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Effects of fungicide and herbicide on a non-target butterfly performance

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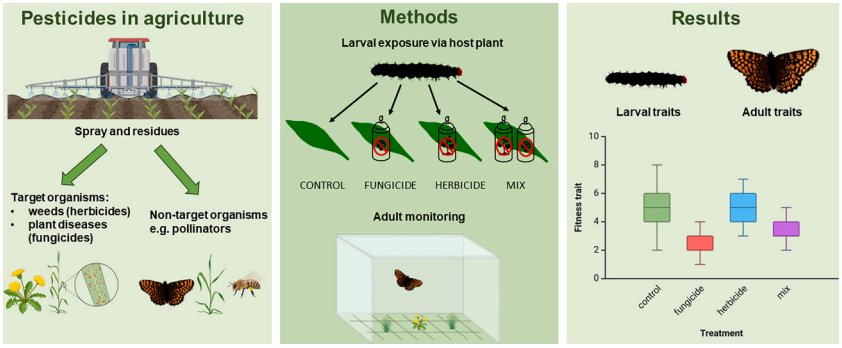
HIGHLIGHTS

- Pesticide use has been linked to biodiversity loss, particularly effecting pollinators
- We studied impacts of a herbicide and a fungicide on a non-target butterfly species
- Fungicide alone and in mix with herbicide reduced larval survival and adult size
- Larval treatment to mix of fungicide and herbicide reduced adult female fitness

GRAPHICAL ABSTRACT



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ABSTRACT

Agricultural intensification is one of the key drivers of biodiversity loss. Intensified agriculture is often associated with increased use of pesticides and the use of pesticides could negatively impact also non-target species living in the vicinity of agricultural lands. We exposed larvae of the Glanville fritillary butterfly (*Melitaea cinxia*) to a short-term exposure of a herbicide, a fungicide, or a mix of the two via their larval host plant (*Plantago lanceolata*). Survival and performance of the larvae was recorded and potential carry-over effects on adult butterfly fitness traits were assessed under semi-natural conditions in an outdoor enclosure. Our results showed significantly higher mortality of 60 % in the larvae exposed to the fungicide and 22 % mortality in the fungicide-herbicide mix treatment, with both treatments also impacting adult morphology. Adult female butterflies exposed to the mix of fungicide and herbicide treatment during their development also had lower lifetime reproductive success than the other treatment groups, suggesting that the combined chemical load had stronger carry-over effects into adulthood. Our results demonstrate clear negative impacts of a commonly used fungicide on a non-target butterfly species. The combined effect of two pesticides, while less lethal to larvae directly, seem to have a more profound carry-over impact on fitness of adult female butterflies. The mechanisms underlying the effects of a fungicide alone and in interaction with the herbicide, as well as the relatively minor impacts of herbicide alone on the specialist insect warrant further investigation in the role of pesticides in natural populations.

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1. Introduction

Agricultural intensification is one of the main drivers behind the current loss of biodiversity (Raven and Wagner, 2021). Intensified land use for agricultural purposes has led to loss and fragmentation of natural habitats for many species, negatively impacting pollinators such as bees and butterflies (Zabel et al., 2019). Intensified agricultural practices have also increased the amount of pesticides used in farming (Botías et al., 2016; Aktar et al., 2009). Herbicides, fungicides and insecticides are pesticides commonly used to control weeds, fungal plant diseases and insect pests, respectively. Pesticides and their combinations are used on many different crops and their spray can cause residues to spread also outside the actual crop growing area (Olaya-Arenas and Kaplan, 2019; Silva et al., 2019). Globally over 3,5 million tonnes of pesticides are used annually, and the most used active substance is the herbicide glyphosate (FAO, 2024). Recent studies have speculated on the role of pesticides in aggravating the current loss of biodiversity and suggested that excessive use of pesticides could also negatively impact non-target species living in the vicinity of agricultural lands (Brühl et al., 2021; Geiger et al., 2010). This may be particularly relevant for pollinator species such as bees, but also species such as butterflies that can find suitable habitats at agricultural landscapes where pesticide runoff is likely. Most studies have focused on bees as the study system (Jütte et al., 2023; Nicholson et al., 2023), due to their importance in providing pollination for crops and less focus has been on other non-target organisms.

Butterflies (superfamily Papilionoidea) are a well-studied group of organisms which makes them popular study species for different ecological questions (van Swaay et al., 2016; An and Choi, 2020). The current biodiversity decline is also evident in butterflies. Several reports have indicated declining population trends around the world (see for example: Wepprich et al., 2019; Warren et al., 2021; Van Strien et al., 2019; Conrad et al., 2006). Many butterfly species prefer open grassland habitats and consequently are often found at different types of agricultural landscapes (Van Swaay et al., 2006). Intensified agricultural practices are considered to be the primary driver of butterfly declines (Habel et al., 2019). Still, the contribution of different drivers associated with intensified agriculture is not well understood. Most studies assessing butterfly population trends in agro-environments have focused on the impacts of habitat loss and fragmentation. However, since suitable habitats for butterflies can often be found in the vicinity of agricultural lands, this could expose butterflies to pesticides, both via direct exposure during the spraying but also indirectly through their food at both the larval (exposure via plants used for feeding) and adult stage (exposure via nectar). Yet, little is known about the impacts of pesticides and how they might affect different butterfly species that occur in these habitats.

