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Phenotypic biomarkers of climatic impacts on declining insect populations: a key role for decadal drought, thermal buffering and amplification effects and host plant dynamics

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Abstract

1. Widespread population declines have been reported for diverse Mediterranean butterflies over the last three decades, and have been significantly associated to increased global change impacts. The specific landscape and climatic drivers of these declines remain uncertain for most declining species.
2. Here we analyse whether plastic phenotypic traits of a model butterfly species (*Pieris napi*) perform as reliable biomarkers of vulnerability to extreme temperature impacts in natural populations, showing contrasting trends in thermally exposed and thermally buffered populations.
3. We also examine whether improved descriptions of thermal exposure of insect populations can be achieved by combining multiple information sources (i.e. integrating measurements of habitat thermal buffering, habitat thermal amplification, host plant transpiration, and experimental assessments of thermal death time (TDT), thermal avoidance behaviour (TAB) and thermally induced trait plasticity). These integrative analyses are conducted in two demographically declining and two non-declining populations of *P. napi*.
4. The results show that plastic phenotypic traits (butterfly body mass and wing size) are reliable biomarkers of population vulnerability to extreme thermal conditions. Butterfly wing size is strongly reduced only in thermally exposed populations during summer drought periods. Lab rearing of these populations documented reduced wing size due to significant negative effects of increased temperatures affecting larval growth. We conclude that these thermal biomarkers are indicative of the population vulnerability to increasing global warming impacts, showing contrasting trends in thermally exposed and buffered populations.
5. Thermal effects in host plant microsites significantly differ between populations, with stressful thermal conditions only effectively ameliorated in mid-elevation populations.

In lowland populations we observe a six-fold reduction in vegetation thermal buffering effects, and larval growth occurs in these populations at significantly higher temperatures. Lowland populations show reduced host plant quality (C/N ratio), reduced leaf transpiration rates and complete aboveground plant senescence during the peak of summer drought. Amplified host plant temperatures are observed in open microsites, reaching thermal thresholds that can affect larval survival.

6. Overall, our results suggest that butterfly population vulnerability to long-term drought periods is associated to multiple co-occurring and interrelated ecological factors, including limited vegetation thermal buffering effects at lowland sites, significant drought impacts on host plant transpiration and amplified leaf surface temperature, as well as reduced leaf quality linked to the seasonal advance of plant phenology. Our results also identify multi-annual summer droughts affecting larval growing periods as a key driver of the recently reported butterfly population declines in the Mediterranean biome.

Introduction

Declines in butterfly populations across diverse species over the last three decades have been described in the Mediterranean basin (Stefanescu *et al*., 2004, 2011ab; Wilson *et al*., 2005, 2007; Carnicer *et al*., 2012, 2013; Zografou *et al*., 2014; Melero *et al*., 2016). Negative effects of land use changes and global warming have been proposed as the main drivers of the observed declining trends (Stefanescu *et al*., 2004, 2011ab; Wilson *et al*., 2005, 2007). These negative demographic trends affect both habitat generalist and specialist butterfly species in the Mediterranean biome, and the spatial diversity of most functional groups (e.g. host-plant use, dispersal capacity, habitat specialisation and thermal niche) is negatively associated with increased temperatures and aridity (Stefanescu *et al.,* 2011ab, Carnicer *et al*., 2013). Furthermore, the available evidence suggests that global-warming induced population responses are intimately linked to complex interactions with habitat features and host plant dynamics (Merrill *et al*., 2008; Suggitt *et al*., 2012; De Frenne *et al*., 2013; Oliver *et al*., 2014, 2015; Carnicer *et al*., 2017). In line with this idea, it has been suggested that specific habitat attributes can modify global warming impacts on butterfly populations, triggering both positive and negative demographic responses. For example, it has been shown that the densification of forest habitats associated with land abandonment can cool local microclimates, buffering the impacts of global warming in some plant and insect populations and resulting in positive or neutral demographic responses to global warming (De Frenne *et al*., 2013; Nieto-Sánchez *et al*., 2015). On the other hand, populations inhabiting sites lacking effective habitat thermal buffering could experience increased negative impacts of extreme temperatures, resulting in substantial long-term demographic declines (Parmesan, *et al*. 2000). In addition to the effects of habitat thermal buffering, the thermal exposure of butterfly populations can be crucially determined by other key processes, such as the seasonal variation of host plant transpiration and leaf water content during summer drought, the operation of thermal amplification processes in microhabitats or the display of thermal avoidance behaviours in the insect larvae allowing the selection of cool microsites at the host plant (Carnicer et al. 2017). These key co-acting processes are often not measured and their complex interactions remain poorly described. To understand the relative importance of all these processes, integrative studies combining multiple information sources in intensively studied populations are warranted.

Here we provide an integrative study of the thermal exposure in four populations of *Pieris napi,* combining multiple sources of information (demographic and climatic data, phenotypic trait data, measurements of habitat thermal buffering, host plant traits, and experimental assessments of thermal responses). Furthermore, we explore whether temperature-responsive phenotypic traits can be applied as reliable biomarkers of the different vulnerability to increased temperatures in these intensively studied populations. Ample experimental evidence supports that diverse life history and functional traits of butterflies are highly responsive to temperature variation and show predictable responses to extreme temperature treatments (Jones *et al*., 1982; Sheridan & Bickford, 2011; Bauerfeind & Fischer, 2013ab, 2014; Nail *et al*., 2015). In particular, wing and body size measures have been identified as traits highly responsive to temperature variation and climate change impacts (Atkinson, 1994; Atkinson & Sibly, 1997; Nygren *et al*., 2008; Kingsolver, 2009; Talloen *et al*.; 2009; Sheridan & Bickford, 2011; Forster *et al*., 2012). Therefore, it is likely that an extensive quantification of plastic phenotypic traits in declining and non-declining natural populations could indicate their different vulnerability to warmer conditions. In other words, if a specific morphological trait of a species is known to respond plastically and in a linear manner to thermal conditions, then we can potentially deduce, for specific populations, the exposure to these thermal conditions by quantifying its morphology. Moreover, if we measure extreme thermal conditions in a target population, which should induce a negative morphological response, and find non-altered biomarker values, we can suspect that the population is buffered from stressful conditions by microhabitat effects.
In the Mediterranean region, summer drought periods and increased summer temperatures are tightly linked and significantly associated (Fig. S1). Therefore, during extreme summer drought periods we expect phenotypic traits to be affected by extreme temperature impacts. In this

context, those population sites lacking effective habitat thermal buffering effects should present a significant negative response in temperature-responsive biomarker traits. In contrast, we expect that populations characterised by effective microsite buffering mechanisms should present non-significant trends in temperature-responsive phenotypic biomarkers (see supplementary text S1 for a formal definition of the term biomarker and a simple mathematical framework supporting this definition).

To test this hypothesis and develop an integrative analysis of thermal exposure in a butterfly species, we address the following five research objectives using the green-veined white *Pieris napi* as a species model: i) to analyse whether plastic phenotypic traits perform as reliable biomarkers of vulnerability to extreme temperature impacts in natural populations, by comparing phenotypic trait responses in four populations of *Pieris napi*; ii) to experimentally estimate thermal death time responses (thermal susceptibility (z) and critical thermal limit (CT_{max})) and the thermal threshold for avoidance behaviour (TAB) for this model species, iii) to quantify thermal buffering in microsites, assessing whether they provide non-stressful thermal habitats only in specific localities; iv) to evaluate whether host plant resource dynamics qualitatively differ between the studied populations, and v) to assess whether increased drought impacts could explain the reported long-term population declines in the selected model species.

