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Article

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Abstract

Non-insecticidal pesticides, such as fungicides and herbicides, are widely used in agriculture, yet their effects on insects remain poorly understood. We investigated the Moselle Apollo butterfly (*Parnassius apollo vinningensis*), an endemic subspecies on the brink of extinction in Germany's viticultural Moselle Valley. In early spring, even before seasonal pesticide spraying began, its larval host plant, *Sedum album*, carried complex pesticide mixtures: we detected 18 active ingredients, comprising 14 fungicides and four herbicides, but no insecticides, with up to nine compounds per sample. Larvae feeding on contaminated plants carrying pesticide mixtures with a higher diversity of modes of action had lower body masses after 31 days, with predicted final body mass declining by about one-third across the observed gradient. By contrast, experimental exposure to a simulated regional aerial fungicide regime had no detectable effect on larval survival or body mass, whereas Luna® Experience, a widely used fungicide containing the PFAS fluopyram, strongly reduced survival to adult emergence. Together, our findings show that pesticides not designed to control insects can nevertheless adversely affect them, and suggest that exposure to complex pesticide mixtures at the landscape scale may represent an underestimated ecological threat, potentially contributing to population declines in already endangered insect species.

Introduction

Pesticide contamination of agricultural landscapes is increasingly recognized as a potential driver of widespread declines in insect biomass, abundance, and diversity[1–3]. While research has historically focused on insecticides, growing evidence suggests that non-insecticidal compounds, including fungicides, may also pose ecological risks to insects[4–6]. This is particularly relevant in perennial cropping systems such as viticulture, where fungicides are applied intensively and frequently occur as components of complex pesticide mixtures in agricultural landscapes and beyond[7–10]. Yet, the consequences of chronic exposure to fungicides and observed complex pesticide mixtures for non-target insects remain poorly understood. Moreover, experimental studies on pesticide mixture toxicity in insects have largely relied on artificially reconstructed mixtures, even when these were derived from environmental monitoring data[11]. Experimental assessments in which non-target insects are exposed through naturally contaminated host plants remain scarce, leaving uncertainty about whether effects observed with reconstructed pesticide mixtures adequately reflect those arising from real-world exposure.

A particularly relevant system in which to investigate these questions is the Moselle Apollo butterfly (*Parnassius apollo vinningensis* Stichel, 1899), a narrowly endemic subspecies of the Apollo (*Parnassius apollo*) restricted to the Moselle River valley upstream of Koblenz, Germany[12]. The Moselle Apollo is strictly protected under the EU Flora–Fauna–Habitat Directive[13] and depends on open, rocky slopes supporting its larval host plant, white stonecrop (*Sedum album*)[12]. In the Moselle Valley, these habitats are closely associated with viticulture, which can help maintain suitable open conditions by preventing shrub encroachment (Fig. 1,[14]). This close association creates a potential conservation trade-off: viticulture contributes

to maintaining suitable habitat while simultaneously creating the potential for pesticide exposure.

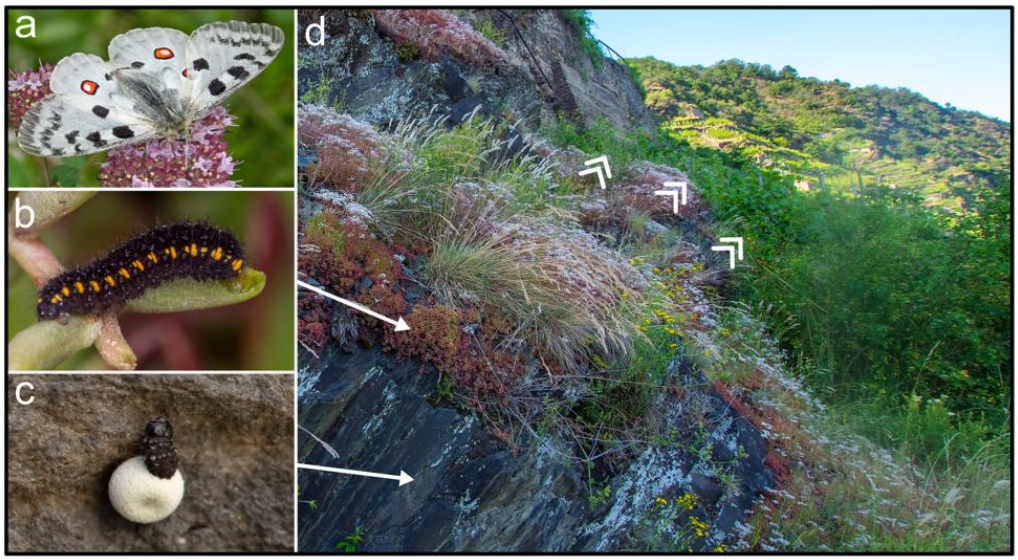

Figure 1: Life stages and natural habitat of *Parnassius apollo vinningensis* in the Moselle valley. (a) adult; (b) larva feeding on *Sedum album*; (c) larva hatching from egg after overwintering in the L1 stage; (d) south-facing slope covered with *S. album* in bloom (white flowers). Caret-shaped symbols indicate adjacent viticulture. Photos: Tim Laußmann (a, b, d), Daniel Müller (c).

Despite the role of viticulture in maintaining suitable open habitats, the Moselle Apollo is now threatened with extinction[15,16]. Its population declined sharply in 2012 and again in 2019, followed by continued low abundance and no apparent recovery[14,15,17,18]. Whereas more than 100 individuals could be recorded on suitable survey days in 2008, only a few or no individuals were observed across the same sites in 2019[17]. Several factors have been proposed to explain this decline, including habitat overgrowth, traffic-related mortality, climate change, and pesticide exposure associated with viticulture[12,14]. Notably, unlike other remaining Apollo populations in Germany, which are currently considered stable, the Moselle population directly overlaps with areas of intensive viticulture[12,14].

While viticulture may promote the availability of larval host plants, it may simultaneously expose them to pesticide contamination. In contrast to most other German wine-growing regions, fungicides in the Moselle valley are applied aerially, traditionally by helicopter, eight to ten times during the growing season. Notably, aerial insecticide applications in the region were discontinued in 1992[19] to reduce exposure of non-target organisms, shifting aerial pesticide application to fungicides only. *P. a. vinningensis* overwinters as eggs, with larvae typically hatching in March–April and feeding on *S. album* during early spring before pupating between May and June[14], although weather conditions can advance hatching to February[20]. Aerial fungicide applications generally begin in early May and continue through August, including up to nine application flights with up to four pesticide products per flight[21], creating potential temporal overlap with late larval development, pupation, and the adult flight period. In addition, residues remaining from previous applications may result in exposure outside the immediate spraying period.

The predominance of fungicides in Moselle viticulture is particularly relevant because several widely used fungicide classes have modes of action with the potential to affect non-target

insects. Sterol biosynthesis inhibitors (SBIs, e.g. Tebuconazole) can interfere with insect detoxification pathways and modify the toxicity of co-occurring pesticides[5,22], while adverse effects of individual SBIs on insect physiology have also been reported[23,24]. Succinate-dehydrogenase inhibitors (SDHIs, e.g. Fluopyram) likewise warrant attention because their molecular target is highly conserved across taxa[25], and experimental evidence indicates that some SDHIs can disrupt cellular metabolism and adversely affect non-target insects[26,27]. Both fungicide classes can be systemically taken up and translocated within plants[28–30], providing a direct route of exposure to herbivorous insects. Their repeated detection in environmental matrices and, for some compounds, prolonged environmental persistence further raise the possibility that herbivores encounter fungicides not only during application but also as components of complex residue mixtures[7–10,31,32].

