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ORIGINAL ARTICLE

Experimental evidence for heritable reproductive time in 2 allochronic populations of pine processionary moth

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Abstract Phenology allows organisms to overcome seasonally variable conditions through life-cycle adjustment. Changes in phenology can drastically modify the evolutionary trajectory of a population, while a shift in the reproductive time may cause allochronic differentiation. The hypothesis of heritable reproductive time was experimentally tested, by studying a unique population of the pine processionary moth *Thaumetopoea pityocampa* (Den. & Schiff.) which has a shifted phenology, and however co-occurs with the typical population following the classical life cycle. When populations of both types were reared under controlled conditions, the reproductive time was maintained asynchronous, as observed in the field. The shifted population was manipulated in the laboratory to reproduce later than usual, yet the offspring emerged in the next year at the expected dates thus "coming back" to the usual cycle. Hybrids from crosses performed between the 2 populations showed an intermediate phenology. From the emergence times of parents and offspring, a high heritability of the reproductive time ($h = 0.76$) was observed. The offspring obtained from each type of cross was genetically characterized using microsatellite markers. Bayesian clustering analysis confirmed that hybrids can be successfully identified and separated from the parental genetic classes by genotyping. Findings support the hypothesis that, for this particular population, incipient allochronic speciation is due to a heritable shift in the reproductive time that further causes assortative mating and might eventually cause ecological adaptation/maladaptation in response to environmental changes.

Key words allochronic speciation; experimental crossings; heritability; phenology; reproductive isolation; *Thaumetopoea pityocampa*

Introduction

Phenology, that is, the timing of life-cycle events, is decisive for most organisms as it allows for life-cycle adjustments to seasonally variable environments and can strongly shape evolutionary processes (Forrest & Miller-Rushing, 2010). Genetic background and environmental cues generally act together to determine individual phenology (Danks, 2006). Variations in phenological traits could result from genetic differences and/or phenotypic plasticity (Forrest & Miller-Rushing, 2010). At the population level, both could be considered as adaptation to face heterogeneous or unpredictable environments. Populations remain genetically cohesive unless disruptive selection acts, or drastic phenological change cause

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disruption in the mating periods (Yamamoto & Sota, 2009, 2012). A shift in phenology may lead to critical asynchronies between suitable conditions and the timing of the reproductive or developmental periods, subjecting the organisms to adverse climatic conditions and/or poor resources. Alternatively, the "shifted" individuals could experience a selective advantage due for instance to reduced competition or natural enemy release, eventually compensating for a less favorable reproductive season (Maron *et al.*, 2001). Under such circumstances, a shift in the phenology may configure a new evolutionary opportunity, ultimately causing reproductive isolation due to nonselective assortative mating by time. Such a situation can lead to allochronic speciation, in which species become reproductively isolated because of distinct reproductive times, without geographical or habitat barriers, as first proposed by Alexander and Bigelow (1960). Allochronic separation has been documented for plants and animals, such as in cicadas (Ritchie, 2001), butterflies (Scriber & Ording, 2005), palm trees (Savolainen *et al.*, 2006), and birds (Moore *et al.*, 2005; Friesen *et al.*, 2007).

This work presents experimental research conducted to investigate the heritability and stability of a phenological shift, observed in a unique population of the pine processionary moth *Thaumetopoea pityocampa* Den. and Schiff. (Lepidoptera, Notodontidae), discovered in 1997 in Leiria National Forest, Portugal (Pimentel *et al.*, 2006; Santos *et al.*, 2007).

The winter pine processionary moth is a common conifer defoliator presently distributed around the Mediterranean basin and further north up to latitudes of around 48º N in Europe (Roques *et al.*, 2015). Over its wide distribution range, the moth typically presents one generation per year. The adults have a lifespan of 1–3 d and reproduce in the same, or in the following day after emergence (Zhang & Paiva, 1998). Each female lays a single egg batch, generally consisting of 100–200 eggs (Santos *et al.*, 2013) and the neonate larvae hatch about 1 month later. Larvae are gregarious and their development normally takes place during the autumn and winter, as enabled by the mild Mediterranean climate. By February–March, the larvae leave the nest forming head-to-tail processions in search of a pupation site underground, remaining in the soil until metamorphosis and adult emergence in the following summer.

Gradual variations in the phenology directly linked to the climatic fluctuations recorded along latitudinal and altitudinal ranges are known for *T. pityocampa* (Démolin, 1969). In particular, adult emergence and egg laying can occur as early as June at high altitudes and latitudes, while taking place in August, or September, at the southern limits of the species range (Battisti *et al.*, 2015). So far, phenological variations were known only for allopatric populations and for all known cases larval development occurs in the winter. Even when prolonged diapause is occasionally observed in this species, in which case the adult emergence can take place between one and a few years later, it always occurs in the summer and is synchronized with the emergence of the local next generation (Démolin, 1969).

