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Additive and interactive effects of anthropogenic stressors on an insect herbivore

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The pressures of global change acting on wild plants and animals include exposure to environmental toxins, the introduction of non-native species, and climate change. Relatively few studies have been reported in which these three main classes of stressors have been examined simultaneously, allowing for the possibility of synergistic effects in an experimental context. In this study, we exposed caterpillars of the Melissa blue butterfly (*Lycaeides melissa*) to three concentrations of chlorantraniliprole, under three experimental climates, on a diet of a native or a non-native host plant throughout larval development in a fully factorial experiment. We find that high pesticide exposure and a non-native diet exhibit strong negative effects on caterpillars, resulting in 62% and 42% reduction in survival, respectively, while interactive effects tend to be weaker, ranging from 15% to 22% reduction in survival. Interactive effects have been shown to be strong in other contexts, but do not appear to be universal; however, our study shows that the cumulative effects of stressors acting in isolation (additively) are sufficiently strong to severely reduce survival and by extension population persistence in the wild.

1. Introduction

Populations of plants and animals are increasingly confronted by the adverse impacts of multiple anthropogenic stressors, including habitat loss, habitat degradation, and climate change [1,2]. In recent years, it has become increasingly clear that insects are being negatively impacted by all of these processes, as highlighted by losses of insect biomass and diversity in many parts of the world [3–8]. The mechanisms implicated in these declines are many and it has been suggested that some of the most severe effects could be the result of interdependence among stressors, such that the effects of one are sensitive to or affected by the action of another factor [9,10]. One common expectation is that threats will have synergistic negative effects [11], however, antagonistic interference between variables could moderate negative impacts or have complex outcomes that are difficult to predict [12]. Thus, understanding additive and interactive effects of anthropogenic stressors is critical for predicting the susceptibility of species to continued global change as well as for developing conservation actions [13]. In this study, we experimentally evaluate interactions among three stressors relevant to the conservation of butterflies: non-native hosts, pesticide exposure, and climate change.

At the broadest scales, these threats have been identified as factors in insect declines across the world [14]. For butterflies, the introduction of non-native plants to novel ecosystems has reduced native host availability and is a risk to many insects [15–17]. Even in cases where butterflies have successfully expanded their diet to incorporate novel hosts, such switches are often associated with reduced survival and performance [18]. Meanwhile, non-target effects of pesticide use and overuse also pose threats to many butterflies, often in landscapes already transformed by non-native plants [19,20]. While

the use of neonicotinoids, in particular, has often been singled out, these are not the only harmful compounds present on landscapes at concentrations that are biologically relevant for butterflies [21,22]. Yet another threat being faced by butterflies is the accelerating influence of climate change. While heterogeneous and context dependent, the impacts of climate change on butterflies are being realized in changing distributions [23], shifting phenologies [24], and population declines in natural areas [25]. Collectively, it is likely that almost every butterfly on Earth is experiencing at least one of these threats and many face all three simultaneously.

While the threats are large, the number of studies that consider the impacts of multiple stressors and their interactions on insects remains small, especially when compared to the number of studies that consider only a single stressor [26]. In studies using data from long-term monitoring, cases have been identified where insects are impacted by interactive effects of temperature and pesticides [27] and cases where only additive effects are found despite testing for interactions [19]. When considering multiple stressors, even butterflies, the most widely studied of all major insect groups, suffer from a paucity of research. In experimental settings, interactions between host plant species and pesticide exposure have been demonstrated in monarch butterflies (*Danaus plexippus*), where caterpillars that experience high neonicotinoid exposure on low cardenolide milkweed species experience reduced survival and smaller body size compared to other treatment combinations [28]. Relative to the few studies investigating interactions with pesticides, controlled studies testing for interactions between temperature and host plant are slightly more common and have identified cases of interaction between diet and climate [29,30] and cases where an interaction is either not detected or is weak compared to additive effects [31–33]. In an example of the former, monarch caterpillars experienced improved survival on a non-native milkweed (that contained higher cardenolides) compared to the native host under current climates, but saw a reversal of this effect under an experimental future climate [30]. We are unaware of any experimental study that has tested for interactions between climate and pesticide exposure and thus, to our knowledge, this is the first study that examines the additive and interactive impacts of diet, pesticide exposure, and climate change on a butterfly.

Butterfly populations in the American west are experiencing complex and variable combinations of these stressors [34,35]. Many butterflies in this region have incorporated non-native hosts into their diet, and in some cases, populations are wholly dependent on the novel host [36]. Additionally, pesticide use in some areas of the western USA is high and caterpillars are probably being exposed to many compounds simultaneously [37,38]. Chlorantraniliprole is one such compound and is used in agricultural regions of the USA [37,38] for its ability to disrupt muscle contraction in caterpillars through contact or ingestion [39]. A study from northern California found chlorantraniliprole in most leaves sampled in settings where the sampled plants were contaminated as a by-product of application, for example through pesticide drift into the weedy edges of agricultural margins [21]. It was found at concentrations known to be lethal to monarch butterflies [21]; however, there is little known about lethal concentrations in other non-target butterflies, many of which use agricultural margins and weedy spaces that are frequently exposed to pesticides. Finally, the western USA is also experiencing rapid warming which is associated with declining abundances of butterflies from long-term monitoring sites [40,41].