Here, we investigated whether pesticide exposure associated with viticulture could adversely affect the Moselle Apollo by combining environmental residue analysis with complementary exposure experiments. First, we quantified pesticide residues on *S. album* collected directly from Apollo habitats and reared larvae on these plants to assess chronic oral exposure to real environmental pesticide mixtures. Second, we experimentally reproduced a local aerial fungicide spray regime to test the effects of direct exposure of food plants and larvae under controlled conditions. Finally, we assessed the toxicity of Luna® Experience, a commercial formulation combining the SDHI fluopyram and the SBI tebuconazole, two fungicides relevant to regional viticulture. Together, these approaches allowed us to contrast biological responses to pesticide mixtures encountered on host plants in the field with those arising from experimentally reconstructed fungicide exposure.

2. Methods

2.1 General Experimental Procedures

Eggs and larvae of *P. a. vinningensis* were provided by the “Moselle Apollo conservation breeding project”. Unless stated otherwise, larvae were reared on *S. album* under long-day conditions (16:8 h light:dark cycle) and maintained in ventilated 1-L plastic cups covered with a fine insect mesh. Experiments were conducted in the greenhouse or climate chamber under conditions approximating those of the Moselle Valley. When outdoor temperatures exceeded 10 °C and weather conditions were suitable (no precipitation or strong winds), greenhouse trolleys were temporarily moved outdoors during the day. Host plants were provided ad libitum and replaced when consumed or desiccated. Feeding and maintenance were performed simultaneously across experimental units to standardize disturbance. Additional details on environmental conditions, larval handling, and housing are provided in the Supplementary Methods.

2.2 Field-Collected Host Plant Experiment

Experimental Design

Sedum album was collected on 29 February and 1 March 2024 at four sites in the Moselle Valley, three of which were adjacent to vineyards (Tab. 1). At these sites, plants were sampled both within vineyards and at varying distances from vineyard boundaries along the slopes. Plants were individually bagged and stored at 4 °C until use. Sampling locations are provided in Supplementary Table S1.

Approximately 500 overwintered eggs of *P. a. vinningensis* originating from the 2023 breeding season were transferred from cold storage to long-day conditions at 20 °C to induce hatching. Larvae were selected within 24 h of hatching and had not been fed before experimental

exposure. Each *S. album* accession constituted one experimental unit and was assigned to three larvae housed together in a ventilated 1-L plastic cup covered with insect netting and perforations in the walls and the bottom for ventilation. Cups were randomly distributed across greenhouse trolleys under semi-natural conditions. Larvae were reared on their assigned host-plant accession for 31 days, with food provided ad libitum, by the end of which the available host-plant material had been fully consumed. Body mass was recorded after 17, 24, and 31 days, and survival was assessed on day 31. Further details are provided in Supplementary Methods S1–2.

Table 1: Overview of *S. album* sampling in the Moselle Valley. The exact locations of the individual *S. album* accessions within locations were subsequently reconstructed using GPS data from supporting photos and satellite images and are documented in Supplementary Table S1. GPS data were not included in statistical analysis.

Location	GPS-Position	Viticulture in Direct Proximity	Number of *S. album* Plant Samples	Total number of Larvae Tested
Rosenberg	50°19'01"N 7°27'49"E	yes	26	78
Brauselay	50°08'32"N 7°11'20"E	yes	15	45
Calmont	50°06'16"N 7°08'12"E	yes	10	30
Dortebach	50°10'08"N 7°12'45"E	no	7	21

Pesticide Residue Analysis

At the start of the experiment, plant material from each *S. album* accession was sampled for pesticide residue analysis. Composite samples were frozen at −80 °C, freeze-dried, homogenized, and extracted using validated multi-residue methods following Bakanov et al.[33]. Samples were screened for 93 current-use pesticides (36 fungicides, 36 herbicides, and 21 insecticides) using HPLC-ESI-MS/MS. Positive detections were confirmed based on retention times, qualifier ion ratios, and signal-to-noise thresholds according to SANTE guidelines[34]. Detailed analytical procedures and data are provided in Supplementary Methods S3 and Tables S2-S6. For each sample, we calculated total pesticide concentration, number of detected pesticides, and the diversity of herbicide and fungicide modes of action (MoA) according to Herbicide Resistance Action Committee (HRAC) and Fungicide Resistance Action Committee (FRAC) classifications[35,36]. Residues below the limit of quantification were included in pesticide counts but excluded from concentration-based analyses.

To characterise potential variation in host-plant quality, nutrient concentrations were additionally determined in the field-collected *S. album* plants. Carbon and nitrogen were quantified by elemental analysis, while additional elements were determined by ICP-OES following microwave-assisted acid digestion. Detailed analytical procedures are provided in Supplementary Analysis S1.

2.3 Helicopter Spray Experiment

Experimental Design

A fungicide regime used for aerial applications in vineyards between Cochem-Cond and Valwig in 2023 was experimentally reproduced (Tab. 2), including field-rate and drift-rate exposure scenarios. Two larval cohorts were used: larvae previously reared on field-collected *S. album* in the host- plant experiment ("pre-exposed cohort") and pesticide-naive larvae reared on *S. album* originating from a remote, non-viticultural site. Larvae were predominantly in the third larval instar at treatment onset.

For the pre-exposed cohort, 15 larvae reared on *S. album* collected from each of the four sampling sites (Rosenberg, Brauselay, Calmont, and Dortebach) were allocated equally among the field-rate, drift-rate, and water-control treatments. Thus, each treatment included five larvae from each sampling site (n = 20 per treatment; 60 larvae in total). For the pesticide-naive cohort, 75 larvae were allocated equally among the same three treatments (n = 25 per treatment). Larvae were individually housed and assigned unique IDs.
Fungicides were applied in a precision spray chamber, with larvae and host plants sprayed simultaneously to account for direct exposure and subsequent exposure via treated plants. Field treatments reproduced the application rates of the documented aerial spray regime, while drift treatments were scaled according to published helicopter drift-deposition estimates for vineyards at a distance of 20 m[37]. Detailed spray calculations and application parameters are provided in Supplementary Methods S5 and Tables S7–S10. Larvae were weighed immediately before the first application and transferred to trays containing untreated *S. album* for treatment. Following simultaneous application to larvae and plants, larvae were maintained individually on treatment-specific host plants under semi-natural greenhouse conditions (see Suppl. Methods). Applications were repeated according to the documented vineyard spray intervals after 11, 21, and 31 days. Larvae were weighed before each application, and mortality was recorded throughout the experiment. Because a short-term temperature spike in the greenhouse caused high pupal mortality after the final application, analyses were restricted to larval body mass and survival to pupation.

Table 2: Helicopter based fungicide regime in viticulture between Cochem-Cond and Valwig in 2023 replicated in the experiment. All listed fungicides were applied in the same combination and at the same intervals as listed. Applied amounts were downscaled to the spray chamber area. The Fungicide marked in red is no longer manufactured and was therefore not included. Source: ADD Rhineland-Palatine; https://add.rlp.de/themen/landwirtschaft-und-weinbau/pflanzenschutz/pflanzenschutzrechtliche-genehmigungen/drohnenspritzung-1.

