EUROPEAN JOURNAL OF ENTOMOLOGY
ISSN (online): 1802-8829
http://www.eje.cz
Eur. J. Entomol. **116**: 320–329, 2019
doi: 10.14411/eje.2019.035
ORIGINAL ARTICLE

Pupal traits and adult emergence in the pine processionary moth *Thaumetopoea pityocampa* (Lepidoptera: Notodontidae) are affected by pupal density

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Key words. Lepidoptera, Notodontidae, *Thaumetopoea pityocampa*, cocoon, gregariousness, pupal density, pupal size, emergence, *Pinus*

Abstract. The expectation is that the phenology of an herbivore is influenced by abiotic factors and its own population density during development. In this study, we investigated how the pupal density affected the pupal traits and emergence of *Thaumetopoea pityocampa* moths over a period of 3 years in two clearings in a pine forest. The pupae were larger in years when the pupal density was high and in the clearing exposed to less solar radiation. There was no relationship between the time of pupation and pupal size. Large pupae were positively correlated with an early emergence of adult moths and a longer period of adult emergence. Up to 13.9% of the pupae developed without cocoons, especially in years when they were abundant, but this did not affect the emergence of the moths. Incidence of pupal diapause was density-dependent and only occurred at a low level in dense populations. Overall, our results indicate that gregariousness confers important fitness-related advantages in this species.

INTRODUCTION

In some Lepidoptera, such as the pine processionary moth (PPM; *Thaumetopoea pityocampa* [Denis & Schiffermüller, 1775]), the pupae develop in the soil close to the trees that were inhabited by the larvae. The PPM is a univoltine species and is widespread in the Mediterranean, including southwest Europe, the Balkans and North Africa (Roques, 2015). This species is one of the most important pests of various types of pines and cedars, and can hinder the growth of infested trees (Jacquet et al., 2012). The spread of this pest into new areas requires the presence of appropriate host plants and is facilitated by climate warming or a high-population density (Hodar & Zamora, 2004; Battisti et al., 2005; Castagneyrol et al., 2016).

Pine processionary moth lays its eggs on pine needles during summer. The eggs hatch 30 to 45 days later (Aydın et al., 2018). Thus, the larvae feed throughout autumn and during winter and spring depending on temperature. The larvae are gregarious from early on in their development and spin conspicuous tents in tree tops. After completing their feeding, generally during late winter, larvae typically form a procession in which they walk head to tail in a column to reach their pupation sites on the ground. They then burrow into the soil to a depth of a few centimeters, spin a silken cocoon in which they pupate. The duration of the period from larval emergence to pupation varies from year to year and site to site, and the total period may be up to 2 months (Demolin, 1971; Buxton, 1990; Bonsignore et al., 2015; Robinet et al., 2015). The larvae select sites well exposed to sun, e.g., the edge of a forest, where the thermal conditions and/or the microclimatic conditions in the soil are favourable (Dulaurent-Mercadal et al., 2011; Samalens & Rossi, 2011). The habitat influences pupation success and survival in PPMs (Markalas, 1989; Dulaurent-Mercadal et al., 2011; Torres-Muros et al., 2017), and the pupae suffer additional mortality due to natural enemies, such as parasitoids (Bonsignore et al., 2015), predacious birds (Barbaro & Battisti, 2011) and entomopathogenic fungi (Er et al., 2007). The adults emerge during summer, but some pupae can spend 2 or more years in diapause and the adults generally disperse over short distances (Bonsignore & Manti, 2013; Roques, 2015).

Pupation has a physiological basis as it is associated with reaching a particular size (D’Amico et al., 2001; Davidowitz et al., 2003). Traits such as pupal weight or size are considered important because they can affect the longevity of females and fitness of adults (Calvo & Molina, 2005; Fantinou et al., 2008). For example, pupal size may affect male phenotype, which could influence-male mating success (Fox et al., 1995). The relationships between pupal traits and pupal density in gregarious Lepidoptera may vary according to the population dynamics of a species and are not easy to understand. Pine processionary moth infestations are not evenly distributed within pine forests and mainly occur at the edges of pine stands (Samalens & Rossi, 2011). Several studies have shown that the adults are more likely to oviposit near the edges of pine stands (Samalens & Rossi, 2011) than more than 25 m from the edges (Parlak et al., 2019). Thus, clearings within forests are suitable sites for PPMs as they create edges and open areas where their processioning larvae can pupate in sun-exposed soils. Pine forests with suitable pupation sites for supporting large aggregations of PPM occur in southern Italy (Bonsignore et al., 2011, 2015).

The relationships between pupal traits, population density and adult phenology in PPM need to be determined in order to better understand the mechanisms that determine the relationships between their development and environmental variables. Over a period of 3 years we investigated two clearings within a pine forest where pupae developed. We studied PPM from when the larvae pupated in the soil until the moths emerged. We aimed to determine whether pupal traits such as size and presence or absence of a cocoon varied in time and with population density (number of pupae in each clearing) in order to obtain a better understanding of development during the pupal stage and its effect on the adult phenology of PPM.

MATERIALS AND METHODS

Experimental site

Two pupation areas located in two clearings in a *Pinus nigra* forest in Aspromonte National Park, Calabria (South Italy) (38°04′10″N and 15°49′38″E, at 1200 m above sea level) were studied for three years (2010–2012). The vegetation in both clearings (designated as A and B) consisted of grassland and low shrubs. Area A (ca 175 m^2) had a south-easterly aspect and a slope of 5%. Area B (about 403 m^2) had a south-westerly aspect and a slope of 4%. The distance between the two sites was about 70 m and they were also separated by a dirt road between the pine trees, which limited the movement of larvae between the two areas (Fig. 1). The soil in areas A and B did not differ in terms of pH (5.8–6.0) and texture (sand 88%, silt 5–9%, clay 3–7% and total limestone 1%) and the average organic matter content in the top 15-cm layer was 22.4–26.4%.

