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Diapause in the pine processionary moth (*Thaumetopoea pityocampa*): ecological significance

Diapausa nella processionaria del pino (*Thaumetopoea pityocampa*): importanza ecologica

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To Api, without your patience I could not continue.

To Battisti-family, you never made me feel away from home.

Contents

SUMMARY

Pine processionary moth (PPM, *Thaumetopoea pityocampa*) is an active range expanding pest species having significant economic impact in terms of tree growth and public health. A lot is known about its natural history although one important aspect of its life history, diapause, has been so far neglected in spite of its acknowledged importance in pest establishment and dynamics. A few questions related to diapause have been addressed in this PhD project.

Although the existence of prolonged diapause (PD) was reported long ago, no study provided its maximum duration. We provide evidence of existence of at least 7 years of PD in one mountain population of PPM, and establish the importance of PD individuals in the sustenance of population density. This study underscores the importance of long-term surveillance of population for understanding the dynamics of PD.

Although several studies have been conducted on the incidence of PD in PPM across it natural range, they were often published as forest service or forest health reports and not available to the large public. A collection of these reports, their validation and comparison with data from papers allowed to make a synthesis that included most of the pest range, including the sibling species *Thaumetopoea wilkinsoni* in the Near East. We suggest that winter conditions at the larval stage influence this decision. Data analysis suggests a strong U-shaped relationship between the incidence of PD and average winter temperature and a linear positive relationship between mortality and proportion of individuals in PD. The result contributes to the advancement of knowledge on the ecology, population dynamics and distribution of the PPM.

Previous phenological models of PPM lack the description of dormant prepupal stage and its significance in synchronization of emergence. Besides, no study reported on the occurrence of diapause in PPM in a stage other than pupa. We show how with a weekly sampling during the two-month long procession period of pine processionary prepupae, it was possible to discover that prepupae differentially regulate their development time in such a way that moth emergence of short-lived adults resulted to be concentrated and synchronized in less than one month. Early descending individuals don’t pupate immediately, rather wait as diapausing prepupae for their late counterparts, whereas late-descending prepupae develop into pupae much faster. The finding of prepupal diapause and its significance in synchronization of emergence may improve the existing model of phenology and provide managers with a new tool to handle this pest.

Diapause termination mechanism in both univoltine and prolonged diapausing individuals of PPM has been previously hypothesized based on the occurrence of a key period during the pupal stage. By using three proxies of metabolic activity, such as body temperature, O_2 consumption, and weight loss, we confirm the hypothesis of existence of a key period (termination) in univoltine and prolonged diapausing individuals of PPM. This finding is a starting point for the study of diapause development in PPM from an ecological point of view.

Compared to simple phenomenon of diapause, field of prolonged diapause in insects per se is not vast. A lot of questions regarding the regulation of prolonged diapause are still unresolved. When we consider PPM, the gap of knowledge in this regard is gigantic. Being an important pest of Mediterranean forests, PPM deploys diapause for its success. We know almost nothing about the regulation of diapause in this species. Despite that, this thesis attempted to start the effort of answering a few of thousands of questions on the regulation of diapause in PPM. This project has prepared the ground for several possible future works. Among them, some are: testing the findings in other populations, understanding genetic regulation of diapause and prolonged diapause, understanding the importance of climate change in regulating diapause, improving phenology models and using for predicting the effects of climate change under different scenarios.

RIASSUNTO

La processionaria del pino (PPM, *Thaumetopoea pityocampa*) è una specie in fase di espansione dell'areale e che causa perdite importanti di crescita degli alberi nonché problemi sanitari all'uoma e altri animali. Nonostante molti studi siano stati condotti su questa specie, alcuni aspetti relativi alla diapausa sono stati finora trascurati anche se riconosciuti come importanti per l'affermazione della specie. Questa tesi si pone l'obiettivo di chiarirne alcuni.

La presenza di diapausa prolungata (PD) in *Th. pityocampa* è nota da tempo ma mancano dati precisi sull'effettiva durata del fenomeno. Con questo studio si è potuto dimostrare che in una valle alpina al limite dell'areale la diapausa si estende fino a 7 anni, con ripercussioni significative sulla dinamica di popolazione. Ciò indica la necessità di una sorveglianza estesa delle popolazioni dell'insetto.

Nell’areale della processionaria del pino sono stati svolti vari studi in cui la presenza di diapausa è stata accertata e quantificata, e alcuni di questi sono stati inclusi in rapporti interni di difficile reperibilità. L’accesso a questa informazione e la sua verifica puntuale hanno consentito di produrre una sintesi dei risultati, includendo anche la specie sorella *Th. wilkinsoni* diffusa in Asia Minore. I risultati mostrano che un fattore importante è rappresentato dalle temperature invernali cui sono esposte le larve. E’ stata infatti trovata una relazione a U tra temperatura invernale e frequenza della diapausa, associata a una maggiore mortalità per gli individui diapausanti. Tale risultato rappresenta un passo in avanti nella documentazione dell’effetto dei fattori ecologici sull’ecologia e sulla dinamica dell’insetto.

I modelli di sviluppo disponibili per la processionaria del pino non hanno mai considerato lo stadio di prepupa e l’esistenza di una quiescenza/diapausa che porti a una migliore sincronizzazione degli sfarfallamenti. Grazie a un campionamento settimanale durante il periodo delle processioni di interramento è stato possibile accertare che il periodo di discesa è lungo circa il doppio rispetto allo sfarfallamento, e che le prepupe presentano una diapausa mirata a sincronizzare l’uscita degli adulti e quindi gli accoppiamenti. Il meccanismo si basa su un arresto dello sviluppo nei primi individui che scendono al suolo, che si riduce progressivamente durante il periodo. Queste nuove conoscenze sono di notevole importanza nella definizione di modelli di sviluppo e di previsione delle popolazioni dell’insetto.

La conclusione della diapausa pupale in individui univoltini o in diapausa prolungata è stata associata alla presenza di un periodo chiave durante il quale l’individuo decide se proseguire nello sviluppo ad adulto o se rimanere in diapausa. Grazie a misure di alcune variabili non distruttive (temperatura superficiale, consumo di ossigeno, peso corporeo) è stato possibile mettere in evidenza per la prima volta l’esistenza di tale periodo in individui mantenuti a varie condizioni ambientali. Questo risultato rappresenta un enorme passo in avanti nelle conoscenza sulla regolazione della diapausa in questa specie.

Nonostante la diapausa degli insetti sia in generale ben nota, le informazioni sulla diapausa prolungata sono scarse e molte domande giacciono irrisolte. Nel caso specifico della processionaria del pino questo ritardo è notevole e allo stesso tempo importante per la regolazione della dinamica di popolazione e quindi dei danni a piante e animali. Questo lavoro contribuisce a chiarire alcune aspetti importanti e apre la strada a studi mirati a conoscere la regolazione genetica della diapausa, gli effetti del cambiamento climatico, e lo sviluppo di modelli in grado di prevedere con affidabilità l’andamento demografico.

CHAPTER 1

Introduction

Background and definitions

Surrounding environment of insects is full of uncertainties. As such, they use “if-else” statements along with continuously debugging decisions, like computer programmers do, throughout their life cycle. Understanding such evolutionary solutions of insects to various ecological problems has been long-standing thirst for biologists. Among the problems faced ecologically by insects, seasonality accounts for a large set of problems. Therefore, study of seasonality in invertebrates has been an important field of search for biologists.

At the beginning, temperature was assumed to be the main factor of seasonal activity and development of animals and plants. Afterwards, scientists started thinking on daylength to be the key factor of plant development. Soon after Garner and Allard (1920, 1923) invented the term photoperiod and photoperiodism, other scientists started finding the similar occurrence in other life forms including insects (see for insects: Marcovitch 1923, Sabrosky et al. 193, Kogure 1933, Müller 1960, for birds: Rowan 1926). Photoperiodism could explain so many features of plants and animals that Danks (1987) wrote in his book referring to that period, “Photoperiodism captured the imagination of the scientific world....”. However, physiologists afterwards discovered the role of hormone in diapause. Although, huge excitement on insect diapause over the decades of 1960s and 1970s resulted vast information on hormonal and environmental aspect of diapause, soon after, the field “entered into diapause” (Denlinger 2008). Subsequently, breakthroughs in molecular biology widened scope for discussing evolutionary and genetic aspect of diapause, along with evolution of life cycles in insect (Danks 1987).

Dormancy was a common term to describe inactivity in the animals. Thinking evolved with the observations, and scientists started to think about the difference between mere inactivity for low temperature and other types of arrests. Wheeler used the term “diapause” for the first time in 1893. Thereafter, the umbrella term dormancy was divided into quiescence and diapause. Early researchers thought the diapause as total arrest of development. But, again observations and thought changed with time. Considering the observation that diapause is a state of total inactivity, rather a dynamic stage, Andrewartha (1952) wrote his landmark paper. He called that state as “diapause development”. Over time, scientists conceded that diapause stage is not an arrested state, rather a stage of suppressed development. Nevertheless, owing to different physiological and ecological nature of the stage in different insects, scientist defined their own terms for different phases of diapause development. Several attempts were made to define those phases (Tauber et al. 1986; Danks 1987; Hodek 2002). Last among those attempts was the paper by Košťál (2006), where he standardized the terminologies for successive eco-physiological phases of diapause (see chapter 5). He defined dormancy as a generic term that covers any state of adaptive suppressed development, generally accompanied with metabolic suppression. Quiescence and diapause in his paper was defined as:

Quiescence: "An immediate response (without central regulation) to a decline of any limiting environmental factor(s) below the physiological thresholds with immediate resumption of the processes if the factor(s) rise above them."

Diapause: "A more profound, endogenously and centrally mediated interruption that routes the developmental programme away from direct morphogenesis into an alternative diapause programme of succession of physiological events; the start of diapause usually precedes the advent of adverse conditions and the end of diapause need not coincide with the end of adversity."

Though the study of diapause stems mainly from the curiosity to unveil the mystery of the world, scientists and technologists soon started to find out practical implication of the knowledge (Danks 1987). Denlinger (2008) wrote an excellent entry on the potentiality of studying diapause. He argued that diapause is not only necessary for understanding life history of insect, but also potential tool for inventing new technologies, in addition to provision of fundamental insights into critical issue in development. The areas he underscored as the direct benefit of diapause researches comprised population modelling, manipulation of diapause for control, exploitation of diapause traits for pest management, managing domesticated species, increasing shelf-life and utility of biological control agents, cryopreservation, foundational studies of insect hormones, role in developmental biology, links to aging research, model for obesity, role in disease transmission, and pharmacological prospecting. Danks (1987) also exemplified few prospects of diapause research in his monograph on insect dormancy (see page 2).

Regulation of diapause

Diapause is regulated at several levels. Interaction of environment and genetic pre-programming combines with physiological process. Fortunately, we have excellent entries on the environmental (Tauber et al. 1986, Danks 1987), hormonal (Denlinger et al. 2005), and genetic (Denlinger 2002) regulation of diapause. In addition, Saunders et al. (2002) discussed in detail the role of photoperiod in the regulation of diapause.

Seasonality is very important for the diapause of temperate insect species. Some physical environment factors regulating diapause are photoperiod, thermoperiod, temperature level, moisture, sunshine, and wind. Non-physical environment cues include occurrence of other organisms, food, and metabolic state of host (Tauber et al. 1986, Danks 1987). Interaction among factors is also common and worth considering. Nevertheless, photoperiod seems to be the most reliable cue for the diapause across the insect taxa (Danks 1987, Saunders et al. 2002).

Apart from environmental regulators, several key hormones are involved in the regulation of diapause. Those important are juvenile, diapause, and prothoracicotropic hormones (Denlinger et al. 2005). Moreover, works on the molecular regulation of diapause are producing significant insights into the dynamic process of diapause, such as changes in the expression patterns throughout the development of diapause (Denlinger 2002, Yocum et al. 2011).

Prolonged diapause

Emergence after diapause is not always restrained in the same reproduction year. Cases are common where insects remain in the diapause for more than one adverse reproductive season. Different authors have named this phenomenon differently, e.g. prolonged diapause, extended diapause, perennial diapause, superpause, super diapause, remanance, überliegen etc. (Danks 1987; Saulich 2011). Powell (1986) reported cases of such prolonged diapause (henceforth PD) in Lepidoptera. Danks (1987) adequately enlisted in his monograph many species that enters PD. Saulich (2011) reported over 150 PD species from different taxa. PD has been identified as one of the four mechanisms for long life cycle in insects (see review by Danks 1992 and Saulich 2011). Although the length of PD varies across species, common length is around 2 to 3 years. Yet, it may extend to more than a decade in extreme cases (Danks 1987, 1992; Hanski 1988). Powell reported the longest case: 30 years of prolonged diapause in some yucca moth larvae.

PD is a characteristic of numerous phytophagous insects with wide fluctuations of abundance and periodical outbreaks (Danks 1992; Saulich 2011). The number of PD pronymphs of the European pine sawfly varied not only from season to season, but also in different age of planted pine stands. Older stands provided more favourable condition for PD than younger plantation (Minder 1973). Unfavourable diet (old needles or spring pine shoots) was found to be more favourable than usual diet (previous year pine needles) for entering PD (Stadnitskii 1964).

Characteristics of prolonged diapausing individuals

Intensity of respiratory metabolism during PD was found to be 10-40 times less than during active state in Colorado potato beetle (Ushatinskaya 1983). Loss of weight in *Chrysopa regalis* and *Chrysopa dorsalis* after PD was found to be less than that of after hibernation (Volkovich 2007). Body mass of prolonged diapausing pronymph of the spruce seed moth *Cydia (Laspeyresia) strobilella* (Nesin 1984) and older instar larvae of the fungus weevil *Exechesops leucopis* (Matsuo 2006) was higher. Pale pupal cuticle was used as an indicator of PD in pharate adults of *Barbara colfaxiana* (Sahota et al. 1985). The literature of fitness between individuals with PD and without PD is contrasting across different species. For example, Saulich (2011) stated that females of Colorado potato beetle after PD had level of fecundity similar to one-year diapausing females, but Wang et al. (2006) found the female of *Colaphellus bowringi* with PD to be more fecund than their regular diapausing counterparts. Nevertheless, lower fecundity and higher mortality in the individuals with PD have also been reported (see for example, Sullivan and Wallace 1967, Sims 1983). Despite insufficient study conducted on PD, available data advocate that PD differs from annual diapause in a deeper level of metabolism suppression as a means of increasing chance of avoiding adverse external conditions (Saulich 2011).

Regulation of prolonged diapause

Most of the species show decreasing pattern emergence in the successive years, where most of the individuals emerge after only one winter or summer (for example, the sawfly *Gilpinia hercyniae* (Prebble 1941), the blueberry maggot *Rhagoletis mendax* (Lathrop and Nickels 1932), the walnut husk fly *Rhagoletis completa* (Boyce 1931), the gall midge *Contarinia sorghicola* (Baxendale and Teetes 1983), and the cabbage beetle *Colaphellus bowringi* (Wang et al. 2006). However, Barnes (1958) reported anomaly of the previous case in gall midge *Sitodiplosis mosellana.* This uncertain pattern of emergence was assumed to be because of response to environmental cues (Danks 1987): abnormally hot dry summer after the first year in the apple maggot *Rhagoletis pomonella* (Allen and Fluke 1933), cool summer temperature in the larch sawfly *Pristiphora erichsonii* (Drooz 1960), low temperature and high humidity in eonymphs of *Neodiprion sertifer* (Minder 1981), density in the same species (Eichhorn 1983), lower temperature in laboratory experiment but low cone crop in the following year in field experiment in cone moth *Barbara colfaxiana* (Hedlin et al. 1982), year with lower cone crop production in several species of cone and seed feeding insects (Hedlin et al. 1981), photoperiod in *Neodiprion abietis* (Wallace and Sullivan 1974), photoperiod and temperature in *Neodiprion* *sertifer* (Sullivan and Wallace 1967), and light sandy soils in *Leptinotarsa decemlineata* (Ushatinskaya 1966, 1972). Moreover, prolonged diapause may be induced in the Swaine jack pine sawfly *Neodiprion swainei* according to the depth of the soil at which the cocoons are located (Price and Tripp 1972). In addition, Volkovitch (2007) found high temperature as favourable factor for PD of lacewings in the temperate zone of Russia. Miller and Hedlin (1984) found no correlation with parental diapause habits while Ushatinskaya (1966, 1972) found heavy, moist and clay soil to prevent PD in *Leptinotarsa decemlineata.* Though there might be correlation between incidence of PD occurrence and the individual factors, it's almost impossible to find out a factor playing a key role (Saulich 2011).

Internal physiological factors may also determine PD in insect. Soula and Menu (2005) identified the role of initial lipid content in PD of the chestnut weevil. Researching with the same species, Menu and Desouhant (2002) found that bigger chestnut weevils emerge late and enter PD. They hypothesize that variability in life cycle duration relies on the metabolic resource, such as lipids, of each individual.

Apart from environmental and physiological factors discussed above, genetic component is an important regulating factor PD (e.g., Eichhorn 1977; Sims 1983; Tauber et al. 1986), as the onset and duration of diapause is a result of interaction between environmental cues (Danks 1987) and genetic programming (Mousseau and Roff 1987). Genetic differences control the onset or duration of PD in many species (Danks 1992). In addition, conditions during induction may determine the duration of PD in some species (for example, Sullivan and Wallace 1967; Minder 1981). Yet PD duration may not be predetermined during onset of diapause (Higaki et al. 2010). Hadlington (1965) suggested moderate levels of sex linkage for the incidence of PD. Point to consider that, in the case of sex linkage, phenotype of a male offspring is entirely dictated by the maternal allele on X chromosome (Morgan 1909). French et al. (2012) demonstrated the heritability of PD trait and strong influence of female parent in Northern corn rootworm.

Termination of prolonged diapause

Environmental cues, such as drought (Ingrisch 1986) or higher temperature (Miller and Ruth 1986) in the preceding summer may terminate PD. In some tropical silkmoths, wetting may influence emergence from diapause (Danks 1992). Powell (1974) experimented on individuals in PD for 33 months, where artificial watering made them break their PD. He thus suggested sporadic rainfalls to be a terminator of PD. PD can be terminated through repeated temperature cycle in several species (e.g., Neilson 1962; Higaki and Ando 2000; Higaki 2005, 2006). Intensity (defined as the depth of response to environment during diapause) of PD in chestnut weevil is dynamic and depends on the condition of ambient temperature (Higaki et al. 2010). In case of winter diapause, relatively warm temperature (such as 20-25 °C) is more effective than cold treatment in terminating PD in several species, provided it might take longer time (2-3 years) (Higaki and Ando 2000; Higaki 2005).

Theory of prolonged diapause

Recently-late entomologist Hanski (1988) did an excellent review on theories of extra-long diapause that comprises both premature and prolonged diapause. I found his review very helpful, thus next few paragraphs will mostly reflect on this paper. Since our concern is about PD rather than premature diapause, I will restrain myself in this theme.

Powell (1974) categorized PD into two types. In one type, all individuals in the population and in the other type, only a fraction of the population enters PD. Entrance into PD by all the individuals may occur if the resource availability or some essential environmental factors show strong multiannual cyclic component (Hanski 1988).

PD may be density-dependent or density-independent (Hanski 1988). Density-dependent diapause is necessarily extra-long diapause (premature or PD), while simple diapause is characteristically density-independent induced by short day-length and low temperature (Tauber et al. 1986; Danks 1987). Despite having less study, it can be assumed that density-dependent prolonged diapause may occur in some forest insects with occasional outbreaks (Hanski 1988).

Cohen (1966) worked on annual plants of deserts, where rainfall is highly variable and unpredictable. He assumed “risk spreading” PD to be a better solution than simple diapause. Dispersal is an alternative to PD. A few models predicted that frequency of PD is negatively correlated with dispersal rate. However, if the fluctuations are synchronized well across the population, it is supposed that the natural selection will favour the extra-long diapause instead of dispersal (Gadgil 1971, Hanski 1988). But, this phenomenon is not tested that much in case of insects (Hanski 1988). Based on the list of insect species with PD provided by Danks (1987), Hanski (1988) concluded about three categories of insects with PD in terms of micro- and macrohabitat occurrence. In the first category, species from the tundra, arctic and arid regions were considered. He assumed that this occurrence of PD was due to the high year-to-year temporal variability than other regions like temperate one. He showed demonstration in favour of his assumption by drawing example of *Pegomya* flies breeding in *Leccinum* mushrooms, where the occurrence of PD was found rarely only in one species in southern Finland (see Hackman 1976), but all seven common species of Finnish Lapland showed incidence of PD (see Ståhls 1987). More year-to-year variability in mushroom production in the northern region than that of south region has been presumed as the probable reason for this variability in occurrence of PD. The second group comprised the species that breeds in patchy and ephemeral microhabitats, such as cones, fruits, nuts, galls, mushrooms, and host individuals. Dramatic temporal variation in availability may also describe this phenomenon. According to Bulmer's (1984) model, species that use more variable resource should have more occurrence of PD than species that use constant resource. Annila (1982) experimented on *Megastigmus* supporting this view. Hanski (1988) hypothesized, from the "predator satiation hypothesis" of Janzen (1971), that PD in insects is a counter-adaptation to seed predation that results in fluctuation of seed production in many trees. Insects must have learnt to "predict" the size of cone crop in the following year (Hanski 1988, for example, see Bakke 1963; Stadnitski et al. 1978; Annila 1981; Hedlin et al. 1982). Temperature might be one of the environmental cues in this regard (Miller and Hedlin 1984). However, PD in the third and largest group of insects, who are herbivorous, cannot be explained well by the theory of temporal variability. However, two taxa in this case are sawflies, many of which are familiar outbreak species, and Lepidoptera, whose inclusion may be a bias as they are well studied (Hanski 1988).

Density-dependent PD is a rare phenomenon in the nature probably because of its independence from adaptive response to most kinds of temporal variability (Hanski 1988). An interesting example of density-dependence is the pine-feeding sawfly *Diprion pini*. In this species, resource depletion at high density is associated with a change of the life cycle from bivoltine to univoltine, with a further switch to prolonged diapause at the end of outbreak (Eichhorn 1982). Thus, PD can avoid many negative factors (food shortage, disease, egg and larval predation, parasitism) occurring at the end of outbreak, and increase the chance of survival (for example, see Kolomiets et al. 1979). Parasitoids of pine sawflies enters PD as well in response to the decrease in host population, although, variability in resource, rather than density-dependence, remains the main element of this strategy in parasitoids. Density-dependent extra-long diapause should be selected evolutionarily where it happens not because of environmental variability, but because of population dynamics. This extra-long diapause helps individuals to exploit the favourable breeding opportunity, which is not possible in the case of density-independent extra-long diapause. Density-dependent extra-long diapause may reduce population fluctuations that led to evolution of extra long diapause at first place (Hanski 1988).

Apart from density-dependent and density-independent characteristics of extra long diapause, Hanski (1988) discussed about multispecies interaction in his review. Though the theoretical work is absent, he drew few examples of interspecific competition and host-parasitoid interaction and discussed on the matter. Hanski (1988) showed two reasons in answering the cause of differential length of PD in four dominant competing species of seed and cone insects of Norway spruce *Picea abies*. First, he identified severe competition among seed and cone insects, which might have restricted the number of coexisting species. There is a possibility of death of nearly all cones and cone-breeding insects, if the emerging population is large compared to number of cones. Second, the mutual adaptation of different PD behaviour that increased the chance of avoiding competition thus increasing probability of survival. However, polymorphism may allow a species exploiting resource regardless of temporal variability.

PD may occur simultaneously in insects and their parasitoids. In a few cases, emergence of parasitoid was well synchronized with that of hosts. Annila (1981) suggested the same external cue being responsible for same timing. But Solbrek and Widenfalk (2012) reported that two parasitoids of gall midge *Contarinia vincetoxici* always broke their PD in advance of breaking of PD in their host. Nevertheless, emergence of parasitoid was sometimes found to be belated than that of their hosts (see for example, Kolomiets 1979). Hanski (1988) threw two questions in this regard. First, whether the phenomenon of longer length of PD in parasitoid than that of their hosts is general or adaptive. Second, whether the host-parasitoid or predator-prey dynamics only can make host and/or parasitoid enter PD. If the density of host population reaches in equilibrium owing to parasitoid, there is narrow chance for the evolution of PD because of little temporal variation in fitness. According to Bulmer’s (1984) model, however, chances should be increased if the dynamics are cyclic or chaotic. Yet, work of Corley et al. (2004) showed modest contribution of PD in the stability and persistence of host-parasitoid interactions.

In the population level, prolonged diapause is important for the marginal and poorly adapted populations, who are prone to variable environment. In addition, when the fluctuation is entirely due to population dynamics, prolonged diapause can increase the stability of the population (Hanski 1988).

What strategy led the insects to adopt PD remains an interesting question. Studies suggest an “adaptive bet-hedging” (Philippi and Seger 1989) strategy or “coin-flipping strategy” (Walker 1986) in the following species (see for chestnut weevil, Menu and Debouzie 1993; Menu 1993; Menu et al. 2000; Soula and Menu 2003; for bee *Perdita portalis,* Danforth 1999; for *Pegomiya* flies, Hanski andStåls 1990; for *Papilio zelicaon*, Sims 1983 etc.). According to the concept of “spreading the risk” (see den Boer 1968), individual females in PD spread the risk of their reproductive effort over more than one season that decreases the risk of elimination of entire year-class from unpredictable catastrophic events (Griffiths 1959; Sims 1983; Menu et al. 2000; Sterns 1976). From an evolutionary point of view, the long-term benefits of PD outweigh the disadvantage of increased mortality rate (Sims 1983). Another type of PD is predictive or weather-dependent where diapausing individuals wait for an appropriate environmental signal representing favourable conditions. A basic difference between risk-spreading and predictive diapause lies on target individual. In the former case, fitness of breeding mothers is affected only; none of its diapausing offspring take part in spreading the risk individually. While, predictive diapause let all diapausing individuals make their own decision and increase their own reproductive success. A third type of PD is disturbance-broken dormancy, although it is not present or very rare in insect. This type arises owing to the lack of suitable condition for reproduction (Hanski 1989).

Prolongation of life cycle into several years was assumed to be a simple extension of normal winter diapause (Ushatinskaya 1984; Tauber et al. 1986; Danks 1987; Hanski 1988; Menu 1993; Danforth 1999). However, no real evidence is present behind this hypothesis. Therefore, Soula and Menu (2005) challenged the statement and presented evidence for their alternative hypothesis of “late switch” against previously assumed “early switch” hypothesis. According to “late switch” hypothesis, short and long-cycle insects (chestnut weevil) resume their development after winter diapause and long-cycle insects re-enter a second diapause afterwards. (See chapter 5 for related discussion).

Diapause and prolonged diapause in the context of insect life cycle, population dynamics and species evolution

Role of diapause in insect life cycle

Natural selection helped insects wield evolutionary solutions to virtually a gigantic number of ecological problems in the nature. Among the problems insect faces, seasonality encompasses the vital set of problems. Thus, phenological strategy, the timing of active and inactive phases in relation to annual cycling of both abiotic and biotic factors, is the most important adaptive strategy of insect.

Reproduction, growth and development, dormancy, and movement and migration comprise the life cycle of each species. Insects must time these phases as though they avoid unfavourable physical factors and synchronize with favourable physical conditions. Diapause per se is the timing mechanism. The timing is achieved through arrest of growth, arrest of development and modification of rates of growth (see chapter 4 for PPM). Diapause interacts dynamically with life-history parameters that comprise fitness (Istock 1976).

Insects are found to enter diapause in any metamorphic stage. However, different taxa have their biases to certain stages. The stage should be better able to withstand extreme weather conditions for a long period. The stage chosen generally reflects the life-styles and developmental characteristics of insect. As such, diapause acts as a mechanism for better survival during harsh periods.

One fascinating way with which insects utilizes diapause as a bet-hedging device is prolonged diapause. It secures any population from going extinct in case of extreme events through spreading the risk of mortality over several reproductive seasons. Semivoltine life-cycle may arise out of such temporal modification. One putative example could be the Corsican population of PPM (Géri 1983).

Insects, as well as their parasites and parasitoids, synchronize their emergence through control of diapause (Härkönen and Kaitala 2013). Thus, the use of diapause as a timing/ synchronizing mechanism is more diverse than generally perceived. Interaction between internal programs and external factors give rise to a large set of alternative developmental pathways that could be controlled directly or by cues. Cued control diapause is more flexible than the direct control because it provides innumerable options through developmental switches, gates, and response thresholds (Danks 1987).