Date of Application in Field	Application Day in the Experiment	Product Name	Amount Applied in Field [kg/ha or l/ha]	Active Ingredients
19.05.2023	0	Delan® Pro	3	dithianon, potassium phosphite, potassium phosphate
		Netzschwefel	4.5	sulfur
30.05.2023	11	Delan® Pro	3	dithianon, potassium phosphite, potassium phosphate
		Vivando®	0.2	metrafenone
		Netzschwefel	4.5	sulfur
09.06.2023	21	Profiler®	2.34	fluopicolide, fosetyl-aluminium
		Talendo® Extra	0.31	proquinazid, tetraconazole
		Netzschwefel	6	sulfur
19.06.2023	31	Zorvec Zelavin®	0.3	oxathiapiprolin
		Flovine®	1.5	folpet
		Luna® Experience	0.47	tebuconazole, fluopyram

2.4 Luna® Experience Toxicity Experiments

Two sequential experiments evaluated the effects of the commercial fungicide formulation Luna® Experience, containing fluopyram and tebuconazole, on *P. a. vinningensis* development and survival. In both experiments, larvae and *S. album* host plants were simultaneously treated in the spray chamber at a field application rate of 0.47 L ha^{-1} in 150 L ha^{-1} water, corresponding to the rate used in the helicopter spray experiment. Larvae were reared on *S. album* at 28 °C under long-day conditions before treatment and subsequently maintained on treatment-specific plants under controlled climate chamber conditions until adult emergence. Adults were frozen 24 h after eclosion, weighed, and sexed. Detailed spray parameters and experimental conditions are provided in Supplementary Methods S4 and S6.

In the first experiment, predominantly third-instar larvae were individually housed and randomly assigned to Luna® Experience or the water-control treatments (n = 50 per treatment). Larval body mass was recorded repeatedly throughout development. *S. album* originated from the same site as the control plants used in the helicopter spray experiment. Climate-chamber conditions were adjusted during the experiment to improve host-plant quality and reduce background mortality; detailed settings are provided in Supplementary Methods S6.

Because control mortality reached 63%, a pattern also observed by experienced breeders when *P. a. vinningensis* is reared under laboratory conditions, a second experiment was conducted using group housing to provide a more structurally heterogeneous environment and greater microhabitat choice. Larvae were grouped by developmental stage in 15-L plastic boxes (400 × 300 × 180 mm). Within each stage, equal numbers of larvae were randomly assigned to Luna® Experience or control treatments, resulting in 10 L3, 50 L4, and 13 L5 larvae per treatment. L3 and L5 larvae were housed in one box per treatment, whereas L4 larvae were distributed across four boxes per treatment. Boxes were covered with insect mesh and randomized across two identical climate chambers maintained at 28 °C, 40% relative humidity, and a 16:8 h light cycle). *S. album* was supplied by Rieger-Hofmann GmbH (Blaufelden, Germany) and originated from a protected area away from active agricultural land under official permit. Larval body mass was not recorded to minimize disturbance.

In both experiments, newly formed pupae remained in their housing containers for 1-2 days to allow cuticular hardening before being transferred to climate chambers maintained at elevated relative humidity (80%). Structures facilitating adult emergence were provided in both individual and group housing.

A third experiment using individually housed larvae was terminated before pupation because severe mortality across treatments indicated abnormal cohort development. Methods and data from this trial are provided in Supplementary Methods S7.

2.5 Statistical Analysis

General Statistical Analyses

All analyses were conducted in R 4.4.3[38] using RStudio[39]. Mixed-effects and generalized linear models were fitted using lme4[40]. Model assumptions were evaluated using residual diagnostics implemented in the performance[41] and DHARMa[42] packages. Fixed effects were evaluated using analysis-of-variance frameworks implemented in the car[43] and lmerTest[44] packages where appropriate. Estimated marginal means (EMMs) were calculated using emmeans[45]. Graphs were generated in R and refined in Inkscape 1.4[46]. All statistical code is provided in the Supplementary Information.

Field-Collected Host Plant Experiment

To assess associations between chronic oral pesticide exposure and larval performance, pesticide contamination was described using (i) total pesticide concentration, (ii) number of detected pesticides, and (iii) presence and diversity of fungicide and herbicide MoA groups based on HRAC/FRAC classification. Predictors were analysed separately to avoid collinearity.

Larval growth across sampling days (17, 24, and 31 days after exposure) and final larval body mass (day 31) were analysed separately using LMMs. Sampling location was included as a fixed covariate, and plant ID was fitted as a random effect to account for non-independence among larvae reared on the same host plant accession. Time since exposure was included as linear and quadratic terms in growth analyses. For each pesticide predictor, candidate models of varying complexity, including additive and interaction structures, were compared against corresponding null models using Akaike's Information Criterion (AIC), with models differing by $\Delta AIC \leq 2$ considered similarly supported[47]. Models including total pesticide concentration were analysed separately because residues below the limit of quantification reduced the number of observations. Larval survival was analysed using binomial generalized linear models (GLMs; logit link) including sampling location and pesticide predictors and following the same model screening approach described above. Survival was defined as the proportion of larvae alive per plant ID after 31 days of exposure. Total pesticide concentration was again analysed separately. Individual MoA groups and herbicide MoA diversity were excluded from survival analyses because of limited variation in predictor distributions. A detailed description of the statistical analysis is provided in Supplementary Methods S4.

Because variation in host-plant nutritional quality could contribute to differences in larval performance, we additionally examined the relationship between plant nutrient composition and final larval body mass. Mean final larval body mass per host plant was correlated with nitrogen, phosphorus, copper, sulfur, and potassium concentrations using Spearman's rank correlations, with p-values adjusted for multiple testing using the Benjamini–Hochberg procedure. For nitrogen, the only nutrient significantly associated with final larval body mass, an additional Spearman's rank correlation was used to assess its relationship with pesticide mode-of-action (MoA) diversity. Further statistical details are provided in Supplementary Analysis S1.

Helicopter Spray Experiment

Larval body mass data were available for 133, 46, and 8 individuals at 11, 20, and 29 days after the first fungicide application, respectively. Because extensive pupation had occurred by day 29, the final sampling day was excluded from analysis. Analyses focused on mean body mass over time rather than individual growth curves because only 46 individuals were measured repeatedly. A linear model was fitted to larval body mass including treatment group, centred sampling time (day 20 = 0), and their interaction. Additional covariates were log-transformed baseline body mass (T0), cohort origin (uncontaminated vs. contaminated host plant origin), and housing cup position within the greenhouse. Larval survival (i.e. successful pupation) was analysed using a binomial GLM (logit link) including treatment, cohort, their interaction, and baseline body mass. Treatment effects within cohorts were assessed using EMMs.