An exceptional situation was found *ca*. 20 years ago in the region of Leiria, Portugal, where some *T. pityocampa* were found to develop following a "shifted" life cycle, in which adults emerge in the spring, starting in early (May–June) and the larvae develop throughout the summer forming the so-called summer population (SP) as opposed to the classical winter populations (WP; Pimentel *et al.*, 2006; Santos *et al.*, 2007). Several life history traits are affected by the phenological shift. In particular, as larvae are exposed to high summer temperatures, the larval growth rate is higher and the larval development time shorter in comparison with those of the winter populations. Larval development is generally completed by the end of September, when the larvae are observed seeking a pupation site. Pupal diapause then occurs in the autumn and lasts through the winter (that is, when the WP are in the larval stages), taking longer than in the WP. In Leiria National Forest, SP and WP individuals co-occur in the same stands and pine trees. Monitoring of adult activity during several years showed that SP and WP adult emergence curves never overlapped and consistently showed a "temporal gap" between the 2 populations. Moreover, population genetic data evidenced a strong differentiation between the SP and the WP in Leiria, suggesting an ongoing speciation process (Santos *et al.*, 2011a).

In the Northern Hemisphere, phenological shifts have been attributed to climate change across taxonomic groups, such as earlier bud burst or flowering time in plants, breeding seasons in birds and flight period in insects (Gordo & Sanz, 2005; Visser & Both, 2005; Cleland *et al.*, 2007; Doi, 2008). Genetic divergence resulting from disruption of reproductive time due to climatic conditions was actually observed in another winter moth (Yamamoto & Sota, 2009, 2012). Consequently, it could be hypothesized that climate change could have driven the phenological shift in *T. pityocampa* by causing some individuals to pupate earlier due to warmer autumn and eventually emerge earlier in the following year. In effect in France in years with exceptional hot autumns, such as in 2009, pupation was observed to occur as early as October in "typical" WP, while it usually occurs during the winter (Christian Burban, personal observation).

Alternatively, it may be assumed that a few individuals could have experienced in historical or ancient times a sudden shift in the phenology due to a mutation in one or more genes regulating diapause (Wadsworth *et al.*, 2013). Further, the "shifted" individuals would still have encountered suitable developmental conditions in the coastal region of Leiria. In the Mediterranean region autumn and winter are mild, which might ease switches between spring and autumn/winter development. Effectively, closely related species co-occurring in Mediterranean areas display larval development in distinct seasons. This is the case of different species of the genus *Thaumetopoea* (Simonato *et al.*, 2013) whose larval development occur either in the winter or spring, as for 2 bark-beetle sister species *Tomicus piniperda* and *T. destruens*, the first breeding in spring and the second in the autumn/winter (Vasconcelos *et al.*, 2006).

In this work we aimed to determine: (i) if the phenological differences between the SP and the typical *T. pityocampa* WPs would still be expressed under controlled conditions; (ii) whether the new phenology of the SP is genetically inherited, by analysing the dates of emergence of the offspring resulting from intra- and interpopulation crosses and assessing the heritability of the reproductive time; (iii) whether crosses between SP and WP individuals would produce hybrids and if their phenotypes and genotypes could be characterized, which would allow to detect the presence of hybrids in the field; (iv) if hybrids would present an intermediate phenology; since there is a narrow gap in the field during which no males are flying (Santos *et al.*, 2011a), hybrids could "fill the temporal gap" and eventually back cross with parental populations.

Materials and methods

Sampling sites

The 2 sympatric populations described above, namely SP and Leiria WP (hereafter LWP), were sampled from Leiria National Forest, 39º47′N 8º58′W, 30-m above sea level (a.s.l.). A second typical *T. pityocampa* WP was sampled in Apostiça (hereafter, AWP), located *ca*. 100 km south of Leiria at a similar longitude and altitude (38º34′N 9º07′W, 50-m a.s.l.). All individuals were collected from maritime pine *Pinus pinaster* Aiton trees to eliminate possible host plant effects.

Reproductive time in the laboratory

Adult emergences in the laboratory were monitored using individuals collected every year, between 2008 and 2011 in Leiria (SP and LWP) and in 2003 and 2008 in Apostiça (AWP). In Leiria, where SP and WP populations are syntopic, correct assignation of the sampled individuals to both populations was guaranteed by collecting pupae of the SP from the soil underneath the pines canopy in December/January, when the LWP was still in the larval stage inside the nests. Conversely, individuals of the LWP were collected in February as 5th instar larvae about to pupate, while the SP was already in the pupal stage, underground.