The temperatures during the period of the study were obtained from a weather station (ARPACAL, Regional Agency for Environmental Protection of Calabria) located close to the sites studied (see Supplementary Fig. S1). Microclimatic differences between the two areas, including solar radiation, temperature and soil moisture, were recorded for one season (from April 1 to July 30, 2014, while pupae were in the soil) at an interval of 15-min at a height of 1.80 m above the ground and 10 cm deep in the

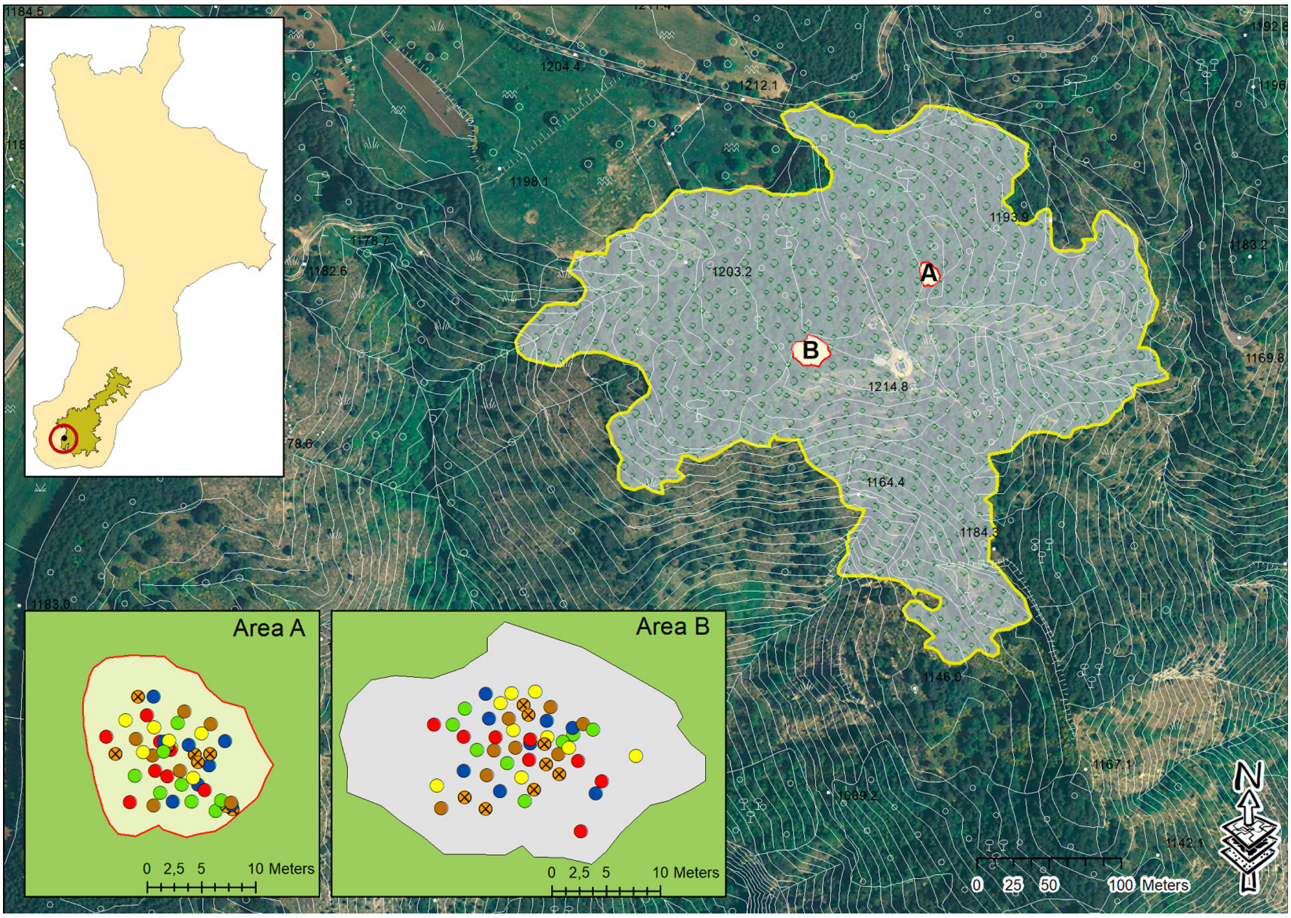

Fig. 1. Map showing the geographical locations of areas A and B in which the marked colonies pupated. Circles of different colours in boxes indicate the flags used for identifying the different pupal colonies.

soil using Micro Station HOBO® data loggers (Onset Computer Corporation, Bourne, USA). The assessment of solar radiation showed that area A was less exposed than area B. The average solar radiation (day and night) (W/m^2) for area A was 72.69 and that for area B was 193.30. Area A was cooler and moister than area B, with an average temperature of 13.95°C for area A and 14.57°C for area B (see Supplementary Fig. S2) and the soil moisture (volume of water per volume of soil, m^3/m^3) in the first 10 cm was 0.1529 for area A and 0.062 for area B.