Methods

Study species

Pieris napi is a widely distributed Holarctic butterfly, common across most of North America and Europe, though only locally in North Africa. Throughout its distribution, it shows a clear preference for humid habitats, such as wetlands, riparian forests and irrigated agricultural land. In Catalan lowland areas, there is a succession of 4-5 generations from early spring (March-April) to autumn (October-early November), with overwintering in the pupal stage. Maximum abundance is typically recorded in early summer, in coincidence with the peak of the third generation. This peak is followed by a period of 1-2 months when abundance is much reduced, in coincidence with summer drought. Butterflies then reappear by the end of September, in what normally constitutes the last annual generation. In mountain areas, where the phenology is constrained by colder temperatures, a succession of three generations from April to September is the most common pattern. At most montane sites, abundance increases all over the season and reaches its maximum in the third and last annual generation. Eggs are laid singly on a wide range of wild Brassicaceae, *Lepidium draba* and *Brassica nigra* being the two most common host plants in lowland areas, and *Alliaria petiolata*, *Arabis glabra* and *Cardamine pratensis* those mostly used in mountain habitats. Other secondary host plants have been recorded over the region (García-Barros *et al*., 2013).

Study zone

We studied two lowland declining populations (sites 1 and 2) and two mid elevation non-declining populations (sites 3 and 4) in Catalonia, NE Spain. Sites 1 and 2 were located at two protected coastal wetlands (Delta del Llobregat and Aiguamolls de l'Empordà, 133 km apart). In contrast, sites 3 and 4 were located at higher elevations, also in natural protected areas (Zona Volcànica de la Garrotxa (503 m a.s.l.) and Montseny (1031 m a.s.l.), 41 km apart). Mid elevation sites were characterised by a heterogeneous mosaic of different habitat types, including open fields, small wetland and riverine areas, and temperate and evergreen forests. A more detailed summary of the geographic, climatic and ecological attributes of the selected sites is provided in Table S1 and Fig. S2. To quantify long term demographic trends, sites were surveyed from 1994 to 2012 as part of the Catalan Butterfly Monitoring Scheme (www.catalanbms.org) via weekly butterfly counts along fixed transect routes from March to September (a total of 30 recording weeks per year). All individuals seen within 2.5 m on each side and 5m in front of the recorder were counted, using the standard methodology of the Butterfly Monitoring Schemes (Pollard & Yates, 1994; Schmucki *et al*., 2016). For site 1 demographic surveys were available only for seven years distributed in two discrete periods

[1994-1997, 2007-2009]. An annual index calculated as the sum of weekly counts was used as the measure of population abundance at each season.

Population trends, climate and landcover data and model selection approach

Climate factors and landscape use changes have been identified as the main drivers of long-term butterfly population trends in the Mediterranean biome (Stefanescu *et al*., 2011ab). However, detailed models combining climatic and dynamic landscape data are still warranted to quantitatively assess the relative contribution of these two factors to long-term butterfly demographic declines. For this purpose, we compiled a database integrating butterfly annual abundance indices, monthly climatic rainfall and temperature data for the 1994-2012 time period (Domingo-Marimón, 2015), and landcover dynamics data for 1994-2012. To study landscape dynamics, aerial orthoimages (1:25000) for 1993, 2001, 2006 and 2012 were digitised using MiraMon, a geographic information system (Pons, 2002). The images were provided by the Cartographic Institute of Catalonia (http://www.icgc.cat/en/). We selected a circular area (2 km of diameter) around the field transect sites and quantified the changes in the total surface (m^2) of the following nine landcover types: wetland and continental water (L1), dense forest (L2), sparse forest (L3), shrubland (L4), grassland and herbaceous meadows (L5), urbanised land (L6), bare land (L7), road/lane areas (L8) and beach area (L9). A continuous annual sequence of estimated land cover changes for 1994-2012 was obtained applying spline fits using JMP (SAS Institute, 2012) and saving predicted values between consecutive orthoimages in the time series.

To analyse the observed temporal trends in the butterfly annual abundance of the four populations over 1994-2012 spline fits and ordinary least squares models (OLS) were implemented. Two model selection approaches were sequentially applied, first using only climatic variables (approach 1) and subsequently combining land and climatic variables in an integrated model (approach 2). The first modelling approach was simply used to reduce the large number of climatic variables analysed (a total of 28 monthly temperature and precipitation variables). In other words, we first selected monthly climatic variables significantly associated with the observed butterfly demographic trends (OLS step-wise approach, (SAS Institute, 2012)) and then we combined the selected climatic variables and dynamic landscape data in an integrated model selection approach. All possible models computable in each approach were contrasted in terms of their corrected Akaike's Information Criterion (AIC_c) and Bayesian Information Criterion (BIC), and the models with the lowest values were selected. The explanatory power of competing variables was contrasted by the stepwise selective approach and by comparing the estimates for the selected predictors (JMP package, SAS Institute, 2012). Digitised orthoimages were not available for site 4, precluding the inclusion of this site in the landscape modelling analyses. We included in the model selection approach monthly climatic variables of two consecutive years in order to account for the previous autumn growing period of winter diapausing generations (i.e. current and previous year climatic data).

Phenotypic biomarkers of population vulnerability

In order to identify phenotypic traits that could perform as climate-extreme biomarkers, butterfly populations were intensively sampled with weekly resolution during 2014 and 2015, covering the whole flying period (early spring to late autumn). Weekly samples were composed of a minimum of four males and four females. Supplementary samples were collected during seasonal abundance peaks. A total of 1265 butterflies were finally collected (see Table S2 for further details). The following phenotypic traits and their seasonal variance were quantified in the four selected populations: dry body mass, dry wing mass, wing size (i.e. length and area). Dry wing mass and wing area variables were significantly correlated and were considered synonymous descriptors (R^2=0.40; p<0.0001). The same was true for dry body mass and wing area measures (R^2=0.41; p<0.0001). In addition, we also quantified wing melanism, and whole body ∂13C, ∂15N, %N and %C. However, these variables were not strongly related to climate variability and were discarded.

To quantify wing size (length and area), wing samples were photographed using standardised settings (fixed Nikon D7100 with a SigmaMacro objective at a height of 41.5 cm). The quantification was performed with ButterflyPhotoGUI, a Matlab algorithm (developed by Hedrick, T.; Kingsolver lab, University of North Carolina), so that wing size corresponded to the number of pixels in the area defined by three fixed landmarks in the hindwing (tip of the vein M1, tip of the vein CuA_1, and the intersection of the veins CuA_1-CuA_2, Fig. S3). The wing-vein naming system applied is described in Wahlberg et al. (2014). In addition, wing length (mm) and area (mm^2) were measured independently in a subset of standardised photographs. All these measures (wing area in pixels, wing area in mm^2 and wing length in mm) were strongly correlated and, thus, were considered related descriptors ($R^2>0.80$; $p<0.0001$). Wing size was finally chosen to present our graphical results (i.e. *wing length 1* in Fig. S3).

To track the impacts of extreme summer climatic conditions on wing size, we focused on the weekly variation of this trait during the spring, summer and autumn periods. To more precisely quantify the effect of climatic variables on phenotypic trait variability, we modelled the variation of wing size using ordinary least squares models (OLS) and introducing the following predictor variables: site, year (2014/2015), mean temperature during the larval and pupal growth period (25 days previous to the adult collection), accumulated rainfall previous to adult collection (60 days), mean relative humidity previous to adult collection (60 days), photoperiod (mean of 25 days previous to adult collection), and sex (male/female). The interactions between the predictor variables were examined and significant interactions were retained. For each climatic variable, different possible temporal spans were assessed, ranging from 5 to 120 days (with a 5-day resolution), compiling subsets of related climatic variables. The climatic variables that were finally selected were characterised by higher correlation coefficients with the modelled variables (wing size) in multivariate correlation analyses (JMP, SAS Institute, 2012). We excluded the first generation, i.e. winter-diapausing individuals, from the modelling analyses. Photoperiod was calculated following Kirk (1994). We randomly collected both freshly emerged and older, worn individuals, and estimated adult age by quantifying wing condition state using an ordinal scale (Fig. S5). No significant effects of wing condition were observed on wing size models

Common-garden experiment

To assess whether the selected biomarker traits were reliable predictors of direct temperature effects on the phenotype, we performed a common garden split-brood experiment. Five female lines from the lowland site 2 were initiated, with eggs reared under photoperiod conditions inducing direct development (13:11 L:D). The offspring (eggs) were divided in two temperature treatments (20 ºC, 25ºC). As illustrated in Fig. S6, the 20 ºC treatment corresponds to the observed mean daily temperatures in June or late August. In contrast, the 25 ºC treatment corresponds to the warmest mean daily temperatures of June, July and August in the study period (Fig. S6, and see Bauerfeind & Fischer 2013ab, 2014 for additional experimental studies in this species).