Role of diapause in the population dynamics

Insect population interacts with biotic and abiotic factors around. Manifold interactions among the trophic levels make the dynamics of a population complex. Climate or seasonality itself regulates life cycle of organisms. Insects are directly affected by various components of the climate. How they face such challenge and adapt themselves has already been discussed. Climate also affects directly the host tress of insects on which they directly rely for their food and shelter. Synchronizing feeding stage with phenology of host trees bears immense importance for survival of insects (Asch and Visser 2007). Insects overcome the complex effects, directly by host tress, and indirectly by climate through impacting hosts, utilizing the powerful timing and regulating mechanism of dormancy (for detail, see Tauber et al. 1986 and Danks 1987).

Diapause potentially stabilizes the dynamics of complex host-parastioid-hyperparasite system. Stability is gained through appropriate diapause rate where low diapause rate makes the host population suffer from high parasitism risk and high diapause rate goes against the parasitoids growth (Zhang and Zhang 2013).

Already discussed above the contribution of prolonged diapause in the stability and persistence of host-parasitoid interaction (Corley et al. 2004). Prolonged diapause is particularly important for the marginal and poorly adapted populations facing variable environment. Population stability increases in the case when the fluctuation arises completely from population dynamics (Hanski 1988).

Finally, interaction of insect with human can also happen in an indirect way. Predictive capability is the key to pest management. Accurate forecasting of life-history events helps conducting appropriate pest-suppression measures at times when they are most effective. Modelling onset and termination of diapause and the non-diapause developing phases of an organism is a way of making decision when to apply suppression measure. Diapause is the phase when organism interacts least with the surrounding environment. In fact, one of the most important aspects of diapause is survival through inactivity. Some insects don’t respond to insecticides in the diapausing phase. As such, regulating the population density of the pest becomes challenging.

Role of diapause in the species evolution

Allopatric speciation (reproductive isolation between populations as a result of geographical divergence) is opined to be the most prevalent form of speciation (Mayr 1963). Geographical divergence tends to create some potential reproductive isolation between the populations in such a manner that at a later point even if two isolates mix each other, they create various forms of selection that strengthen their difference. Divergence in diapause may play such reinforcing role (Tauber et al.1986).

Parapatric speciation (reproductive barriers between adjacent populations that vary clinally along a gradient) may arise both because of selection of mating barriers and selection of climatic differences that adapt each adjacent subpopulation to local condition. The term climatic speciation (Masaki 1983) was used for the latter sense to imply a situation in which different seasonal conditions along the climatic gradient may select for different types of life cycle. Parpatric speciation is probably more prevalent than previously thought.

Sympatric speciation (reproductive isolation occurring within a single population without geographical isolation) is a controversial alternative to allopatric speciation. Seasonal isolation (allochrony/ isolation through time) is an important mechanism giving rise to such kind of speciation. Although a matter of debate, several authors have advocated existence of this mode of speciation in different species, one commonly used example being field crickets. Complex races may arise from subtle difference in diapause, foodplants and other adaptations. Such races may become spontaneously isolated just because of a switch between the diapause occurring stage resulting from genetic mutation. In addition, untimely termination of diapause in a group of diapausing insect due to any microclimatic factor may shift the phenology of the group. Such a phenological race has been discovered in Portugal, where some winter pine processionary moth allochronically shifted their phenology and became an independent summer population.

Prolonged diapause in the pine processionary moth

PD is present in many species under the order Lepidoptera. Danks (1987) enlisted insect species from different families that enter PD mainly during pupal stage (Danks 1987, pp. 181-183). However, prepupal and larval stages are also common in the list. PD is supposed to be typical of Thaumatopoeinae, a subfamily of Notodontidae (Halperin 1969). The trait has been recorded in the species *Thaumetopoea pinivora* (Biliotti et al. 1964), *Th. processionea* (Biliotti 1953), *Th. wilkinsoni* (Wilkinson 1926), *Th. solitaria* (Halperin 1983) and *Th. pityocampa* (Biliotti 1953, Besceli 1965). Pupal diapause may persist for six years in *Th. wilkinsoni* (Halperin 1969). PD of 1-2 year is a common phenomenon in *Th. solitaria* (Halperin 1983). An interesting finding was variation of persistence of PD in different conditions. When he took the descending prepupae in the pots, PD either did not occur or lasted only for one year. But, transfer into the caged soil plot resulted in 2-year PD. The observation was consistent with the study with *Th. wilkinsoni* (Halperin 1983). On the other hand, Halperin's (1990) experiment with *Th. wilkinsoni*, a sister species of *Th. pityocampa,* revealed 1-9 years of PD and fluctuating emergence in the species.

Pine processionary moth *Th. pityocampa* [(Denis & Schiffermüller)] (henceforth PPM) is a major defoliator of pine in the Mediterranean basin. Impact of PPM larvae was reported long ago during the Roman period. Knowledge on the species has been enriched greatly in the last two centuries. To know detail on the history of the study on PPM and the contributors to the studies, refer to Roques (2015). A large number of studies were conducted on the population dynamics, life history, expansion process, impact on host plant, medical significance, and control method of PPM.

Owing to its sensitivity to even weak variation of temperature and resulting range expansion due to increase in winter temperature, IPCC (Intergovernmental Panel on Climate Change) has marked PPM as an indicator of global warming (Rosenzweig et al. 2007).

Life history traits of PPM are highly variable. It is generally a univoltine species. Semivoltine population was found in the Corsican Island (Géri 1983). Although the defoliating Lepidopterans feed during the summer, PPM feeds through the winter. Therefore, temperature of winter plays a big role in the adult phenology. Adult emergence spans from early June in the colder sites to September in the warmer sites (up to October and November in the sister species *Th. wilkinsoni*).

PPM is an oligophagous species feeding on native and introduced *Pinus* and *Cedrus* species. Both stand edges and isolated tress are preferred, however they can spread in the inner part as well in the case of high density. Geographic range of the species falls encompassing southern Europe to northern Africa and Atlantic coast to western part of Turkey.

Adults are short lived; females having a life span of 1 to 2 days and males living for a week (Zhang and Paiva 1998). Dispersal capacity of the adults are limited though some individuals may fly up to or more than 10 km. Males fly more distance. Depending on environmental condition, females lay about 150-300 eggs, which may vary in size. Eggs are laid in batches on needles and twigs and covered with scales of the female. Larvae build silk tents right after the hatching and move to sun exposed parts of the tree. One colony may build 2 to 3 tents before building the final one. Larvae go through five instars. Young larvae start feeding on current-year needles, but later switch to old needles. Larvae feed on night provided that the temperature at night is above 0°C and the colony temperature at day reached around 9°C (Battisti et al. 2005). Setae in the dorsal part of the abdomen is urticating from the third instar. Larval stage spans Autumn and Winter.

In the beginning of the spring, well fed prepupae descend from trees in groups and start a typical head-to-tail procession in search of suitable pupation site e.g., open areas and forest edges (Démolin, 1971). They dig into the soil. The procession period spans around two months. Early descending prepupae enters diapause to synchronize with late descending prepupae (see chapter 4). After pupation (metamorphosis into pupa), pupae enter an obligatory short diapause. And finally, they emerge in the summer. But, interestingly, a portion of pupae may not emerge in the current year, rather prolong their diapause into the next year/s (figure 1). As adults are short-lived, synchronizing emergence is important. Natural enemies (predators, parasitoids and pathogens) are present in all the stages (Biliotti 1958, Vago 1959, Battisti et al. 2000, Triggiani and Tarasco 2002, Barbaro and Battisti 2011).

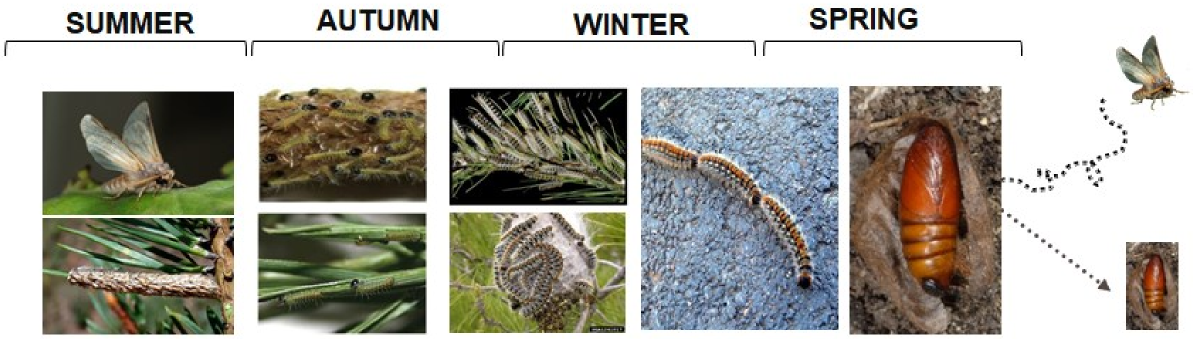

Figure 1: Life-cycle and three types of diapause of pine processionary moth

In addition to reducing tree growth (Jacquet et al 2013), it causes severe skin reaction to human and animals (Battisti et al. 2011, 2017). PPM enters PD at high elevation and latitudes (Démolin 1969). PD may occur in this species after biotic and abiotic trouble in population growth (Démolin 1969). Predicting population density and developing predictive risk model for PPM was not entirely possible possibly because of PD, in addition to other factors. For example, Tamburini et al. (2013) assumed PD to be an explanation of negative density feedback in the PPM population dynamics. Although impact of population density on the frequency and duration of PD in PPM has never been tested. PD could be a reason for cyclicity of population dynamics in PPM (Li et al. 2015). In explaining smaller effect of climatic drivers in the population dynamics of PPM in France, Toïgo et al. (2017) assumed the buffering of environmental variation by the individuals at PD. Despite having vast importance, biotic and abiotic factors behind the PD of this species remain unknown. Understanding PD mechanism in PPM is particularly critical because PD has been described as a factor increasing invasion speed in stochastic environments (Mahdjoub & Menu 2008). PPM has been used as a model both for range expansion and invasion (Roques 2015). Existence of PD may help spreading this obnoxious species of medical importance in its range edge or elsewhere.

Existence of PD in insect has been thought to be negligible. However, as I already discussed, evidence speaks the contrary. I concede with Danks (1987) that occurrence of PD in insect is not rare. Danks (1987, pp-179-184) established his view by giving a large list of such phenomenon in insect taxa. Classic view of not considering prolonged diapause being common stemmed from a number of reasons: first, studying prolonged diapause is time consuming. For example, an attempt of finding out prolonged diapause is PPM took 10 years of continuous monitoring before the experiment finished (chapter 2). Even if anybody agrees to work, getting a fund for so long a period is hard. A PhD student may not complete the entire experiment within the timeframe (such happened to me). As such, publication on the fact is rare, although I heard researchers express their feeling of existence of the trait in the species they work.

Objectives

Despite the existence of challenges, my PhD project focused on understanding the ecological role of diapause in PPM, and especially of PD. Almost all the questions regarding diapause regulation in PPM are still unanswered. Nevertheless, my work attempted to answer a few basic questions on the existence and regulation of diapause, both short and prolonged, in PPM. One of the main objective was to understand the importance of PD in the regulation of population dynamics in a range edge population of PPM. What is the longest duration PPM can diapause was another question to answer. The second chapter will discuss the findings. Literature of PD in PPM is scattered and mostly unpublished. One of the objectives was to collect and survey available literature on the topic and understanding the role of winter-temperature on the incidence of PD in the whole geographic distribution of PPM. It will be discussed in the third chapter. Another important question was whether diapause is present only in the pupal stage, or it is available in other stages of PPM. If available, what is the role it plays. I will discuss these issues in the fourth chapter. Finally, my project wanted to understand the termination mechanism and other related eco-physiological phases during diapause development. Whether prolonged diapause in PPM is a continuation of obligatory or it has different mechanism was another aspect of my study. All these issues will be elaborated in the fifth chapter.

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CHAPTER 2

Prolonged pupal diapause drives population dynamics of the pine processionary moth (*Thaumetopoea pityocampa*) in an outbreak expansion area

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Abstract

Due to its negative impact on tree growth and human health, pine processionary moth is a major concern for forest managers, especially in recent outbreak expansion areas. As some individuals have prolonged diapause for more than a single year, population dynamics of this species is hard to understand. To decipher the mechanism of prolonged diapause and its role in population dynamics, we started a pest surveillance program in 1999 in a pine processionary population in the southern-central Alps of northern Italy, a recent range expansion zone for the species. The 16-year study used a pheromone trap network and four large rearing cages. We found some individuals could diapause for up to a maximum of seven years. With few exceptions, prolonged pupal diapause increased and retrieval rate of moth in cages decreased with increasing elevation. Moreover, we found prolonged diapausing individuals to emerge in advance of non-diapausing individuals. This trait allowed us to infer the proportion of prolonged diapausing individuals caught in the pheromone traps. Prolonged diapause was responsible for maintaining high population density for eight years in spite of annual applications of the biocontrol agent *Bacillus thuringiensis kurstaki*. This sustained density relied on individuals from cohorts before the application of insecticide started, and from cohorts not completely suppressed by the insecticide applications.

Key words: Pinus nigra; Bacillus thuringiensis kurstaki; pheromone trap; range edge; Alps; prolonged diapause

1. Introduction

Diapause plays a key role in the life history of many insect species (Tauber et al. 1986). As an alternative, or sometimes in addition, to escape in space through flight, insects avoid environmental extremes in time through diapause (Dingle 1978). Prolonged diapause (synonymously called extended diapause, perennial diapause, superdiapause, superpause) is the extension of diapause over several reproductive seasons or years. At least 150 insect species, including a few pests, of different taxa show this feature (Powell 1986, Danks 1992, Saulich 2010). Insects with prolonged diapause are mostly phytophagous and show wide fluctuations of abundance and periodical outbreaks (Danks 1992, Saulich 2010). Their preferred feeding substrates include leaves, cones, seeds and roots (Danks 1987). A number of studies have been conducted on different aspects of prolonged diapause (Danks 1987), but the impact on population dynamics remains mostly unexplored. Although MacDonald (1976), Takahashi (1977) and Corley et al. (2004) approached the problem using mathematical modeling, empirical data and studies appear to be limited to seed-associated insects (Maeto & Ozaki 2003).

Pine processionary moth (*Thaumetopoea pityocampa*), PPM, is a pest with large impacts on tree growth and human health, and shows prolonged diapause as a pupa (Battisti et al. 2015). Prolonged diapause is supposed to be common in Thaumetopoeinae, a subfamily of Notodontidae (Battisti et al. 2015, Halperin 1969). In addition to *Th. pityocampa* (Biliotti 1953), the phenomenon has been recorded in *Th. pinivora* (Biliotti et al. 1964), *Th. processionea* (Biliotti 1953), *Th. wilkinsoni* (Wilkinson 1926), and *Th. solitaria* (Halperin 1983).

Population dynamics of PPM has been studied using long-term data in various parts of its range (Geri et al. 1985, Hellrigl 1995, Zamoum et al. 2007, Sbabdji & Kadik 2011, Tamburini et al. 2013, Li et al. 2015). Potential mechanisms behind the density fluctuations were inferred to be deterioration of host quality, decrease of food quantity, increased mortality caused by natural enemies and pathogens, and prolonged diapause. Researchers often identified prolonged diapause as noise in predicting dynamics of PPM populations (Masutti and Battisti 1990, Aimi et al. 2006, Tamburini et al. 2013, Li et al. 2015). Deciphering the prolonged diapause strategy of PPM is particularly critical since it has been described as a factor increasing invasion speed in stochastic environments for other model species (Mahdjoub & Menu 2008).

Development of PPM is largely affected by temperature because of the winter feeding habit of the larvae (Démolin 1969). In a cold site, larvae take more time to feed and the univoltine cycle is not possible, as at least 40 days are required at 20°C for pupal development (Démolin 1990). Consequently, emergence is postponed to the next year, resulting in a semivoltine life cycle typically observed in populations of PPM at high elevation forests of Corsica (Geri 1980, 1983), or to following years (Battisti et al. 2015). Thus, prolonged diapause results in a semivoltine life cycle or it can extend the duration of the life cycle over more years.

Here we report the duration of pupal diapause in a population of PPM in an alpine valley at the northern edge of its range (Venosta/Vinschgau, Italy), where temperature is the major limiting factor for larval development (Battisti et al. 2005), and thus we expect a high frequency of prolonged diapause. We address the issue of differences in timing of emergence between diapausing and non-diapausing individuals in the population. Finally, we demonstrate the importance of prolonged diapause in the population dynamics of this species.

2. Materials and Methods

2.1 Study site

The Venosta/Vinschgau Valley is situated amidst the Southern Alpine region of South Tyrol at the borders of Italy, Austria and Switzerland. It has an east–west orientation with north-south facing slopes (Battisti et al. 2005). Venosta Valley experiences a unique arid climate in the Alps, having more than 300 days of sun per year and less than 600 mm of annual precipitation (C3 Alps 2014).

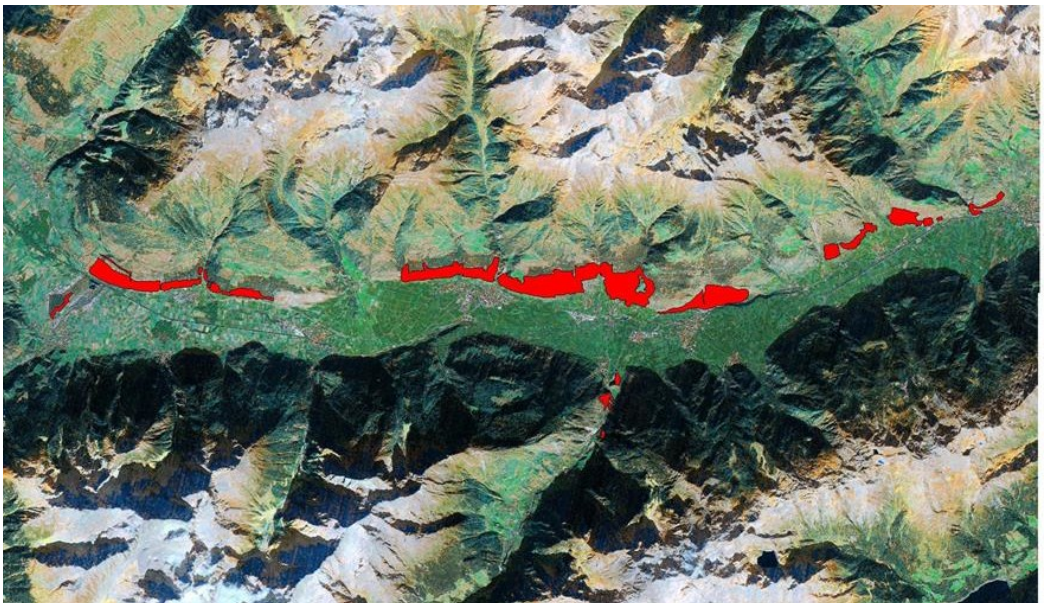

Figure 1. Map of Venosta/Vinschgau valley showing the attacked pine forest between 1999 and 2014 in red. The oval includes the experimental area of Silandro/Schlanders, located on the southern facing slope of the valley within the elevation span of 700-1400 m. Background map courtesy of Forest Service of Bolzano/Bozen Province.

Venosta valley represents the upper edge of the range of PPM in the southern-central Alps (Figure 1). The attacked stands are located on the southern-facing slope, where native Scots pine (*Pinus sylvestris*) has been mixed with the introduced black pine (*Pinus nigra*) since the beginning of the 20th century, with the aim of protecting soil from erosion and for timber. Although historical occurrence of the insect is documented during the 20th century, it was only since 1998 that a large outbreak started and the population colonized the upper parts of the valley and the upper slopes, reaching an elevation as high as 1500 m on the south-facing and 1200 m on the north-facing slope (Battisti et al. 2005). Since then the population has fluctuated in density (Zovi et al. 2006), and plant protection measures were adopted (Aimi et al. 2006). Climate change has been invoked to explain survival at elevations that were considered prohibitive for the insect based on previous knowledge. Recent work, however, has documented that the thermal ecology of this species is more complex than expected and that local adaptation to climate can be observed (Hoch et al. 2009).

As soon as the outbreak occurred in 1998, the local foresters started pest surveillance. They mapped the infested areas, deployed a pheromone trap network (Jactel et al. 2006), and installed four large cages for monitoring the emergence from prolonged diapause in the 1999 cohort. The latter was done to clarify whether the life cycle of the population was univoltine, semivoltine, or pluriannual.

2.2 Catch data from the pheromone trap

Fifteen pheromone traps were deployed for capturing male moths in three different sites close to the town of Silandro/Schlanders, namely Vetzaner Leiten (around 800 m of elevation, later called Vetzaner), Priel (around 900 m), and Kortsch (around 1,000 m), with 5 traps in each site. Capture started in 1999 and has continued till present. In 1999, the experiment started in each site with three traps developed by the Forest Service of Trento district, one was a funnel-trap with large wings above it, and two traps marketed by Serbios as Super Green (http://www.serbios.it/), which is a funnel-trap for large moths. The Serbios traps progressively replaced the Forest Service traps so that from 2007 only Serbios traps were used. Foresters visited traps every week during the emergence period and recorded the number of trapped males and pheromone was changed every 4 weeks.

2.3 Prolonged diapause experiment

During winter 1999, three large cages (2x2x2 m) were constructed at Vetzaner over an elevation gradient (822, 910, 1073 m) and one cage was set up at Kortsch (near pheromone traps, with an elevation of 963 m). The cages were located at the edge of the forest in order to mimic the natural pupation site of PPM (Démolin 1969). In the results we will refer to each by its height, e.g. cage at 822 m is the cage located at 822 m, etc. The cages had a wooden frame and a wire net (mesh 2 mm) and the walls entered the soil down to a depth of 30 cm. In April 1999, a total of 300 tents with larvae in the fifth instar were collected from the same site at an elevation of 800—1000 m. Tents were then randomly assigned to each of the four cages assuring that all the cages receive about the same number of larvae. This allocation was done based on random counting of the larvae in a subset of 180 tents, which resulted in the average of 77 larvae/tent (SE 3.9), and on the assumption that tent size is positively related to the number of larvae. Overall, about 5,000 larvae were added to each cage. The larvae were provided regularly with pine branches as food until pupation, which was inferred from the time when no more needles were eaten. The leftover was then removed and the cages were regularly inspected once a week during the rest of spring and summer. Emergences were then checked with the same frequency during each of the following years, by counting and removing individuals irrespective of the gender, and stopped when no emergences were observed for three years (in 2009). Soil samples were then taken from inside the cages and no intact cocoons were found. In addition, another cohort was started in 2013 with the same method. In order to measure temperature inside and outside of each cage, data loggers (HOBO U23 Pro v2 with a RS1 solar radiation shield, Onset Corp, Bourne, Massachussets) were deployed and data were retrieved with an hourly frequency.

2.4 Spraying data

A population suppression campaign started in 1999 and was based on aerial applications of *Bacillus thuringiensis kurstaki* (*Btk*) (Foray, Valentbioscience). As the larvae started to feed during summer, the damage became detectable in the early autumn. At that time, foresters mapped the infested area as that having one early tent per tree on the average, which was sprayed in the autumn with a dose of 2 L/ha, designed for second to third instar larvae (Démolin & Martin 1998). During the winter, the same area was inspected again and if an average density higher than 1 tent/tree was detected it was sprayed at the end of the winter, generally late February to early March. The second application had a dose of 4 L/ha because it was directed at fourth to fifth instar larvae (Démolin & Martin 1998). *Btk* applications are usually very effective, killing almost all of the larvae (Zovi et al. 2006, unpublished data from Forest Service).

2.5 Data analysis

To distinguish non-diapausing from diapausing individuals in a population, we analysed catch data in pheromone traps and used time of emergence observed in the cages in different years as a discriminating trait. Emergence in the cages in 1999 and 2013 corresponded to the current year, non-diapausing individuals, while emergence in subsequent years represented the diapausing individuals. We compared the first emergence date in the cages with that of the first catch in the pheromone traps.

To maintain consistency, the same person checked the cages and pheromone traps on the same day throughout the emergence period. He counted both alive and dead individuals, the emergence of which was attributed to the days since the last check. We standardized the weekly trap data into daily ones by dividing the number of caught individuals by the days since the last check. Our dataset contained two types of traps that we called “Trento Forest Service” and “Serbios”. As the number of catches clearly varied between the types, we standardize both of them into “Serbios” type in order to be able to compare the yearly data. We calculated the average daily catch ratio of Trento Forest Service and Serbios traps for each year and site, and divided the average daily catch of all Trento Forest Service traps by their corresponding ratios.

All the statistical and graphical analyses have been conducted using R (R Core Team 2014) and MS Excel 2011 for Mac (Microsoft 2010).

3. Results

3.1 Intensity of prolonged diapause and rates of emergence of adults in the cages

Three out of four cages produced moths, although they showed a different retrieval rate of moths and a different distribution of emergence through time (Table 1). The cage at 1073 m did not produce any adults. There was no first year emergence in the cage at 963 m then emergences extended over the next six years. Emergence was observed in the first year at both the cages at 910 m and 822 m, and it extended over seven and four years, respectively. We report the length of prolonged diapause without considering the emergence of first year (e.g., thus although the cage at 910 m produced moths for 8 years, we estimate the prolonged diapause in this cage being 7 years).

Table 1: Emergence and estimated retrieval rate of moths from the rearing cages established in 1999 at four different elevations

Year	822 m		910 m		963 m		1073 m	
	No. emerged moth	*% emergence*	*No. emerged moth*	*% emergence*	*No. emerged moth*	*% emergence*	*No. emerged moth*	*% emergence*
1999	9	0.18	2	0.04	0	0	0	0
2000	402	8.04	20	0.4	13	0.26	0	0
2001	100	2	25	0.5	87	1.74	0	0
2002	11	0.22	241	4.82	20	0.4	0	0
2003	7	0.14	406	8.12	70	1.4	0	0
2004	0	0	100	2	7	0.14	0	0
2005	0	0	38	0.76	1	0.02	0	0
2006	0	0	9	0.18	0	0	0	0
2007	0	0	0	0	0	0	0	0
2008	0	0	0	0	0	0	0	0
Retrieval rate of moths	10.58%		16.82%		3.96%		0%	

Percentages of individuals emerging in the year of pupation (univoltine) were 0.18%, 0.04%, and 0% in the cages at 822, 910 and 963 m, respectively. Distribution of emergence over years in the cage at 822 m was positively skewed, while the cage at 910 m showed more or less normally distributed. Cage at 963 m displayed a bimodal distribution. Retrieval rate of the moths decreased with the increase of elevation with an exception, in the lowest cage, where the rate was higher than the cage at 963 m, but lower than the cage at 910 m.

As expected, based on the lapse rate associated with elevation, temperature decreased with increasing elevation. The lowest cage (822 m) experienced the highest hourly mean air temperature in all seasons (spring 13.46°C, summer 19.68°C, autumn 9.24°C and winter 3.58°C) while the highest cage (1073 m) the lowest temperature (spring 9.57°C, summer 17.24°C, autumn 6.47°C and winter 1.24°C), with the other cages in between. The temperature inside and outside the cages was similar, with the largest difference observed from mid-January to mid-May of 2015 in the cage at 822 m, when the hourly mean air temperature inside and outside was 6.74°C and 6.64°C, respectively.

3.2 Btk spray

After the outbreak took place in 1998-1999, aerial spraying of *Bacillus thuringiensis kurstaki* (*Btk*) over the infested area continued until 2007-2008 (Figure 2). There was no spraying in 2008-2009 because the density was low. Spraying started again in the following years but it was limited to a few spots, and was stopped completely from 2011-2012. Although, the sprayed area varied over years, Vetzaner (where the cage at 822 m and 910 m are located) were sprayed regularly.