Luna® Experience Toxicity Experiments

For individually housed larvae, growth across sampling days (7, 13, and 19 days after exposure) was analysed using a LMM including log-transformed larval body mass, centred sampling time (linear and quadratic terms), treatment, and initial larval body mass. Larval ID was included as a random effect to account for repeated measurements. To assess treatment effects on final larval body mass (day 19) independently of the growth analysis, a linear model including treatment and centred initial body mass was fitted. A treatment × initial-body mass interaction was tested but did not improve model fit; therefore, inference was based on the additive model. An interaction term was not included in the growth model to avoid unnecessary complexity. Adult male body mass was compared between treatments using a Wilcoxon rank-sum test. Females were excluded because they emerged only in the control treatment. Survival (i.e. successful adult emergence) was analysed using a binomial GLM (logit link) including treatment and initial larval body mass. Treatment contrasts were derived from EMMs. Pairwise comparisons were calculated on the logit scale, exponentiated to odds ratios (ORs) with 95% confidence intervals (CIs), and treatment-specific survival probabilities were obtained on the response scale.

For grouped larvae, adult body mass was analysed using an LMM including treatment and larval stage at treatment onset as fixed effects and housing box as a random effect. Survival was analysed using a Fisher's exact test because of limited biological replication. Individuals originating from L3 larvae were excluded because of complete mortality across treatments. In both experiments, sex ratios were not analysed statistically because of small sample sizes but are presented in Supplementary Figure S1.

Results

3.1 Field-Collected Host Plant Experiment

Across all *Sedum* samples (N = 58), 18 pesticides were detected, 15 of which occurred above the LOQ, including 14 fungicides (Fig. 2). At least one active ingredient was found in 67% of the samples, with an average of 3.1 pesticides detected per sample and up to nine pesticides in individual samples. Nine different modes of action (MoA) were represented, comprising six fungicide and three herbicide MoA groups (one below LOQ, i.e. not shown in Fig. 2). Succinate dehydrogenase inhibitors (SDHIs) were the most prevalent group, with at least one SDHI detected in 35 out of 58 samples (60%). The most frequently detected compounds were fluopyram (n = 33), fluxapyroxad (n = 26), fluopicolide (n = 25), dimethomorph (n = 24), and cyflufenamid (n = 21). These five fungicides co-occurred in 17 of the 39 pesticide-positive samples (44 %), frequently alongside additional active ingredients. Pesticide compositions across samples are shown in Fig. 2.

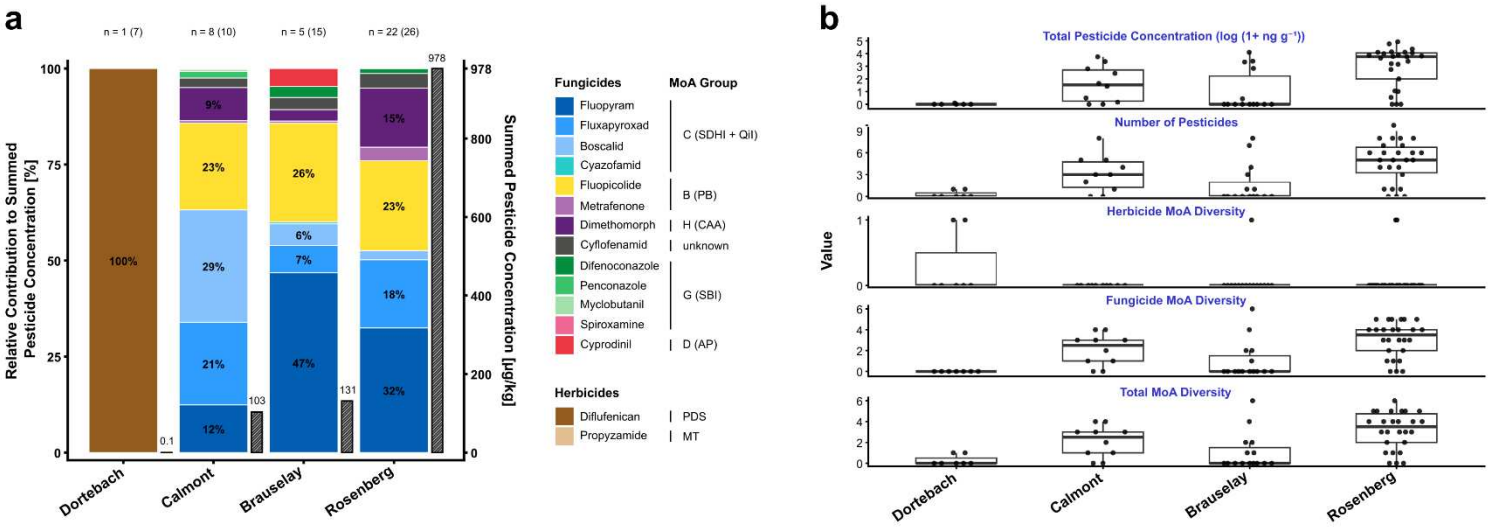

Figure 2: Distribution of pesticide residues and exposure metrics in field-collected *Sedum album* across sampling locations. Panel a: Composition and summed concentrations of pesticide active ingredients. Stacked bars show the relative contribution of each active ingredient to the total pesticide concentration summed across all plant samples from the respective location. Numbers within bars indicate these relative contributions; only values >5% are labelled. Adjacent hatched bars show the total pesticide concentration summed across all plant samples at each location. Values above bars indicate the number of pesticide-positive plant samples, with the total number of analysed plant samples given in parentheses. Colours indicate Mode of Action (MoA) groups based on FRAC and HRAC classifications[35,36]. Letters in the legend indicate MoA group codes, with subgroups indicated in parentheses: SDHI = succinate dehydrogenase inhibitors, QiI = quinone inside inhibitors, PB = pyridinylmethyl-benzamides, CAA = carboxylic acid amides, SBI = sterol biosynthesis inhibitors, AP = anilino-pyrimidines, PDS = inhibition of phytoene desaturase, and MT = microtubule interference. Propyzamide was detected only at Rosenberg, where it accounted for 0.04% (0.4 ng/g) of the summed pesticide concentration and is therefore not visible in the stacked bar. n = number of pesticide-positive plant samples (total number of plant samples). Only concentrations above the LOQ were included; consequently, 15 of the 18 detected pesticides are shown. **Panel b:** Distribution of pesticide exposure metrics (blue) in *S. album* across sampling locations used in the statistical analysis. Total pesticide concentration was log-transformed using log(1+x) prior to visualization. MoA diversity indices represent the number of distinct mode-of-action groups detected per plant sample based on the HRAC and FRAC classifications. Points represent individual plant samples; boxplots display medians and interquartile ranges. n = 7 (6), 10, 15 (14), and 26 (25) for Dortebach, Calmont, Brauselay, and Rosenberg, respectively (values in parentheses indicate sample sizes for the total pesticide concentration metric after exclusion of <LOQ cases).

Higher pesticide MoA diversity is associated with lower final larval body mass

Final larval body mass at 31 days was negatively associated with total pesticide mode-of-action (MoA) diversity. The best-supported model included total MoA diversity (β = −3.22, 95% CI [−6.3, −0.13]), whereas the corresponding null model had an AIC value 2.2 units higher. Model-predicted body mass decreased from 59.1 mg (95% CI [51.1, 67.1]) in the absence of detected MoA groups to 39.8 mg (95% CI [24.3, 55.2]) at the highest observed diversity of six MoA groups, corresponding to a 32.7% decline across the observed gradient (Fig. 3).

