diapause induction. *Entomologia Experimentalis et Applicata*, *140*(1), 69-76.

Sander, J. D., & Joung, J. K. (2014). CRISPR-Cas systems for editing, regulating and targeting genomes. *Nature biotechnology*, *32*(4), 347-355.

Saunders, D. S. (2010). Photoperiodism in insects: migration and diapause responses. *Photoperiodism: The biological calendar*, 218-257.

Shirai, Y., Ohde, T., & Daimon, T. (2021). Functional conservation and diversification of yellow-y in lepidopteran insects. *Insect Biochemistry and Molecular Biology*, *128*, 103515.

Shreeve, T. G. (1986). Egg-laying by the speckled wood butterfly (Pararge aegeria): the role of female behaviour, host plant abundance and temperature. *Ecological Entomology*, *11*(2), 229-236.

Siemers, I., von Schmalensee, L., Ziesemer, S., Gotthard, K., & Lehmann, P. (2024). Predictable local adaptation in butterfly photoperiodism but not thermal performance along a latitudinal cline. *Ecosphere*, In Press

Sturtevant, A. H. (1920). Genetic studies on Drosophila simulans. I. Introduction. Hybrids with Drosophila melanogaster. *Genetics*, *5*(5), 488.

Suwa, A., & Gotoh, T. (2006). Geographic variation in diapause induction and mode of diapause inheritance in Tetranychus puerariсola. *Journal of Applied Entomology*, *130*(5), 329-335.

Söderlind, L., & Nylin, S. (2011). Genetics of diapause in the comma butterfly Polygonia c-album. *Physiological Entomology*, *36*(1), 8-13.

Tauber, C. A., Tauber, M. J., & Nechols, J. R. (1977). Two genes control seasonal isolation in sibling species. *Science*, *197*(4303), 592-593.

Tauber, M. J., Tauber, C. A., & Masaki, S. (1986). *Seasonal adaptations of insects*. Oxford University Press, USA.

Tison, J. L., Edmark, V. N., Sandoval-Castellanos, E., Van Dyck, H., Tammaru, T., Välimäki, P., Dalén, L. & Gotthard, K. (2014). Signature of post-glacial expansion and genetic structure at the northern range limit of the speckled wood butterfly. *Biological Journal of the Linnean Society*, *113*(1), 136-148.

Traut, W., & Marec, F. (1996). Sex chromatin in Lepidoptera. *The Quarterly Review of Biology*, *71*(2), 239-256.

Urbanski, J., Mogi, M., O'Donnell, D., DeCotiis, M., Toma, T., & Armbruster, P. (2012). Rapid adaptive evolution of photoperiodic response during invasion and range expansion across a climatic gradient. *The American Naturalist*, *179*(4), 490-500.

Välimäki, P., Kivelä, S. M., Mäenpää, M. I., & Tammaru, T. (2013). Latitudinal clines in alternative life histories in a geometrid moth. *Journal of Evolutionary Biology*, *26*(1), 118-129.

Wang, D., Chen, J., Yuan, Y., Yu, L., Yang, G., & Chen, W. (2023). CRISPR/Cas9-mediated knockout of period reveals its function in the circadian rhythms of the diamondback moth Plutella xylostella. *Insect Science*, *30*(3), 637-649.

Wang, X., Cao, X., Jiang, D., Yang, Y., & Wu, Y. (2020). CRISPR/Cas9 mediated ryanodine receptor I4790M knockin confers unequal resistance to diamides in Plutella xylostella. *Insect Biochemistry and Molecular Biology*, *125*, 103453.

Wiklund, C., Persson, A., & Wickman, P. O. (1983). Larval aestivation and direct development as alternative strategies in the speckled wood butterfly, Pararge aegeria, in Sweden. *Ecological Entomology*, *8*(2), 233-238.

Wiklund, C., Wickman, P. O., & Nylin, S. (1992). A sex difference in the propensity to enter direct/diapause 553 development: a result of selection for protandry. *Evolution*, *46*(2), 519-528.

Williams, C. T., Barnes, B. M., Kenagy, G. J., & Buck, C. L. (2014). Phenology of hibernation and reproduction in ground squirrels: integration of environmental cues with endogenous programming. *Journal of Zoology*, *292*(2), 112-124.

Yamada, H., & Yamamoto, M. T. (2011). Association between circadian clock genes and diapause incidence in Drosophila triauraria. *PLoS One*, *6*(12), e27493.

Paper I

Repeated evolution of photoperiodic plasticity by different genetic architectures during range expansions in a butterfly

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Abstract

Local adaptation can cause variation in phenotypic traits. In cases of recurrent range expansions originating from the same base population, it is commonly expected that repeated phenotypic adaptation is caused by similar changes in genetic variation. However, it is also becoming increasingly clear that this prediction is not always upheld and that similar phenotypic variation may evolve by alternative genetic pathways. Here we are exploring the repeated evolution of plasticity for diapause induction across several Swedish populations of the Speckled wood butterfly *Pararge aegeria*. Winter diapause in this species is induced by short photoperiods in advance of the onset of cold conditions, and the photoperiodic reaction norm varies adaptively among populations from different latitudes. Previous work shows that *P. aegeria* has colonized Scandinavia at least twice and that the results of these two colonizations are still largely geographical separated, in the very south and more northern regions of Sweden, respectively. Moreover, adaptive variation in photoperiodic plasticity has evolved independently after both these colonization events and is now present as variation across populations. We build on previous genomic result showing that one of the candidate regions that is associated with variation in photoperiodism is situated on the Z-chromosome and assay hybrid crosses between populations for effects on the photoperiodic reaction norm. We show that while a cross between populations that are from the two different colonization events show a strong sex-dependent inheritance of photoperiodic plasticity, a similar cross between populations within the oldest colonization show no such effect. This suggests that similar adaptive variation in diapause induction has evolved through partly different genetic variation across the species range in Sweden. Therefore, we conclude that the genotype-phenotype map varies across these populations in a trait that evolve relatively rapidly to form local adaptations across populations. We speculate that this may be because the standing genetic variation for photoperiodic plasticity differed in the two separate expansions into Sweden and that natural selection "solved" the same environmental challenge by altering somewhat different genomic regions.

Introduction

Environmental conditions and resource availability often vary considerably across a species range and can result in spatial variation in natural selection (Hereford, 2009; Savolainen et al, 2013; Agrawal, 2020). Variation in local adaptation between populations is one of the major sources for generating diversity within species (Savolainen et al., 2013). Parallel changes in environmental conditions, for instance due to repeated dispersal events, are expected to

lead to repeated evolution of similar adaptive phenotypic differences between populations (Haldane, 1932; Gompel & Prud'homme, 2009). A common expectation is that these adaptive phenotypic differences are due to similar changes in genetic variation across multiple occasions of local adaptation, termed "gene-reuse" (Conte et al., 2012; Bohutínská & Peichel, 2023). However, it is possible that more complex phenotypic adaptations may evolve in parallel by different, alternative, changes in genetic variation (Barghi et al., 2020). If so, the genotype-phenotype map will vary across populations.

The evolution of similar phenotypes across similar environments is common across all life (Yokoyama & Yokoyama, 1990; Losos et al., 1998; Blackledge & Gillespie, 2004; Donley et al., 2004; Harrison et al., 2005; Wooding et al., 2006; De Busschere et al., 2012). Similar adaptive phenotypes are expected to be caused by natural selection (Losos, 2011) and are often studied to investigate the underlying mechanism and predictability of local adaptation (Gompel & Prud'homme, 2009; Elmer & Meyer, 2011; Conte et al., 2012). A classic example is the repeated evolution of a reduction in armor plates across the body when the marine three-spined stickleback (*Gasterosteus aculeatus)* colonized several freshwater lakes and streams. For this trait, the decrease in armor plates was found to predominantly be caused by repeated fixation of the same alleles in one single gene (Colosimo et al., 2005). However, in similar cases of parallel evolution phenotypic differences evolves repeatably though different genetic variation (Borowsky, 2008; Ravinet et al., 2015). For instance, the rough periwinkle snail (*Littorina saxatilis*) has two distinct ecotypes that have adapted in parallel at several different shores, where one ecotype is adapted to strong predation risk from crabs and the other to heavy wave action. Despite a distance of less than 10 km between different, parallel expansions to the two habitats, the amount of shared genomic variance within ecotypes is limited across three separate expansions in Sweden (Ravinet et al., 2015). These types of differences in genotype-phenotype associations across study systems may be explained by variation in the time since divergence (Conte et al., 2012).

Range expansions across latitudes is a common process that is expected to happen in parallel across regions, and have led to the evolution of similar adaptive phenotypic differences, both within and between species (Parmesan et al., 1999; Hickling et al., 2005; Lancaster, 2016). An example of such phenotype is plasticity for diapause induction in insects. Diapause is a type of dormancy that allow insects to survive periods not suitable for growth and reproduction, such as cold winters. The current data suggests that winter diapause have evolved repeatedly across species, as

insects have expanded and shifted their range from the southern tropical regions into the northern temperate zone (Ragland & Keep, 2017). As many temperate insects may produce several generations per year, they have the capacity to express both non-diapause and diapause development. The expression of these two alternative developmental pathways is controlled by adaptive plasticity in relation to seasonal cues such as photoperiod and temperature (Tauber et al, 1986; Denlinger, 2002). The life stage(s) where diapause can be expressed is typically species-specific but may vary across species (Gill et al., 2017). During diapause the individual will suppress its metabolism and arrest development until conditions improve, for instance the arrival of spring (Tauber et al., 1986). The decision to induce winter diapause is typically made in late summer or early autumn, well before the onset of cold conditions (Clark & Platt, 1969; Nylin et al., 1995). Since the length of the growth season varies depending on latitude and altitude, populations of the same species often differ in the photoperiod that induces diapause (Bradshaw, 1976; Nylin, 1995; Kato, 2005; Aalberg Haugen & Gotthard, 2015). A frequently used metric to assess this variation across populations is the critical photoperiod, which is the daylength where 50% of a population enters diapause (Danilevskii, 1965; Hut et al., 2013; Lankinen et al., 2021).

While the diapause phenotype appears conserved across insect species its genetic background seems to vary (Ragland et al, 2019). For instance, a study of the flesh fly (*Sarcophaga bullata*) suggests that the diapause response is controlled by a single gene or a small gene cluster (Han & Denlinger, 2009) while a study of the speckled wood butterfly (*Pararge aegeria*) indicate a combination of several genes with high and low effect size (Pruisscher et al., 2018). Additionally, some studies find that the genetic background is sex-linked (King et al., 1974; Pruisscher et al., 2017, 2018) while others find it to be almost exclusively determined by autosomal alleles (Tauber et al., 1977; Han & Denlinger, 2009; Söderlind & Nylin, 2011). However, if similar phenotypic variation in diapause induction may evolve by different genetic pathways also in the case of repeated evolution within species is unknown (Barghi et al., 2020).

Interestingly, variation in the seasonal timing of diapause is often associated with variation at circadian clock genes, which often are found both on sex chromosomes and on autosomes (Han & Denlinger, 2009; Gotthard & Wheat, 2019; Pruisscher et al., 2018, 2021). The circadian clock is a mechanism to time the day-night cycle and although there is substantial variation across species, the timer is often also associated with photoperiodism (Tauber & Kyriacou, 2001; Koštál, 2011; Saunders & Bertossa, 2011; Goto, 2013; Meuti & Denlinger, 2013; Saunders, 2020). It has been proposed that the circadian clock's light-dark cycle may provide information about the change in daylength across

time necessary for photoperiodism (Bünning, 1936; Bünsow, 1953). Still, the potential connection between the circadian clock and photoperiodism of diapause induction is not well understood (Goto, 2022) and the clock genes that are associated with the diapause response varies between species (Han & Denlinger, 2009; Pruisscher, 2018, 2021). If the circadian clock can contribute in different ways to photoperiodism between species, it may also contribute differently to variation among populations.

In this study we aim to investigate if variation in induction of winter diapause has the same genetic background across two expansions of the speckled wood butterfly (*Pararge aegeria*) into Sweden. The genetic relationships between populations and entomological records both suggest that the species has expanded into Sweden twice since the last glaciation. Present-day central and northern Swedish populations are descendants from an early expansion that occurred before there are any records while the most southern populations are the result of a second more recent expansion less than 100 years ago (~ year 1930) (Nordström, 1955; Tison et al., 2014) (se figure 1). By comparing the underlying genetic variation causing a difference in the response to photoperiod between and within these two present-day distributions it is possible to assess if repeated adaptive variation in the phenotype relies on the same or a different genetic variation. Importantly, *P. aegeria* has a well-studied cline in the critical photoperiods in Sweden (Aalberg Haugen & Gotthard, 2015) that is regulated by several genes (Pruisscher et al., 2018).

A previous study of the genetic background to adaptive divergence in the critical photoperiod investigated population crosses between two distant populations of *P. aegeria* in Sweden, and found a strong association between phenotypic variation in the critical photoperiod and two genomic regions, one being sex-linked (on the Z-chromosome) and the other autosomal, that included the central clock genes *period* and *timeless,* respectively. These two populations originated from the two different expansion events, one from the oldest expansion (a population at the species most northern rage margin, Sundsvall) and one from the more recent expansion (the most southern population, Skåne) (Pruisscher et al., 2018). To explore if a similar phenotypic divergence found among two different populations, both originating from the oldest expansion event, has evolved by similar genetic changes we here tested for the presence of a similar sex-linked effect. We did this by exploring phenotypic differences of the reciprocal F1 crosses between these two more closely related populations, and compared the results to the original data of the study by Pruisscher et al. (2018). A lack of sex-linkage of the diapause response between these two closely related populations from the first expansion would suggest that variation in critical photoperiod within the oldest expansion is not dependent on

variation at the Z-chromosome, and therefore does not rely entirely on the same genetic variation as within the second expansion.

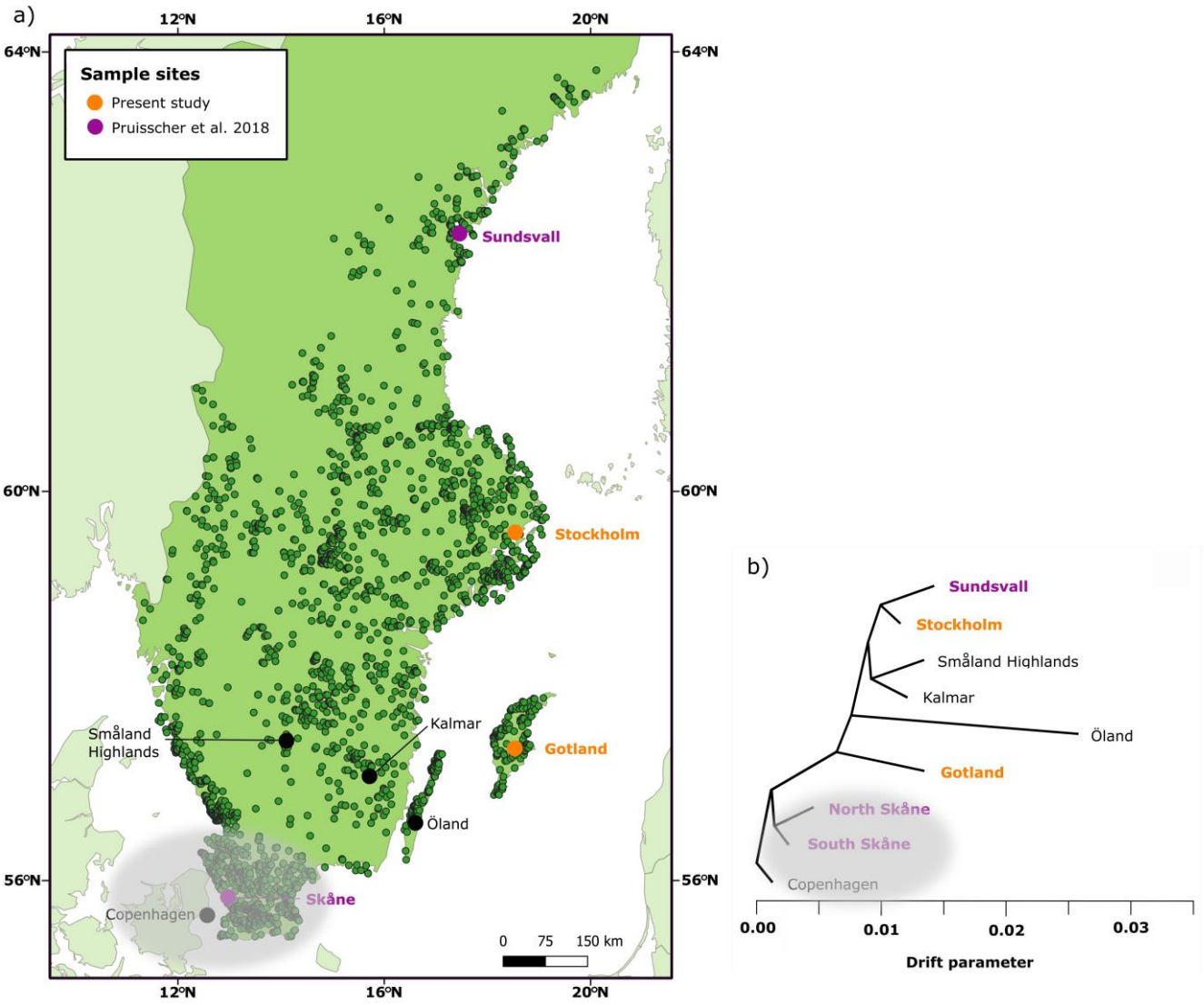

Figure 1. a) Map of Sweden highlighting the sample sites. The green points are where adult P. aegeria *have been observed from January 2017 to May 2023 according to Artportalen.se. The orange and purple points show sample sites; the orange points are the sample sites from the present study (Stockholm and Gotland) and the purple points are the sample sites from Pruisscher et al. 2018 (Sundsvall and Skåne). The grey bubble covering south of Sweden and parts of Denmark shows where the populations from the most recent expansion presently occur. b) Phylogeographic relationship of seven populations of* P. aegeria *in Sweden based on figure 1b in Lindestad et al. 2022. The phylogeographic is based on whole-genome sequences and branch length indicates amount of genetic drift. The populations sampled in the present study is marked with orange and the populations sampled in Pruisscher et al. (2018) are in purple. The most recent expansion is covered with a grey bubble.*