Fresh leaves of the host plant species *Lepidium draba* were provided to the larvae *ad libitum*. A total of 143 adult individuals belonging to 5 different families were finally obtained (see Table S3 for detailed numbers). The experiment allowed testing the effects of treatment, sex and family on wing size. The heritability of the measured traits was estimated using MCMCglmm (Hadfield 2010; Aalberg Haugen *et al*., 2012). To assess putative differences between populations in plastic phenotypic responses between low-elevation and mid-elevation sites, 32 additional adult individuals, belong from mid-elevation site 3 were assessed in replicated experimental split-brood conditions (20/25 ºC treatments, two female lines).

Host plant microsite climatic measures

Maximum summer temperatures often surpass the critical thermal limits of invertebrate ectotherms in multiple biomes (Sunday *et al*., 2014), and as a result a key role of thermal buffering processes has been identified for population persistence (e.g. Ashton *et al*., 2009;

Sunday *et al*., 2014; Suggitt *et al*., 2015; Pateman *et al*., 2016). Consequently, a robust evaluation of climate impacts on butterfly populations requires quantifying microclimatic thermal variability and habitat buffering effects at the host plant level during larval growth periods. In addition, the analysis of the temporal variation of host plant traits over the season allows the identification of critical periods of resource scarcity and changes in host plant quality. In the studied system, the dominant host plants were *Lepidium draba* at lowland wetland areas and *Alliaria petiolata* at mid elevation sites. For *L. draba* and *A. petiolata* six host plant microsites were selected at lowland and mid elevation mountain ranges, respectively (see Table S4 for details). In each microsite we installed an automatic temperature and humidity sensor (LascarElectronics EL-USB-2-LCD) recording hourly climatic variability over 2014 and 2015. In the lowlands, *L. draba* was mostly distributed in open microsites (open meadows and grassland areas) and more rarely under tree canopy and/or shrub cover. Egg-laying by *P. napi* has been recorded on plants growing in both conditions. Four microsite sensors were therefore distributed in the most representative open meadow microsites, and two sensors were located at closed-canopy host plant microsites to quantify the effect of canopy cover on temperature and humidity records. An additional and commonly used host plant, *Brassica nigra*, was also present in lower numbers at lowland site 2 (inhabiting open microsites, along ditches). Two sensors were located at *Brassica nigra* microsites to quantify the observed trends for this host plant. At mid elevation sites, a single dominant host plant (*A. petiolata*) was preferentially located and used for egg-laying at closed-canopy sites. However, a comparatively smaller number of host plants were distributed in open meadow and/or grassland microsites. Four sensors were located at the dominant and representative conditions (closed-canopy sites) and two additional sensors were located at the more unusual open grassland microsites. To contrast microsite climatic measures and standard measures, daily temperature and rainfall records were obtained from four meteorological stations located nearby the four surveyed transects (2-5 km) and at the same altitudinal range (Table S5). The automatic temperature and humidity sensors (LascarElectronics EL-USB-2-LCD) were located at 25 cm height above the soil surface using metal stakes, and were protected from direct solar radiation by a plastic envelope sustained by a wire-mesh cylinder (installed 5 cm above the sensor and thus precluding the direct incidence of solar radiation). The sensors were surrounded by the host plant leaves and were also covered by abundant herbaceous vegetation (the herbaceous layer ranged between 50-120 cm of height). At closed sites, the sensors were in addition directly affected by the shadows of the surrounding shrubs and trees. The sensors estimated the air temperature, relative humidity and dew point with hourly resolution.

Thermal avoidance behaviour (TAB) and thermal death time (TDT) experiments

To avoid the exposure to critical thermal temperatures, butterfly larvae may display thermal avoidance behaviors (i.e. short movements to cooler microsites of the host plant). However, the thermal thresholds for these behaviours remain poorly quantified and experimentally studied in most butterfly species. We experimentally assessed thermal avoidance behaviour in 149 larvae of *Pieris napi* (last instar, site 2), assessing a total thermal range of 28-48 ºC. Larvae were firstly placed on a leaf of a potted *Alliaria petiolata*, and acclimated at an ambient temperature of 22 ºC for 5 minutes. Then, we experimentally raised leaf surface temperatures to a selected Celsius degree treatment (in the thermal range of 28 - 48 ºC) using a 70 W light lamp and carefully controlling the leaf surface temperature with a HANNA HI935005N thermal sensor. Larval behaviour was recorded for 2 minutes, annotating three types of responses: thermally neutral, thermally positive and thermal avoidance movements to cooler microsites. For each Celsius degree treatment, we assessed 5-7 larvae. Each larvae was used in a single thermal trial. A two parameter logistic model was fitted to model the changes in the frequency of thermal avoidance behaviour ($f(T)$) with increasing leaf surface temperature (T):

$$f(T) = \frac{1}{1 + e^{-aT - b}}$$

where a is the growth rate of the function and b is the thermal inflection point (ºC) in which we observed a 0.5 frequency of thermal avoidance behaviours.

Thermal death time experiments (TDT) allow predicting from first principles when environmental temperatures may affect larval survival (Deutsch et al 2008, Rezende et al 2014). To assess the upper critical thermal limit (CT_{max}) in *Pieris napi* we implemented a static thermal death time experiment with three static thermal treatments (40, 42 and 44 ºC; Rezende et al. 2014). We estimated CT_{max} and thermal susceptibility (z) from the equation:

$$T_{ko} = CT_{max} - z\log_{10} t$$

where t is the observed time to death of last instar *Pieris napi* larvae in static thermal experimental treatments, T_{ko} corresponds to the constant stressful temperature levels applied, CT_{max} is the temperature that would result in knowckdown or death at 1 min ($\log_{10} t = 0$) and z is the constant of thermal susceptibility describing how thermal tolerance decays with the duration of the heat challenge. The experiment was implemented in 60 individuals from 6 family lines collected at site 2. 20 individuals were assessed in each thermal treatment (40, 42 and 44 ºC).

Plant trait measurements

To evaluate whether host plant resource dynamics qualitatively differed between populations we quantified the weekly variation of leaf %N and leaf C/N. Previous empirical works have shown that nitrogen strongly determines butterfly host plant quality of mature leaves (Mattson, 1980; Slansky & Feeny, 1977; Scriber & Slansky, 1981; Myers, 1985; Kaitaniemi *et al*., 1998). Moreover, leaves containing less nitrogen constrain insect performance and reduce pupal mass in field experiments (Myers, 1985; Kause *et al*., 1999, but see Fischer & Fiedler 2000). Plant phenology and drought have been identified as key drivers of leaf nitrogen variation (Kause *et al*., 1999; Grant *et al*., 2014). Notably, drought and phenology should produce qualitatively different effects on the selected nitrogen-related traits (%N, C/N). In the case of phenology, a progressive reduction on the quantity of nitrogen in the leaves should be expected with plant maturation and leaf ontogeny due to the translocation of nitrogen-rich resources to flowers, fruits and rhizomes (Kause *et al*., 1999; Jacobs, 2007). Overall, phenology should promote a progressive reduction of leaf %N and an increase of leaf C/N over the late spring and early summer period. In contrast, in the case of drought-induced effects on plant ecophysiology, an increase in the quantity of nitrogen in the leaves could be expected (e.g. Bauerfeind & Fischer 2013b; Grant *et al*., 2014; Valim *et al*., 2016). To perform the weekly leaf measurements five plants were sampled per week and site, collecting 5-8 leaves per plant for the analyses (Table S6). Seasonal trends for C/N ratio and %N were modelled applying ordinary least squares for linear trends and spline fits for non-linear trends (JMP package (SAS Institute 2012)).