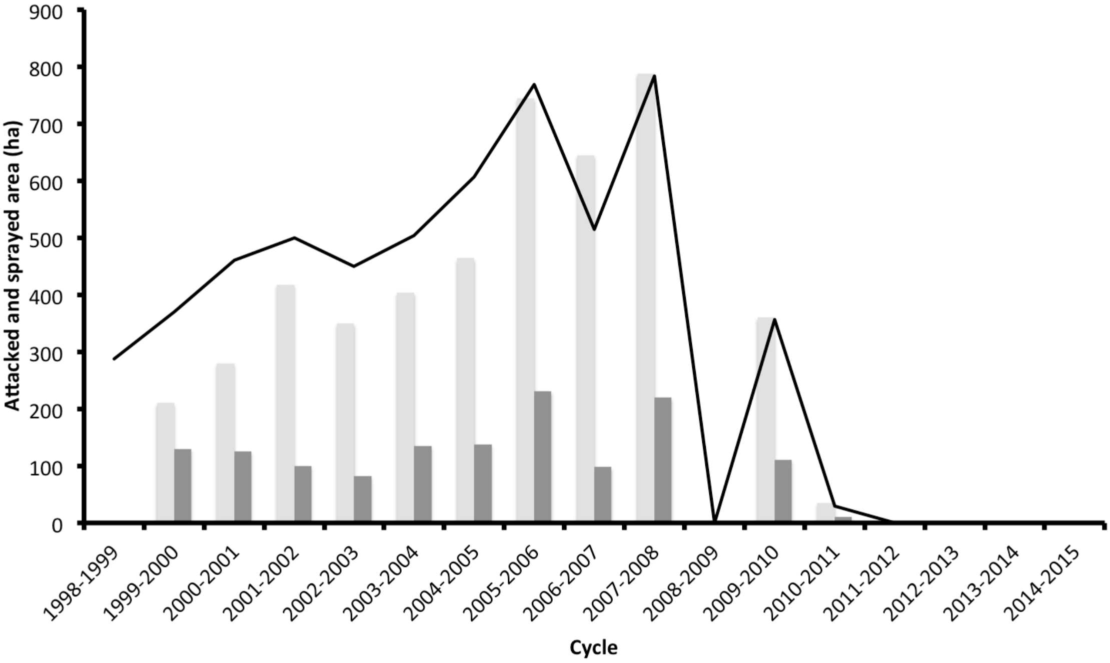

Figure 2: Amount of *Btk* sprayed area in each year. Light grey and dark grey bars represent amount of spray in total area and Vetzaner, respectively. Black line corresponds to attacked area each year.

3.3 Emergence in diapausing and non-diapausing individuals

Comparing the emergence time in the first year of emergence in the cages with the catch in the pheromone traps of that year, we may infer if the emergence of non-diapausing individuals is delayed. In 1999, the first moth in the cage at 822 m was found on 27th of July. The first catch in the pheromone traps of corresponding site was recorded in 30th of June (Figure 3). In the same year, the first moth was caught in the cage at 910 m 41 days after the first moth was captured in the corresponding pheromone traps. A similar result was found in the cage experiment commenced in 2013. In that year, the first moths emerged on 23rd and 30th of July in the cages at 910 m and 822 m, respectively, while pheromone traps on their corresponding sites caught the first moth on 24th of June and 1st of July, respectively. However, the first emergence of prolonged diapause individuals in cages and the first catch in pheromone traps almost overlapped (Figure 3) in both cohorts.

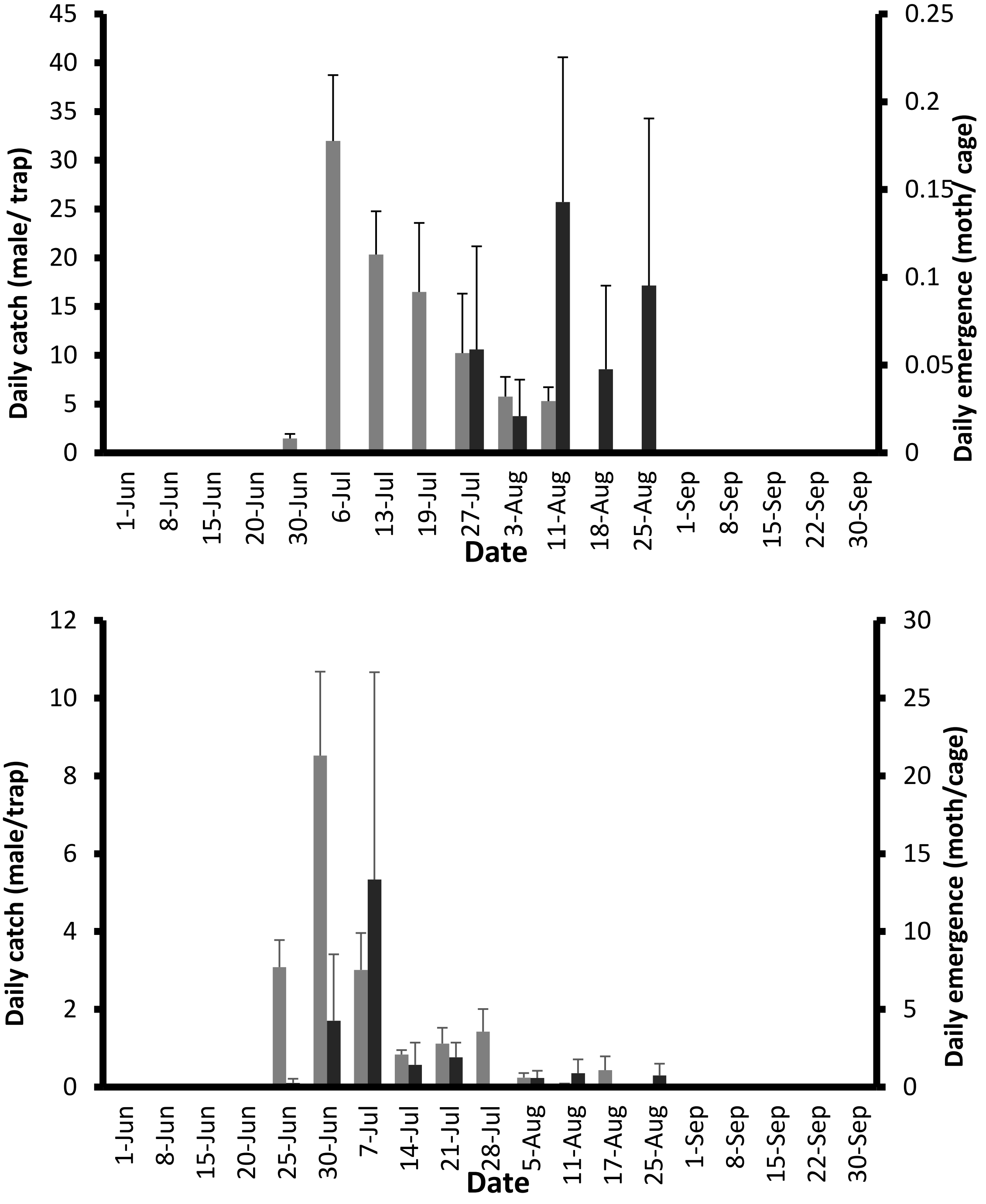

Figure 3. Average daily catch of males in pheromone traps for Vetzaner (grey bar) and daily emergence of moths in the cage at 822 m (black bar) with positive standard error for the year 1999 (above) and 2000 (below).

Non-diapausing individuals emerged on average four weeks after the first emergence of prolonged diapausing individual. The males caught in the traps in that period should have gone through prolonged diapause. Even if we use three instead of four weeks, the contribution of prolonged diapausing individuals always remained higher than 50% and varied throughout time with an irregular pattern (Supplementary material, Figure S1).

Population density, as revealed by trap catch, was very high in 1999 and it dramatically dropped in the following years because of the spraying, although it remained above the threshold for several years, due to the continuous release of moths from the diapausing stock (Supplementary material, Table S1 and Figure S2 and S3). Number of catches in pheromone traps decreased with increasing elevation.

4. Discussion

A newly started outbreak at the upper range edge of PPM in the Alps provided a unique opportunity to investigate how important prolonged diapause is in the population dynamics. As pest suppression through *Btk* application started almost immediately after the increase of density, and was applied for nine consecutive years, we may conclude that individuals emerging from prolonged diapause mainly caused yearly pest resurgence. The presence of large numbers of individuals in diapause can be seen as a way to escape environmental extremes common in the expansion areas (Mahdjoub & Menu 2008) but as a consequence represent a problem for forest managers. Our study provides a clear example of a sustained outbreak linked to prolonged diapause and offers tools, based on standard surveillance techniques, to understand how important prolonged diapause is in the dynamics of this pest.

Our experimental results in the cages indicate that PPM in Venosta extends its pupal diapause over several years. Démolin (1969) reported the diapause of PPM to be extending up to six years under controlled, semi-field conditions. The variation in prolonged diapause observed among cages can be explained by temperature, with individuals exposed to higher temperature ending the prolonged diapause earlier than those at lower temperature. Halperin (1990) found a similar effect of elevation, a proxy of temperature, in the duration of prolonged diapause in *Th. wilkinsoni* , a sister species of PPM. In his experiment, Halperin (1990) collected 500 larvae from Ramallah (850 m) and 336 larvae from Ramat Gan (50 m) and reared them in their corresponding habitats. Individuals that were reared at higher elevation had prolonged diapause, up to nine years, whereas those reared in lower elevation showed only six years of diapause. These effects could be confounded, however, by other non-controlled factors. For example, our cage at 963 m showed lower intensity in prolonged diapause than that of the cage at 910 m. This anomaly could arise from higher mortality. For instance, Halperin (1990), consistent with our result, documented in his study higher mortality in the upper elevation site (47%) than the lower elevation site (22%). Although retrieval rate of moths was higher in the cage at 910 m than that of at 963 m one, interestingly, the cage at 822 m showed an intermediate value of retrieval. This oddity in retrieval rate can occur because of increased fungal and nematode activity arising from a combination of higher temperature and higher moisture level in the soil (Markalas 1989, Dulaurent et al. 2011, Hódar et al. 2012). Increased energy loss owing to high temperature could be another reason why prolonged diapause at 822 m did not last as long as in the two relatively colder sites. In addition, deviations in retrieval rate of moths could have happened because of uncertainty in the estimation of initial number of individuals and different microclimatic conditions of the cages, which we were not able to control. We are aware that our data refer to a temperature treatment concerning mature larvae and pupae, so we cannot exclude that local populations which are adapted to different temperature respond in a different way. This issue has to be tested with a design involving reciprocal transplants of populations (Kawecki & Ebert 2004).

Although it was observed in nature (Battisti et al. 2000), extremely high mortality of PPM in our cages could raise doubts about of findings. Total mortality in the cage at 1073 m could have happened due to the fact that the cage was beyond the local upper range and the individuals taken from lower elevation were not adapted. Therefore, environmental extreme might not have allowed the individuals to survive. Variable intensity of diapause in different cages within a very short distance and temperature variation, along with putative energetic constraint stemming from altered food source in our rearing cages, lead us to expect some deviation from natural conditions. However, we believe our method provided the best possible estimation as soil and climatic variables were not modified by the cage setup. We are aware that the limitation in the number of samples does not allow us to draw definitive conclusions and a more intense sampling is required.

Very little or no emergence in the first year but highest emergence in the second year in the cage at 822 m may imply a semivoltine life cycle, as hypothesized by Geri (1983) at high elevation stands of Corsica. However, the five-year delay in the cage at 910 m for producing the highest number of adults casts doubt about semivoltinism. Similar results were obtained in the cage at 963 m, despite low retrieval rate of moths. Fluctuation of this kind suggests plasticity in the duration of the life cycle of the individuals belonging to the same cohort. The emergence pattern of prolonged diapausing insects of one cohort across time can be decreasing or fluctuating, depending on environmental cues (Danks 1987). We found a fluctuating pattern in the experimental cages of our study site. A similar fluctuating pattern was also evident in *Th. wilkinsoni* (Halperin 1969).

Because of very efficient spraying of insecticide, we assume the contribution of non-diapausing individuals in the population density of PPM remained low after 1999. Length of diapause in the cages implies that individuals that emerged until at least 2006 came mainly from prolonged diapause of 1999 cohort. In addition, cohorts before 1999 could have contributed to the emergence as well, but as outbreak took place in 1998 and peaked in 1999, contribution from those previous cohorts should be minimal. It is unlikely that the area may have been colonized from outside because the spraying was extended to the whole area, which extends over more than 70 km east to west, and the nearest pine stands infested are far away from the valley (Bolzano area is more than 50 km from the nearest stand of Venosta), well beyond the flight range of female moth (Sauvard & Yart 2015). Instead, recolonization of the specific study site within Venosta could be possible, although the population was suppressed in the whole valley and so the contribution of the survivors should be minimal. The same applies to the high elevation areas close to the study site. Those areas were not sprayed because the density was very low. In addition to the spray, the establishment of natural enemies may have contributed to the collapse of the population density. Zovi et al. (2006) documented an increase in the density of egg parasitoids of PPM (mainly *Baryscapus servadeii*) starting from 2003, which resulted around 50% increase in mortality of eggs of the population in the following years. Despite the combination of successful establishment of parasitoids from 2003 and spray of *Btk* since 1999, we observed sustained high density of the population until 2007. This density can only be explained by the prolonged diapause of PPM spreading out from the cohort of 1999 and possibly the earlier ones.

The phenomenon of earlier emergence of prolonged diapausing individuals is consistent with the work of Menu (1993) on chestnut weevil. Since the number of current-year moths emerged in the first year of 1999 cage experiment was small, one might doubt the proposed emergence gap between diapausing and non-diapausing individuals. However, indirect evidence may come from the overlap of the emergence of non-diapausing individuals in 2013 cage experiment with the start of second peak in the pheromone trap catch graph (cf. 23rd of July against 2014 graph of Supplementary material, Figure S3). Considering the strengths and weakness of evidences and surveillance frequency, we conservatively assume the gap of emergence between diapausing and non-diapausing individuals being three to four weeks.

Whether the earlier emergence of prolonged diapausing individuals is adaptive is not clear. Factors that select for emergence timing and pattern are: availability of resources for an adult and its progeny; availability of opposite sex for reproduction; and temporal predictability of the resources (Waldbauer 1978). Any of the factors may have selected for the trait of early emergence. Temporal resource use of PPM adults need to be understood before any conclusion is reached. Whether early emergence and early progeny would survive better is a matter of local condition of the population. We studied the phenomenon of early emergence in a sub-alpine population only. Other populations should be tested for the occurrence of such phenomenon.

Partitioning of insects into two emergence groups may help escape various detrimental factors occurring stochastically at any time of the season. Seasonal advantage itself varies with populations and their climatic conditions.

Another possibility is that there could be no such adaptation at all. Early emerge of PD individuals may occur for a termination mechanism that coarsely, not finely, adjust emergence in PPM. Current-year insects might not have chance of such early emergence simply because they need to go through some specific phases that requires a certain period of time. Whereas PD individuals already have advantage of being in a stage suitable for terminating diapause anytime soon. However, long emergence period should be a problem for mate-finding for short-lived adults as PPM. Nevertheless, this problem would arise only in a density that starts Allee effect.

The striking contribution of prolonged diapausing individuals in our study site throughout the study period implies how this species may overcome the possibility of extinction from lethal temperature and starvation in every cohort through spreading the risk of reproduction over several years, thus making it a good performer under the extreme conditions typical of range edges (Battisti & Larsson 2015).

Acknowledgements

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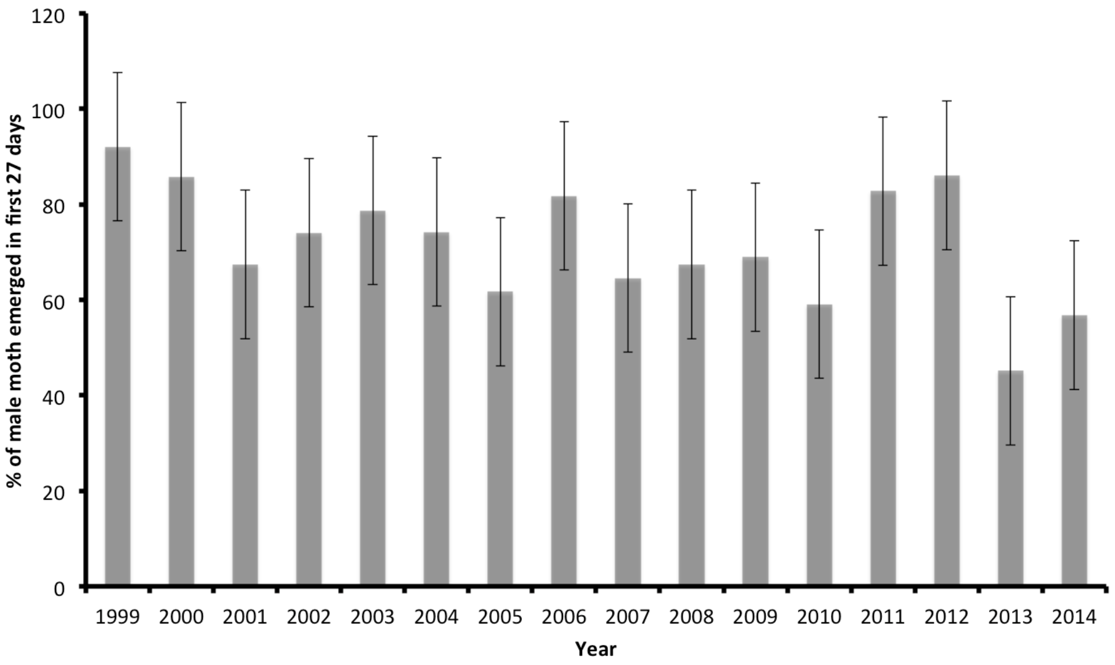
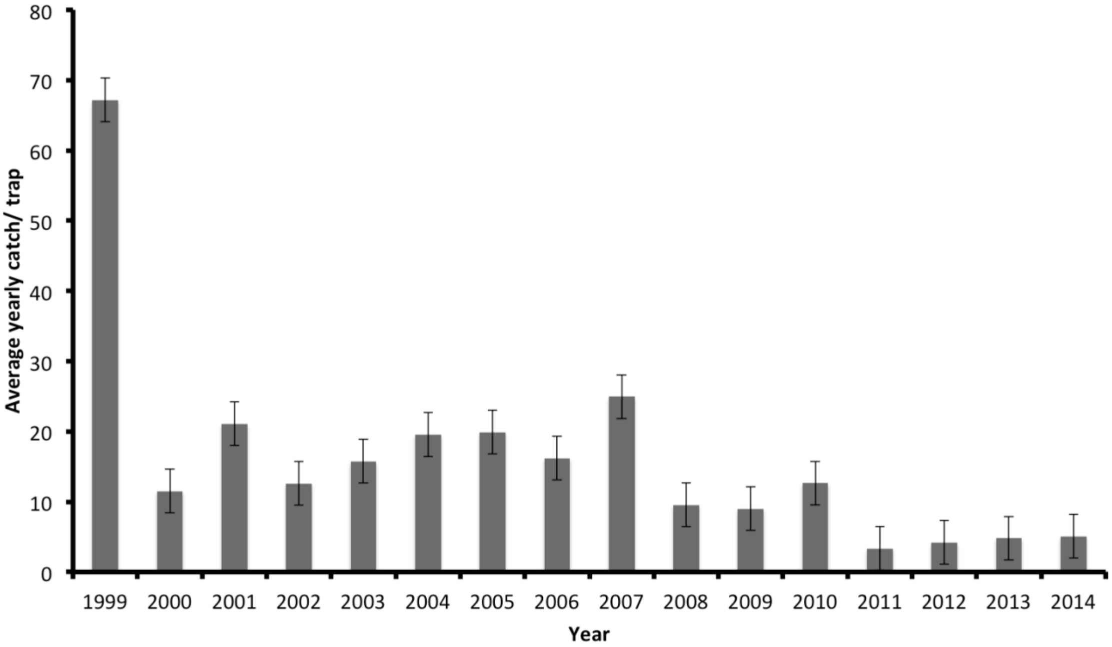
Supplementary material

Supplementary Table S1: Peak characteristics of yearly catch of male moths in pheromone traps in Vetzaner.

Peak characteristics	Year
One earlier major, upright peak	1999; 2000; 2003; 2009; 2012
One earlier major, flat peak	2006
One earlier major peak + one later minor peak	2005; 2008; 2010; 2013; 2014
One earlier major peak + several later minor peaks	2002; 2004; 2007; 2011
Two earlier major peak+ two later minor peak	2001

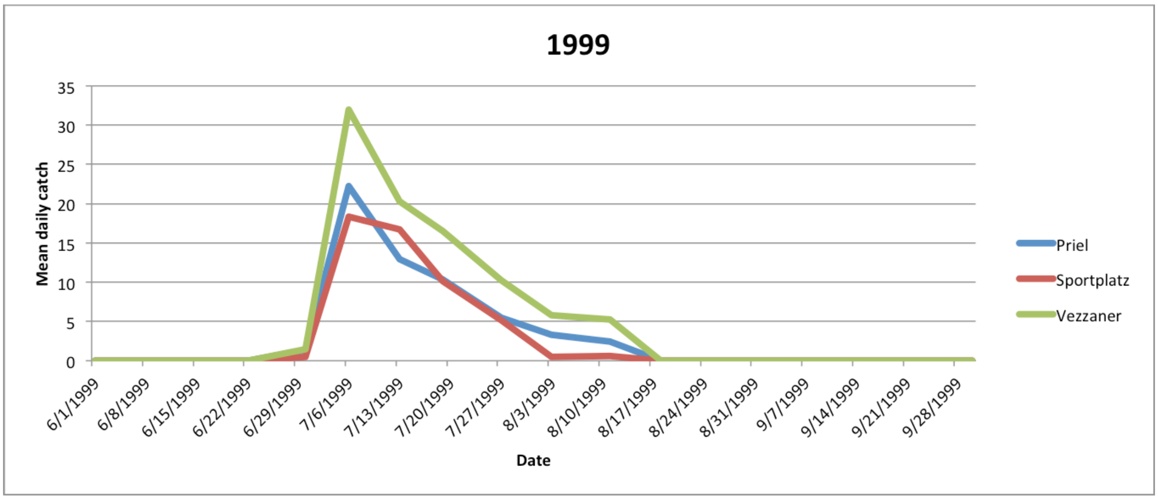


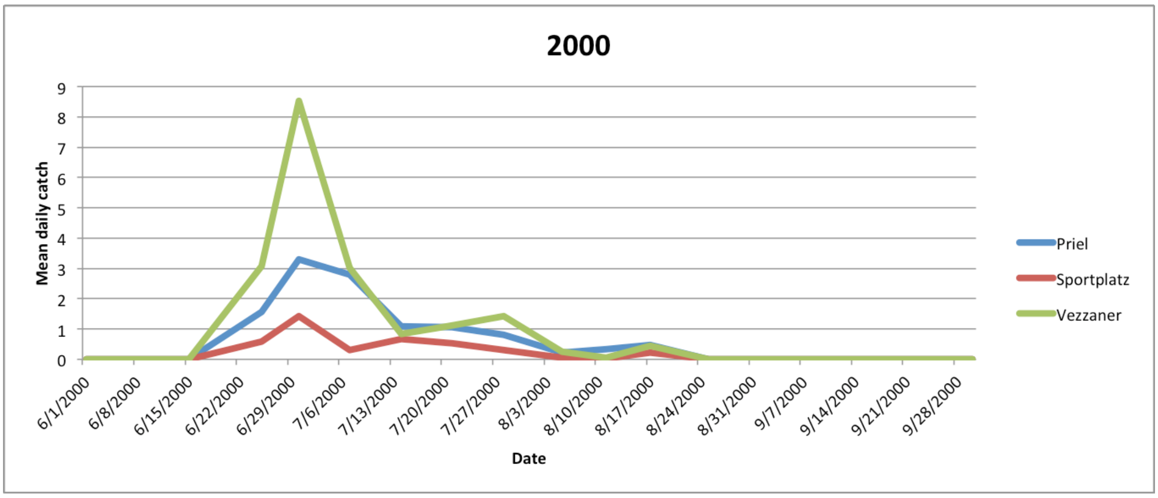
Supplementary Figure S1: Mean percentage of male moths caught in pheromone traps in the first 27 days of trap catch for the experimental area of Silandro/Schlanders, with standard error. These individuals are likely belonging to the prolonged diapausing cohorts.

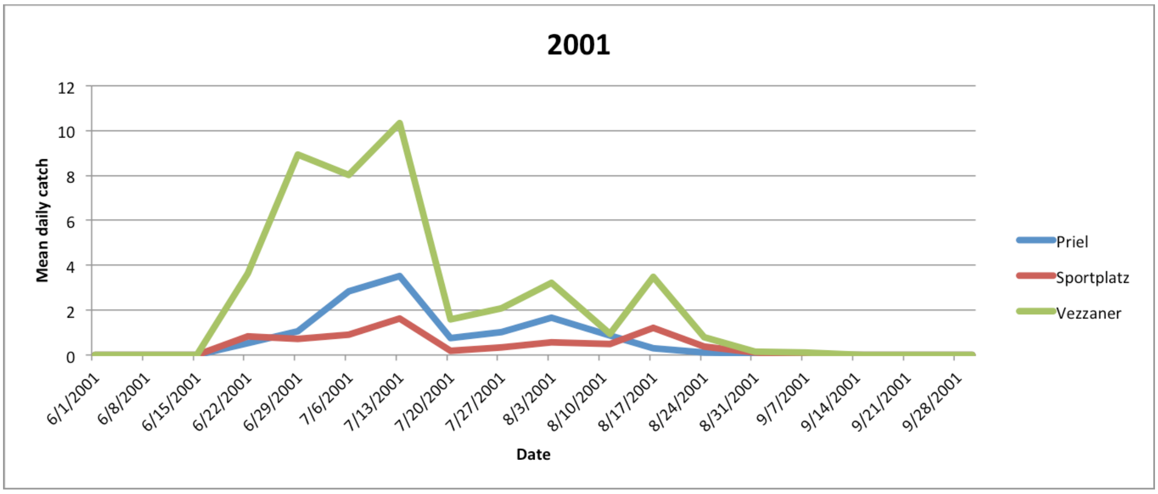


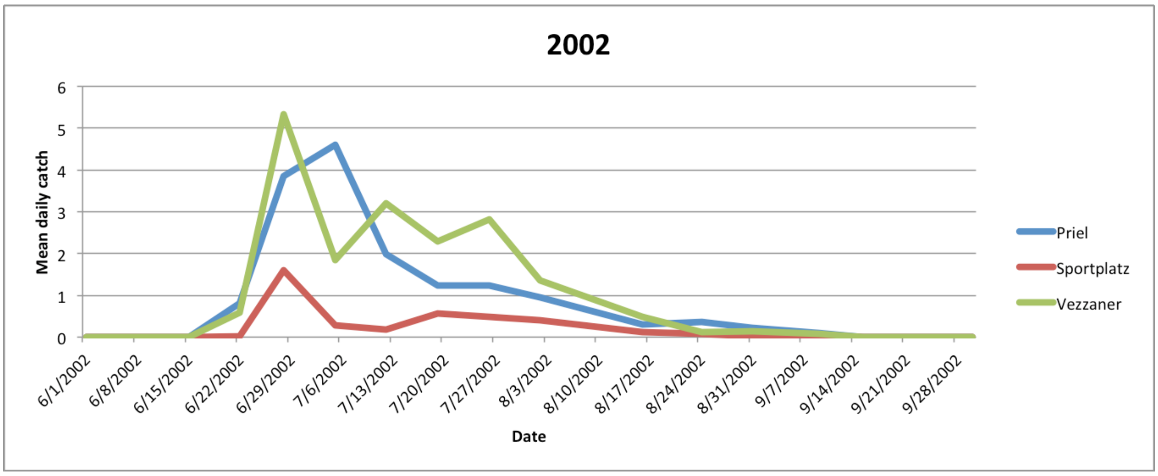
Supplementary Figure S2: Average number of male moths caught each year in pheromone traps in the experimental area of Silandro/Schlanders (n = 15). Bars indicate standard error.