Methods

The speckled wood butterfly (*Pararge aegeria*) is distributed across Europe and North America (Livraghi et al., 2018). It is a woodland species whose larvae feed on a diversity of grass species, including *Dactylis glomerata* and *Poa annua* (Shreeve, 1986). The species is a well-studied model organism in regards to seasonal adaptation and voltinism (Wiklund et al., 1983; Shreeve, 1986; Merckx & Van Dyck, 2006; Aalberg Haugen & Gotthard, 2015; Pruisscher et al., 2018; Lindestad et al., 2021). In Sweden it is distributed across a latitudinal cline with genetically distinct populations showing local adaptation in voltinism (Lindestad et al., 2019, 2021). The variation in voltinism is controlled by seasonal plasticity in response to photoperiod and in particular to the induction of diapause in response to shorter days in late summer (Lindestad et al., 2019). The populations across Sweden have different critical photoperiods (Aalberg Haugen & Gotthard, 2015), which is defined as the photoperiod resulting in 50% of the individuals in a population entering diapause (Koštál, 2011). From the southern part of the Swedish distribution (Skåne) to the northern range margin (Sundsvall) the critical photoperiod differs by approximately 2.7h (16.3h to 19h) (Aalberg Haugen & Gotthard, 2015). The variation in critical photoperiod is accompanied by variation in voltinism where the southern populations have two annual generations (bivoltine) while the northern populations only have one generation per year (univoltine) (Lindestad et al., 2019). While *P. aegeria* is capable of winter diapause during both the larval and pupal stages (Wiklund & Friberg, 2011) in Sweden it mainly diapauses as pupa (Wiklund et al., 1983).

The two populations of *P. aegeria* that was the main focus of this study are from just north of Stockholm and from the Island of Gotland, respectively. They are situated approximately 245 kilometers apart and are relatively closely related. A population genomic analysis suggests that there has been gene flow from Stockholm to Gotland (Lindestad et al., 2022). Despite this, the two populations have a substantial difference in their critical photoperiod of around 1.4h (Gotland≈17.2h and Stockholm≈18.6h) (Aalberg Haugen & Gotthard, 2015). The main aim of the present study was to investigate if this difference in the critical photoperiod is dependent on genetic variation on the Z-chromosome, which was found to be the case in the comparison of the more distantly related populations from southern Sweden and the northern range margin in Sweden. For this we created F1 reciprocal hybrid crosses of the Stockholm and Gotland populations. As females are the heterogametic sex in butterflies (ZW) the females of the F1 crosses only have one Z-chromosome that they have inherited from their father, whereas they are heterozygotes on all loci on the autosomes. The males which are ZZ are consequently heterozygotic for all loci on all chromosomes in

these F1 hybrids. Therefore, we wanted to test if the diapause response of the F1 females differed between the two reciprocal hybrid crosses, which is predicted if genetic variation on the Z-chromosome to some degree influences the differences between our two original populations.

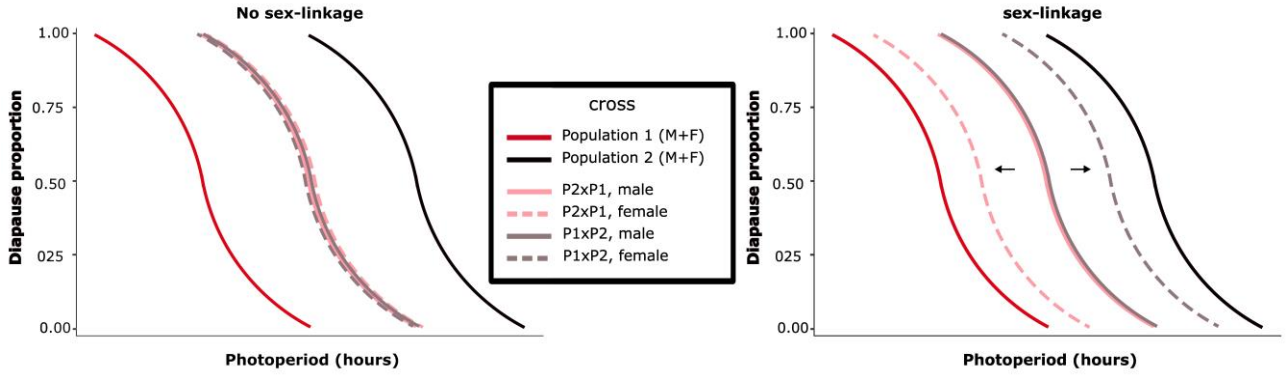

Figure 2. Illustration of expected results when analyzing diapause proportions of reciprocal crosses in case of a) no sex-linkage and b) a sex-linkage explaining part of the difference in the diapause response between the two original populations. The reciprocal males are expected to have a reaction norm that is intermediate to the two original populations. The same is expected of the reciprocal female crosses if there is no sex-linkage. If the reciprocal female crosses differ it would indicate that genetic variation at the Z-chromosome influence the difference in the critical photoperiod of the two original populations.

In June 2021 wild mated females of the speckled wood butterfly (*Pararge aegeria*) were collected from the Baltic Island of Gotland (57.40 N, 18.52 E, 15 wild females) and from the mainland north of Stockholm (Riala) (59.60 N, 18.54 E, 16 wild females). All females were allowed to oviposit individually in 0.5 L plastic cups with access to the host plant, *Poa annua*. At hatching, larvae were reared in 30x40x50 cm net cages in long-day conditions causing non-diapause development in both populations (22:2h light:dark, 23°C). Within each cage were up to 15 larvae from each of two to three females from the same population and they were fed ad libitum a second host plant *Dactylis glomerata*. The resulting adults (157 Gotland (G) and 99 Stockholm (S)) were used to produce F1 offspring of the two natural populations (SS and GG) as well as reciprocal F1 population hybrids (SG and GS, female first). In total 13 GG, 15 SS, 22 GS, 21 SG successful crosses were completed, with none of the females mating with the same male. The F1 larvae from all crosses were placed individually in 0.5 L plastic cups on the grass *Poa annua* and distributed among five climate cabinets with a standard temperature of 18°C and one of the following photoperiods: 17h, 17,4h, 17,8h, 18,2h and 18,6h, which are covering the range of photoperiods intermediate to the critical photoperiods of the two original populations (Gotland≈17.2h and Stockholm≈18.6h) (Haugen et al., 2014). Each climate cabinet contained a logger,

confirming correct photoperiod and low variation in temperature throughout the experiment. Each photoperiod treatment contained 15 GG, 16 SS, 44 GS and 46 SG individuals evenly distributed from the individual crosses and with maximum three siblings per treatment. All individuals were grown individually in the cups and were checked daily. Whenever the quality of the plant had deteriorated or was consumed by the larva, they were supplied with fresh host plants, ensuring ad libitum food throughout the experiment. For each individual the following was recorded: start date of the experiment, date of pupation, pupal weight, sex, adult eclosion date and adult weight. Pupal and adult weight were measured on a Precisa 205 A SCS balance with a precision of 0.1 mg. Pupal weight was recorded two days after pupation to secure sufficient hardening of the cuticle before handling. Sex of each individual was determined in the pupal stage and corroborated in all adults that eclosed (Friberg et al., 2011).

The populations of *P. aegeria* in this study enter winter diapause in the pupal stage (Aalberg Haugen & Gotthard, 2015). The decision to diapause is a two-step process and involves two major deviations from non-diapause development. First a decision to prolong larval development in order to time pupation before the start of winter followed by a second and final decision to postpone adult eclosion until the following spring (Nylin et al. 1989, Lindestad et al. 2019; 2021). These are two separate plastic developmental decisions that each have their own photoperiodic reaction norms (Lindestad et al., 2019, 2021) and can evolve independently (Nielsen et al., 2023). The two decisions may both contribute to the phenotypic variation in diapause induction across the butterfly's range in Sweden and, therefore, in this study we analyzed them separately. The decision of larval development time (hereafter termed "larval decision") is made throughout larval development and is especially expressed in 3rd and 4th larval instar (Lindestad et al., 2019, 2021). Larvae that enter non-diapause development typically develops quickly through the larval stage and pupate within 20-37 days after egg hatch (at 17°C -18°C) (Pruisscher et al., 2018; Lindestad et al., 2019) whereas individuals that enter the diapause pathway have markedly longer larval development (Pruisscher et al., 2018; Lindestad et al., 2019). Therefore, in line with previous studies, the larval decision was scored as non-diapause development when larval development time was shorter than 40 days. The decision of whether to delay adult eclosion (from here on termed "pupal decision") occur in the fourth and last larval instar, just before pupation. Individuals that enter non-diapause pupal development will spend 12-20 days in the pupal stage (at 18°C) while pupal diapause lasts up to 8 months (Lindestad et al., 2020). Also, in this case we followed earlier studies and scored the pupal decision as non-diapause development when eclosion occurred within 25 days from pupation. Cases where the

plastic decision of individuals could not be assessed where excluded from the data, for instance individuals that died during larval development (79 individuals in total (13.1%)) as well as individuals that died within the first 25 days of pupal development (5 individuals in total (2%)).

To explore if *P. aegeria's* two separate expansions into Sweden have evolved divergence in critical photoperiods based on a similar sex-linked effect we did a direct comparison of the present study with the data from Pruisscher et al. (2018). As in the present study, Pruisscher et al. (2018) assayed the diapause proportions of the larval decision of F1 offspring and reciprocal F1 population hybrids. The results showed that the hybrid females with a father from the south (Skåne) did have a markedly lower diapause proportion than hybrid females with a northern father (Sundsvall) while the males did not differ between reciprocal crosses. This indicated that genetic variation at the sex-chromosomes influences the difference in the critical photoperiods of the Sundsvall and Skåne populations, which was also supported by whole-genome sequencing of these crosses. By directly comparing the results from the present study to this earlier study it is possible to test if a similar sex-linked effect can explain at least parts of the difference in the critical photoperiods between the Stockholm and Gotland populations. However, while the present study measured the hybrids' diapause response in considerable detail across the entire range of photoperiods between the critical photoperiods of the original populations, Pruisscher et al. (2018) measured this response at one single photoperiod where the diapausing response of the two original populations were at either 0% or 100% diapause. For the direct comparison of the original data from the two studies all data from the five photoperiods used in the present study was pooled within cross, resulting in one treatment per cross.

Statistical analysis

All analyses were carried out using R version 4.1.2 for Windows and a P-value of 0.05 was applied throughout. The larval and pupal developmental decisions were scored using the criteria described above as either non-diapause (0) or diapause (1), adhering to binomial distributions. The link was set as "logit".

In the analysis of the present comparison of Stockholm and Gotland we first tested if the diapause response of the hybrids (independent of reciprocal cross) deviated from the response of the two founding populations by pooling the data from both reciprocal hybrid crosses. A generalized linear mixed-effect model was fitted to the data to test if the response of diapause to variation in photoperiod was dependent on the factors cross (GG, SS and (GS+SG)), treatment

(photoperiod 17, 17.4, 17.8, 18.2, 18.6) and sex (female, male). “Family” was added as a random factor, since there were up to 3 siblings in each treatment. During exploratory model analysis, challenges arose in achieving convergence for models incorporating interactions. Given our specific interest in determining whether the crosses exhibit variation in diapause response, we chose to construct a model containing solely the main factors and the random effect. In case of a significant effect of cross, a Tukey test was performed using the emmeans package (Lenth, 2021) to investigate which crosses differed from each other.

To answer our main question of whether sex-linked genes could explain some part of the difference in critical photoperiod between the natural Gotland and Stockholm populations, we tested if there was a difference in diapause response between the females from the two reciprocal hybrid crosses while we expect no such difference between the males from the reciprocal crosses (i.e. a significant sex*reciprocal cross interaction). We used a generalized linear mixed-effects model with “Family” added as random factor. As explanatory variables we added the factors cross (GS, SG), treatment (photoperiod 17, 17.4, 17.8, 18.2, 18.6) and sex (female, male). Once more, during exploratory model analysis, convergence for the full model and models incorporating several interactions did not converge. Therefore, as the effect of the cross:sex interaction is the direct test of the hypothesis it was the only interaction included in the final model.

Finally, to directly test if the present study showed a pattern of sex-dependent inheritance that differed from the earlier study of the two others, more distantly related, populations (Pruisscher et al. 2018), we compared the data from both experiments. For this we used the original data on F1 reciprocal hybrids from both studies and categorized the populations in each study as either from the north (Sundsvall and Stockholm) or south (Skåne and Gotland) depending of the origin of each individuals’ father. To investigate if the two studies showed similar patterns we were interested in if the sexes had similar diapause responses depending on their father’s origin in the two studies (a three-way interaction). Therefore, we used generalized linear model (GLM) and as explanatory variables we added the factors father’s origin (father originating from the north/south), sex (male/female) and experiment (present/Pruisscher et al. 2018). Model selection was performed by initially including all interactions and subsequently removing insignificant interactions to find the model with the lowest AIC value. However, in the final model we included the interaction father’s origin:sex:experiment, since it is the direct test of the hypothesis.

Since the individuals were scored as non-diapausing if their larval development time was below 40 days and some larvae occasionally gets close to developing for 40 days we performed a sensitively analysis. This was done by repeating all the above-mentioned analyses but with individuals regarded as non-diapausing if their larval development time was below 36 days or 44 days.

Results

When the two reciprocal hybrid crosses were pooled, the diapause response of the F1 offspring was clearly intermediate to the two natural populations across all five photoperiods, both for the larval and pupal decision (figure 3a and 3b). The effect of F1 cross on diapause response was highly significant for both the larval and pupal decision (cross; larval decision: $\chi^2(2) = 35.96$, $P < 0.001$, pupal decision: $\chi^2(2) = 39.51$, $P < 0.001$). Additionally, performing a Tukey test revealed that the difference in diapause response between the crosses (GG, SS and (GS+SG)) was significant in all pairwise comparisons for both decisions (larval decision; GG-SS: $P < 0.001$, SS-(GS+SG): $P < 0.001$, GG-(GS+SG): $P = 0.0012$, pupal decision; GG-SS: $P < 0.001$, SS-(GS+SG): $P < 0.001$, GG-(GS+SG): $P < 0.001$). This suggests that variation in both the larval and pupal decisions have a strong genetic basis. In addition, for both decisions there was a significant effect of sex (larval decision: $\chi^2(1) = 18.71$, $P < 0.001$, pupal decision: $\chi^2(1) = 17.36$, $P < 0.001$) and treatment (larval decision: $\chi^2(4) = 90.93$, $P < 0.001$, pupal decision: $\chi^2(4) = 92.86$, $P < 0.001$). The difference in sex indicates that females generally diapause at a shorter photoperiod than males, which has previously been observed in this and other butterflies (Wiklund et al., 1992).

To investigate the hypotheses of any Z-linked effect on the difference in critical photoperiods between the Stockholm and Gotland populations, we next analyzed the diapause responses of the two reciprocal hybrid crosses. Also, in this analysis we tested the larval and pupal decision separately (figure 3c and 3d). The cross:sex interaction was non-significant for both decisions (larval decision: $\chi^2(1) = 0.07$, p=0.7980, pupal decision: $\chi^2(1) = 0.59$, $P = 0.4426$), indicating that genetic variation at the Z-chromosome did not contribute significantly to the difference in the diapause response between the Stockholm and Gotland populations. In both the larval and pupal decisions the photoperiod treatment (larval decision: $\chi^2(4) = 76.93$, $P < 0.001$, pupal decision: $\chi^2(4) = 80.76$, $P < 0.001$) and sex (larval decision: $\chi^2(1) = 13.75$, $P < 0.001$, pupal decision: $\chi^2(1) = 7.30$, $P = 0.0069$) had significant effects on the diapause response, whereas there was no significant effect of cross in any of the decisions (larval decision: $\chi^2(1) = 2.35$, $P = 0.1250$, pupal

decision: $\chi^2(1) = 0.04$, $P = 0.8366$). The difference between sexes indicates that, similarly to the original populations, the reciprocal hybrid females enter diapause at a shorter photoperiod than the males.