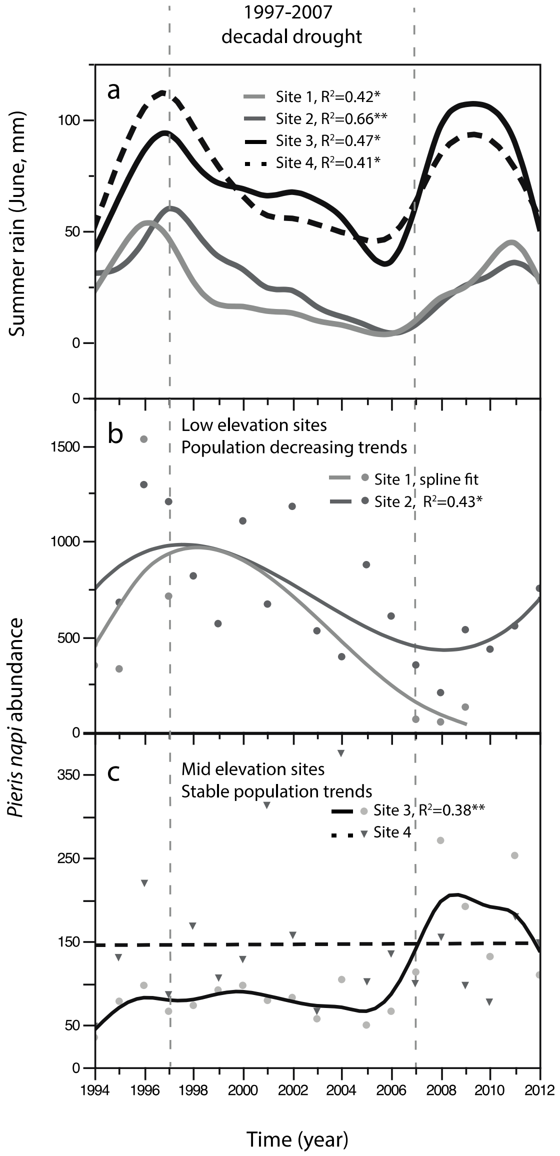
In order to achieve a more detailed assessment of plant responses to drought stress at lowland sites, monthly measures of leaf stomatal conductance and leaf surface temperatures were specifically conducted for plants *L. draba* and *B. nigra* (using a LICOR 6400 portable photosynthesis system). These measurements were restricted to open microsites and were conducted at midday (12.00-14.00) (Aiguamolls de L'Empordà wetlands, site 2). Four replicates were measured for each host plant species. The measurements were conducted in 2015, a year characterised by warm and dry summer conditions (Fig. S7). Stomatal conductance and leaf surface temperatures have been widely applied as integrated indicators of drought and heat stress in herbaceous plants (Munns *et al*., 2010; Anissa *et al*., 2013). Experimental evidence shows that leaf conductance and surface temperature show qualitatively different responses in heat treatments and soil drought experiments (Anissa *et al*., 2013). In conditions of abundant soil moisture, *Brassica* plants respond to strong air temperature stress with an increase in leaf stomatal conductance values, producing in turn positive leaf-to-air temperature differences (i.e. cooler leaf temperatures relative to air temperatures due to increased leaf transpiration (Anissa *et al*., 2013)). In contrast, if plants experience combined soil water and air heat stress, which is probably more common in Mediterranean ecosystems, reduced leaf conductance values and negative leaf-to-air temperature differences are to be expected (i.e. higher leaf temperatures relative to air temperatures due to reduced leaf transpiration). Photosynthetic rates (A) and stomatal conductances (g_s) were measured between 12:00 a.m. and 14:00 p.m. at a quantum flux density (PPFD) of 1080±19 µmol m^{-2} s^{-1} and ambient air temperature under a controlled CO_2 concentration of 400±2 ppm. To conduct the measurements one leaf was enclosed in a clamp-on gas-exchange cuvette of 2 cm^2. We selected healthy leaves that were not affected by insect larvae consumption and/or fungal damages. Air flow through the dynamic cuvette was 732±0.05 ml min^{-1}. A Licor-6400XT (4647 Superior Street P.O. Box 4425 Lincoln, Nebraska USA) gas-exchange system was used.

Results

Climatic and population trends

Model selection analyses using only climatic data identified June rainfall of the current year as the best predictor of the interannual variation of *Pieris napi* abundance (Tables S7 and S8). An analysis of the temporal trends for this climatic predictor (June rainfall) over 1994-2012 identified a decadal period of increasing drought (1997-2007, Fig. 1a). The observed decadal reduction in June rainfall was highly significant at the four sites (Fig. 1a). In line with the reported decadal trend of increasing summer drought stress, butterfly abundance at lowland sites significantly declined, paralleling the trend of June rainfall (Fig. 1b). As a result, lowland populations showed a sharp reduction of more than one order of magnitude respect the initial abundance numbers. In contrast, mid elevation site populations remained fairly stable over the 1997-2007 drought period (Fig 1c), and were therefore not paralleling June rainfall trends as observed at the lowland sites. After the decadal drought period, however, population at site 3 increased significantly (R^2=0.38, p=0.0024, Fig. 1c), and this increase was significantly correlated to an abrupt increase in June rainfall during 2008-2012 (R^2=0.38, p=0.0028). Lowland sites showed significantly lower June rainfall values during the 1997-2007 period (Tukey-Kramer test, R^2=0.35; p=0.0005, Fig. S8).

Figure 1. Climatic and butterfly demographic dynamics over the 1994-2012 period. a) Annual variation of June rainfall at the four population sites. Significant linear rainfall trends are indicated in the 1997-2007 period. b) Observed variation of butterfly annual abundance at lowland sites (Delta del Llobregat (1) and Aiguamolls de l'Empordà (2) protected wetlands). A significant polynomial and a spline fit are illustrated. c) Observed variation of butterfly annual abundance at mid elevation sites (Zona Volcànica de la Garrotxa (3) and Montseny Ranges (4) protected areas). Spline and linear fits were applied. When significant, the variance explained by the linear fit (R^2) is indicated.



The model selection approach combining climatic and landcover data reported that both types of variables significantly contributed to the reported demographic trends. Overall, however, the estimates of the models suggested a stronger and predominant effect for climatic variables in the reported trends (June rainfall, Tables S7–S11). For landscape variables, significant negative effects of reduced meadow cover extent during 1994-2012 were detected at site 2. At mid elevation site 3, a positive effect of increased wetland area was detected. Landscape data were not available for 2013-2015 and therefore these years were excluded from the butterfly annual abundance models. Nevertheless, we examined the observed population trends for an extended period (1994-2015) and the results were fully consistent with the trends reported for 1994-2012 (Fig. S9). Mid elevation site 4 showed a stable population trend (Fig. 1 C), and no significant relationships with climatic variables in OLS models were observed for this site. Population abundances at low elevation sites areas were higher (Fig. 1), presumably due to a much higher spatial density of host plants per unit of surface observed in these wetland areas.

Different population sensitivity to temperature impacts

The analysis of the environmental variation of wing size revealed significantly different trends between lowland and mid elevation populations (Fig. 2a). Of note, these trends were significantly associated to increased summer temperature only at lowland sites (Fig. 2a). Consistent with this, OLS models identified the seasonal variation of temperature during the larval and pupal growth period (25 days previous to the adult emergence and collection) as the principal driver of diverging wing size seasonal trends and detected site x temperature interactions (Table 1). The interactions between site and temperature were highly significant, reporting strong negative effects only at lowland sites (Table 1, and see Fig. 2a).