To date most studies assessing impacts of pesticides on pollinators have focused on insecticides, more specifically neonicotinoids (Van der Sluijs et al., 2013; Stanley et al., 2015). Studies assessing impacts of herbicides often focus on the reduction of native plants as the main consequence of herbicide use, which can negatively impact the insect communities they support (Stenoien et al., 2018). Less focus has been on how herbicides and fungicides impact non-target insects either via oral or contact exposure, though some recent studies have addressed this using beneficial non-target species, mainly pollinators such as bees (Kaila et al., 2023; Helander et al., 2022) but also other species such as insect predators (Defarge et al., 2023). The use of fungicides has increased steadily and over 760,000 t of fungicides are used annually around the world (FAO, 2024). Previous studies have shown that pesticide residues can be found in pollen and nectar of both crops and wild plants, the fungicide azoxystrobin being one of the most often detected active substances (Zioga et al., 2023). However, as residues of pesticides have been shown to have negatively impact some pollinators such as different bee species (Knapp et al., 2023; Nicholson et al., 2023), more information is needed on the impact of pesticides on different butterfly species with different ecological requirements. One previous study on monarch butterflies showed that larval exposure can carry over to the adult stage reducing adult performance (Olaya-Arenas et al., 2020).

In this study we explored the possible consequences of exposing a non-target butterfly larvae to fungicides and herbicides via their larval food plants. Our aim was to assess the impacts of short-term exposure to a fungicide, a herbicide and their combination via food plants on larval developmental traits but also potential carry-over effects on adult performance. Specifically, the questions of interest were: How does a three-day short-term exposure during larval stage affect the mortality and growth rate of larvae? Does the exposure during larval stage have carry-over effects into the adult performance? How do the effects differ between a fungicide and a herbicide, or a combination of these two?

To study these questions, we exposed larvae of the Glanville fritillary butterfly (*Melitaea cinxia*) to two different pesticides; a glyphosate-based herbicide and an azoxystrobin and difenoconazole-based fungicide, or a combination of these two via their larval host plant (*Plantago lanceolata*) leaves. It is plausible that organisms inhabiting agricultural landscapes will be exposed to multiple pesticide products simultaneously, which is why a combination of the two pesticide products was also included. Dilutions of the pesticide products were made following manufacturer instructions in Finland and the exposure was carried out for three consecutive days during the larval development. We were interested in a short-term exposure via host plants. The three-day exposure was chosen to ensure that all larvae would be exposed orally to the pesticides, since our prior experience in *M. cinxia* has shown that occasionally larvae can go for few days without eating. Prior to and after the treatment the larvae were reared using standard methods until adulthood, upon which adult butterflies were released into an outdoor population cage for further monitoring of adult performance. Different individual-level fitness traits such as larval growth rate and survival, adult mobility, lifetime reproductive success and adult lifespan under semi-natural conditions were assessed.

2. Materials & methods

2.1. Study species

The Glanville fritillary butterfly (*Melitaea cinxia*) is a well-studied model species in ecology, that has a wide distribution across Europe and temperate Asia (Hanski, 1999). In Finland, the species occurs only in the Åland islands, an archipelago in south-west Finland, where the butterfly larvae feed on two host plant species, the ribwort plantain (*Plantago lanceolata)* and the spiked speedwell (*Veronica spicata)* (Ojanen et al., 2013). These plants grow in dry meadows or pastures, often in the vicinity of agricultural areas (Kuussaari et al., 2000). Female butterflies lay eggs in clutches of usually 150–200 eggs and larvae live gregariously on the host plants until they go into diapause for the winter inside a winter nest spun from silk (Kuussaari, 1998; Saastamoinen, 2007a). Larvae usually have 7 or 8 instars before they pupate, and the growth rate of the larvae is the highest in the last instars, where they require larger amounts of host plants to feed on (Kuussaari, 1998). Post-diapause larvae are also more mobile and can move from one host plant into another within the same habitat patch (Ojanen et al., 2013). The two host plants that the larvae feed on produce iridoid glycosides as secondary metabolic compounds and the larvae sequester these compounds and use them in their own defence (Saastamoinen et al., 2007). The Glanville fritillary butterfly spends most of its life in the larval stage and adult butterflies fly only for a couple of weeks in the summer where they feed on nectar (Kuussaari, 1998).

2.2. Butterfly rearing

Diapausing butterfly larvae were collected from their natural habitats in the Åland islands in September 2023. Larvae were placed in individual Eppendorf tubes to diapause over the winter in 5 °C, 80 %

relative humidity and 24 h darkness in a growth chamber. Diapause for all larvae was broken in mid-March 2024. Each larva was individually placed in a plastic cup (⌀ 4.3 cm) and fed with ad libitum *P. lanceolata* leaves every day from Monday to Thursday, during which they were kept in growth chambers with a photoperiod of 12:12 at temperatures of 28 °C during the day and 15 °C during the night. From Friday to Sunday larvae were kept inactive under cooler temperatures of 12 °C at day and 8 °C at night with no fresh food. The rearing conditions follow our previous experience on the standard laboratory conditions for *M. cinxia* that have been developed to simulate the natural conditions that larvae encounter (Verspagen et al., 2020; Rytteri et al., 2021). Larvae were reared with these standard practices from diapause until they reached their 7th instar. We chose to do the pesticide exposure during the 7th instar since at this stage larvae grow the most and are eating larger amounts of food making potential pesticide exposure more likely. After diapause mortality and death due to parasitism, 401 larvae reached the 7th instar and were assigned either into one of the three pesticide treatments or into a control treatment. The pesticide exposure was done during the 7th instar stage and after completing the treatment the larvae were reared using the aforementioned conditions until pupation.