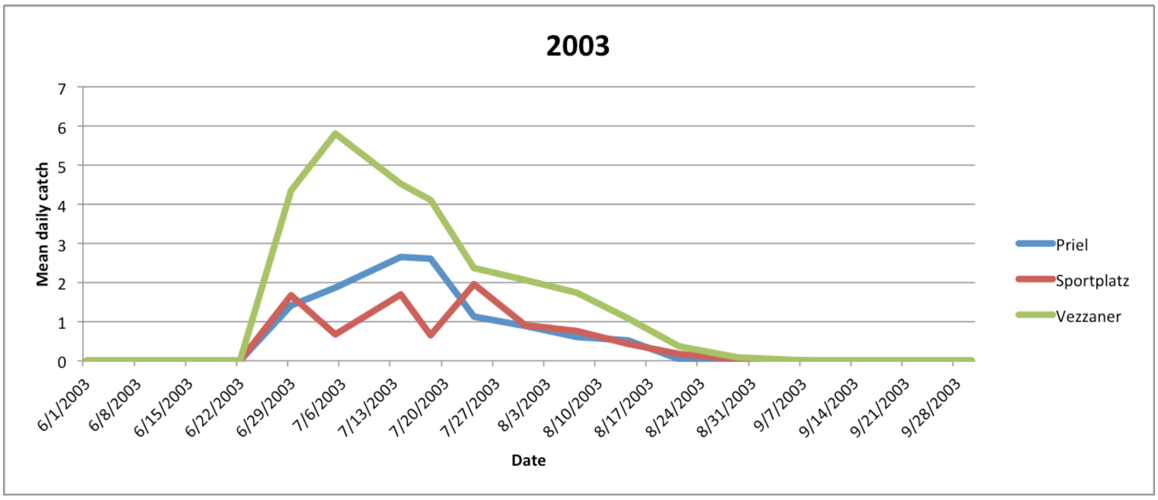
Supplementary Figure S3: Mean daily catch in the pheromone traps at the three experimental sites (5 traps/site) from 1999 to 2014. The scale of Y axis is adjusted to the catch size of each year.








2002
Mean daily catch
0
1
2
3
4
5
6
6/1/2002
6/8/2002
6/15/2002
6/22/2002
6/29/2002
7/6/2002
7/13/2002
7/20/2002
7/27/2002
8/3/2002
8/10/2002
8/17/2002
8/24/2002
8/31/2002
9/7/2002
9/14/2002
9/21/2002
9/28/2002
Date
Priel
Sportplatz
Vezzaner


2003
Mean daily catch
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5
6
7
6/1/2003
6/8/2003
6/15/2003
6/22/2003
6/29/2003
7/6/2003
7/13/2003
7/20/2003
7/27/2003
8/3/2003
8/10/2003
8/17/2003
8/24/2003
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9/28/2003
Date
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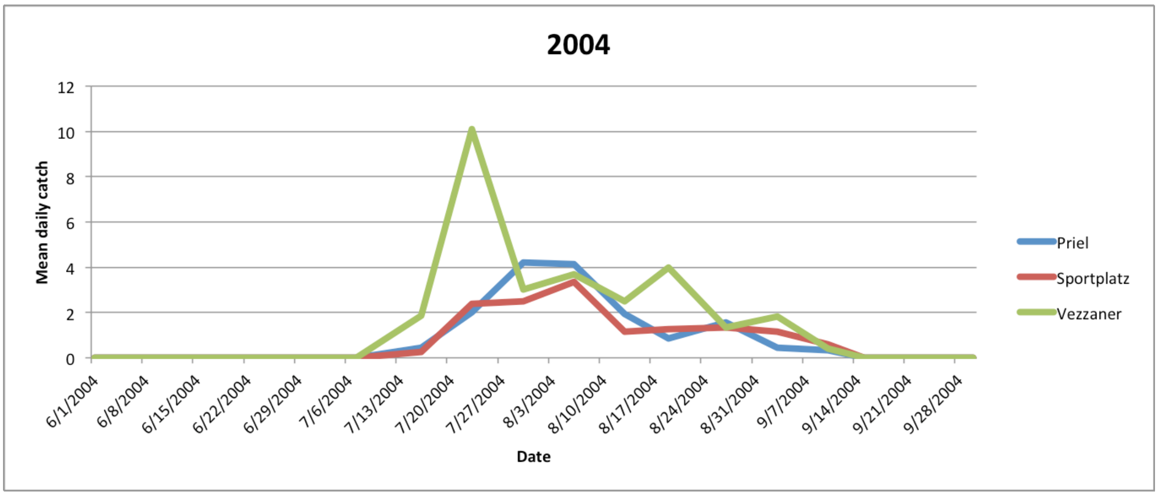
2004
Mean daily catch
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8
10
12
6/1/2004
6/8/2004
6/15/2004
6/22/2004
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7/6/2004
7/13/2004
7/20/2004
7/27/2004
8/3/2004
8/10/2004
8/17/2004
8/24/2004
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9/7/2004
9/14/2004
9/21/2004
9/28/2004
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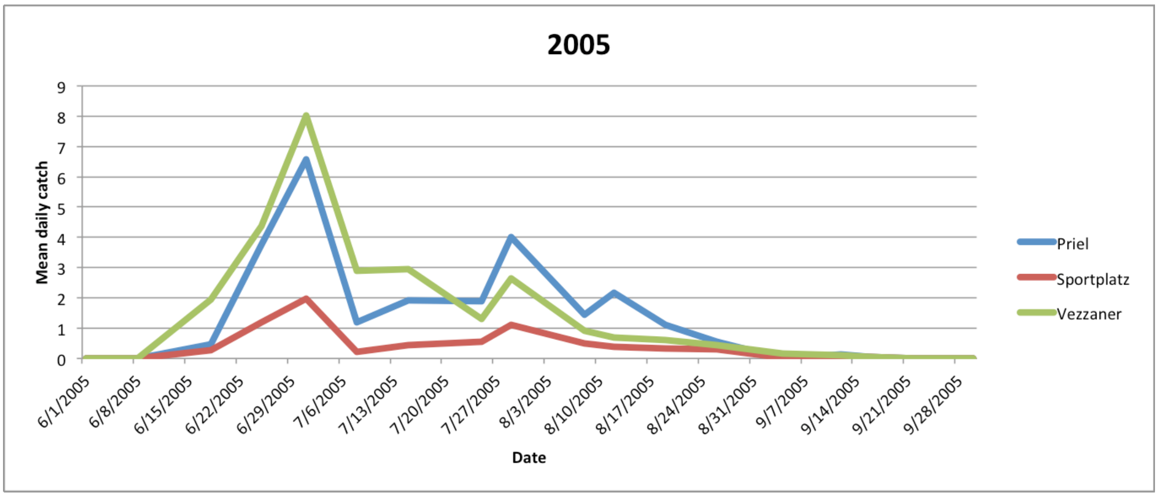
2005
Mean daily catch
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7
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6/8/2005
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6/22/2005
6/29/2005
7/6/2005
7/13/2005
7/20/2005
7/27/2005
8/3/2005
8/10/2005
8/17/2005
8/24/2005
8/31/2005
9/7/2005
9/14/2005
9/21/2005
9/28/2005
Date
Priel
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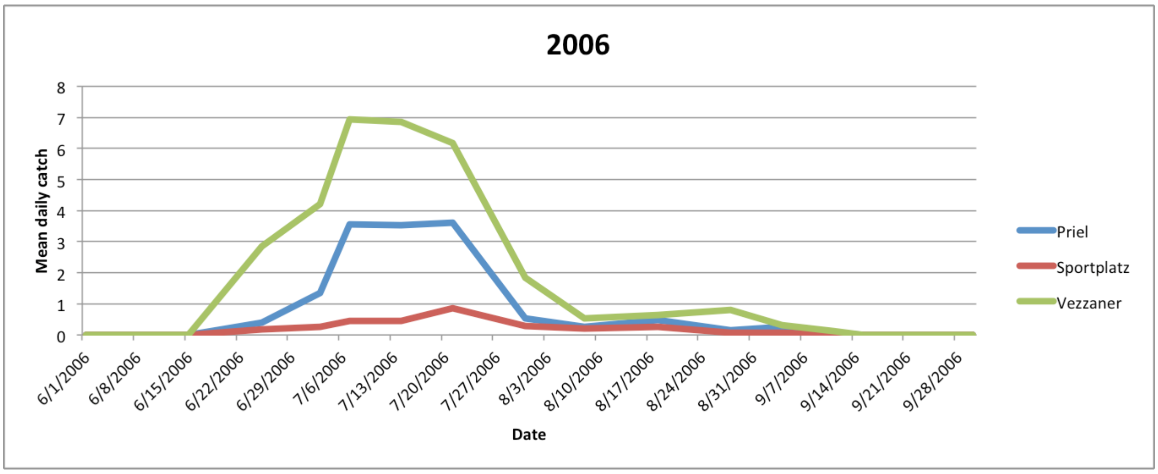
2006
Mean daily catch
0
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6/8/2006
6/15/2006
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6/29/2006
7/6/2006
7/13/2006
7/20/2006
7/27/2006
8/3/2006
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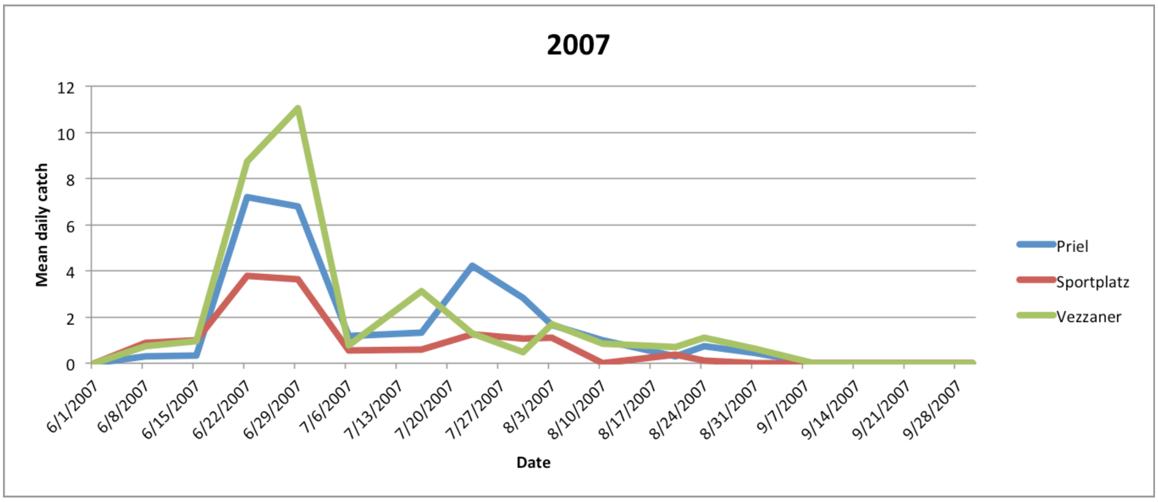
2007
Mean daily catch
0
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4
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8
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12
6/1/2007
6/8/2007
6/15/2007
6/22/2007
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7/6/2007
7/13/2007
7/20/2007
7/27/2007
8/3/2007
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8/24/2007
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9/28/2007
Date
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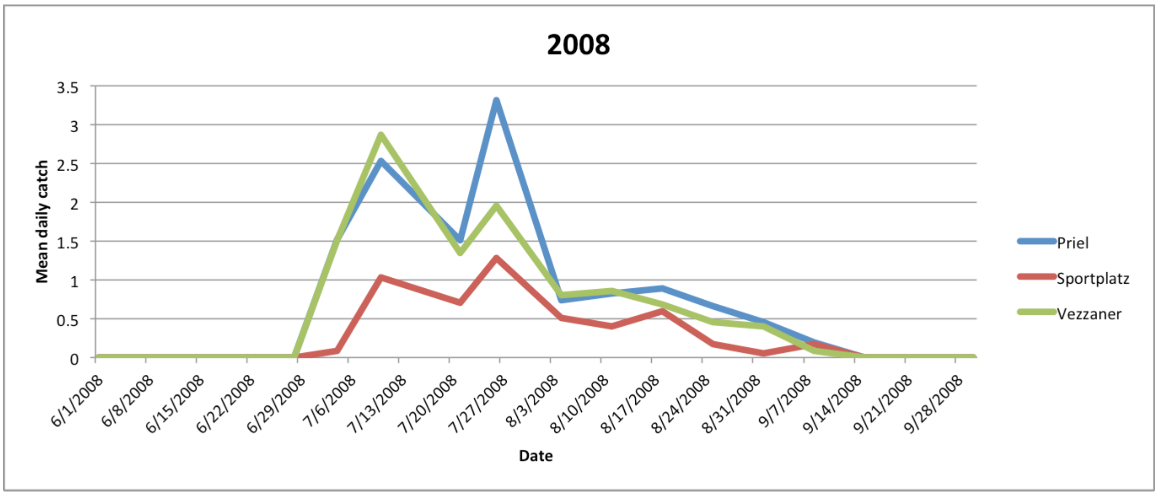
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3.5
3
2.5
2
1.5
1
0.5
0
6/1/2008
6/8/2008
6/15/2008
6/22/2008
6/29/2008
7/6/2008
7/13/2008
7/20/2008
7/27/2008
8/3/2008
8/10/2008
8/17/2008
8/24/2008
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Date
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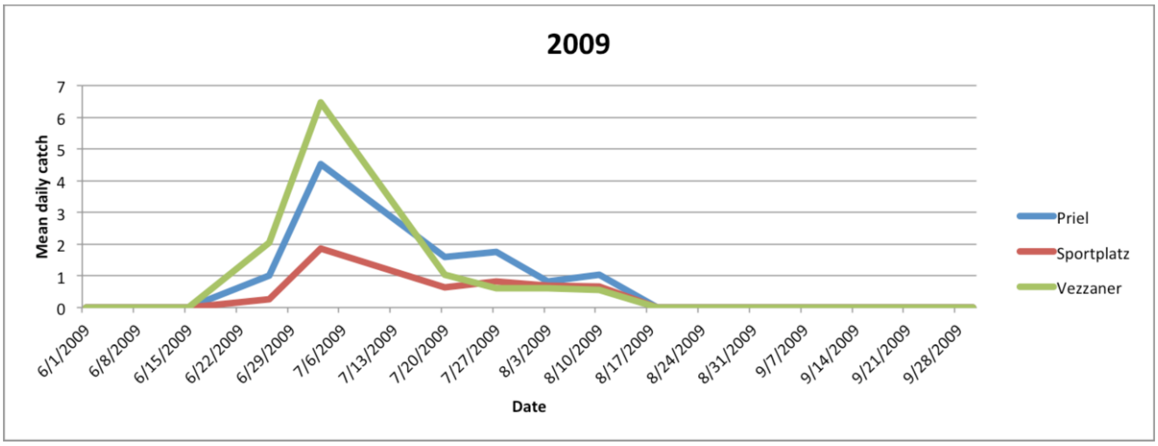
2009
Mean daily catch
7
6
5
4
3
2
1
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6/1/2009
6/8/2009
6/15/2009
6/22/2009
6/29/2009
7/6/2009
7/13/2009
7/20/2009
7/27/2009
8/3/2009
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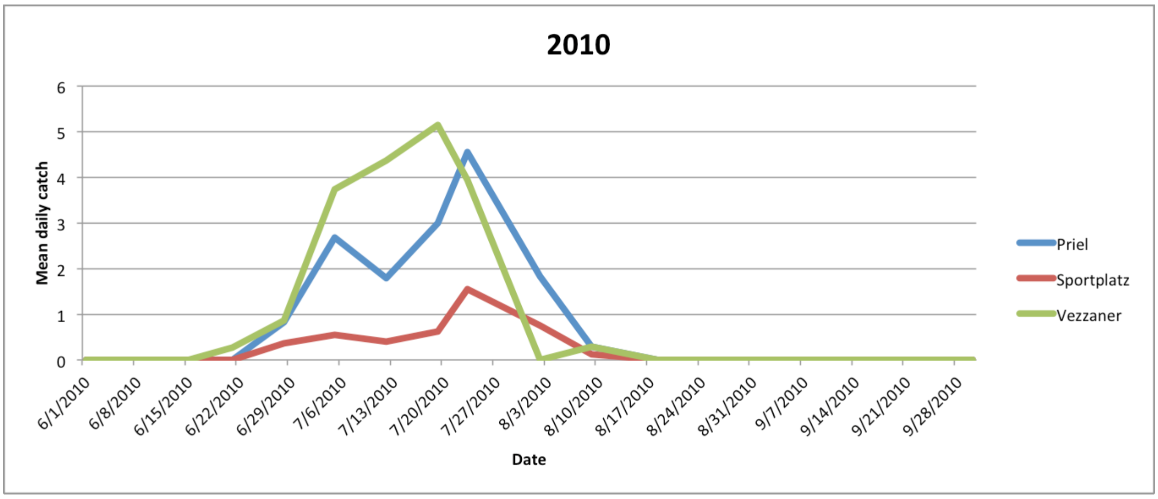
2010
Mean daily catch
6
5
4
3
2
1
0
6/1/2010
6/8/2010
6/15/2010
6/22/2010
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7/6/2010
7/13/2010
7/20/2010
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8/17/2010
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Date
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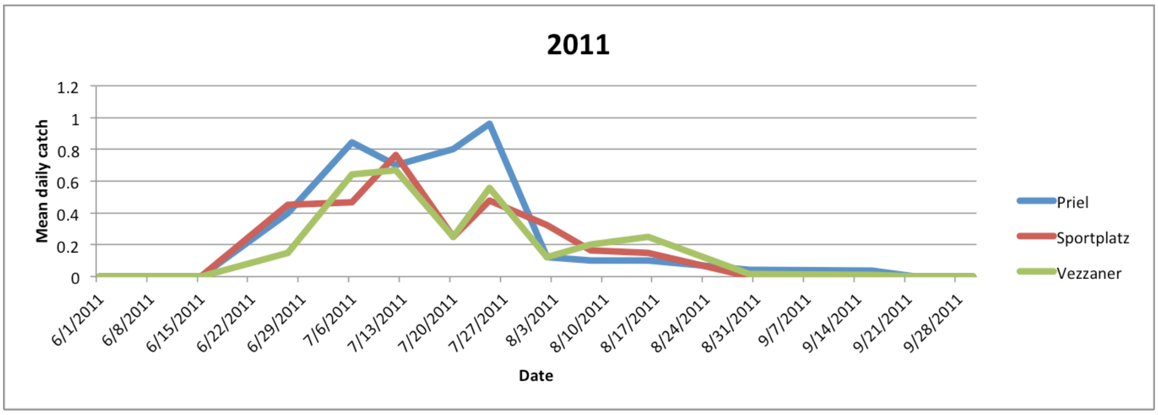
Vezzaner


2011
Mean daily catch
1.2
1
0.8
0.6
0.4
0.2
0
6/1/2011
6/8/2011
6/15/2011
6/22/2011
6/29/2011
7/6/2011
7/13/2011
7/20/2011
7/27/2011
8/3/2011
8/10/2011
8/17/2011
8/24/2011
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9/14/2011
9/21/2011
9/28/2011
Date
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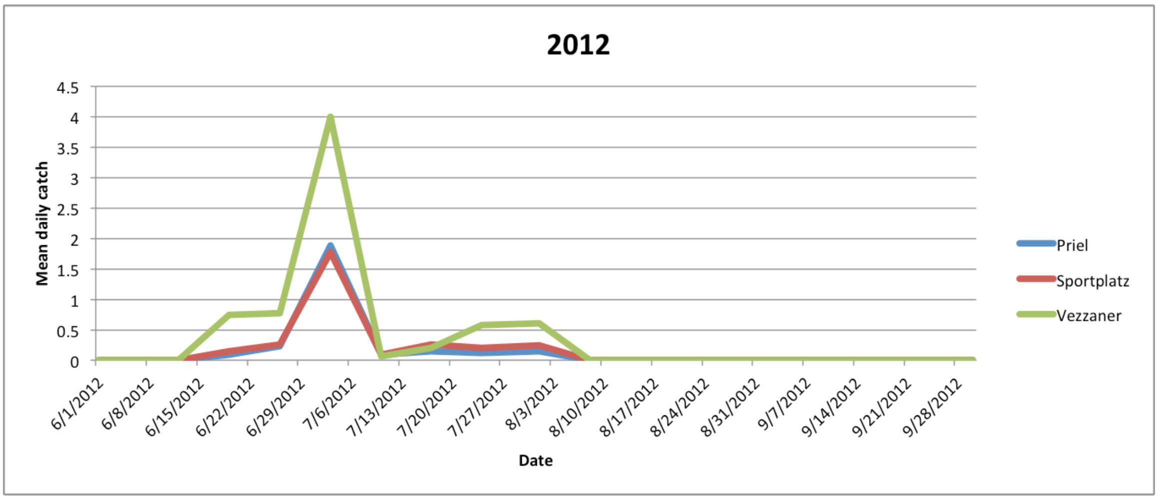
Vezzaner


2012
Mean daily catch
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
6/1/2012
6/8/2012
6/15/2012
6/22/2012
6/29/2012
7/6/2012
7/13/2012
7/20/2012
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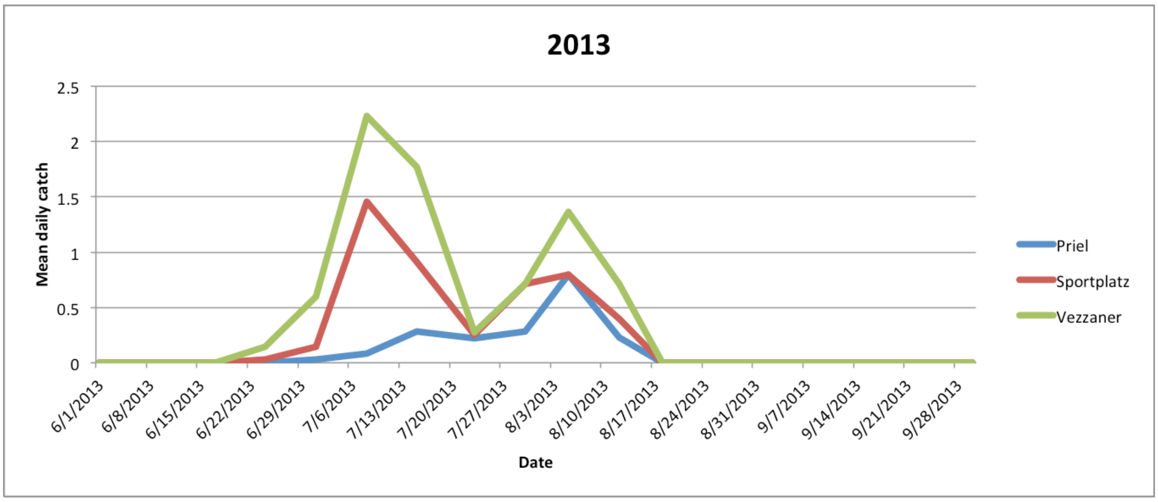
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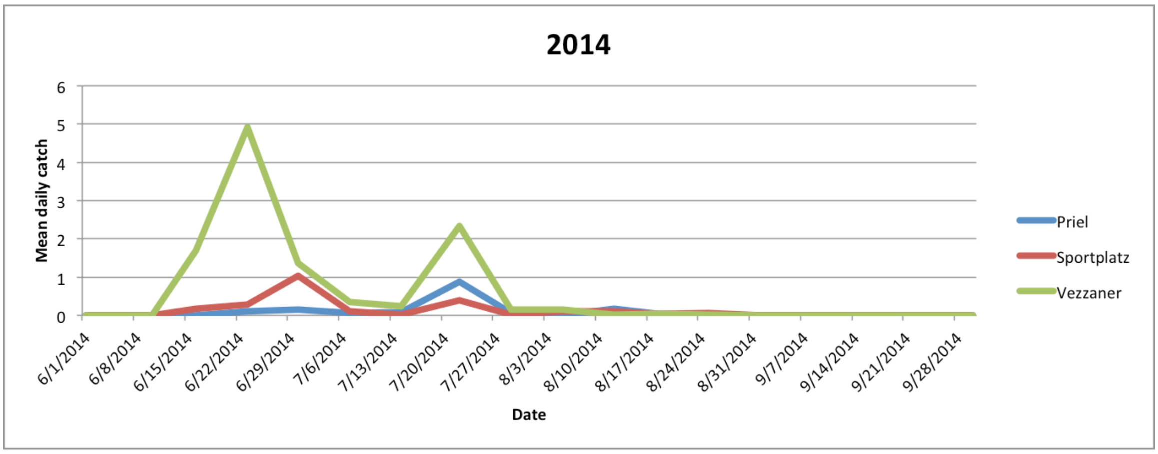
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CHAPTER 3

Winter temperature predicts prolonged diapause in the pine processionary moth across its geographic range

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(Preliminary manuscript, the final version will be submitted to a journal after a new analysis of the dataset)

Abstract

Prolonged diapause (PD) occurs in a number of insects and is interpreted as a way to evade adverse abiotic and biotic conditions. The winter pine processionary moths (*Thaumetopoea pityocampa* and *T. wilkinsoni*) are univoltine, with larvae feeding across the winter and pupating in the spring in the soil. Pupae may enter a facultative PD with adults emerging one or more years later. We tested the effect of spatial variation in winter temperature on the incidence of PD using a total of 117 PD records over the period 1964-2015 for 49 sites in 7 countries, covering most of the geographic range of the species. We found high variation in PD incidence over the species' range. Regions with average winter temperatures lower than 0°C or higher than 10°C were associated with higher PD incidence than regions with intermediate temperatures, which seemed to favour univoltine development. PD may represent a risk-spreading strategy although it is associated with high mortality. Climate change may reduce the incidence of PD at the colder sites whereas it may increase it at the warmer ones.

Keywords: *Pinus*, pest, temperature, pupa, mortality, soil

1. Introduction

Although diapause is recognized as a common strategy among insects to overcome unfavourable periods, prolonged diapause (PD) extending over more than one year remains hardly studied (Danks 1987; Soula and Menu 2005). In general, diapause represents a break in development maximising the chance to survive predictably adverse conditions (Tauber et al. 1986). PD, which is variable within a population or cohort, is generally considered as a way to spread the risk due to variably unfavourable conditions amongst years (Menu and Viala 2000). PD, however, can cause increased mortality (Sims 1983), likely due to longer exposure to adverse factors and enemies, as well as reduced performance (Matsuo 2006) related to extended energetic expenditure.

In Europe, Middle East, and Northern Africa, the winter pine processionary moth is represented by two sister species, *Thaumetopoea pityocampa* and *T. wilkinsoni* (Lepidoptera, Notodontidae), which are pests of conifers and a threat to human and animal health because of urticating setae (Roques 2015). Larvae feed during winter and generally adopt a univoltine life cycle. Temperature influences the duration of both larval and pupal stages (Demolin 1969; Berardi 2015; Robinet et al. 2015), and facultative PD of one or more years may occur in pupae (Demolin 1969; Halperin 1990; Salman et al. 2016). A phenology-based model suggests that PD in processionary moths is a developmental strategy to cope with adverse abiotic conditions, at both high and low elevations and latitudes (Demolin 1969). Additionally, factors such as soil moisture (Torres-Muros et al. 2017), food quality, population density, and natural enemies (Geri 1983) have been invoked to be linked to PD, although without clear support.

Here, to test the abiotic condition hypothesis (Demolin 1969; Halperin 1990), we review all the available evidence of PD across the range of *T. pityocampa* and *T. wilkinsoni* and specifically test the effect of winter temperatures as a driver of PD in this species. We also test if PD implies a cost in terms of mortality and we discuss the role of climate change on PD.

2. Materials and methods

2.1 Data collection

Published and unpublished data were retrieved from scientific databases and from internal reports. Available documents (initial n=42) were screened and data retained only when the total number of individuals, either larvae taken from the trees/ground or pupae taken from the soil, the number of moths emerged in the year of collection and in the following year(s), and the number of dead larvae/pupae were given for a specific year of sampling (namely a cohort). Information was obtained for seven countries from a total of 14 documents and 49 sites (Fig. 1; Table 1; supplement Table S1). These sites belong to 6 out of 11 genetic subclades of the pine processionary moth described by Kerdelhué et al. (2009) and El Mokhefi et al. (2016) (Fig. 1) and cover most of the climatic range of the species (supplement Fig. S1). For 17 sites, more than one year of observations was available, giving a total of 117 individual datasets on the occurrence of PD.

For the year(s) of sampling, the monthly average air temperature of each site was collected from the source document (n=32) or from the nearest weather station (n=17) (supplement Table S1). The average of monthly temperature was calculated for December, January, and February as these three months correspond to the ‘cold period’ of larval development (Roques 2015).

Data related to PD incidence and pupal mortality obtained from samples collected when larvae were in procession on the ground before pupation and when pupae were already buried in the soil (supplement Tables S1) were pooled since no differences were found (t-test, $p>0.05$). The mortality percentage was calculated as the number of individuals that did not produce moths with respect to the initial number of individuals in the cohort. The PD percentage was calculated as the number of individuals that emerged over the year(s) following the year of pupation, or that were still alive when the experiment was over one or more years after pupation (emerged after PD + living pupae at the last check), with respect to the total number of emerged or living individuals (emerged univoltine + emerged after PD + living pupae at the last check).