Consistent with the field observations, the split brood common-garden experiment demonstrated a significant link between temperature and wing size variation (Fig 2b). Tables S12-S15 summarise the results of the split-brood experiment. Significantly different wing size values were observed for the 20 ºC and the 25 ºC treatment, with reduced wing lengths observed for the high temperature treatment. In addition, we observed significant effects of sex and family, with females showing significantly lower wing sizes (Tables S12-S15). The effect of the temperature treatment, however, was dominant and stronger than family and sex effects. Wing size heritability estimate reported by MCMCglmm models was 0.41 (CI=0.12-0.76).

Importantly, the observed wing sizes for the 25 ºC treatment were consistent with the observed range of wing size values in the field dataset in the same range of temperatures (25 ºC [i.e. mean temperature during the growth period (25 days)], Fig. 2a, grey square area). Significant effects of 20/25 ºC thermal treatment on larval developmental times were observed, as reported in Fig.

S10 (R^2=0.71, p<0.0001). Site effects were not significant (Tables S14-S15). Mid altitude sites showed more plastic responses to temperature in experimental treatments (Table S15), indicating that the flat trends in Fig 2a were not related to a lack of thermally induced wing plasticity in mid altitude populations.

Figure 2. Observational (a) and experimental (b) trends in the selected phenotypic biomarker trait (wing size (mm)). a) Observed relationships between butterfly wing size and environmental temperature at the four sites. The grey square represents the mean wing size and the 95% confidence interval of the thermal stress treatment (25 ºC), matching the field observational values (lines) at lowland sites. b) Observed differences in wing size measurements between two experimental temperature treatments (20 / 25 ºC). The line across each diamond represents the treatment mean. Diamond plots indicate the 95% confidence interval for each treatment (vertical span) and mean (midpoint line). Green dots represent mid-elevation individuals. Red dots represent low-elevation individuals. We concluded that experimental and field results were in agreement, suggesting a key role of stressful temperatures at lowland sites in the reported wing size trends. c) Estimated thermal inflection point for behavioural avoidance responses in last instars of *Pieris napi*. d) Observed thermal death time (TDT) in static thermal treatments in last instar larvae of *Pieris napi.* The line within the box represents the median sample value. The ends of the box represent the 25th and 75th quantiles.

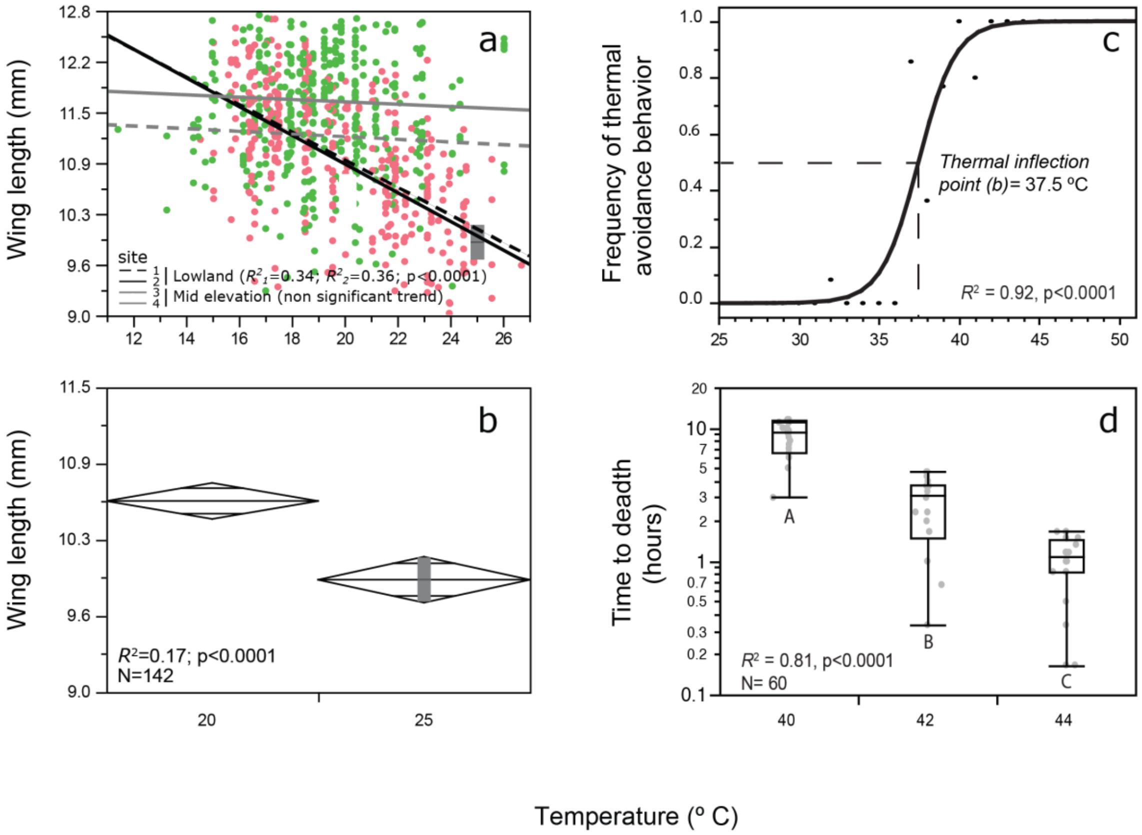

a
Wing length (mm)
12.8
12.2
11.5
10.9
10.3
9.6
9.0
12
14
16
18
20
22
24
26
site
1/2 | Lowland (R^2_1=0.34; R^2_2=0.36; p<0.0001)
3/4 | Mid elevation (non significant trend)
b
Wing length (mm)
11.5
10.9
10.3
9.6
9.0
R^2=0.17; p<0.0001
N=142
20
25
c
Frequency of thermal avoidance behavior
1.0
0.8
0.6
0.4
0.2
0.0
Thermal inflection point (b)= 37.5 ºC
R^2 = 0.92, p<0.0001
25
30
35
40
45
50
d
Time to deadth (hours)
20
10
7
5
3
2
1
0.7
0.5
0.3
0.2
0.1
A
B
C
R^2 = 0.81, p<0.0001
N= 60
40
42
44
Temperature (º C)

Table 1. OLS model of the variation of wing size. Values in bold highlight the principal effect of temperature variation and site*temperature interactions (negative in lowland, declining populations; and positive at mid elevation sites).

Wing size **Model fit:** R^2=0.38, p<0.0001, AIC$_c$=18232.3, BIC=18300.8				
	Estimate	**Std Err**	**t**	**p**
Intercept	317368.16	190999.1	1.66	0.0970
Temperature (Temp)	**-9164.388**	**1321.999**	**-6.93**	**<.0001**
Site 1	-13424.82	8506.542	-1.58	0.1150
Site 2	-23883.71	8205.615	-2.91	0.0037
Site 3	32545.087	10085.57	3.23	0.0013
Site 4	4763.4443	6388.543	0.75	0.4561
Sex (female)	-14702.02	2308.956	-6.37	<.0001
Year	-153.3687	4089.377	-0.04	0.9701
Photoperiod	10464.797	5677.805	1.84	0.0657
Rainfall	764.4488	7598.182	0.10	0.9199
Relative Humidity	2546.9183	2090.97	1.22	0.2236
Temp*site1	**-6824.945**	**1318.059**	**-5.18**	**<.0001**
Temp*site2	**-7078.373**	**1352.495**	**-5.23**	**<.0001**
Temp*site3	**4663.1566**	**1517.146**	**3.07**	**0.0022**
Temp*site4	**9240.1615**	**1799.712**	**5.13**	**<.0001**
Temp*sex (female)	-1274.876	854.2187	-1.49	0.136