Finally, we compared the findings from our reciprocal hybrid crosses (GS and SG) with the reciprocal hybrid crosses of the Sundsvall and Skåne populations investigated by Pruisscher et al. (2018). Since that study only measured the diapause response of the hybrids at one photoperiod, the five photoperiods from the present study were pooled together for comparison. If the effect of genomic regions at the Z-chromosome on the diapause response identified by Pruisscher et al. (2018) would also be involved for explaining the difference between the two present populations we would expect no significant three-way-interaction between the origin of the father (north or south), sex and experiment. However, for the larval decision we did find a significant three-way-interaction ($\chi^2(4) = 10.92$, $P = 0.0274$), indicating that the results in the two experiment did differ (figure 4a). Hence, although parts of the difference in the critical photoperiod between the Sundsvall and Skåne populations can be explained by a Z-linked effect in their larval decision, the same does not seem to be the case for the difference in critical photoperiods between the Stockholm and Gotland populations. When testing the pupal decision, no significant three-way-interaction was found ($\chi^2(3) = 3.00$, $P = 0.3917$), indicating that for the pupal decision the reciprocal hybrids reacted similarly in the two studies, showing no Z-linked effect on diapause response (figure 4b). Instead, a significant two-way interaction was found between the origin of the father and experiment ($\chi^2(1) = 21.40$, $P < 0.001$), suggesting that the diapause responses of individuals with a father from the south or the north did differ between the experiments but that this difference did not depend on the sex of the tested individual. Having a father from Skåne compared to Sundsvall (south and north in Pruisscher et al. (2018)) effected the diapause response more than having a father from Gotland compared to Stockholm (south and north in the present study).

To secure that the results for the larval decision were robust a sensitivity analysis was performed. In the present study individuals were regarded as non-diapausing if their larval development time was below 40 days. In the sensitivity analysis the cutting point was set to below 36 days or 44 days. This caused no difference in the results in any of the above-mentioned analysis (table S4).

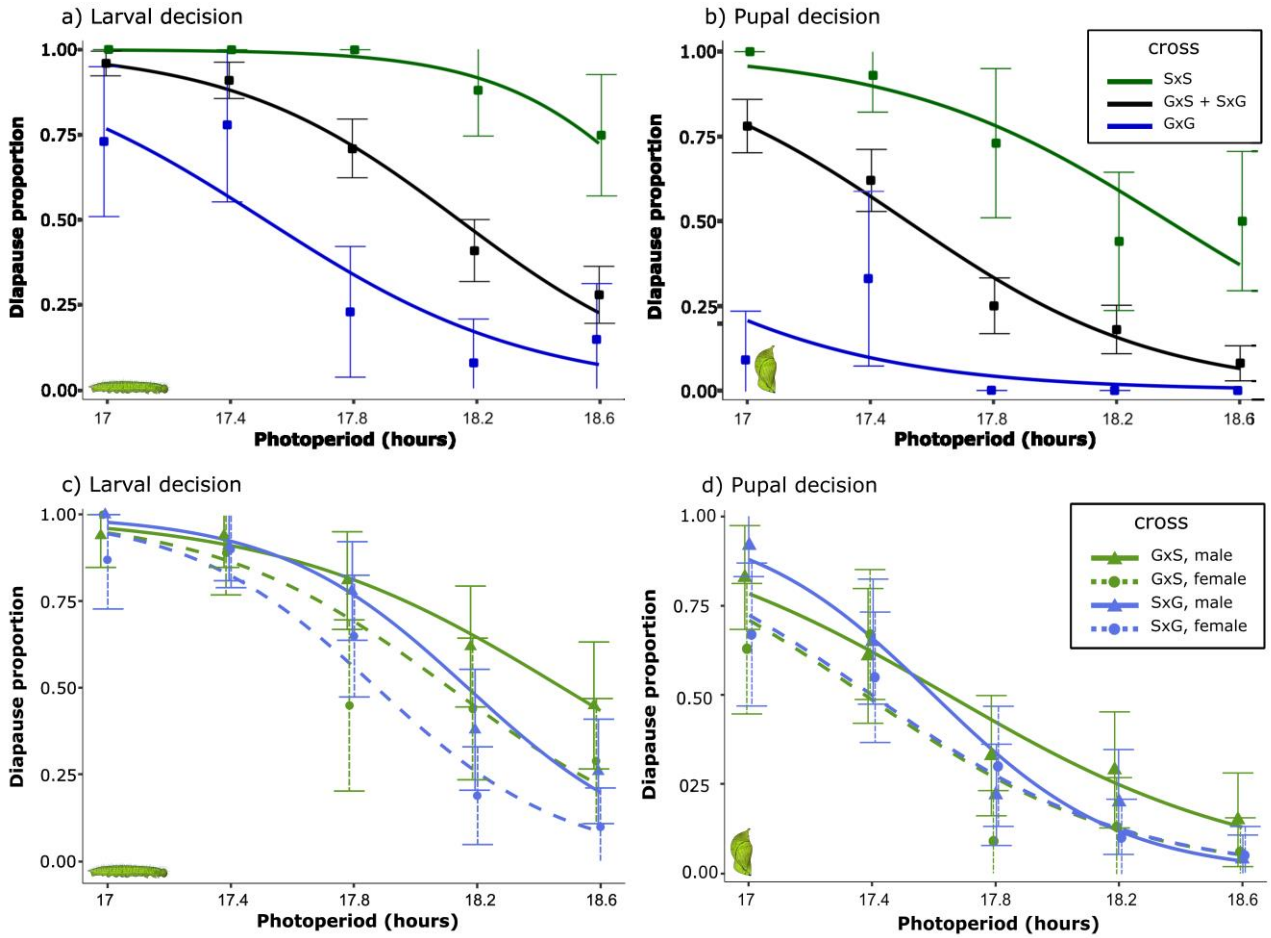

Figure 3. *Proportion of diapausing individuals at the photoperiods 17, 17.4, 18.2, 18.2 and 18.6 (left to right). All cabinets had a constant temperature of 18°C. Bars show 95% confidence intervals of actual proportions. a) Larval diapause response of the two original populations (GxG, Gotland, males and females, dark blue; SxS, Stockholm, males and females, dark green) and the reciprocal hybrid crosses pooled (GxS+SxG, males and females, black solid line). Means are depicted as squares. b) Same results but for the pupal decision. c) Larval diapause response for the two reciprocal crosses (GxS, father from Stockholm, light green; SxG, father from Gotland, light blue). Males have solid lines and means depicted as triangles while females have strippled lines and means depicted as circles. d) Same results but for pupal decision.*

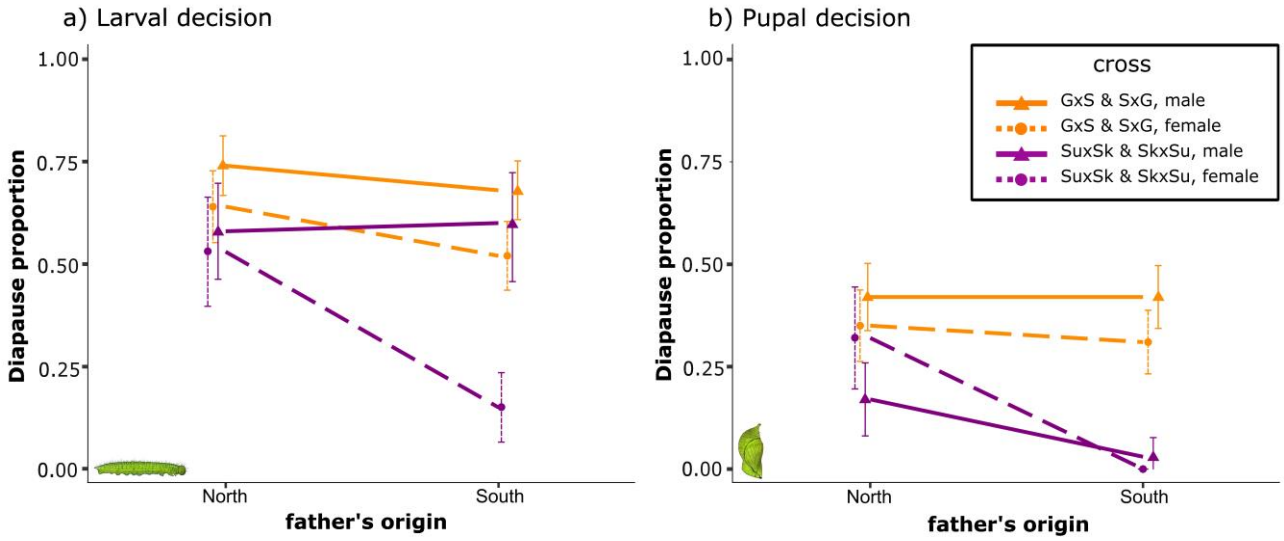

Figure 4. *Proportion of diapausing individuals in reciprocal hybrid crosses from the present (orange) and the study by Pruisscher et al. (2018) (purple). Within the studies individuals are divided by where their father originates from (Stockholm=North, Gotland=South; Sundsvall=North, Skåne=South) and by sex (Females, stippled lines and dots; Males, solid lines and triagles). Bars show 95% confidence intervals of actual proportions. a) depicts larval decision and b) depicts pupal dicision.*

Discussion

By analyzing hybrid crosses between populations of *P. aegeria* we here demonstrate that genetic variation segregating at the Z-chromosome does not explain phenotypic variation in the diapause induction between the two quite closely related populations from Stockholm and Gotland. This is contrasting to a different F1 cross between two populations that diverged much earlier in phylogeny of *P. aegeria* (Skåne and Sundsvall). A direct comparison between the two different population crosses (Gotland*Stockholm, Skåne*Sundsvall) confirmed that similar adaptive phenotypic variation in photoperiodic plasticity that has evolved during separate range expansions appear to be partly due to different genetic variation.

The present and previous studies clearly show that local phenotypic variation in photoperiodic plasticity for diapause induction is to a large degree due to genetic variation across populations of *P. aegeria* (Aalberg Haugen & Gotthard, 2015; Pruisscher et al., 2018; Lindetad et al., 2019; Nielsen et al., 2023). This is true for both of the photoperiodic plasticity of larval and pupal development, that together constitute the decision to induce “winter diapause” in the pupal stage in *P. aegeria*. Under common-garden conditions (photoperiods and temperature) the reaction norms of

the photoperiodic response for the Stockholm and Gotland populations clearly differ (figure 3a and 3b). In line with the adaptive prediction, the critical photoperiod is highest for the population at the highest latitude (Stockholm) meaning that individuals of this population enter diapause development earlier in the summer compared to the Gotland population (Hut et al., 2013). In both the larval and pupal plasticity, the F1 hybrid crosses have an intermediate reaction norm (figure 3a and 3b) which is in accordance with predictions of polygenic inheritance (Danilevskii, 1965; Tauber et al., 1986; Beck, 2012).

The sex-linked effect explaining part of the difference in the photoperiodic response between the two more distantly related populations was only detected in the larval reaction norm (figure 4a). In none of the population crosses we found evidence for a sex-linked effect in the pupal reaction norm (figure 4b). This suggests that the two decisions rely on partly different genetic mechanisms. The larval and pupal reaction norms have previously been shown to evolve at different rates in this species (Nielsen et al., 2023). Over more than 30 years of climate change the larval reaction norm had evolved significantly, while no difference in the pupal reaction norm was detected in two separate Swedish populations (Nielsen et al., 2023). The fact the diapause induction in *P. aegeria* is a consequence of two photoperiodic reaction norms that depend on partly different genetic mechanisms is likely to increase the probability that adaptive phenotypic variation can evolve by different genetic variation.

Previous whole-genome sequencing has demonstrated that variation in the critical photoperiod across populations of *P. aegeria* in Sweden is associated with variation at genomic regions containing genes of the circadian clock (Pruisscher et al., 2018, Lindestad et al 2022), which is also a pattern found in many other insects (Han & Denlinger, 2009; Yamada & Yamamoto, 2011; Ikeno et al., 2013; Pruisscher et al., 2021). In *P. aegeria*, genomic regions containing the genes *period* and *timeless* are associated with the difference in critical photoperiod between the Skåne and Sundsvall populations (Pruisscher et al., 2018). With particular relevance for the present study the *period* gene is located on the Z-chromosome in *P. aegeria* and variation within this gene may partly explain the sex-linked effect demonstrated in the study of Pruisscher et al. (2018). In contrast, the present study suggests that any influence of genetic variation at the Z-chromosome is of less importance for explaining the difference in photoperiodic plasticity between the two more closely related populations from Stockholm and Gotland. The other circadian clock gene, *timeless,* is instead situated on an autosomal chromosome (Pruisscher et al., 2018) and interestingly, the Gotland

population is fixed for a relatively large deletion in the *timeless* gene not found in any of the other populations Lindestad et al., 2022). It is interesting to speculate that this deletion may influence the photoperiodic response of the Gotland population. If so, it is possible that genetic variation for different clock genes may contribute to similar phenotypic variation across populations of *P. aegeria*, and that natural selection have "solved the problem" by favoring somewhat different combinations of genetic variants in different locations.

There is a relatively high probability that repeated phenotypic variation is caused by variation within the same genes especially across populations of the same species (Conte et al., 2012). This is likely a consequence of closely related populations sharing the same pool of standing genetic variation on which natural selection may act. However, as time since divergence increase gene reuse is likely to decrease (Bohutínská & Peichel, 2023). In the present case we do not know the time since divergence, but the photoperiodic response for diapause induction appears to have evolved through selection on partly different genetic variation across populations of *P. aegeria* in Sweden. It is conceivable that the photoperiodic response for diapause induction is influenced by a large number of genes of small effect that were segregating in the ancestral populations, making it more likely that the same phenotypic variation may evolve by different genetic variation (Gompel & Prud'homme, 2009). Alternatively, the difference in demographic history across these populations may have resulted in a difference in the standing genetic variation at the time of the two expansions into Scandinavia. For instance, it is possible that the very recent invasion into southern Sweden may have brought genetic variation at the Z-chromosome that evolved further south in Europe, after the initial expansion into Scandinavia. The first and much older expansion would then have adapted through selection on standing genetic variation at other chromosomes or mutations that arose during the northward expansion. It should be noted that there is a quantitative difference in how the timing of diapause induction varies between the two population pairs (difference in the critical photoperiod between Stockholm-Gotland ≈ 1.4 hours, and between Sundsvall-Skåne ≈ 3 hours). It is possible that the divergence in the photoperiodic timing of diapause induction of the magnitude seen between the two extreme populations in Sweden requires the evolution of Z-linked genes. Future studies of the phenotypic effects of alternative alleles at the autosomes using genetic backcrosses or gene editing in this quite trackable system, could offer valuable insight into the genotype-phenotype map of photoperiodism in *P. aegeria* and for insects in general.

Conclusion

Although it is frequently observed that closely related populations and species show parallel genetic backgrounds to parallel adaptive phenotypes, it is clear that alternative genetic variation may also underpin similar phenotypes (Conte et al., 2012; Bohutínská & Peichel, 2023). This last option seems to be the case for photoperiodic plasticity for diapause induction in *P. aegeria*. We could build on previous genomic insights and the fact that candidate regions are situated on the Z-chromosome, to explore how genetic variation at this chromosome affects variation in photoperiodic plasticity. As the differences in the diapause response was Z-linked in one population pair but not the other, we can conclude that evolution of similar phenotypic variation has evolved by partly different genetic mechanisms. This shows that the genotype-phenotype map may not be constant across a species range even for traits that appears to evolve relatively rapidly to form local adaptations across populations. This may be particularly likely for more complex traits such as adaptive plasticity evolving repeatedly during range expansions.

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References

Aalberg Haugen, I. M., & Gotthard, K. (2015). Diapause induction and relaxed selection on alternative developmental pathways in a butterfly. *Journal of Animal Ecology*, *84*(2), 464-472.

Agrawal, A. A. (2020). A scale-dependent framework for trade-offs, syndromes, and specialization in organismal biology. *Ecology*, *101*(2), e02924.

Beck, S. D. (Ed.). (2012). *Insect photoperiodism*. Elsevier.

Barghi, N., Hermisson, J., & Schlötterer, C. (2020). Polygenic adaptation: a unifying framework to understand positive selection. *Nature Reviews Genetics*, *21*(12), 769-781.

Blackledge, T. A., & Gillespie, R. G. (2004). Convergent evolution of behavior in an adaptive radiation of Hawaiian web-building spiders. *Proceedings of the National Academy of Sciences*, *101*(46), 16228-16233.

Bohutínská, M., & Peichel, C. L. (2023). Divergence time shapes gene reuse during repeated adaptation. *Trends in ecology & evolution*.

Borowsky, R. (2008). Restoring sight in blind cavefish. *Current biology*, *18*(1), R23-R24.

Bradshaw, W. E. (1976). Geography of photoperiodic response in diapausing mosquito. *Nature*, *262*(5567), 384-386.

Bünning, E. (1936). *Die Entstehung der Variationsbewegungen bei den Pflanzen* (pp. 235-347). Springer Berlin Heidelberg.

Bünsow, R. (1953). Endogene Tagesrhythmik und Photoperiodismus bei Kalanchoe blossfeldiana. *Planta*, *42*, 220-252.

Clark, S. H., & Platt, A. P. (1969). Influence of photoperiod on development and larval diapause in the viceroy butterfly, Limenitis archippus. *Journal of Insect Physiology*, *15*(10), 1951-1957.