Two additional models were within ΔAIC < 2 of the best supported model. A model including fungicide MoA diversity (ΔAIC = 0.6)] showed a similar negative association, although its 95% CI included zero (β = −3.1, 95% CI [−6.3, 0.12]). A model including the total number of detected pesticides (ΔAIC = 1.7) likewise provided no clear evidence for an association with final body mass (β = −1.6, 95% CI [−3.6, 0.4]). Models including pesticide × location interactions received substantially less support (all ΔAIC > 4).

Because the location Rosenberg contributed the largest number of samples (26 of 58 plant samples, see Tab. 1) and encompassed a comparatively broad range of pesticide contamination (Fig. 2), we conducted an exploratory within-location analysis to assess whether the association between MoA diversity and final larval body mass was also evident independently of variation among sampling locations. The model included total MoA diversity as a fixed effect and plant ID as a random intercept, with model fit as described above. Within Rosenberg, total MoA diversity was negatively associated with final larval body mass (β = −5.08, 95% CI: [−8.39, −1.77]). Predicted body mass decreased from 58.8 mg (95% CI: [46.13, 71.55]) at zero detected MoA groups to 28.4 mg (95% CI: [16.4, 40.31]) at the highest observed MoA diversity, corresponding to a 51.8% decline across the observed gradient (Fig. 3).

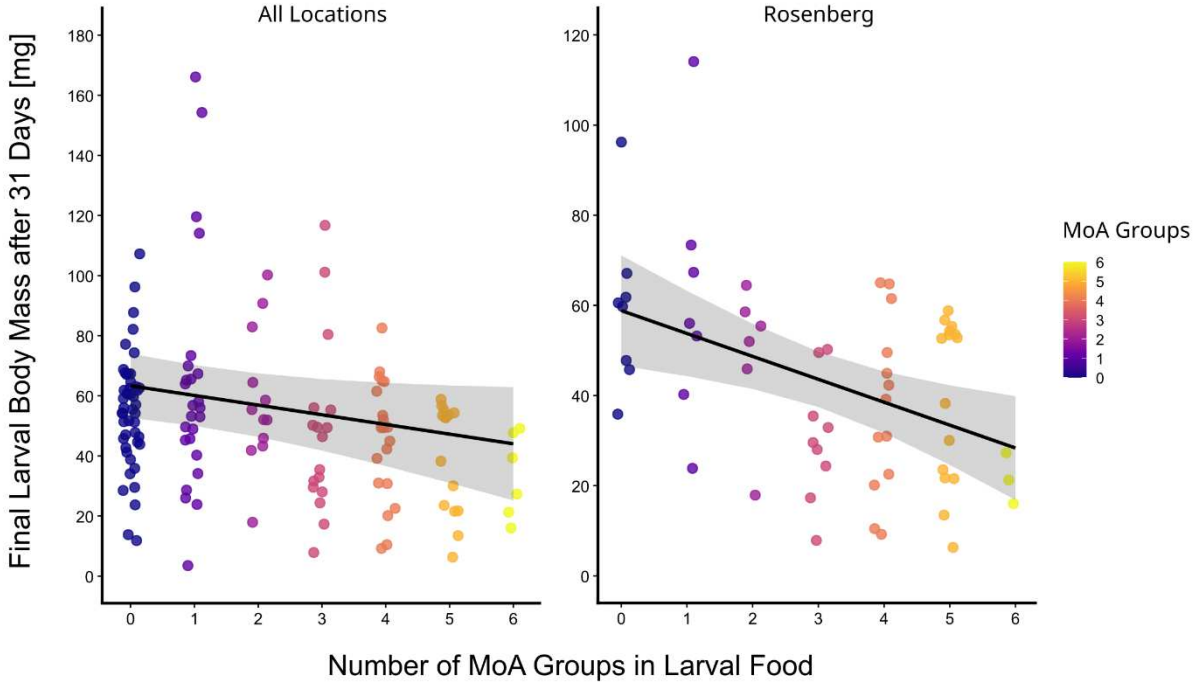

Figure 3: Model-predicted effect of total pesticide mode-of-action (MoA) group diversity on *P. a. vinningensis* larval body mass after 31 days of exposure for all sampled locations (left) and Rosenberg only (right). The lines represent the marginal predicted effect of *MoA diversity* on final body mass (±95% CI) based on the linear mixed effects models. Points represent individual larval measurements coloured by the number of distinct FRAC/HRAC MoA groups present in each plant sample. For the left graph, points represent individual larvae (N = 143) from 57 plant samples (= biological replicates); sample sizes per *MoA diversity* level (i.e. biological replicates) were 19, 10, 4, 8, 8, 6, and 2 for 0–6 MoA groups, respectively. For the right graph, points represent individual larvae (N = 61) from 25 plant samples (= biol. replicates); sample sizes per *MoA diversity* level (i.e. boil. replicates) were 3,

3, 2, 5, 5, 6, and 1 for 0–6 MoA groups, respectively. In both analysis, plant ID was included as a random intercept in the models.

To assess potential associations between host-plant nutritional quality and larval performance, plant nutrient contents were additionally examined. Final body mass was positively associated with plant nitrogen content, whereas no clear associations were detected for phosphorus, copper, sulfur, or potassium (Supplementary Table S17). Nitrogen content was not associated with pesticide MoA diversity (Supplementary Fig. S6), suggesting that the negative association between MoA diversity and final larval body mass was unlikely to be attributable to variation in plant nitrogen content.

Pesticide predictors were not supported as drivers of larval growth curves

Across repeated measurements, pesticide predictors (i.e. *number of pesticides*, *individual MoA groups*, and *MoA number*) showed limited evidence of associations with larval body mass. The best-supported pesticide model included *total MoA number* (ΔAIC = 1.9 relative to the corresponding pesticide-free model), with a negative estimated association whose 95% CI narrowly included zero (β = −0.056, 95% CI [−0.112, 0.0002]). Models including interactions between pesticide predictors and time received less support (all ΔAIC > 2), providing no clear evidence that temporal changes in body mass differed with pesticide exposure.

Total pesticide concentration was not supported as a predictor of larval body mass

Total pesticide concentration showed no evidence of an association with growth curves or final body mass. For both growth and final body mass, the null model received the strongest AIC support. The best competing growth model included an interaction between pesticide concentration and time (ΔAIC=1.5), but the estimated interaction was small and its 95% CI included zero ($\beta = -5.17 \times 10^{-4}$, 95% CI [−0.002, 0.001]). Similarly, the best competing model for final body mass included *total pesticide concentration* as an additive predictor (ΔAIC = 1.41), but the estimated association was uncertain (β = −2.4, 95% CI [−8.6, 3.8]). All remaining pesticide models received substantially less support (ΔAIC > 3.2).

Pesticide exposure metrics were not supported as predictors of larval survival

Larval survival after 31 days was high overall (mean: 82%) and was not clearly associated with any pesticide exposure metric. In both candidate model sets, the null model received the strongest AIC support. For the pesticide-concentration dataset, the best competing model included total pesticide concentration (ΔAIC = 1.4), but the estimated association with survival was uncertain (OR = 1.1, 95% CI: [0.7, 1.8]). Similarly, models including fungicide MoA diversity, total MoA diversity, or the number of detected pesticides (all ΔAIC ≤ 1.5) showed no clear associations with survival (all ORs ≈ 1.1, 95% CIs including 1).