In this study, we examine the impacts of these stressors on *Lycaeides melissa*, the Melissa blue butterfly, a widespread species across the American west that has been used in previous studies focused on the ecology and genetics of host plant range [42–44]. In the experiment we report here, caterpillars were exposed to multiple concentrations of the insecticide chlorantraniliprole, under three different temperature treatments, on a diet of either a native or non-native host plant in a fully factorial experiment. The parameters of this experiment are calibrated to form a realistic representation of threats facing many western butterflies. Here, we use *L. melissa* as an example of a species that is potentially exposed to our focal chemical (chlorantraniliprole), and first investigate the relative importance of additive effects of temperature, diet, and pesticide exposure on survival, larval development time (LDT), and adult mass as a proxy for fitness. We then consider the interactive effects of diet, pesticide, and temperature treatments on those same variables and ask if they act synergistically or antagonistically. The independence or overlap of threats has not been explicitly quantified for our focal butterfly in the wild. However, as a species that uses an exotic host, often along the margins of agricultural fields in the arid west (experiencing rapid climate change), it is likely that populations of *L. melissa* are frequently exposed to two and could be exposed to three of the stressors that are being studied here. Thus, we suggest that a consideration of all potential interactions is important, for both understanding our focal species specifically, but to also advance our knowledge of global change impacts on non-target butterflies in general.

2. Methods

(a) Study organism

The Melissa blue butterfly, *L. melissa* (family Lycaenidae) is distributed across much of the American west in a population structure that is patchy with low gene flow among individual locations [43]. Regional monitoring indicates that *L. melissa* is among a group of at-risk species, and that the family Lycaenidae in general contains a high concentration of declining species [45]. Additionally, although not occurring in the west, the related Karner blue butterfly (*Lycaeides samuelis* or *Lycaeides melissa samuelis)* is a federally listed endangered species [46]. The populations from which we collected adults for this study are multivoltine and adult butterflies are seen from May to September. The butterfly specializes on larval host plants from the Fabaceae family, including the native genera *Astragalus*, *Glycyrrhiza*, and *Lupinus* [47]. Within the last 200 years, *L. melissa* has incorporated the non-native alfalfa, *Medicago sativa*, into its diet and many populations are dependent on it as the only local host plant [48]. Compared to individuals reared on the native plant *Astragalus canadensis*, *L. melissa* reared on *M. sativa* have reduced larval performance, lower adult fecundity, and reduced immune function in response to host-specific variation in phytochemistry and microbial diversity [48,49]. As caterpillars, *L. melissa* develop over four instars over the course of approximately one month. In the wild, *L. melissa* caterpillars frequently engage in a facultative mutualism with ants, in which sugary secretions are exchanged for protection that is effective against generalist predators but apparently not from parasitoids [44,50].

(b) Experimental design

Gravid females were collected from two sites in Verdi, Nevada, on the east side of the Sierra Nevada Mountains and the western edge of the Great Basin Desert, and were placed into outdoor oviposition chambers with *A. canadensis* to obtain eggs following methods used previously [48]. From these eggs, a total of 450 caterpillars hatched and were placed individually into Petri dishes for a total of 25 replicates per treatment combination. When caterpillars were placed in dishes, they were randomly assigned to a climate treatment and were placed in one of three growth chambers (based on climate assignment). In each chamber, temperatures oscillated between a daytime and a night-time temperature, spending 11.5 h at each (with 30 min transitions between them). Light cycles were timed to match the daytime and night-time temperatures. In the chamber meant to represent current conditions, the daytime temperature was set to 26°C and the night-time temperature was set to 17.5°C. The other two chambers were hotter and increased both day and night temperatures by 3°C and 6°C, respectively (referred to as the warmer and hot climates). We chose these warming intervals based on regional projections of future climates in 2100. The warmer condition (3°C) is within a range of expected warming under a low emissions scenario (representative concentration pathway (RCP) 4.5), and the hot climate condition (6°C) is within the range of expected temperatures under a high emission scenario (RCP 8.5) [51]. These climates were informed by data collected by a thermochron iButton placed in one of the collection locations near Verdi, Nevada that has supported an *L. melissa* population for at least two decades [52]. The iButton was placed in a low tree adjacent to the ground to capture the range of ambient temperatures that a caterpillar might be exposed to while feeding on host plants. The daytime temperature used in experiments was the mean across days in June 2021 and the night-time was the mean temperature across nights in the same month. The caterpillars assigned to the current climate were raised in a Percival growth chamber (model I-36LLVL), the warmer climate caterpillars in a Percival growth chamber (model E-36HO), and the hot climate caterpillars in a Darwin growth chamber (model IN034). After the experiment, the temperatures in the control and hottest chambers were measured by iButtons to confirm that those chambers met their programmes, and those data are shown in the electronic supplementary material, figure S1. To standardize light exposure across chambers, a lamp was placed in each and was connected to a timer. The distance from the light to the Petri dishes was equidistant in each chamber. Petri dishes were randomly reshuffled each day in every chamber to account for within chamber light differences over the length of the experiment.

At the same time, neonate caterpillars were assigned a climate treatment, they were also placed on one of two diets, either an ancestral and native host plant *A. canadensis* or the non-native host *M. sativa*. *Astragalus canadensis* was collected from a site approximately 2 miles from Hallelujah Junction, California, where it grows in a natural area and is not near any agricultural cultivation. *Medicago sativa* was collected from the town of Verdi, Nevada, where it grows along an unmanaged roadside on the outskirts of the small town and is also not near any agricultural systems or residences. Plants were obtained from these same locations for the entirety of the experiment. Both sources of host plants have been used in previous studies on *L. melissa* over many years, and the Verdi location has been visited every two weeks during the warm months by one of us (M.L.F.) since 2014. To our knowledge, neither site has been exposed to pesticides. Plants were stored in a standard refrigerator in the laboratory and provided to caterpillars in the form of small clusters of leaves with the stems wrapped in damp Kimwipes to minimize drying. Plants in each dish were assessed daily and were either watered or replaced as needed.

