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Article

Common pheromone use among host-associated populations of the browntail moth, *Euproctis chrysorrhoea*, displaying different adult phenologies

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With 3 figures and 1 table

Abstract: The diversity of herbivorous insects may arise from colonization and subsequent specialization on different host plants. Such specialization requires changes in several insect traits, which may lead to host race formation if they reduce gene flow among populations that feed on different plants. Behavioural changes may play a relevant role in host race formation, for example if different races evolve distinct sexual communication signals or adult phenology. Previous research has revealed differences in larval phenology in different host-associated populations of the browntail moth, *Euproctis chrysorrhoea* (Lepidoptera: Erebidae). Here, sex pheromones among populations of this species are compared, and pheromone trapping data obtained is used in the field to build a phenological model that tests whether populations that feed on different plants differ in their adult flight period. The chemical and electrophysiological analyses revealed that two *E. chrysorrhoea* populations (on *Prunus* and on *Arbutus unedo*) use the same sex pheromone component for mate finding. Our trapping data, however, showed that males fly on average 25 days earlier in populations whose larvae feed on *A. unedo* compared to those whose larvae feed on *Quercus* species. Although the shifted phenology described here may underlie host-plant specialization in *E. chrysorrhoea*, and adults of this species are short-lived, the use of a common sexual pheromone and a large overlap in flight periods suggest that host race formation via allochronic isolation is unlikely in this moth.

Keywords: allochronic speciation; electroantennography; host races; *Euproctis*; stable isotopes; local adaptation

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1 Introduction

For more than a century, biologists have been aware that differences in selection pressures among local populations can be a source of diversity if they promote divergence (Rundle & Nosil 2005). Spatial isolation has traditionally been seen as the main cause promoting divergence, but currently there is much interest in understanding how ecological factors can also contribute to population differentiation (Bolnick & Fitzpatrick 2007, Nosil 2012). Studies on a variety of model systems have provided insights into the mechanisms whereby divergence is promoted, and have revealed that a critical step required for ecological speciation is the reduction of gene flow among populations (Coyne & Orr 2004, Gavrilets 2004, Nosil 2012). Insect herbivores are excellent models for studying ecological speciation, and comparative phylogenetic studies suggest that ecological interactions with their host plants are the underlying force driving their diversification process (Nyman et al. 2010).

Many species of herbivorous insects are able to survive on more than one species of host plant, but in most cases they are adapted to those that are regionally abundant (e.g. Mitter et al. 1979, Kerslake & Hartley 1997). Specialisation on a particular host plant is commonly associated with changes in several life-history traits, which may lead to host race formation if they reduce gene flow among populations that feed on different plants (Berlocher & Feder 2002). The causes leading to host race formation have deep implications for understanding insect diversity because they represent early stages of divergence where the causes reducing gene flow can be studied. Behavioural changes can play a relevant role in host race formation if adults tend to mate on the same plant they developed on as larvae, or if different races evolve distinct sexual communication signals (Nosil 2012). In nocturnal moths, females rely on species-specific sex pheromones to attract males, and divergence in such sexual signals has been demonstrated for host races of the larch budmoth *Zeiraphera griseana* (Priesner & Baltensweiler 1987), the European corn borer *Ostrinia nubilalis* (Thomas et al. 2003, Lassance & Löfstedt 2009), and the fall armyworm *Spodoptera frigiperda* (Groot et al. 2010), among others.

Although relatively less studied, differences in phenology (i.e. the periodic occurrence of various life cycle events) among populations can also promote divergence. In herbivorous insects, phenology is tightly linked to the availability of suitable host plant tissue (van Asch & Visser 2007). Colonisation of a new host may thus require a shift in phenology, which can reduce gene flow between populations feeding on the derived host versus the ancestral one. This mechanism is known as allochronic isolation, and although it has great potential in reducing gene flow among populations that feed on different host plants, its role in host race formation and ecological speciation has only been demonstrated in a few species (Coyne & Orr 2004, Nosil 2012). One of the first examples was provided by the apple maggot fly *Rhagoletis pomonella*. This fly switched from feeding on hawthorn fruits to feeding on domesticated apples when these fruits were introduced in America. This switch led to specialisation on this new host, which was associated with changes in diapause termination that allowed the insects to adapt to earlier production of fruits in apple trees (Feder et al. 1988, 2003, McPheron et al. 1988). Allochronic speciation has also been hypothesized in *Strobilomyia* sp. cone flies infesting successive development stages of larch cones (Sachet et al. 2006). Host race formation has often been documented in insects that feed on a few, and usually closely related, host plants (e.g. Drès & Mallet 2002, Coyne & Orr 2004, Svensson et al. 2005). More studies based on highly polyphagous species are therefore needed to better understand how lineages can colonise plant species that are phylogenetically distant. This information might be important to understand major evolutionary transitions in the radiation process of herbivorous insects.

Euproctis (Lepidoptera: Erebidae) is a highly diverse moth genus mostly distributed throughout Africa and Eurasia. The browntail moth, *Euproctis chrysorrhoea* (L.), is a common species in northern Africa and Eurasia, which invaded eastern North America more than a century ago (Elkinton et al. 2006). This species has been observed to feed on 26 genera of broadleaved plants belonging to 13 different families in its native range (Cabi International 2005), and inhabits a large variety of forested habitats. *Euproctis chrysorrhoea* has often been reported as a pest in agricultural and forested areas, and its larvae represent a health threat as they can cause serious skin rashes, and acute allergic reactions (Battisti et al. 2011). A recent study on eight *E. chrysorrhoea* populations feeding on nine different host plants in Europe, has revealed that genetic divergence within *E. chrysorrhoea* populations is mostly influenced by the geographic origin of the populations studied, but also by the host plant from where the insects were collected (Marques et al. 2014). Field and laboratory studies in the Iberian Peninsula (south western Europe) also revealed that populations on the strawberry tree *Arbutus unedo* (Ericaceae) have a shifted larval phenology. This moth is univoltine, and has a particular life history because early instars are gregarious and larvae enter diapause at the beginning of autumn inside communal winter nests. Diapause is obligatory in this species (Frago et al. 2009) but its termination may depend on the host plant on which larvae feed. In sites dominated by the evergreen *A. unedo*, diapause is limited to two to three months, whereas diapause lasts at least seven months on deciduous hosts (Frago et al. 2010). This phenological shift may imply that adults from evergreen hosts appear earlier in the season, which may reduce encounters with adults from deciduous hosts as adults have a short lifespan (Torossian et al. 1988, Sterling & Speight 1989). This shifted larval phenology thus suggest that gene flow between populations feeding on the evergreen *A. unedo*,

and on deciduous hosts might be reduced due to allochronic isolation.