3.2 Helicopter Spray Experiment

Larval body mass differed between cohorts but not among spray treatments

Mean initial larval body mass was 312 mg (SE ±174) in pesticide-naive larvae (N = 75) and 261 mg (SE ±126) in pre-exposed larvae from the *Field-Collected Host Plant Experiment* (N = 60). In a linear model of body mass measured 11 and 20 days after the onset of fungicide applications, initial body mass was strongly associated with subsequent body mass ($F_{1,170}$ = 130.08, $p < 0.001$). After accounting for initial body mass, pre-exposed larvae had approximately 4% lower body mass than pesticide-naive larvae ($F_{1,170}$ = 3.90, $p = 0.049$). By

contrast, body mass did not differ clearly among spray treatments drift and full ($F_{2,170}$ = 1.22, p = 0.298), and there was no clear treatment × time interaction ($F_{2,170}$ = 2.39, p = 0.095). Because prior pesticide exposure could modify responses to subsequent fungicide exposure, we additionally tested a treatment × cohort interaction; this interaction was not supported ($F_{2,168}$ = 1.04, p = 0.357).

Fungicide spray treatments did not reduce larval survival

The association between spray treatment and survival to pupation was cohort-dependent, but within-cohort comparisons provided no evidence of reduced survival under either fungicide exposure regime. This cohort-dependent response was supported by improved model fit when including a treatment × cohort interaction (likelihood-ratio test: χ^2_2 = 9.93, p = 0.007). We therefore evaluated treatment effects separately within each cohort using contrasts of estimated marginal means (EMMs). In pesticide-naive larvae, estimated survival probabilities were 1.00 in the control, 0.90 (95% CI: [0.68, 0.97]) under drift-rate exposure, and 0.97 (95% CI [0.80, 0.99]) under field-rate exposure (Fig. 4). In the pre-exposed larvae, estimated survival probabilities were 0.73 (95% CI [0.48; 0.89]) in the control, 0.97 (95% CI [0.80; 0.99]) under drift-rate exposure, and 0.89 (95% CI [0.68; 0.97]) under field-rate exposure. Despite the treatment × cohort interaction, pairwise contrasts among treatments within cohorts provided no evidence for reduced survival under either fungicide exposure regime relative to the respective control.

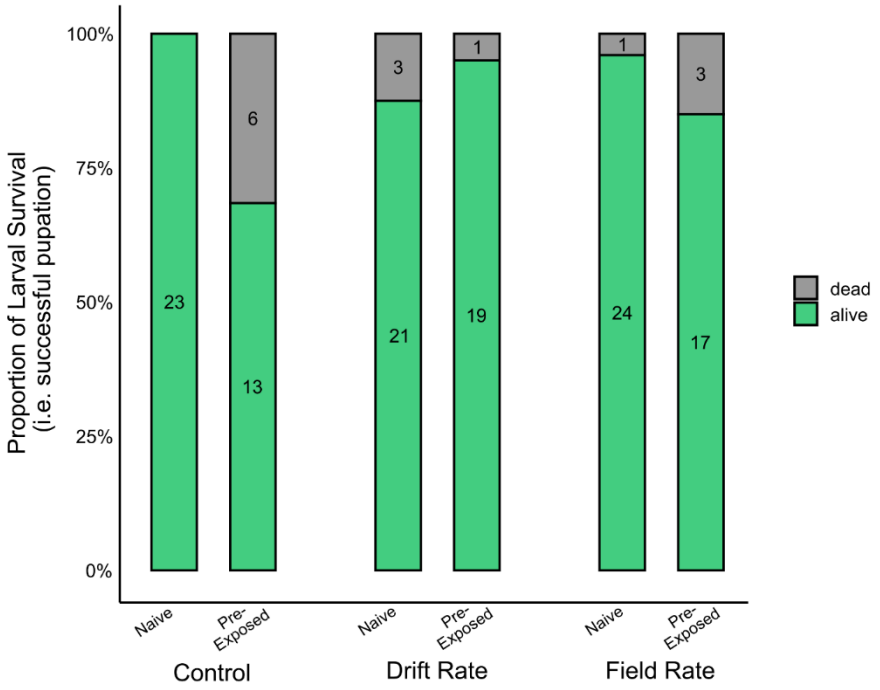

Figure 4: Larval survival to pupation in the *Helicopter Spray Experiment*. Proportions of *P. a. vinningensis* larvae that successfully pupated (green) or died before pupation (grey) following exposure to the water control, drift-rate, or field-rate treatment. Numbers within bars indicate the number of surviving or dead larvae. Pesticide-naive larvae were reared on control host plants before the experiment, whereas pre-exposed larvae originated from the *Field-Collected Host Plant Experiment*. Survival was analysed using a binomial generalized linear model including treatment, cohort and their interaction, treatment effects were evaluated separately within cohorts using contrasts of estimated marginal means (EMMs).

3.3 Luna® Experience Toxicity Experiments

Luna® Experience reduced survival to adult emergence

In the experiment with individually housed larvae, Luna® Experience significantly reduced survival to adult emergence. Of 49 larvae per treatment, 18 control individuals (37%) successfully emerged as adults, compared with only four individuals (8%) exposed to Luna® Experience (Fig. 5). The odds of surviving to adult emergence were 6.5 times higher in the control than in the Luna® Experience treatment (OR = 6.5, 95% CI [2.00, 21.17], p = 0.002). Model-based estimates likewise indicated a 4.6 times lower probability of survival under Luna® Experience exposure, with estimated survival probabilities of 0.37 (95% CI [0.24, 0.51]) in the control and 0.08 (95% CI [0.03, 0.20]) in the Luna® Experience treatment.
Of the four adults emerging from the Luna® Experience treatment, two exhibited pronounced hindwing deformities, whereas no deformities were observed among the 31 control adults (Supplementary Fig. S2). Because only four treated individuals reached adulthood, this observation was not analysed statistically.

The grouped-larvae experiment showed a similar directional pattern, although the estimated treatment effect was uncertain (Fisher's exact test: OR = 0.47, 95% CI [0.19, 1.14], p = 0.10). Survival to adult emergence was 21 of 63 individuals (33%) in the control and 12 of 63 individuals (19%) following Luna® Experience exposure (Fig. 5). The experiment comprised five experimental units per treatment. L3 larvae were excluded from the analysis because mortality was complete in both treatments. Stage-specific treatment effects were not analysed because replication at the experimental-unit level was insufficient for such comparisons.