Survey of pupation sites and pupae collection

Each year, from the last 10 days of February, both areas were monitored on a weekly basis to determine when the larvae commenced their processions to the pupation sites. When a procession was found on the ground, the point where an individual larva entered the soil was identified using a coloured identification flag (Fig. 1). The field observations were continued until the second week after the last procession was observed, which generally occurred in April or May. Pine processionary moth pupae from each of the marked colonies were extracted from the soil starting 1.5 months after the larvae buried themselves. The pupae collected may have included individuals in diapause from previous years, but this was considered a minor problem because very few individuals were reported entering diapause in a previous study at these pupation sites (Bonsignore et al., 2011). The pupae were grouped according to the colony they were in and taken to a laboratory where the numbers and presence/absence of cocoons were recorded. The pupae were then weighed individually to the nearest 1 mg (Kern EWJ 300-3) and their lengths were measured with precision digital calipers (0.01 mm resolution). Healthy pupae with no damage due to birds or mammals, or parasitized, were individually placed in labelled polystyrene containers and sealed with cotton wool. They were then inspected daily until the moths emerged. The containers housing pupae were maintained in darkness in a naturally ventilated room kept under ambient conditions at temperatures that ranged between 5.5–25°C during the study period. The containers were placed at new positions in the room each week to avoid positional effects. Field and laboratory studies were conducted wearing protective clothing in order to avoid contact with the urticating setae.

Moth emergence

The pupae were examined daily during the adult emergence period in each year (2010–2012), but only once each week during the remainder of the study period. Observations on the pupae in diapause continued until the adults emerged. After emergence, each individual was identified and sexed. To determine larval investment in cocoon production, we counted the number of pupae without cocoons and weighed 50 cocoons, which were sampled arbitrarily from the two sites in each year. We only used cocoons that contained no residue after adult emergence.

Data analysis

Pupal phenology and density

The individual measures were processed to exclude outliers based on tests of normality, where single extreme values were visualized using box and whisker plots before the tests of normality and exclusion based on standard procedures in SPSS v23 (IBM Corp., Armonk, N.Y., USA, 2015). The number of pupae retrieved from each marked colony in both clearings during the 3 years of observations was compared using analysis of variance (ANOVA).

Pupal traits and adult emergence

The pupal length and weight in each year were analyzed using Pearson's correlation coefficient. The effect of pupation date on mean pupa length was analyzed using Spearman's correlation coefficient. Pupal length and weight were analyzed independently using ANOVA. The factors included in the full factorial model included the year and clearing. Linear regression was used to determine the relationship between the mean pupal length and the total number of individuals that pupated on each date.

The percentage of pupae without cocoons was calculated for each year and compared with the density of individuals at each pupation site. For all years, the percentages were compared using a chi-squared (χ^2) test of the equality of distributions. The weights of the cocoons in different years were compared using ANOVA. The null hypothesis was that the total investment in cocoon production was independent of the number of larvae in the aggregations.

The relationships between pupal length and weight, sex, and the date of adult emergence were assessed using a CHAID classification tree analysis with Bonferroni adjusted *P*-values. This exploratory analysis allowed us to identify possible differences in the continuous data given the probable sizes of the groups relative to the emergence of adults. Due to the low numbers of adult moths that emerged after 1 or more years in diapause, we performed classification and regression tree analysis to obtain a binary tree and verify the relationships between pupal size and the date of adult emergence.

All analyses were performed using SPSS v23 (IBM Corp., Armonk, N.Y., USA, 2015).

RESULTS

Pupal phenology and density

The time of pupation was early (March and April) and synchronized in the first two years of the investigation and occurred later (April and May) in 2012, when lower temperatures were recorded in January and February (Fig. S1).

The total number of pupae decreased greatly during the study period (Table 1). In 2010, 3634 healthy pupae, some with and some without cocoons, were used in subsequent experiments. Most of the remainder were damaged or showed signs of fungal infection. In 2011 and 2012, the numbers of healthy pupae were 2018 and 646, respectively. The number of pupae retrieved from the marked colonies decreased significantly over time with no differences between areas (Table 1). Similarly, the number of healthy pupae used to determine when the moths emerged from each marked colony decreased from 78.98 (SE 14.49, n = 98) in 2010 to 43.30 (SE 10.49, n = 99) in 2011 and 17.78 (SE 3.06, n = 96) in 2012.

Table 1. Mean number of pupae retrieved from each marked colony in each year and study area. N – number of marked colonies; No. tot – total number of pupae; h.p. % – percentage of healthy pupae. Different letters indicate significant differences among years and areas [univariate analysis test, year (F = 14.293, *P* < 0.001); area (F = 0.042, *P* = 0.837), N = 293)].

Year	Area	Mean	SE	N	No. tot	h.p. %
2010	A	131.8 a	18.36	42	12,662	28.70
	B	129.2 a	21.91	56		
2011	A	89.1 b	8.91	49	8,358	24.14
	B	81.6 b	10.95	50		
2012	A	55.1 c	6.08	48	5,440	10.88
	B	58.3 c	6.00	48		

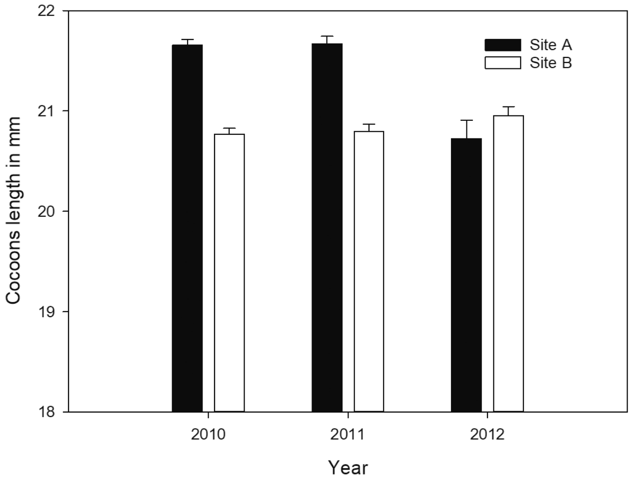

Fig. 2. *Thaumetopoea pityocampa* pupal lengths (+SE) in each year and pupation area (A and B).