2.3. Pesticide exposure

The day after each larva reached the 7th instar, they were assigned into one of four treatment groups: control ($N = 105$), herbicide treatment (N = 105), fungicide treatment ($N = 97$) and pesticide mix treatment ($N = 94$). Control larvae were fed *P. lanceolata* leaves with no pesticide on them. For herbicide treatment, the commercial product Glyphomax 480® (Finnish registration number 3102) which includes 480 g of the active substance glyphosate in one litre was used and diluted per the Finnish label instructions at the maximum concentration 6 L of product in 200 L of water (14,4 g of active substance in 1 L of water). For fungicide treatment the commercial product Amistar Gold® (Finnish registration number 3321) including 125 g of the active substances azoxystrobin and difenoconazole in one litre of product and this was diluted again per the Finnish label instructions with 1 L of product in 200 L of water (0,6 g of active substance in 1 L of water). Pesticide mix was created by diluting equal amount of both products (6 L of Glyphomax 480/200 L of water and 1 L of Amistar Gold/200 L of water) in water. These products were chosen as glyphosate is the most used herbicide globally (Benbrook, 2016) and azoxystrobin residues have been commonly found in pollen and nectar (Zioga et al., 2023). Both products are also commonly used in Finland and were readily available from retailers. Pesticides were applied to one side of *P. lanceolata* leaves with a paintbrush under a fume hood, covering the top side of the leaf with the diluted pesticide product. The amount spread on each leaf was not standardized, and as such it is possible that the exact amounts of product applied differed slightly between the plant leaves. Leaves were left to dry under the fume hood until surface was dry and then added to the containers with larvae. This was repeated for three days for each larva, after which they were fed with normal leaves ad libitum until pupation. Larvae in all treatment groups were observed to eat at least on one of the treatment days, but the amount consumed by each larva was not quantified.

2.4. Adult butterfly enclosure

Once larvae pupated, the pupal weight and pupation date were recorded. Pupae were kept in cool conditions (12 °C/8 °C) until about a week before the planned starting date of the outdoor enclosure experiment with the aim of synchronising eclosion for the outdoor cage without large variation. To identify each butterfly in the outdoor enclosure, newly eclosed butterflies were sexed and marked individually by writing a number with permanent marker on the underside of the hind wing. In addition, their forewing length was measured with a caliper to the closest 0.5 mm. All but three malformed adults were released in one outdoor enclosure in the evening of the day of eclosion. The outdoor enclosure was 32 × 26 × 3 m in size and covered with mesh (Hanski et al., 2006). The ground of the enclosure was similar to a small dry meadow, the usual habitat of the butterfly. Altogether 215 individuals were released in the enclosure within a period of 6 days starting on 15th of May 2024. Due to late spring, there was a limited number of naturally occurring flowers providing nectar to adult butterflies in the enclosure, so fresh dandelions (*Taraxicum* sp.) were provided as additional nectar resources for the first few days until more nectar flowers grew on the ground. 150 potted larval host plants (*P. lanceolata*) were placed into the enclosure for ovipositing females (for details see fig. A1. in appendix A). The butterflies were observed in the enclosure continuously while butterflies were active, for a total of 19 sunny and warm days. Systematic censuses of an 8 × 8 m grid were carried out every two hours between 9 am and 5 pm. Additional observations were done to observe mating pairs and ovipositions also in between the censuses. The butterflies mated freely within all treatment groups. Host plants were also inspected at the end of each day for any additional egg clutches where the female laying the eggs might have not been detected during the day. All egg clutches were collected and transferred to the growth chamber with same settings as used for the rearing of the post-diapause larvae. Number of eggs and the number of larvae hatching for each clutch were counted. Dead individuals found from the enclosure were recorded and stored if the individual ID number was still visible.

2.5. Statistical analysis

Larval survival after receiving the treatment was analysed using the "survival" R package (Therneau, 2024), and a survival object that included survival time and whether or not the individual died before pupation was created and survival curves were fitted for each treatment following the Kaplan-Meier method. For individuals that pupated successfully survival time ends at pupation. A log rank test was performed using the "survdiff" function after which pairwise comparisons between each treatment group were made using the Benjamini-Hochberg correction for multiple comparisons.

To calculate larval growth rate, pupal weight was divided by the development days from the 7th instar into pupation. For development days only the days when larvae were exposed to warm development conditions were counted (i.e. excluding days when larvae were kept in cool conditions). Adult lifespan was calculated based on recorded death date from the outdoor enclosure or in the case of no recorded death date, derived from the last observation during the censuses. Individual mobility index was calculated as a residual from the regression of the number of grid cells in which the butterfly had been observed against the number of observations during the censuses in the enclosure (Saastamoinen, 2007b). Individual mobility index was calculated for entire lifespan and for early life only (first 3 days after release), as previous studies have shown that early life mobility is a particularly relevant movement index for females (Hanski et al., 2006).

Generalized linear mixed models were fitted using the R package glmm (Brooks et al., 2022) with pupal weight, growth rate, forewing length, adult lifespan and mobility indices as the response variables, treatment, sex and their interaction as explanatory variables, and sample origin (collection location) as a random factor. The interaction of sex and treatment was kept in the model for pupal weight and mobility but removed for all of the other traits based on AIC comparison. Pupal weight, growth rate and forewing length were normally distributed so a gaussian family was used in the model. A negative binomial model was used for lifespan and mobility data was log transformed to achieve normality.

Mating success was analysed by fitting a general linear mixed model with a binomial family including whether or not individual mated as the response variable with treatment and sex and their interaction as explanatory variables. Lifetime reproductive success was measured as

the number of larvae produced by the individual. General linear mixed models were fitted to analyse the total number of larvae between the treatment groups and analysed separately for females and males. Since this data was count data and not normally distributed a negative binomial family was used and treatment was used as the explanatory variable. Male fecundity was analysed as the total number of larvae that were sired by the male. Males often mate multiple times, so all the larvae from different females that the male mated with were added together. In cases where females were observed to mate more than once it was assumed that the larvae were sired by the last male that the female mated with (Sarhan and Kokko, 2007).

Sample origin (collection location) was included in all of the generalized mixed models as a random factor. Type III ANOVAs were run for each of the generalized linear mixed models by using the "car" package (Fox et al., 2023) and the estimated marginal means (least-squares means) were computed using the "emmeans" function (Lenth et al., 2022) for each response variable. Pairwise comparisons were then conducted for each treatment and both sexes, with Tukey method *p*-value adjustment for comparing the four treatments. All statistical analysis were performed using R version 4.3.1 (R core team, 2023).