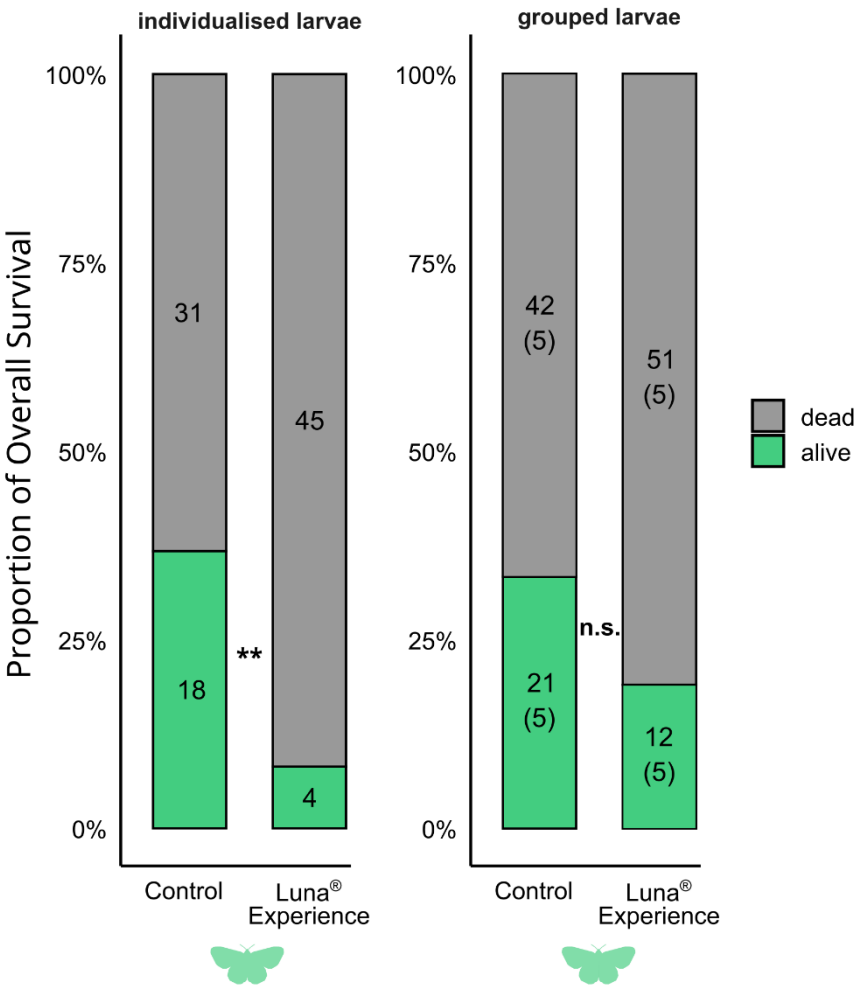

Figure 5: Survival of *P. a. vinningensis* in the Luna® Experience assays. Numbers inside stacked bars indicate the number of individuals that died (grey bar) or survived (green bar; i.e. hatched adults) within treatments. Where this count differs from the number of biological replicates, the latter is given in brackets. Top labels indicate the experiment. Data was analyzed using a generalized linear mixed model (individual housing) and a Fisher's exact test (grouped housing). ** = significance at p<0.01, n.s. = not significant.

No clear effect of Luna® Experience on body mass among surviving individuals

Among surviving larvae in the individually housed experiment, body mass across repeated measurements did not clearly differ between treatments ($F_{1,81.66}$ = 0.36, p = 0.553; Supplementary Fig. S3). There was no evidence for a treatment × linear-time interaction ($F_{1,58.95}$ = 0.13, p = 0.723), whereas the treatment × quadratic-time interaction was estimated with greater uncertainty ($F_{1,45.66}$ = 3.93, p = 0.053). At day 19, model-estimated larval body mass was 819 mg (95% CI [712, 927]) in controls and 768 mg (95% CI [610, 927]) in Luna® Experience-treated larvae, with no clear treatment difference ($F_{1,38}$ = 0.29, p = 0.59).
Among adults from the individually housed experiment, male body mass showed no detectable treatment difference (Wilcoxon rank-sum test: W = 27.5, p = 0.72); females could not be compared because none emerged from the Luna® Experience treatment. In the grouped-larvae experiment, adult body mass likewise showed no detectable treatment difference after accounting for sex and larval stage at treatment onset ($F_{1,7.1}$ = 1.62, p = 0.24).

4. Discussion

The Moselle Apollo butterfly is threatened with extinction, and pesticide exposure associated with viticulture has been proposed as one potential main contributor to its decline[12,14,15]. Our results show that agricultural pesticide exposure can adversely affect an insect of high conservation concern, even when the dominant pesticides are not insecticides. Early-stage larvae reared on contaminated host plants from four habitat sites had lower final body mass with increasing pesticide mixture complexity, while experimental exposure to the fungicide product Luna® Experience substantially increased mortality. By contrast, a reconstructed aerial fungicide regime produced no detectable effects on larval body mass or survival in older larvae. Together, these findings highlight the importance of environmentally realistic exposure pathways and show that pesticides not intended to control insects can nevertheless adversely affect them. Given the frequent detection of residues from persistent fungicides, particularly per- and polyfluoroalkyl substances (PFAS; fluopyram, fluxapyroxad, fluopicolide, and cyflufenamid), in larval food plants, their use warrants particular caution.

4.1 Complex pesticide mixtures in host plants are associated with impaired larval development

The main larval host plant of *P. a. vinningensis*, *S. album*, carried diverse pesticide mixtures even when collected approximately two months before the onset of annual aerial fungicide applications or eight months after the last application in the previous year. Residues were detected both in plants within vineyards and in surrounding habitats, including plants up to 76 m from the nearest vineyard edge, and were dominated by fungicides spanning six modes of action. This pattern is consistent with landscape-scale studies showing that pesticide contamination can extend beyond treated fields and persist outside periods of active application[9,10]. Because sampling preceded the annual aerial spray period, the detected residues indicate that Apollo larvae can encounter pesticide contamination through their host plants from the onset of larval feeding in early spring, well before current-season spraying begins.

When freshly hatched larvae were reared on these field-collected plants, final body mass declined with increasing mode-of-action diversity. Across the contamination gradient, predicted final body mass was approximately one-third lower at the highest MoA diversity than in the absence of detected MoA groups. This association was also evident in an exploratory analysis restricted to the Rosenberg location, indicating that the overall relationship was not solely attributable to differences among sampling locations. Because pesticide contamination was not experimentally manipulated, the association cannot establish that pesticide mixture diversity itself caused the reduction in body mass.

Host-plant nutritional quality provides one obvious alternative explanation. Supplementary analyses showed that larval body mass increased with plant nitrogen content, whereas no clear relationships were detected for phosphorus, copper, sulfur, or potassium. Importantly however, nitrogen content was not associated with pesticide MoA diversity (Supplementary Analysis S1), making variation in this key nutritional parameter an unlikely explanation for the negative association between MoA diversity and final larval body mass.

The finding that MoA diversity, rather than total pesticide concentration, best described variation in final body mass is consistent with growing evidence that the biological effects of pesticide mixtures cannot always be inferred from summed concentrations alone. Environmentally realistic agrochemical mixtures have previously been shown to alter

development and physiology in *Drosophila melanogaster* and *Vanessa cardui*[11] in laboratory assays. Our results extend this evidence to an authentic field-exposure setting in which larvae consumed naturally contaminated host plants rather than experimentally reconstructed mixtures. Several mechanisms could contribute to such mixture effects. Simultaneous exposure to multiple xenobiotics may increase detoxification demands and physiological stress, diverting resources away from somatic growth[48,49]. SDHI fungicides were particularly prevalent in the field-collected *Sedum* samples and may additionally interfere with mitochondrial metabolism and energy production in insects[25]. These mechanisms remain hypothetical in the present system, but they provide plausible routes by which chronic exposure to chemically diverse mixtures could affect larval performance.

