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RESEARCH ARTICLE

Larval parasitism in a specialist herbivore is explained by phenological synchrony and host plant availability

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Abstract

1. Parasitism is a key factor in the population dynamics of many herbivorous insects, although its impact on host populations varies widely, for instance, along latitudinal and altitudinal gradients. Understanding the sources of geographical variation in host-parasitoid interactions is crucial for reliably predicting the future success of the interacting species under a context of global change.
2. Here, we examine larval parasitism in the butterfly *Aglais urticae* in south-west Europe, where it is a mountain specialist. Larval nests were sampled over 2 years along altitudinal gradients in three Iberian mountain ranges, including the Sierra Nevada, home to its southernmost European population. Additional data on nettle condition and adult butterflies were obtained in the study areas.
3. These data sources were used to investigate whether or not differences in parasitism rates are related to the geographical position and phenology of the host, and to the availability of the host plants.
4. Phenological differences in the host populations between regions were related to the severity of summer drought and the corresponding differences in host plant availability. At the trailing-edge of its distribution, the butterfly's breeding season was restricted to the end of winter and spring, while in its northern Iberian range the season was prolonged until mid-summer. Although parasitism was an important source of mortality in all regions, parasitism rates and parasitoid richness were highest in the north and lowest in the south. Moreover, within a region, there was a notable increase in parasitism rates over time, which probably led to selection against an additional late summer host generation in northern regions. Conversely, the shorter breeding season in Sierra Nevada resulted in a loss of synchrony between the host and one important late season parasitoid, *Sturmia bella*, which may partly explain the high density of this butterfly species at the trailing-edge of its range.

5. Our results support the key role of host phenology in accounting for differences in parasitism rates between populations. They also provide insights into how climate through host plant availability affects host phenology and, ultimately, the impact of parasitism on host populations.

KEYWORDS

altitudinal gradient, host plant availability, host–parasitoid interactions, Mediterranean basin, nettle-feeding butterfly, parasitism rate, phenology, trailing-edge populations

1 | INTRODUCTION

Parasitoids and parasitism have long been recognized as key factors driving the population dynamics of herbivorous insects (Cornell & Hawkins, 1995; Varley et al., 1973). However, the impact of parasitism on host populations is highly variable and depends on multiple factors including the behavioural and physiological characteristics of the host (Greeney et al., 2012), the habitat structure and resources used by the host (Hansen et al., 2017; Lill et al., 2002; Singer et al., 2014), the time elapsed since the host population became established (Menéndez et al., 2008), and climatic conditions experienced by both the host and its parasitoids (Jefferies & Lewis, 2013; Stireman III et al., 2005). It is thus to be expected that the impact of parasitism will vary over a host's geographical range since these factors will combine in different ways, for instance, over latitudinal or altitudinal gradients (Santos & Quicke, 2011). Although understanding the sources of geographical variation in host–parasitoid interactions is important for reliably predicting the future success of the interacting species under a context of global change, few studies to date have addressed this topic (but see Audusseau et al., 2021; Hódar et al., 2021; Maunsell et al., 2015), probably because of the difficulty of obtaining comprehensive data from these complex natural systems.

Latitudinal and altitudinal gradients are likely to produce decoupling in host–parasitoid interactions and to affect parasitism rates due to at least two factors, namely, the difficulty parasitoids have when tracking their host's distribution (i.e. spatial tracking) and the loss of phenological synchrony between interacting pairs under different climatic conditions (i.e. temporal tracking). In both cases, a mismatch between the host and some of its parasitoids may lead to a phenomenon of 'enemy release' or 'enemy escape' (Keane & Crawley, 2002), which may benefit some host populations. The failure of parasitoids to track spatially and temporally their hosts has been documented in several systems (e.g. Hance et al., 2007), especially in range-margin populations of the host (Jefferies & Lewis, 2013; Menéndez et al., 2008) and in specialized parasitoids with generally poorer dispersal abilities than generalist species (Elzinga et al., 2007; Roy et al., 2011). Phenological asynchrony may occur due to divergences in thermal preferences between host and parasitoids (Hance et al., 2007), which are more likely under the extreme climatic conditions experienced by range-margin populations (Gröbler & Lewis, 2008; Hódar et al., 2021; Klock et al., 2003). Moreover,

a loss of temporal tracking along spatial gradients leading to lower parasitism rates may occur when the number of host generations declines under more extreme conditions given that parasitism levels in multivoltine hosts often increase in successive generations (Askew & Shaw, 1986; Stefanescu et al., 2012).

Here we address these questions by focusing on a Lepidopteran species, the small tortoiseshell, *Aglais urticae*, and its main parasitoids at its south-western Palaearctic margin, where this butterfly behaves as a mountain specialist. Although European butterflies figure among the best studied of all herbivorous insects from an ecological viewpoint (Settele et al., 2009), data on parasitism remain relatively scarce and, for many species, purely anecdotal (Shaw et al., 2009). A notable exception is the guild of nettle feeders in the Nymphalini tribe (*Aglais io*, *A. urticae*, *Vanessa atalanta*, *Araschnia levana*), which has been the subject of much research that has generated comparatively thorough knowledge of the parasitoid assemblages associated with each species (Audusseau et al., 2021; Pyörnilä, 1976a, 1976b, 1977; Rice, 2012; Shaw et al., 2009). In spite of this, the geographical patterns of parasitism have only begun to be investigated recently by Audusseau et al. (2021), who studied this question over a 500-km latitudinal gradient in Sweden, with a focus on apparent competition (i.e. how parasitoids shared between co-occurring host species influence population dynamics and abundance). Our work, however, takes a different approach by focusing on a single host and how its parasitism rate is affected by the environmental conditions experienced by increasingly marginal populations at the southern, trailing-edge of its host's distribution.

Over 2 years, we systematically recorded the condition of nettles and sampled larval nests of the small tortoiseshell along a latitudinal gradient comprising three Iberian mountain ranges, including the Sierra Nevada, where the southernmost European population of this species is found. This allowed us to identify the parasitoid complexes associated with each butterfly population and to use this study system to investigate whether or not differences in parasitism rates could be related to the geographical situation of host populations and to various environmental conditions, including the quality of host plants.

We specifically tested (a) whether or not the southernmost and highly isolated host population has an impoverished parasitoid community due to the inability of some parasitoid species to track host distribution or to maintain populations under extreme climatic conditions; (b) whether or not environmental conditions over the

latitudinal gradient impose phenological constraints in some of the host populations (e.g. a reduction in the number of generations) leading to phenological decoupling and to differences in parasitism rate; and (c) to what extent host plant condition could cascade upwards and affect the host and its associated parasitoid populations.

2 | MATERIALS AND METHODS

2.1 | The small tortoiseshell as a model study

The small tortoiseshell, *Aglais urticae*, is a specialist butterfly that feeds on nettles, *Urtica dioica*, in its larval stage. It is widely distributed throughout the Palaearctic and Europe, although towards the southern limits of its range it is restricted to high mountains. In the Iberian Peninsula, it is widely distributed in the northern mountain ranges (Pyrenees and the Cantabrian range), but it is much more local in the central mountains of the Sistema Central (e.g. Sierra de Guadarrama) and the Sistema Ibérico (García-Barros et al., 2013). It reaches the southernmost limit of its western-European range in Sierra Nevada in Andalusia (Figure 1).