3. Results

3.1. Larval mortality and development

In total 401 larvae were used for the experiment, of which 235 completed pupation and 215 eclosed successfully as adult butterflies. Larval mortality varied between the treatments with mortality being highest in the fungicide treatment (60 %), followed by the pesticide mix treatment (22 %), herbicide (4 %) and control (3 %). The fungicide and pesticide mix treatments larvae had significantly higher mortality compared to both herbicide and control treatments ($p < 0.0001$; Fig. 1), whereas there was no statistical difference in mortality between control (3 individuals of $N = 105$) and herbicide (5 individuals of N = 105) treatments ($p = 0.18$).

Larval growth rates (pupal weight/development time) were significantly higher in females than in males for all treatments (Fig. 2.). This is mainly due to females being significantly heavier at pupation with average pupal weight of 217 mg for females and 167 mg for males (table A1. in appendix A). Growth rates of female larvae exposed to the fungicide (mean 7.1 mg/day) and pesticide mix (mean 7.8 mg/day) treatments were lower in comparison with larvae exposed to control (mean 9.2 mg/day) and herbicide (mean 8.7 mg/day) treatment. Similar patterns were observed for males where the average growth rates were lower in fungicide (6.0 mg/day) and mix treatments (6.7 mg/day) in comparison to control (8.3 mg/day) and herbicide (7.7 mg/day). Even though the growth rates were lower in all treatments in comparison to control, the differences were statistically significantly lower in the fungicide and mix treatments for both sexes in comparison to control and herbicide ($p < 0.0001$; Fig. 2.). Similarly, pupal weights were significantly lower in the fungicide and mix treatments compared to control and herbicide for both sexes ($p < 0.01$), with the exception that in males there was no significant difference in the pupal weight between herbicide and mix treatments ($p = 0.12$). There were no significant differences ($p > 0.05$) between the fungicide and pesticide mix treatments or between control and herbicide treatments in the growth rate in either sex.

3.2. Adult life history traits

In total 215 adults (control $N = 67$, fungicide $N = 26$, herbicide $N = 68$, mix $N = 54$) were released into the outdoor enclosure. Forewing lengths were longer on average in females than in males ($p < 0.001$), which is usual for this species since females generally have larger body size. Females exposed to fungicide and pesticide mix treatment during their development had shorter forewing length (16.3 and 16.6 mm, respectively) in comparison to females from control and herbicide treatment groups (17.3 and 17.1 mm, respectively). In males the pattern was similar with shorter forewings in fungicide and mix (14.2 and 14.1 mm, respectively) compared to control and herbicide (15 and 14.8 mm,

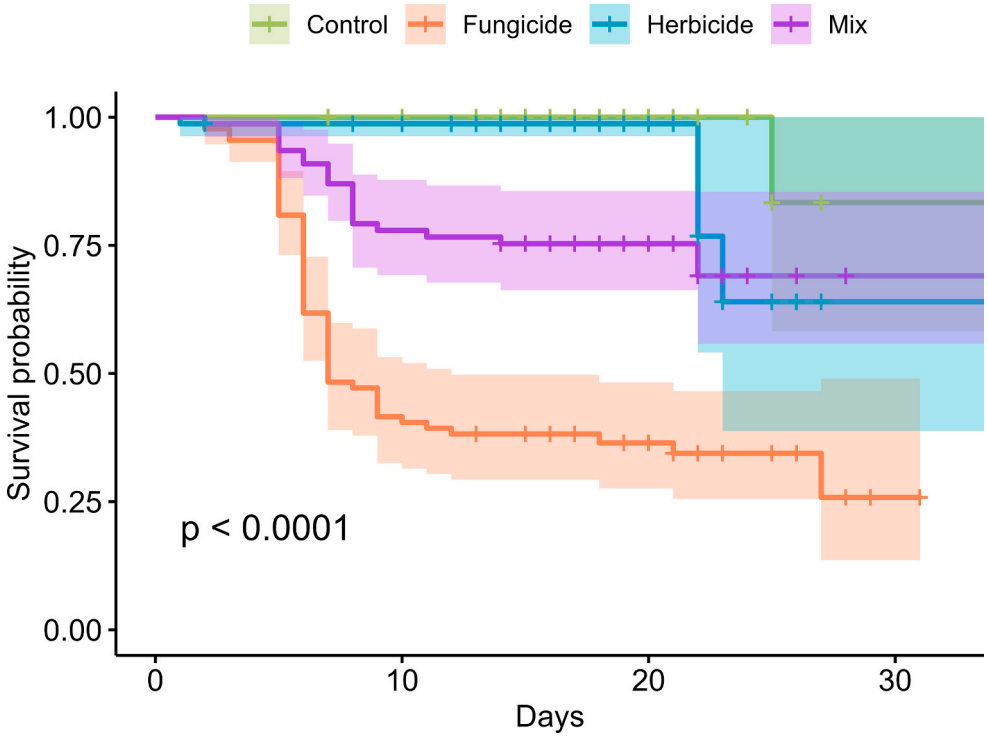

Fig. 1. Larval survival probability between the treatment groups (represented by different colours). Crosshatches on curves represent larvae that pupated. *P*-value <0.0001 indicates significant effect of treatment on survival.