Pupal traits and adult emergence

Pupal length and weight were positively related in each year (2010: r = 0.738, n = 3636, $P < 0.001$; 2011: r = 0.762, n = 2017, $P < 0.001$; 2012: r = 0.552, n = 646, $P < 0.001$). There was no correlation between burial date and average pupal size (2010: $r_s = 0.029$, n = 6, $P = 0.957$; 2011: $r_s = 0.257$, n = 6, $P = 0.623$; 2012: $r_s = 0.486$, n = 6, $P = 0.329$). Overall, the pupae were larger in 2010 and 2011 than in 2012 ($F_{2,\ 6297} = 5.195$; $P = 0.006$) (Table 2). There were also differences in pupal size between areas A and B in both 2010 and 2011, but not in 2012 ($F_{1,\ 6297} = 33.438$, $P < 0.001$, Fig. 2). An interaction was found between year and site ($F_{2,\ 6297} = 10.195$, $P < 0.001$), in which the pupal length decreased over time at site A and increased over time at site B.

Only in area A was there a linear relationships between pupae length and pupal density on the date of burial (Fig. S3). The weight of the pupae in different years and different areas matched the differences in length, with heavier pupae in 2010 and 2011 (Table 2).

We found that 13.9% (n = 679) of the pupae lacked cocoons in the three years of the study, i.e., 17.45 ± 3.1% in 2010 (total = 3633), 11 ± 1.36% in 2011 (total = 2018), and 3.25 ± 1.37% in 2012 (total = 646). The χ^2 test detected significant differences among years ($\chi^2_{2,\ 6297} = 113.476$, $P < 0.001$). The distribution of adult emergence times from the pupae without cocoons were comparable to those from the pupae with cocoons and they showed very similar trends (Fig. 3). The cocoon weight did not differ among years (ANOVA: $F_{2,146} = 0.747$, $P = 0.476$), with an average of 28.3 ± 2.6 mg in 2010, 24.1 ± 1.9 mg in 2011 and 26.12 ± 1.4 mg in 2012.

Table 2. Average pupal lengths in mm (± SE) in 2010, 2011 and 2012. Mean fresh weight (g) in each year and study area (A, B). N = number of pupae. [Univariate analysis test, year (F = 184.943, $P < 0.001$); area (F = 31.995, $P < 0.001$); year* area (F = 7.721, $P < 0.001$)].

Year	Area	Mean length	SE	Mean weight	SE	N
2010	A	21.2	0.04	0.53	0.004	1868
	B			0.50	0.004	1765
2011	A	21.2	0.05	0.59	0.006	992
	B			0.58	0.006	1026
2012	A	20.9	0.08	0.46	0.018	156
	B			0.40	0.007	490

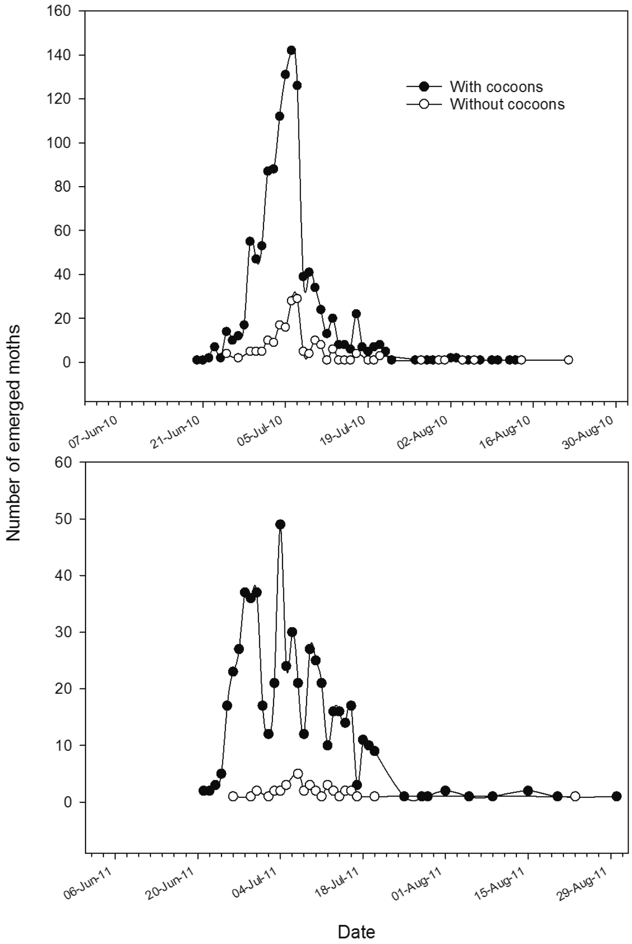

Fig. 3. Times of adult emergence from pupae with and without cocoons during summer in 2010 and 2011.

Large pupae (in length or weight) produced moths earlier, which were present for longer in 2010 (two groups identified) and 2011 (three groups identified), but not in 2012 (zero groups identified) (Table 3; Supplementary Table S1; and S5 show the complete statistics). In addition, those moths that emerged from large pupae after a long diapause emerged earlier than those from small pupae (Table 3). A 2-year long diapause was recorded for the 2010 cohort and 1-year diapause for the 2011 cohort, with percentages of 1.53% and 0.33% in 2010 and 2011, respectively, but no diapause was recorded for the 2012 cohort (Table 3).