The phenology of this butterfly varies throughout its range (Dennis, 1985). Although many European populations are typically bivoltine (e.g. Asher et al., 2001; Audusseau et al., 2021), up to three generations have been recorded under favourable conditions (Dennis, 1985); conversely, strictly univoltine populations seem to occur in some northern areas (e.g. in parts of Scotland; Pullin, 1988). The adult overwinters and, in late winter and early spring, the butterflies that have remained in diapause—typically, in dark crevices, hollow trees or old buildings (Thomas & Lewington, 2016; Wiklund et al., 2008)—become active and mate. Subsequently, females lay egg

clusters on nettles and a first generation of adults is produced in late spring. Depending on the population and weather conditions, a second (or even a third) generation is then produced over the summer.

Females lay eggs on the underside of nettle leaves in clusters of about 200–300. Larvae live in communal silken webs on the host plant until the fifth and final instar, when they become solitary. Pupae are suspended more or less aerially in the leaf litter, under rocks or, more rarely, directly on the host plant. Both larvae and pupae are commonly attacked by hymenopteran and dipteran parasitoids, which can inflict important losses on populations (Audusseau et al., 2021; Rice, 2012; Shaw et al., 2009). On the other hand, egg parasitism seems to be negligible (Pyörnilä, 1976a, 1976b). Parasitoids include both specialists (that are usually shared with other Nymphalini species feeding on *Urtica*; Rice, 2012; Audusseau et al., 2021) and generalists with wide host repertoires (Shaw et al., 2009).

2.2 | Study system

We studied the complex of larval parasitoids of *A. urticae* in three regions encompassing the whole of its latitudinal range in the Iberian Peninsula (Figure 1): the Pyrenees in the north of the Iberian Peninsula, the Sierra de Guadarrama in central Spain and Sierra Nevada in southern Spain (see Supplementary methods for details of these areas). Sampling sites were established along an altitudinal gradient in each region that covered most of the altitudinal range in which this species breeds. Nine sites were sampled for parasitoids in the Pyrenees at 1,127–2,560 m a.s.l., seven sites in the Sierra de Guadarrama at 1,150–2,004 m a.s.l. and six sites in Sierra Nevada at 975–2,532 m a.s.l. As part of a larger project aimed at investigating various aspects of the ecology of *A. urticae*, additional sites were

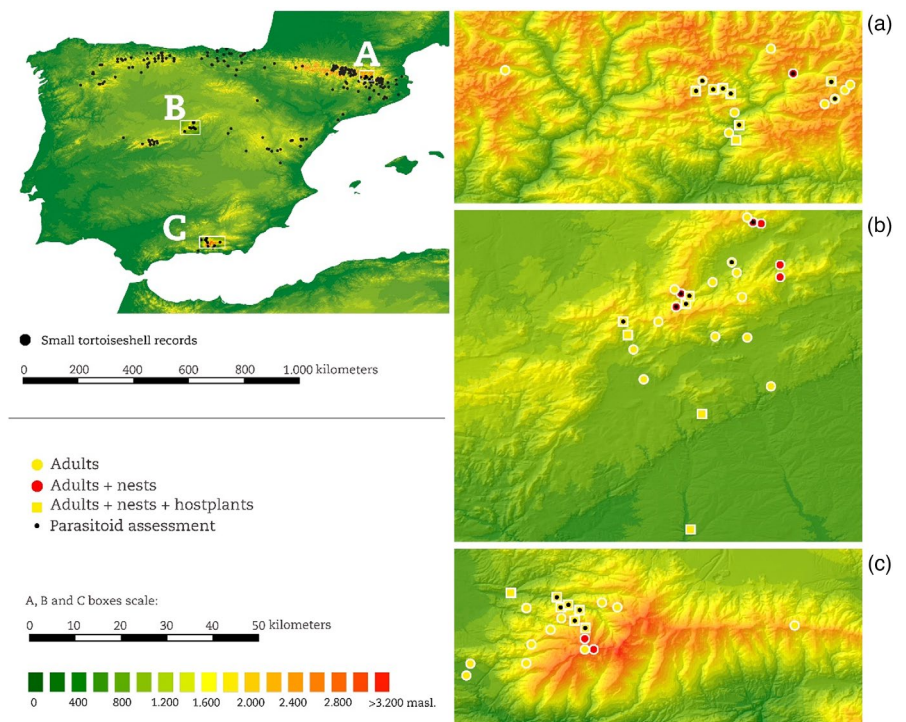


FIGURE 1 General distribution of *Aglais urticae* in the Iberian Peninsula (records from García-Barros et al. (2004), GBIF and ornitho.Cat), and site distribution in the three studied areas (a: Andorra, Pyrenees; b: Sierra de Guadarrama; c: Sierra Nevada). Symbol colours show different combinations for the sampling of adults, larval nests and host plants. GBIF.org (16 August 2018) GBIF Occurrence Download <https://doi.org/10.15468/dl.uw8mbd>

surveyed in each region. The information gathered at these additional sites was used in this work to improve knowledge of the phenology of this butterfly (Figure 1; see below).

No ethical approval was required for this work.

2.3 | Field sampling

To study the phenology of *A. urticae* adults, we used 500-m transects on which butterflies were counted every 2 weeks from March to September (a total of 15 sampling visits), following the standard methodology of the Pollard walks (Pollard & Yates, 1993). Butterflies were classified either as overwintered or freshly emerged based on wing colouration (i.e. dull or brightly coloured, respectively). We based our classification on previous work on a related butterfly species and considered as freshly emerged butterflies those corresponding to the first two categories of a categorical system of five (Stefanescu et al., 2016, 2021). Transects were walked at 15, 24 and 14 sites in 2016, and at 16, 24 and 20 sites in 2017, in the Pyrenees, Sierra de Guadarrama and Sierra Nevada, respectively (Figure 1).

To study the phenology of *A. urticae* larvae, we counted all larval nests found in four (exceptionally, just two and three at two sites) focal *U. dioica* patches in a subsample of the sites used for adult counts in each region (Figure 1). The focal patches were randomly selected along the butterfly transects and, if not available, at other accessible sites that were as close as possible to the transect route. The focal patches were visited every 2 weeks from March to September, whenever possible during the same visits as for the adult transect counts.

To study larval parasitism, larval nests detected at focal *U. dioica* patches (see above) were marked and, if larvae were in the third or later instars, 20 individuals were collected to assess parasitism. Because the total number of larvae per nest was sometimes less than 20, the overall average number of larvae per sample (\pm SD) was 16.2 ± 6.9 . Moreover, given that the opportunistic parasitoid, *Cotesia vestalis*, is known to parasitize first instar larvae of the small tortoiseshell and to emerge mainly from the second instar (Audusseau et al., 2021), in 2017 we also collected eight samples of five second instar larvae in all three regions (three samples in the Pyrenees, two in the Sierra de Guadarrama and three in Sierra Nevada). We did not assess pupal parasitism, even if it may be important (Pyörnilä, 1977; Shaw et al., 2009) because pupae are difficult to locate in the field, thereby precluding any reliable estimates of mortality.