After 14 days, caterpillars were given a single leaf that was treated with chlorantraniliprole with concentrations of either a control, 9.5 ppb, or 95 ppb. At this stage, caterpillars were either third or fourth instar. We waited two weeks for the pesticide exposure to avoid killing an excessive number of the most vulnerable early instars which would probably have ended the experiment. This design also mimics realistic conditions in which caterpillars in the field might be exposed to a pesticide during only part of development as a consequence of intermittent application (and drift) in adjacent agricultural fields. The concentrations were chosen based on a field study where chlorantraniliprole was found at an average of 9.5 ppb and in one instance at 95 ppb, thus these concentrations are chosen to represent concentrations caterpillars could encounter in the field [21]. In that study, concentration was measured in ppb (ng pesticide per g leaf). To match these concentrations, we weighed leaves to determine the average weight of a leaf being given to a caterpillar (20 mg). Using this as the denominator, we then calculated the amount of active ingredient needed to achieve the target ppb per 20 µl pipette application. A stock solution was created using acetone as the solvent and was serially diluted to obtain the target concentrations. Twenty millilitres of the diluted solutions were then pipetted onto a leaf disc using an Eppendorf Research Plus pipette. Caterpillars assigned to the control group received leaves treated with 20 µl of acetone. These methods have successfully been used in other pesticide bioassays on caterpillars [53]. Prior to being given pesticide treated leaves, caterpillars were starved for 24 h and then weighed. The pesticide contaminated leaves were not changed until either the full leaf disc was consumed, or the leaf dried out. All subsequent leaves fed to caterpillars were untreated. Following the application of pesticide, caterpillars continued to develop as before in their assigned temperature treatment and on their assigned diets. Upon pupation, caterpillars were weighed, the date was recorded, and they were returned to the growth chamber. Finally, all surviving adults were sexed and weighed.

(c) Statistical analyses

We modelled survival, adult weight, and time to pupation using Bayesian linear models, where temperature, pesticide, and host plant treatments were categorical variables. We considered additive effects, all two-way interactions, and three-way interactions among the treatment variables. We also included covariates for mass before being given pesticides, as well as source population (one of two locations from which females were collected) for all models. Sex was included as a covariate for the adult mass and development time models. Survival was modelled from a Bernoulli distribution as the data are measured as a binary category. The priors on the covariates for this model were chosen to be as uninformative as possible (mean = 0, s.d. = 1.5) for a Bernoulli distribution [54]. Adult weight was treated as normally distributed, as the variable is continuous and far removed from zero, with a variance drawn from a gamma distribution (rate = 2, shape = 0.1). The mean for adult weight was modelled as a function of covariates that were chosen to be vaguely informative (mean = 0, s.d. = 10 000). Time to pupation was drawn from a Poisson distribution, as this is an integer and cannot be negative, with vaguely informative covariates as in previous models (mean = 0, s.d. = 10 000). The intercept for each model was made to be our *a priori* prediction of the best treatment combination and was the linear combination of the control group for pesticide, the current climate, and the native host. Each model was run with four separate search chains, each for 25 000 iterations with a burn-in phase of 5000. Models were assessed using the Gelmin–Rubin diagnostic and by examining posterior traceplots. All models were run using the jagsUI package [55] in R.

To inform our choice of model (with respect to the inclusion or not of higher-order interactions along with simple effects), we also performed a simulation to examine the changes in inference

caused by including versus excluding two- and three-way interactions in models. We generated three types of datasets: a dataset where the response variable is the result of only additive covariates, a dataset where additive and two-way interactions are present (but three-way interactions are not), and finally, a dataset where both types of interactions contribute to the response variable. These datasets contained the same number of categorical covariates as our data with the same numbers of treatment levels and are meant to represent data we could have collected given our experimental design. Each of the three dataset types was generated 1000 times with effect sizes (coefficients for terms in the models) being randomly selected from a uniform distribution (from −5 to 5) for each dataset. We then ran three types of models on each of these datasets: a model with only additive terms, a model with additive and two-way interactions, and a model with all types of effects including the three-way interaction. For each iteration, we determined the difference between the true simulated effect sizes and the model estimates, whether a sign was estimated in the correct direction, and the *p*-value associated with each term in the model. Using this approach, we were able to determine the structure of the model that most consistently found the simulated answer; that model was then used as a guide in structuring the model we used with the empirical data, as discussed below.

3. Results

Across all treatments, 132 *L. melissa* caterpillars out of 312 that made it to the pesticide treatment stage of the experiment survived to adulthood. Survival was influenced by both additive and interactive effects (table 1; electronic supplementary material, table S1 and figure 1*a*; electronic supplementary material, figure S2). High pesticide concentration and a diet of non-native alfalfa both had negative effects on survival estimated with greater than 99% credibility. Specifically, the high pesticide treatment reduced the probability of survival by 62% and alfalfa reduced survival by 42% compared to caterpillars reared on *A. canadensis* without pesticides. We did not detect an effect of the low pesticide concentration, at least not when considered across all levels of other factors (interactions are discussed below). We also observed a highly credible (greater than 99% probability) positive effect of the weight of the caterpillar at the middle stage of the experiment, where every 15 mg increased survival by 12% (table 1). We did not observe any additive effects of temperature treatment on caterpillars that made it to the pesticide treatment stage of the experiment.

While 312 caterpillars were assigned a pesticide treatment, originally, 450 were placed in dishes and subjected to a diet and climate treatment upon first hatching. When considering these additional caterpillars that died before being assigned a pesticide treatment (and limiting analyses to caterpillars that received a control pesticide treatment), we did see additive, negative effects of both temperature treatments and host plant on overall caterpillar survival. Specifically, caterpillars that were assigned to the 3°C warming treatments were 12% less likely to survive and caterpillars that were assigned to the 6°C warming treatment were 14% less likely to survive, while alfalfa reduced survival by 43% (table 2; electronic supplementary material, table S2). Each of these results was estimated with a probability of effect over 90% (table 2).