DISCUSSION

The adaptive advantages of gregarious and collective behaviour are well documented for many Lepidoptera, in which they are associated with feeding facilitation in some instars and better direct or indirect defense against predators (Costa & Pierce, 1997; Denno & Benrey, 1997; Hunter, 2000; Ronnås et al., 2010; McClure & Despland,

Table 3. Classification accuracy for pine processionary moth pupal length when the date of adult emergence and sex were used as independent variables. The results were obtained by running a single decision-tree algorithm, which was adapted for the pupal length data set (growing method: CHAID with Bonferroni adjusted *P*-values).

Cohort	Adult emergence	N	Pupa length (mm)	F	P	Sex	N	Pupa length (mm)	F	P
2010	≤ 05-Jul-10	712	21.68	24.9	< 0.001	Female	319	23.15	362.6	< 0.001
						Male	393	20.50		
	> 05-Jul-10	642	21.06			Female	273	22.70	355.6	< 0.001
						Male	369	19.84		
2011	≤ 30-Jun-11	193	21.89	11.9	< 0.001	Female	84	23.57	105.6	< 0.001
						Male	109	20.59		
	30-Jun-11 to 09-Jul-11	231	21.28			Female	117	22.88	230.6	< 0.001
						Male	114	19.65		
	> 09-Jul-11	179	20.73			Female	77	22.33	150.2	< 0.001
						Male	102	19.53		
2012		51	21.08							
2011 (2010 cohort)	≤ 07-Jun-11	10	21.18				1	24.04		
	> 07-Jun-11						9	20.86		
2012 (2010 cohort)	≤ 20-Jul-12	11	21.457				8	21.97		
	> 20-Jul-12						3	20.09		

2011; Despland, 2013). In this study, for the first time, we showed that a decrease in the pupal density over successive years at two pupation sites was associated with a decrease in pupal size (Fig. 2 and Table 2). Pérez-Contreras et al. (2003) identified a minimum threshold density for *Thaumetopoea pityocampa* larval colonies, but did not determine a proportional relationship between density and body size. The hypothesis that group size can enhance the feeding activity of larvae and increase their growth over time has been confirmed in other species of Lepidoptera, which also form aggregations during the larval stages, such as *Euselasia chrysippe* (Lepidoptera: Riodinidae) (Allen, 2010).

Aggregation behaviour of larvae of Lepidoptera is frequently recorded and often starts with the deposition of eggs in clusters (Courtney, 1982; Hunter, 2000). The general benefits identified in previous studies include improved conditions and possible cooperation between larvae during feeding, as well as enhancing the water balance during the aggregative phase (Klok & Chown, 1999; Bryant, Thomas & Bale, 2000). Pimentel et al. (2010) propose that a large colony is very important for PPM larvae at high latitudes in providing an obvious advantage via thermoregulation and tent building in areas where larvae have to survive harsh conditions during winter. The thermal advantages reported for some caterpillar species are unlikely to be important for pupae of PPM in the Mediterranean area. In addition, gregariousness is maintained during the selection of the pupation site, which may indicate that there are other benefits of living in large groups, such as facilitating soil penetration and better protection from enemies, or bringing the sexes together after emergence to facilitate the reproductive success of the species. Linear relationships between pupal length and population density on each burial date were only recorded in area A (Fig. S3). This requires confirmation based on investigations over longer periods because the high pupal mortality in both areas reduced the number of individuals measured and thus the relationship between colony size and pupal size also needs to be verified. In the areas sampled, the population was declining following a previous outbreak (Bonsignore, unpublished data).

The larger pupae in the clearing where the incidence of solar radiation was lower and soil humidity higher is difficult to explain because this insect clearly performs better in warm and dry conditions (Roques, 2015). However, the study site is located at the southern edge of the continental range for this species and the environmental conditions differ from those in the northern range where most previous studies were conducted.

The time of larval burial was fairly well synchronized in the first 2 years of the investigation, but the first processions were detected later in 2012 and taking longer to reach maturity did not affect the size of the pupae. Similarly, there is no relationship between development time and pupal mass in *Malacosoma disstria* Hübner (Lepidoptera: Lasiocampidae) a species in which pupation occurs after exceeding the size threshold (Etilé & Despland, 2008). The patterns in the variability in the development time and pupal size were shaped by the requirement for reaching a threshold size before pupation and thus there was no relationship between development time and the size that the pupae reached.

Large body size in adult insects of the same species of Lepidoptera is associated with high fertility (Kamata & Igarashi, 1995; Allen, 2010), greater mating success (Bonsignore & Jones, 2013) and a higher capacity for dispersal (Trochet et al., 2013). That adults started to emerge earlier from large pupae may also be considered in this context and our results confirm those of a previous 3-year study (Bonsignore & Manti, 2013); thus, the earlier emergence in high density years could have been due to better performance and larger body size. The same results were also obtained for the pupae that entered diapause in 2010 and 2011. Our previous study carried out over the period 2003–2005 (Bonsignore & Manti, 2013) reports a high incidence of flight associated with a high population density, which is supported by the current results. Relationships between pupal size and time of adult emergence have not been reported previously in PPM and the differences in the time of adult emergence are related only to climatic factors with a general advance in flight with increase in altitude (Bonsignore & Manti, 2013). Understanding the highly complex relationships between temperature, size and individual fitness will require further study of systems in specific ecological contexts (Kingsolver & Huey, 2008). The gregariousness of this species during the final juvenile stage has direct implications for the phenology of PPM and when adults are present in spring, but not the thermoregulation of aggregations of moth pupae.