Similar to many other moth species, *E. chrysorrhoea* relies on a long-range sex pheromone that females produce for mate attraction. This pheromone was identified by Leonhardt et al. (1991) as the compound (*Z*,*Z*,*Z*,*Z*)-7,13,16,19-docosatetraen-1-ol isobutyrate (hereafter 22:4 OiBu), and was obtained from *E. chrysorrhoea* populations that feed on deciduous trees in the genus *Prunus* in North America. Here, whether populations feeding on the evergreen *A. unedo* also use this sex pheromone is explored, and the factors that could contribute to reproductive isolation between *E. chrysorrhoea* populations feeding on *Quercus* and *Prunus* species versus those feeding on *A. unedo* are analysed. First, the sex pheromone between two populations that feed on two different host plants are compared, using chemical and electrophysiological analyses. Second, pheromone trapping is used to build a phenological model and to test whether these different host-associated populations differ in their flight phenology, a potential mechanism underlying host race formation via allochronic isolation.

2 Materials and methods

2.1 Collection and rearing of insects

Overwintering third instar *E. chrysorrhoea* larvae were collected from communal nests in 2011 on *A. unedo* in Sierra Nevada, Spain (Table 1), and in 2012 on *Prunus* spp. in High Head Road, Pilgrim Heights, Truro, MA, USA. These two populations were chosen to select an evergreen and a deciduous host, but also based on the availability of larvae in the same period [larval densities of this outbreaking insect can greatly vary between years (Torossian et al. 1988, Sterling & Speight 1989, Frago et al. 2011)]. In addition, these two populations are among the most distant (Spain and USA) so that pheromone differentiation between them is more likely than between closer ones. Larvae were then transported in hermetic containers to Lund (Sweden) where they were stored in darkness at 5°C. Batches of five larvae per nest, which are usually offspring of the same female, were later transferred to another climate chamber under a 18:6h light:dark regime, and a fluctuating temperature of 20°C during daytime and 15°C at night. Relative humidity was kept constant at 60%. Larvae were placed in small plastic cups and fed a bean-based artificial diet (Zhu et al. 1996). After pupation, the cocoons were carefully dissected, pupae were sexed based on the last three abdominal segments, and then separated. Two- to three-days-old adult females and males were used for chemical and electrophysiological analyses, respectively (see details below).

2.2 Sex pheromone extraction

To analyse the female-produced sex pheromone, four females from the Spanish population and six females from the North American population were used. Pheromone glands of two- to three-days-old virgin females were dissected and individually extracted after 2–3 h into the scotophase under a vacuum bench to prevent urticating reactions. The pheromone glands of *E. chrysorrhoea* are located in the dorsal membrane between the 8th and 9th abdominal segments. To dissect them, the brown hairs and the abdominal tip on the last segment were removed and the intersegmental membrane was cut off. The pheromone was then extracted by immersing the membrane into 100 µl hexane for 30 min (Leonhardt et al. 1991). Pheromones were extracted similarly from the abdominal tip and the remaining part of the cuticle of the last two abdominal segments associated with the brown hairs.

Table 1. Location, study year and total number of males caught in each *Euproctis chrysorrhoea* population studied. Populations were identified based on larval presence and males subsequently collected with traps baited with a synthetic female pheromone. Note that the first population (Truro) was used to obtain larvae for pheromone analyses, and not to place pheromone traps.

Pop.	Site	Country	Host plant	Host plant type	Latitude	Longit.	Year	Total number of males
1	Truro	USA	*Prunus* spp.	Deciduous	42.06	–70.11	2012	NA
2	Satu Mare	Romania	*Quercus robur*	Deciduous	47.70	22.87	2011	24
3	Orleans	France	*Q. robur*	Deciduous	47.90	2.36	2013	1
4	St. Guilhem le Désert	France	*Arbutus unedo*	Evergreen	43.74	3.55	2013	7
5	Halle Peissnitz	Germany	*Q. robur*	Deciduous	51.29	11.56	2011	0
6	Macchia Pisana	Italy	*A. unedo*	Evergreen	43.80	10.27	2011	4
7	La Verna	Italy	*Quercus cerris*	Deciduous	43.69	11.94	2011	48
8	Vall d'Uixó	Spain	*A. unedo*	Evergreen	40.08	0.04	2011	20
9	Sierra Huétor	Spain	*Q. faginea*	Deciduous	37.23	–3.45	2013	13
10	Sierra Nevada	Spain	*A. unedo* and *Q. faginea*	Both	37.11	–3.41	2013	11

2.3 Gas chromatography/mass spectrometry

An Agilent 5975 mass-selective detector coupled to an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) was used to analyse the pheromone extracts. An HP-INNOWax column (30 m × 0.25 mm i.d., and 0.25 µm film thickness; J&W Scientific, Folsom CA, USA), and a non-polar column HP-5MS (30 m × 0.25 mm i.d., and 0.25 µm film thickness; J&W Scientific) were used. The carrier gas was helium, which was provided at a constant flow of 0.8 ml/min corresponding to linear velocity of 33 cm/s. The oven temperature was programmed as in the GC-EAD analysis (see below). The pheromone compound was identified based on comparison of the retention time and mass spectrum with a synthetic reference on both columns. The pheromone titre was quantified by comparing the abundance of the base ion at *m*/*z* 79 with the same ion from the synthetic reference with a known concentration.