Thermal avoidance behaviour (TAB) and thermal death time (TDT) experiments

The results of the thermal avoidance behaviour experiment are shown in Figure 2c. The observed behavioural response of thermal avoidance was well described by a two parameter logistic model (R^2=0.92, p<0.0001) with the following parameters: growth rate a = 0.86±0.24, thermal inflection point b =37.46±0.37 ºC. These results indicate a rapid shift to behavioural avoidance responses at temperatures above 37.5 ºC in the last instar larvae of *Pieris napi*. Fig. 2d synthesises the results of the static thermal death time experiments. Thermal death time experiments (TDT) reported an estimate of the temperature resulting in death at 1 min of exposure (CT_{max}) of 51 ºC, and a thermal susceptibility constant (z) of 4.11±0.33 (ºC). The observed TDT relationships for 100% and 50% of mortality are illustrated in Fig. S11a. The thermal threshold for a time of exposure equal to the whole larval period was estimated in 32.5 ºC (Fig S11b). For a daily exposure of 6 hours to maximum daily temperatures (TE6h, 10 am-16 pm), the TDT curve indicates a thermal threshold of 34.5 ºC (Fig. S11b). These thermal thresholds were achieved in warm summer days characterised by mean daily temperature ≥ 25 ºC in 2014-2015 (Fig. S11c).

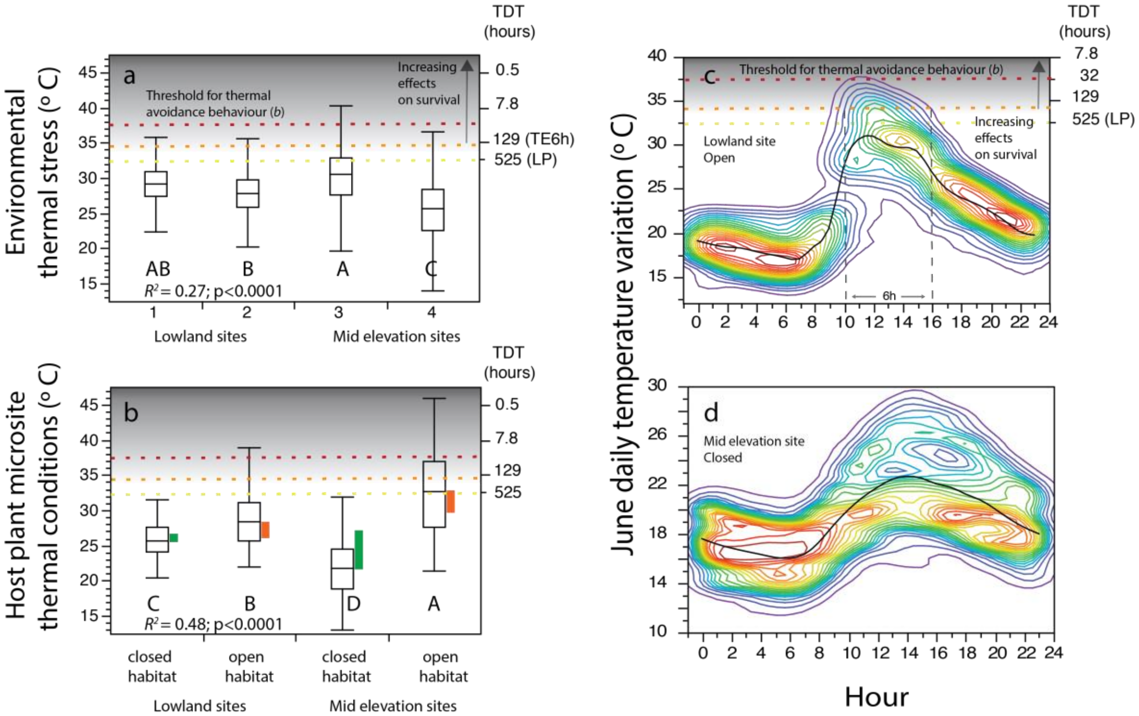
Thermal stress during summer drought and microsite effects

Analysis of meteorological data during 2014-2015 for the four population sites found that mean June maximum temperatures were in the range of 28-30 ºC at three sites (1-3) and around 25 ºC at site 4 (Fig. 3a). Maximum daily temperatures reached the experimental TDT thermal thresholds in warm summer days (i.e. for thermal values higher than the quantile 75th, Fig 3a). Next, we examined whether host plant microhabitat buffering effects at the four sites could allow reduced maximum temperature values. Analysis of host plant microsite climatic data (2014-2015) revealed strong buffering effects only at mid elevation populations (-5.2±0.17 ºC), and only for those plants located at closed microsites (Fig. 3b, green rectangles; Table S16). In contrast, in low elevation sites we observed limited cooling effects of canopy cover at closed sites (-0.79±0.32 ºC, Fig. 3b, Table S16). Open microsites were characterised by amplified mean maximum June temperatures (1.9±0.18 ºC in lowland sites and 3.04±0.28 ºC in mid altitude sites; orange rectangles in Fig. 3b). The analysis of daily cycles of temperature variation showed that temperatures of warm summer days reached values higher than the experimental TDT thresholds for several hours in open sites (Fig. 3c). In contrast, these range of thermal values were not achieved in closed microsites of mid elevation sites (most values < 30 ºC, Fig. 3d). Overall, we conclude that significantly different thermal buffering effects were observed at lowland and mid elevation sites, in line with the previously reported trends for butterfly demographic declines and for phenotypic biomarkers (wing size responses).

Host plant resource dynamics

The analysis of the seasonal patterns of host plant availability and quality (C/N ratio) revealed important differences between mid elevation and lowland sites. Mid elevation sites were characterised by a continuous availability of fresh *Alliaria petiolata* leaves during the whole summer period and, consequently, by more stable temporal C/N ratios (Fig. 4a). In contrast, at lowland sites, the leaves of the two host plants *Lepidium draba* and *Brassica nigra* presented a significant linear increase in the C/N ratio (indicating a progressive reduction of host plant quality with the advance of summer and plant phenology). This trend culminated in total leaf senescence at the end of June – early July (Fig. 4b).

Figure 3. Comparison of June maximum temperature measurements (i.e. mean of the daily maximum temperatures during June) at standardised meteorological stations and at host plant microsites for 2014-2015. a) Meteorological data. The line within the box represents the median. The ends of the box represent the 25th and 75th quantiles. The lines that extend from the box indicate the following distances: 25th quantile - 1.5*(interquartile range) and 75th quantile + 1.5*(interquartile range). The plane yellow dotted line indicates a thermal threshold of 32.5 ºC calculated from the TDT relationship, corresponding to a time of thermal exposure equivalent to whole larval period (LP, see Fig. S11). The orange dotted line indicates a 34.5 ºC threshold, corresponding to the TDT for 6 hours of daily exposure to maximum temperatures over the larval period (TE6h). The red dotted line indicates the experimental threshold for thermal avoidance behaviour (TAB) of 37.5 ºC. Different capital letters indicate significantly different means (Tukey-Kramer test). The grey surface area illustrates the logarithmic decrease of the thermal death time with linearly increasing temperatures. b) Temperature-humidity host plant sensor data. Green rectangles indicate the observed habitat buffering effect in Celsius degrees at host plant microsites relative to standardized meteorological records. Orange rectangles indicate the observed thermal amplification of host plant microsites relative to standardized meteorological records. c and d) Observed daily variation of June temperatures at two host plant microsites characterised by contrasting buffering trends (c, open microsite, lowland site; d, closed microsite, mid elevation site). A spline fit (black line) indicates the mean trend observed. A smooth surface illustrating the density of data points is provided. Red contour lines indicate maximum point density. The contour lines are quantile contours in 5% intervals (i.e. 5% of the temperature measurements are below the lowest (blue) contour, 10% are below the next contour. The highest (red) contour has about 95% of the thermal values below it).