Colosimo, P. F., Hosemann, K. E., Balabhadra, S., Villarreal Jr, G., Dickson, M., Grimwood, J., Schmutz, J., Myers, R. M., Schuter, D., & Kingsley, D. M. (2005). Widespread parallel evolution in sticklebacks by repeated fixation of ectodysplasin alleles. *science*, *307*(5717), 1928-1933.

Conte, G. L., Arnegard, M. E., Peichel, C. L., & Schluter, D. (2012). The probability of genetic parallelism and convergence in natural populations. *Proceedings of the Royal Society B: Biological Sciences*, *279*(1749), 5039-5047.

De Busschere, C., Baert, L., Van Belleghem, S. M., Dekoninck, W., & Hendrickx, F. (2012). Parallel phenotypic evolution in a wolf spider radiation on Galápagos. *Biological Journal of the Linnean Society*, *106*(1), 123-136.

Danilevskii, A. S. (1965). Photoperiodism and seasonal development of insects. *Photoperiodism and seasonal development of insects.*

Denlinger, D. L. (2002). Regulation of diapause. *Annual review of entomology*, *47*(1), 93-122.

Donley, J. M., Sepulveda, C. A., Konstantinidis, P., Gemballa, S., & Shadwick, R. E. (2004). Convergent evolution in mechanical design of lamnid sharks and tunas. *Nature*, *429*(6987), 61-65.

Elmer, K. R., & Meyer, A. (2011). Adaptation in the age of ecological genomics: insights from parallelism and convergence. *Trends in ecology & evolution*, *26*(6), 298-306.

Friberg, M., Aalberg Haugen, I. M., Dahlerus, J., Gotthard, K., & Wiklund, C. (2011). Asymmetric life-history decision-making in butterfly larvae. *Oecologia*, *165*, 301-310.

Gill, H. K., Goyal, G., & Chahil, G. (2017). Insect diapause: a review. *J. Agric. Sci. Technol*, *7*, 454-473.

Gompel, N., & Prud'homme, B. (2009). The causes of repeated genetic evolution. *Developmental biology*, *332*(1), 36-47.

Goto, S. G. (2013). Roles of circadian clock genes in insect photoperiodism. *Entomological Science*, *16*(1), 1-16.

Goto, S. G. (2022). Photoperiodic time measurement, photoreception, and circadian clocks in insect photoperiodism. *Applied Entomology and Zoology*, *57*(3), 193-212.

Gotthard, K., & Wheat, C. W. (2019). Diapause: circadian clock genes are at it again. *Current Biology*, *29*, R1245–R1246

Haldane, J. B. S. 1932. The causes of evolution. Longman, London.

Han, B., & Denlinger, D. L. (2009). Mendelian inheritance of pupal diapause in the flesh fly, Sarcophaga bullata. *Journal of Heredity*, *100*(2), 251-255.

Harrison, C. J., Corley, S. B., Moylan, E. C., Alexander, D. L., Scotland, R. W., & Langdale, J. A. (2005). Independent recruitment of a conserved developmental mechanism during leaf evolution. *Nature*, *434*(7032), 509-514.

Hereford, J. (2009). A quantitative survey of local adaptation and fitness trade-offs. *The American Naturalist*, *173*(5), 579-588.

Hickling, R., Roy, D. B., Hill, J. K., & Thomas, C. D. (2005). A northward shift of range margins in British Odonata. *Global change biology*, *11*(3), 502-506.

Hut, R. A., Paolucci, S., Dor, R., Kyriacou, C. P., & Daan, S. (2013). Latitudinal clines: an evolutionary view on biological rhythms. *Proceedings of the Royal Society B: Biological Sciences*, *280*(1765), 20130433.

Ikeno, T., Ishikawa, K., Numata, H., & Goto, S. G. (2013). Circadian clock gene Clock is involved in the photoperiodic response of the bean bug Riptortus pedestris. *Physiological Entomology*, *38*(2), 157-162.

Kato, Y. (2005). Geographic variation in photoperiodic response for the induction of pupal diapause in the Aristolochia-feeding butterfly Atrophaneura alcinous. *Applied entomology and zoology*, *40*(2), 347-350.

King, A. B. S. (1974). Photoperiodic induction and inheritance of diapause in Pionea forficalis (Lepidoptera: Pyralidae). *Entomologia Experimentalis et Applicata*, *17*(3), 397-409.

Koštál, V. (2011). Insect photoperiodic calendar and circadian clock: independence, cooperation, or unity?. *Journal of insect physiology*, *57*(5), 538-556.

Lancaster, L. T. (2016). Widespread range expansions shape latitudinal variation in insect thermal limits. *Nature climate change*, *6*(6), 618-621.

Lankinen, P., Kastally, C., & Hoikkala, A. (2021). Nanda-Hamner curves show huge latitudinal variation but no circadian components in Drosophila montana photoperiodism. *Journal of Biological Rhythms*, *36*(3), 226-238.

Lenth, R. V. (2021). emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.7.1-1. https://CRAN.R-project.org/package=emmeans

Lindestad, O., Aalberg Haugen, I. M., & Gotthard, K. (2021). Watching the days go by: Asymmetric regulation of caterpillar development by changes in photoperiod. *Ecology and Evolution*, *11*(10), 5402-5412.

Lindestad, O., Nylin, S., Wheat, C. W., & Gotthard, K. (2022). Local adaptation of life cycles in a butterfly is associated with variation in several circadian clock genes. *Molecular Ecology*, *31*(5), 1461-1475.

Lindestad, O., von Schmalensee, L., Lehmann, P., & Gotthard, K. (2020). Variation in butterfly diapause duration in relation to voltinism suggests adaptation to autumn warmth, not winter cold. *Functional Ecology*, *34*(5), 1029-1040.

Lindestad, O., Wheat, C. W., Nylin, S., & Gotthard, K. (2019). Local adaptation of photoperiodic plasticity maintains life cycle variation within latitudes in a butterfly. *Ecology*, *100*(1), e02550.

Livraghi, L., Voda, R., Evans, L. C., Gibbs, M., Dinca, V., Holland, P. W., Shreeve, Tm. G., Vila, R., Dapporto, L., & Breuker, C. J. Historical and current patterns of gene flow in the butterfly Pararge aegeria. *Journal of Biogeography*, *45*(7), 1628-1639.

Losos, J. B. (2011). Convergence, adaptation, and constraint. *Evolution*, *65*(7), 1827-1840.

Losos, J. B., Jackman, T. R., Larson, A., Queiroz, K. D., & Rodríguez-Schettino, L. (1998). Contingency and determinism in replicated adaptive radiations of island lizards. *Science*, *279*(5359), 2115-2118.

Merckx, T., & Van Dyck, H. (2006). Landscape structure and phenotypic plasticity in flight morphology in the butterfly Pararge aegeria. *Oikos*, *113*(2), 226-232.

Meuti, M. E., & Denlinger, D. L. (2013). Evolutionary links between circadian clocks and photoperiodic diapause in insects. *Integrative and Comparative Biology*, *53*(1), 131-143.

Nielsen, M. E., Nylin, S., Wiklund, C., & Gotthard, K. (2023). Evolution of butterfly seasonal plasticity driven by climate change varies across life stages. *Ecology Letters*, *26*(9), 1548-1558.

Nordström, F. (1955). De fennoskandiska dagfjärilarnas utbredning. Lepidoptera, Diurna (Rhopalocera & Hesperioidea). *Kungliga Fysiografiska Sällskapets Handlingar (N.F.)*, 66, 1–157.

Nylin, S., Wickman, P. O., & Wiklund, C. (1989). Seasonal plasticity in growth and development of the speckled wood butterfly, Pararge aegeria (Satyrinae). *Biological Journal of the Linnean Society*, *38*(2), 155-171.

Nylin, S., Wickman, P. O., & Wiklund, C. (1995). Life-cycle regulation and life history plasticity in the speckled wood butterfly: are reaction norms predictable?. *Biological Journal of the Linnean Society*, *55*(2), 143-157.

Parmesan, C., Ryrholm, N., Stefanescu, C., Hill, J. K., Thomas, C. D., Descimon, H., Huntley, B., Kaila, L., Kullberg, J., Tammaru, T., Tennent, W. J., Thomas, J. A., & Warren, M. (1999). Poleward shifts in geographical ranges of butterfly species associated with regional warming. *Nature*, *399*(6736), 579-583.

Pruisscher, P., Larsdotter-Mellström, H., Stefanescu, C., Nylin, S., Wheat, C. W., & Gotthard, K. (2017). Sex-linked inheritance of diapause induction in the butterfly P ieris napi. *Physiological Entomology*, *42*(3), 257-265.

Pruisscher, P., Nylin, S., Gotthard, K., & Wheat, C. W. (2018). Genetic variation underlying local adaptation of diapause induction along a cline in a butterfly. *Molecular ecology*, *27*(18), 3613-3626.

Pruisscher, P., Nylin, S., Wheat, C. W., & Gotthard, K. (2021). A region of the sex chromosome associated with population differences in diapause induction contains highly divergent alleles at clock genes. *Evolution*, *75*(2), 490-500.

Ragland, G. J., & Keep, E. (2017). Comparative transcriptomics support evolutionary convergence of diapause responses across I nsecta. *Physiological Entomology*, *42*(3), 246-256.

Ragland, G. J., Armbruster, P. A., & Meuti, M. E. (2019). Evolutionary and functional genetics of insect diapause: a call for greater integration. *Current opinion in insect science*, *36*, 74-81.

Ravinet, M., Westram, A., Johannesson, K., Butlin, R., André, C., & Panova, M. (2016). Shared and nonshared genomic divergence in parallel ecotypes of L ittorina saxatilis at a local scale. *Molecular ecology*, *25*(1), 287-305.

Saunders, D. S. (2020). Dormancy, diapause, and the role of the circadian system in insect photoperiodism. *Annual Review of Entomology*, *65*, 373-389.

Saunders, D. S., & Bertossa, R. C. (2011). Deciphering time measurement: the role of circadian 'clock'genes and formal experimentation in insect photoperiodism. *Journal of Insect Physiology*, *57*(5), 557-566.

Savolainen, O., Lascoux, M., & Merilä, J. (2013). Ecological genomics of local adaptation. *Nature Reviews Genetics*, *14*(11), 807-820.

Shreeve, T. G. (1986). Egg-laying by the speckled wood butterfly (Pararge aegeria): the role of female behaviour, host plant abundance and temperature. *Ecological Entomology*, *11*(2), 229-236.

Söderlind, L., & Nylin, S. (2011). Genetics of diapause in the comma butterfly Polygonia c-album. *Physiological Entomology*, *36*(1), 8-13.

Tauber, C. A., Tauber, M. J., & Nechols, J. R. (1977). Two genes control seasonal isolation in sibling species. *Science*, *197*(4303), 592-593.

Tauber, E., & Kyriacou, B. P. (2001). Insect photoperiodism and circadian clocks: models and mechanisms. *Journal of Biological Rhythms*, *16*(4), 381-390.

Tauber, M. J., Tauber, C. A., & Masaki, S. (1986). *Seasonal adaptations of insects*. Oxford University Press, USA.

Tison, J. L., Edmark, V. N., Sandoval-Castellanos, E., Van Dyck, H., Tammaru, T., Välimäki, P., ... & Gotthard, K. (2014). Signature of post-glacial expansion and genetic structure at the northern range limit of the speckled wood butterfly. *Biological Journal of the Linnean Society*, *113*(1), 136-148.

Wiklund, C., & Friberg, M. (2011). Seasonal development and variation in abundance among four annual flight periods in a butterfly: a 20-year study of the speckled wood (Pararge aegeria). *Biological Journal of the Linnean Society*, *102*(3), 635-649.

Wiklund, C., Persson, A., & Wickman, P. O. (1983). Larval aestivation and direct development as alternative strategies in the speckled wood butterfly, Pararge aegeria, in Sweden. *Ecological Entomology*, *8*(2), 233-238.

Wiklund, C., Wickman, P. O., & Nylin, S. (1992). A sex difference in the propensity to enter direct/diapause development: a result of selection for protandry. *Evolution*, *46*(2), 519-528.

Wooding, S., Bufe, B., Grassi, C., Howard, M. T., Stone, A. C., Vazquez, M., Dunn, D. M., Meyerhof, W., Weiss, R. B., & Bamshad, M. J. (2006). Independent evolution of bitter-taste sensitivity in humans and chimpanzees. *Nature*, *440*(7086), 930-934.

Yamada, H., & Yamamoto, M. T. (2011). Association between circadian clock genes and diapause incidence in Drosophila triauraria. *PLoS One*, *6*(12), e27493.

Yokoyama, R., & Yokoyama, S. (1990). Convergent evolution of the red-and green-like visual pigment genes in fish, Astyanax fasciatus, and human. *Proceedings of the National Academy of Sciences*, *87*(23), 9315-9318.

Paper II

CRISPR/Cas9-mediated high-efficiency knock-out of *yellow-y* gene and germline mutations unveils promising applications in the speckled wood butterfly *Pararge aegeria*

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Abstract

The CRISPR/Cas9 gene modifying tool has tremendous potential for research in ecology and evolutionary biology. The possibility of inducing site-specific gene manipulation enables examination of the causal connection between genetic variation and adaptive phenotypic variation. The technique is especially promising in the research of lepidopterans where the widely used genetic tool RNAi is not universally effective. However, the CRISPR/Cas9 technology has only been tested for its efficiency and ability to induce germline mutations in a limited number of ecological model species. The ability to induce germline mutations is crucial, since it ensures the transformation of all targeted cells as well as allows for the generation of numerous individuals for phenotypic analysis. Therefore, we applied the CRISPR/Cas9 technology to an ecological model species, the speckled wood butterfly (*Pararge aegeria*). We targeted the *yellow-y* gene, which is necessary for the production of black melanin, hoping to induce mutations that are easy to phenotype. We employed four alternative treatments, including variation in sgRNAs and their concentrations, to explore what factors may affect efficiency of transformation. Color changes in the head capsule of first larval instar as well as adult wing color were used as indicators of successful knockouts. Highly transformed individuals were mated and allowed to produce F1 offspring in which we investigated the presence of germline mutations. Our findings show that the CRISPR/Cas9 technique was highly efficient in knocking out the *yellow-y* gene in *P. aegeria*. Across all treatments, nearly 80% of adults exhibited mosaic knockout phenotypes with altered coloration or appeared fully transformed. Additionally, all matings between highly transformed adults resulted in fully transformed offspring, revealing high incidence of germline mutations. The results demonstrated relatively limited variation in mutation efficiency between treatments suggesting low sensitivity to the factors varied in this study in *P. aegeria*. With the ability to induce germline mutations in ecological model species it will be possible to explore the genotype-phenotype map also for ecologically relevant genetic variation across natural populations.

Introduction

CRISPR/Cas9 is a comparatively simple, cost-effective and efficient genetic tool for creating site-specific gene manipulations (Baci et al., 2022). Despite being a relatively new genetic tool, only applied in biological research since 2013 (Cong et al., 2013), CRISPR/Cas9 has rapidly gained widespread usage and has demonstrated effectiveness across

diverse systems. The technology has been successfully applied in bacteria (Jiang et al., 2013), human cells (Cong et al., 2013; Mali et al., 2013), plants (Schindele et al., 2018), invertebrates (Douris et al., 2020; Fleming et al., 2021), as well as vertebrates (Inui et al., 2014; Liang et al., 2022). The extensively used RNAi method for exploring gene function is not universally effective, and in particular it has relatively low efficiency in lepidopterans (Perrimon et al., 2010; Terenius et al., 2011). Therefore, in genetic research involving lepidopterans the CRISPR/Cas9 technology holds great promise for advancing the research. The technology has already increased the understanding of gene function in lepidopteran development, metamorphosis, pigmentation as well as mating and reproduction (Li et al., 2021; Ficarrotta et al., 2022; Han et al., 2022; Okamura et al., 2022; Xu et al., 2022; Chakraborty et al, 2023; Hanly et al, 2023; Shirk et al., 2023; Tunström et al., 2023; Li et al., 2024; Li S. et al., 2014; Tendolkar et al., 2024). However, CRISPR/Cas9 is still only widely used in the highly domesticated silkworm (*Bombyx mori*) while the practice in other lepidopterans is relatively limited (Li et al., 2021). There are research areas that can benefit from extending the application of this technique to ecological model species showing natural genetic variation for ecologically relevant traits. However, the challenge in performing CRISPR/Cas9 on a new species is that the technique has to be modified and tested for its efficiency and potential side-effects. Moreover, microinjection of CRISPR/Cas9 construct into embryos can result in mosaic individuals (Perry et al., 2016; Bi et al., 2019). Therefore, for researching traits not phenotypically apparent, it is essential to be capable of creating germline mutations to ensure mutations in the targeted tissue. Additionally, creating germline mutations also allows for the generation of many individuals for phenotyping. However, the induction of germline mutations using the CRISPR/Cas 9 method in lepidopteran ecological model species is still relatively rare (Shirai et al., 2021; Connahs et al. 2022; Li et al., 2022; Okamura et al., 2022; Wang et al., 2023).