In addition to the many simple (or additive) effects, we detected interactive effects of specific treatment combinations on caterpillar survival, albeit with lower confidence than the additive effects (table 1 and figure 1*c–e*). As shown in figure 1*c*, there was an additional 22% reduction in the probability of survival for caterpillars that experienced both the high pesticide treatment and 3°C warming (92% probability of effect; figure 1*c*). Meanwhile, figure 1*d* shows that the

Table 1. Coefficient estimates from Bayesian models predicting survival (on a logit scale). Medians are the median of the posterior distribution and the 95% credible intervals are shown in parentheses. The probability of effect indicates the area of the posterior in the direction of the median effect that is not overlapping zero. A full table that includes Gelman–Rubin diagnostics and effective samples sizes can be found in electronic supplementary material, table S1.

predictor variable	median (2.5%, 97.5%)	prob. of effect
population	0.13 (−0.49, 0.74)	0.66
pre-treatment weight	1.04 (0.52, 1.61)	> 0.99
temperature		
3°C warmer	−0.13 (−1.07, 0.84)	0.61
6°C warmer	0.05 (−1.15, 1.29)	0.53
pesticide		
low	0.06 (−0.90, 1.04)	0.55
high	−2.94 (−4.04, −1.92)	> 0.99
host plant		
alfalfa	−1.93 (−2.91, −0.98)	> 0.99
temperature × host plant		
3°C warmer * alfalfa	−0.41 (−1.74, 0.90)	0.73
6°C warmer * alfalfa	−0.79 (−2.17, 0.53)	0.88
pesticide × host plant		
low pesticide * alfalfa	0.14 (−1.12, 1.38)	0.59
high pesticide * alfalfa	0.74 (−0.89, 2.27)	0.82
temperature × pesticide		
3°C warmer * low pesticide	0.49 (−0.84, 1.87)	0.76
6°C warmer * low pesticide	−0.58 (−2.09, 0.96)	0.77
3°C warmer * high pesticide	−1.10 (−2.72, 0.43)	0.92
6°C warmer * high pesticide	0.30 (−1.19, 1.80)	0.65
temperature × pesticide × host plant		
3°C warmer * low pesticide *alfalfa	−0.48 (−2.12, 1.14)	0.72
6°C warmer * low pesticide * alfalfa	−0.04 (−1.76, 1.65)	0.52
3°C warmer * high pesticide * alfalfa	−0.22 (−2.39, 1.85)	0.58
6°C warmer * high pesticide *alfalfa	0.68 (−1.15, 2.47)	0.77