Larvae were reared indoors in transparent plastic containers (155 × 105 × 45 mm) in groups of up to five individuals, all from the same sample. To avoid possible contamination, larvae were always reared with nettle leaves collected from their original nettle patch; if not available, nettles were harvested from sites where *A. urticae* and its closest congener, *A. io*, were absent, since some common parasitoids (e.g. the tachinids *Sturmia bella* and *Pales pavidus*) lay microtype eggs on nettle leaves that can infect caterpillars if they eat these leaves. When a larva or pupa (in the case of larva–pupal parasitoids) produced a parasitoid, we recorded the stage at which the host was

killed and kept the parasitoid individually in a vial until the adult emerged. Adults were preserved in pure ethanol for identification (Ichneumonidea by M.R.S., Tachinidae by D.H.). Although hatching success was generally poor for most tachinids, careful inspection of puparia allowed for correct identification in almost all cases.

To examine host plant availability, we recorded the growing condition (i.e. quality level and height) of nettles over the season at a subsample of sites used for larval nest counts in each region. At each visit, two stems were randomly selected from each nettle patch. Their height was measured (in cm) and they were given a categorical value from 1 (worst quality) to 4 (best quality) in which (1) corresponds to already dry or withered plants, with senescent leaves; (2) to flowering plants and plants with green but not fresh leaves; (3) to old plants in which regrowth leaves were beginning to become visible (a common situation at the end of summer after rain or after herbivory); and (4) to vigorous plants, with fresh leaves. Category ranking was based on previous work showing how nettles in these various phenological stages differently affect larval growth rates, pupal and adult weights in the small tortoiseshell (Pullin, 1987) and the map butterfly, *Araschnia levana* (Mevi-Schütz & Erhardt, 2005).

2.4 | Analyses

2.4.1 | Host phenology

A combination of the standardized adult and larval count data was used to define the phenology of the species. GAM models were fitted to the adult (overwintering and fresh butterflies separately) and larval nest counts, which allowed us to extract the Julian day corresponding to each peak of abundance in a given region and season. GAM models were built using the package `MGCV` in R (Wood, 2011). In these models, we used pooled data from 2016 and 2017 to increase the sample size and to improve the overall phenological picture in each of the study regions.

To investigate the potential altitudinal delay in larval phenology, we regressed separately the timing of larval nest appearance against site altitude for each year and region. The timing of larval nest appearance was summarized as the weighted mean appearance date (hereafter, mean date; Equation 1). This statistic is widely used in studies of butterfly phenology and has been shown to be more reliable than other phenological estimators based on first appearance date (Moussus et al., 2010).

$$\text{Mean date} = \frac{(\text{number of larval nests per visit} \times \text{date})}{\text{annual total number of larval nests}} \quad (1)$$

2.4.2 | Host plant phenology

We tested for differences in the phenology of nettles between regions using GAMM models, in which either nettle condition or height were the response variables and altitude, region, year, visit number (i.e. the timing of the season, used as the smoothing term) and the

interactions of region with both visit number and altitude were the predictors. In these models, each individual stem was used as a data point and 'nettle patch' was entered as a random factor.

2.4.3 | Impact of parasitism on host populations

To test whether or not the number of parasitoid species was comparable between regions (because sample sizes differed greatly between regions, see below), we computed the most common non-parametric estimators of species richness for each region and year separately (based on all recorded parasitoid species and genera) using the SPADER package in R (Chao & Chiu, 2016).

To assess the impact of parasitoids on host populations, we calculated the parasitism rate for each larval nest as the number of larvae killed as a result of parasitism, after discounting those that died for unknown reasons (i.e. our calculations were always based on effective larval samples). To avoid biases resulting from low sample sizes, the parasitism rate was calculated for effective samples of ≥ 5 larvae. We obtained very similar results (not shown) when models of parasitism rate were built following a more restrictive criterion of effective samples of ≥ 10 larva.

The parasitism rate was modelled with generalized linear models (GLMs) using a binomial distribution and logit link function, with region, Julian day (date of nest collection) and altitude as predictors. However, because altitude and Julian day were highly correlated ($r = 0.76$), only models with just one of these two variables were retained in the end. Models were built separately for 2016 and 2017 because the sampling sites in Andorra differed slightly between the 2 years. All possible models were built using the package LME4 in R (Bates et al., 2015); the best models were selected using the MuMIn package in R (Barton, 2015), with model selection being based on the Akaike Information Criterion (AIC). Models that differed by < 2 points from the lowest AIC ($\Delta AIC < 2$) were considered the top-ranked models (statistically equivalent to the best model of the set).

2.4.4 | Phenological overlap between the host and its main parasitoids

The overlap (i.e. temporal co-occurrence) between the host and its two main parasitoids, *Pelatachina tibialis* and *Sturmia bella*, was estimated using the Overlap Parasitoid-Host index (OPH), as described by Audusseau et al. (2020). This index was calculated for all possible combinations of site and year in each region.

The OPH (Equation 2) is bounded from zero (i.e. no overlap) to 1 (i.e. complete overlap), and is calculated as the sum over the sampling events of the minimum between the standardized abundance values of the parasitoid (either *P. tibialis* or *S. bella*) and the host. Standardized abundance data for the parasitoid (Equation 3) refer to the number of individuals (NP) collected for a given sampling fortnight k at site j and year i , expressed as the proportion of the total number of individuals of that species collected at that site and year.

For the butterfly host, standardized abundance data (Equation 4) refer to the number of nests (NH) collected for a given sampling week k at site j and for year i , expressed as the proportion of the total number of nests collected at that site and year.

$$OPH_{ij} = \sum_{k=1}^n \min(P_{ij,k}, H_{ij,k}), \quad (2)$$

$$P_{ij,k} = \frac{NP_{ij,k}}{\sum NP_{ij}}, \quad (3)$$

$$H_{ij,k} = \frac{NH_{ij,k}}{\sum NH_{ij}}. \quad (4)$$

The maximum value of 1 is obtained in the hypothetical case when all individuals recorded in a given season, both of the parasitoid and the host, are concentrated in the same sampling event k . In this case, the standardized value of abundance (i.e. the proportion of individuals in this sample to all individuals) is one for the parasitoid and one for the host, and is also the minimum common value that appears in the only term in Equation 2 other than zero. The opposite situation occurs when in all available samples one of the interacting species is always missing. In this case, the standardized minimum value of abundance is zero in any of the terms in Equation 2.

To understand which factors explain the degree of overlap between the parasitoid and the host, we used GLM models with a binomial distribution and a logit-link function. For each parasitoid species and year, we applied a model in which OPH was the dependent variable and site altitude and region were the variable predictors.

All models were run in R (R Core Team, 2018).