CRISPR/Cas9 has been used to create gene knockouts as well as insert new sequences in insects and other arthropods (review, Sun et al., 2017). However, the effects of the Cas9-sgRNA concentration and number of sgRNAs per treatment may vary between different genes and systems (Bassett et al., 2013; Cong et al., 2013; Pattanayak et al., 2013; Wang et al., 2013; Mohr et al., 2016; Liu et al., 2020). Therefore, when applied to a new system, protocol optimization is necessary. Moreover, off-target effects (cleavage at unintended sites) are of great concern when utilizing CRISPR/Cas9 (Fu et al., 2013; Hsu et al., 2013; Cho et al., 2014). Several factors can influence the risk of off-target effects (Naeem et

al., 2020). For instance, while increasing the concentration of Cas9-sgRNA in the injected construct can increase mutation efficiency (Bassett et al., 2013), it can also increase the risk of off-target effects (Pattanayak et al., 2013).

The application of CRISPR/Cas9 technology offers substantial benefits to the fields of ecology and evolutionary biology (Gudmunds et al., 2022). The technology enables the exploration of the genetic foundations of adaptive traits, bridging the gap between genotype variations, adaptive phenotypic changes, and ultimately, fitness (Bono et al., 2015). For instance, the speckled wood butterfly (*Pararge aegeria*), which is investigated in this study shows substantial genetic variation in several traits related to flight morphology, mating behavior and seasonal life cycle regulation across its European range (Shreeve et al., 1987; Nylin et al., 1989, 1995; Merckx & Van Dyck, 2006; Berwaerts et al., 2008; Aalberg Haugen & Gotthard, 2015; Pruisscher et al., 2018; Taylor-Cox et al., 2020; Lindestad et al., 2019, 2022). Recent genomic studies have repeatedly shown associations between adaptive variation in traits for seasonal timing and genomic regions that contain central genes of the circadian clock (Pruisscher et al., 2018; Lindestad et al., 2022). However, the possibility of providing functional genomic insights through direct genetic manipulations in this system has still not been explored.

To measure the mutation efficiency of CRISPR/Cas9 several studies have created knock-outs of the *yellow-y* gene, a melanin-promoting factor (Perry et al., 2016; Wang et al., 2021). While the exact function of the *yellow-y* protein in the melanin synthesis pathway is still unclear it is necessary for the production of black melanin in the cuticle (body and wings) of many adult insects as well as in the pupa, larvae and larval head capsule in some species (Futahashi & Fujiwara, 2007; Futahashi & Osanai-Futahashi, 2021; Shirai et al., 2021; Wang et al., 2021). By targeting a pigment-influencing gene, mutations induced in the microinjected generation will be visually apparent and the proportion of transformed cells per individual can be evaluated. First instar *P. aegeria* larvae have a dark brown head capsule and adult wings also have dark brown background color. Like for other lepidopterans, the *yellow-y* gene most likely influences the dark color in one or both of these areas as the influence of *yellow-y* on pigmentation appears to be evolutionarily conserved across lepidopteran taxa (Shirai et al., 2021). The *yellow-y* gene is also a safe “first target” as it has already been successfully knocked-out in several lepidopterans (Perry et al., 2016; Liu et al., 2020; Shirai et al., 2021). However, the *yellow-y* gene can have pleiotropic effects and has been shown to influence hatching success and larval segmentation and molting in *Spodoptera litura* (Liu et al., 2020; Shirai et al., 2021) as well as male courtship

behavior in *D. melanogaster* and the butterfly *Bicyclus anynana* (Drapeau et al., 2003; Connahs et al., 2022). Therefore, pleiotropic effects from knocking-out the *yellow-y* gene may additionally occur in *P. aegeria*.

Here, we investigated the mutation efficiency of CRISPR/Cas9 in the butterfly *P. aegeria*. We targeted a presumed color influencing gene, *yellow-y*, making it possible to easily phenotype successful mutations. Both color differences in first instar head capsule and adult wing color were assessed. In one treatment we additionally targeted a different gene, *sepiapterin reductase* (*SPR)*, in combination with a different experiment on genes possibly influencing color in this species (Andrade & Carneiro, 2021). However, in this study we only focused on the phenotypic changes caused by a knock-out of the *yellow-y* gene. To detect potential differences in mutation efficiency depending on methodology, we created four treatments varying in the concentration of Cas9-sgRNA in the construct, the utilized sgRNAs and the number of sgRNAs per construct. Importantly, we wanted to test if we could induce germline mutations that would allow us to produce a second generation carrying the target mutation, by breeding on individuals of the microinjected generation that showed a high degree color transformation. Finally, we wanted to investigate potential side-effects of microinjections and pleiotropic effects of the *yellow-y* gene on different life history traits.

Methods

The study species

The speckled wood butterfly (*P. aegeria*) is a palearctic woodland butterfly (Livraghi et al., 2018) with a variety of grass species as host plants, including *Dactylis glomerata* and *Poa annua* (Shreeve, 1986). Newly hatched first instar larvae are white with a dark brown head capsule, but after feeding on host plant the white body becomes green making the larvae very cryptic on the host (figure 1). As the larva moults into the second instar it drops the dark head capsule and the head and body is green throughout the remaining 3 larval instars (4 in total). The pupa can be either green or brown and is well camouflaged in the natural environment (Van Dyck et al., 1998). In Sweden and most of Europe the adult has a dark brown background color, yellow and orange patches and eye-spots on both the fore- and hind wings (figure 1) (Packer, 1984; Van Dyck et al., 1997; Van Dyck et al., 1998; Talloen et al., 2009). The adult's morphology can vary slightly in how pale the background color is, the size of the patches, and number of eye-spots depending on factors like the time of the season, sex, population and larval food stress (Packer, 1984; Van Dyck et al., 1997; Windig

et al., 2004; Talloen et al., 2009). In both sexes the background color and size of patches are intermediate to highly genetically determined (Van Dyck et al., 1998). In the present study we hypothesize that both the dark brown head capsule and adult background color necessitate *yellow-y* gene function, and that a knock-out of this gene will cause significant color changes. The pupal color was not observed as the larvae were kept on fresh green grass which should result in mainly green pupae (Van Dyck et al., 1998). The green color is not expected to be influenced by the *yellow-y* gene.

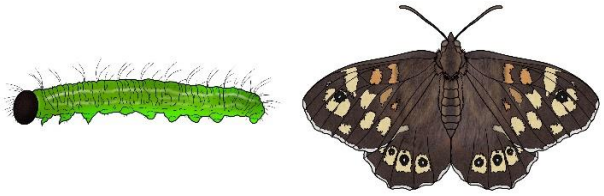

Figure 1. As a first instar the larva has a dark brown head capsule. The adult has a brown background color, yellow and/or orange patches and eye spots on both fore- and hind wings. The larval head capsule and adult background colors may be influenced by a knock-out of the yellow-y gene.

Identification of the yellow-y *gene*

Utilizing the reference genome and annotation data from Lohse et al. (2021), we determined the cDNA sequence of the *yellow-y* gene in *P. aegeria*. The *yellow-y* gene has the gene ID ENSPAGG00005015484 and is located on chromosome 5 at position 7853744-7868807. In addition to the provided annotation, a validation of the gene was conducted using the Basic Local Alignment Search Tool (BLAST) (Camacho et al., 2009), comparing it with the orthologs the painted lady butterfly (*Vanessa cardui*) (GCA_022405095.1 (Zhang et al., 2021)) and the mycalesine butterfly (*Bicyclus anynana*) (GCA_900239965.1 (Nowell et al., 2017)). Notably, in both these species researchers have previously achieved successful G_0 mosaic knock-out of the *yellow-y* gene (Perry et al., 2016; Matsuoka & Monteiro, 2018). The risk of affecting potential off-target sites was reduced by examining the *P. aegeria* reference genome and the two orthologs for duplicates or closely related genes. The same procedures were performed for the *SPR* gene (included in combination with another experiment) using sequences from the ortholog the silkworm (*Bombyx mori*) (SPR=C0STP5).

Design of sgRNAs

The sgRNA target sequences were identified and generated manually following the protocol outlined by Perry et al. (2016). The process involved several steps, beginning with the manual identification of PAM-sites (NGG sequences) within exons. Recognition and binding of the Cas9 to the targeted DNA sequence rely on the presence of a PAM-site (Sternberg et al., 2014). In the next step, we identified which among these PAM-sites had flanking sequences with GC-ratios ranging from 40% to 60%. Finally, we determined the exclusivity of these sequences to the target DNA sequences by blasting them against the *P. aegeria* reference genome using NCBI BlastN (with the BlastN-short flag and an e-value filter set at 0.01). Three distinct sgRNA target sequences for the *yellow-y* gene were selected (table 1 and figure 2), along with a single sgRNA target sequence for the *SPR* gene (table 1).

Figure 2. Target locations of the sgRNAs y1, y2 and y3 in the yellow-y *gene. The blue blocks are exons and the target sites are marked with red arrows. The gene is visualized in the 5' to 3' direction.*

Table 1: Name of sgRNAs used in this experiment and their target sequences.

Name of sgRNA	Target gene	Target sequence
Pae_y_1	*Yellow-y*	CGGAGACTTTAACATAGC
Pae_y_2	*Yellow-y*	GCAAAACGCTTTACCTGT
Pae_y_3	*Yellow-y*	ACAACAGGCCCTCCAGAC
Pae_s_4	*SPR*	ATCTATCTACTGCATCCG

Generating sgRNAs

Each sgRNA target sequence (excluding the PAM-site) was combined with the remaining structure of an sgRNA sequence and then produced as DNA from IDT (Coralville, Iowa, USA), hereafter referred to as a 'gBLOCK' (synthesized

DNA fragment). These gBlocks contained an additional two primer binding sites (P505 and M13f) flanking the sgRNA sequence to facilitate DNA amplification through PCR, and a T7-promoter sequence was included internal to these for the transcription of the DNA sequence into RNA. In sum gBlocks comprised the following elements: a M13F sequence, a T7-promoter sequence, a spacer sequence, **the target sequence**, a Cas9 binding site, and a P505 sequence.

PCR amplification of the gBlocks was conducted employing the primers M13f (GTAAAACGACGGCCAG) and P505 (AAAAAAAAGCACCGACTCGGTGCC), along with Platinum Taq (Invitrogen, cat. 10966-034). The thermal cycling program was: 94℃x120s, 35 cycles of '94℃x30s, 62℃x30s, 72℃x30s' followed by 72℃x4min. For every sgRNA, a total of four 50 µL PCR reactions were run and then combined. The combined PCR products were purified using the MinElute PCR Purification Kit with a MinElute spin column (cat. 28004, Venlo, Netherlands) following the manufacturer's protocol. The resulting templates were subsequently transcribed into RNA to generate the sgRNAs, adhering to the protocol provided in the Lucigen AmpliScribe T7-Flash Transcription kit from Epicentre/Illumina (cat. ASF3507, Madison, WI, USA). Lastly, the transcribed sgRNAs were purified using the previously mentioned purification kit. The resulting products were resuspended in Qiagen buffer EB, quantified using Qubit, and subsequently diluted to a concentration of 1000 ng/µL before being stored at -20℃ until use. In preparation for injection, the sgRNA(s) were combined with Cas9-NLS protein (PNA Bio, Newbury Park, CA, USA) at a 1:1 ratio, and the construct was diluted to its final concentration. Each day of the experiment one of the given treatments was utilized for injections over a span of 4 consecutive days. The treatment construct was kept on ice the day of injections.

Treatments

To test for the most effective solution we created four different treatments of Cas9-sgRNA constructs, that varied in sgRNA(s) as well as concentration of Cas9-sgRNA:

1. y1+y3 (500 ng/µL)
2. y1+s4 (250 ng/µL)
3. y1 (500 ng/µL)
4. y2 (250 ng/µL)

The 's4' sgRNA was chosen as a secondary target site in combination with a different experiment on a possible function of SPR on color in this species.

Collection and breeding of P. aegeria

In June 2021 wild mated females of *P. aegeria* were collected from the Baltic Island of Gotland (57.40 N, 18.52 E, 15 females). The F1 offspring were raised in cycles of 22h light:2h darkness and 23°C to induce non-diapause development while the F2 generation were reared under a set of daylengths (shorter than 18.6h light) and 17-18°C that is known to induce pupal diapause in this population. The diapausing pupae were subsequently moved to a 2°C climate cabinet with constant darkness to stimulate winter. In February 2022, after diapausing for 139-167 days, 104 pupae were moved to a climate room (12h light:12h darkness, 17°C) to induce adult eclosion. This resulted in 92 adults that could be used to produce F3 offspring. In total 35 independent matings were successfully completed after which, the mated females were placed individually in 0.5 L plastic cups with grass for oviposition. At all times the females had access to sugar water on a piece of cotton. The oviposition room had a cycle of 8h light:16h darkness (light period temperature: 27°C, dark period temperature: 18°C).

Egg preparation

To secure short time until injection of the eggs, each female's cup was exchanged for a new cup with grass every 2 hours. The cups containing eggs on grass were immediately brought to a lab where the eggs were placed on double-sided adhesive tape on ethanol-cleaned microscope slides. When placing the eggs on the double-sided tape they were preferably still on the grass, but if that was not possible because the eggs fell off the host plant, they were placed directly on the tape. Afterwards, an effort was made to cover the remaining tape as well as possible with grass to keep the larvae from getting stuck after hatching. Each plate was marked with the slide number, treatment and date of injection.

Injection

The slide with eggs was placed under the microscope and the eggs were injected with the CRISPR/Cas9 construct using a thin glass needle created using a Narishige PC-10 Dual Stage Glass Micropipette Puller. After injection, the maximum time until injection (from the time the female was given the cup with grass to the injection was done for a full slide) as well as the number of eggs on the slide were noted. The slides with injected eggs were then placed in a petri dish next to a piece of paper towel with distilled water to keep the humidity high. The petri dish was sealed with parafilm and

placed in a climate room with a 20h light:4h dark cycle and 23°C. A total of 54 slides were created with between 1 and 22 eggs in total and the maximum injection time varied from 2 hours and 20 min to 5 hours and 10 min. In total 555 eggs were injected (table 4). As controls, 34 eggs remained on the grass in the original cup from their mother (non-injected control). In addition, we added a control treatment where 114 eggs were injected with nuclease-free water (water control), to address if microinjection affects survival (table 4). We used water as it was the main ingredient in the treatment constructs. To mimic the CRISPR/Cas9 treatments water was added to an Eppendorf tube and placed on ice before use.

After injection

Every day the slides were checked for hatchings. As soon as one egg hatched on a slide the larva was scored as being a knock-out or not depending on the head capsule looking black/dark brown or light brown. The open containers had distilled water added to the paper towel daily to keep humidity high inside. The hatched larvae were placed 1-3 individuals in plastic cups with *Poa annua* grass and fed ad libitum until pupation. The cups were checked for pupation regularly. Pupation day was noted and the pupae were given 1-2 days to harden, after which they were weighted and placed individually in 0.2 L plastic containers. After eclosion and release of meconium the adults were weighed on a Precisa 205 A SCS balance (precision 0.1 mg). The color of each adult was scored and assigned to one of four categories: **wildtype**, if the background color was brown as the wildtype; **100% yellow**, if the background color appeared fully yellow; **more than 50% yellow** or **less than 50% yellow**, when an individual showed a mosaic of colors on its wings. The background color of both the dorsal and ventral side of the wings were assessed. One adult from the non-injected control treatment was scored as a more than 50% yellow. We assume that this individual was misplaced and removed it from all analyses. Three larvae from each yellow treatment that were scored as knock-outs based on their head capsules were followed until adult eclosion to assess any correlation between knock-out head capsule and adult morphology.

Assessment of germline mutations

Approximately 25 adults from treatment y1+y3, Y1+s4 and y1 belonging to the two categories with the highest penetrance (100% yellow, > 50% yellow) were placed in two 30x40x50 cm net cages containing the grass *Dactylis glomerata* in a climate room with a 20h light:4h dark cycle and 23°C. Two matings were positively observed (a female

from treatment y1+y3 and a female from treatment y1 mated with the same male from treatment y1+y3). The females that were observed mating were removed from the cages and placed individually in 0.5 L plastic cups with *Poa annua* grass to lay eggs. After hatching 15 larvae from each of the two females were randomly chosen and placed in separate plastic cups with ad libitum *Poa annua* grass. During development each individual was scored for pupation date, adult weight and sex as well as the adult phenotype.

Unobserved matings in the mating cages also produced fertile eggs that hatched and in total 45 larvae from this group were randomly chosen, scored for color of the head capsule and placed in two 30x40x50 cm net cages with *Dactylis glomerata* (20 or 25 larvae in each of the two net cages). As adults each individual was phenotyped as either **wildtype** or **yellow** depending on if their background color appeared dark brown as the wildtype or yellow due to a knock-out of the *yellow-y* gene.

Statistical analysis

The analyses were conducted utilizing R version 4.1.2, with a significance level set at 0.05 throughout. All significant effects in general linear models were further examined by Tukey tests using the emmeans package (Lenth, 2021) to identify which of the treatments differed significantly.

We first analyzed if the CRIPSR/Cas9 technique targeting the *yellow-y* gene overall had any significant effect on the morphology of the larvae or adults, separately. This was done using a chi-square test and comparing the frequency of "wildtype" to "yellow knock-out" between all four *yellow-y* treatments (treatment y1+y3, y1+s4, y1 and y2 pooled) and the two controls (water-injected control and non-injected control pooled). Individuals were considered "yellow knock-out" if they belonged to any of the three categories showing any level of transformation, 100% yellow, more than 50% yellow and less than 50% yellow.