We did not measure the dispersal abilities of early-emerging adult moths in this study. It is possible that a high capacity for dispersal could be an evolutionary advantage in this species in contributing to its greater persistence over time.

Another interesting aspect of pupal size is its possible link with diapause. The few results indicate that the individuals that entered diapause tended to be larger than those that did not, possibly because of the higher metabolic cost of diapause (Danks, 1987). The evidence for density-dependent induction of diapause in PPM is of ecological interest. At a high density, some PPM pupae may enter a "reserve" of individuals in diapause that will emerge in following seasons. Thus, the initiation of diapause in PPM may be driven by density-dependent processes and result in a reserve of individuals when the population is below a certain density (Salman et al., 2016, 2019). In this study, the pupae that produced adults several years later (although stored in a laboratory) characteristically emerged between June and July. This synchronization could facilitate mating. Unfortunately, the frequency of diapause was too low at the study site for further analysis.

This study provided an opportunity to identify and evaluate the incidence of PPM pupae without cocoons. Indeed, a significant number or pupae without cocoons may imply a reduced biological cost of pupation. There are no known implications of this trait in the life history of moths. The adults that emerge from pupae without cocoons may exhibit differences in traits associated with fitness, such as a greater capacity for flight and dispersal (for further details, see Davis & Stamps, 2004), or the possible preservation of memory by moths throughout metamorphosis (Blackiston et al., 2008). In cold environments, silk cocoons play important roles in the development of pupae or they may provide individuals with the ability to cope with environmental changes (Guerra & Reppert, 2017), but these effects may differ in very similar species (Lyon & Cartar, 1996). In high population density conditions, there may be a relationship between thermoregulation and heat dissipation during pupation. The PPM may reduce its investment by not forming a cocoon, especially in the largest clusters, as also reported by Tagawa & Satoh (2009) in some species of parasitoids. In group-living Lepidoptera, such as the forest tent caterpillar (*Malacosoma disstria*), a reduced investment in silk is advantageous for young larvae (Despland & Le Huu, 2007). The relationships between the benefits of exogenous silk production and investment in silk cocoons in PPM require further investigation.

CONCLUSION

The relationship between pupal size and the emergence of adults tended to favour the development of large adults because it increased the period over which the adults emerged. The safety benefits of living in large larval aggregations may have reduced the cost of investment in cocoons, and gregarious behaviour also had other effects that facilitated the presence of PPM in the pine forests, which may help to explain the success of this species in Mediterranean areas.

ACKNOWLEDGEMENTS. We thank the National Park of Aspromonte for providing support during monitoring and for supporting the financial cost (decision number 270 of the 07.ix.2012 to C.P. Bonsignore). The authors confirm that they have no financial interests to report and that the experiments adhere to the animal ethics requirements under Italian law.

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Received July 4, 2018; revised and accepted September 23, 2019

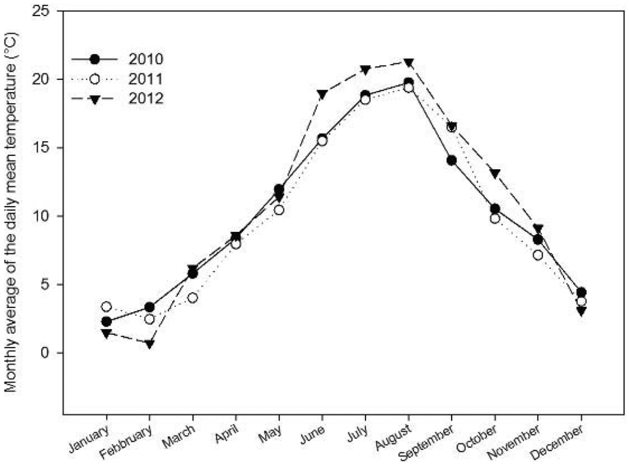


Fig. S1. Monthly temperature recorded in the area of the study over the three years of the investigation.

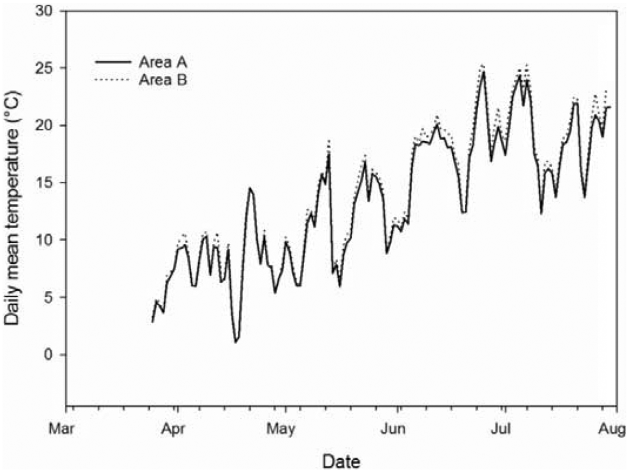


Fig. S2. Mean daily temperature from April to July 2014 in the two areas monitored (Area A and B).