2.4 Electrophysiological analyses

An Agilent 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a polar HP-INNOWax column (30 m × 0.25 mm i.d., and 0.25 µm film thickness; J&W Scientific, Folsom CA, USA), and coupled to a flame ionization detector (FID) and to an electroantennographic detector (EAD) was used. The inlet and FID temperatures were set at 250°C and 280°C, respectively, and the transfer line leading to the EAD was maintained at 255°C. Hydrogen was used as the carrier gas at a flow rate of 1 ml/min, and 2 µl samples of pheromone gland extracts, or synthetic 22:4 OiBu (purity 94.7%) were injected under the splitless mode. The GC oven temperature was programmed at 80°C for 1 min and increased by 10°C/min up to 220°C, held for 10 min, and then increased by 10°C/min up to a final temperature of 230°C, which was maintained for 10 min. The GC effluent was carried to the FID and EAD using a split ratio of 1:1. For the EAD, an antenna was cut off from an unmated 2- to 3-days old male anaesthetised with CO_2 and mounted on a PRG-2 EAG (10x gain) probe (Syntech, Kirchzarten, Germany) using conductive gel (Blågel, Cefar, Malmö, Sweden). The antenna was exposed to an air stream that was filtered through charcoal and humidified. The air passed over the antenna via a glass tube outlet positioned at 0.5 cm distance from the preparation. The synthetic 22:4 OiBu at a dose of 35 ng and the gland extracts from both Spanish and North American females were used to stimulate the antennae of Spanish and North American males. Data were analysed with the GC-EAD Pro Version 4.1 software (Syntech, Kirchzarten, Germany).

2.5 Pheromone trapping

Synthetic 22:4 OiBu was used in traps set in 2011 and 2013 at nine European sites in which *E. chrysorrhoea* populations were present to assess its attractiveness to males, and to monitor their flight period. The sites, which were located in five different European countries (France, Germany, Italy, Spain and Romania) were selected based on larval presence in the previous year (Table 1). To ensure that adults came from specific host plants, and given the little dispersal capabilities of *E. chrysorrhoea*, all potential host plants in a site were screened for the presence of winter nests. This was done in winter as nests are easily spotted in deciduous trees, which have lost their leaves. Selected sites included five sites in which larvae were found feeding on *Quercus* spp, three on *A. unedo*, and one site in Sierra Nevada where larvae feed on *Q. faginea* and *A. unedo* in sympatry. The forest in these sites were dominated by these trees. In each location, three traps loaded with the pheromone and one blank control trap were placed in the vegetation, 1.5 m above the ground. Each trap was at least 10 m away from the others. RAG (Sticky "delta") traps (Csalomon, Budapest, Hungary) were used and loaded with the 22:4 OiBu. The pheromone was dissolved in hexane and 100 µl of this solution was applied on a red rubber septa (11 × 5 mm, catalogue no. 224100-020, Wheaton Science Products, Millville, NJ, USA). In 2011 and 2013 each dispenser contained 275 and 250 µg of 22:4 OiBu, respectively. In 2013, 1% butylated hydroxytoluene was added to the solution to reduce UV sensitivity. Based on *E. chrysorrhoea* phenology (Frago et al. 2010), lures were placed in the field from early May to late July, and trapped males were counted every other week. Lures were replaced after approximately one month and sticky bottoms when needed. Males trapped in Sierra Nevada were stored in ethanol at –20°C for further isotope analyses.

2.6 Analysis of allochronic isolation

The degree of allochronic isolation (AI) was estimated using the following formula developed by Feder et al. (1993) and Hood et al. (2015):

$$AI = 1 - \sum x_i y_i / \sqrt{\sum x_i^2 \sum y_i^2}$$

where x_i and y_i are the proportion of moths from populations feeding on *A. unedo* and *Quercus* spp., respectively, on day i. To estimate such proportions, male moths obtained every 2-week period were used. To fit this model the following assumptions were considered: (i) adults have a short lifespan (i.e. one week) and (ii) become reproductively active immediately after emergence from pupae, (iii) the developmental time from egg to adult is similar between sexes (Torossian et al. 1988, Sterling & Speight 1989, Frago et al. 2009), and (iv) adult phenology does not vary among years.

2.7 Isotope analyses

The different food sources used during larval development are known to correlate with isotope signatures in adult insects (Boecklen et al. 2011, Kraus et al. 2014). Isotope signatures of males caught in the pheromone-baited traps in Sierra Nevada (where moths develop on both evergreen and

deciduous hosts) were analysed to determine their larval host plant, by comparing these signatures with that of larvae collected in the same population and year on known hosts. All samples were dried for three days at 65°C, and approximately 1 mg of moth tissue per sample was weighed and individually placed into tin capsules. The whole body of third instar larvae was used, whereas for adults only the heads were used. Isotope ratios of $\delta^{15}N$ and $\delta^{13}C$ were determined simultaneously from the same sample by a coupled system consisting of an elemental analyser (NA2500; CE-Instruments, Rodano, Milano, Italy) and an isotopic gas mass spectrometer (Delta plus, Finnigan MAT, Bremen, Germany). Isotopic contents are presented in δ units, which represent the relative difference between the sample and the standard following Peterson and Fry (1987). The standard for $\delta^{15}N$ was atmospheric nitrogen, whereas for $\delta^{13}C$ a marine limestone known as Vienna PeeDee belemnite was used. After analysing each batch of ten samples, the system was calibrated with Acetanilide (Merck, Darmstadt, Germany).