LWP and AWP larvae were placed inside plastic boxes (30 cm × 40 cm × 20 cm) with a layer of about 10 cm of sandy soil collected in the pine forest, until pupation occurred. One month after the burial of all larvae the pupae were extracted from the soil, the cocoons removed and the pupae sexed and counted. Only healthy pupae, identified by their color and consistency, were selected. In total, 1057, 1300, 1010, and 1278 pupae were obtained for SP, and 391, 983, 1125, and 1036 for LWP, in 2008, 2009, 2010, and 2011, respectively. For AWP, 2227 pupae were collected in 2003 and 391 in 2008. Pupae were kept at natural room temperature (RT), natural photoperiod and 55%–65% humidity, until emergence of the adults. The room temperature was on average 22.0 ºC (SE = 0.58).

Experimental crosses

Protocols were prepared to obtain offspring from different types of crosses performed in the laboratory, using as parental lines insects collected from SP, LWP, and AWP.

In order to obtain "hybrids" between the 2 typical WPs and the phenologically shifted SP, it was necessary to delay the date of emergence of SP individuals and synchronize it with that of WP adults in order to enable mating. For most species, it is possible to delay diapause termination under laboratory conditions through temperature manipulation (Hodek & Hodková, 1998; Denlinger, 2002). In absence of previous studies, preliminary experiments were conducted to establish successful protocols. Separate batches of SP pupae were subjected to low temperature treatments in order to delay adult emergences. In 2008, SP pupae were exposed to 3 different temperatures 15, 13, and 12 °C, from February until the emergence of the first adult in each group, to determine which thermal treatment would allow the synchronization of SP adults emergence with that of LWP and AWP.

The highest rate of emergence of SP adults (75%) was obtained at 15 ºC, however, the date of emergence was insufficiently delayed to render mating possible with WP adults. Pupae kept at 15 ºC emerged only about 2 weeks later than pupae kept at room temperature. The time shift needed was achieved both at 12 and 13 ºC. Yet, at 12 ºC only 2% of individuals survived until emergence and those were mostly unable to fully spread wings and fly. Although mortality, or abnormal emergences, still approached 50% at 13 ºC, this temperature was applied to delay the emergence of SP adults (hereafter named SPd for delayed SP) used in all hybrid crosses over the following years. Finally, for SP individuals, adults were obtained in April–May from pupae kept at RT (SP) and in July from pupae kept at 13 °C (SPd).

All experiments were performed inside large mating chambers, made of wooden frames (1.2 m × 0.6 m × 0.5 m) covered with net (mesh 0.5 mm). The types of crosses performed, described as ♀ × ♂, were (number of egg batches in brackets): (1) SP × SP (121); (2) SPd × SPd (28); (3) SPd × LWP (33); (4) LWP × SPd (123); (5) AWP × SPd (7); (6) LWP × LWP (120), and (7) AWP × AWP (9). It was not possible to obtain SP females with sufficiently delayed phenology to allow for crosses with AWP males, so that SPd × AWP crosses could not be performed.

For each type of cross, the pupae obtained as indicated above, were separated by population and sex before being introduced into the corresponding mating chamber. The well ventilated chambers remained indoors in front of large windows which were kept open, thus allowing adult flight to take place and pheromone plumes to form. Fresh branches of *P. pinaster* were placed inside the chambers, and replaced every 2–3 d, for egg laying. Wooden sticks (length 12 cm), inserted in a base, were also added to each mating chamber acting as alternative oviposition supports.

Larval rearing

The egg batches obtained from the different types of crosses were kept individually in separate vials in the laboratory, as each one corresponded to 1 full-sib family. After hatching, the neonate larvae from each egg batch were kept in acrylic boxes and fed with fresh maritime pine branches, which were renewed 3 times per week. The boxes were cleaned weekly and the larval development assessed until the 5th instar was reached. Approximately 10 cm of sandy soil, collected from the pine forest and sieved, was then added to each box to facilitate pupation. The proportion of colony mortality during larval development was very high: 77%, 93%, 85%, 94%, 86%, 94%, and 67%, respectively for crosses 1–7.

About 1 month after the burial of all larvae, the sand from each box was sieved to collect the pupae which were then stripped from their outer cocoons, counted, and sexed. Pupae from different families were kept separately and in the following year, the date of emergence of each adult was registered. The adults were kept in absolute alcohol for further genetics analyses (see below). The temperature inside the laboratory was recorded with a datalogger. The average monthly mean temperature (±SE) remained relatively stable across the years: 22.4 (± 1.10) in 2008, 21.8 (± 1.22) in 2009, and 21.9 (± 1.24) in 2010. Inside the room, which had very large windows, the photoperiod was not manipulated, so that the insect cultures were exposed to natural light conditions.