A recurring group of five fungicides – fluopyram, fluxapyroxad, fluopicolide, cyflufenamid (all recognized as PFAS pesticides) and dimethomorph – occurred in 24% of host-plant samples. Associations with individual compounds could not be disentangled because of their strong co-occurrence, and models including all five compounds simultaneously provided little basis for assigning effects to any single active ingredient. Fluopyram and fluxapyroxad are SDHIs that target mitochondrial respiration, although sensitivity to SDH inhibition differs among taxa[25,27,50]. Evidence for the remaining compounds in insects is more limited. The present data therefore support an association with mixture composition but do not identify which individual compounds, combinations, or interactions were responsible.

The reconstructed aerial fungicide regime produced a contrasting result. Neither field-rate nor drift-rate exposure had detectable adverse effects on larval body mass or survival. Several differences between the experiments may explain this contrast. Most importantly, exposure in the aerial-spray experiment began when larvae were already considerably more advanced in development (L3–L4), whereas larvae in the field-collected host-plant experiment were exposed immediately after hatching. Susceptibility may therefore differ substantially between these developmental windows, with younger and smaller larvae potentially being more sensitive. Moreover, exposure from hatching provided a broader developmental window for contact with pesticide residues, whereas later applications in the simulated spray sequence increasingly coincided with advanced larval development or pupation. In addition, the field-collected plants represented accumulated environmental contamination and potentially a broader chemical profile than what was reproduced by the simulated spray sequence, including residues or transformation products not represented in the experimental applications. The two experiments should therefore be viewed as complementary exposure scenarios rather than direct tests of the same chemical mixture.

Temporal overlap between fungicide application and Apollo larval development may also vary among years because phenology is strongly weather-dependent[14,20]. The exposure window reproduced in our experiment may therefore not capture years in which larval development occurs later and overlaps more extensively with fungicide applications. Potential delayed or carry-over effects into adults of the reconstructed spray regime could not be assessed because pupal development was disrupted by an unexpected heat event.

4.2 Luna® Experience causes substantial mortality under direct and dietary exposure

Luna® Experience combines the SDHI fluopyram with the SBI tebuconazole and is relevant to the Moselle Apollo system because of its use in regional viticulture (up to nine aerial applications per year[21]) and the detection of fluopyram in 57% of field-collected *Sedum* samples. In the individually housed experiment, exposure to Luna® Experience reduced survival to adult emergence to 8% compared to 37% in the control. The grouped experiment

showed the same directional pattern, although the treatment effect was not significant. These findings do not conflict with the absence of detectable effects in the reconstructed aerial fungicide regime, as the experiments represented different developmental and exposure scenarios. In the simulated aerial-spray sequence, Luna® Experience was applied only during the final application, when many larvae had already pupated, whereas the targeted experiments exposed developing larvae directly and subsequently via treated host plants. Because Apollo phenology varies with weather conditions[14,20], greater overlap between active larvae and fungicide applications may occur in some years. Moreover, the frequent detection of fluopyram in host plants before seasonal aerial spraying indicates that dietary exposure may also extend beyond the period immediately following application.

Both Luna® experiments were characterised by high control mortality, but mortality was higher under Luna® Experience in both cases: 63% vs 92% in individually housed larvae and 67% vs 81% in the grouped experiment. High control mortality should be considered when interpreting effect magnitude and limits direct quantitative comparison between experiments; a statistically significant treatment effect was detected only in the individually housed experiment. Among surviving larvae, body mass did not differ between treatments. However, increasing differential mortality reduced later measurements to the subset of individuals that survived exposure, and the absence of a detectable mass difference therefore does not exclude adverse effects on more susceptible larvae.

The mechanisms underlying the observed mortality were not examined directly. Both active ingredients nevertheless have modes of action that could affect insect physiology: fluopyram can interfere with mitochondrial energy metabolism[25], whereas tebuconazole has been associated with effects on detoxification pathways, oxidative stress, immune responses, and hormonal regulation in insects[23,51]. Effects of interactions between the active ingredients or formulation components also cannot be excluded. Determining the relative contribution of these factors would require experiments with the individual active ingredients and their combinations.

Experimental information on Luna® Experience in Lepidoptera remains limited. Regulatory assessments classify the product as slightly harmful to several tested beneficial arthropods[52], but these assays differ substantially from the present study in species, exposure route, duration, and endpoints. It especially important that under current regulation no herbivorous insect is included. Our results therefore provide complementary evidence that prolonged direct and dietary exposure of a herbivorous butterfly larva can produce adverse responses that may not be represented by standard surrogate-species tests. Such variation in pesticide sensitivity among insect species and exposure scenarios has been reported previously[53].

4.3 Broader implications for Moselle Apollo conservation and pesticide risk assessment

The Moselle Apollo illustrates an important conservation tension. Viticulture helps maintain the open, xerothermic habitats required by the butterfly and its host plant, yet at the same time introduces pesticide exposure into those habitats. Although aerial fungicide application is a distinctive feature of Moselle viticulture, the broader exposure pathway is not unique: herbivorous insects in many agricultural landscapes encounter pesticide residues through their host plants, often as complex mixtures and beyond periods of active spraying[8,10,54].

Our study moves beyond documenting environmental contamination by linking authentic field-derived pesticide mixtures to biological performance in an endangered insect. The association

between mixture diversity and larval body mass suggests that the composition and functional diversity of pesticide mixtures may contain ecologically relevant information that is not captured by total residue concentration alone. Importantly, this field association is complemented by experimental evidence that a fungicide formulation used in viticulture can cause substantial mortality under direct and dietary exposure.

These findings do not establish pesticide exposure as the sole or primary cause of the recent decline of the Moselle Apollo. Climate- and habitat change, host-plant availability, and other pressures have also been proposed as contributing factors[12,14] but are so far not evaluated systematically. Our experimental results identify pesticide exposure as the first validated plausible stressor in this discussion, whose importance is likely to depend on the timing, composition, and duration of exposure. Such interactions may be particularly relevant in small and declining populations, where sublethal effects on development and growth may reduce individual fitness and, together with episodic increases in mortality, contribute to cumulative population-level stress[48].

More broadly, our findings emphasize that pesticides not designed to kill insects can nevertheless affect them, and that exposure to environmental mixtures may differ substantially from exposure to individual compounds under standardized conditions. Current pesticide testing frameworks rely necessarily on surrogate species and defined exposure scenarios[55–59], but these approaches may not capture all chronic, mixture-based, species-specific, or life-stage-specific effects encountered in agricultural landscapes. Incorporating environmentally realistic mixtures and dietary exposure into ecotoxicological research should therefore help clarify when and where such effects translate into ecological risk. Our findings support reducing aerial fungicide applications as a measure to limit habitat contamination and reduce pesticide exposure of the remaining Moselle Apollo population.

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Statements and Declarations

Competing Interests

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Data Availability

All data supporting the findings of this study will be made publicly available upon acceptance for publication, without access restrictions.

Contributions

J.E.S., E.G., C.A.B. and G.P designed the experiments. T.L. and D.M. provided information on *P. apollo* rearing and helped optimizing experimental designs. J.E.S., E.G., C.D., D.K. and S.R. conducted the experiments. J.E.S. performed the analysis of the experiments. J.E.S. wrote the original draft of the manuscript and created the figures. L.W. conducted the ecotoxicological analysis and wrote the respective methods section. G.P. , C.A.B., D.K., and E.G. edited the manuscript. G.P. and C.A.B. supervised the research.

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