2.8 Phenological model

The Julian date at which an individual male was caught in a pheromone-baited trap was estimated as the median between the two dates a specific trap was checked. This value was then correlated with the accumulated daily degree-days in each location and year of sampling. Degree-days express insect growth in response to daily temperatures and are an effective way to predict development in ectothermic organisms. Insects only develop above a certain temperature known as the lower threshold, which is specific for every species and 12°C for *E. chrysorrhoea* (Pantyukhov 1962). This threshold, as well as maximum and minimum daily temperatures at each location, were used to estimate accumulated daily degree-days (or physiological time) based on the single triangle method (Higley et al. 1986). Accumulated degree-days were calculated from the 1st of January, and corresponded to five to eight months. Temperatures were retrieved from the nearest sampling point in the global database NCEP (National Centre for Environmental Prediction) with the package RNCEP in R (Kemp et al. 2012).

2.9 Statistical analyses

All analyses were performed in R 3.2.4 (http://www.r-project.org/). Pairwise differences of male flight periods between populations were evaluated with the non-parametric Kruskal-Wallis test. Differences in isotope signatures between larvae collected from *Q. faginea* and *A. unedo* were tested using analysis of variance (ANOVA). Differences in the flight period (i.e. Julian days at which males were caught) between populations feeding on deciduous *Quercus* spp. or on the evergreen host *A. unedo* were analysed by fitting a linear mixed effects model assuming a Gaussian error distribution using the function lmer from the package nlme (Pinheiro et al. 2015). To account for the non-independence of males collected in the same population and year, year and year nested within study site were included as random factors, while host-plant type (i.e. deciduous vs. *A. unedo*) was modelled as a fixed effect. Clines in insect phenology along latitudinal gradients are common (e.g. Dambroski & Feder 2007), and our previous studies revealed that latitude was an important factor in the genetic structure of *E. chrysorrhoea* (Marques et al. 2014). To account for this, latitude was also included in the model as a fixed effect. A similar model was built to test for the effect of accumulated degree-days on males' flight period with accumulated degree-days and latitude included as a continuous fixed factor, and host type as a categorical fixed factor. In this model the interaction term between host type and degree-days was also included. A significant interaction would indicate that temperature effects on *E. chrysorrhoea* male phenology depend on which host the larvae developed. In particular, an earlier flight period in *E. chrysorrhoea* populations from *A. unedo* was expected so that a lower amount of accumulated degree-days would be required for adult emergence. In the mixed models, the presence of data points with high influence was checked by calculating Cook's distances, and model fit was checked by visual inspection of the residuals. Significance of fixed terms in linear mixed models was tested with the likelihood-ratio chi-square test.

3 Results

3.1 Electrophysiological and chemical analyses

The antennae of *E. chrysorrhoea* males from both *Prunus* spp. (USA) and *A. unedo* (Spain) populations showed a clear response to synthetic 22:4 OiBu. The antennae also responded to a single compound found in the gland extracts from females feeding on both host types, which had the same retention time as the 22:4 OiBu (Fig. 1), and was further identified as 22:4 OiBu via GC-MS analysis. No other compounds in the gland extracts elicited antennal responses. The pheromone compound was found in all female extracts including the pheromone gland tissue, the surrounding abdominal cuticle, and brown hairs, with an overall titre in glands of 31.6 ± 16.7 ng/female in *Prunus* spp. population (n=6) and 43.9 ± 24.1 ng/female in *A. unedo* population (n=4).

3.2 Allochronic isolation and flight period of *E. chrysorrhoea*

Two populations [Halle Peissnitz (Germany) and Orleans (France)] were excluded from the analysis because of the very low catches at these sites (Table 1). For the remaining seven populations, 127 males were captured with catches within populations ranging from four to 48 (Table 1), while no moths were captured in the unbaited traps. The overall flight period peaked from early June to early August (Fig. 2).