Genetic analysis

Previous studies using population genetics approaches with a limited number of markers, showed that SP and WPs were highly differentiated and could be genetically identified (Santos *et al.*, 2011a). In the present work, offspring obtained from each experimental cross was genetically characterized using recently designed microsatellite markers (Burban *et al.*, 2012) to: (a) ensure the genetic origin of each progeny analyzed, thus validating the results of the rearing procedures; (b) confirm that hybrids can also be successfully identified by genotyping.

One to two individuals per family, from 49 out of the 53 sib families that produced adult offspring (samples from 4 families could not be analyzed due to poor DNA preservation), were genotyped amounting in a total of 86 individuals. Thirteen microsatellite loci (MS-Thpit7 to MS-Thpit19) were amplified in 4 multiplex. Details of DNA extraction, amplification, and molecular protocols for genotyping are described in Burban *et al.* (2012).

Individual assignations were completed using Bayesian model-based clustering analysis with NewHybrids (Anderson & Thompson, 2002), using Jeffreys-like priors, and 50 000 MCMC simulation steps after burn-in of 10 000. The genetic frequency class file was modified to take into account the 2 parental and the F1 hybrid categories only. This method computes the posterior probability that each individual will belong to each parental lineage and F1 hybrid cluster without requiring prior information on pedigree.

Statistical analysis

A generalized linear model (GLM) was used to model adult emergence times in the laboratory, from pupae collected in the field using as dependent variable the Julian day of emergence and considering the explanatory factors population, sex, and year nested within population. Normal distribution and maximum likelihood estimates were used. The dates of emergence of the offspring from laboratory crosses were analysed by linear mixed model (LMM) considering the type of cross as a fixed effect and family as a random effect. The sources of phenotypic variance were then decomposed by fitting the following equation:

$$P_{ij} = \mu + C_i + F_j + \varepsilon_{ij}, \qquad (1)$$

where P represents the phenotypic individual observation; μ is the overall mean; C_i is the fixed effect of ith type of cross (fixed); F_j is the effect of jth family (random); and ε is the residual error. Variance components for parental family ($\sigma^2{}_p$) and residual errors ($\sigma^2{}_\varepsilon$), with the respective associated standard errors, were estimated by restricted maximum likelihood (REML). A singularity matrix was used for random components and for estimates of covariance parameters.

An estimate of broad-sense heritability (H^2) was calculated from the components of variance as the fraction of phenotypic variance that is not due to environmental effects ($H^2 = \sigma^2{}_p/\sigma^2{}_{\text{Total}}$) (Lynch & Walsh, 1998), where $\sigma^2{}_p$ represents the genetic variance due to parental families and $\sigma^2{}_{\text{Total}}$ is the phenotypic variance plus the error term.

Estimates of fixed effect, that is, cross types, were obtained and compared pairwise by least significant differences (LSD). Estimates of fixed effect for offspring development time (in days) was also compared among crosses using the LMM procedure.

Since both parents and offspring phenotypic values for the day of emergence were available, narrow-sense heritability (h^2) was also estimated (Lynch & Walsh, 1998):

$$h^2 = \frac{2\text{Cov}(P, O)}{\text{Var}(P)} = 2b, \qquad (2)$$

where Cov(P,O) represents the covariance parents-offspring, Var(P) represents the total phenotypic variance of parents, and b is the regression coefficient (slope) of offspring emergence on parents emergence day. SPSS software 21.0 was used for the statistical analysis.

Results

Emergences in the laboratory

A total of 2134, 1503, and 3599 individuals emerged in the laboratory from pupae kept at RT, originating, respectively, from Apostiça, Leiria SP, and Leiria WP (Online Resource S1). Emergence times differed significantly among populations ($z = 195154.8$, df $= 2$, $P < 0.001$). The average date of emergence for the SP was 109.6 ± 0.22 Julian days, and the emergence dates never overlapped with those of LWP (200.5 ± 0.18) or AWP (221.6 ± 0.18) (Fig. 1). Significantly earlier emergence dates were also recorded for LWP in comparison with AWP ($P < 0.001$). Also, significant variations in the dates of emergence were found among years, within populations ($z = 5403.5$, df $=$ 11, $P < 0.001$). The interaction term population × sex was significant ($z = 61.4$, df $= 2$, $P < 0.001$), with males emerging on average earlier than females for both winter populations (LWP and AWP), whereas an opposite trend was observed for the SP.