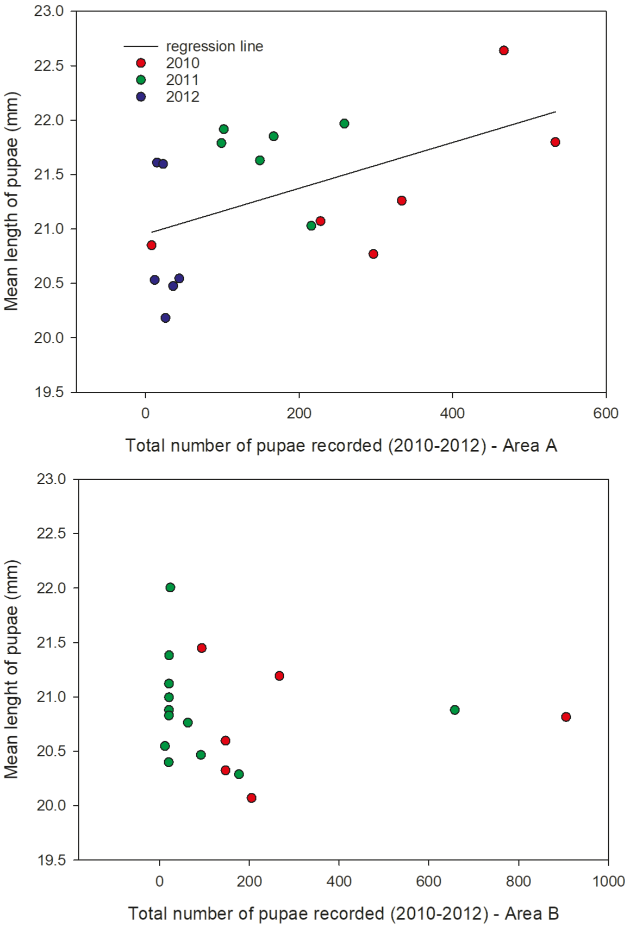


Fig. S3. Relationship between mean pupal length and total number of larvae in the group that pupated on the same date. Equation fitted to data only for area A: pupa length = f = y0+a*x; y0 = 20.9528 (SE 0.2021); a = 0.0021 (SE 0.0009); R = 0.5122; Rsqr = 0.2623; F = 5.69; *P* = 0.0298, N = 17.

Table S1. Classification accuracy for pine processionary moth pupal weight, when date of adult emergence and sex were used as independent variables. The results were obtained by running a single decision-tree algorithm, which was adapted for the pupal weight dataset (growing method: Chaid with Bonferroni adjusted p-values).

Cohort	Adult emergence	N	Pupa weight (g)	F	P	Sex	N	Pupa weight (g)	F	P
2010	≤ 08-Jul-10	905	0.551	7.52	0.08	Female Male	381 524	0.699 0.443	1453.57	< 0.001
	> 08-Jul-10	213	0.52			Female Male	388 125	0.659 0.422	315.83	< 0.001
2011	≤ 30-Jun-11	193	0.661	16.109	< 0.001	Female Male	84 109	0.825 0.535	243.25	< 0.001
	30-Jun-11 to 09-Jul-11	231	0.603			Female Male	117 114	0.748 0.422	670.71	< 0.001
	> 09-Jul-11	179	0.558			Female Male	77 102	0.709 0.449	289.01	< 0.001
2012		51	0.532							