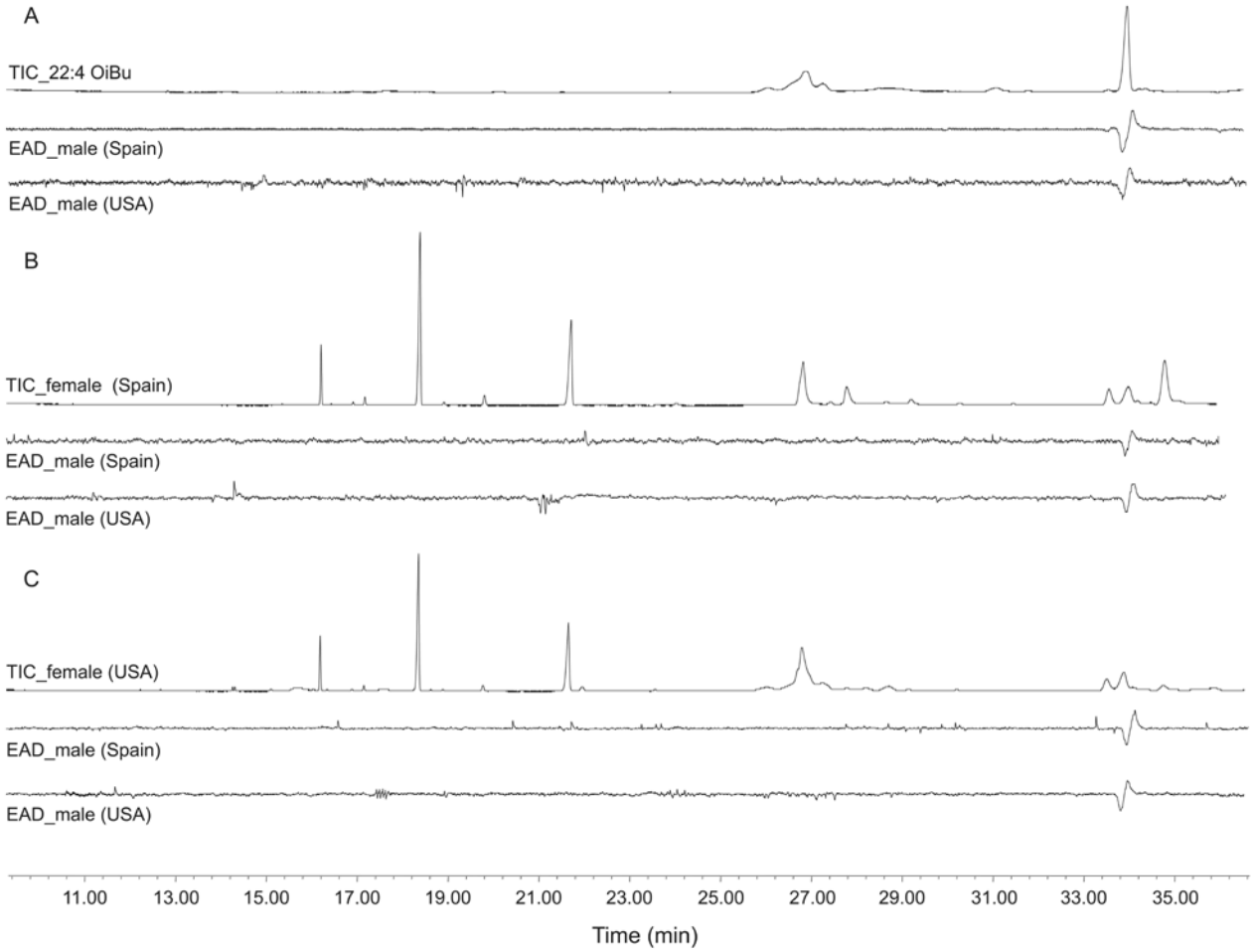

Fig. 1. Coupled gas chromatographic-electroantennographic analysis of synthetic compounds and pheromone gland extracts from female *Euproctis chrysorrhoea*. In each panel the upper trace is the total ion chromatogram (TIC) monitored with flame ionization detector (FID), and the middle and lower traces are the antennal responses of conspecific males from the same or different geographical populations. **A.** Male antennal response to the synthetic pheromone compound (*Z*,*Z*,*Z*,*Z*)-7,13,16,19-docosatetraen-1-ol isobutyrate (22:4 OiBu); **B.** Male antennal response to the female pheromone gland extract of the Spanish population on *Arbutus unedo*; **C.** Male antennal response to the female pheromone gland extract of North American population on *Prununs* spp.

When excluding the data obtained from the sympatric population in Sierra Nevada, Spain (males could not be associated to a particular host plant), adults from populations feeding on deciduous *Quercus* spp. trees occurred on average 25 days later than those feeding on *A. unedo.* This difference was significant (mixed-model host plant type effect, X^2_1 = 14.15, $P < 0.001$), even when the effect of latitude was taken into consideration in the model (mixed-model latitude effect, $X^2_1 = 0.18$, $P = 0.670$). On a finer spatial scale, pairwise comparisons between nearby populations monitored the same year revealed that in central Italy in 2011, males feeding on *A. unedo* flew significantly earlier than those feeding on *Quercus cerris* (Kruskal–Wallis $\chi^2 = 12.32$, 1 df, $P < 0.001$). Similarly, in southern Spain in 2013, males feeding on *Q. faginea* in Sierra Húetor flew significantly later than in Sierra Nevada where the moth develops on this same host but also on *A. unedo* (Kruskal–Wallis $\chi^2 = 17.77$, 1 df, $P < 0.001$) (Fig. 2). In Sierra Nevada (Spain), only 11 males were trapped with no apparent bimodal distribution of catches over the season, which suggests similar flight periods for the two populations. In addition, such phenology analysis was limited because trapped males could not be assigned to a particular host plant based on isotope analyses (see below). Excluding the population from Sierra Nevada, and assuming that males and females have similar development times (Torossian et al. 1988, Sterling & Speight 1989, Frago et al. 2009) and that flight periods do not differ among years, the degree of allochronic isolation was estimated. It was found that reproductively active *A. unedo* and *Quercus* spp. adults would have overlapped for 81.1% of their flight period. On a finer spatial scale the overlap was 67.3% between nearby populations feeding on *A. unedo* and *Quercus* spp. in Italy,