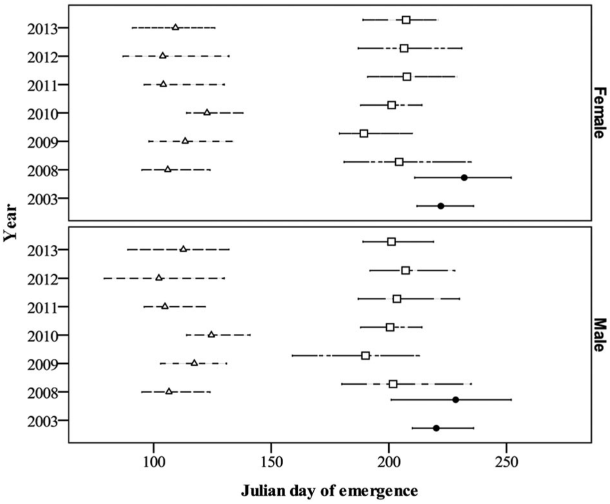

Fig. 1 Range (minimum to maximum, bars) and mean date of emergence for *Thaumetopoea pityocampa* females (above) and males (below), observed in the laboratory from pupae/mature larvae collected in the field in late winter, from 3 populations: summer population Leiria LSP △, winter population Leiria LWP □, and Apostiça AWP ● for several years of observations.

Offspring development and emergence date

From the 7 types of experimental crosses, 738 individuals originating from 53 full-sib families could be successfully reared until adult emergence in the laboratory. Emergences occurred in 2009, 2010, 2011, and 2012 from crosses performed in 2008, 2009, 2010, and 2011. The offspring from both SP × SP and SPd × SPd emerged mostly in May–June, in all years (Fig. 2). Two individuals obtained from an SP × SP cross emerged as early as the 26th and 30th of April, respectively. Most of the hybrids emerged between June and July. Yet, 3 individuals, obtained from 1 hybrid family LWP × SPd, emerged particularly early on the 27th, 28th, and 29th of April, respectively. The opposite tendency was observed for 3 individuals of another LWP × SPd family, which emerged at the end of July, while the remaining 18 individuals from the same family emerged in June. Finally, the

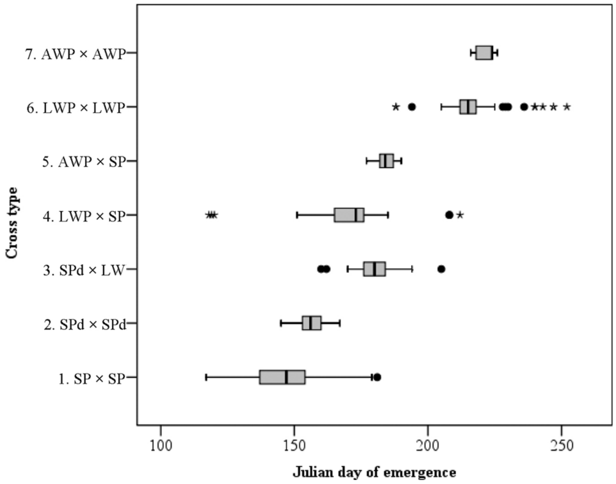

Fig. 2 Boxplot for the emergence of the offspring of *Thaumetopoea pityocampa*, obtained from 7 types of crosses (female × male) conducted in the laboratory in the previous year. SP, Leiria naturally occurring "summer" population (parents from April–May); SPd, SP adults with manipulated delayed emergence (parents from July–August); LWP, Leiria naturally occurring "winter" population (parents from July–August); AWP, Apostiça naturally occurring "winter" population (parents from July–August).

offspring from WP crosses (both LWP and AWP) emerged from mid-July to September (Fig. 2). There were no differences in the development time between males and females within each type of cross ($F_{21,127} = 0.166$; $P = 0.684$). Additional data are given in Online Resource S2.

Prolonged diapause, that is, causing emergence to be delayed by 1 year occurred in 35 individuals from 4 SP × SP families and in 5 individuals from 1 SPd × SPd family from crosses made in 2009, emergences taking place in 2011. All 40 adults emerged in early June 2011, within the expectable time period for SP × SP progeny (Table 1).

Two individuals of 1 SP × SP family, obtained in May 2011, did not undergo pupal diapause and emerged in November of the same year. The 2 individuals were genotyped and correctly assigned to SP × SP, being removed from further analysis due to their aberrant life-cycle for which no explanation can be offered.