2.2 Data analysis

To test the effect of temperature on PD incidence, we used a linear mixed-effect model for Normal distribution. The model included linear and quadratic terms of temperature as fixed effects and site nested within genetic subclade as random effects. The consideration of subclade and site as random effects accounted for the biological and spatial dependence in the data. The genetic structure of the subclades was considered as a grouping factor in statistical analyses (linear mixed models). It is important to stress that our model tested the effect of spatial variation in winter temperature across the species range considered, and not the within-site temporal variation. The analyses were run using the *lme* package in *R*. A model with the same random structure was estimated to test the association between the incidence of PD and pupal mortality.

3. Results

PD occurred in all genetic subclades of *T. pityocampa* and *T. wilkinsoni* for which data were available (Fig 2a). In the Corsica subclade, all individuals were fixed on a semivoltine life cycle, with further years of PD. As more work is needed to clarify this different pattern, the Corsica samples were excluded from analyses. The incidence of PD in the first year after pupation varied greatly among sites and cohorts within sites. Cold winters (temperature of December, January, and February below 0°C) as well as warm winters (temperature above 10°C) resulted in high PD incidence, while the optimal conditions for univoltine development were identified between 0 and 10°C, irrespective of subclades (Fig. 2a). Pupal mortality was positively and significantly correlated to PD incidence (Fig. 2b). Mortality varied greatly among sites and cohorts within sites. It reached the highest values at both cold (lower than 0°C) and warm (higher than 10°C) winter sites, indicating that individuals entering PD are more likely to die than those with univoltine development. The mortality factors were not systematically addressed in each study, so it was not possible to analyse for these effects.

4. Discussion

Winter temperature experienced by larvae is a reliable predictor of the PD incidence in the processionary moth. PD may have a clear adaptive value in this species, since the risk of being exposed to acute unfavourable conditions associated with extreme cold weather is diluted among years (Danks 1987). Diapause durations up to 8-9 years have been found at a few sites (Halperin 1990, Salman et al. 2016), while in most cases the duration varied between 1 and 3 years (Table S1) but we could not test for factors associated with duration.

High incidence of PD was observed at the southern edge of the range, where winter temperature can be above 10°C. A potential explanation for this finding is that winter temperature is a proxy of another thermal stressor, i.e. the summer temperatures that can impair the development of young larvae at these sites (Santos et al. 2011). Thus, entering PD at the southern edge of the range would also allow risk spreading through time and contribute to persistence of populations.

Irrespective of the temperature conditions associated with PD occurrence, pupal mortality increased with PD incidence likely because of a longer exposure to chronic mortality factors (Sims 1983), desiccation and depletion of the energy reserves (Hahn and Denlinger 2011). The higher mortality risks associated with PD challenge the hypothesis that PD of processionary moth pupae represents a diversifying risk-spreading strategy for the species, although more attention should be given to climatically non-typical years when benefits of such a strategy would be expected (Menu et al. 2000). PD could, however, limit the risks of local extinction after the generally high mortality observed at outbreak density, enabling the survival of the species (Salman et al. 2016; Tamburini et al. 2013; Li et al. 2015). In addition, the phenology of the pine processionary moth is severely constrained by climatic factors Robinet et al. (2015), and it could be that in certain years the required physiological conditions to complete univoltine development are not met, thus leaving no alternative to PD.

Climate change may affect PD in different ways. As winter is experienced in two different life stages, the growing larva and the diapausing pupa, it is likely that stressful conditions differ between stages; larvae feeding on trees (e.g. exposed to low, fluctuating temperatures) and pupae in the soil (e.g. buffered against temperature extremes), with complex interactions. Observations carried out between 1999 and 2016 in the coldest edge of the species' geographical range indicated that PD incidence markedly decreased as a consequence of climate warming (Salman et al. 2016). Such changes in PD, and related mortality, have to be taken into consideration when modelling population dynamics and range expansion, since PD can alter the success of a population in a given area (Li et al. 2015). Our study has shown temperature variation across most of the species range to be an important extrinsic factor for geographic variation in PD incidence, although the identification of the precise mechanism requires further work.

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Table 1. Distribution of the 49 study sites among genetic clades and subclades of pine processionary moths. The time period of sampling and the pine host species are also given

Clade	Subclade	No. of sites	Years	Host *Pinus*
Pityocampa	Rest of Europe	16	1971-2015	*brutia* *nigra* *pinaster*
Pityocampa	Corsica	10	1966-1974 2015-2016	*laricio*
Pityocampa	N Algeria N Morocco	13	1988	*halepensis*
Pityocampa	S Algeria S Morocco	3	1988-1995	*halepensis*
ENA	E Algeria Tunisia	4	1983	*halepensis*
Wilkinsoni	Israel	3	1964-1970	*halepensis*

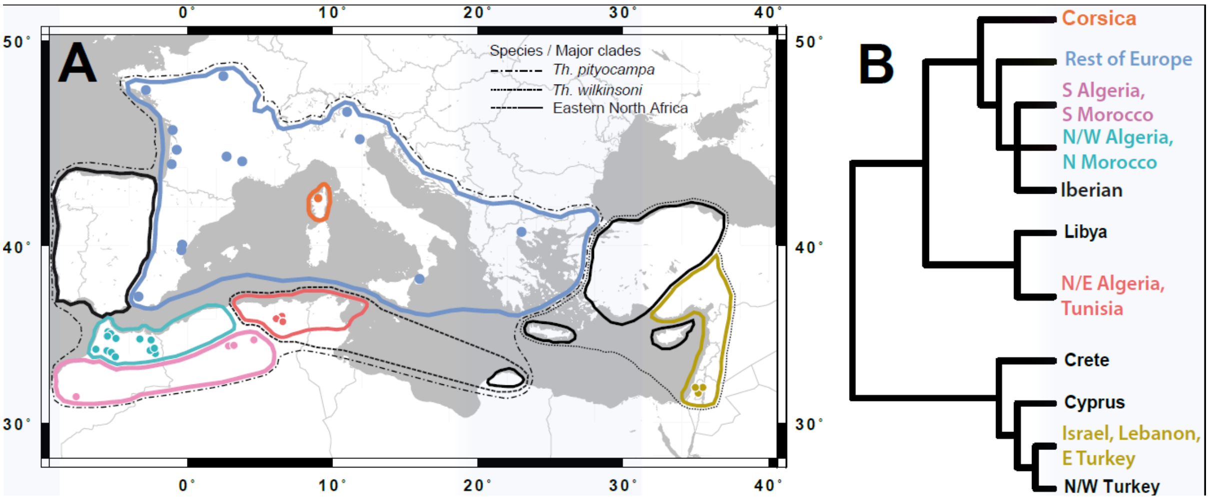


Fig. 1. Sites used for the analysis of prolonged diapause at the pupal stage, indicated with coloured dots within each species and subclade of the pine processionary moth (a), and tree of genetic structure based on Kerdelhué et al. (2009) and El Mokhefi et al. (2016). Data were not available for the subclades indicated in black.

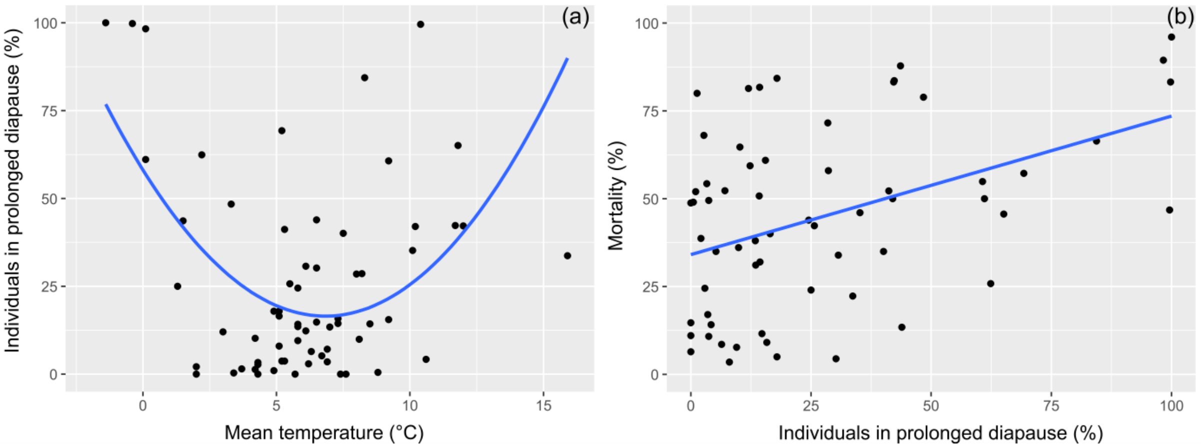
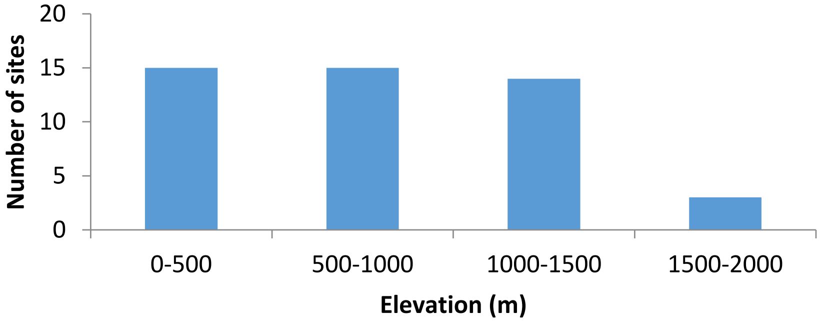
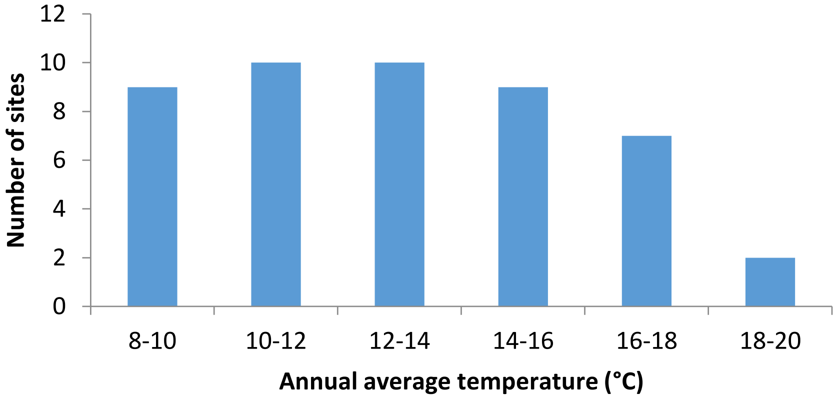
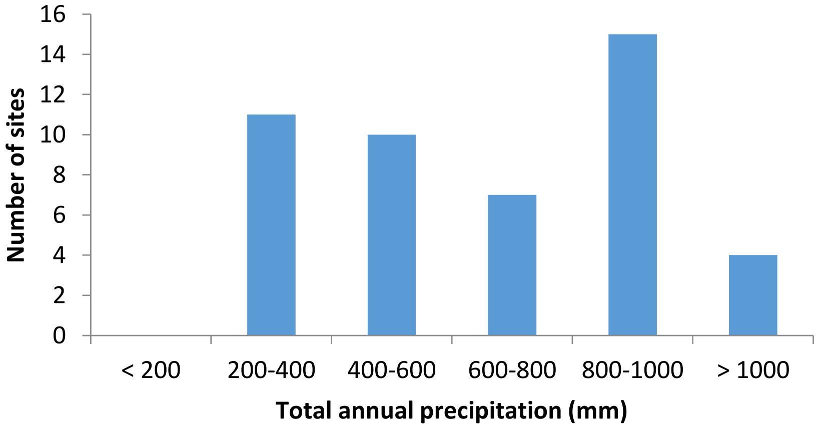


Fig. 2. (a) Relationship between the daily mean temperature during the cold period of the larval development (December, January, and February) and the percentage of prolonged diapause in the cohorts of pupae originating from larvae experiencing those temperatures. The fitted line represents predictions of a mixed model including subclade and site as random effects (Diapause = 67.02 - 12.50 Temp + 0.85 Temp^2, both terms $P<0.001$, $n=65$). (b) Relationship between the percentage of individuals entering prolonged diapause and the pupal mortality observed in the first year of prolonged diapause. The fitted line represents a prediction of a mixed model including subclade and site as random effects (Mortality = 37.88 + 0.364 Diapause, $P<0.001$, $n=65$).

Fig. S1. Distribution of the 49 study sites among classes of elevation, annual average temperature, and annual precipitation (data obtained from WorldClim). WorldClim is a set of global climate grids with a spatial resolution of about 1 square kilometre (www.worldclim.org),and provides average climatic data for 30 years (1970-2000). These grids can be imported into GIS (geographic information system) applications. The monthly average temperature (in °C) and the monthly average rainfall (in mm) were imported into Qgis software. Then, in Qgis, we used the coordinates of each sample site to extract the relevant climate data.







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Year	Country	Site	Latitude	Longitude	Elevation	Host	Clade	Sub-Clade	Tmean-DJF-WC-(1950-2000)	Mean-Rainfall-AMJ-WC-(1950-2000)	Tmean_n	Tmean_min_n	Rainfall-AMJ-local-year-n	Density	Sampling	N.individuals	Condition	Diapause-%-(from-total-initial-number)	Mortality	Diapause	Reference
1983	Algeria	Ain-Yagout	3577255	641094	895	Pinus-halepensis	ENA	ENA	6.5	35	6.9	NA	31.3	NA	L	176	outdoor	3.4	52.3	7.1	Bertella 1987
1983	Algeria	Bouilef	3558086	620339	1100	Pinus-halepensis	ENA	ENA	5.4	30.3	5.8	NA	31.3	NA	P	935	field/lab	9.3	31.1	13.5	Bertella 1987
1983	Algeria	Bouilef	3558086	620339	1100	Pinus-halepensis	ENA	ENA	5.4	30.3	5.8	NA	31.3	NA	L	244	outdoor	7	50.8	14.2	Bertella 1987
1983	Algeria	Bou-Saƒda	3514982	407504	883	Pinus-halepensis	ENA	ENA	6.4	24.3	7	NA	31.3	NA	L	229	outdoor	8.3	38	13.4	Bertella 1987
1983	Algeria	Ich-Ali	3551961	612813	1146	Pinus-halepensis	ENA	ENA	5.1	29.7	5.5	NA	31.3	NA	L	196	outdoor	14.8	42.3	25.7	Bertella 1987
1994	Algeria	Moudjebara	3462361	332389	1210	Pinus-halepensis	Pityocampa	South-Algeria-South-Morocco	4.9	30.3	5.2	1.3	6.1	high	L	2567	outdoor	1.9	49.5	3.7	Zamoum 1998
1994	Algeria	Moudjebara	3462361	332389	1210	Pinus-halepensis	Pityocampa	South-Algeria-South-Morocco	4.9	30.3	5.2	1.3	6.1	high	P	1048	outdoor	29.7	57.2	69.3	Zamoum 1998
1994	Algeria	Senalba-Chergui	3464944	319528	1253	Pinus-halepensis	Pityocampa	South-Algeria-South-Morocco	4.7	31.3	4.9	1.1	6.1	low	L	1220	outdoor	0.5	52	1	Zamoum 1998
1995	Algeria	Moudjebara	3462361	332389	1210	Pinus-halepensis	Pityocampa	South-Algeria-South-Morocco	4.9	30.3	6.1	1	21	high	L	4997	outdoor	5	59.4	12.3	Zamoum 1998
1995	Algeria	Moudjebara	3462361	332389	1210	Pinus-halepensis	Pityocampa	South-Algeria-South-Morocco	4.9	30.3	6.1	1	21	high	P	292	outdoor	17.5	33.9	30.7	Zamoum 1998
1995	Algeria	Senalba-Chergui	3464944	319528	1253	Pinus-halepensis	Pityocampa	South-Algeria-South-Morocco	4.7	31.3	5.8	0.8	21	low	L	1783	outdoor	13.8	43.9	24.5	Zamoum 1998
1966	France	PK51	4230639	895528	950	Pinus-laricio	Pityocampa	Corsica	4.6	52	4.8	NA	73.3	medium	L	700	lab	51	49	100	Geri 1980
1966	France	PK51	4230639	895528	950	Pinus-laricio	Pityocampa	Corsica	4.6	52	4.8	NA	73.3	medium	P	5000	field/lab	19	81	100	Geri 1980
1966	France	Mezzanule	4229083	893444	1030	Pinus-laricio	Pityocampa	Corsica	4	54.3	4.4	NA	73.3	low	L	700	lab	64	36	100	Geri 1980
1966	France	Mezzanule	4229083	893444	1030	Pinus-laricio	Pityocampa	Corsica	4	54.3	4.4	NA	73.3	low	P	5000	field/lab	18	82	100	Geri 1980
1966	France	Pente-des-Genˆts	4227778	890250	1150	Pinus-laricio	Pityocampa	Corsica	3.7	55.7	3.7	NA	73.3	high	L	700	lab	53	47	100	Geri 1980
1966	France	Pente-des-Genˆts	4227778	890250	1150	Pinus-laricio	Pityocampa	Corsica	3.7	55.7	3.7	NA	73.3	high	P	5000	field/lab	27	73	100	Geri 1980
1966	France	Castellacce-Bas	4230750	889361	1350	Pinus-laricio	Pityocampa	Corsica	3.1	57.7	2.6	NA	73.3	high	L	700	lab	75	25	100	Geri 1980
1966	France	Castellacce-Bas	4230750	889361	1350	Pinus-laricio	Pityocampa	Corsica	3.1	57.7	2.6	NA	73.3	high	P	4000	field/lab	45	55	100	Geri 1980
1966	France	Castellacce-Haut	4230833	889167	1390	Pinus-laricio	Pityocampa	Corsica	3.1	57.7	2.4	NA	73.3	medium	L	700	lab	59	41	100	Geri 1980
1966	France	Castellacce-Haut	4230833	889167	1390	Pinus-laricio	Pityocampa	Corsica	3.1	57.7	2.4	NA	73.3	medium	P	2000	field/lab	15	85	100	Geri 1980
1968	France	PK51	4230639	895528	950	Pinus-laricio	Pityocampa	Corsica	4.6	52	3	NA	95.2	medium	L	300	lab	42	58	100	Geri 1980
1968	France	PK51	4230639	895528	950	Pinus-laricio	Pityocampa	Corsica	4.6	52	3	NA	95.2	medium	P	1400	field/lab	11	89	100	Geri 1980

1968	France	Mezzanule	4229083	893444	1030	Pinus-laricio	Pityocampa	Corsica	4	54.3	2.5	NA	95.2	low	L	300	lab	49	51	100	Geri 1980
1968	France	Mezzanule	4229083	893444	1030	Pinus-laricio	Pityocampa	Corsica	4	54.3	2.5	NA	95.2	low	P	2100	field/lab	2	98	100	Geri 1980
1968	France	Pente-des-Genˆts	4227778	890250	1150	Pinus-laricio	Pityocampa	Corsica	3.7	55.7	1.9	NA	95.2	high	L	300	lab	38	62	100	Geri 1980
1968	France	Pente-des-Genˆts	4227778	890250	1150	Pinus-laricio	Pityocampa	Corsica	3.7	55.7	1.9	NA	95.2	high	P	2100	field/lab	18	82	100	Geri 1980
1968	France	Castellacce-Bas	4230750	889361	1350	Pinus-laricio	Pityocampa	Corsica	3.1	57.7	0.7	NA	95.2	high	L	300	lab	41	59	100	Geri 1980
1968	France	Castellacce-Bas	4230750	889361	1350	Pinus-laricio	Pityocampa	Corsica	3.1	57.7	0.7	NA	95.2	high	P	2800	field/lab	23	77	100	Geri 1980
1968	France	Castellacce-Haut	4230833	889167	1390	Pinus-laricio	Pityocampa	Corsica	3.1	57.7	0.5	NA	95.2	medium	L	300	lab	74	26	100	Geri 1980
1968	France	Castellacce-Haut	4230833	889167	1390	Pinus-laricio	Pityocampa	Corsica	3.1	57.7	0.5	NA	95.2	medium	P	2800	field/lab	26	74	100	Geri 1980
1970	France	PK51	4230639	895528	950	Pinus-laricio	Pityocampa	Corsica	4.6	52	2.5	NA	42.9	medium	L	300	lab	36	64	100	Geri 1980
1970	France	PK51	4230639	895528	950	Pinus-laricio	Pityocampa	Corsica	4.6	52	2.5	NA	42.9	medium	P	1500	field/lab	30	70	100	Geri 1980
1970	France	Mezzanule	4229083	893444	1030	Pinus-laricio	Pityocampa	Corsica	4	54.3	2.1	NA	42.9	low	L	300	lab	2	98	100	Geri 1980
1970	France	Mezzanule	4229083	893444	1030	Pinus-laricio	Pityocampa	Corsica	4	54.3	2.1	NA	42.9	low	P	700	field/lab	1	99	100	Geri 1980
1970	France	Pente-des-Genˆts	4227778	890250	1150	Pinus-laricio	Pityocampa	Corsica	3.7	55.7	1.4	NA	42.9	low	L	300	lab	5	95	100	Geri 1980
1970	France	Pente-des-Genˆts	4227778	890250	1150	Pinus-laricio	Pityocampa	Corsica	3.7	55.7	1.4	NA	42.9	low	P	2800	field/lab	19	81	100	Geri 1980
1970	France	Castellacce-Bas	4230750	889361	1350	Pinus-laricio	Pityocampa	Corsica	3.1	57.7	0.3	NA	42.9	high	P	3000	field/lab	14	86	100	Geri 1980
1970	France	Castellacce-Haut	4230833	889167	1390	Pinus-laricio	Pityocampa	Corsica	3.1	57.7	0	NA	42.9	medium	P	3200	field/lab	20	80	100	Geri 1980
1970	France	Casamaccioli	4231361	900694	950	Pinus-laricio	Pityocampa	Corsica	4.8	51	2.5	NA	42.9	NA	P	1200	field/lab	23.5	76.5	100	Geri 1980
1970	France	Calasima	4232944	894889	1050	Pinus-laricio	Pityocampa	Corsica	4.4	53	1.9	NA	42.9	NA	P	2600	field/lab	5.9	94.1	100	Geri 1980
1970	France	Popaja	4228417	892222	1100	Pinus-laricio	Pityocampa	Corsica	4.2	53.7	1.7	NA	42.9	NA	P	1600	field/lab	15.7	84.3	100	Geri 1980
1970	France	Castellacce	4230778	889083	1370	Pinus-laricio	Pityocampa	Corsica	3	58	0.2	NA	42.9	NA	P	1600	field/lab	13.7	86.3	100	Geri 1980
1971	France	Les-Mathes-La-Palmyre	4569861	-119778	10	Pinus-pinaster	Pityocampa	Rest-of-Europe	6.5	59.3	6.5	NA	59.3	low	P	42	field/lab	28.9	4.4	30.2	Abgrall 2001
1971	France	Vielle-St-Girons	4395528	-134389	20	Pinus-pinaster	Pityocampa	Rest-of-Europe	7.5	89.3	5.8	1.2	114.9	low	P	91	field/lab	8.8	7.7	9.5	Abgrall 2001
1972	France	Berder	4757500	-2.8900	10	Pinus-pinaster	Pityocampa	Rest-of-Europe	6.2	55.3	6.3	3.6	63.7	high	P	169	field/lab	5.9	8.5	6.4	Abgrall 2001
1972	France	Les-Mathes-La-Palmyre	4569861	-119778	10	Pinus-pinaster	Pityocampa	Rest-of-Europe	6.5	59.3	6.5	NA	59.3	low	P	52	field/lab	38	13.4	43.9	Abgrall 2001
1972	France	St-Germain-de-Calberte	4421889	380361	600	Pinus-nigra	Pityocampa	Rest-of-Europe	3.5	63.7	4.9	1.2	89.8	medium	P	100	field/lab	17	5	17.9	Abgrall 2001

1972	France	Vielle-St-Girons	4395528	-134389	20	Pinus-pinaster	Pityocampa	Rest-of-Europe	7.5	89.3	6.7	2.4	66.4	low	P	88	field/lab	3.4	35	5.2	Abgrall 2001
1972	France	PK51	4230639	895528	950	Pinus-laricio	Pityocampa	Corsica	4.6	52	6.1	NA	107.1	low	L	NA	lab	63.1	36.9	100	Geri 1980
1972	France	PK51	4230639	895528	950	Pinus-laricio	Pityocampa	Corsica	4.6	52	6.1	NA	107.1	low	P	3000	field/lab	60	40	100	Geri 1980
1972	France	Casamaccioli	4231361	900694	950	Pinus-laricio	Pityocampa	Corsica	4.8	51	6.1	NA	107.1	NA	L	NA	lab	73.8	26.2	100	Geri 1980
1972	France	Casamaccioli	4231361	900694	950	Pinus-laricio	Pityocampa	Corsica	4.8	51	6.1	NA	107.1	NA	P	3000	field/lab	35.3	64.7	100	Geri 1980
1972	France	Calasima	4232944	894889	1050	Pinus-laricio	Pityocampa	Corsica	4.4	53	5.5	NA	107.1	NA	L	NA	lab	72.8	27.2	100	Geri 1980
1972	France	Calasima	4232944	894889	1050	Pinus-laricio	Pityocampa	Corsica	4.4	53	5.5	NA	107.1	NA	P	3000	field/lab	19.6	80.4	100	Geri 1980
1972	France	Popaja	4228417	892222	1100	Pinus-laricio	Pityocampa	Corsica	4.2	53.7	5.2	NA	107.1	low	L	500	lab	77.8	22.2	100	Geri 1980
1972	France	Popaja	4228417	892222	1100	Pinus-laricio	Pityocampa	Corsica	4.2	53.7	5.2	NA	107.1	low	P	3000	field/lab	23.6	76.4	100	Geri 1980
1972	France	Castellacce	4230778	889083	1370	Pinus-laricio	Pityocampa	Corsica	3	58	3.7	NA	107.1	medium	L	NA	lab	71	29	100	Geri 1980
1972	France	Castellacce	4230778	889083	1370	Pinus-laricio	Pityocampa	Corsica	3	58	3.7	NA	107.1	medium	P	3000	field/lab	15.7	84.3	100	Geri 1980
1973	France	Berder	4757500	-2.890	10	Pinus-pinaster	Pityocampa	Rest-of-Europe	6.2	55.3	6.2	3.1	54.5	low	P	45	field/lab	2.2	24.5	2.9	Abgrall 2001
1974	France	Berder	4757500	-2.890	10	Pinus-pinaster	Pityocampa	Rest-of-Europe	6.2	55.3	7.4	4.2	52.6	low	P	164	field/lab	0	48.8	0	Abgrall 2001
1974	France	Les-Mathes-La-Palmyre	4569861	-119778	10	Pinus-pinaster	Pityocampa	Rest-of-Europe	6.5	59.3	6.5	NA	59.3	low	P	199	field/lab	13.1	11.6	14.8	Abgrall 2001
1974	France	St-Germain-de-Calberte	4421889	380361	600	Pinus-nigra	Pityocampa	Rest-of-Europe	3.5	63.7	5.1	1.2	73	low	P	642	field/lab	7.8	3.5	8	Abgrall 2001
1974	France	Vielle-St-Girons	4395528	-134389	20	Pinus-pinaster	Pityocampa	Rest-of-Europe	7.5	89.3	6.9	2	80.9	low	P	235	field/lab	2.9	17	3.5	Abgrall 2001
1974	France	PK51	4230639	895528	950	Pinus-laricio	Pityocampa	Corsica	4.6	52	3	NA	42.9	low	L	NA	lab	74.7	25.3	100	Geri 1980
1974	France	PK51	4230639	895528	950	Pinus-laricio	Pityocampa	Corsica	4.6	52	3	NA	42.9	low	P	3000	field/lab	29	71	100	Geri 1980
1974	France	Casamaccioli	4231361	900694	950	Pinus-laricio	Pityocampa	Corsica	4.8	51	3	NA	42.9	low	L	NA	lab	94.2	5.8	100	Geri 1980
1974	France	Casamaccioli	4231361	900694	950	Pinus-laricio	Pityocampa	Corsica	4.8	51	3	NA	42.9	low	P	3000	field/lab	35.3	64.7	100	Geri 1980
1974	France	Calasima	4232944	894889	1050	Pinus-laricio	Pityocampa	Corsica	4.4	53	2.4	NA	42.9	low	L	NA	lab	77.1	22.9	100	Geri 1980
1974	France	Calasima	4232944	894889	1050	Pinus-laricio	Pityocampa	Corsica	4.4	53	2.4	NA	42.9	low	P	3000	field/lab	34.3	65.7	100	Geri 1980
1974	France	Popaja	4228417	892222	1100	Pinus-laricio	Pityocampa	Corsica	4.2	53.7	2.1	NA	42.9	medium	P	3000	field/lab	21.6	78.4	100	Geri 1980
1974	France	Castellacce	4230778	889083	1370	Pinus-laricio	Pityocampa	Corsica	3	58	0.6	NA	42.9	medium	L	NA	lab	41.8	58.2	100	Geri 1980
1974	France	Castellacce	4230778	889083	1370	Pinus-laricio	Pityocampa	Corsica	3	58	0.6	NA	42.9	medium	P	3000	field/lab	19.6	80.4	100	Geri 1980
1975	France	St-Germain-de-Calberte	4421889	380361	600	Pinus-nigra	Pityocampa	Rest-of-Europe	3.5	63.7	5.7	1.4	52.1	low	P	100	field/lab	0	11	0	Abgrall 2001