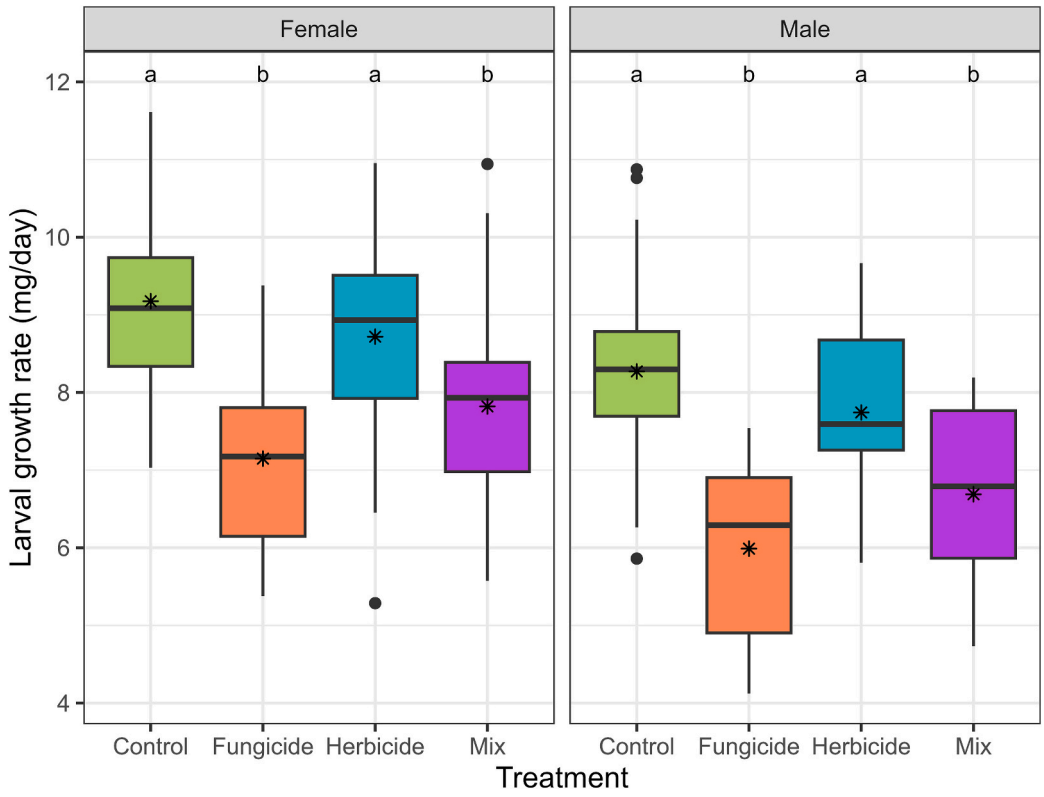

Fig. 2. Larval growth rate (mg/day) per treatment and sex. Horizontal lines in the box plots represent the median, asterisks show the mean and circles represent outliers. Different letters indicate a significant difference between treatment groups ($p < 0.0001$).

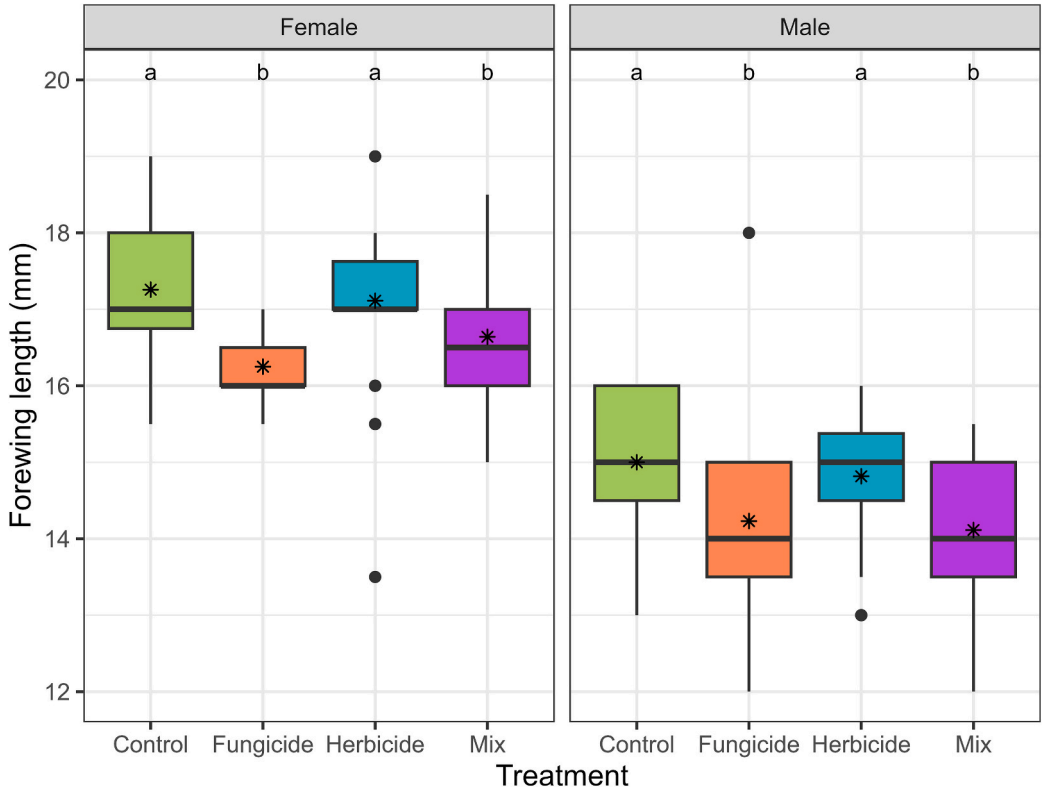

Fig. 3. Forewing length (mm) per treatment and sex. Horizontal lines in the box plots represent the median, asterisks show the mean and circles represent outliers. Different letters indicate a significant difference between treatment groups ($p < 0.01$).

respectively) even though the differences were slightly smaller for males. The differences between fungicide and mix in comparison to control and herbicide were statistically significant for both sexes ($p < 0.01$; Fig. 3.). No significant difference was found between control and herbicide or between fungicide and pesticide mix treatment groups ($p > 0.8$) for either sex. There were no differences in the lifetime mobility index between any of the treatment groups in either sex ($p > 0.05$, appendix A, table A1.). However, a small but significant difference was evident between females exposed to control and herbicide treatment during their development for early life mobility ($p = 0.02$) with females exposed to herbicide treatment having higher early-life mobility (4.3) than control females (3.3). No differences in early-life mobility were found between the other treatment groups ($p > 0.05$).