Secondly, we investigated if the four CRISPR/Cas9 treatments differ in their efficiency. This was done by scoring the larval and pupal phenotypes as either "wildtype" or "yellow knock-out", adhering to binomial distributions. The category "yellow knock-out" again included individuals from all three knock-out categories (<50% yellow, >50% yellow, and 100% yellow). A generalized linear model was fitted to the data to test if the response variable "yellow knock-out" phenotype was dependent on the factor treatment (y1+y3, y1+s4, y1 and y2).

Since we were primarily interested in how successfully we could induce germline mutations and that adults assessed as 100% knock-outs are most likely to carry the mutation in the germline, we tested if the four CRISPR/Cas9 treatments differ in the frequency of individuals appearing as fully knock-out. This was done with a generalized linear model as mentioned above but where "yellow knock-out" only included individuals from the 100% yellow category. For this analysis all individuals in the categories >50% yellow and <50% yellow were pooled with the "wildtype" category.

Microinjections as well as injection with a CRISPR/Cas9 construct may affect survival. Therefore, survival of eggs (hatching rate) and survival to adult eclosion (proportion of hatched larvae that eclosed as adults) were compared between the treatments. For both traits each individual was scored as having survived or not. To test the effects on survival we fitted a generalized linear model to the data and investigated if the response survival (both as egg and to adult eclosion) was dependent on the factor treatment (y1+y3, y1+s4, y1 and y2, water-injected control and non-injected control).

Knock-out of the *yellow-y* gene may affect survival by itself. Therefore, survival to adult eclosion was compared between the non-injected control (G0) and the germline knock-outs ($G1^{-yellow\text{-}y}$). We could not test for egg survival, as we did not register egg survival in the germline knock-outs. Each larva was scored as surviving or not to adulthood. Only the germline knock-outs from the two observed matings were included as they were reared in separate cups as the control. Again, a generalized linear model was fitted to the data to test if survival differed between the control and germline knock-outs (G0 and $G1^{-yellow\text{-}y}$).

Knock-out of the *yellow-y* gene may have pleiotropic effects on other life history traits. Therefore, we performed two-way Anova tests with the response variable being one of the life history traits, larval development time, pupal development time, pupal weight or adult weight and the explanatory factors being the control and germline knock-outs as well as sex. Again, only the germline knock-outs from the two observed matings were included.

Results

Injected generation

The CRISPR/Cas9 treatments had a significant effect on the phenotypes of both larvae and adults (Larvae: $\chi^2_{(1,289)}$ = 111.29, $P < 0.001$; Adults: $\chi^2_{(1,114)} = 50.98$, $P < 0.001$). Across the four CRISPR/Cas9 treatments 83 out of 146 larvae (56.85%) had knock-out head capsules, 69 out of 87 adults (79.31%) had at least some cells transformed and 24 out of 87 adults (27.59%) appeared fully transformed (figure 3, table 2).

The four CRISPR/Cas9 treatments differed significantly in efficiency in regards to the proportion of larvae that had a knock-out head capsule ($\chi^2(3) = 17.14$, $P < 0.001$). The treatment y1+y3 with concentration 500 ng/ul was significantly more successful than the two treatments with a concentration of 250 ng/ul, the y1+s4 treatment ($P = 0.0101$) and y2 treatment ($P = 0.0031$).

The four CRISPR/Cas9 treatments did not differ significantly in the efficiency of adult transformation to a yellow knock-out phenotype ($\chi^2(3) = 2.83$, $P = 0.4181$). However, the treatments did differ in how many individuals were in the category 100% yellow ($\chi^2(3) = 25.65$, $P < 0.001$). Similarly as for larval knock-out efficiency, treatment y1+y3 with concentration 500 ng/ul was significantly more successful than the two treatments with a concentration of 250 ng/ul, the y1+s4 treatment ($P < 0.001$) and y2 treatment ($P = 0.0181$).

From each of the four treatments with a CRISPR/Cas9 construct, 3 larvae scored as knock-outs based on their head capsules were followed to adult eclosion to assess for a correlation between knock-out head capsule and adult phenotype. Out of the 12 larvae in total 9 survived to adulthood. Out of these 4 appeared 100% yellow, 4 were more than 50% yellow and one appeared wildtype (table 3). Hence, the correlation between the two knock-out phenotypes was very high.

The treatments significantly differed in eggs survival ($\chi^2(5) = 81.47$, $P < 0.001$). The non-injected control had a significantly higher egg survival than each of the injected treatments (water-injected control $P < 0.001$, y1+y3 $P < 0.001$, y1+s4 $P < 0.001$, y1 $P < 0.001$ and y2 $P < 0.001$) and the water-injected control additionally had a higher egg survival than the y1+s4 treatment ($P = 0.0022$). However, from egg hatching to adult eclosion there was no significant difference in survival between the treatments ($\chi^2(5) = 3.71$, $P = 0.5923$) (table 4).

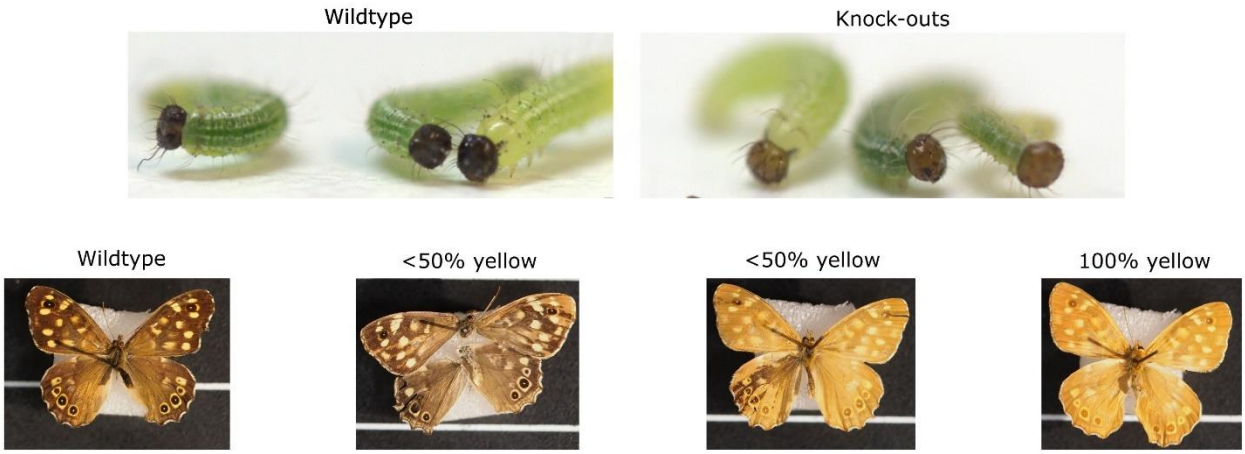

Figure 3. Pictures illustrating the phenotypic categories for larvae and adult yellow-y *knock-outs. The wildtype first instar head capsule is dark brown and was scored as knock-out if it appeared light brown. The wildtype adult has a dark brown background color, and was scored as wildtype, less than 50% yellow, more than 50% yellow and 100% yellow depending on the proportion of the background color that appeared yellow.*

Table 2. The number of larvae and adults scored in each phenotypic category in each CRISPR/Cas9 treatment. In brackets are proportions that for egg survival are based on the no. of eggs injected, for larvae the no. of eggs that survived and for adults the no. of adults that eclosed.

Treatment	Concentration (ng/µL)	Eggs injected	Egg survival	Knock-out larvae	Adults	Wildtype	Less than 50% yellow	More than 50% yellow	100% yellow
y1+y3	500	137	45 (0.33)	35 (0.78)	27	3 (0.11)	2 (0.07)	5 (0.19)	17 (0.63)
y1+s4	250	263	54 (0.21)	25 (0.46)	31	7 (0.23)	10 (0.32)	11 (0.35)	3 (0.10)
y1	500	57	21 (0.37)	14 (0.67)	13	3 (0.23)	2 (0.15)	5 (0.38)	3 (0.23)
y2	250	98	26 (0.27)	9 (0.35)	16	5 (0.31)	3 (0.19)	7 (0.44)	1 (0.06)

Table 3. Categorization of adult phenotype from larvae with knock-out head capsules followed to eclosion.

Treatment	Concentration (ng/µL)	Larvae	Adults	Wildtype	Less than 50% yellow	More than 50% yellow	100% yellow
y1+3	500	3	2				2
y1+s4	250	3	1			1	
y1	500	3	3			2	1
y2	250	3	3	1		1	1

Table 4. Egg survival and survival until adult eclosion of each treatment, including the two controls. In brackets is egg survival (in proportion of eggs injected) and adult eclosion (in proportion of eggs that survived).

Treatment	Concentration (ng/µL)	Eggs Injected/started	Egg survival	Adult eclosion
y1+y3	500	137	45 (0.33)	27 (0.60)
y1+s4	250	263	54 (0.21)	33 (0.61)
y1	500	57	21 (0.37)	13 (0.62)
y2	250	98	26 (0.27)	17 (0.65)
Water Control	0	114	45 (0.39)	14* (0.78)
Non-injected Control		34	32 (0.94)	14* (0.78)

*Only 18 larvae from each of the control treatments were followed to adult eclosion.

Germline generation

Matings were performed between adults classified as >50% and 100% transformed coming from three different treatments (y1+y3, y1+s4 and y1). In total 75 larvae were phenotyped and all of them where scored as knock-outs. Out of these, 59 larvae survived to adulthood and all adults appeared fully yellow (table 5).

There was no significant difference in adult survival between the germline *yellow-y* gene knock-outs ($G1^{-yellow\text{-}y}$) and the control (G0) ($\chi^2(1) = 1.31$, $P = 0.2533$). Additionally, there was no significant difference in larval development time ($F_{(1,39)} = 0.22$, $P = 0.6401$), although males developed faster than females in both categories ($F_{(1,39)} = 17.39$, $P < 0.001$). In some contrast, pupal development time was significantly longer for the germline transforms ($F_{(1,39)} = 30.73$, $P < 0.001$) while there was no significant difference in the pupal development time between the sexes ($F_{(1,39)} = 0.07$, $P = 0.796$). Pupal weights of the germline transforms was lower than the controls ($F_{(1,39)} = 37.15$, $P < 0.001$), with males weighting significantly less than females in both categories ($F_{(1,39)} = 18.86$, $P < 0.001$). Similarly, adults of the germline generation was significantly lighter in weight than the control ($F_{(1,32)} = 11.21$, $P = 0.0021$), with males of both categories weighing less than the females ($F_{(1,32)} = 21.49$, $P < 0.001$).

Table 5. Germline transformations of larvae and adults from the two observed matings as well as unobserved matings.

Parents (F x M)	Larvae	Knock-out larvae	Survival to adult eclosion	100% yellow
Mating 1 (y1+y3 x y1+y3)	15	15	14	14
Mating 2 (y1 x y1+y3)	15	15	13	13
Cage Matings*	45	45	32	32

***Cage with unobserved matings between adults from treatment y1+y3, y1+s4 and y1.**

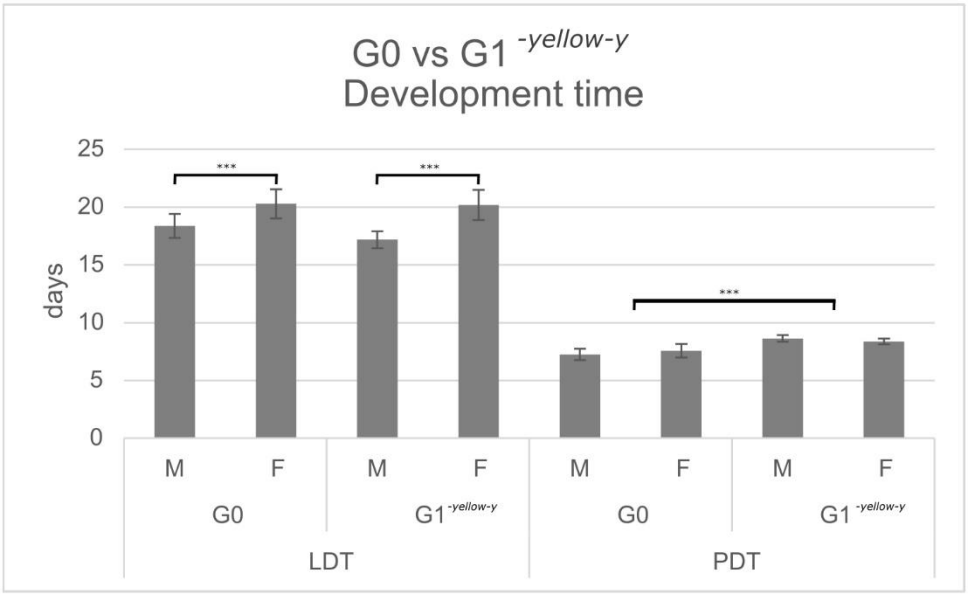

Figure 4. The mean and CI of the development times in days for the control (G0) and germline transformed ($G1^{-yellow-y}$) split by sex (M: male, F: female). The black lines signal a significant difference between the two sexes or the two groups (G0 or $G1^{-yellow-y}$). LDT: larval development time. PDT: pupal development time.

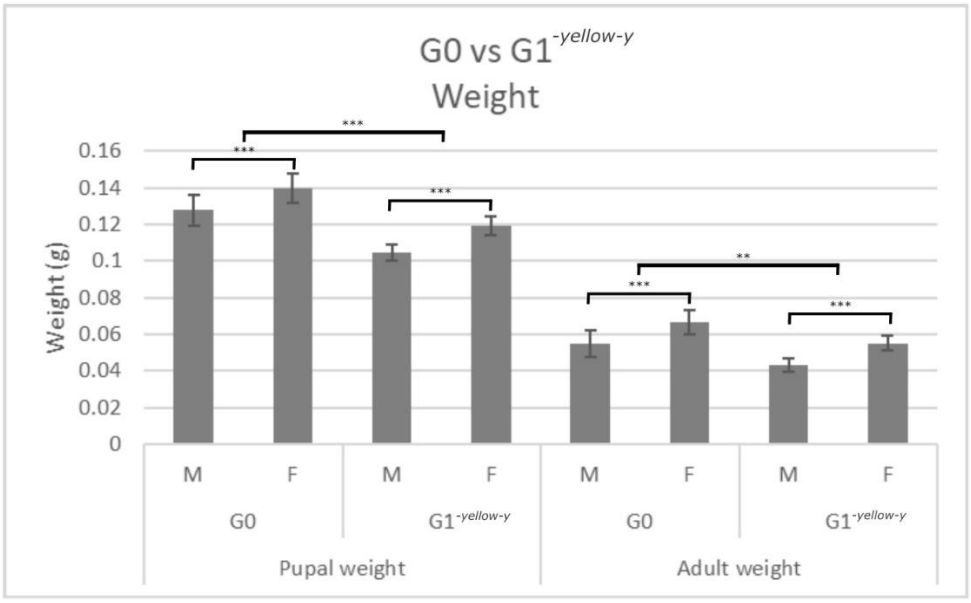

Figure 5. The mean and CI of the weights in gram of the control (G0) and germline transformed ($G1^{-yellow-y}$) split by sex (M: male, F: female). The black lines signal a significant difference between the two sexes or the two groups (G0 or $G1^{-yellow-y}$).

Discussion

Here we show that the CRISPR/Cas9 technology is highly efficient and capable of inducing germline mutations in the ecological model species *P. aegeria*. Targeting the *yellow-y* gene, the injected generation had more than 50% of the larvae exhibiting knock-out head capsules, and nearly 80% of surviving adults displayed at least partial transformation. The correlation between individuals showing the larval and adult knock-out phenotypes was very high, suggesting they were due to the same genetic transformation. In the following generation that resulted from matings between highly transformed individuals, all individuals were fully *yellow-y* knock-outs, both as larvae and as adults, indicating that all mated individuals carried homozygotic germline mutations.

The four CRISPR/Cas9 treatments were all successful and did not differ significantly in the proportion of adults with visible mutations. However, a significantly higher proportion of adults appeared 100% transformed in the treatment with the highest concentration of Cas9-sgRNA in the construct (500 ng/µL) and two different sgRNAs targeting the *yellow-y* gene. It should be noted that it is not possible to separate the effects of concentration and number of sgRNAs in the present study, but the increase in effectiveness is in line with previous findings (Bassett et al. 2013; Wang et al., 2013). In the silkworm (*Bombyx mori*) combining two sgRNAs likewise increased the observation of highly transformed

individuals markedly, when targeting the gene *BmBLOS2*, compared to the combined results of the two sgRNAs separately (Wang et al., 2013). Additionally, an increase in overall efficiency as a result of an increase in the concentration of Cas9-sgRNA in the construct has previously been shown for *Drosophila melanogaster* (Bassett et al., 2013).

Not so surprisingly, microinjections have the side-effect of causing a lower hatching success. As there was no general difference between the injection of CRISPR/Cas9 constructs and the water-injected control this seemed to be due to the injection itself, possibly due to loss of egg yolk. While practice performing microinjections may decrease egg mortality, the hatching success achieved here (between 21% and 37%) is similar to results from other butterflies (Li et al., 2015; Matsuoka & Monteiro, 2018). After hatching, survival until adult eclosion was not significantly affected by the treatments, consistent with observations in the black cutworm (*Agrotis ipsilon*) (Cao et al., 2018).