1975	France	Vielle-St-Girons	4395528	-134389	20	Pinus-pinaster	Pityocampa	Rest-of-Europe	7.5	89.3	8.2	3	105	low	P	309	field/lab	12	58	28.6	Abgrall 2001
1976	France	Vielle-St-Girons	4395528	-134389	20	Pinus-pinaster	Pityocampa	Rest-of-Europe	7.5	89.3	5.3	0.9	37.2	low	P	240	field/lab	3.3	10.8	3.7	Abgrall 2001
1977	France	Berder	4757500	-2.8900	10	Pinus-pinaster	Pityocampa	Rest-of-Europe	6.2	55.3	7.6	4.2	61.9	medium	P	47	field/lab	0	6.4	0	Abgrall 2001
1977	France	St-Germain-de-Calberte	4421889	380361	600	Pinus-nigra	Pityocampa	Rest-of-Europe	3.5	63.7	4.3	0.6	89.8	low	P	231	field/lab	0	14.7	0	Abgrall 2001
1977	France	Vielle-St-Girons	4395528	-134389	20	Pinus-pinaster	Pityocampa	Rest-of-Europe	7.5	89.3	7.3	3.1	132	low	P	251	field/lab	14.3	9.1	15.8	Abgrall 2001
2008	France	Landes-de-Gascognes	4473333	-0.76667	59	Pinus-pinaster	Pityocampa	Rest-of-Europe	5.9	73	8.1	4.2	101.7	NA	L	2700	field	6.3	36.1	9.9	Dulaurent et al. 2011
2014	France	Marcillac-en-Vallon	4447818	2482817	400	Pinus-nigra	Pityocampa	Rest-of-Europe	4.5	66.7	5.1	1.1	69.2	high	L	999	field/lab	9.7	40	16.5	Abgrall 2001
2015	France	Vienne-en-Val	4780580	2120900	102	Pinus-nigra	Pityocampa	Rest-of-Europe	3.7	55	4.3	1.2	37.7	low	P	1667	outdoor	1.5	54.3	3.3	Laparie 2016
2015	France	Corsica	4225268	8817917	913	Pinus-laricio	Pityocampa	Corsica	5.1	51.7	5.1	NA	51.7	low	P	228	field/lab	53.5	28.5	74.8	Geri 1980
1984	Greece	Thessaloniki	4063556	2298278	247	Pinus-brutia	Pityocampa	Rest-of-Europe	5.1	39	5.1	NA	62	NA	L	1029	field	2.82	84.26	17.9	Markalas 1989
1964	Israel	Eshtaol	3178278	3499250	360	Pinus-halepensis	Wilkinsoni	East-Turkey-Lebanon-Israel	13.3	6.3	11.7	7.6	5	NA	L	800	field	6.9	83.6	42.3	Halperin 1969 and 1990
1969	Israel	Ramallah	3190333	3521083	850	Pinus-halepensis	Wilkinsoni	East-Turkey-Lebanon-Israel	9.2	8.7	10.4	7.3	10.2	NA	L	500	field	53	46.8	99.6	Halperin 1969 and 1990
1970	Israel	Ramat-Gan	3206583	3483139	50	Pinus-halepensis	Wilkinsoni	East-Turkey-Lebanon-Israel	13.7	6	15.9	12.9	8.8	NA	L	336	field	26.2	22.3	33.7	Halperin 1969 and 1990
1993	Italy	Colle-Calbarina	4527306	1173722	136	Pinus-nigra	Pityocampa	Rest-of-Europe	4	75.3	3.3	-1.3	37.3	high	P	294	outdoor	10.2	78.9	48.4	Ghiraldo 1994, Battisti et al. 2000
1994	Italy	Colle-Calbarina	4527306	1173722	136	Pinus-nigra	Pityocampa	Rest-of-Europe	4	75.3	4.2	0.1	64.7	medium	P	139	outdoor	3.6	64.7	10.2	Ghiraldo 1994, Battisti et al. 2000
1995	Italy	Colle-Calbarina	4527306	1173722	136	Pinus-nigra	Pityocampa	Rest-of-Europe	4	75.3	4.3	0.5	84.1	low	P	462	outdoor	0.9	68	2.7	Ardesi 1996, Battisti et al. 2000
1996	Italy	Colle-Calbarina	4527306	1173722	136	Pinus-nigra	Pityocampa	Rest-of-Europe	4	75.3	3	-0.1	72	low	P	495	outdoor	2.2	81.4	12	Ardesi 1996, Battisti et al. 2000
1999	Italy	Venosta	4663278	1079833	1073	Pinus-nigra	Pityocampa	Rest-of-Europe	-1	71	-1.4	-6.1	63.9	high	L	5000	field	4	96	100	Salman et al. 2016
1999	Italy	Venosta	4662972	1079833	910	Pinus-nigra	Pityocampa	Rest-of-Europe	-1.1	71	-0.4	-5.2	63.9	high	L	5000	field	16.8	83.2	99.8	Salman et al. 2016
1999	Italy	Venosta	4662750	1080306	822	Pinus-nigra	Pityocampa	Rest-of-Europe	-1	71	0.1	-4.7	63.9	high	L	5000	field	10.4	89.4	98.3	Salman et al. 2016
2010	Italy	Calabria	3806944	1582722	1200	Pinus-nigra	Pityocampa	Rest-of-Europe	NA	NA	3.7	1.2	NA	NA	P	3634	NA	0.6	NA	1.5	Bonsignore et al. 2015
2011	Italy	Calabria	3806944	1582722	1200	Pinus-nigra	Pityocampa	Rest-of-Europe	NA	NA	3.4	0.8	NA	NA	P	2018	NA	0.1	NA	0.3	Bonsignore et al. 2015
2012	Italy	Calabria	3806944	1582722	1200	Pinus-nigra	Pityocampa	Rest-of-Europe	NA	NA	2	-0.2	NA	NA	P	646	NA	0	NA	0	Bonsignore et al. 2015
2013	Italy	Venosta	4662750	1080306	860	Pinus-nigra	Pityocampa	Rest-of-Europe	-1	71	0.1	-3.7	61.1	low	P	6000	field/lab	23	50	61.1	Salman et al. 2016

2014	Italy	Venosta	4662750	1080306	860	Pinus-nigra	Pityocampa	Rest-of-Europe	-1	71	1.3	-2.1	51.8	low	P	621	field/lab	19	24	25	Salman et al. 2016
2015	Italy	Venosta	4662750	1080306	860	Pinus-nigra	Pityocampa	Rest-of-Europe	-1	71	2	-1.9	46	medium	P	2158	field/lab	1.3	38.7	2.1	Salman et al. 2016
1988	Morocco	Ain-B.Mathar	3401084	-2030275	900	Pinus-halepensis	Pityocampa	North-Morocco	8	24.7	8	NA	24.7	NA	L	345	field	8.1	71.6	28.5	Graf 1988
1988	Morocco	Ain-Rami	3513250	-528222	500	Pinus-halepensis	Pityocampa	North-Morocco	10.2	37	10.2	NA	37	NA	L	800	field	21	50	42	Graf 1988
1988	Morocco	Bab-Taza	3506111	-521194	820	Pinus-halepensis	Pityocampa	North-Morocco	8.8	50	8.8	NA	50	NA	L	400	field	0.05	49	0.5	Graf 1988
1988	Morocco	Bouhachem	3524889	-542389	1157	Pinus-halepensis	Pityocampa	North-Morocco	7.5	48	7.5	NA	48	NA	L	388	field	26	35	40.1	Graf 1988
1988	Morocco	Boutroubay	3345028	-504222	1910	Cedrus-atlantica	Pityocampa	North-Morocco	2.2	60	2.2	NA	60	NA	L	600	field	46.3	25.8	62.4	Graf 1988
1988	Morocco	El-Ghomra	3411722	-496889	500	Pinus-halepensis	Pityocampa	North-Morocco	10.1	41.7	10.1	NA	41.7	NA	L	800	field	19	46	35.2	Graf 1988
1988	Morocco	Issaguen	3491361	-457556	1548	Pinus-halepensis	Pityocampa	North-Morocco	4.2	54.3	4.2	NA	54.3	NA	L	400	field	0.05	80	1.3	Graf 1988
1988	Morocco	Jerrada	3429167	-215500	1000	Pinus-halepensis	Pityocampa	North-Morocco	7.3	28	7.3	NA	28	NA	L	1370	field	9.8	32	14.4	Graf 1988
1988	Morocco	J'Zerhoun	3403944	-550361	800	Pinus-halepensis	Pityocampa	North-Morocco	8.3	46	8.3	NA	46	NA	L	2385	field	28.4	66.4	84.4	Graf 1988
1988	Morocco	Oued-Beth	3388028	-591639	250	Pinus-halepensis	Pityocampa	North-Morocco	12	30	12	NA	30	NA	L	1838	field	7.3	83.2	42.2	Graf 1988
1988	Morocco	Saka	3461378	-3417656	750	Pinus-halepensis	Pityocampa	North-Morocco	9.2	31	9.2	NA	31	NA	L	820	field	5.9	60.9	15.5	Graf 1988
1988	Morocco	Tafoughalt	3480889	-240306	800	Pinus-halepensis	Pityocampa	North-Morocco	9.2	30.7	9.2	NA	30.7	NA	L	1241	field	27.3	54.9	60.7	Graf 1988
1988	Morocco	Tahanaout-	3132468	-7947678	1000	Pinus-halepensis	Pityocampa	South-Algeria-South-Morocco	8.5	35.7	8.5	NA	35.7	NA	L	405	field	2.6	81.7	14.3	Graf 1988
1988	Morocco	Tiflet	3390088	-6303508	300	Pinus-halepensis	Pityocampa	North-Morocco	11.8	29.3	11.8	NA	29.3	NA	L	1560	field	35.4	45.6	65.1	Graf 1988
2000	Spain	Los-Serranos	3993111	-106944	1238	Pinus-nigra	Pityocampa	Iberian	3	58.7	5.3	-0.1	34	low	P	88	outdoor	19.7	52.2	41.2	Lopez Sebastian 2014
2000	Spain	Sierra-Calderona	3969722	-0.54333	300	Pinus-halepensis	Pityocampa	Iberian	9.4	35.7	10.6	5.1	34	low	P	49	outdoor	3.6	14.1	4.2	Lopez Sebastian 2014
2010	Spain	Garden-of-La-Cortijuela	3710806	-34825	1600	Pinus-nigra	Pityocampa	Iberian	3.5	54	1.5	-3.7	18.3	NA	L	9000	field	3.7	87.8	43.6	Torres-Muros et al. 2017

Header Explanation:

Header	**Explanation**
Year	The year of sampling
Country	Country from which data originate
Site	Study site
Latitude	Latitude of study site
Longitude	Longiture of study site
Elevation	Elevation reported in the documents
Host	Host plant
Clade	Genetic clade within which population of the study site falls
Sub-Clade	Sub-clade within which population of the study site falls
Tmean-DJF-WC-(1950-2000)	Average of mean monthly temperature (°C) for the months of December, January and February across 50 years, obtained from the WorldClim
Mean-Rainfall-AMJ-WC-(1950-2000)	Average of mean monthly rainfall (mm) for the months of April, May and June across 50 years, obtained from the WorldClim
Tmean_n	Average of mean monthly temperature (°C) for the months of December, January and February, obtained from source document
Tmean_min_n	Average of minimum monthly temperature (°C) for the months of December, January and February, obtained from source document
Rainfall-AMJ-local-year-n	Average of mean monthly rainfall (mm) for the months of April, May and June, obtained from source document
Density	Standardized population density obtained from source document. low: <1 tent/tree or defoliation of <20%; medium: between 1 and 6 tents/ tree or defoliation 20-60%; high: >6 tents/ tree or defoliation >60%
Sampling	Stage of individuals during collection
N.individuals	Number of individuals
Condition	Rearing condition of the individuals
Diapause-%-(from-total-initial-number)	Percentaged calculated as the number of individuals that emerged following the year of pupation, with respect to the total number of individuals emerged
Mortality	Percentage calculated as the number of individuals that did not produce moths with respect to the initial number of individuals in the cohort
Diapause	Percentage calculated as the number of individuals that emerged over the year(s) following the year of pupation, or that were still alive when the experiment was over one or more years after pupation, with respect to the total number of emerged or living individuals
Reference	Source document from which data were obtained

CHAPTER 4

Prepupal diapause synchronizes adult emergence in the pine processionary moth *Thaumetopoea pityocampa* (Lepidoptera: Notodontidae)

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Abstract

1. Insects with short-lived adults must synchronize their emergence to maximise fitness.
2. Yet, pre-imaginal development time often varies among individuals due to exposure to varied abiotic and biotic factors, therefore mechanisms adjusting pre-imaginal development time are expected.
3. Larvae of the pine processionary moth feed throughout the winter and conclude their development with characteristic processions in spring, leaving the trees to pupate in soil.
4. The procession period can be as long as two months in some regions due to prior desynchronization of larval colonies, whereas the emergence period of the adults in summer remains short (less than one month).
5. Through weekly sampling of prepupae leaving trees to pupate in soil and subsequent rearing under field and laboratory conditions, it was observed that early prepupae waited longer than late prepupae before moulting into pupa.
6. The differential duration of the prepupal stage was independent of temperature conditions and allowed resynchronization of colonies and overlapping emergences.
7. The prepupal stage therefore seems critical to understand the regulation of adult emergence of this important pest insect.

Key words: phenology, pupa, colony, holometabolous insect, univoltinism.

Introduction

Individuals of a species should time their adult emergence to maximize mate-finding and to coincide with environmental conditions suitable for the whole reproduction process (Chapman, 2013). Mechanisms of emergence synchronization have been explored in a number of species and have been often shown to be important for species fitness (e.g. Holliday, 1985; Topp & Kirsten, 1991; Härkönen & Kaitala, 2013). This seems particularly important for species showing a long pre-imaginal stage (Brown, 1983), during which delays in the development of individuals, or part of the population, may accumulate due to variable environmental conditions (Danks, 1987). These individuals/sub-populations are in strong need of synchronization mechanisms in order to successfully reproduce, especially if adults have a short lifespan.

In holometabolous insects, diapause in the prepupal and pupal stages generally synchronizes the life cycle with conspecifics, and with favourable environmental conditions can level out differences in the time taken by the larvae to achieve the critical weight for metamorphosis (Stålhandske *et al.*, 2015; Hironaka & Morishita, 2017). Delays in pre-imaginal development can be observed either in uni- or in semivoltine insect species, and synchronization of adult emergence within the year (seasonal diapause of univoltine species) or across years (prolonged diapause of semivoltine species) is essential for the performance of these species (Danks, 2007). Pupal diapause is a generally well-known mechanism to adjust emergence time of adults (Tauber *et al.*, 1986), but little is known about the mechanism of entry into pupal stage by larvae that have developed under different environmental conditions. In these cases, prepupal and pupal diapause can be invoked to adjust the time of entry into pupal and later, the adult stage.

The prepupa is a stage of mature larva that has completed larval growth and is followed directly by pupation without further feeding or moulting (Beck, 1980). The prepupal stage is a common diapausing stage in holometabolous insects (Danks, 1987), although it is less studied than the pupal stage (Koštál, 2006). In plant feeding insects, prepupal diapause has been demonstrated in Lepidoptera (e.g. Koštál & Hodek, 1997; Shintani *et al.*, 2015) and Hymenoptera (Battisti, 1994). While prepupal stage is mainly motionless, in some Lepidoptera, it can be quite mobile, as evidenced by Fitzgerald (2003) during the pupation procession of the pine processionary moth (*Thaumetopoea pityocampa* ([Denis et Schiffermüller]) Lepidoptera: Notodontidae). In this species, the gregarious larvae live in a silk tent and feed throughout the winter; the prepupal stage starts when mature larvae stop feeding on their host tree in spring and look for suitable pupation sites in the ground. This is when the typical 'procession' happens, i.e. the prepupae from a colony walk one after the other in a single-individual line. This procession continues until suitable pupation conditions are found, and may vary from just less than an hour up to 6-7 days (Halperin, 1990; Fitzgerald, 2003). In the latter case, phases of active search usually alternate with rest in protected sites (typically litter and upper soil layers), until the final burial site has been found (Démolin, 1971). The process is ruled by environmental factors such as temperature, light, and presumably humidity and soil nature, which interact with the social organization of the colony and possibly with regulatory physiological mechanisms (Démolin, 1971).

The cues governing prepupae movement on the ground appear to differ from those regulating the larvae while foraging on the tree, as the thigmotactic stimuli (head-to-tail contacts) among the individuals are more important during processions than the trail pheromone or the silk strand that prevail during larval growth (Fitzgerald, 2003). This may imply an easier breaking up of the procession, with the subdivision of the initial colony into subgroups, as well as the merging of different groups when they encounter (Démolin, 1971; Fitzgerald, 2003). During and right after processions, prepupae are exposed to a number of natural enemies that are important regulating factors of population density (Buxton, 1990; Battisti *et al.*, 2000; Bonsignore *et al.*, 2015; Tarasco *et al.*, 2015), so minimizing the duration of site selection is crucial to their survival.

Both the timing and duration of the period from first to last prepupal processions are highly variable from site to site, and total period may extend up to two months in some sites (Démolin, 1971; Buxton, 1990; Bonsignore *et al.*, 2015; Robinet *et al.*, 2015). This makes the prepupa a conspicuous active stage of the species, and also the most likely to get in direct contact with people and domestic animals and causing allergic reactions (Moneo *et al.*, 2015; Battisti *et al.*, 2017). The developmental steps following soil penetration, i.e. cocoon spinning and moulting into pupae, are poorly known in the pine processionary moth. Based on general literature on Lepidoptera, after cocoon spinning the prepupa loses its normal shape, becoming more compact and not any longer able to walk (Beck, 1980). The duration of this latter phase is unknown.

Given the short adult lifespan and univoltine development of the species with a relatively short mating period, Robinet *et al.,* (2015) have suspected that the high local variation in the timing of prepupal processions may negatively impact fitness if there are no mechanisms that resynchronize individuals before they emerge. The hypothesis of a resynchronizing mechanism occurring at the prepupal or pupal stage is supported by the much narrower period of emergences as compared to the period of processions at the same local scale (Robinet *et al.*, 2015).

In order to better understand the prepupal stage in the pine processionary moth, we here study it in a population characterized by recent range expansion (Battisti *et al.*, 2005), and whose phenology has been intensely studied over several years (Salman *et al.*, 2016), with special reference to pupal diapause. We intercepted prepupal processions descending from trees during the whole procession period and then maintained them under both field and laboratory conditions to know the fate, phenology and size of resulting moths. The overall aim was to understand whether (i) a mechanism regulating the duration of the prepupal phase does exist, and (ii) if it acts to synchronize the emergence pattern of individuals that differ in timing of the prepupal procession.

Materials and methods

Study site

The study site was located at Vezzano/Vetzan, on the southern facing slope of the Venosta/Vinschgau valley that is part of Bolzano/Bozen province of northern Italy. The valley is situated in the southern Alps and borders with Switzerland and Austria. Climate is continental with an annual mean temperature of (10.7 ±0.5°C) and annual rainfall of (556±111 mm) (period 1988-2016). A reforestation process was in action between 1884 and 1960 on the southern slope over a few thousand hectares with introduced *Pinus nigra* and native *Pinus sylvestris* (Aimi *et al.*, 2006). Population density of *T. pityocampa* remained low until 1998, when an outbreak started and the population expanded onto the upper parts of the valley and the upper slopes (Battisti *et al.*, 2005). The density later decreased (Salman *et al.*, 2016) and when the current experiment was carried out (spring 2015) it was estimated as (2.3±1.6 tents/tree) based on 33 trees randomly selected for the collection (see later). This is a low-medium density at the study site, corresponding to 20 to 40% defoliation.

Collection of prepupae

Three sites (A, B, and C) were selected (coordinates intervals 46°37′40′′- 46°37′56′′N, 10°47′30′′- 10°48′06′′E, elevation 835 - 1047 m). Three dataloggers (HOBO Pro v2) located in a solar radiation shield (HOBO RS3) were installed at each site for logging air (1.5 m from the ground) and soil (10 cm deep) temperature. Prepupae were intercepted on the tree stems during their way down to the ground with 33 Ecopiège® collar traps (www.ecopiege-boutique.com) (11 trees at site A, 10 at site B, and 12 at site C) deployed on the trees in January 2015 (Ecopiège® collar traps were slightly modified as shown in supplementary material Fig. S1). Pine trees carrying white silk tents (revealing the presence of larval colonies) were haphazardly selected. The traps were checked weekly from the end of February, which was the presumable starting time for pupation procession, until no procession was found for two consecutive weeks (18 May 2015). At each check, the collection pots were changed and individuals still occurring in the collection pipes and collars were added to the pots.

Treatments of prepupae and pupae

The collected prepupae were exposed to either field or laboratory conditions. This allowed us to test how development was affected by (i) constant temperature in the laboratory (Berardi *et al.*, 2015), and (ii) fluctuating temperature in the field. To achieve this, on each collection date, half of the pots were kept on site, under trees with semi-shade conditions, whereas the other half was taken to the laboratory at room temperature (20 ± 2°C). Based on experience from previous year's observations on prepupal development, they were inspected every 4-5 days. On the first check when cocoons were found, 6 of them were randomly selected from different pots collected on the same date, opened to assess the developmental stage, and then discarded. For the pots of each collection date, the monitoring continued until the first fully developed pupa was found among the 6 individuals, and that day was considered as the pupation date for the individuals collected on the same date. The number of days between the date of collection and the first occurrence of pupae provided an estimate of the time required for pupation for individuals of each collection date. As a fume hood was not available in the field for safe manipulation of urticating insect material (Moneo *et al.,* 2015), the inspection of the pots left on site was limited to a subsample of three pots per collection date. When the pupal stage was reached in all the pots kept in the laboratory (June the 1st, 2015), the pots kept in the field were taken to the laboratory for an exhaustive check. Every cocoon containing pupa was removed from the soil, cleaned with forceps and brush and transferred to polystyrene trays (21.8 x 21.8 cm) with 96 wells, each well with a diameter of 1.7 cm and depth of 2.2 cm hosting a cocoon. Trays were covered with small mesh nets with the help of a rubber band.

The cocoons, be they from the laboratory or from the field, were then assigned to one of two temperature treatments. Half were left at the same laboratory conditions as described above, while the other half were transferred to a basement with about the same average temperature but under naturally fluctuating conditions (basement: 19.5±3.7°C, forest soil: 19.3±3.6°C, average±SD from June 1st to July 15th). Emergence of adult moths was checked every two or three days until no more moths were found for 15 days. All emerged individuals were immediately placed into individual vials and stored at – 20°C in a freezer. Later on, all moths were sexed and a subsample (N = 376) dried in a laboratory oven (Jouan, Astel, France) at 70°C for 24 hours and weighed to the nearest 0.1 mg (Mettler Toledo AT460).

At the end of the emergence period, the condition of all the remaining cocoons was checked to determine whether they were alive and in prolonged diapause or dead (due to desiccation, pathogens, parasitoids, although it was not possible to discriminate among mortality factors).

Statistical analysis

Hourly air and soil temperatures from all the sites were averaged for each interval of prepupae collection dates. Three sites sharing origin (plantation), age (70-80 year-old), aspect and soil were pooled because of many 0 values across time (supplementary material Fig. S2) combined with a low number of trees (10 per site). Simple regression analyses were carried out separately to test the association between: (i) days of collection of prepupae and days needed to observe the first pupa in the pots; (ii) ordinal collection date and ordinal emergence date; (iii) days of collection of prepupae and moth dry weight, and (iv) weekly catch of prepupa in the traps and temperature. Adequacy of the residuals to Normality was confirmed by a Shapiro-Wilk test and a QQ plot. Individuals for which the date of emergence could not be identified with precision (within 3 days) were excluded from the analysis, for a total of 215 out of more than 2000 individuals. A χ^2 test was used to assess differences in the emergence time of male and female moths in the same treatment. To remove treatment effect in regression between collection and emergence date, we grouped individuals that were exposed to the same conditions right after the collection, i.e. laboratory and field + field-mimicking conditions. The association between prepupal collection date and moth dry weight was tested separately for females and males due to sexual dimorphism in body size in the pine processionary moth. All levels of significance were set to $\alpha=0.05$ and the dispersion of data from the mean is given by standard deviation. The R statistical software environment (v. 3.3.0, R Core Team 2016) was used for all analyses.

Results

The prepupae were caught in traps over two months (from March 8th to May 10th), with a peak in the central part of the period (April 8th) (Fig. 1). Average hourly air and soil temperatures steadily increased during the catch period (Fig. 1) at all sites and did not show any clear relationship to the number of prepupae leaving the trees (linear regression, air: $F = 0.02$, $P = 0.88$; soil: $F=0.01$, $P=0.93$).

Prepupae taken to the laboratory right after being caught spent between 18 and 49 days as prepupae before they started to moult into pupae (Fig. 2). Although weekly sampling of the pots kept in the field did not allow to get precise data on pupation time, when the pots were taken to the laboratory for careful inspection on June the 1st, 2015, all the individuals had already pupated, as observed in the pots kept in the laboratory. Individuals from early processions spent significantly more time as prepupae than those leaving the trees later in the season, even if they were all kept under the same constant temperature after collection (Fig. 2). The regression showed that an individual going into procession n days after another spends n/2 days less as a prepupa.

Moth emergence in the four treatments is shown in Fig. 3. Sexes were pooled because no difference between them could be detected (upper left: n = 275, female % = 46.2, χ^2 = 1.6, df = 1, *P* = 0.21; upper right: n = 108, female % = 53.7, χ^2 = 0.6, df = 1, *P* = 0.44; lower left: n = 113, female % = 54.9, χ^2 = 1.1, df = 1, *P* = 0.31; lower right: n = 198, female % = 45.5, χ^2 = 1.6, df = 1, *P* = 0.23). Individuals experiencing constant laboratory temperature as prepupae (Fig. 3 upper panels) emerged 12.5 days earlier than those experiencing field conditions (Fig. 3 lower panels), irrespective of the temperature at which the pupae were later kept. Additionally, the emergence period spanned 32 days for prepupae kept in the laboratory, while it was 34.5 days for the individuals originating from field prepupae, irrespective of the temperature at which pupae were later kept (laboratory vs basement). The longest duration of the emergence period (44 days) was observed for the individuals that were kept in the field as prepupae and then under field-mimicking conditions as pupae.

No association was found between collection date (a proxy of tree descent date) and emergence date (n = 280, *F* = 0.43; *P* = 0.51) in the prepupae and pupae kept in the laboratory until emergence (supplementary material, Fig. S3). The same was observed for field prepupae transferred to fluctuating basement temperatures as pupae (n = 206, *F* = 0.53; *P* = 0.47) (supplementary material, Fig. S3).

Interestingly, the collection date of the prepupae was negatively related to the dry weight of the moths of both sexes (Fig. 4), with individuals that left trees earliest as prepupae being almost twice as heavy at the adult stage as those that left latest. No such relationship between adult dry weight and emergence date was detected (n = 376, *F* = 0.03, *P* = 0.85), which further supports that the date of prepupal procession is not linked to the date of moth emergence.

The cocoons that did not produce moths were found to contain few prolonged diapausing pupae (0.9%) and several dead pupae (38.4%). A total of 60.7% cocoons produced moths during the first summer monitored in this study.