Within each type of cross, a low variance for the day of emergence was observed. There were no significant differences in the dates of emergence between the different "F1 hybrids" (crosses types 3, 4, and 5), or between the 2 winter populations LWP and AWP (crosses types 6 and 7, Fig. 2). Therefore, for the LMM analysis and heritability estimate the hybrids were grouped together and the 2 WP crosses merged, so that the following 4 types of crosses were obtained: SP × SP; SPd × SPd; F1 hybrids, and WP × WP. This procedure further resulted in an increase of the number of families per type of cross.

Emergence dates from SP × SP, F1 hybrids, and WP × WP crosses differed significantly among them (Table 1). The hybrids emerged significantly later than the offspring from SP × SP and significantly earlier than the offspring from WP × WP (Table 1). Additionally, the dates of emergence did not differ in relation to the offspring from SP × SP and SPd × SPd (Table 1). On the other hand, the offspring from SPd × SPd emerged significantly earlier than those from WP × WP, despite the fact that these 2 crosses were made in the same period. An earlier emergence date for the progeny of SPd × SPd than for F1 hybrids was observed, however, differences were not significant.

Developmental time, that is, the number of days elapsed from oviposition until adult emergence was the highest for SP × SP crosses, being about 2 weeks longer than the expectable duration of 1 year (Table 1). Developmental times for WP × WP crosses corresponded to the expected duration of 1 year. On the contrary, for the descendants of F1 hybrids, as well as for those of SPd × SPd crosses, that is, involving parents with delayed emergence by exposure of SP pupae to 13 °C, the time of development was significantly shorter (Table 1). Even for individuals with prolonged diapause, which emerged 2 years after egg laying, the time of development was significantly shorter for the offspring of SPd × SPd by comparison to SP × SP crosses and both types of offspring emerged at similar dates (Table 1).

Heritability

The variance components estimated by LMM for the offspring of the 4 types of crosses allowed for the estimation of broad-sense heritability H^2 regarding the day of emergence, which was found to be 0.760 with 95% CI = [0.650–0.845].

Considering all crosses performed, except those including one or both parents manipulated by cold to delay emergence, that is, SPd × SPd and crosses to obtain hybrids, the regression of offspring phenotypic values on parental phenotypic values allowed us to estimate the 95% CI of the narrow sense heritability $h^2 = [0.652–0.727]$.

Experimental crosses genetic assignation

Eleven microsatellite loci were taken into account for genetic assignation, that is, after removal of MS-Thpit14 and 19, which produced ambiguous profiles. Graphical summary of the assignation of the 86 genotyped individuals is provided in Online Resource S3. NewHybrids

Table 1 Total number of offspring of *Thaumetopoea pityocampa* for each type of cross reared until adulthood, number of families, average (±SE) development time, and emergence day estimated by generalized linear mixed models.

Cross type (female × male)	Number of families	Number of emerged offspring	Development time	Emergence (in Julian days)
Without prolonged diapause				
SP × SP	28	411	396.2 ± 3.45^{a}	148.2 ± 2.40^{a}
SPd × SPd	2	57	311.7 ± 12.72^{c}	157.7 ± 8.75^{ab}
F1 hybrids	13	159	330.2 ± 5.06^{c}	172.9 ± 3.49^{b}
WP × WP	10	111	372.4 ± 5.76^{b}	217.6 ± 3.97^{c}
F			$F_{5,47} = 46.754$	$F_{3,47} = 76.279$
P value			$P < 0.001$	$P < 0.001$
With prolonged diapause				
SP × SP	4	35	765.0 ± 8.91^{a}	160.3 ± 3.00^{a}
SPd × SPd	1	5	678.0 ± 17.59^{b}	161.0 ± 5.84^{a}
F			$F_{1,2.5} = 19.449$	$F_{1,3.5} = 0.012$
P value			$P = 0.032$	$P = 0.918$

Different letters within columns indicate significant differences (Post hoc test, $P < 0.05$). SP, individuals from the summer population; SPd, SP individuals with delayed emergence; WP, individuals from the winter populations (Leiria and Apostiça).

pertinently assigned the offspring to their expected cluster, either parental SP (SP × SP and SPd × SPd), WP (LWP × LWP and AWP × AWP) or "F1 hybrids" between SP and WPs (SPd × LWP, LWP × SPd, and AWP × SPd) with a posterior probability above 0.99. It is noticeable that the presence of 2 different origins for WP parents, Leiria and Apostiça, did not distort the clustering. Genetic analyses therefore confirmed that each progeny, obtained in the laboratory, was correctly identified and the phenology of the 7 types of crosses could be further studied.