Here we show that, in resemblance to other lepidopterans, the *yellow-y* gene influence the color of the first instar larval head capsule and adult background color of *P. aegeria* (Perry et al., 2016; Liu et al., 2020; Shirai et al., 2021). There were no strong pleiotropic effects in life history traits of knocking-out the *yellow-y* gene. While significant differences were found between the germline transformed and the control, the variation in the life history traits was not much larger than normally found between lines that are not reared in parallel (Aalberg Haugen et al., 2021). Whether the *yellow-y* gene influences growth and development may vary between species, as the gene for instance influences hatching success in the tobacco cutworm (*Spodoptera litura*) (Liu et al., 2020; Shirai et al., 2021) but not in the diamondback moth (*Plutella xylostella*) (Wang et al., 2021).

With the CRISPR/Cas9 technology effective in the ecological model species *P. aegeria*, it will be possible to do direct manipulations of candidate genes that effects ecologically important traits. For instance, in Sweden *P. aegeria* has a latitudinal cline in the daylength inducing winter diapause (a dormancy found in insects) (Aalberg Haugen & Gotthard, 2015). Moreover, parts of this phenotypic variation is associated with variation at identified genomic regions that include relevant candidate genes (Pruisscher et al., 2018; Lindestad et al., 2022). This includes SNPs highly associated with seasonal timing in the *period* gene and a region including the gene *timeless*. Interestingly, a relatively large deletion in the *timeless* gene has been shown to be fixed in an isolated population and this mutation may be associated with photoperiodism for seasonal timing (Lindestad et al., 2022). The CRISPR/Cas9 technology presents a completely new and exciting opportunity to explore the phenotypic effect of such ecologically relevant mutations

(Gudmunds et al., 2022). However, the development of gene knockouts results in unpredictable DNA repairment (Sander & Joung, 2014). For precise gene manipulations it will be necessary to perform CRISPR homology-directed repair (HDR), where a donor plate is provided in the CRISPR/Cas9 construct containing the desired mutation (Sander & Joung, 2014; Gudmunds et al., 2022). This method is under development (Di Stazio et al., 2021) but has been tested for its ability to induce germline mutations in lepidopteran species (Wang et al., 2020; Heryanto et al., 2022). Potentially this type of more precise gene editing using CRISPR/Cas9 can soon be applied to ecological model species. As our findings indicate that the CRISPR/Cas9 technology is highly efficient in creating germline mutations in this species we anticipate an exciting future for ecological genetics and evolutionary biology.

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References

Aalberg Haugen, I. M., Berger, D., & Gotthard, K. (2012). The evolution of alternative developmental pathways: footprints of selection on life-history traits in a butterfly. *Journal of Evolutionary Biology*, *25*(7), 1377-1388.

Aalberg Haugen, I. M., & Gotthard, K. (2015). Diapause induction and relaxed selection on alternative developmental pathways in a butterfly. *Journal of Animal Ecology*, *84*(2), 464-472.

Andrade, P., & Carneiro, M. (2021). Pterin-based pigmentation in animals. *Biology Letters*, *17*(8), 20210221.

Baci, G. M., Cucu, A. A., Giurgiu, A. I., Muscă, A. S., Bagameri, L., Moise, A. R., Bobiș, O., Rațiu, A. C., & Dezmirean, D. S. (2022). Advances in editing silkworms (Bombyx mori) genome by using the CRISPR-cas system. *Insects*, *13*(1), 28.

Bassett, A. R., Tibbit, C., Ponting, C. P., & Liu, J. L. (2013). Highly efficient targeted mutagenesis of Drosophila with the CRISPR/Cas9 system. *Cell reports*, *4*(1), 220-228.

Berwaerts, K., Matthysen, E., & Van Dyck, H. (2008). Take-off flight performance in the butterfly Pararge aegeria relative to sex and morphology: a quantitative genetic assessment. *Evolution*, *62*(10), 2525-2533.

Bi, H. L., Xu, J., He, L., Zhang, Y., Li, K., & Huang, Y. P. (2019). CRISPR/Cas9-mediated ebony knockout results in puparium melanism in Spodoptera litura. *Insect science*, *26*(6), 1011-1019.

Bono, J. M., Olesnicky, E. C., & Matzkin, L. M. (2015). Connecting genotypes, phenotypes and fitness: harnessing the power of CRISPR/Cas9 genome editing. *Molecular ecology*, *24*(15), 3810-3822.

Chakraborty, M., Lara, A. G., Dang, A., McCulloch, K. J., Rainbow, D., Carter, D., Ngo, L. T., Solares, E., Said, I., Corbett-Detig, R. B., Gilbert, L. E., Emerson, J. J., & Briscoe, A. D. (2023). Sex-linked gene traffic underlies the acquisition of sexually dimorphic UV color vision in Heliconius butterflies. *Proceedings of the National Academy of Sciences*, *120*(33), e2301411120.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. L. (2009). BLAST+: architecture and applications. *BMC bioinformatics*, *10*, 1-9.

Cao, Y., Zhan, S., Zhang, Y., Tan, A., & Huang, Y. (2018). Identification of yellow gene family in Agrotis ipsilon and functional analysis of Aiyellow-y by CRISPR/Cas9. *Insect Biochemistry and Molecular Biology*, *94*, 1-9.

Cho, S. W., Kim, S., Kim, Y., Kweon, J., Kim, H. S., Bae, S., & Kim, J. S. (2014). Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome research*, *24*(1), 132-141.

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W., Marraffini, L. A., & Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science*, *339*(6121), 819–823.

Connahs, H., Tan, E. J., Ter, Y. T., Dion, E., Matsuoka, Y., Bear, A., & Monteiro, A. (2022). The yellow gene regulates behavioural plasticity by repressing male courtship in Bicyclus anynana butterflies. *Proceedings of the Royal Society B*, *289*(1972), 20212665.

Di Stazio, M., Foschi, N., Athanasakis, E., Gasparini, P., & d'Adamo, A. P. (2021). Systematic analysis of factors that improve homologous direct repair (HDR) efficiency in CRISPR/Cas9 technique. *PLoS One*, *16*(3), e0247603.

Douris, V., Denecke, S., Van Leeuwen, T., Bass, C., Nauen, R., & Vontas, J. (2020). Using CRISPR/Cas9 genome modification to understand the genetic basis of insecticide resistance: Drosophila and beyond. *Pesticide biochemistry and physiology*, *167*, 104595.

Drapeau, M. D., Radovic, A., Wittkopp, P. J., & Long, A. D. (2003). A gene necessary for normal male courtship, yellow, acts downstream of fruitless in the Drosophila melanogaster larval brain. *Journal of Neurobiology*, *55*(1), 53-72.

Ficarrotta, V., Hanly, J. J., Loh, L. S., Francescutti, C. M., Ren, A., Tunström, K., Wheat, C. W., porter, A. H., Counterman, B. A., & Martin, A. (2022). A genetic switch for male UV iridescence in an incipient species pair of sulphur butterflies. *Proceedings of the National Academy of Sciences*, *119*(3), e2109255118.

Fleming, T. J., Schrankel, C. S., Vyas, H., Rosenblatt, H. D., & Hamdoun, A. (2021). CRISPR/Cas9 mutagenesis reveals a role for ABCB1 in gut immune responses to Vibrio diazotrophicus in sea urchin larvae. *Journal of Experimental Biology*, *224*(7), jeb232272.

Fu, Y., Foden, J. A., Khayter, C., Maeder, M. L., Reyon, D., Joung, J. K., & Sander, J. D. (2013). High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nature biotechnology*, *31*(9), 822-826.

Futahashi, R., & Fujiwara, H. (2007). Regulation of 20-hydroxyecdysone on the larval pigmentation and the expression of melanin synthesis enzymes and yellow gene of the swallowtail butterfly, Papilio xuthus. *Insect biochemistry and molecular biology*, *37*(8), 855-864.

Futahashi, R., & Osanai-Futahashi, M. (2021). Pigments in insects. *Pigments, pigment cells and pigment patterns*, 3-43.

Gudmunds, E., Wheat, C. W., Khila, A., & Husby, A. (2022). Functional genomic tools for emerging model species. *Trends in Ecology & Evolution*.

Han, W. K., Yang, Y. L., Si, Y. X., Wei, Z. Q., Liu, S. R., Liu, X. L., Yan, Q., & Dong, S. L. (2022). Involvement of GOBP2 in the perception of a sex pheromone component in both larval and adult Spodoptera litura revealed using CRISPR/Cas9 mutagenesis. *Insect Biochemistry and Molecular Biology*, *141*, 103719.

Hanly, J. J., Francescutti, C. M., Loh, L. S., Corning, O. B., Long, D. J., Nakatani, M. A., Porter, A. H., & Martin, A. (2023). Genetics of yellow-orange color variation in a pair of sympatric sulfur butterflies. *Cell Reports*, *42*(8).

Heryanto, C., Hanly, J. J., Mazo-Vargas, A., Tendolkar, A., & Martin, A. (2022). Mapping and CRISPR homology-directed repair of a recessive white eye mutation in Plodia moths. *Iscience*, *25*(3), 103885.

Hsu, P. D., Scott, D. A., Weinstein, J. A., Ran, F. A., Konermann, S., Agarwala, V., Li, Y., Fine, E. J., Wu, X., Shalem, O., Cradick, T. J., Marraffini, L. A., Bao, G., & Zhang, F. (2013). DNA targeting specificity of RNA-guided Cas9 nucleases. *Nature biotechnology*, *31*(9), 827-832.

Inui, M., Miyado, M., Igarashi, M., Tamano, M., Kubo, A., Yamashita, S., Asahara, H., Fukami, M., & Takada, S. (2014). Rapid generation of mouse models with defined point mutations by the CRISPR/Cas9 system. *Scientific reports*, *4*(1), 5396.

Jiang, W., Bikard, D., Cox, D., Zhang, F., & Marraffini, L. A. (2013). RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nature biotechnology*, *31*(3), 233-239.

Lenth, R. V. (2021). emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.7.1-1. https://CRAN.R-project.org/package=emmeans

Li, J. J., Shi, Y., Wu, J. N., Li, H., Smagghe, G., & Liu, T. X. (2021). CRISPR/Cas9 in lepidopteran insects: Progress, application and prospects. *Journal of Insect Physiology*, *135*, 104325.

Li, J. L., Li, S. S., Luo, Z. J., Lu, J., Cai, X. M., Luo, Z. X., Bian, L., Xiu, C. L., Fu, N. X., Liu, N. Y., & Li, Z. Q. (2024). CRISPR/Cas9-mediated ebony knockout causes melanin pigmentation and prevents moth Eclosion in Ectropis grisescens. *Pesticide Biochemistry and Physiology*, 105810.

Li, S., Lao, J., Sun, Y., Hua, X., Lin, P., Wang, F., Shen, G., Zhao, P., & Xia, Q. (2024). CRISPR/Cas9-Mediated Editing of BmEcKL1 Gene Sequence Affected Silk Gland Development of Silkworms (Bombyx mori). *International Journal of Molecular Sciences*, *25*(3), 1907.

Li, X., Fan, D., Zhang, W., Liu, G., Zhang, L., Zhao, L., Fang, X., Chen, L., Dong, Y., Chen, Y., Ding, Y., Zhao, R., Feng, M., Zhu, Y., Feng, Y., Jiang, X., Zhu, D., Xiang, H., Feng, X., Li, S., Wang, J., Zhang, G., Kronforst, M. R., Wang W.

(2015). Outbred genome sequencing and CRISPR/Cas9 gene editing in butterflies. *Nature communications*, *6*(1), 8212.

Li, Y., Liu, Z., Liu, C., Shi, Z., Pang, L., Chen, C., Chen, C., Chen, Y., Pan, R., Zhou, W., Chen, X. X., Rokas, A., Huang, J., & Shen, X. X. (2022). HGT is widespread in insects and contributes to male courtship in lepidopterans. *Cell*, *185*(16), 2975-2987.

Liang, W., He, J., Mao, C., Yu, C., Meng, Q., Xue, J., Wu, X., Li, S., Wang, Y., & Yi, H. (2022). Gene editing monkeys: Retrospect and outlook. *Frontiers in Cell and Developmental Biology*, *10*, 913996.

Lindestad, O., Nylin, S., Wheat, C. W., & Gotthard, K. (2022). Local adaptation of life cycles in a butterfly is associated with variation in several circadian clock genes. *Molecular Ecology*, *31*(5), 1461-1475.

Lindestad, O., Wheat, C. W., Nylin, S., & Gotthard, K. (2019). Local adaptation of photoperiodic plasticity maintains life cycle variation within latitudes in a butterfly. *Ecology*, *100*(1), e02550.

Liu, G., Zhang, Y., & Zhang, T. (2020). Computational approaches for effective CRISPR guide RNA design and evaluation. *Computational and structural biotechnology journal*, *18*, 35-44.

Liu, X. L., Han, W. K., Ze, L. J., Peng, Y. C., Yang, Y. L., Zhang, J., Yan, Q., & Dong, S. L. (2020). Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 mediated knockout reveals functions of the yellow-y gene in Spodoptera litura. *Frontiers in Physiology*, *11*, 615391.

Livraghi, L., Vodă, R., Evans, L. C., Gibbs, M., Dincă, V., Holland, P. W., Shreeve, T. G., Vila, R., Dapporto, L., & Breuker, C. J (2018). Historical and current patterns of gene flow in the butterfly Pararge aegeria. *Journal of Biogeography*, *45*(7), 1628-1639.

Lohse, K., Taylor-Cox, E., & Darwin Tree of Life Consortium. (2021). The genome sequence of the speckled wood butterfly, Pararge aegeria (Linnaeus, 1758). *Wellcome Open Research*, *6*(287), 287.

Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., DiCarlo, J. E., Norville, J. E. & Church, G. M. (2013). RNA-guided human genome engineering via Cas9. *Science*, *339*(6121), 823-826.

Matsuoka, Y., & Monteiro, A. (2018). Melanin pathway genes regulate color and morphology of butterfly wing scales. *Cell reports*, *24*(1), 56-65.

Merckx, T., & Van Dyck, H. (2006). Landscape structure and phenotypic plasticity in flight morphology in the butterfly Pararge aegeria. *Oikos*, *113*(2), 226-232.

Mohr, S. E., Hu, Y., Ewen-Campen, B., Housden, B. E., Viswanatha, R., & Perrimon, N. (2016). CRISPR guide RNA design for research applications. *The FEBS journal*, *283*(17), 3232-3238.

Naeem, M., Majeed, S., Hoque, M. Z., & Ahmad, I. (2020). Latest developed strategies to minimize the off-target effects in CRISPR-Cas-mediated genome editing. *Cells*, *9*(7), 1608.

Nowell, R. W., Elsworth, B., Oostra, V., Zwaan, B. J., Wheat, C. W., Saastamoinen, M., & Blaxter, M. (2017). A high-coverage draft genome of the mycalesine butterfly Bicyclus anynana. Gigascience 6: 1–7.

Nylin, S., Wickman, P. O., & Wiklund, C. (1989). Seasonal plasticity in growth and development of the speckled wood butterfly, Pararge aegeria (Satyrinae). *Biological Journal of the Linnean Society*, *38*(2), 155-171.

Nylin, S., Wickman, P. O., & Wiklund, C. (1995). Life-cycle regulation and life history plasticity in the speckled wood butterfly: are reaction norms predictable?. *Biological Journal of the Linnean Society*, *55*(2), 143-157.

Okamura, Y., Dort, H., Reichelt, M., Tunström, K., Wheat, C. W., & Vogel, H. (2022). Testing hypotheses of a coevolutionary key innovation reveals a complex suite of traits involved in defusing the mustard oil bomb. *Proceedings of the National Academy of Sciences*, *119*(51), e2208447119.

Packer, L. (1984). The ecological genetics of the speckled wood butterfly, Pararge aegeria L.—A preliminary study. *Heredity*, *52*(2), 179-188.

Pattanayak, V., Lin, S., Guilinger, J. P., Ma, E., Doudna, J. A., & Liu, D. R. (2013). High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nature biotechnology*, *31*(9), 839-843.

Perrimon, N., Ni, J. Q., & Perkins, L. (2010). In vivo RNAi: today and tomorrow. *Cold Spring Harbor perspectives in biology*, *2*(8), a003640.

Perry, M., Kinoshita, M., Saldi, G., Huo, L., Arikawa, K., & Desplan, C. (2016). Molecular logic behind the three-way stochastic choices that expand butterfly colour vision. *Nature*, *535*(7611), 280-284.

Pruisscher, P., Nylin, S., Gotthard, K., & Wheat, C. W. (2018). Genetic variation underlying local adaptation of diapause induction along a cline in a butterfly. *Molecular ecology*, *27*(18), 3613-3626.

Sander, J. D., & Joung, J. K. (2014). CRISPR-Cas systems for editing, regulating and targeting genomes. *Nature biotechnology*, *32*(4), 347-355.

Schindele, P., Wolter, F., & Puchta, H. (2018). Transforming plant biology and breeding with CRISPR/Cas9, Cas12 and Cas13. *FEBS letters*, *592*(12), 1954-1967.

Shreeve, T. G. (1986). Egg-laying by the speckled wood butterfly (Parargе aegeria): the role of female behaviour, host plant abundance and temperature. *Ecological Entomology*, *11*(2), 229-236.

Shreeve, T. G. (1987). The mate location behaviour of the male speckled wood butterfly, Pararge aegeria, and the effect of phenotypic differences in hind-wing spotting. *Animal Behaviour*, *35*(3), 682-690.