Discussion

The results provide evidence of fitness-related variation in the duration of the prepupal stage in the pine processionary moth, just before cocoon spinning and pupation. Individuals ending their larval development early spent a longer time as prepupa than later ones, which tended to decrease the desynchronization at the adult stage. Diapause induction is generally related to photoperiod and temperature (Tauber *et al.*, 1986; Danks, 1987; Koštál, 2006), and our sampling design allowed us to test for both factors independently. First, the regular weekly sampling took into account the effect of the photoperiod to which the individuals were exposed before and during the procession, a process that can take up to 6-7 days between the departure from the tent and burial for later pupation (Démolin, 1971). Second, since we transferred half of the pupae to a distinct constant temperature at the laboratory right after the weekly sampling we could control for the effect of temperature on diapause development. Since the prepupae behaved similarly in the laboratory and the field, temperature seemed weakly if at all associated with prepupal diapause. Instead, photoperiod could be responsible for the observed diapause pattern, as the duration of prepupal diapause was inversely related with increasing day length. Thus photoperiod during the last period of larval development may have influenced prepupal diapause duration (Beck, 1980), and the high number of individuals sampled within the same month (80% In April) suggests that such putative effect would be sensible, as described in the literature for numerous species (Denlinger, 1986).

The prepupal diapause described here for the pine processionary moth is probably important for the synchronization of adult moth emergence, since it facilitates co-emergence of individuals from early to late processions, which often co-exist with a relatively high variation compared to the emergence period (Robinet *et al.*, 2015). This probably has large fitness consequences, since it increases the probability that adults encounter conspecifics of the alternate sex (Shintani *et al.*, 2015) as well as limits the exposure to natural enemies through predator satiation when preys are concentrated in a short-time window (Holling, 1965). This is particularly true for species with short-lived females, such as the females of the pine processionary moth which can live from just a few hours to at most two days (Démolin, 1969; Zhang & Paiva, 1998). As larval development may take up to 9 months under cold conditions, any variation that can be expected based on abiotic and biotic factors may tremendously affect the time when the critical weight for metamorphosis is achieved (Hironaka & Morishita, 2017), leading to variation in procession timing. Thus, a mechanism of synchronization based on the prepupal diapause period responds very well to this purpose, as hypothesized by Robinet *et al.,* (2015). Lyons and Griffiths (1962) reported that *Neodiprion sertifer* male and female synchronize emergence even though males have one less larval instar and spin their cocoons earlier than females. As an explanation, they found that the number of days spent in the cocoons were negatively associated with the date of spinning.

Synchronizing life cycles through variation in development rates is one of two broad roles of diapause (Tauber & Tauber, 1978). However, this dormant period alone does not explain the mix-up during emergence of individuals originated from different collection dates. The slope of the linear equation linking procession date and pupation date (Fig. 2) indicates that their inverse relation can compensate only half the phenological mismatch among larvae going into procession at distinct times. In other words, individuals starting the prepupal stage by descending to the soil two days earlier than others stay as prepupae one day longer, and reciprocally (Fig. 2). If the slope had explained one-to-one compensation, then the prepupal dormancy could fully account for the mixture during emergence. This implies that early individuals should compensate more during the pupal period to synchronize the emergence event with their late counterparts. This assumption should be tested following our experimental design with more populations and employing physiological investigation during pupal stage immediately after pupation. Interestingly, a population tested in 2016 (located in Italy, Liguria Ruta) from the core range of the pine processionary moth, confirmed the pattern observed, with early prepupae diapausing longer than late prepupae (gap in collection: 25 days; gap in pupation: 10 days), and showing the same emergence period of 28 days (unpublished data).

The observation that early descending prepupae produced larger moths, irrespective of when they emerged, can be explained by different factors. Primarily, the slope of the regression is similar to that of the duration of the prepupal rest before moulting to pupa, so one could infer that the two processes are associated and that larger prepupae need more time to develop into pupae. Unfortunately, our data do not allow us to test for this as we cannot relate the individual mass to the duration of the prepupal stage in each date of collection. Leather (1984) found adult weight of *Panolis flammea* (Noctuidae) to be positively associated with pupal length. Faster rate of prepupa-to-pupa development of the late-descending individuals could also be ascribed to their exposure to warmer temperature at the initiation of development, as found in *Mamestra configurata* (Noctuidae) at the pupal-adult metamorphosis (Turnock *et al.,* 1986). Other factors possibly involved are related to the performance of the colonies, depending on the number of individuals (Pimentel *et al.*, 2010), the quality of the host plant (Battisti, 1988; Stastny *et al.*, 2006), and the activity of an important natural enemy, the tachinid parasitoid *Phryxe caudata*

(Buxton, 1990). Large pupae were found deeper in the soil than small pupae, and for this reason they better escaped predation by natural enemies, namely those with a searching area restricted to the upper soil layer, like the hoopoe *Upupa epops* and two hymenopteran parasitoids (Battisti *et al.*, 2000). More work is required to understand these data.

The precise mechanisms that determine the timing of moth emergence and thus set the duration of both prepupal and pupal stages still remain to be understood. It is known that individuals from populations originating in locations characterized by different climatic conditions, and which have found an optimal timing for the local conditions will maintain their intrinsic timing even when translocated elsewhere or raised in the laboratory under constant conditions (Berardi *et al.*, 2015; Santos *et al.*, 2011; Petrucco-Toffolo *et al.*, 2017). This strongly suggests there is a genetic basis for the prepupal diapause duration. The abacus produced by Démolin (1969) was based on this observation and has been widely used to predict the life cycle of the moth in different parts of its range. However, the study lacked details in some parts, which has limited its usefulness. Démolin (1969) went from larval pupation procession straight to pupal diapause, without considering intermediate stages such as the prepupa, shown in the present study to be of great importance. Studying prepupal development in populations characterized by different emergence time of the moths and duration of the larval stage would allow us to verify the strengths of prepupal diapause as a mechanism of adjusting the emergence time within a population.

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Author contribution

AB and MHRS conceived the study and carried out the experiments. FG, ML, and PL contributed to data analysis and interpretation. All authors contributed to the writing.

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Figures

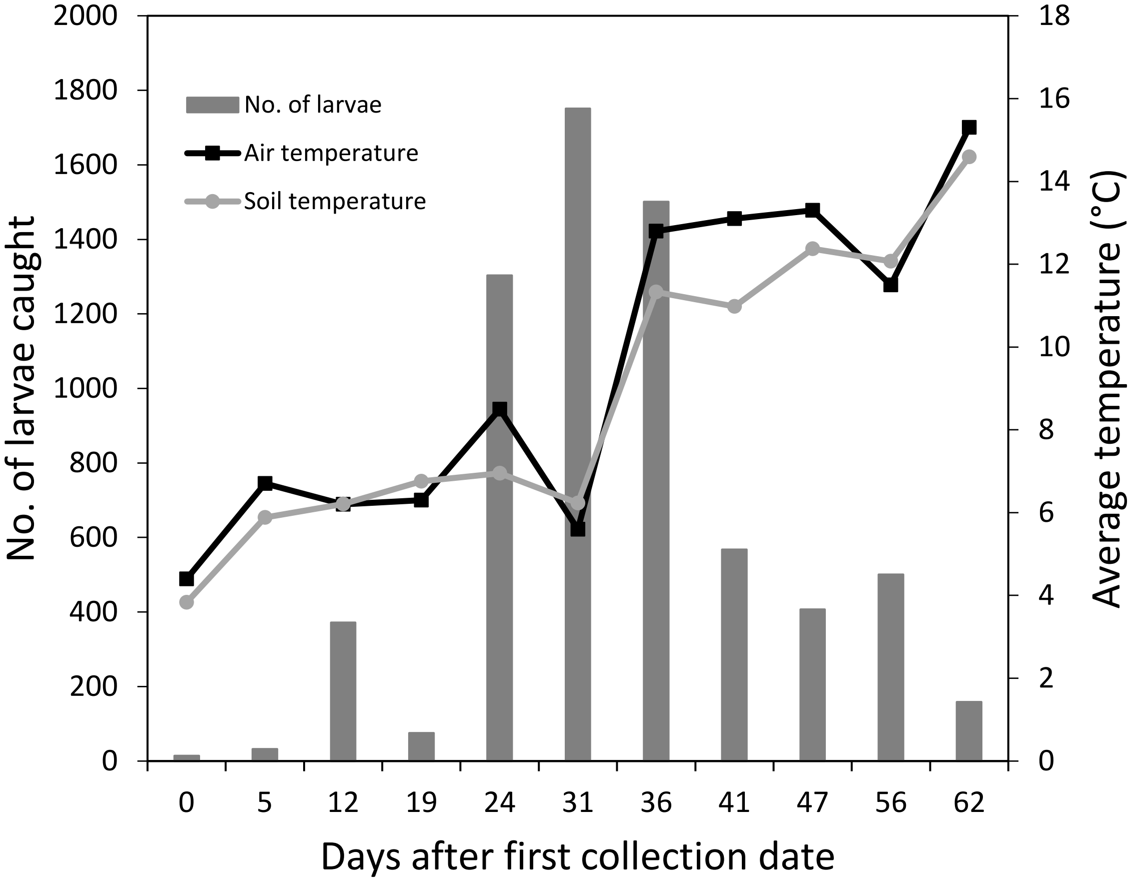

Figure 1. Number of prepupae descending from trees and caught in trunk traps during the procession period (0: March 9th to 62: May 10th, 2015) (total of the three sites, left y axis), with the hourly average temperature of the air and soil in the days preceding the check (average of three sites, right y axis).

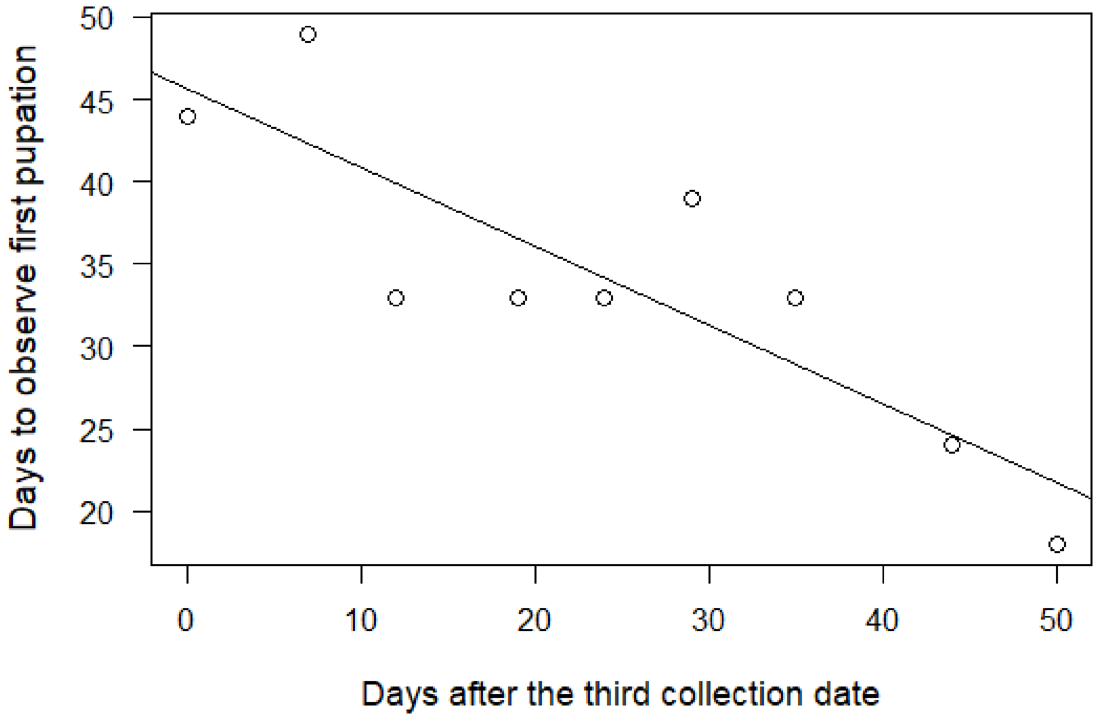

Figure 2. Relationships between the time of prepupae catch in traps (0: March 21st to 50: May 10th, 2015) and the number of days needed to detect the first pupae in the pots collected on each specific date and immediately brought into laboratory conditions at constant temperature. Available field data were unsuitable to run a model, thus not included. The two earliest catch dates (March 9th and 15th) were excluded because of low sample sizes (<3 colonies). Regression equation: $Y = -0.48x + 45.71$, *Adjusted* $R^2 = 0.69$, $F = 18.81$, $P = 0.003$.

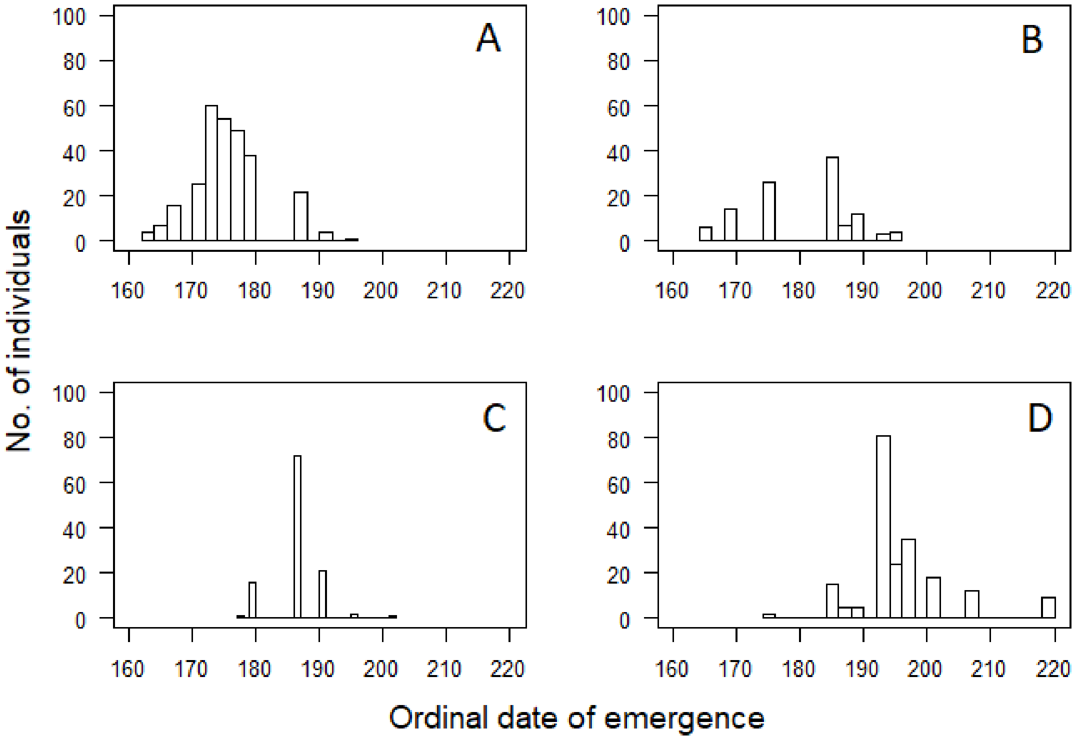

Figure 3. Number of moths emerged through time (ordinal date 160: June 9, 220: August 8) from pupae obtained and kept under different conditions: A) prepupae in the laboratory, emergence in the laboratory; B) prepupae in the laboratory, emergence at conditions mimicking field; C) prepupae in the field, emergence in the laboratory; and D) prepupae in the field, emergence at conditions mimicking field.

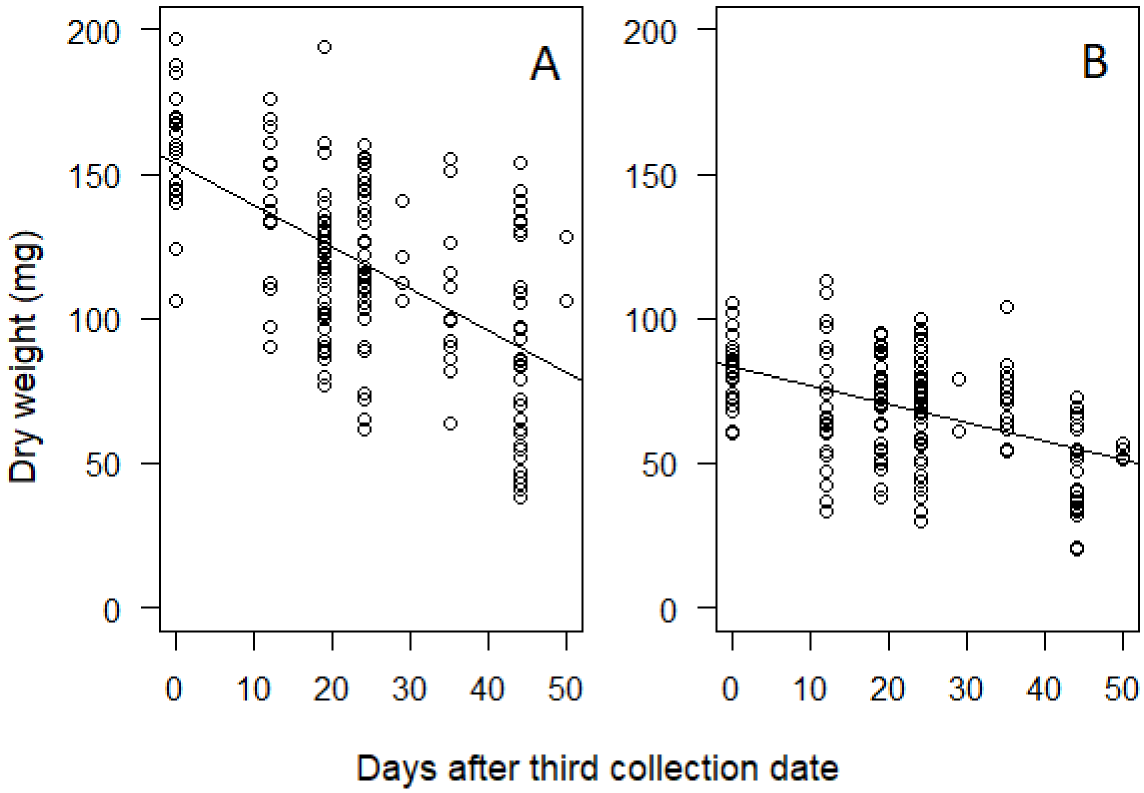

Figure 4. Dry weight of moths (A: females, B: males) emerged from groups of prepupae collected from traps (0: March 21st to 50: May 10th, 2015). Two earliest catches (March 9th and 14th) were excluded because of low sample size. Regression equations (females: $y = -1.45x + 153.7$, $F = 105.8$, *adjusted* $R^2 = 0.36$, $P < 0.01$; males: $y = -0.65x + 83.4$, $F = 53.04$, *adjusted* $R^2 = 0.22$, $P < 0.01$).

Supplementary material

Figure S1: Modified version of Ecopiège® collar traps (www.ecopiege-boutique.com) to intercept prepupae leaving trees and convey them into bags filled with sand fitted into 5 L pots.

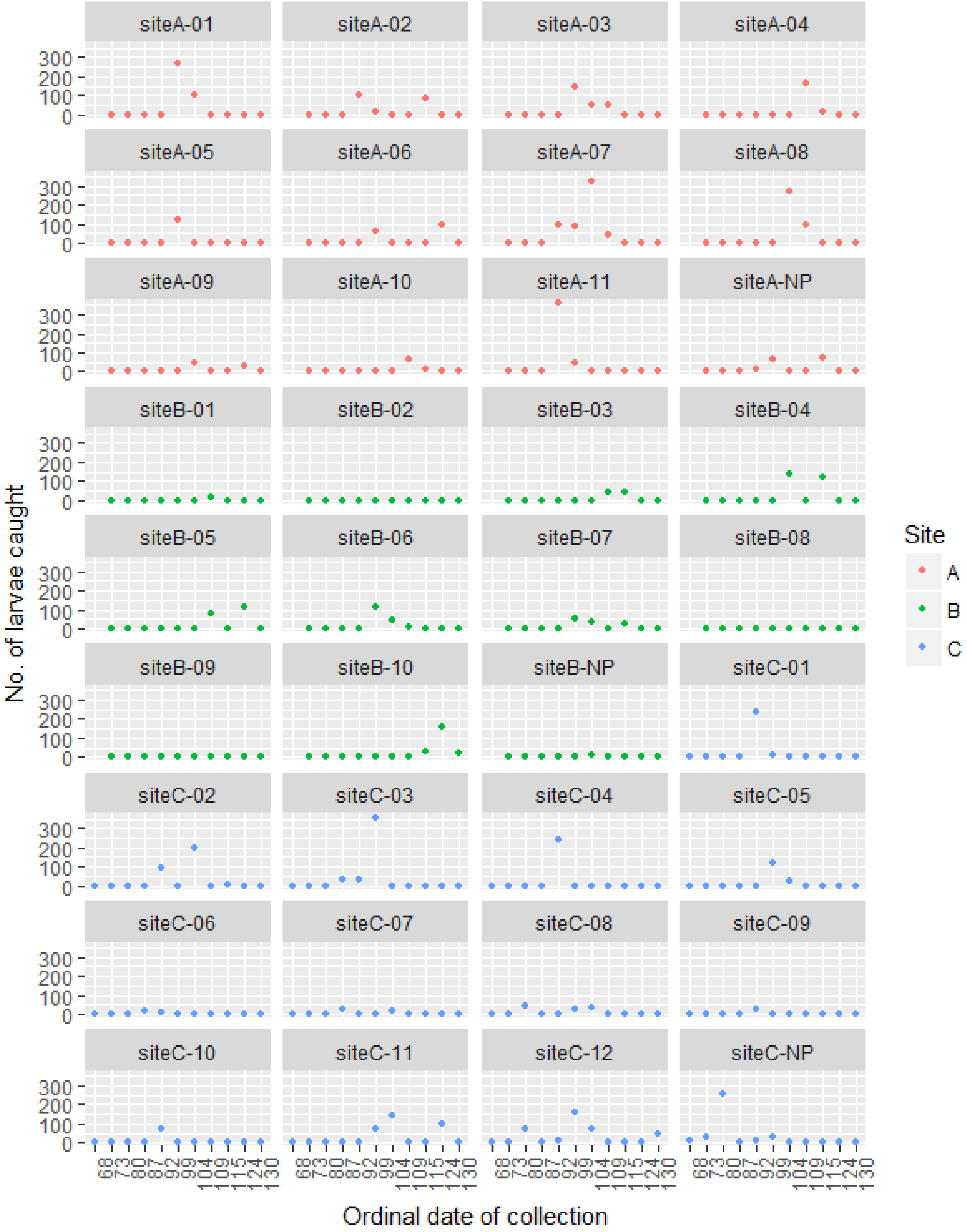

Figure S2: Number of larvae caught on each tree at the three sites (A, B, C) and natural procession on ground (NP) during the procession period (March 9th to May 10th, 2015) in Venosta.

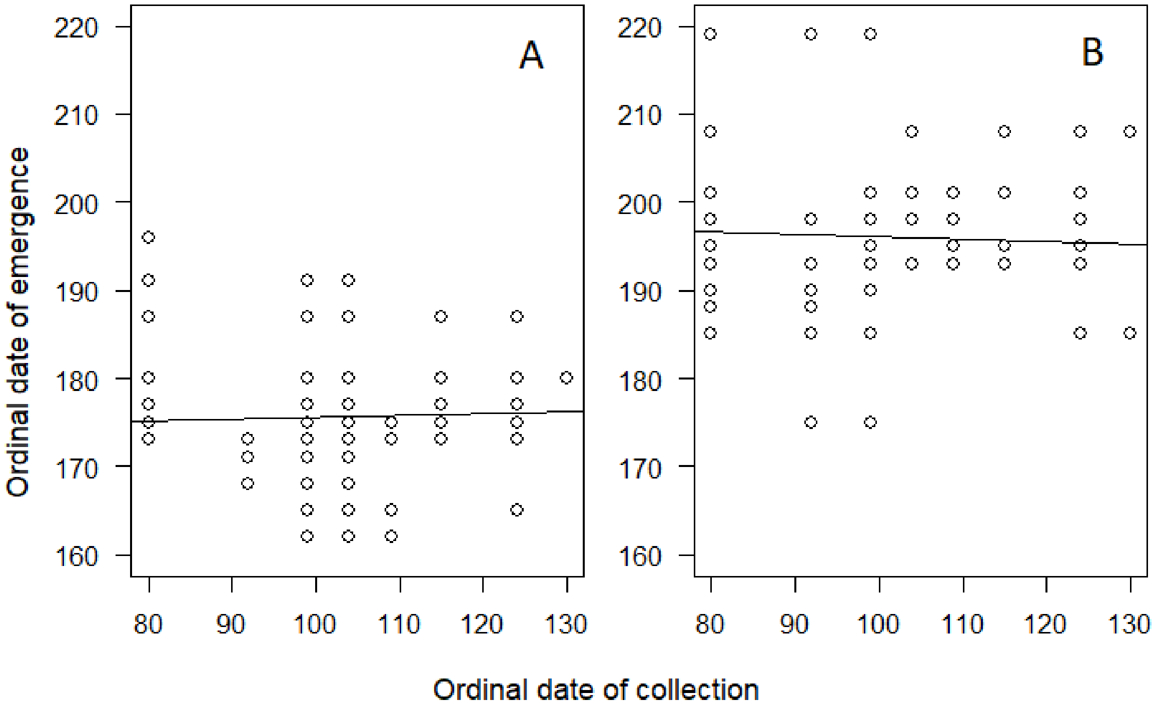

Figure S3: Comparison of time of collection of the larvae descending from trees (x axis: pupation processions) with time of emergence of moth (y axis) in the same year. A) Pupation and emergence in the laboratory under constant temperature (n = 280, *F* = 0.43, *P* = 0.51) B) Pupation in the field and emergence under semi-field condition of fluctuating temperature (n = 206, *F* = 0.53, *P* = 0.47).

CHAPTER 5

Diapause termination in the pine processionary moth

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(manuscript in preparation)

Abstract

Diapause development is a complex process involving several ecophysiological phases. Understanding of these phases, especially termination, is vital for acquiring knowledge on the life history of the studied species and employing suitable management tools (e.g., spray of insecticide) at an appropriate time. Pine processionary moth (PPM) is a major defoliator of pine and vertebrate health hazard in the Mediterranean region, which displays simple and prolonged diapause in the pupal stage. Nevertheless, study on the diapause of PPM is rare. The present study aimed to physiologically characterize pre-termination, termination and post-diapause phase of PPM pupae at simple and prolonged diapause at different temperature conditions. We used three proxies of metabolic activity (pupal body temperature, pupal O_2 consumption, and pupal weight loss) to detect termination phase, difference among pre-termination, termination and post-termination, and the nature of development trajectory of prolonged diapausing individuals. All the proxies detected clearly the termination point in the diapausing pupae. Metabolic activity in the emerged individuals kept increasing from pre-termination via termination to post-termination. Prolonged diapausing individuals terminated their diapause repeatedly each year, although they went back to pre-termination activity state following termination, if not emerged. This study is a starting point in understanding ecophysiologically the diapause development process in PPM.

Keywords: *Thaumetopoea pityocampa,* diapause development, repeated termination, prolonged diapause, O_2 consumption, weight loss, thermocouple.

1. Introduction

Diapause is a dynamic process (Tauber *et al.* 1986; Danks 1987; Košťál 2006). Historically, phases of this process have been defined by different authors mostly subjectively, thus remained burdened with full of ambiguity. However, attempts of standardizing the terminology for the successive phases of diapause have been made in the recent decades (Tauber 1986; Danks 1987; Hodek 1996; Hodek 1999; Košťál 2006). At latest, Košťál (2006) standardized the terminologies characterizing three main phases and their sub-phases. Apart from pre-diapause and post-diapause, diapause phase consists of initiation, maintenance and termination.

Induction of diapause usually occurs in insect during a specific time (sensitive period) by such token stimuli that reliably signal for the future unfavourable environmental conditions. The stimuli for induction include both abiotic (photoperiod, oxygen level, and temperature) and biotic (semiochemicals, diet quality) factors depending on the environment of the organism and the reliability and slowness of the stimulus in predicting seasonal changes. Activities such as migration, aggregation, search for suitable habitat, and accumulation of energy reserve through feeding are general characteristics of the preparation phase in some species with early embryonic or pupal diapause (Kostal 2006). Initiation phase of diapause is characterized by cessation of direct development and regulated decrease of metabolic rate. In the maintenance phase, metabolic rate is relatively low and constant. Intensity of diapause decreases with time and sensitivity to diapause terminating condition increases. Following termination (discussed later), insects may maintain a post-diapause quiescence because the environment still remains unfavourable to development. Abiotic factors such as temperature, humidity, or presence of liquid water may regulate this phase.