3 | RESULTS

3.1 | Host phenology

There were considerable differences in the phenology of the small tortoiseshell between the three regions (Figure 2). In the Pyrenees, the life cycle was essentially bivoltine, as evidenced by two distinct peaks of larval nests and fresh adults (Figure 2a). Overwintered adults were seen from mid-March to early June, being most abundant in mid-May. A first peak of larval nests was recorded in late April, and was followed by a strong first generation of butterflies in June. Larval nests became very abundant 1 month later, probably produced by a mixture of the offspring of the first generation and of the late overwintered butterflies. Although the emergence of a second generation was evident in late July and early August, it was numerically much less important than the first generation, which suggests low larval survival rates. Butterflies from this second generation did not reproduce, as deduced from the complete absence of larval nests in August and September. Instead, they entered into diapause and were completely absent from the late summer counts.

The phenology in the Sierra de Guadarrama is more difficult to interpret, partly because of the generally lower population

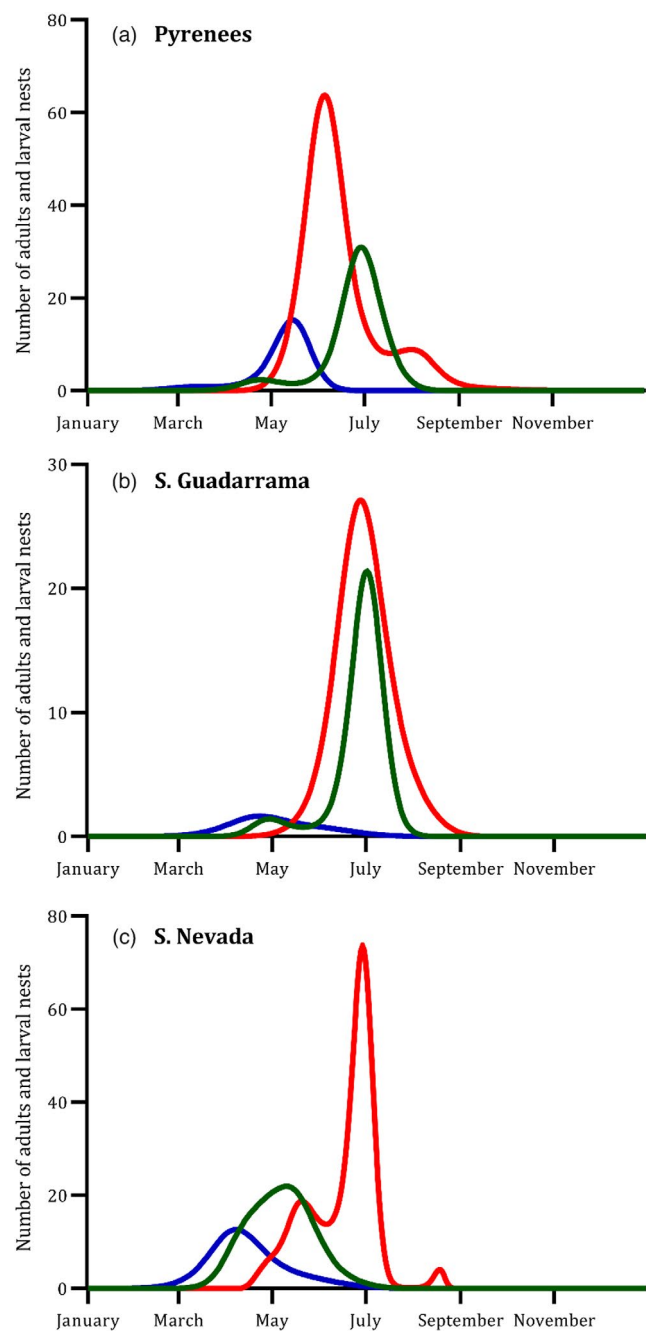


FIGURE 2 Phenology of the small tortoiseshell in the three studied regions in 2016–2017: (a) Pyrenees (Andorra); (b) Sierra de Guadarrama (Central Spain); (c) Sierra Nevada (SE Spain). Blue line: Overwintering adults; green line: Larval nests; red line: Fresh adults

levels in this central region (note the smaller values on the Y-axis in Figure 2b). Wintering adults and a small first peak of larval nests were recorded in low numbers in late April and early May. Then, a single strong peak of fresh adults was recorded 2 months later at the end of June and in early July. Quite surprisingly, the timing of the emergence of this presumed first generation almost completely overlapped with a second and much more prominent peak of larval nests (Figure 2b). The great overlap between the larval and adult curves is puzzling and suggests that larvae that

should produce a second generation have such low survival rates that adults of this hypothetical second generation are very rare and that their flight periods completely overlap those of the first generation.

In Sierra Nevada, the southernmost region, overwintered adults were recorded from the beginning of March—or even earlier—to mid-June (with a peak in early April); larval nests showed a unimodal curve from late March to late June, with a prolonged smooth peak centred on May (Figure 2c). Fresh adults had two distinct peaks, the first in mid-May and the second in late June and early July (Figure 2c), suggesting a bivoltine cycle with two generations. However, when models were fitted separately to each year, only a single peak of fresh adults was observed in 2017 (Figure S1), raising the possibility that variable voltinism occurs (i.e. one or two annual generations) depending on the conditions of that year. In this southern region, butterflies disappeared rapidly from the transect counts early in the summer and were only recorded occasionally after the first week of July. This suggests that butterflies entered into diapause soon after their summer emergence, a fact corroborated by the complete absence of larval nests from the beginning of July onwards. Given the lack of larval nests in the preceding weeks, the small peak of fresh butterflies recorded in mid-August after about 1 month and a half with almost no adult sightings (Figure 2c) most probably indicates that some adults broke their diapause for a short period rather than the occurrence of a small partial additional generation.

The distribution of larval nests indicates a trend towards breeding sites at greater altitudes as the season progresses, which was significant in the Pyrenees (F -test = 21.83, p = 0.0095 in 2016; F -test = 16.45, p = 0.01 in 2017) and in Sierra Nevada (F -test = 31.83, p = 0.0049 in 2016; F -test = 18.82, p = 0.0074 in 2017) (Figure 3). In these regions, the first larval nests found in early spring were located at around 1,000 m a.s.l. at the lower end of the studied altitudinal range. Conversely, late in the season, larval nests were found towards the upper altitudinal end, at 2,300–2,600 m a.s.l. For most of the remaining breeding season, larval nests were found in both regions at intermediate altitudes of 1,400–2,000 m a.s.l. In the Sierra de Guadarrama, this altitudinal breeding trend was significant in 2016 (F -test = 8.859, p = 0.03) but not in 2017 (F -test = 0.459, p = 0.55; Figure 3). However, in this second year, larval nests were found only in a very small altitudinal range of 242 m (1,766–2,008 m a.s.l.), which thus strongly limited the power of the analysis.