Shirai, Y., Ohde, T., & Daimon, T. (2021). Functional conservation and diversification of yellow-y in lepidopteran insects. *Insect Biochemistry and Molecular Biology*, *128*, 103515.

Shirk, B. D., Shirk, P. D., Furlong, R. B., Scully, E. D., Wu, K., & Siegfried, B. D. (2023). Gene editing of the ABC Transporter/White locus using CRISPR/Cas9-mediated mutagenesis in the Indian Meal Moth. *Journal of Insect Physiology*, *145*, 104471.

Sternberg, S. H., Redding, S., Jinek, M., Greene, E. C., & Doudna, J. A. (2014). DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Biophysical Journal*, *106*(2), 695a.

Sun, D., Guo, Z., Liu, Y., & Zhang, Y. (2017). Progress and prospects of CRISPR/Cas systems in insects and other arthropods. *Frontiers in physiology*, *8*, 608.

Talloen, W., Van Dongen, S., Van Dyck, H., & Lens, L. (2009). Environmental stress and quantitative genetic variation in butterfly wing characteristics. *Evolutionary Ecology*, *23*, 473-485.

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*, e10352.

Tendolkar, A., Mazo-Vargas, A., Livraghi, L., Hanly, J. J., Van Horne, K. C., Gilbert, L. E., & Martin, A. (2024). Cis-regulatory modes of Ultrabithorax inactivation in butterfly forewings. *ELife*, *12*, RP90846.

Terenius, O., Papanicolaou, A., Garbutt, J. S., Eleftherianos, I., Huvenne, H., Kanginakudru, S., Albrechtsen, M., An, C., Aymeric, J. L., Barthel, A., Bebas, P., Bitra, K., Bravo, A., Chevalier, F., Collinge, D. P., Crava, C. M., de Maagd, R. A., Duvic, B., Erlandson, M., Faye, I., Felföldi, G., Fujiwara, H., Futahashi, R., Gandhe, A. S., Gatehouse, H. S., Gatehouse, L. N., Giebultowicz, J. M., Gómez, I., Grimmelikhuijzen, C. J., Groot, A. T., Hauser, F., Heckel, D. G., Hegedus, D. D., Hrycaj, S., Huang, L., Hull, J. J., Iatrou, K., Iga, M., Kanost, M. R., Kotwica, J., Li, C., Li, J., Liu, J., Lundmark, M., Matsumoto, S., Meyering-Vos, M., Millichap, P. J., Monteiro, A., Mrinal, N., Niimi, T., Nowara, D., Ohnishi, A., Oostra, V., Ozaki, K., Papakonstantinou, M., Popadic, A., Rajam, M. V., Saenko, S., Simpson, R. M., Soberón, M., Strand, M. R., Tomita, S., Toprak, U., Wang, P., Wee, C. W., Whyard, S., Zhang, W., Nagaraju, J., Ffrench-Constant, R. H., Herrero, S., Gordon, K., Swevers, L., Smagghe, G. (2011). RNA interference in Lepidoptera: an overview of successful and unsuccessful studies and implications for experimental design. *Journal of insect physiology*, *57*(2), 231-245.

Tunström, K., Woronik, A., Hanly, J. J., Rastas, P., Chichvarkhin, A., Warren, A. D., Kawahara, A. Y., Schoville, S. D., Ficarrotta, V., Porter, A. H., Watt, W. B., Martin, A., & Wheat, C. W. (2023). Evidence for a single, ancient origin of a genus-wide alternative life history strategy. *Science Advances*, *9*(12), eabq3713.

Van Dyck, H., Matthysen, E., & Wiklund, C. (1998). Phenotypic variation in adult morphology and pupal colour within and among families of the speckled wood butterfly Pararge aegeria. *Ecological Entomology*, *23*(4), 465-472.

Van Dyck, H., Matthysen, E. R. L. K., Windig, J. J., & Dhondt, A. (1997). Seasonal phenotypic variation in the speckled wood butterfly (Pararge aegeria L.): patterns in and relationships between wing characters. *Belgian journal of zoology*, *127*, 167-178.

Wang, D., Chen, J., Yuan, Y., Yu, L., Yang, G., & Chen, W. (2023). CRISPR/Cas9-mediated knockout of period reveals its function in the circadian rhythms of the diamondback moth Plutella xylostella. *Insect Science*, *30*(3), 637-649.

Wang, Y., Huang, Y., Xu, X., Liu, Z., Li, J., Zhan, X., Yang, G., You, M., & You, S. (2021). CRISPR/Cas9-based functional analysis of yellow gene in the diamondback moth, Plutella xylostella. *Insect Science*, *28*(5), 1504-1509.

Wang, Y., Li, Z., Xu, J., Zeng, B., Ling, L., You, L., Chen, Y., Huang, Y., & Tan, A. (2013). The CRISPR/Cas system mediates efficient genome engineering in Bombyx mori. *Cell research*, *23*(12), 1414-1416.

Wang, X., Cao, X., Jiang, D., Yang, Y., & Wu, Y. (2020). CRISPR/Cas9 mediated ryanodine receptor I4790M knockin confers unequal resistance to diamides in Plutella xylostella. *Insect Biochemistry and Molecular Biology*, *125*, 103453.

Windig, J. J., Veerkamp, R. F., & Nylin, S. (2004). Quantitative genetic variation in an island population of the speckled wood butterfly (Pararge aegeria). *Heredity*, *93*(5), 450-454.

Zhang, L., Steward, R. A., Wheat, C. W., & Reed, R. D. (2021). High-quality genome assembly and comprehensive transcriptome of the painted lady butterfly Vanessa cardui. *Genome Biology and Evolution*, *13*(7), evab145.

Xu, X., Chen, J., Du, X., Yao, L., & Wang, Y. (2022). CRISPR/Cas9 mediated disruption of seminal fluid protein Sfp62 induces male sterility in Bombyx mori. *Biology*, *11*(4), 561.

Paper II: Supplementary material

Table S1: The number of adults in each treatment, including the controls, scored for their phenotype. In brackets are the proportions of all adults that eclosed in each treatment.

Table S1

Treatment	Concentration (ng/µL)	Adults analyzed for phenotype
y1+y3	500	27 (1.00)
y1+s4	250	31 (0.94)
y1	500	13 (1.00)
y2	250	16 (0.94)
Water-injected Control	0	14 (1.00)
Non-injected Control		13 (0.93)

Table S2: The number of adults per treatment (including the germline generation) that failed to eclose with fully developed wings and may not have been capable of mating. In bracket are the proportions of all adults that eclosed in each treatment. When scoring the phenotype of the adults all eclosed adults were included, independently if they eclosed with all wings fully formed.

Table S2

Treatment	Concentration (ng/µL)	Malformed adults
y1+y3	500	5 (0.19)
y1+s4	250	3 (0.09)
y1	500	2 (0.15)
y2	250	3 (0.18)
Water-injected Control	0	1 (0.07)
Non-injected Control		3 (0.21)
Germline		7 (0.26)

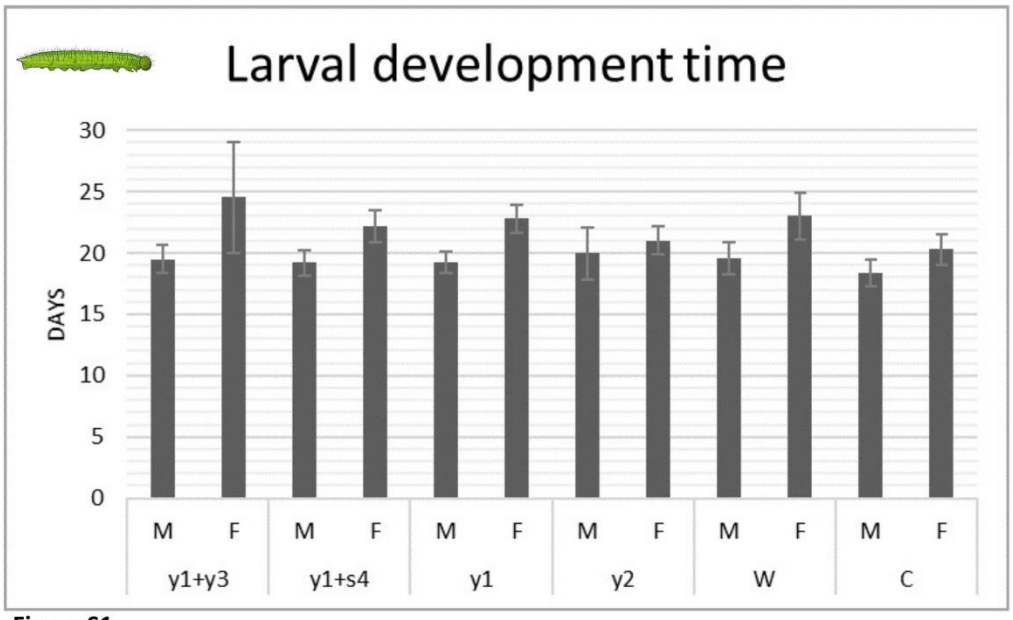

Figure S1

Figure S1 Larval development time (mean and CI) in days of the CRISPR/Cas9 treatments, the water-injected control (W) and non-injected control (C) split by sex (M: male, F: female). The treatments did not differ significantly in larval development time ($F(5) = 1.20$, $P = 0.366$) but males developed significantly faster than the females across treatments ($F(1) = 28.86$, $P < 0.001$).

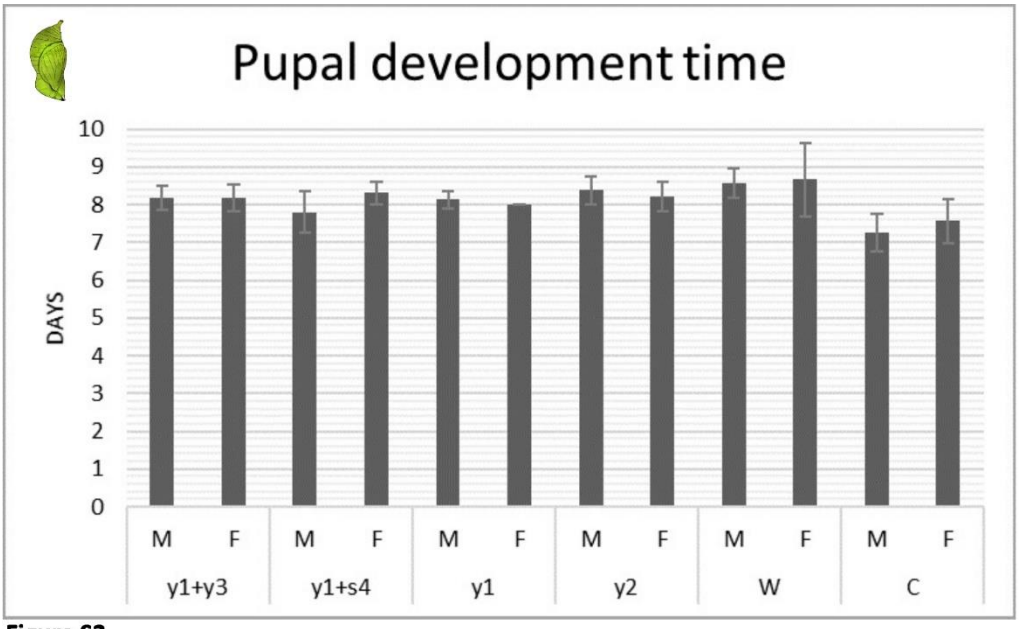

Figure S2

Figure S2 Pupal development time (mean and CI) in days of the CRISPR/Cas9 treatments, the water-injected control (W) and non-injected control (C) split by sex (M: male, F: female). The pupal development time differed significantly between treatments ($F(5) = 4.55$, $P < 0.001$) but there was no significant difference between the sexes ($F(1) = 1.90$, $P = 0.171$). The pupal development time of the non-injected control was significantly shorter than the water-injected control ($P < 0.001$) as well as the CRISPR/Cas9 treatments y1+y3 ($P = 0.0099$), y1+s4 ($P = 0.0482$) and y2 ($P = 0.0103$).

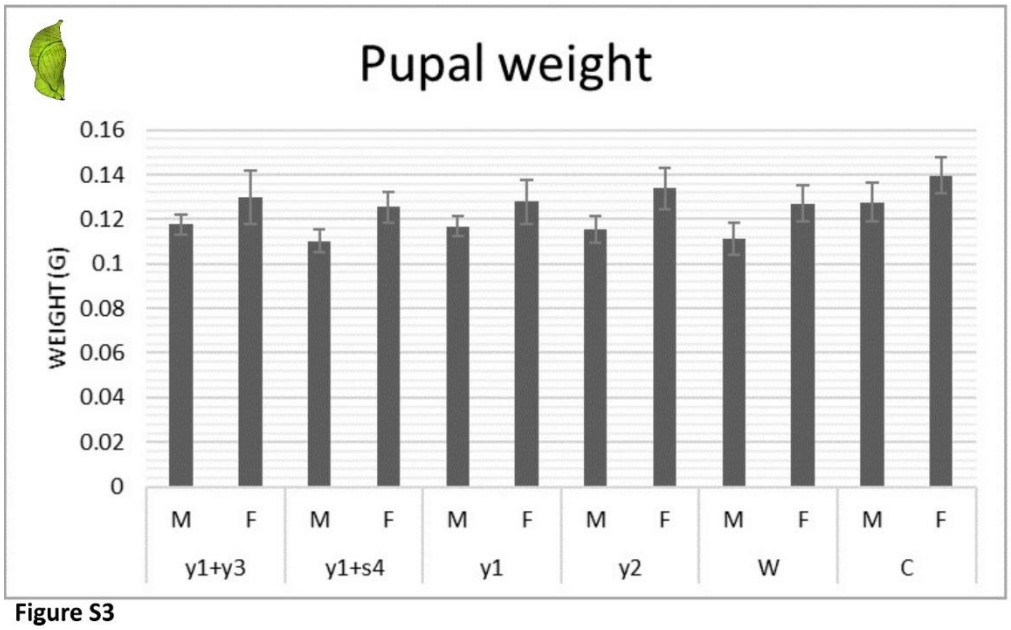

Figure S3

Figure S3 Pupal weight (mean and CI) in gram of the CRISPR/Cas9 treatments, the water-injected control (W) and non-injected control (C) split by sex (M: male, F: female). There was a significant effect of treatment ($F(5) = 4.23$, $P = 0.0015$) as well as sex ($F(1) = 41.40$, $P < 0.001$). The pupal weight was significantly higher in the non-injected control than the water-injected control ($P = 0.0198$) and the CRISPR/Cas9 treatment y1+s4 ($P = 0.0004$). Across treatments males weighed less than females.

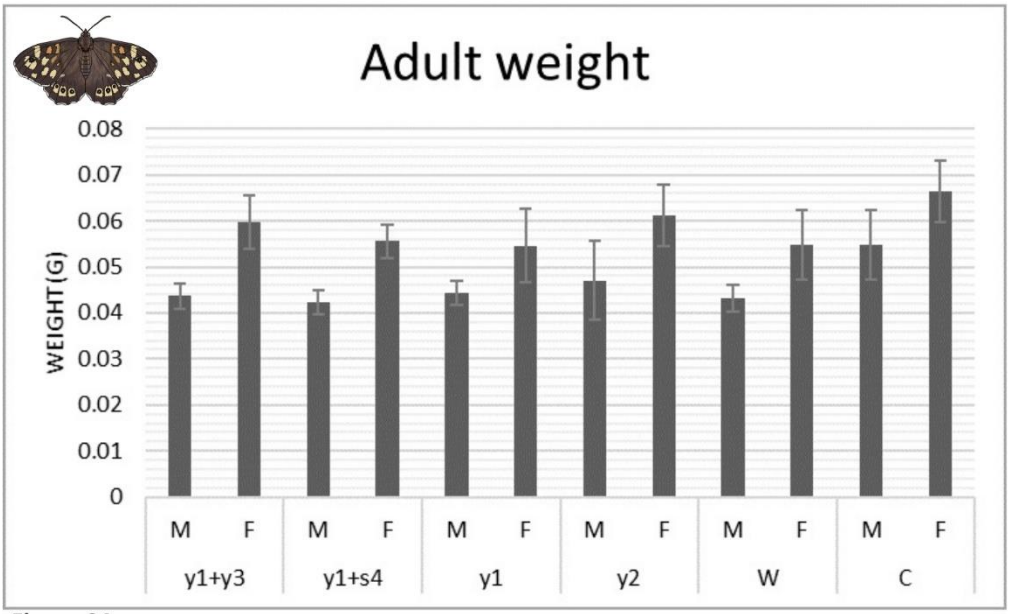

Figure S4

Figure S4 Adult weight (mean and CI) in gram of the CRISPR/Cas9 treatments, the water-injected control (W) and non-injected control (C) split by sex (M: male, F: female). There was a significant effect of treatment ($F(5) = 3.79$, $P = 0.0037$) and sex ($F(1) = 72.14$, $P < 0.001$). The non-injected control was significantly heavier than the water-injected control ($P = 0.0029$) and the CRISPR/Cas9 treatments y1+y3 ($P = 0.0126$), y1+s4 ($P = 0.0004$) and y1 ($P = 0.0089$). Females were significantly heavier than males across all the treatments.