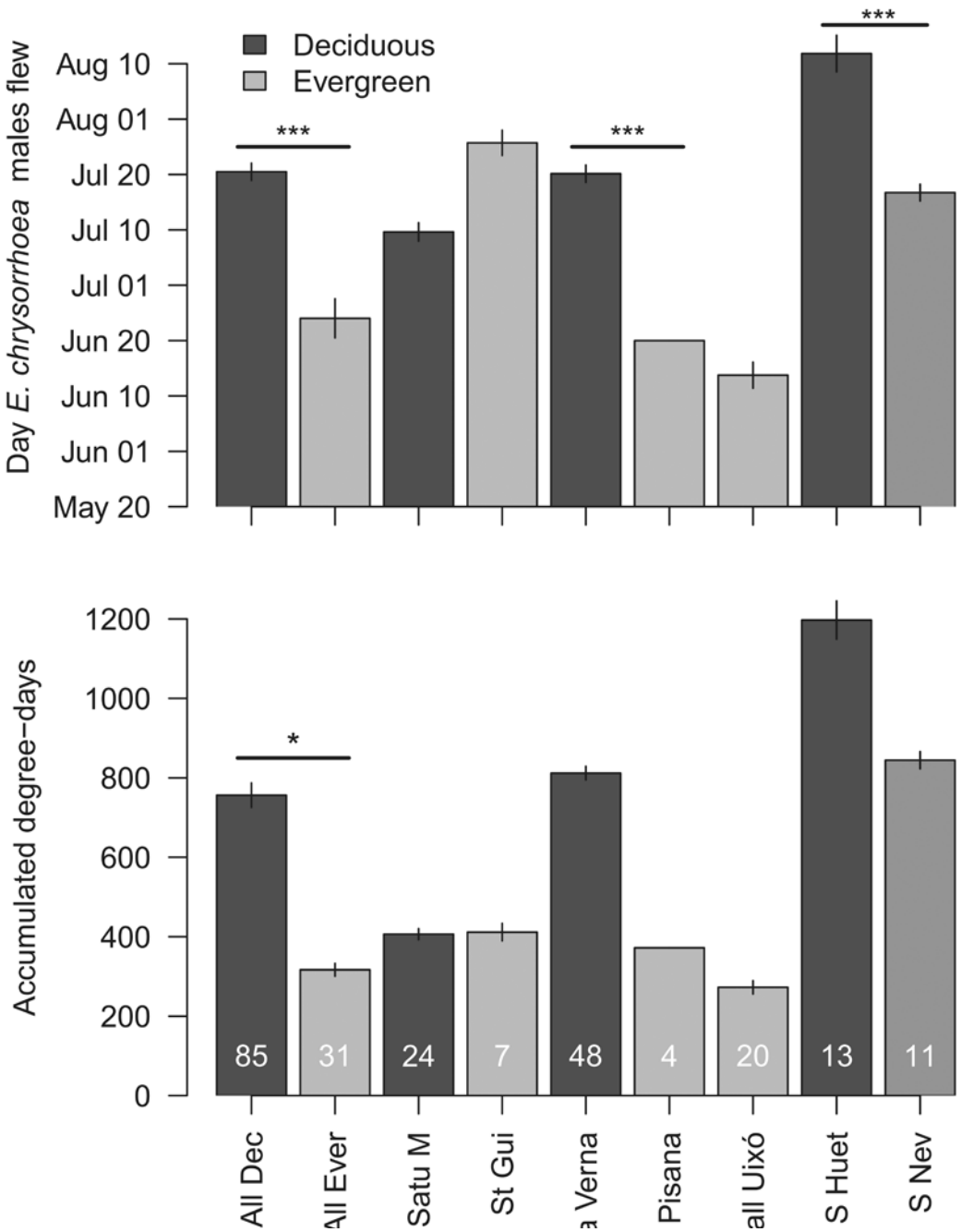

Fig. 2. Male *Euproctis chrysorrhoea* flight period (top) and degree-day accumulation (bottom). Average day or degree-days (±SE) individual males were caught in pheromone traps in the different populations studied. Dark-grey bars represent populations feeding on deciduous trees in the genus *Quercus* and light-grey bars represent populations feeding on the evergreen *Arbutus unedo.* The population in Sierra Nevada is represented by an intermediate tone as in this location *E. chrysorrhoea* larvae were found attacking both host plant types in sympatry. The first two columns comprise all populations on each tree type pooled together (excluding the population in Sierra Nevada). The following *a priori* hypothesised pairwise comparisons are shown at the top of the bars. All populations on deciduous trees compared with all populations on *A. unedo*, analysed with mixed effect models with year and year nested within study site as random factors. Nearby populations in Italy and nearby populations in the south of Spain compared with the Kruskal-Wallis test. The number of males caught in each population is shown at the bottom of each bar. *** $P < 0.001$, * $0.01 < P < 0.05$. Satu M (Satu Mare), St Gui (St. Guilhem le Désert), M pPisana (Machia Pisana), S Huet (Sierra Huétor) and S Nev (Sierra Nevada), more details on the studied localities can be found in Table 1.