Out of the total of 120 females released into the population enclosure 85 % ($N = 104$) mated, whereas in males the mating rates were lower (56 %) due to some males mating multiple times. Most of the mated females ($N = 81$) laid eggs. Of these, the majority ($N = 66$) were observed to mate whereas mating was unobserved for 15 of the females that laid eggs, and 22 females were observed to mate but never laid eggs. There were no significant differences between the treatment groups in likelihood of mating for either sex or laying eggs for females (appendix A, table A1.).

Total lifetime fecundity for females, analysed as the total number of larvae varied between 0 and 1118 larvae. The total number of larvae varied significantly between females from herbicide (314 larvae on average) and pesticide mix (192 larvae on average) treatment groups ($p = 0.04$; Fig. 4.). No differences were observed in the fecundity of males between the different treatments ($p > 0.16$) for the number of larvae they sired.

The average lifespan of all individuals was 5.5 days and 5.2 days for females and males, respectively and there were no differences in lifespan between the adults exposed to different treatments during their development for either sex ($p > 0.4$, appendix A, table A1.).

4. Discussion

In this study we show that short-term pesticide exposure affects both larval and adult stages of the specialist Glanville fritillary butterfly, a non-target species that occurs in agricultural landscapes. Specifically, we assessed how short-term pesticide exposure affected larval mortality, growth rate, and various adult fitness traits such as mobility, fecundity and longevity. We found that a fungicide containing the active substances azoxystrobin and difenoconazole, as well as mixture of the fungicide and glyphosate-based herbicide impaired the fitness of the butterflies when larvae were exposed to the pesticide via their host plant.

4.1. Fungicide but not herbicide drastically impacted larval survival and development

Our results show that fungicide exposure during the final larval instar significantly induced a 60 % mortality rate of the butterfly larvae. In addition to reduced survival, the fungicide treatment also reduced the pupal weightand growth rates of the larvae who survived the exposure. The fungicide used in this study was a broad-spectrum systemic fungicide, which contained two active substances: azoxystrobin and difenoconazole. Azoxystrobin affects the plant pathogen by inhibiting mitochondrial respiration by blocking electron transport (Becker et al., 1981) and difenoconazole inhibits demethylation during ergosterol synthesis (Liu et al., 2021). The mitochondrial respiration target site of azoxystrobin is also present in animals and previous studies incorporating azoxystrobin have shown that it can negatively affect Monarch butterfly larval survival (Olaya-Arenas et al., 2020) and may even affect bumblebee memory retention (Kaila et al., 2023). Our results align well with some previous research suggesting that fungicides can have detrimental effects on non-target arthropods, likely due to their broad-spectrum activity, which can interfere with crucial biological processes (Sánchez-Bayo, 2021). The reduced larval growth rate in fungicide-treated groups can be attributed to potential disruptions in

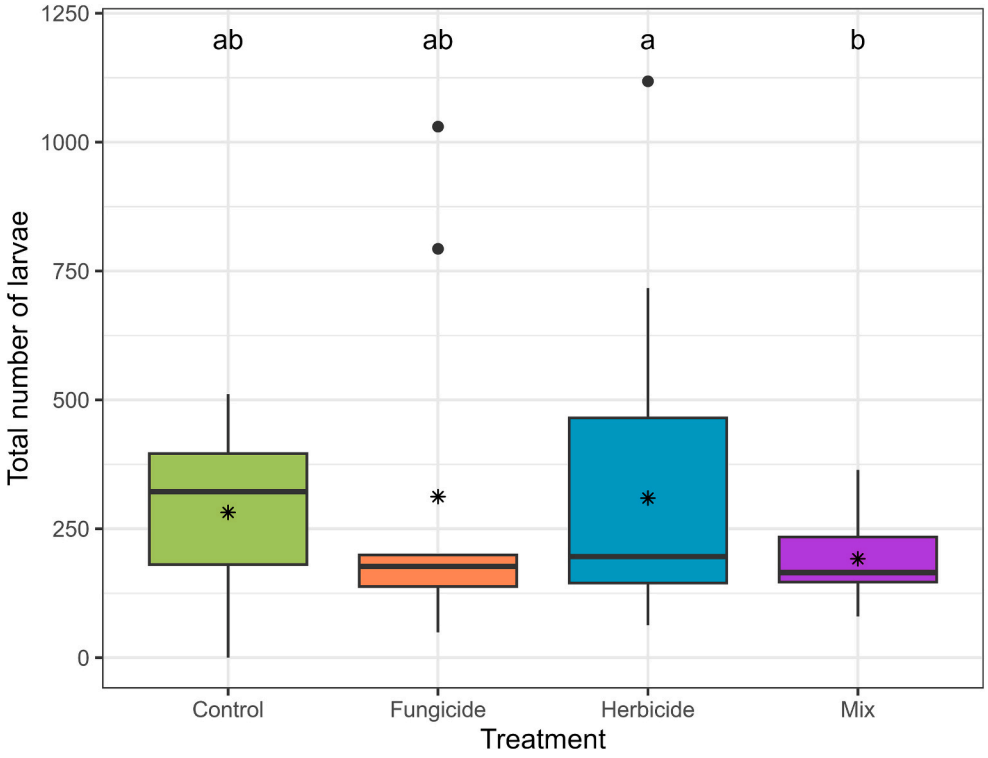

Fig. 4. Female lifetime reproductive success (lifetime number of larvae produced) of each treatment group. Horizontal lines in the box plots represent the median, asterisks show the mean and circles represent outliers. Different letters indicate a significant difference between treatment groups ($p = 0.04$).

nutrient absorption or metabolic functions, as fungicides may impair gut flora or physiological mechanisms critical for growth (Huang et al., 2023). The gut microbiota of *M. cinxia* has been shown to be highly variable among individuals (Minard et al., 2019) and it is possible that differences in the gut microbiome could help some individuals tolerate the fungicide exposure, but this warrants further studies.