3.2 | Host plant phenology

The phenology and growing condition of the nettles varied greatly across the regions. First, the quality of the nettles assessed by our categorical system differed between regions depending on the timing of the season (Figure 4a–c). In the Pyrenees, high-quality nettles for larval development (i.e. stems with values equal or greater than 3; Pullin, 1987) were available from early

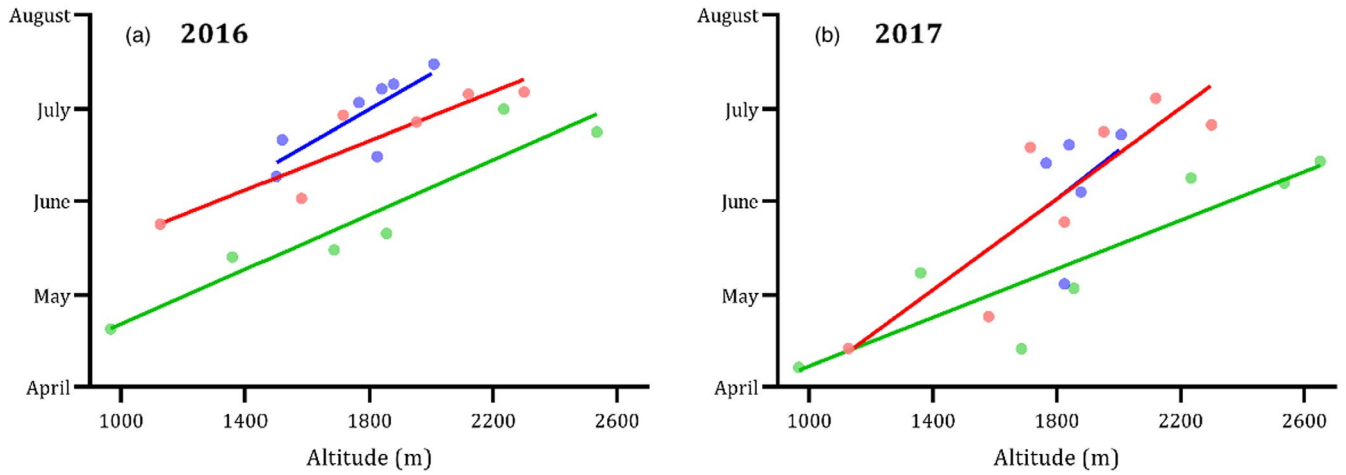


FIGURE 3 Mean date on which larval nests were recorded along the altitudinal gradient in the three study regions in (a) 2016 and (b) 2017. Green circles: Sierra Nevada; blue circles: Sierra de Guadarrama; red circles: Pyrenees. Continuous lines indicate significant linear trends

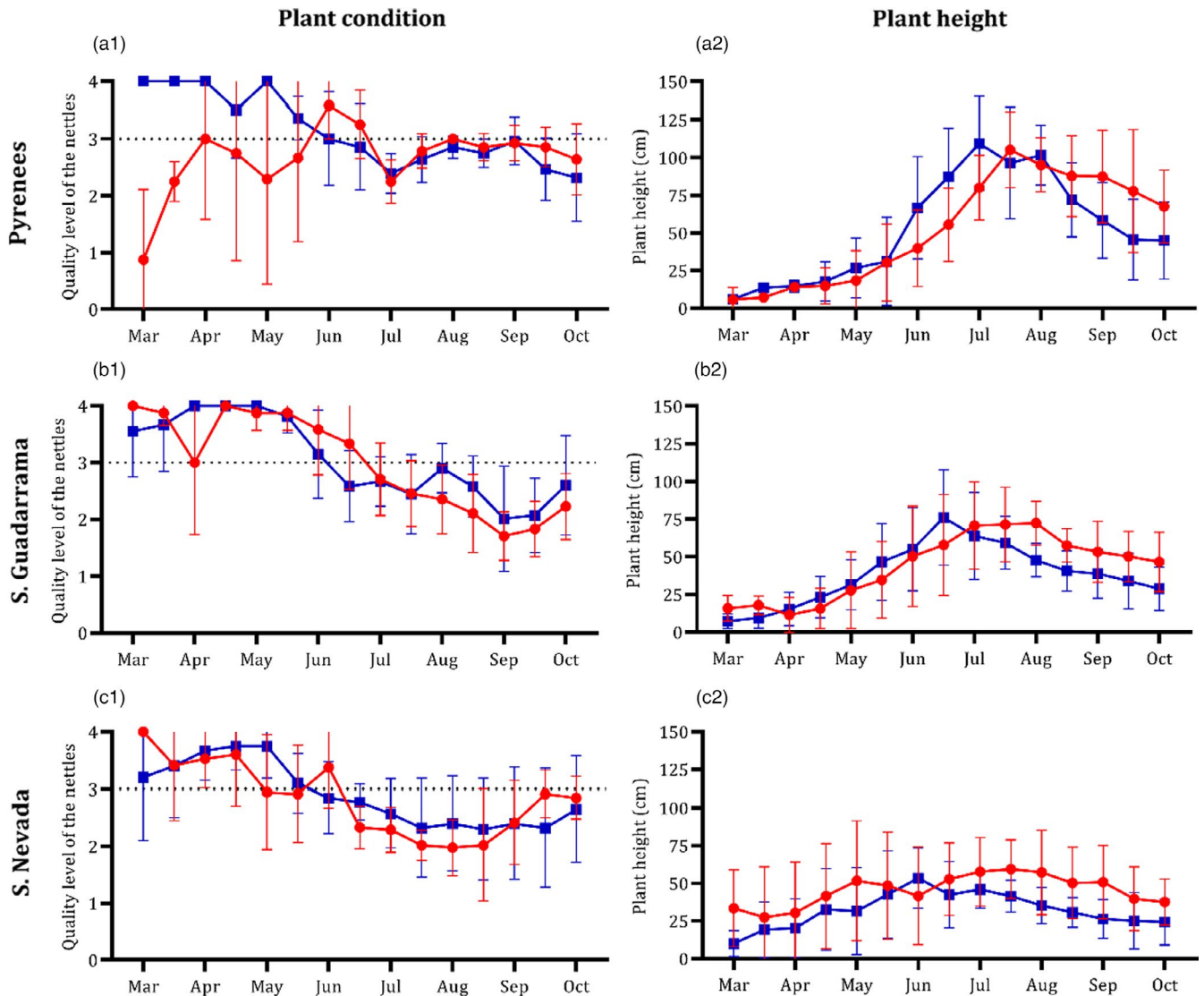


FIGURE 4 Phenology of nettles as measured by (1) plant condition on a categorical scale from 1 (least quality) to 4 (best quality); and (2) height. (a) Pyrenees; (b) Sierra de Guadarrama; (c) Sierra Nevada. Red line: 2016; blue line: 2017. Bar errors show the standard deviation between sites

March (2017) or early April (2016) to early October, except for a short period around the end of June (Figure 4a). In summer, they mostly consisted of regrowth shoots that could still support larval development. By contrast, in Sierra Nevada, there was a steady decrease in nettle quality from May onwards, with a long period of almost 3 months (June–August) when all plants were in poor condition for larval development (Figure 4c). The situation in the Sierra de Guadarrama was intermediate between that of the other two regions. Nettles were in best condition from the beginning of the season to the end of June, after which they withered quickly and remained of low quality for most of the rest of the season (Figure 4b).

Second, there was a clear latitudinal gradient in nettle height, with plants increasing in height with latitude (Figure 4a–c). As well, the growing season was earlier in the southern region than in the other two regions. Thus, plants were taller in Sierra Nevada than in the Pyrenees and the Sierra de Guadarrama until the end of May, but beyond this point in the season the reverse was true and, above all in the Pyrenees, plants were eventually almost double the size of those in Sierra Nevada.