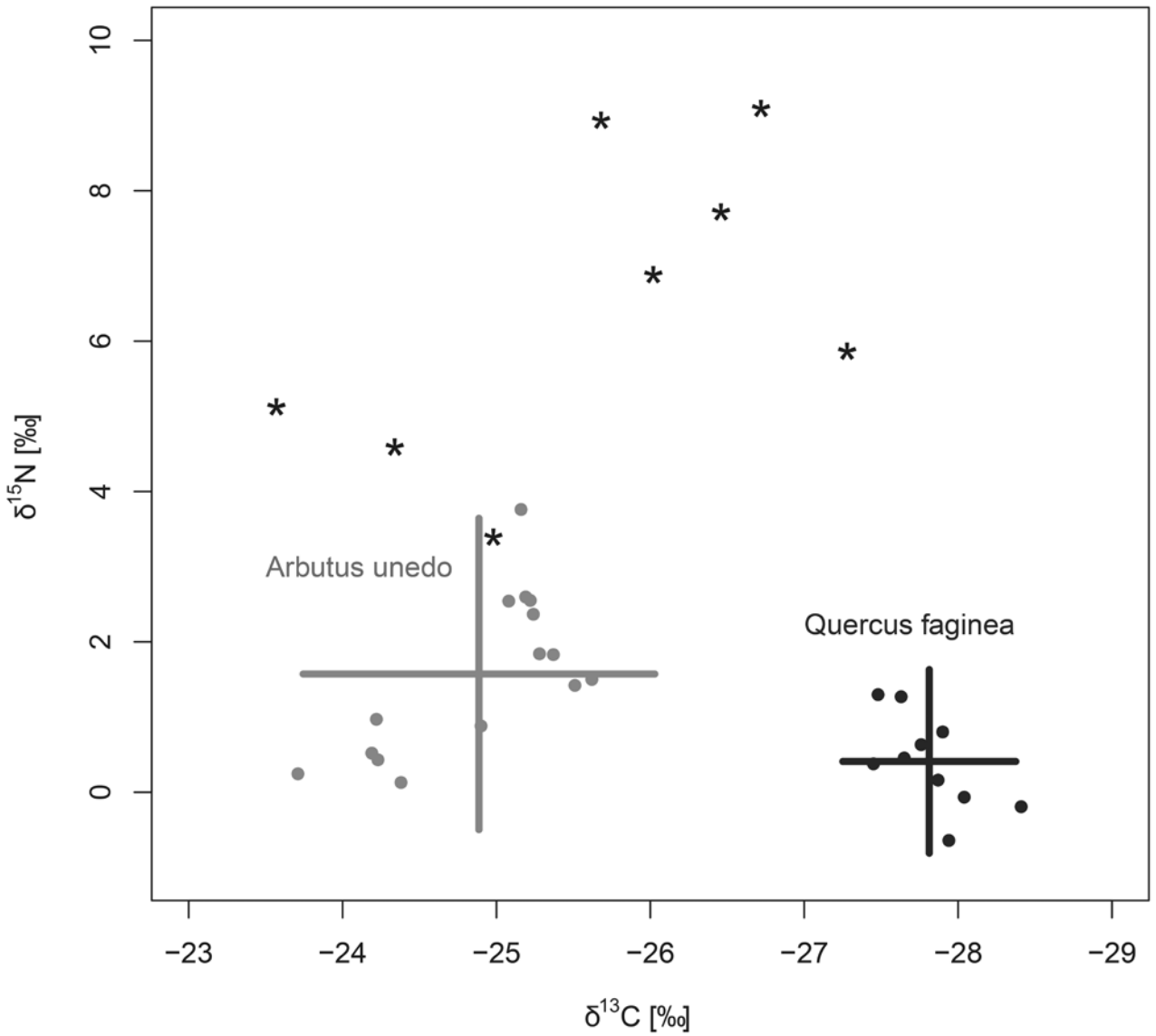

Fig. 3. $\delta^{15}N$ and $\delta^{13}C$ values [‰] of larvae and adult *Euproctis chrysorrhoea* in Sierra Nevada (Spain) where the species feeds in sympatry in both the evergreen *Arbutus unedo* and the deciduous *Quercus faginea*. Isotope signals from larvae collected in *A. unedo* are depicted in light grey, from larvae collected in *Q. faginea* in dark grey and from adult moths with asterisks. The 95% confidence intervals of the mean for larval samples are also shown.

and 83.5% between the two populations from southern Spain.

3.3 Isotope analysis of sympatric *E. chrysorrhoea* populations in Sierra Nevada

Both $\delta^{15}N$ and $\delta^{13}C$ signatures differed significantly between the ten larvae collected on *Q. faginea* and the 15 larvae collected on *A. unedo* in Sierra Nevada (ANOVA for $\delta^{15}N$, $F_{1,23} = 9.75$, $P = 0.004$; ANOVA for $\delta^{13}C$, $F_{1,23} = 215$, $P < 0.001$; Fig. 3). The 95% confidence intervals for the mean, however, largely overlapped in $\delta^{15}N$ signatures, but not in $\delta^{13}C$ signatures (Fig. 3). The former values can therefore not be used to distinguish larvae feeding on either host. When analysing the eight adult males captured in the pheromone traps in this same area, the $\delta^{13}C$ signature fell within the 95% confidence interval of the mean larval signatures in only four individuals. Adult isotope signature can thus not be used in *E. chrys-*

orrhoea to undoubtedly discern the host plant on which their larvae developed.

3.4 Phenological model

Excluding the population from Sierra Nevada, *E. chrysorrhoea* males from populations feeding on deciduous trees flew on average when 756±31 (mean±SE) degree-days accumulated after the 1st of January, whereas males from populations feeding on the evergreen *A. unedo* flew after 317±16 degree-days. In the model built to test this difference, both host type and latitude had a significant effect on accumulated degree-days (mixed-model host type effect, $X^2_1 = 11.007$, $P < 0.001$; latitude effect, $X^2_1 = 7.03$, $P = 0.008$; Fig. 2). Average accumulated degree-days for male flight in the different populations paralleled the mean dates at which males flew, except in the populations in Satu Mare (Romania) and St. Guilhem le Désert (France). In these sites accumulated degree-days were relatively lower than the mean dates at which males were caught (Fig. 2). Accumulated degree-days from the 1st of January were positively correlated with the Julian day at which males were caught in the pheromone-baited traps (mixed-model accumulated degree-days effect, $X^2_1 = 449.4$, $P < 0.001$). In this model, host type had no significant effect on flight period (mixed-model host type effect, $X^2_1 = 2.33$, $P = 0.127$), but the interaction term between host type and accumulated degree-days was highly significant ($X^2_1 = 67.2$, $P < 0.001$). This is in agreement with our hypothesis as it suggests that the effect of temperature on male flight period depends on the host where larvae developed. In these model flight periods were negatively related to latitude (mixed-model latitude effect, $X^2_1 = 4.60$, $P = 0.032$), which reveals a latitudinal cline on *E. chrysorrhoea* phenology. Similar analyses performed with degree-days accumulated from the 1st of September [when all *E. chrysorrhoea* populations enter diapause, Frago et al. (2010)] led to similar results.

4 Discussion

The chemical and electrophysiological analyses performed in the laboratory revealed that for two geographically isolated *E. chrysorrhoea* populations (i.e. USA and Spain) feeding on a deciduous *Prunus* species and on the evergreen *A. unedo* use the same sex pheromone component (22:4 OiBu) for mate finding. Antennae of males from populations associated to both hosts only responded to this single compound in GC-EAD recordings using gland extracts from females associated with these hosts. Pheromone trapping also revealed that traps baited with 22:4 OiBu were attractive to male moths that developed as larvae on different host plants. Assortative mating based on different sex pheromones can thus be ruled out as a mechanism facilitating reproductive isolation between the populations of *E. chrysorrhoea* feeding on different host plants, unless assortative mating is determined by other less abundant and not yet detected components in the pheromone blend. In one site in Germany, *E. chrysorrhoea* winter nests were spotted but adults were not caught. Although it is possible that the population in this site has evolved a different pheromone signal, it is more likely that adult population densities dropped below detection levels. This situation is quite likely given the large changes in density that this moth suffers from year to year, and the large mortality that larvae suffer from diapause to adulthood (Torossian et al. 1988, Sterling & Speight 1989, Frago et al. 2011). This lack of pheromone evolution contrasts to other moth species where different host-associated populations have diverged in their sex pheromone communication channel leading to host race formation. For example, both *O. nubilalis* and *Z. diniana* harbour host-specific races that use different ratios of their sex pheromone components, thereby avoiding cross attraction (Priesner & Baltensweiler 1987, Thomas et al. 2003, Lassance & Löfstedt 2009).