Therefore, in contrast to mid elevation sites, summer drought at the lowland sites produced a relatively large period (45-65 days) in which we observed a total absence of fresh leaves due to the complete senescence of the aboveground organs (leaves and shoots, corresponding to Julian days 190 (July the 9th) - 235 (August the 23rd) in Fig. 4b). In agreement with these observations, a significant reduction of leaf conductance values was observed during summer drought (Fig. 5a). The observed values were below 0.2 mol/m^2 s (*Brassica nigra*: 0.176±0.034 mol/m^2 s; *Lepidium draba:* 0.137±0.021 mol/m^2 s). These values matched the range of conductance values reported in water stress experiments for *Brassica* species in stressful conditions (Anissa *et al*., 2013; Guo *et al*., 2015). Complementary results for photosynthetic rates (*A*) and sub-stomatal CO_2 concentrations (c_i) for *Lepidium draba* are reported in Fig. S12.

In accordance with these trends, significantly higher temperatures at the leaf surface in relation to air temperature were recorded during the peak of summer drought (Tukey-Kramer test, *L. draba*, R^2=0.64, p<0.0001; *B. nigra*, R^2=0.56, p<0.0001, Fig. 4c and d). Similarly, with the onset of summer season, midday leaf temperatures significantly increased (*L. draba* temperature increase: $T_{June} - T_{May} = 14.5$ ºC; *B. nigra*, $T_{June} - T_{May} = 14.4$ ºC; Tukey-Kramer test, $p<0.0001$). As a result, midday leaf temperatures in June reached 37.56±0.35 ºC in *Brassica nigra* and 38.16±0.52 in *Lepidium draba* (Fig. 5c and d). In summary, during the peak of summer drought the results for lowland plants indicated significant reductions on leaf quality (increased C/N ratios), significantly reduced conductance values ($g_s < 0.2$ mol/m^2 s) and significantly increased leaf surface temperatures.

Figure 4. Observed annual variation of leaf host plant quality (C/N content ratio). Higher C/N ratio corresponds to lower host plant quality. a) Observed trends at mid elevation site 3. b) Low elevation sites 1 and 2. The grey surface area illustrates the summer drought period.

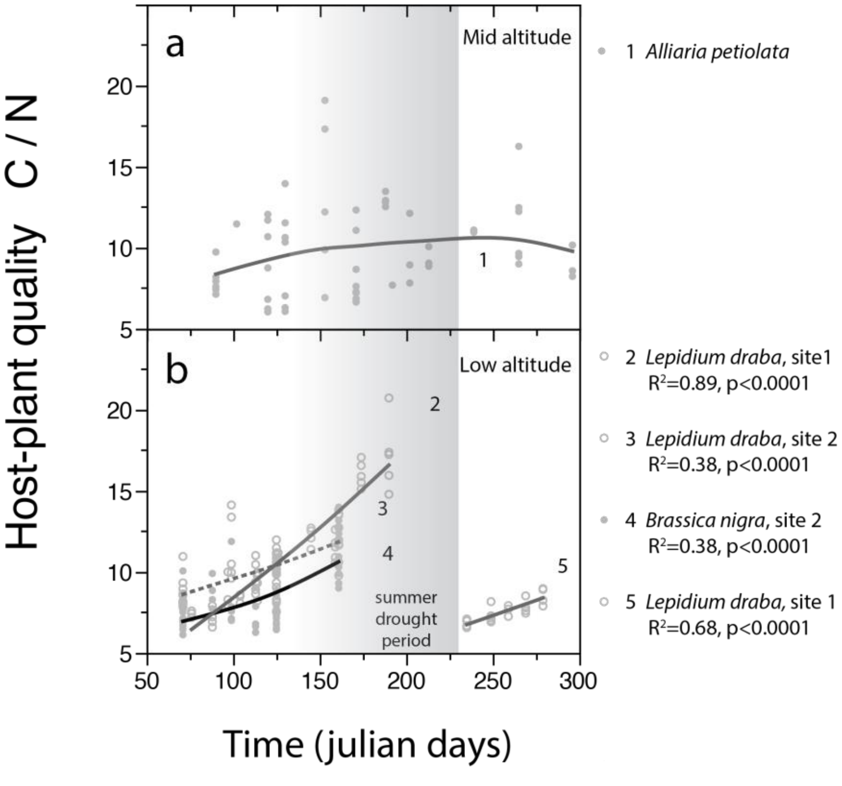
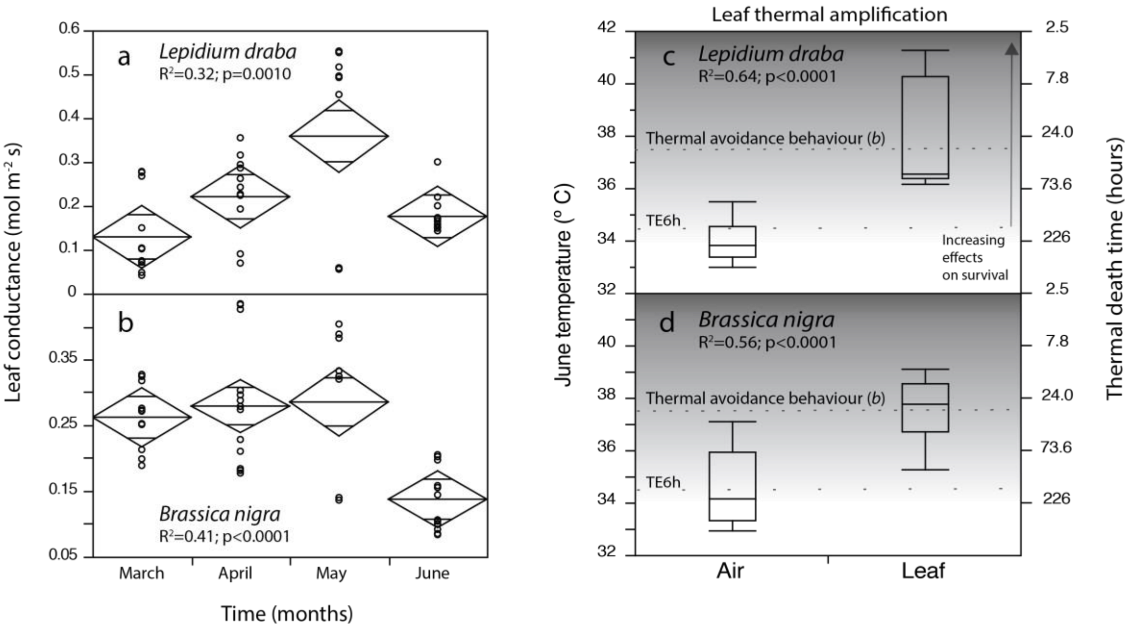


Figure 5. Observed monthly variation of leaf conductance for lowland plants *Lepidium draba* (a) and *Brassica nigra* (b). The line across each diamond represents the mean. The vertical span of each diamond represents the 95% confidence interval. Leaf conductance is linked to plant transpiration and leaf energy balance, and hence to the ability of the plant to cool itself under heat stress. c and d) Observed midday June temperatures of the leaf surface of low elevation host plants *Lepidium draba* (upper panel) and *Brassica nigra* (lower panel). Temperatures were significantly higher than air temperatures synchronously recorded using LICOR 6400 portable photosynthesis system (indicated as "*Air*" in the panels). Expected thermal death time for *Pieris napi* is provided in the right axis for the amplified leaf surfaces temperatures. The line within the box represents the median sample value. The ends of the box represent the 25th and 75th quantiles. The grey surface area illustrates the logarithmic decrease of the thermal death time with linearly increasing temperatures.