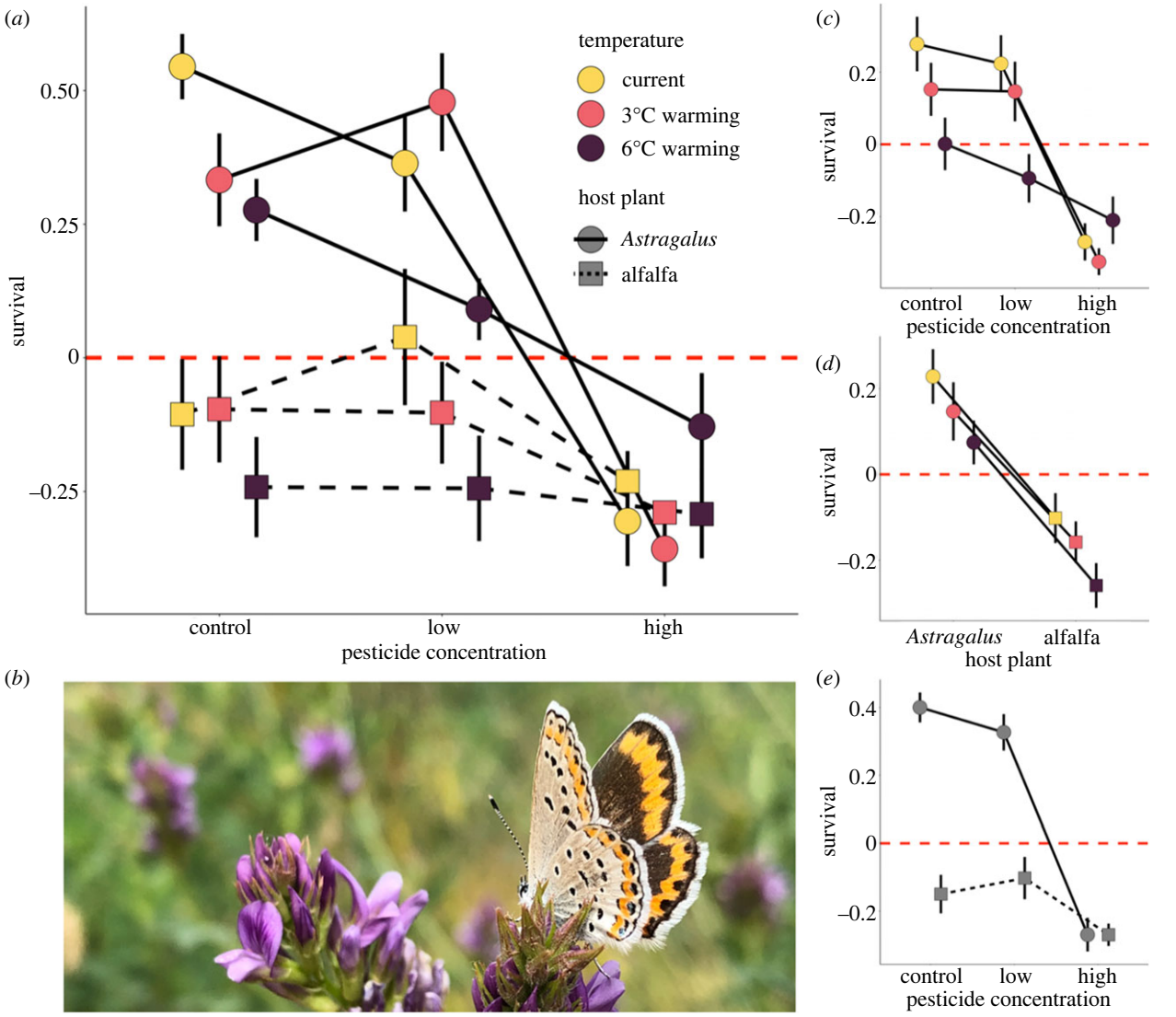

Figure 1. The partial effects of host plant, pesticide, and temperature on survival after conditioning on population and pre-pesticide treatment weight. Weight is positively associated with survival and thus we present the impacts of the three target variables after accounting for difference in initial caterpillar size. Raw results without the effect of population or pre-pesticide weight removed can be found in the electronic supplementary material, figure S2. Treatment combinations above the red line are positive (relative to the mean) while combinations below the red line are negative. (*a*) The mean and standard error of each partial effect. (*b*) An adult female *Lycaeides melissa* on an alfalfa plant. Photo credit: M.L.F. (*c*) The partial effects of pesticide and temperature (averaging over host plants). (*d*) The partial effects of host plant and temperature (averaging over pesticide treatments). (*e*) The partial effects of pesticide and host plant (averaging over temperature treatments). Note that values on the graphs were jittered with respect to the *x*-axis for ease of visualization.

caterpillars subjected to the warmest climate on a diet of alfalfa saw an additional reduction of survival by 15% after accounting for additive effects (88% probability of effect; figure 1*d*). By contrast and perhaps surprisingly, we found no interactive effects on caterpillars exposed to high pesticides and 6°C warming. All parameters, both additive and interactive, in the survival model converged upon examination of the Gelman–Rubin diagnostics, effective sample sizes, and the traceplots (electronic supplementary material, tables S1 and S2).

Among the 132 caterpillars that survived to adulthood, we observed an additive effect of host plant and interactive effects of temperature and pesticides on adult weight (table 3 and figure 2), which is a proxy for fitness both through its effects on egg-laying potential in females [48] and the probability of finding a mate in males [56]. Caterpillars that were reared on alfalfa were on average 17 mg lighter compared to those reared on *A. canadensis* in the control climate (greater than 99% probability of effect) (table 3). We also found that caterpillars reared in the hot climate and that received either the low or high pesticide treatment weighed less upon eclosion (90% and 95% probability of effect, respectively). Specifically, 6°C warming and low pesticides resulted in a reduction of adult mass of 2.78 mg, while 6°C warming and high pesticides resulted in a reduction of 3.23 mg. We also observed the effects of population, sex, and LDT on adult mass. Females weighed more than males by an average of 3.14 mg and increased development time resulted in larger adults (greater than 99% probability of effect). We also investigated associations between treatments and LDT but estimated fewer of these relationships with confidence compared to effects involving survival or adult mass (table 4 and figure 2). We found that caterpillars develop more slowly on alfalfa (96% probability of effect) and that caterpillars develop faster in the 6°C warming treatment (88% probability of effect). Additionally, males developed more quickly than females (90% probability of effect). All parameters, both additive and interactive, in the mass and LDT models converged upon examination of the Gelman–Rubin diagnostics,

Table 2. Coefficient estimates from Bayesian model survival across the entire lifespan of a caterpillar, including those dead prior to pesticide treatment. (Medians are the median of the posterior distribution and the 95% credible intervals are shown in parentheses. The probability of effect indicates the area of the posterior in the direction of the median effect that is not overlapping zero. A full table that includes Gelman–Rubin diagnostics and effective samples sizes can be found in the electronic supplementary material, table S2.)

predictor variable	median (2.5%, 97.5%)	prob. of effect
population	0.09 (−0.54, 0.74)	0.62
temperature		
3°C warmer	−0.53 (−1.34, 0.25)	0.91
6°C warmer	−0.62 (−1.44, 0.18)	0.94
host plant		
alfalfa	−2.31 (−3.26, −1.43)	> 0.99
temperature* × *host plant		
3°C warmer * alfalfa	0.08 (−1.31, 1.42)	0.55
6°C warmer * alfalfa	−0.00 (−1.40, 1.33)	0.50

number of effective sample sizes, and the traceplots (electronic supplementary material, table S1).

The models we ran included both two- and three-way interaction terms, as suggested by results from our simulations (electronic supplementary material, figures S3 and S4). Specifically, analyses of simulated data using a model that included two- and three-way interaction terms (given our sample sizes) returned the coefficient estimates closest to the simulated target values (electronic supplementary material, figure S3). Interestingly, if a simulated dataset contained a three-way interaction, but a model that contained only two-way interactions was used to estimate coefficients, this caused instances with large increases in the error of model coefficient estimates, including cases where important effects were estimated in the wrong direction (electronic supplementary material, figure S4). Our simulation revealed that a model which includes a three-way interaction is most effective at accurately detecting lower-order effects across different simulated datasets. This was true even when it was difficult (given our experimental design and a range of sample sizes) to detect the three-way interaction itself in simulated data. Thus, with our empirical data, we used a model that included three-way interactions.