Discussion

Under laboratory conditions 2 sympatric populations of *T. pityocampa* were found to be reproductively isolated by time as indicated by consistently separated reproductive periods observed over repeated years, both for individuals collected as larvae or pupae in the field, and for reared individuals. Results are in agreement with previous field studies revealing a gap of 15–41 d between the flight periods of both populations (Santos *et al.*, 2007, 2011a). Previous phylogeographic studies showed that the 2 sympatric populations from Leiria are closely related grouping together within the western Iberian clade defined for this species (Rousselet *et al.*, 2010). Both SP and LWP share common haplotypes not present in other regions (Kerdelhué *et al.*, 2015). Still, analyzing microsatellites allelic frequencies, a strong genetic differentiation was inferred between SP and LWP (Santos *et al.*, 2007, 2011a). The genetic distances between SP and LWP from several years of sampling as well as those calculated between SP and other allopatric WPs from Portugal and Spain were always high and significant (Santos *et al.*, 2007, 2011a). Altogether results suggest that the SP originated locally from some ancestral population and that an incipient allochronic speciation process is ongoing. Allochronic speciation has been rarely documented. Still, recent study cases covering unrelated taxa showed that this mechanism of speciation might act more frequently than previously thought (Simon *et al.*, 2000; Savolainen *et al.*, 2006; Friesen *et al.*, 2007; Yamamoto & Sota, 2012).

So far the origin of the phenological change undergone by the shifted population and the mechanisms allowing its perpetuation in time has not been clarified. By manipulating pupae we could produce hybrids and demonstrate that reproductive times are, to a large extent, genetically inherited ($H^2 = 0.76$). A high heritability of the reproductive time was also observed in other organisms, such as flowering time in plant species (Johannes *et al.*, 2009), thus indicating that this speciation model may operate across different taxa.

While variation within year could originate both from phenotypic plasticity and genetic variation, important variations in the day of emergence recorded within populations among years (Fig. 1) might reflect plasticity. This could correspond to environmental annual variations of the climatic variables, such as temperature, which is known to partly rule insect development and diapause (Denlinger, 2002; Visser *et al.*, 2010). Indeed, our results showed that we could delay SP emergence by manipulating temperature during pupation, even though this caused a high mortality rate. Phenotypic plasticity was previously evidenced by monitoring males with pheromone traps in Leiria, which showed variability among years in the flight period of both populations and in the "reproductive gap,", that is, the time during which no males were trapped (Santos *et al.*, 2011a). This variability could be due to some plasticity and variation in climatic conditions, but data are still needed to explore this issue. Larval and pupal developments occur in different seasons for SP and WP. As summer and winter temperatures may vary from year to year in opposite direction, that is remain below average values in one of the seasons and above average in the other, the 2 populations may be pulled in different directions. In consequence the gap between the periods of emergence of SP and WP can either become larger or shorter, separating or bringing closer their reproductive times (Fig. 1). Therefore, it is possible that in exceptional years, some SP individuals emerging late could still mate with early WP. This would also indicate that limited gene flow may occasionally take place. The produced hybrids would in this case possibly emerge at intermediate dates and back-cross with either parental populations.

Still, even when reproductive isolation is incomplete, the genetic variation responsible for the shifted phenology, simultaneously produce nonrandom mating that may lead to speciation, even if some gene flow still takes place (Servedio *et al.*, 2011). Indeed, previous assignment tests suggested that some individuals may shift from SP to WP phenology, possibly causing some hybridization (Santos *et al.*, 2011a). In this work, hybrids were experimentally obtained in the laboratory, thus supporting the possibility that adults from the SP and WP might actually mate and produce viable progeny whenever the emergence curves of the 2 populations partially overlap. It has been previously established that SP males are attracted to female sex pheromone lures, developed, and commercialized for the typical *T. pityocampa* WP. Additionally, it has been demonstrated that the 2 populations use the same substance in olfactory sexual communication, since the composition of the SP female pheromone is identical to that used in commercial lures developed for the typical WP (Kerdelhué *et al.*, 2015). Furthermore, the hybrids emerged at intermediate times between those of the 2 parent populations, thus suggesting a form of codominance for the genes ruling this trait. The occurrence of back crosses between hybrids emerging in the field at intermediate dates, with both parental lines (either late SP or early WP adults) is thus expectable, further favoring some gene flow. Characterization of actual hybridization rates in the field is now needed. Irrespectively of which parent, male or female, belonged to either population, SP or WP, the hybrids obtained had similar developmental times and emergence dates. Results strongly suggest that maternal/paternal effects appear not to be relevant and that the reproductive times are genetically determined, remaining stable even under controlled conditions.