The above general patterns are actually more complex if studied at site level (Figure S2). Thus, the average values shown in Figure 4 may give a false impression of a widespread availability of plants at particular times in the season, which in fact only occurred at some of the study sites. Nevertheless, regional differences were fully confirmed by our phenological models (Table S1). Nettle condition was significantly affected by all predictors except for year, although the effect was especially strong for the timing of the season (i.e. visit number) and the interaction between the region and the timing of the season. The model for plant height

showed a significant effect for the same factors but also for the 'year' factor, with plants being c. 30% taller in 2017 than in 2016 in the Pyrenees and the Sierra de Guadarrama (but not in Sierra Nevada, where there were no differences between years). Plant height was strongly affected by the timing of the season (Figure 4a–c.2) and, secondarily, by altitude (with plants decreasing in size at higher altitudes).

3.3 | Parasitoid complexes

In the 2 years of study, we found 201 larval nests at the sampling sites and collected 2991 larvae to assess parasitism (Table S2). One-third (33.1%) of the larvae died for unknown reasons during the rearing process, so the effective sample for assessing parasitism was reduced to 2002 larvae. Parasitism was an important source of mortality in both years, with 32.6% and 39.4% of the effective larval sample being killed by parasitoids in 2016 and 2017, respectively (Table S2).

Overall, 12 species of parasitoids were obtained, with more Tachinidae than Hymenoptera, both in terms of species richness (9 vs. 3 species) and, above all, host larvae parasitized (97.4% vs. 2.6% of the 726 larvae that died from parasitism) (Table 1).

Some differences in the parasitoid complexes between regions were noted (Figure 5; Table 1). Parasitoid richness was higher in Pyrenean (9 species) than in the southern and central Spanish host populations (7 and 6 species, respectively). This difference was confirmed with a nonparametric estimation of species richness accounting for differences in the sample size between regions. Different estimators all suggest that the number of parasitoid species in the

TABLE 1 The parasitoid species and number of small tortoiseshell larvae from which they were reared in the three studied regions in 2016 and 2017. Note that the total number of larvae ($n = 766$) does not coincide with the number provided in Table 1 ($n = 726$) because some of the larvae were parasitized by more than one species of parasitoid (i.e. multiparasitism)

Parasitoid species	Order	Pyrenees		Sierra de Guadarrama		Sierra Nevada	
		2016	2017	2016	2017	2016	2017
<i>Compsilura concinnata</i>	Diptera		2		18	1	2
<i>Exorista segregata</i>	Diptera	43	8			1	
<i>Masicera sphingivora</i>	Diptera	1				8	7
<i>Pales pavidus</i>	Diptera	2	18	2	2		
<i>Pelatachina tibialis</i>	Diptera	112	169		9	11	16
<i>Phryxe</i> sp.	Diptera	1					
<i>Sturmia bella</i>	Diptera	111	154		43		
Tachinidae	Diptera				2	3	
<i>Voria ruralis</i>	Diptera	1					
<i>Cotesia vanessae</i>	Hymenoptera	2	7	2	1		
<i>Cotesia vestalis</i>	Hymenoptera					2	
<i>Phobocampe confusa</i>	Hymenoptera					3	2
Total larvae parasitized/ Effective sample		273/517	358/603	4/117	75/207	29/283	27/275

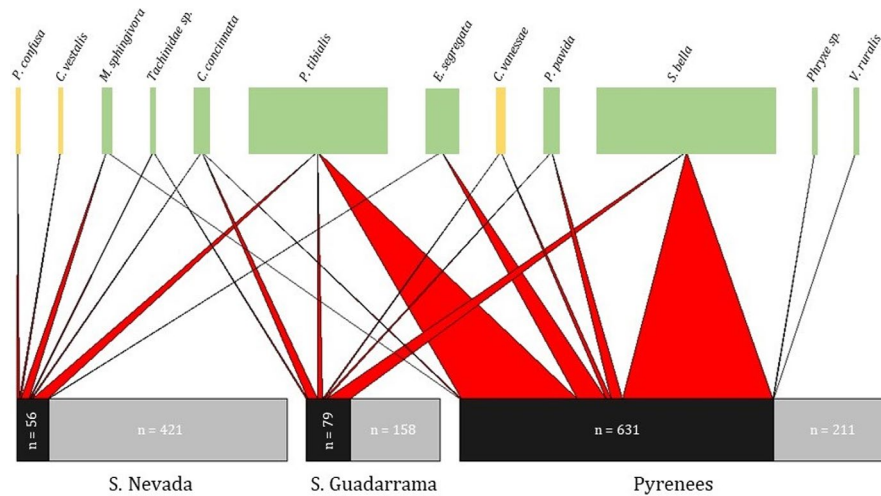


FIGURE 5 Bipartite plots showing quantitative associations between larvae of the small tortoiseshell and its parasitoids (orange: Hymenoptera, Ichneumonidae and Braconidae; green: Diptera Tachinidae) in three regions along a latitudinal gradient in the Iberian Peninsula (from south to north, Sierra Nevada, Sierra de Guadarrama and the Pyrenees). The bottom boxes represent, for each region, the number of larvae that were parasitized (black rectangle) and the number of larvae that developed into adults (grey rectangle). For each species of parasitoid, the size of the box is proportional to its abundance in the samples (i.e. the number of larvae that were parasitized). The BIPARTITE package in R version 3.6.3. (Dormann et al., 2009) was used to represent the parasitoid complexes in each region

Pyrenees is indeed twice or more than in Sierra Nevada (Table S3). On the other hand, when using these estimators, species richness was only slightly higher in the Sierra de Guadarrama than in Sierra Nevada.

The tachinid *Sturmia bella* was the parasitoid causing the higher larval mortality on Pyrenean and central Spanish host populations, but was totally absent from Sierra Nevada. Another tachinid, *Pelatachina tibialis*, was recorded as a regular parasitoid in all three regions, with a serious impact especially in the Pyrenees and Sierra Nevada. The ichneumonid, *Phobocampe confusa*, was rare and only appeared in Sierra Nevada. We recorded several instances of multiparasitism between various species of tachinids, accounting for c. 3% of the larvae that were parasitized. Specifically, *S. bella* was reared from single larvae together with four other species of tachinids (*P. tibialis*, *E. segregata*, *C. concinnata* and *P. pavidus*), *E. segregata* with three others (*S. bella*, *P. pavidus* and *M. sphingivora*) and *P. pavidus* also with three others (*S. bella*, *E. segregata* and *C. concinnata*).