4. Discussion

Insects are facing a multitude of threats across the world, including the loss of native hosts, the widespread use of pesticides, and climate change [3,14]. In regions where two or all three of these pressures coexist, it is critical to understand both their additive and interactive impacts [26]. In this study, we show that *L. melissa*, whose distribution covers areas where all three pressures occur, responds negatively to all three threats in a mostly additive fashion. Caterpillars that received the highest concentration of pesticide saw a large reduction in survival, and caterpillars that were raised on the non-native alfalfa also saw lower survival. Both temperature treatments reduced survival in early developing caterpillars, before the pesticide-pulse phase of the

Table 3. Coefficient estimates from Bayesian models predicting adult mass (mg). (Medians are the median of the posterior distribution and the 95% credible intervals are shown in parentheses. The probability of effect indicates the area of the posterior in the direction of the median effect that is not overlapping zero. A full table that includes Gelman–Rubin diagnostics and effective samples sizes can be found in the electronic supplementary material, table S1.)

predictor variable	median (2.5%, 97.5%)	prob. of effect
population	7.59 (−6.80, 21.63)	0.85
pre-treatment weight	−2.70 (−21.54, 16.11)	0.61
sex	−3.14 (−4.43, −1.84)	> 0.99
LDT	0.21 (−0.05, 0.47)	0.94
temperature		
3°C warmer	0.92 (−1.19, 3.02)	0.81
6°C warmer	−0.75 (−3.30, 1.80)	0.72
pesticide		
low	0.81 (−1.32, 2.90)	0.78
high	0.61 (−3.75, 4.98)	0.60
host plant		
alfalfa	−13.90 (−17.85, −9.99)	> 0.99
temperature* × *host plant		
3°C warmer * alfalfa	−2.67 (−8.43, 2.99)	0.82
6°C warmer * alfalfa	1.68 (−3.57, 4.98)	0.74
pesticide* × *host plant		
low pesticide * alfalfa	−0.14 (−4.74, 4.44)	0.56
high pesticide * alfalfa	−0.56 (−7.96, 6.97)	0.52
temperature* × *pesticide		
3°C warmer * low pesticide	−0.40 (−3.57, 2.74)	0.60
6°C warmer * low pesticide	−2.78 (−6.07, −0.52)	0.95
3°C warmer * high pesticide	3.53 (−4.01, 11.10)	0.82
6°C warmer * high pesticide	−3.23 (−8.23, 1.77)	0.90
temperature* × *pesticide* × *host plant		
3°C warmer * low pesticide *alfalfa	0.23 (−6.75, 7.31)	0.53
6°C warmer * low pesticide * alfalfa	1.50 (−5.49, 8.57)	0.66
3°C warmer * high pesticide * alfalfa	–	–
6°C warmer * high pesticide *alfalfa	−0.32 (−9.52, 8.75)	0.53

Table 4. Coefficient estimates from Bayesian models predicting larval development time. (Medians are the median of the posterior distribution and the 95% credible intervals are shown in parentheses. The probability of effect indicates the area of the posterior in the direction of the median effect that is not overlapping zero. A full table that includes Gelman–Rubin diagnostics and effective samples sizes can be found in the electronic supplementary material, table S1.)

predictor variable	median (2.5%, 97.5%)	prob. of effect
population	1.61 (−12.30, 15.48)	0.59
pre-treatment weight	−5.51 (−9.10, −1.96)	> 0.99
sex	−0.05 (−0.13, 0.03)	0.90
temperature		
3°C warmer	0.00 (−0.14, 0.13)	0.53
6°C warmer	−0.11 (−0.28, 0.07)	0.88
pesticide		
low	0.02 (−0.11, 0.16)	0.63
high	0.09 (−0.22, 0.36)	0.72
host plant		
alfalfa	0.21 (−0.02, 0.44)	0.96
temperature × host plant		
3°C warmer * alfalfa	−0.15 (−0.53, 0.22)	0.79
6°C warmer * alfalfa	−0.06 (−0.42, 0.30)	0.63
pesticide × host plant		
low pesticide * alfalfa	0.04 (−0.25, 0.33)	0.60
high pesticide * alfalfa	−0.16 (−0.69, 0.35)	0.72
temperature × pesticide		
3°C warmer * low pesticide	−0.01 (−0.22, 0.19)	0.56
6°C warmer * low pesticide	−0.01 (−0.24, 0.22)	0.53
3°C warmer * high pesticide	−0.04 (−0.56, 0.45)	0.56
6°C warmer * high pesticide	−0.03 (−0.37, 0.33)	0.57
temperature × pesticide × host plant		
3°C warmer * low pesticide *alfalfa	0.09 (−0.37, 0.56)	0.65
6°C warmer * low pesticide * alfalfa	−0.16 (−0.65, 0.33)	0.73
3°C warmer * high pesticide * alfalfa	–	–
6°C warmer * high pesticide *alfalfa	0.24 (−0.42, 0.91)	0.76

experiment. Once pesticides were administered in the later stage, temperature did not have any further additive effect on survival. We found additional effects of host plant on both adult weight and larval development time. We also observed interactive effects of specific treatment combinations, especially in combinations of temperatures and host plants, and combinations of temperature and pesticide levels, both of which further reduced survival and adult mass above the action of additive effects alone.