The Bayesian analysis of the genetic data allowed for the pertinent assignation of SP and WP individuals to their respective phenological populations, as well as for the correct identification of F1 hybrids (SP × WP). Further, the rearing procedures could be *a posteriori* controlled, as well as the classes of offspring analyzed. Similar molecular tools could be useful to address the question of the existence of hybrids in natural populations.

In general, *T. pityocampa* undergoes an obligate 1-year life cycle (Démolin, 1969), so that any shift in the time of reproduction would imply a similar shift in the next generation. In accordance, present results show that the progeny from SP × SP and WP × WP crosses completed about 1-year cycle under laboratory conditions. Yet, the progeny of delayed SPd × SPd crosses had a shorter life cycle lasting for only 10 months, thus resulting in the synchronization of adult emergences with those originated from SP × SP crosses, which were conducted about 2 months earlier. Similarly, for individuals experiencing a prolonged diapause which emerged 2 years later, the dates of emergence were similarly synchronized between SP × SP and SPd × SPd families. The fact that life cycles can be completed in less than 1 year leads us to infer that should, in a given year, extreme environmental conditions alter the timing of adult emergence, the next generation would still "return" to the typical phenology rather than originating a shifted cycle.

We do not know the underlying mechanism that prompts the emergence of SP and WP on their respective "right season." In many insects, induction of diapause is triggered by a critical photoperiod in the sensitive stage (Denlinger, 2002). However, in the present case, it does not seem plausible that the larval stage might be critically affected by the photoperiod, since SPd and SP larvae were reared at separate times of the year and yet, in the following year, synchronized emergences of the 2 populations took place. Although variations of the photoperiod could play a role in diapause termination, this cue should influence SP and WP differently. The existence of separate cues (such as photoperiod and temperature) for diapause onset, diapause termination, and metamorphosis might be plausible and need to be disentangled. Further work will be needed to investigate the physiological mechanisms regulating diapause in this insect species.

The data obtained for adult emergence in the laboratory (Fig. 1) show significant differences between the reproductive times of the 2 *T. pityocampa* WP, emergences taking place later at the warmer location of Apostiça than in Leiria in accordance with Démolin (1969) and Simonato *et al.* (2013) as well as with the results of adult trapping reported in Santos *et al.* (2011a). Yet, when the offspring was obtained and reared for a complete 1-year cycle under laboratory conditions, the time of emergence did not differ between the 2 WP types of crosses (Fig. 2). This suggests that the differences observed between the 2 WP, which are separated by 1º17′ latitude, might be environmentally determined thus corresponding to plasticity that is not expressed when the insects are fully reared under controlled conditions.

In conclusion, the *T. pityocampa* SP population offers a unique opportunity to analyze the hypothesis of allochronic speciation model through a shift in the reproductive time. Present data strongly suggest the occurrence of a genetically determined shift that might have originated from a sudden evolutionary step related to mutations in one or more genes. This hypothesis is supported by findings of Wadsworth *et al.* (2013), who demonstrated that a phenological divergence in the life cycle of 2 strains of the European corn borer, resulted from a delay in the timing of the diapause termination phase, was caused by allelic variation at a single locus. The phenological shift may further provide opportunities for ecological adaptation in response to environmental changes as life-cycle events occur in different seasons. In fact, previous works demonstrated that divergence between the 2 sympatric populations had already occurred regarding the thresholds of temperature for larval survival (Santos *et al.*, 2011b) and reproductive traits (Santos *et al.*, 2013) which might, in part, result from adaptive processes. Although the 2 sympatric populations, SP and LWP, are reproductively isolated, some hybridization might occur that may shape subsequent evolutionary processes. In effect, a previous study allowed us to hypothesize that natural hybridization between the 2 sympatric *T. pityocampa* populations may eventually occur (Santos *et al.*, 2011a). Further work is needed to understand if hybridization is naturally occurring as well as its consequences for the ongoing evolutionary process.

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Disclosure

There was no financial or other commitment that might potentially had bias our work and there is no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Online Resource S1. Total number of Thaumetopoea pityocampa adult emergences obtained from pupae collected in the field and kept in the laboratory at room temperature (22 ± 1 ºC) for the 3 studied populations.

Online Resource S2. Description for each individual offspring of the cross type, family identification, date of mating, date of emergence, sex, and development time.

Online Resource S3. NewHybrids Bayesian clustering of the offspring of Thaumetopoea pityocampa determined from microsatellite genotyping (red: parental summer population, SP; green: parental winter population, WP; blue: F1 hybrids). Individuals were obtained from experimental crosses: 1–52: SP × SP and SPd × SPd; 53–68: WP × WP; 69–86: SP × LWP and LWP × SP. WP parent(s) of individuals 65–68 and 85–86 originated from Apostiça, and all others from Leiria.