3.4 | Factors accounting for parasitism

Overall, the parasitism rate was higher in the Pyrenees than in the other two regions (Figure 6). Both in 2016 and 2017, the complete model for explaining parasitism rate (e.g. including the three predictors: 'region', 'Julian day' and 'altitude') had the lowest AIC value (Table 2). However, a simpler model including only 'region' and 'Julian day' was equally good in 2016 and nearly as good in 2017. The same models were selected when considering effective samples of 10 instead of 5 larvae. Given the high correlation between 'altitude' and 'Julian day' ($r > 0.7$), and the fact that models including only 'region' and 'altitude' had consistently much higher AIC values than models

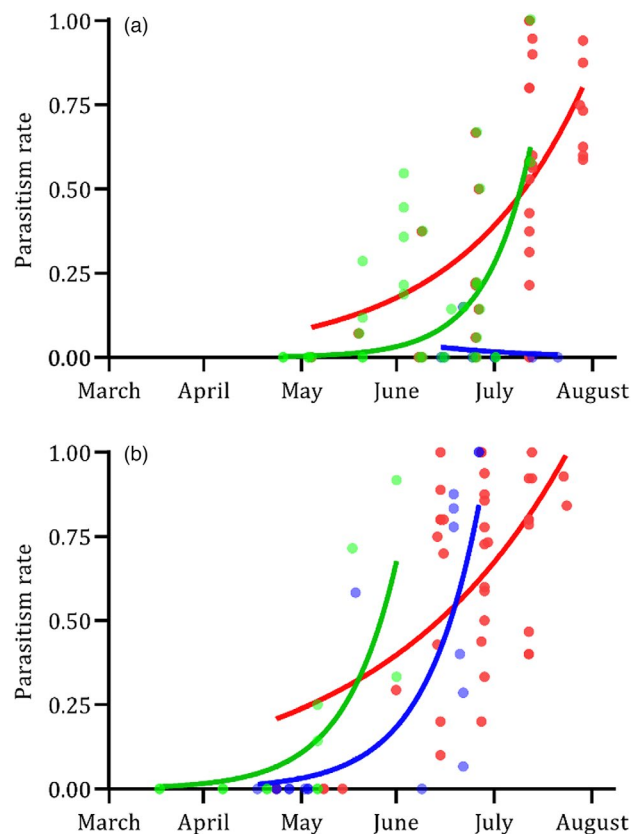


FIGURE 6 Seasonal increase in parasitism rates of the small tortoiseshell in the three studied regions. (a) 2016; (b) 2017. Green circles: Sierra Nevada; blue circles: Sierra de Guadarrama; red circles: Pyrenees. Continuous lines indicate significant trends

with 'region' and 'Julian day' (Table 2), we consider that the latter is the best combination for explaining parasitism rate in our studied system.

Study year	Model	Predictors included in the model			AICc	Δ AICc
		Julian day	Altitude	Region		
2016	A.1	0.049**	0.001	+**	353.7	0
	A.2	0.056**		+**	354	0.3
	A.3		0.002**	+**	407.5	53.8
	A.4	0.045**	0.001**		407.8	54.1
	A.5	0.057**			417.9	64.2
	A.6			+**	473.9	120.2
	A.7		0.004**		485.2	131.5
	A.8				690.1	282.4
2017	B.1	0.077**	-0.001**	+**	371.8	0
	B.2	0.068**		+**	374.4	2.7
	B.3	0.07**	-0.001*		379.2	7.4
	B.4	0.058**			383.8	12
	B.5		0.003**	+**	595.7	223.9
	B.6		0.004**		623.5	251.7
	B.7			+**	682.7	311
	B.8				885.8	514

TABLE 2 Models for explaining parasitism rate for effective samples of ≥ 5 larvae. The same best models (Δ AIC $<$ 2) were selected when using effective samples of ≥ 10 larvae. Significance levels: * p -value $<$ 0.01; ** p -value $<$ 0.001. Beta coefficients for each factor are given

Parasitoid species	Year	Model	Factors included in the model		AICc	Δ AICc
			Altitude	Region		
<i>Pelatachina tibialis</i>	2017	B.1		+**	15.7	0
		B.2			16.2	0.5
		B.3	0.002		17	1.4
		B.4	-0.001	+*	21.7	6
<i>Sturmia bella</i>	2017	C.1		+*	13.7	0
		C.2			14.9	1.3
		C.3	0.003		16.3	2.6
		C.4	0.002	+*	18	4.3

TABLE 3 Models for explaining phenological overlap (OPH) between the small tortoiseshell and its two main larval parasitoids, *Pelatachina tibialis* and *Sturmia bella*, in 2017. Sample sizes: *P. tibialis*: Pyrenees, $n = 6$; Sierra de Guadarrama, $n = 2$; Sierra Nevada, $n = 2$. *S. bella*: Pyrenees, $n = 6$; Sierra de Guadarrama, $n = 3$. Significance levels: * p -value $<$ 0.05; ** p -value $<$ 0.01. Beta coefficients for each factor are given

The best models indicate a strong effect of Julian day, with the rate of parasitism increasing continuously over the season and reaching its highest values in the final samples each year. The effect of the region was highest in Sierra Nevada, although this effect was overridden by the effect of Julian day and the longer larval season in the other two regions (Figure 6; Table 2).

3.5 | Phenological overlap between the host and its main parasitoids

Due to limited sample size, models of phenological overlap (OPH) could only be run for year 2017. The best models consistently selected 'region' as the best predictor, always with a significant effect (Table 3). OPH was higher in the Pyrenees than in the other two regions (Figure S3).

4 | DISCUSSION

As in previous studies carried out in northern and central Europe, we found that parasitism is a major source of mortality in Iberian populations of *A. urticae*. Overall, about 35% of the larvae sampled over the 2 years died as a result of parasitism. Indeed, the impact of parasitism on its butterfly populations must be considerably higher than this given that pupal parasitism (e.g. by the generalist chalcidoid *Pteromalus puparum*) is regularly observed at the study sites (CS pers. obs.; see also Pyörnilä, 1977) and may be an important additional mortality factor. Parasitism thus represents a strong mortality factor on *A. urticae*, as seems to be the case for many other butterfly species (Choutt et al., 2011; Ohsaki & Sato, 1994; Shaw et al., 2009).

The parasitoid complexes consisted mainly of a few specialists that are known to attack exclusively or predominantly butterflies in

the tribe Nymphalini. Among these, the two dominant species were the tachinids *Pelatachina tibialis* and *Sturmia bella*; on the other hand, the ichneumonid *Phobocampe confusa*, which represents the main enemy of northern and central European populations (Audusseau et al., 2020; Rice, 2012; Shaw et al., 2009), was very rare and only appeared in low numbers in Sierra Nevada. Although the parasitoid complexes in all three sampled regions were fairly similar in composition, species richness was clearly higher in the Pyrenees, especially after accounting for differences in sample size. Thus, our data agree with the impoverishment in the number of parasite species towards the edges of hosts' distributions that has been found in other studies (Hódar et al., 2021; Jeffs & Lewis, 2013; Menéndez et al., 2008).

The most remarkable difference between regions was, however, an apparent absence of *S. bella* from Sierra Nevada. Given the high impact of this tachinid on populations in the Pyrenees and the Sierra de Guadarrama (where it accounted for about half of the recorded losses by parasitism), its absence from Sierra Nevada could partly explain the high density that this butterfly reaches in this mountain range despite being its southernmost population in western Europe. In fact, Gripenberg et al. (2011) suggested a direct relationship between the abundance of *A. urticae* and the presence of *S. bella*, with a declining trend of butterfly populations in the UK having occurred following the recent northward expansion of this parasitoid and its colonization of the British Isles, although convincing evidence of this causality is as yet scarce.