The strongest observed effect across all treatments was the additive negative effect of the high chlorantraniliprole treatment, which reduced survival by 62% compared to reference caterpillars that were fed the native host, were given no pesticides, and were raised in the current climate. The concentration of the high pesticide treatment, 95 ppb, was the maximum observed concentration in the leaves of milkweed plants along an agricultural margin in the North Central Valley of California in 2019 [21]. While the Melissa blue does not use milkweed as a larval host, both alfalfa and *Astragalus* can grow along agricultural margins and could be exposed in a similar fashion. The mean concentration detected in leaves from that same study was 9.5 ppb and was not found to affect survival of *L. melissa* caterpillars; however, there are some notable caveats. We exposed caterpillars to the compound only once because chlorantraniliprole is relatively short lived on leaves, within the range of a few days [57,58], and a single pulsed approach was thought to be sufficient for capturing the primary adverse effects. Of course, in a field setting caterpillars could be exposed more than once and chronic exposures to chlorantraniliprole versus a single exposure increases the compound's lethality [59]. We also chose to expose caterpillars in the second half of their development rather than the first half out of concern for killing too many early instar caterpillars. Other work with chlorantraniliprole has shown that late instar monarch caterpillars are more robust by multiple orders of magnitude compared to early instar caterpillars [60], so while we do not find that a single exposure to 9.5 ppb at a late instar is a lethal combination, the possibility remains that such concentrations could still have biological effects in other contexts. Furthermore, we only tested a single compound, while larval host plants in agricultural settings often contain many pesticides, including compounds meant to synergize with other pesticides. Such experiments that test multiple compounds against other factors would be fruitful, but also pose unique challenges as factorial designs become unwieldy and the statistical power to detect important interactions becomes harder to achieve. In all, the pesticide results from this experiment are probably conservative and present the lower bound of risk to caterpillars.

The most consistent effect across all response variables was the alfalfa treatment, which reduced survival and adult size, while increasing larval development time. A general expectation is that longer development times leads to greater adult mass [61], but here we show the opposite. This is probably owing to development time being driven by host plant and not temperature, alfalfa being a lower quality host and caterpillars taking longer to consume nutritionally equivalent amounts of leaf mass compared to the native host. One major drawback from our collection of plant material directly from the field is the implicit assumption that the plants are not already contaminated when being fed to the caterpillars, as such effects would confound any other host plant effects. The collection sites are both far from active agriculture or other known inputs of environmental contaminants and have been used in past studies in this same system. This is also not the first time that *M. sativa* and *A. canadensis* have been compared with this butterfly, and these findings (with respect to host plant effects on caterpillar performance) are consistent with past studies that have used different sources

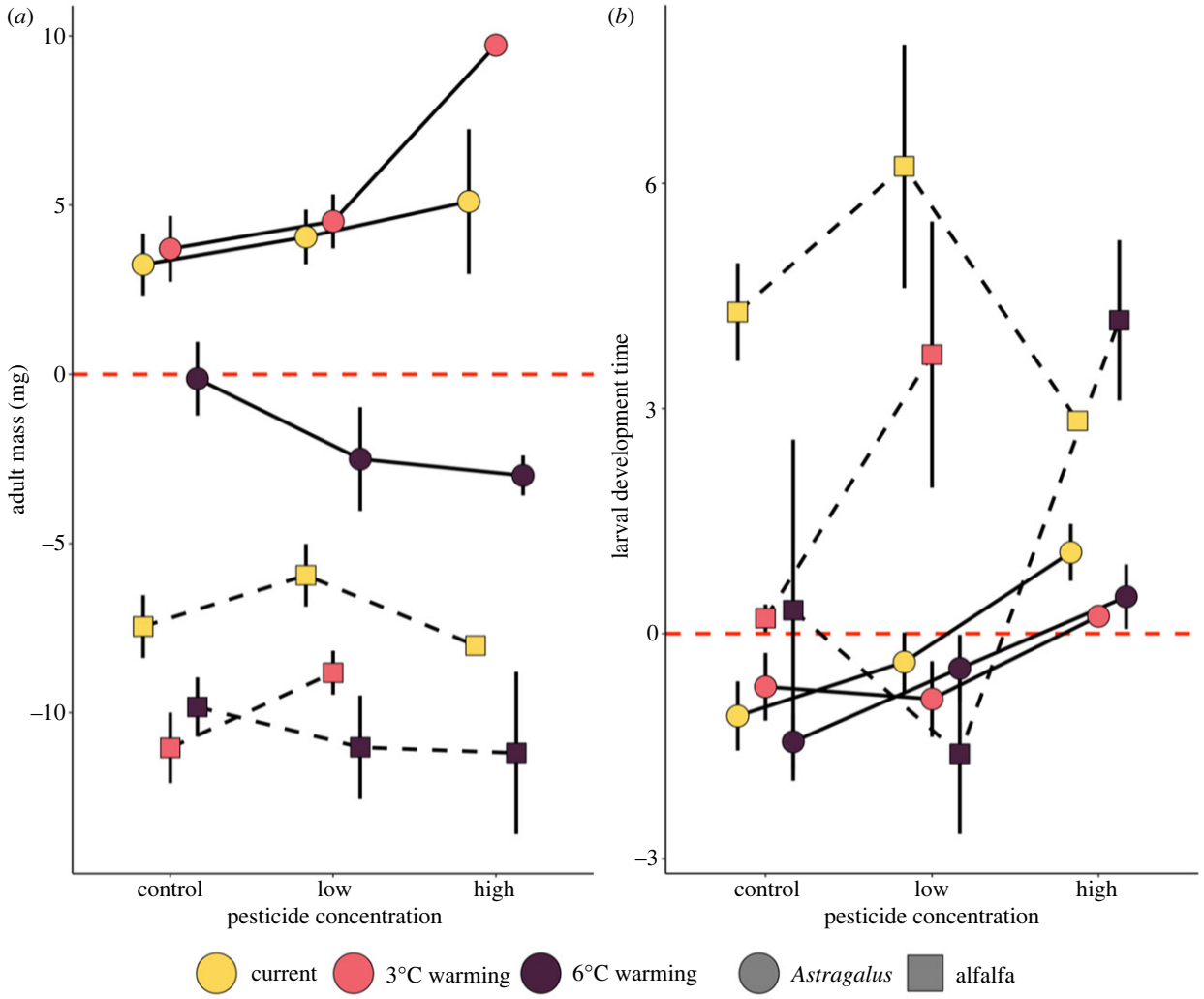

Figure 2. The partial effects of host plant, pesticide and temperature on (*a*) adult mass and (*b*) larval development time after removing the effects of population and pre-pesticide treatment weight and sex. Partial effects are shown with the mean and standard error. Treatment combinations above the red line are positive while combinations below the red line are negative relative to the mean.