Our trapping data showed that males fly on average 25 days earlier in populations whose larvae feed on *A. unedo* compared to populations whose larvae feed on *Quercus* spp. To assess whether this difference in flight period was caused by host use or by abiotic conditions, a phenological model was built. This model explored the thermal requirements (or accumulated degree-days) for *E. chrysorrhoea* larvae to develop into flying adults. In agreement with our hypothesis, the phenological model revealed that, on average populations that feed on *A. unedo* need significantly fewer degree-days to reach the adult stage than populations that feed on *Quercus* spp. The phenological model was particularly useful to understand catch data in some populations. For instance, in the population feeding on *A. unedo* in Saint Guilhem le Désert, males were caught much later than in the other *A. unedo* populations. Catch data based on accumulated degree-days, however, resembled that of the other populations on this same host. This suggests that in this particular population or year, temperatures were lower than average and hence a longer period was required for larvae to develop into adults. Our results from the trapping experiments thus confirm our hypothesis that the known shifted phenology in larval development between populations feeding on *Quercus* spp. versus *A. unedo* (Frago et al. 2010) is translated into a shifted phenology also at the adult stage.

Allochronic isolation is a powerful mechanism by which populations can become isolated but it has been described in only a few species. For example, in the European corn borer, *O. nubilalis*, adults fly on average ten days earlier in the mugwort race than in the maize race, which might contribute to host race formation in this species (Thomas et al. 2003). Allochronic isolation can lead to genetic differentiation even when different populations use the same host plant, as shown in the pine processionary moth, *Thaumetopoea pityocampa* (Santos et al. 2007), and in *Strobilomyia* spp. larch cone flies (Sachet et al. 2006). *Thaumetopoea pityocampa* larvae develop during winter, but an atypical population has

recently been discovered in Portugal where larvae develop during summer. This phenological shift during the larval stage also affected adult phenology, with subsequent differentiation at the genetic level even if pheromones were not altered (Santos et al. 2007). In the fall armyworm, *S. frugiperda*, phenological differences between races occur at an even finer temporal scale as the corn race is active early at night, while the rice race is active late at night (Groot et al. 2010). A study with 400 species of neotropical skippers (Hesperiidae), revealed that speciation at different taxonomic levels (including subfamilies, genera, and species) is potentially caused by differences in diel flight activity (Devries et al. 2008). All these examples suggest that speciation through phenological differences may be more common than is currently appreciated.

Our study, however, does not provide any strong evidence for allochronic isolation between *E. chrysorrhoea* populations that feed on *A. unedo* or *Quercus* spp. for several reasons. First, a low degree of allochronic isolation was found as the flight period of moths from both host plant types are likely to overlap for as much as 81%. Second, moths from both host types use the same sex pheromone, which suggests that populations from both host types have non-independent evolutionary trajectories, and that divergence through host plant use is unlikely. Finally, catch data from the single site where both host plant types were attacked by the moth did not reveal a bimodal distribution of adult catches. Although *E. chrysorrhoea* feeds on several host plants in Europe, after more than seven years of research only two sympatric sites have been found, and pheromone traps were placed in only one of these. This site was located in Sierra Nevada where larvae were found on both *Q. faginea* and *A. unedo*. Unfortunately, only 11 males were captured in pheromone traps at this site, and catch data did not cluster into two distinct peaks, which indicates similar flight periods for the different host associated populations. Isotope analyses of trapped males were also used to assess to which host plant they were associated with. Isotope signals revealed that relative to larvae, adult stages were enriched in $\delta^{15}N$, a common pattern in insects (Kraus et al. 2014). Unfortunately, these signals could not be used to assess the host origin of the analysed adults. However, their late flight period and a large degree-day accumulation before the adult stage, suggested that males captured at this site likely originated from *Q. faginea*, which is in fact the most abundant plant species in the area (E. Frago, pers. obs.).

A previous population genetics study revealed that *E. chrysorrhoea* populations are mostly structured at the latitudinal level, but haplotype diversity was relatively higher when insects were found attacking several host plant species in sympatry (including the site in Sierra Nevada). To better understand this, future research is needed to unveil whether gene flow between populations feeding on different host plants is reduced in sympatric sites. These studies may imply finding more sites where *E. chrysorrhoea* attacks several plants so that the fitness consequences of shifting hosts can be studied. This data may explain selection against migrant individuals (i.e. that move from one host plant to another). In the pea aphid *Acyrthosiphon pisum* model system, for example, these types of experiments revealed that although insects perform better on the plants they are adapted to, their degree of specialization also depends on the host plant used by the insect (e.g. Ferrari et al., 2008). *Euproctis chrysorrhoea* is reported to feed on several trees throughout Europe (Cabi International 2005), and it would be very interesting to explore whether diapause duration in this species depends on whether larvae feed on a single host plant or on both evergreen and deciduous plants.

In conclusion, it has been demonstrated both in the field and in the laboratory that different host-associated populations of *E. chrysorrhoea* use the same sex pheromone (22:4 OiBu) for mate finding. Differences in the flight period between populations feeding on *Quercus* spp. and those feeding on *A. unedo* has also been observed. This might generate allochronic isolation, but future research is needed to demonstrate whether this translates into reduced gene flow between populations, and therefore into host race formation. In addition, transplant experiments are needed to evaluate the fitness consequences of female moths ovipositing on the non-native host, which will determine the degree of selection against individuals that shift hosts. *Euproctis* is a genus comprising several pest species, and our results may help designing more environmentally benign control methods using, for example, pheromone traps to monitor the density of this pest, or to eventually mass trap flying adults.

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H-LW, GPS, OA and EF wrote the manuscript; all authors contributed with revisions.

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