The absence of *S. bella* from Sierra Nevada is not the result of the inability of this parasitoid to track this marginal host population because this region is within its geographical range (e.g. there are records from both S Turkey and from S Morocco: Aytar et al., 2021; Stefanescu et al., 2012). A more likely reason is the lack of synchrony between the parasitoid and its host linked to the butterfly's phenology in Sierra Nevada. At the trailing-edge of its distribution, the breeding season of *A. urticae* is constrained by the end of winter and the spring, in contrast to what we observed in its northern Iberian range, where larvae were recorded until the end of July or early August. This difference may be key to explaining the inability of *S. bella* to parasitize *A. urticae* in Sierra Nevada, as this parasitoid was only reared in the Pyrenees and the Sierra de Guadarrama from relatively late larval nests (only one out 308 of records in our study came from a larva collected before the third week of June, a date beyond which larval nests had almost completely disappeared in Sierra Nevada). This differs with the phenology of *P. tibialis*, the other main parasitoid in our study, which is known to have a first generation centred in May and June, also in central and northern Europe (Belshaw, 1993; Tschorsnig & Herting, 1994).

The relevance of the host phenology when accounting for the impact of parasitism is demonstrated by our models, which selected Julian day as the main predictor of the parasitism rate and showed increasing levels of parasitism as the season progresses. The same has been reported in other study systems (Askew & Shaw, 1986; Shaw, 2017; Stefanescu et al., 2012) and, in fact, appears to be a general pattern in host-parasitoid interactions involving multivoltine species. Further evidence of the importance of phenology in

explaining the highest levels of parasitism in the Pyrenees comes from the greater phenological overlap between the host and its two main parasitoids in this region than in the Sierra de Guadarrama and Sierra Nevada. Paradoxically, the more restrictive conditions experienced by *A. urticae* in Sierra Nevada could in fact represent more of an opportunity than a problem. For instance, the growing conditions of its host plant may allow the butterfly to escape from one of its main parasitoids and to stabilize its population at the southern trailing-edge of its distribution. In this area, rainfall is lower, and temperatures are higher in spring and summer than in other Iberian mountain ranges where the butterfly occurs. Our data suggest that the more arid conditions in this region in late spring and early summer have consequences for the nettles on which the larvae feed. For instance, in a study focusing also on the small tortoiseshell, Merckx et al. (2015) showed an increase in the nettle leaf C/N ratios (and hence a presumed lower nutritional quality) in plants growing in field margins compared with those growing in woodland clearings, and this was related to the lower soil moisture and the corresponding lower uptake of available soil nitrogen by plants. In Sierra Nevada, the quality of nettles for supporting larval development decreased rapidly after having reached an optimum in May, and there was a long period of nearly 3 months starting in mid-June in which breeding opportunities were actually very low for this reason. Although the breeding season is curtailed and the higher growth rate that is typically linked to a multivoltine cycle is thus reduced (Kerr et al., 2020), we suggest that this loss is offset by higher larval survival resulting from lower parasitism.

The situation in Sierra Nevada contrasts clearly with that in the Pyrenees, where environmental conditions permit the growth of high-quality nettles throughout most of the summer. In spite of this, this potentially multivoltine butterfly population had, instead, a bivoltine cycle, with no further generations occurring in the second part of the summer, even when there were no thermal restrictions potentially limiting a third generation. We suggest that in this case, the phenology is constrained by increasing levels of parasitism over the season (approaching 100% in some of the late samples in July), which would lead to a mortality rate that was too high to allow for a hypothetical third generation. Indirect support for this hypothesis is provided by the much lower contribution of fresh adults in Pyrenean transect counts at the time of the emergence of the second summer generation compared to counts corresponding to the first spring generation, which could be explained by the high mortality rate experienced by the larvae due to parasitism. Nevertheless, other explanations cannot be ruled out, as, for example, the poorer detectability of second generation adults if they enter into diapause soon after emergence. In any case, our results suggest that the predicted increase in the number of generations in multivoltine species under a scenario of climate warming (Altermatt, 2010; Kerr et al., 2020) could be constrained by the greater impact of parasitism in later generations. In fact, many species of butterflies and moths maintain a univoltine phenology throughout their European range even in the absence of thermal and phylogenetic constraints, suggesting that in some cases there are ecological constraints that

make it disadvantageous to add additional generations even under a suitable climatic regime (Teder, 2020). Our study provides good evidence that increased parasitism rates in late generations could represent one of these ecological constraints in some potentially multivoltine butterfly species. Experimental studies should be designed to confirm this possibility and determine whether intraspecific variation in voltinism in Iberian populations of *A. urticae* is a consequence of genetic differentiation due to local adaptation or reflects phenotypic plasticity as a response to different environmental conditions.

Yet, another aspect that we did not consider in the present study and that could influence the parasitism rates recorded in the different regions is the structure of the local assemblages of butterfly species. As has been widely discussed in the literature, indirect biotic interactions between co-occurring species due to shared parasitoids (i.e. apparent competition) could lead to higher or lower impacts of parasitism among host populations of different species (e.g. Holt & Lawton, 1994; Van Nouhuys & Hanski, 2000). This possibility seemed to be confirmed empirically for nettle-feeding butterflies by Audusseau et al. (2021), who showed that parasitism of Swedish populations of *A. urticae* was higher in samples when larval nests of *Aglais io* were also present in the nettle patches. Interestingly, *A. io* is absent from Sierra Nevada and is only found at low densities in Sierra de Guadarrama, but it is a common species in the Pyrenees. During our field work, five larval nests of *A. io* were found in the same nettle patches used by *A. urticae* in the Pyrenees. However, it has to be noted that spatial and temporal overlap between both species is very limited in this region, as *A. io* generally breeds in more forested habitats in the lower altitudinal range used by *A. urticae*, which means that larvae of both species rarely co-occur at the beginning of the season, when parasitism impact is very low.

To conclude, we found that parasitism plays a key role in the population dynamics of *A. urticae* in the Iberian Peninsula and is probably the main factor precluding a multivoltine cycle in its northern populations because otherwise its impact would be too great. Conversely, we hypothesize that the low level of parasitism allows the southernmost population to reach high densities due to the lack of any phenological overlap between the host and one of its main parasitoids. However, further work is needed to establish a link between population abundance (ideally measured experimentally as the offspring that is eventually produced by a cohort of larvae subjected to different levels of mortality from parasitism) and this mortality factor. Our findings can be probably extrapolated to other species and systems, and highlight the need to consider biotic interactions when modelling changes in species abundance under a context of climate change (van der Putten et al., 2010; Wisz et al., 2013).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTIONS

C.S. and D.G. conceived the ideas and designed the methodology; A.U., A.M., C.S., J.M.B.-A. and D.G. collected the data; M.R.S. and D.H. identified the parasitoids; P.C. analysed the data; C.S. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository <https://doi.org/10.5061/dryad.gb5mkkwrn> (Stefanescu et al., 2022).

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