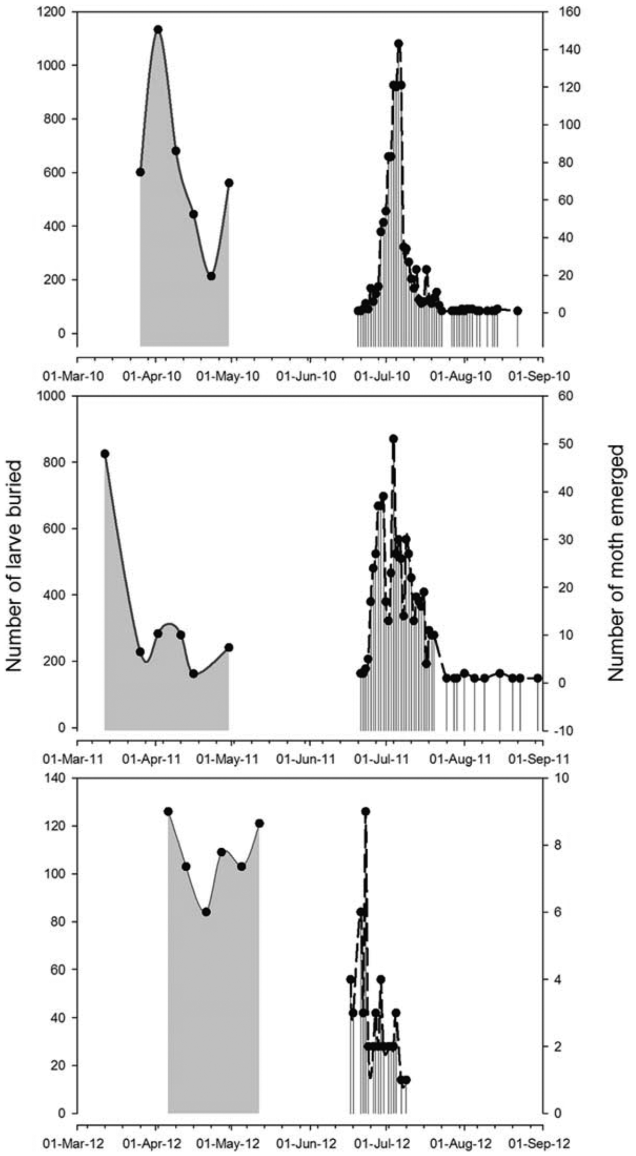
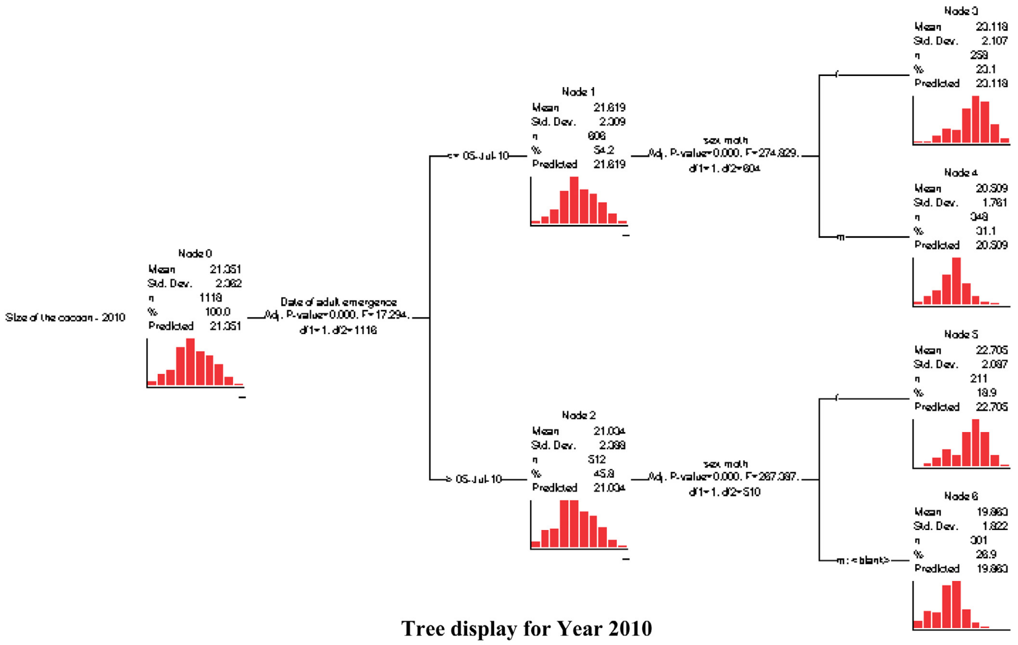
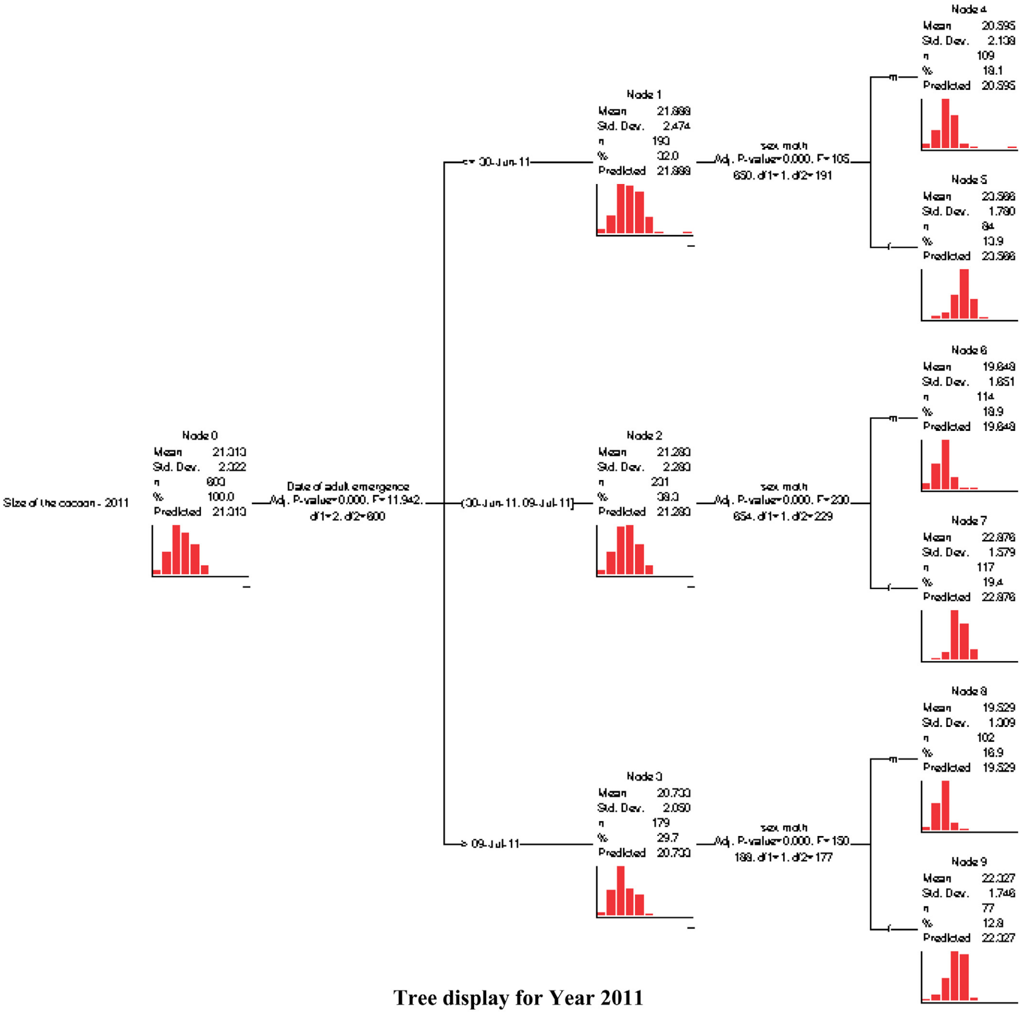


Fig. S4. Examples of the times of larval burial and adult emergence (site A, B, years 2010–2012).

Fig. S5 (on next page). Tree displays of adult emergence according sex and length of pupae.



Tree display for Year 2010



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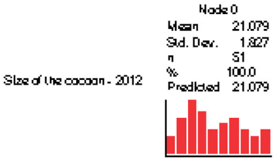

Tree display for Year 2012