of plant material [44,48]. In addition to general nutritional quality, another motivation for this work was the possibility of interactions between a pesticide and secondary metabolites, given both hosts produce distinct phytochemical defences that can impact all the butterfly responses we measured [62] and interactions between host and pesticides have been observed in at least one other butterfly [28]. However, we only find simple, additive effects of these factors, which were highly lethal but not synergistic in action. We also note that our experiments were done on leaves collected from the wild, but no longer connected to a living plant, and we do not know how this might affect any interactions between a pesticide and plant primary or secondary metabolites.

While the introduction of non-native hosts and the non-target effects of pesticides are both current and historical factors affecting populations, butterflies everywhere are increasingly experiencing the impacts of climate change. We did not observe strong additive effects of either temperature treatment on our response variables in later instars. The only additive effect we estimated with any confidence was a decrease in larval development time in caterpillars raised in the 6°C warming treatment, a typical response to increased temperature. However, when we focus instead on the early instars (before the pesticide-pulse phase of our experiment), we do find an effect of both experimental climates, where warmer climates reduce survival on those more sensitive early instars. We also found a large effect of host plant early in development, but no interaction with temperature. As with our pesticide treatment, it should be noted that these temperature results are probably conservative. We presented caterpillars with diurnal cycles that transitioned between a night-time low and a daytime high over a period of 30 min (although the hottest chamber did warm over a slightly longer period) (electronic supplementary material, figure S1). This is of course more realistic than a constant temperature treatment, but it misses the potentially most deleterious aspects of climate change, exposure to extreme temperatures that often happens in narrow windows of time. A limited exposure to temperatures far outside of a tolerable range (such as near or above a critical thermal maximum; CT_{max}) has the potential to be much more disruptive than continued exposure to temperatures slightly warmer than current conditions [63]. If we are to fully understand interactions between climate and other stressors, experimental work on temperature extremes is a critical area in need of further research.

Although the threat of climate change in isolation is of great concern, the ubiquity of its influence vastly increases

the chance for interactions with other stressors. While we did not observe large additive effects of temperature, we did find interactions between temperature and both host plant and pesticide exposure. Caterpillars that received the alfalfa treatment and were part of the 6°C warming treatment group had an additional negative chance of survival after accounting for both the additive components of those variables. We are unsure of the mechanism of interaction here, but as we have already discussed, alfalfa exhibits phytochemical defences that impact *L. melissa* caterpillars so the opportunity for interaction with plant phytochemistry is present and such interactions have been shown in other butterflies [30]. We also observed an interaction between increased temperature and pesticide lethality. Caterpillars exposed to the high pesticide treatment in the more moderate 3°C warming suffered an additional reduction in survival. It is possible that a direct effect of thermal stress or some indirect effect of temperature on caterpillar size mediates the ability of a caterpillar to tolerate a pesticide, however this is complicated in that the high temperature group did not see this same interaction. The lack of interaction with pesticide exposure in the 6°C warming group might be a consequence of the fact that those caterpillars were larger on average than the current and 3°C warming groups (which were not different from each other) when pesticides were applied. This larger size would increase pesticide resistance at that stage of the experiment, however in the long run size does not convey additional benefit over the other temperature treatments. In addition to the caveats raised about extreme temperatures, which also apply here, climate change will probably impact caterpillars indirectly through changes in host quality, but this study assumes that plants will remain the same in future climates. This is a requirement for an experimental design that isolates direct effects on caterpillars, however these indirect temperature and host plant additive and interactive effects may be just as important for impacting butterfly populations. A follow-up study that incorporates knowledge about lethal ranges of pesticide concentrations (such as what was learned here) and heat stress would be informative, whether in this system or another where prior knowledge has been established.

While the details of this study are motivated by our work with butterflies in the western USA, these stressors are not specific to any region. Many butterflies (and other insects) across the world are facing two if not all three of these stressors simultaneously and it is our hope that this study provides a useful data point for understanding interactive threats, especially in a small bodied and non-migratory butterfly. At the organismal level, we found that additive effects are stronger than interactions and that these collective, additive effects alone can result in large reductions in survival. The question remains, however, of whether interactions among stressors might be realized at other ecological scales. By taking a narrow and controlled approach, we focus on how threats impact individual caterpillars, while we miss important outcomes including effects at other life stages, behavioural changes, and other impacts on dynamics that could manifest at the population or metapopulation scale. Work on butterflies from the region using long-term monitoring data suggests that additive effects are indeed stronger [19], however large-scale studies of other insects from other regions have identified important interactions between temperature and pesticides [27]. Thus it is important that future observational studies continue to consider the possibility of interactions between relevant stressors that might only be detectable or primarily detectable with historical datasets at landscape scales. It is through a combination of observational, simulation, and experimental approaches (spanning organismal to landscape scales) that we can understand threats posed by the Anthropocene and design the most effective and scientifically informed conservation strategies for butterflies.

Data accessibility. The data and code used for analyses can be downloaded from Zenodo: https://doi.org/10.5281/zenodo.7401175 [64].

Data is provided in the electronic supplementary material [65].

Authors' contributions. C.A.H.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, supervision, visualization, writing—original draft, writing—review and editing; D.J.Z.: investigation, writing—review and editing; M.L.F.: funding acquisition, investigation, methodology, supervision, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

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