Discussion

Our results indicate that a decadal trend of increased summer drought has triggered long-term declines of *P. napi* populations at lowland sites (Fig. 1). In contrast, the analysis of microclimatic conditions experienced by mid elevation populations suggests a key role for habitat buffering processes in these sites. Mid elevation populations presented significantly stronger thermal buffering effects in closed habitat microsites (a six fold increase), and as a result individuals were characterised by comparatively larger butterfly wing sizes than butterflies from lowland populations exposed to similar conditions of environmental thermal stress without access to thermal buffering micro-refugia (Figs 2 & 3). Moreover, a continuous availability of high quality leaf resources with low C/N ratios was observed only at mid elevation sites, in non-declining populations (Fig. 4). In contrast, lowland host plants showed a progressive seasonal reduction of leaf nitrogen content, possibly associated to the seasonal advance of the flowering and fruiting phenological cycle (Fig. S13). In line with this finding, previous studies have documented a decrease in crude protein and digestible fibre after flowering in *Lepidium draba* (Jacobs, 2007). Finally, consistent effects of summer drought were observed in the leaf conductance of *Lepidium draba* and *Brassica nigra*, the two lowland host plants, resulting in turn in significantly increased leaf surface temperature. In addition to these combined drought and leaf heat stress impacts, lowland populations also experience periods of host plant resource scarcity (during late July-August), which are in turn associated with a decrease in abundance due to pupal aestivation (Fig. 4). Overall, our results suggest that butterfly population vulnerability to long-term drought periods is associated to multiple co-occurring and interrelated ecological factors, including limited vegetation thermal buffering effects at lowland sites, significant drought impacts on host plant transpiration and amplified leaf surface temperature, as well as reduced leaf quality linked to the seasonal advance of plant phenology.

June maximum daily temperature values recorded by host plant thermal sensors at open lowland microsites ranged from 22 to 42 ºC (Fig. 3a-d). Experimental studies in *Pieris* butterflies in thermally variable environments have been conducted (i.e. treatments of short-term heat stress exposure in daily cycles, mimicking natural daily variability). These treatments report strong negative effects on larval growth rates and consumption rates for temperatures above 39 ºC (Kingsolver, 2000; Kingsolver *et al*., 2006). In addition, it has been recently reported that thermal conditions above 35 ºC can significantly increase egg and young larvae mortality in other model species (Klockmann *et al*., 2016). In line with this finding, we observed a significant negative effect of reduced larval size in thermal death time responses in *Pieris napi* (F ratio = 9.67, p=0.031, Table S17), indicating a significantly increased susceptibility of younger larvae to thermal stress. In the case of *P. napi* habitats we observed that at open lowland microsites a large percentage of the maximum daily temperature records (97.5%) were below 37.5 ºC and more than 90% were below 35 ºC (i.e. most of the values were not surpassing the thermal threshold of 34.5 ºC estimated in TDT experiments for a daily exposure of 6h to maximum temperatures). Therefore, and according to the available experimental evidence in *Pieris* butterflies and other species, these thermal regimes should not necessarily impose a strong negative impact on the survival, consumption rates and growth rates of larvae if conditions of optimal host plant quality and reduced leaf drought stress were simultaneously met (Kingsolver, 2000; Kingsolver *et al*., 2006). In line with these findings, we measured reduced leaf quality and water stress conditions in host plants at open lowland microsites during the summer period. We observed that in open exposed sites amplified leaf surface temperatures and reduced transpiration could significantly increase host plant temperature (Fig 4), surpassing the experimental thresholds estimated (i.e. TDT for TE6h and TAB). Of note, previous experimental works demonstrate significant interactions in combined heat stress and altered host plant quality treatments, often resulting in stronger negative impacts on butterfly larval growth rates (e.g. Jones, 1982; Kingsolver, 2000). In addition, pupal mass has been positively associated with fitness and total lifetime egg production in the genus *Pieris*

(Jones, 1982; Wiklund & Kaitala, 1995). Consequently, direct negative impacts of body size reductions on population demography should not be discarded.

Our results also documented summer drought impacts on host plant ecophysiology. The observed reductions of leaf conductances at the peak of summer drought (values < 0.2 mol/m^2 s) are in line with the quantitative values reported for *Brassica rapa* in comprehensive water stress experimental treatments (Fig 5; Anissa *et al*., 2013, Guo *et al*., 2015). Under strong drought stress, host plants are expected to progressively reduce leaf water content and transpiration. This could potentially affect butterfly population demography because leaf-water content is known to be an important factor for larval development (Soo Hoo & Fraenkel, 1966; Scriber, 1977; Slansky & Feeny, 1977). Moreover, leaf transpiration and leaf water content are key characters driving host plant selection by females in *Pieris* butterflies (Wolfson, 1980; Myers, 1985). The same is true for leaf nitrogen content, which also limits larval development and is a key trait in female host plant selection (Myers, 1985). Finally, leaf water and nitrogen content are generally positively correlated in *Brassica* host plants used by *Pieris* species (Mattson, 1980) and are also positively and significantly related to transpiration rates (Myers, 1985). On top of this, our results indicated a key role of decreased June rains on long-term population declines and in addition reported a significant reduction of leaf conductance in the transition from May to June at lowland areas (Fig. 5). Our study also highlights the potential importance of seasonal trends in leaf phenology, which in turn determine C/N content and host plant quality (Kriedeman, 1968; Kause *et al*., 1997). To our knowledge, these factors have been seldom considered as contributing factors determining butterfly population vulnerability to increased drought impacts. Overall this study identifies multiannual trends in summer drought as a primary driver of long-term demographic declines of *Pieris napi*. Crucially, nearly 70% of the butterfly species in this hotspot region for European butterflies are currently affected by significant population declines (Stefenescu *et al*., 2011ab, Melero *et al*., 2016). Landscape changes and climatic drivers have been considered as the principal candidate drivers of these widespread declines but their relative role and the ecological mechanisms implied are still poorly described for most of the species. In this context, our study clarifies the importance of summer drought as a key primary driver in *P. napi* in the studied populations and sheds some light into some of the ecological mechanisms implied (i.e. vegetation thermal buffering, phenology effects on plant quality (C/N) and changes in host plant water transpiration and content). It remains to be assessed whether these mechanisms could also apply to other populations of *P. napi* in Catalonia and to other butterfly species. In this regard, it is important to bear in mind that our analyses are restricted to abundant populations located in protected areas. The reported trends could possibly differ in lowland and mid-land populations currently affected by increased urbanisation pressures, intensified land use changes, pesticide management impacts and land abandonment (Stefanescu et al 2011ab). Moreover, the results do not describe the responses of *P. napi* populations that rely on other host plants in Catalonia (e.g. *Cardamine pratensis, Arabis glabra*). The host plant-specific mechanisms described in the paper may non-necessarily apply to these populations. Finally, our study suggests that wing and body size measures are reliable phenotypic biomarkers of the geographic variability of thermal stress exposure in the studied populations, providing an indirect indicator of limited habitat thermal buffering conditions for these specific populations. In our field and experimental datasets, the percentage of reduction of wing size per degree Celsius (as defined in Forster *et al.*, 2012) was in the range of 1-2% [regression slope for normalised experimental data: -1.56±0.25, $p<0.0001$; regression slope for normalised field data: -1.80±0.11, $p<0.0001$]. This trend is consistent with the experimental slopes reported for body size-temperature relationships in other temperate butterfly species exposed to similar experimental thermal treatments (Forster et al. 2012; see Fig. S14 for some examples). More detailed quantitative studies of the effects of thermal stress on survival and fecundity functions in this species are required to estimate the critical size values associated to negative effects on insect performance and the associated thermal threshold (P^* and T^* values, see supplementary text T1 for further discussion). Moreover, we show that complementary analyses of host plant dynamics are highly informative and necessary, due to the multiple ecological processes that seem to be co-acting and interacting (Nygrin et al., 2008, Talloen et al., 2009). In summary, this study indicates that phenotypic thermal biomarkers are informative as climatic stress indicators but should be complemented, whenever possible, by multi-trait frameworks analysing host plant ecophysiological responses and by detailed microclimatic measurements.

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