In contrast, exposure to glyphosate-based herbicide during development did not significantly impact larval mortality or growth. Glyphosate is a broad-spectrum systemic herbicide, which impacts plants by inhibiting the plant enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSP) (Funke et al., 2006). Since the target site for glyphosate is not present in animals, it has been considered to be non-toxic to animals. Recent studies have, however, raised concerns whether glyphosate can have other non-lethal negative impacts on animals such as impacting the thermoregulation in honeybees (Weidenmüller et al., 2022) and fine-colour discrimination in bumblebees (Helander et al., 2022). Our results suggest that this specific herbicide might not be immediately toxic to specialist Glanville fritillary larvae at the concentrations and short exposure time tested in this study or that there could be another interaction at play that makes the larvae less susceptible to herbicides. The host plants used by the Glanville fritillary butterflies produce secondary compounds called iridoid glycosides (aucubin and catalpol) (Saastamoinen et al., 2007), to chemically defend themselves against herbivores. The specialist larvae have, however, co-evolved to overcome these defences, and even sequester these chemical compounds to protect themselves against insectivorous vertebrates (Suomi et al., 2003; Dobler et al., 2011). The ability of the Glanville fritillary larvae to sequester secondary compounds could potentially influence their response to other chemicals, such as those apparent in herbicides. These compounds might enhance the larvae's ability to detoxify harmful substances, thus reducing the negative impacts of herbicide exposure. Iridoid glycosides have been shown to play a role in the detoxification processes in insects, potentially mitigating the toxic effects of herbicides (Bowers, 1992). For butterfly species that do not sequester secondary compounds from their host plants, such as *Pieris rapae*, herbicides have been shown to negatively impact larval survival (Russell and Schultz, 2010). Also, the short exposure time tested in our study might have masked effects that could be present in longer more chronic exposure studies. Testing more chronic, longer exposure but at lower levels could produce different results. On top of this, comparative studies assessing responses to herbicide in specialist vs. generalist non-target species would further our understanding on the possible mechanisms of these interactions.

The impacts of pesticide 'cocktails' (i.e. more than one pesticide at the time) have traditionally been overlooked in studies assessing impacts of pesticides and in risk assessments, although recently there have been calls for the cocktail impacts to be included in risk assessments (Geissen et al., 2021; Göbölös et al., 2024). Pesticides can interact with each other in different and even unexpected ways, depending on the chemical, the dose, and the surrounding environment (Hernández et al., 2017). The effects of pesticide mixes have mainly been studied in the context of human health and the pesticide residue exposure that humans have via food (Kim et al., 2017). Yet, more recently impacts of pesticide cocktail on bees, particularly honeybees, have indicated that certain fungicide-insecticide combinations can have synergestic effects (Schuhmann et al., 2022). Jansen et al. (2017), for example, reported synergetic effects of insecticides and fungicides in three different non-target arthropods, even when fungicide alone had no toxicity effects. Here, we found some mixed results on the impacts of pesticide 'cocktails'. The mortality of the larvae exposed to two pesticides simultaneously was lower than for larvae exposed to fungicide only. This suggests, potentially an antagonistic interaction between the herbicide and fungicide, as when both pesticides are present, the lethality of the fungicide seems to be lessened by the herbicide. One possibility is that the presence of a herbicide reduces the natural plant defence mechanisms, which in turn results in the plant being easier to digest for the butterfly larva hence reducing the negative impacts of fungicide. Further studies would be needed to confirm this. It would be nevertheless important to consider combined pesticide effects in the context of current biodiversity loss and how they might affect the different non-target species such as pollinators.

4.2. Exposure during development impacted adult morphology and female fecundity

In our study both pupal weight (used as a proxy for body size) and forewing length of the butterflies exposed to either fungicide or the pesticide mix treatment during their development were significantly lower compared to the control and herbicide treatments. Body size can be an indication of the overall fitness of butterflies (De Brito Freire et al., 2023), and thus, alterations in morphology could have consequences for the performance and fecundity of the individual. The results thus suggest that early exposure to certain pesticides can lead to morphological changes that may affect the performance and overall fitness. Even though the amount of food was not specifically assessed in our experiment, we do not think that the difference in body size results from the larvae in those treatment groups (fungicide and mix) not eating or eating less food during the exposure period. This is because previous short-term starvation experiments during the same developmental period have not impacted body size, since food deprived individuals compensate for the starvation via delayed development time (Saastamoinen et al., 2013). Such compensation was not evident in the present study.

Smaller body size can impact different performance traits such as fecundity, heat tolerance and mobility (Berger et al., 2008; Bladon et al., 2020; Kuussaari et al., 2014). Previous studies on other species have similarly shown that larval exposure to a herbicide or a fungicide can negatively impact wing morphology, resulting in shorter and/or smaller wings (Olaya-Arenas et al., 2020; Russell and Schultz, 2010). Wing size and/or length is often used as proxy for dispersal on butterflies (Sekar, 2012), and previous studies have speculated that exposure to fungicides might also negatively impact the dispersal ability via impacts on wing morphology. We did not find support for this as individuals exposed to fungicide treatment did not show reduced lifetime or early-life mobility even though their wing size was significantly smaller. Interestingly, females exposed to herbicide treatment during their development showed increased early-life mobility compared to control females. Whether this would actually reflect increased emigration and/or dispersal under more natural conditions would be an interesting next step to assess.

In female butterflies, the lifetime fecundity (measured as a total number of eggs and larvae produced) of the individuals receiving the pesticide mix treatment was lower on average than for other treatments. This was especially evident between the herbicide and mix treatment, where females exposed to the pesticide mix had significantly lower lifetime fecundity compared to females exposed to the herbicide treatment alone. This suggests that combined pesticide exposure may have amplified the negative reproductive effects in female butterflies. It is possible that there is an interaction between the two pesticides making them more stable when together, and thus remaining in the body of the insects throughout metamorphosis and into adulthood and as such impacting the lifetime reproductive success of females. Interestingly, lifetime fecundity of the females exposed to herbicide and fungicide treatment was also slightly higher than that of the control females, even though this was not statistically significant. This could indicate a compensatory response mechanism where exposed females lay more viable eggs than those in the control group. It is also possible that the pesticide exposure during larval stage filtered out individuals with poorer fitness in the treatment groups, resulting in only "high-quality" individuals being able to survive and reproduce successfully, whereas in the control group also "poorer quality" individuals would have survived until adulthood and reproduced successfully. No differences were observed in fecundity for male butterflies. In general, our results show interesting dynamics in relation to single and multiple-pesticide exposure, but larger sample sizes would be needed to draw further

conclusions.

Finally, no differences were observed in lifespan across the different treatment groups. Overall, the results suggest that while short-term fungicide exposure negatively impacts larval growth, adult morphology and female fecundity these impacts do not seem to extend to adult longevity under the exposure level used in this study. However, even if we did not see carry-over impacts after this level of exposure, it is possible that a longer more chronic exposure over different juvenile stages of the butterfly could have more profound impacts also on the adult stage. It is also possible that any sub-lethal effects at the larval stage can be compensated for during the pupal and early adulthood stage, allowing adults to exhibit similar mobility and lifespan as control butterflies. Similar results were reported by Olaya-Arenas et al. (2020) where only marginally negative impacts of larval pesticide exposure were found on adult monarch butterfly longevity. In fact, monarchs exposed to fungicides as larvae even had extended longevity as adults, compared to control individuals. It is also important to note that the average lifespan of all butterflies in our study was overall much shorter (4.4 to 7 days) in comparison to previous studies under similar setting (Hanski et al. 2005, Saastamoinen et al., 2007, Rosa and Saastamoinen, 2021). This was likely influenced by the prevailing hot conditions during the study, which is known to reduce adult lifespan (Saastamoinen et al., 2007). Generally shorter lifespan may have also masked potential differences between the treatments.

4.3. Limitations and future directions

Due to logistic reasons, we tested only one herbicide and one fungicide from the large pesticide family with different active substances and different modes of actions. In future, more active substances and their combinations should be studied to understand better the impacts of fungicides and herbicides on non-target organisms. The tested commercial fungicide product contained two active substances, azoxystrobin and difenoconazole. While the fungicide affected both the larval and adult stages of the Glanville fritillary butterfly, it should be further studied which active substance impaired the fitness of the life stages or whether it was the combination. Due to the unexpectedly high larval mortality in the fungicide group, the sample size for adults was smaller than for other treatment groups which might have reduced the statistical power in the analysis for the adult traits in this study. Another topic of interest has been different co-formulants of pesticide products and what are their impacts on non-target species. The co-formulants in glyphosate-based herbicides have been shown to have toxic impacts (Defarge et al., 2016; Klátyik et al., 2023) and Straw and Brown (2021) showed that a co-formulant in a commercial fungicide product Amistar® caused lethal and sub-lethal effects on bumble bees. It is also possible that in this study the co-formulant in Amistar Gold® the co-formulant is the actual ingredient causing the larval mortality and other sub-lethal impacts, so this should be considered in future studies.

Future studies should look further into how different dosages of pesticides might impact different non-target species. Exposure levels should be tested at different exposure times and at different life stages of insects. For this, more studies are needed to estimate the amounts of pesticide residuals found in different terrestrial habitat types. In our study we were limited in developing our methods due to very limited data on pesticide residues found within the agricultural areas in the Åland islands and in Finland in general. When it comes to butterflies, it would also be important to include species with different host plant requirements (e.g. generalist v. specialist) especially when looking into impacts of herbicides. This could help shed more light on the potential interactions from hostplant-butterfly coevolution and how the specialised ability of certain larvae to sequester plant defence chemicals might influence these interactions (Bras et al., 2022). It would be important to also further improve our knowledge on how pesticides impact different life stages of a butterfly and preferably also extend the study period to the next generation for potential transgenerational effects.

5. Conclusion

The results of this study underscore the complexity of pesticide impacts on non-target species like the Glanville fritillary butterfly that live in agricultural landscapes. While fungicide exposure poses clear risks to larval survival and growth, the carry-over effects into adulthood, particularly concerning reproduction and mobility, warrant further investigation. The observed morphological changes and altered fecundity patterns suggest that pesticide exposure can have carry-over impacts, potentially affecting population dynamics in natural settings. Given the widespread use of pesticides in agricultural landscapes, pesticide impacts should be considered when developing conservation strategies for butterflies and other non-target organisms. Mitigating pesticide exposure through the use of less harmful alternatives, buffer zones, and integrated pest management practices could help preserve biodiversity and ecosystem health. Future research should focus on long-term and multi-generational studies to fully elucidate the implications of pesticide exposure for non-target insect populations.

CRediT authorship contribution statement

Ulla Riihimäki: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mathijs de Koning:** Writing – review & editing, Investigation. **Lotta Kaila:** Writing – review & editing, Methodology, Conceptualization. **Marjo Saastamoinen:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Parts of graphical abstract were created using biorender.com.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2025.179214.

Data availability

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.1vhhmgr43

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