Diapause termination may be spontaneous or may require specific conditions (Tauber and Tauber 1976). Adversity period may act as reliable token signal and prevents untimely termination of diapause in the field. Therefore, termination as an eco-physiological phase can be characterized by decrease of diapause intensity to its minimum level and enabling of resumption of development. Hodek (1983) characterized termination as either horotelic or tachytelic, *i.e.*, occurring at standard or faster rate, respectively. Besides, genetic factors may also regulate this process (Danks 1987).

Resumption of developmental potential is the principal feature of diapause termination. As diapause development is a dynamic process, characterizing the precise termination point on an individual level is challenging and often arbitrary (but see Košťál, 2017). As a characteristic, metabolic activity is expected to increase as diapause intensity decreases. Since determining the timing of diapause termination is difficult, investigating physiological processes throughout diapause could be a pragmatic approach for determining and understanding its termination in insects (Gray *et al.* 1995; Kostal 2006, 2017; Sgolastra *et al.* 2010; Yocum *et al.* 2011; Lehmann *et al.* 2017a, 2017b, 2018). The pine processionary moth *Thaumetopoea pityocampa* [(Denis & Schiffermüller)] (called PPM henceforth) is a Mediterranean pest species thriving on pine and cedar species and responsible for health problems in humans and endothermic animals (Battisti *et al.,* 2011; 2017). After completion of larval development, groups of prepupae leave the host tree to search for suitable pupation sites, e.g., open areas and forest edges (Démolin, 1971), in a typical head-to-tail procession and dig into the soil down to 5-20 cm. Upon entering an obligatory diapause, usually in spring, part of the individuals complete metamorphosis and emerge during the summer, while a certain proportion of them may enter prolonged diapause and postpone their emergence to the same period of the following years, up to a maximum of eight years (Salman *et al.* 2016). After observing a large number of populations, Démolin (1969) hypothesized that low winter temperatures are associated with longer larval development times and to higher frequency of prolonged pupal diapause. This hypothesis has been recently confirmed with quantitative data from the entire range of PPM (Salman *et al.* in preparation, see Chapter 3).

Adult phenology of PPM is also affected by temperature and spans from June to September. Moths emerge early in colder sites and late in warmer sites. This behaviour, opposite to that observed in most insect species, has been seen as an adaptation to larval feeding across the cold period of the year (Démolin, 1969; Robinet *et al.* 2015; Roques 2015). In this regard, the duration of obligatory diapause plays an essential role in maintaining a univoltine life cycle, as it has to be short in the cold and long in the warm sites. When individuals from populations with different phenology are reared under laboratory conditions, they still exhibit the emergence time typically observed in their region of origin, suggesting a genetic regulation working at population level (Berardi *et al.*, 2015). Although larvae develop faster in the laboratory, the adjustable duration of the prepupal and pupal diapause allows maintaining the natural adult phenology (Salman *et al.* submitted, see chapter 4).

Démolin (1990) identified the termination of the diapause of PPM with what he called key period. In one of the populations that he studied, Mont Ventoux in S France, the key period was reported at about 40 days before emergence when pupae were kept at 20°C. Individuals destined to emerge (later called univoltine) during the ongoing season keep developing from that point onward, while insects deciding to remain in prolonged diapause postpone their emergence. This view was based on the observations by Biliotti *et al.* (1964) on the formation of *corpus luteum* (yellow body) in the ovaries of female pupae after diapause termination. In addition, he suggested that pupae in prolonged diapause break their diapause every year before the emergence season, based on the evidence of accumulation of yellow bodies in the ovaries of pupae diapausing for more than one year (Biliotti *et al.* 1964). This repeated termination of diapause supports the "late switch" hypothesis described by Soula and Menu (2005) as opposed to "early switch" pathway that views prolonged diapause as a simple extension of summer or winter diapause without interruption (Ushatinskaya 1984; Danks 1987; Hanski 1988). It also matches with the hypothesis of an energy status check before continuation of diapause (Hahn & Denlinger 2007, 2011). Framing the problem from a physiological aspect is necessary for deeper understanding of diapause in this species.

This paper focuses on understanding the termination mechanism of obligatory and prolonged diapause in the PPM. We physiologically characterized pre-termination (diapause initiation and maintenance), termination and emergence (post-diapause) phase in PPM at different temperature conditions. Doing that in a non-destructive way is necessary to follow individuals before, during and after the termination phase and unambiguously characterize whether they resume development or return into diapause after the measurements. Therefore, we used three distinct proxies of metabolic activity to monitor individuals through time: i) pupal body temperature (henceforth body temperature), ii) pupal O_2 consumption (henceforth O_2 consumption), and iii) pupal weight loss (henceforth weight loss). Specific questions include whether i) termination of diapause in PPM can be detected, ii) intrinsic metabolic activity differs among pre-termination, termination, and post-termination, iii) prolonged diapause is a continuation of the obligatory diapause or a distinct developmental trajectory.

2. Materials and methods

2.1 Study site and system

The Venosta/Vinschgau Valley lies in northern Italy close to the borders of Austria and Switzerland. It has an east-west orientation and comprises south and north facing slopes. Historically the slopes were characterized by a steppic vegetation with overgrazing by free range animals. As such, soil erosion occurred severely and a series of reforestation programs with native *Pinus sylvestris* or introduced *Pinus nigra* took place since 1900s (Aimi *et al.* 2006). Climate of the valley is continental with annual mean temperature and annual rainfall of 10.7°C and 556 mm, respectively, between 1988 and 2016 (Silandro weather station; Salman *et al.* submitted, see chapter 4). Population density of PPM remained low in the past and pest control in the 1950s mainly involved clipping off and burning winter tents (Hellrigl 1995). However, range expansion and a large outbreak occurred since 1998, as a consequence of climate change (Battisti et al. 2005), and aerial spray of insecticide (*Bacillus thuringiensis kurstaki*) was employed to suppress the population (Salman et al. 2016, see chapter 2). Since the outbreak occurred, population density has been in regular monitoring with pheromone-baited traps. Owing to the spraying efforts, population density dramatically decreased from on average 68 males per trap in 1999 to around 10 males in 2000. Still, population density remained above the threshold of spray decision at least for eight years because of the stock of prolonged diapausing pupae in the soil spreading from the cohort prior to the insecticide application (Salman *et al.* 2016). The density decreased below the threshold of spraying following 2009, although limited amount of spray was applied in a few spots to protect humans living nearby. Our study samples come from the expansion zone on the southern slope.

2.2 Insect collection and maintenance

Insect collection was performed for four years, from 2014 to 2017. Insects were collected in Vetzan Schlanders (coordinates intervals 46°37´40´´- 46°37´56´´N, 10°47´30´´- 10°48´06´´E, elevation 835 - 1047 m). For logging air (1.5 cm above ground) and soil (10 cm deep) temperature, three dataloggers (HOBO Pro v2) with solar radiation shield (HOBO RS3) were installed. To intercept prepupae when they start their procession, we randomly selected 33 pine trees carrying tents in the first year and we maintained them in the following three years because they resulted to be colonised, with the only exception of 2017 when 22 of them were used. On each tree we deployed a trap called Ecopiège® (www.ecopiege-boutique.com), intended to be used on ornamental trees for insect protection. Each trap consisted of a collar around the trunk leading to soil contained either in pots on the ground or in plastic bags hung to the trunk.

We generally started checking traps from the end of February before PPM prepupae start their procession. In 2014, bags containing already pupated insects were collected twice, the first on May the 15th and the second on July the 10th. In 2015, we collected prepupae weekly for the whole procession period for another experiment (Salman et al. revision submitted, see chapter 4). In the two subsequent years, we collected the bags containing prepupae only once (May the 6th for 2016 and April the 11th for 2017).

In 2014, 2016 and 2017, all insects collected at the field were taken on the same day to the laboratory and kept at an average temperature of 21°C (SD=1.2). In 2015, on each weekly collection, half the pots containing prepupae were left on site, but the rest half were taken to the laboratory and kept at the same temperature reported above for the other years. Bags and pots taken to the laboratory were regularly checked under fume hood to assess ontogenic stages of the insects until pupae formed. When pupae formed, their cocoons were removed and they were placed individually into transparent plastic vials (5 ml). Holes (2.5 mm in diameter) in the stoppers of vials were drilled for ventilation. Vials were kept vertically in 96-well polystyrene trays (21.8 × 21.8 cm, wells of 1.7 cm diameter and 2.2 cm depth). Thereafter insects contained in vials were assigned to different temperatures until measurements started (Table 1).

2.3 Temperature monitoring with thermocouples

Measuring heat production using thermocouple is a direct calorimetry (Lighton 2008). We monitored body temperature of pupae with thermocouples for three consecutive years since 2014. E-type (Campbell Scientific) thermocouples with a 0.0762 mm diameter were used. Twenty-five thermocouples were sequentially connected to a 25-channel solid state multiplexer (AM25T) connected to a datalogger (CR23X). The datalogger was programmed to log temperature data every 15 minutes.

The tip of each thermocouple probe was attached to the pupal skin (dorsal abdomen) with paper tape that was twice wrapped around the pupa. Paper tape was used to avoid any injury to the insects. A microtube (1.5 ml) containing water was used as a control onto which a thermocouple probe was attached in a similar manner. Emergence was checked and registered daily. At the end of the experiment, remaining pupae were checked and their status (dead or in prolonged diapause) was recorded.

In 2014, experiment conducted only at high temperature started from July the 24th. First batch of insects collected from the field and subsequently treated in high temperature (table 1) emerged between June the 30th and July the 15th (none emerged after the latter date). Remaining insects from the batch either were dead or in prolonged diapause. Individuals already in prolonged diapause from that batch comprise one of two groups of our experiment. The other group of individuals came from the second batch collected from the field and subsequently kept in low temperature (table 1). As the insects of the second batch were kept in low temperature immediately after collection, they entered quiescence state. We took out a sample from this batch at low temperature, and used in the experiment at high temperature (table 1). Since development of the insects in this batch was suppressed by keeping them in a low temperature condition, they were expected to resume development upon exposing into high temperature condition of the experiment. In 2015, four groups of insects were used for monitoring body temperature at high temperature condition (table 1). In addition to three groups coming from current-year cohort, one group came from 2014 cohort treated at high temperature. Experiment started on June the 26th. In 2016, body temperature was recorded in three temperature conditions and insects came from both current and previous-year cohorts (table 1). Data logging started on May the 13th.

In 2014, a polystyrene box (45 × 35 × 25) with thick walls/side layers (4 cm) and a thick lid (3.75 cm) was used to conduct this experiment (supplementary figure S1.A). Elliptic wells (length= 5.3 cm; width= 2 cm) were dug on the inner side of the lid down to a depth of 1.5 cm by pressing hot glass-vials against the polystyrene. Wells were aligned and numbered according to an 8.75 × 4.5 cm grid on the whole inner surface of the lid, resulting in a total of 40 wells. A hot needle was eventually used to drill a small (diameter of approximately 1.5 mm) channel from each well to the outer face of the lid. The lid was then placed upside down so that the elliptic wells containing pupae faced upwards, and the box was mounted onto the inverted lid to prevent emerging moths from escaping, reduce air flow, and buffer air temperature variations. A circular hole (8 cm in diameter) was drilled at the top of the mounted box and thereafter covered with net (mesh= 1 mm) to provide sufficient ventilation. The tip of each thermocouple probe was inserted through the drilled channels before attaching it to the pupal skin (dorsal abdomen).

In 2015, two 96-well polystyrene trays were used instead (supplementary figure S1.B). Pupae were attached to thermocouple probes the same way as in 2014 and randomly assigned to one of the 192 wells. Each of the trays was covered with a net (mesh= 1mm) attached with pins. The system was kept inside a cabinet experiencing ambient high temperature.

The design was improved in 2016 with pupae placed into 5 ml plastic vials and thermocouple wires passing through a hole in their lid. Each vial was then placed in one of three polystyrene trays, themselves tightly inserted into one of three plastic containers (21×21×9 cm) covered with a net. To further reduce the fluctuation of temperature, water was added into the containers until the water-level reached the lids of the vials (supplementary figure S1.C).

Monitoring both in 2014 and 2015 included two control thermocouples. In 2016, two thermocouples were used in each temperature condition. Two non-functional thermocouples were discarded at the onset in 2016. By the end of each year's experiment, the number of individuals emerged was 3, 18 and 8 for the 2014, 2015 and 2016 cohorts, respectively. The number of pupae found dead was 12, 0, and 5 for the three consecutive years. In addition, 8, 5, and 4 pupae prolonged their diapause during the three years.

Logged data were downloaded using software from the provider company's website (https://www.campbellsci.com/pc200w). Data were then transferred and analysed in Kutools for Excel v 16.50 (Detong Technology Ltd. Hainan, 2017). To obtain biologically relevant data, daily mean temperature was calculated both for test and control thermocouples. The subtraction of the control daily mean values to the pupae values was used as a surrogate of metabolic activity.

2.4 O_2 consumption

We measured O_2 consumption in 2016 and 2017 at different temperature conditions (table 1). Insects used came either from current-year cohort or prolonged diapausing individuals of the previous-year cohorts (table 1). They were kept at constant darkness mimicking the natural condition of the insect.

A 4-channel fibre-optic O_2 meter connected to a computer running the FireSting software (FireStingO_2 by Pyro Science e. K., Aachen, Germany) was used to measure O_2 consumption rate in a stop-flow experiment, in order to quantify aerobic metabolic rate of pupae from all treatment groups. The measuring principle behind the current method is red light excitation and detection in the near infrared spectrum using luminescent O_2 indicators. A glass laboratory thermometer was used to record the temperature during the measurement. Four 2.5 ml plastic vials were used as respirometry chambers. A circular cut on the lid was made so as to fit a rough green sensor spot. A protective plastic tube was then protracted from the cut and glued at the base. A robust O_2 probe composed of black fibre and stainless steel contained the O_2 sensitive part at the very end that needed to enter the protective plastic tube and touch the sensor spot. The respirometry chambers including part of protective plastic tube was immersed in an adapted water bath during measurement to improve thermal stability over time.

The system had to be calibrated before the first measurement. Afterwards, only the temperature section of the software required regular change when measuring at different temperature. When the computer and respirometer were ready, individual insects were put into the chambers and immersed in the water bath. Prior to each measurement, the assay vials were left to stabilize at the water bath temperature for 3 minutes. The O_2 data were recorded in computer every second for half an hour. After every measurement, the measured individuals were weighed to the nearest 0.1 mg (Mettler Toledo AT460).

A series of steps were followed for calculating O_2 consumption rate (MO_2). We used the following equation: $MO_2 = a(V_{resp} w^{-1})$ Where MO_2 is O_2 consumption rate (µmol O_2 g^{-1} h^{-1}), a the slope, V_{resp} the volume of the respirometry chamber minus the volume of the insect, and w is weight of the insect in grams.

We used the package "*respirometry*" in *R* (Birk 2017) for importing the raw O_2 data and convert it from Torr to µmol/L. A linear model was used for each assay to get the slope factor of the linear equation between values of a channel of the O_2 meter in µmol/L and the assay duration in hours (supplementary source code S1).

Pupal volume was calculated by multiplying the individual fresh mass by the slope of a prior calculated standard regression between pupal fresh mass and pupal volume (N = 20 pupae). The volume for the corresponding subsamples was obtained using the water displacement method. The air volume around the insect inside the respirometry chamber was then obtained by subtracting the calculated insect volume from the chamber volume. Final O_2 consumption rate was calculated from the equation above.

Measurement was performed on average once a week for each individual. During the measurement period, if the O_2 consumption rate of a pupa almost doubled or more than doubled in one measurement date compared to the previous measuring date, we considered the pupa to be metabolically active (in other words, in the process of diapause termination) on that measuring day. Similar consumption values of the active period were averaged to get the activity level of the termination period. Values of the O_2 consumption rate at different measuring dates during pre-termination period were averaged to calculate metabolic activity level of the pre-termination phase (comprising diapause initiation and maintenance phase *sensu* Koštál 2006). However, activities immediately following pupation (formation of pupa from prepupa) was ignored because the increased activity most likely was related to metamorphosis rather than preparation for obligatory diapause. Post-diapause (or emergence in our category) metabolic activity comprised the last value of measurement or average of the values of the last and the penultimate measurement prior to emergence. For those that did not emerge, level of post-termination metabolic activity was calculated by averaging consumption values found at measurement dates between receded activity after putative termination effort and the last measurement of the year of experiment.

2.5 Weight loss

Weight loss was measured both in 2016 and 2017 with the individuals coming from different temperature treatment. A subsample of individuals was selected from each treatment and weighed to the nearest 0.1 mg with an analytical balance (Mettler Toledo AT460) every week or two, and the weight loss was calculated as the mass difference divided by the duration between two weighing sessions. The slope of the weight loss was used as a proxy of metabolic activity.

2.6 Statistical analysis

Temperature data from thermocouples were transformed into positive relative values by subtracting the lowest value of each pupa series to all other values in that series before analysis. As individuals at high temperature condition emerged earlier, thus reducing the number of measurement, only the individuals having at least three measurements were included in the analyses both for body temperature and O_2 consumption. One-way ANOVA was used to test the significance of difference among phases. Data were log or square-root transformed before analyses if not found normal and homoscedastic. As the sample sizes of diapausing individuals were small at the high and medium temperatures, we combined individuals from all three temperature treatments (assuming metabolic activity of diapausing individuals being similar at any three temperature). All levels of significance were set to $\alpha=0.05$. The R statistical software environment (v. 3.3.0, R Core Team 2016) was used for all analyses.

3. Results

The maintenance of pupae at different conditions allowed to obtain univoltine and prolonged diapause individuals to be used in the experiments in each of the sampling years (Table 1 and supplementary table S1). Univoltine individuals increased their metabolic activity approximately 20 days before emergence at high temperature in 2014 (Figure 1.A). The existence of a termination point was evident from the spike in temperature generated on August the 1st to 3rd. The individuals progressively kept on increasing their body temperature that peaked just before emergence. Immediately prior to emergence a sudden drop in temperature occurred. Interestingly, individuals in prolonged diapause synchronously increased activity with emerging individuals. Their body temperature quickly increased around the spike during the key period (August the 1st to 3rd), although soon after they decreased their temperature. Another minor spike of increased temperature occurred synchronously in all prolonged diapause pupae exactly when their counterparts emerged. After this second spike, individuals in prolonged diapause progressively lowered their temperature to values corresponding to the pre-termination phase (July 24th to July 30th, Fig. 1.A).

A similar pattern was found when we measured O_2 consumption of the high–temperature group in 2016 (Figure 1.B), although the frequency of measurement was low. The first measurement was taken on May the 16th, approximately one month before emergence of the two individuals that later emerged. During this first measurement, O_2 consumption was very low for all four individuals, irrespective if they were univoltine or prolonged diapausing. However, the second measurement on June the 8th showed dramatically high O_2 consumption in both univoltine individuals. One of them emerged on June the 14th, and the other on June the 20th. Another measurement was made on the second individual before it emerged. In both individuals, high O_2 consumption before their emergence compared to the initial measurement was evident. Individuals destined for prolonged diapause consumed high amount of O_2 at the similar period. O_2 consumption continued to increase for approximately a month after which it decreased to levels reflecting the previous dormant state, although a small spike of consumption could be seen in late August. Two separate termination efforts in the same year seemed plausible.

Another metric reflecting metabolic activity in univoltine or prolonged diapausing individuals is weight loss. Overall we found that weight loss is much faster in univoltine individuals than individuals destined for prolonged diapause (figure 1.C, note that the two groups are plotted on separate y-axes). Increased weight loss in emerging individuals was clear during the key period and immediately prior to emergence. Interestingly, weight loss did not increase progressively from the key period towards emergence as body temperature and O_2 consumption did, but rather dropped for a few days after termination, and finally peaked right before emergence. That some termination effort occurred in the individuals destined for prolonged diapause was clear with this approach as well.

The time between the start of the key period and adult emergence varied depending on temperature. At the high temperature, the duration was 18.7 days (SD=4.5, n=32), but 39.4 days (SD=17.7, n=12) at the medium temperature. Only two individuals emerged at the low temperature, and they took 66 days (SD=0) since the start of the key period. Based on the observation of dynamic patterns in body temperature, O_2 consumption and weight loss at different temperature treatments (figure 1), individuals could be clustered into three categories called: pre-termination, termination, and emergence (for univoltine) or post-termination (for prolonged diapausing individuals). We analysed these three phases for univoltine and prolonged diapausing individuals at different temperature treatments separately. Body temperature (n=12), O_2 consumption (n=33) and weight loss (n=33) varied significantly among the three phases at high temperature (see figure 2). Similar results were seen also at the medium temperature (ANOVA $P<0.001$ for O_2 consumption (n=11) and weight loss (n=9); for body temperature, the sample size was too low (n=2) for analysis) (see supplementary material figure S2). Although low in sample size, average body temperature of the two individuals monitored with thermocouples at the same condition corroborates the result (supplementary material figure S2.A). Of all the physiologically-monitored individuals (n=17) kept at low temperature, only two emerged. Interestingly, their O_2 consumption dynamics suggests that metabolic activity of individuals prior to emergence slows down with declining temperature treatment. Average O_2 consumption values of the two individuals were 0.02 ± 0.02 µmol/g.h during the pre-termination phase, 0.22 ± 0.03 during the termination phase and 0.12 ± 0.03 prior to emergence. Similarly, their weight loss values were 2.04 ± 0.32 mg for pre-termination, 4.38 ± 2.30 mg for termination, and 2.83 ± 1.97 mg prior to emergence.

In individuals undergoing prolonged diapause, body temperature (n=12; $P=0.012$), O_2 consumption (n=19; $P<0.001$) and weight loss (n=12; $P<0.001$) varied significantly among the three phases (see figure 3). Metabolic activity of pre- and post-termination was similar to each other, but dissimilar to the termination phase. We observed this result both in the univoltine at low temperature and prolonged diapausing individuals at low and high temperature. For instance, four individuals in prolonged diapause since 2015 and kept at low temperature showed increased metabolic activity in both 2016 and 2017 (figure 4) during the typical emergence period in their origin location, although the degree of O_2 consumption seemed to be lower in 2017.

4. Discussion

We could detect termination phase (Démolin's key period) in PPM from the increase of body temperature and of O_2 consumption, and from weight loss. In addition, we found that intrinsic metabolic activity differs among pre-termination, termination and post-termination in both emerging and prolonged diapausing individuals at different temperature treatments. Moreover, individuals that underwent prolonged diapause terminated their diapause every year at their population-specific emergence time. The finding of existence of a distinct key period and repeated termination accords with the earlier hypotheses by Démolin (1990), who hypothesized it based on a destructive method of dissecting prolonged diapause individuals, and finding the *corpus luteus,* or yellow body, in the ovaries as a remnant of the attempt to resume development (Biliotti et al. 1964). Unfortunately, no precise documentation of those observations is available. A non-destructive way to monitor metabolic activity is preferable to confirm the phases of diapause development, as dissection cannot determine the future fate of the individuals dissected that could otherwise have stayed in prolonged diapause. We show here that continuous body temperature measurement combined with oxygen consumption and weight loss are reliable proxies of metabolic activity. They could be ideally coupled with modern technology like micro-computed tomography (micro-CT) to detect morphological changes in the development of ovaria in a lepidopteran pupa (Lowe et al. 2013). Another finding was the synchronous termination of diapause in individuals of the same cohort subjected to the same temperature treatment.

Although a distinct termination phase is difficult to characterize, we could clearly detect it with the proxies of physiological activity. The other phases, such as pre-termination, diapause initiation and maintenance, and post-diapause development as conceptualized in previous studies (Tauber *et al.* 1986; Danks 1987; Hodek 1996, 2002; Koštál 2006; Saulich and Musolin 2012), could also be determined. Moreover, repeated termination of PPM in prolonged diapause supports the late-switch hypothesis put forward by Soula and Manu (2005) in contrast to the widely-held view of early-switch hypothesis supported, among others, by Ushatinskya (1984), Hanski (1988), and Powell (2001).

A novel aspect of our finding was detection of termination point with strong signal from continuous body temperature monitoring with thermocouples. Although the use of thermocouple in different biological field is already established (Kuusik *et al.* 1994; Marks *et al.* 1995; Harak *et al.* 1998; Crosthwaite *et al.* 2011; Hanson and Venette 2013), detecting phases of diapause development with thermocouples is not available yet. Measuring principle of direct calorimetry with thermocouple is easy. Moreover, it takes both aerobic and anaerobic metabolism into account (Lighton 2008). In addition, thermocouples allowed the detection of a precise timing of emergence as shown by a typical drop in temperature occurred just prior to emergence probably because of evaporation of water associated with the breaking of the pupal case (figure 1A).

Nevertheless, temperature monitoring with thermocouples was not always clear and was burdened with variability. There are inherent differences among thermocouples that manifest different sensitivity to even very small temperature variation. It is also likely that the contact surface could not be positioned in the exact same way onto every pupa. Moreover, pupae wriggle their abdomen from time to time even when in diapause. Coupled with these sources of nuisance, surrounding temperatures are difficult to control if not taken care of, especially because we are dealing with small temperature differences (0.05-0.1°C). If homogeneous temperature can be maintained throughout the experiment, the difference among phases is expected to be more clear and significant. Improving this method or finding better solution to reduce variability could be a potential future work that might be used both in laboratory and field.

Metabolic activity progressively increased from diapause maintenance to termination to emergence in high temperature. However, the progress was not consistent in the lower experimental temperature (medium and low). O_2 consumption plateaued after termination without any increase at emergence. This fall of activity with lower temperature was likely associated with quiescence of pharate adults as we already have seen in another experiment at natural condition (unpublished data). Nevertheless, it could also be a temperature compensation of metabolic rate. Further investigation is required to understand the trend.

Weight loss is a universal proxy of metabolic activity (Klarke 1956). However, it is a much less direct reflection of metabolic rate than the other proxies, such as heat production or O_2 consumption, as it depends much on the energy metabolites an organism uses (Chown and Nicholson 2004). Despite that, results of weight loss could reflect well the results from the other proxies we used in our study. Nevertheless, weight loss in emerging individuals did not progress linearly following termination as did with body temperature and O_2 consumption (fig 1.C). This implies that after the termination, the individuals didn’t use mass for building their body.. The synchronization in the diapause termination, or in the attempt to, among individuals of the same or different cohorts treated in the same cabinet conditions is prompting hypotheses about mechanisms possibly involved. The phenomenon has been observed for both simple and prolonged diapause individuals. As PPM is a gregarious insect also during the pupal stage in the ground, as the cocoons are clustered in the pupation site (Roques 2015), one could speculate about the occurrence of a communication system working at the pupal stage. Alternatively, it could be hypothesized that the response to external factors, e.g. fluctuation of temperature, is very synchronous, or that a cohort-specific clock is driving the process. Repeated termination of diapause in prolonged diapausing individuals at dark condition and at the time of its field origin may suggest the existence of a strong clock mechanism. Our experimental design did not include the possible occurrence of a synchronization mechanism, and the whole matter should be addressed with new experiments.

The cost related to repeated termination of PD and selective advantage is not clear yet. Energy cost associated with prolonged diapause of pupae in PPM has not been explored. Soula and Menu (2003) found similar adult performance after 1 and 2 years of larval life in the soil, probably because heavier larvae tended to prolong diapause (Menu and Desouhant 2002), although fresh weight of larvae may not be related to the lipid content and use. Whether heavier pupae of PPM tend more to enter prolong diapause is not clear so far, but bigger pupae were found to dig deeper (Battisti et al. 2000). However, this behaviour could be more related to escape from natural enemies than preserving energy. A metabolomics approach to the study of the energetic costs associated with PD is necessary in order to assess the limits imposed by energy availability to the diapause duration. Mechanism underlying the repeated resumption of prolonged diapause by individuals is not clear. A decision threshold could be in effect based on internal energy reserve of the individuals (Hahn and Denlinger 2011).

Overall, we identified three phases of diapause development in PPM as far as our data could support us. However, other sub-phases as discussed in the diapause literature might be possible to distinguish if more care and effort is given in study design and data collection. Although eco-physiological phases are not standardised throughout the insect taxa, and different insects may show different character of diapause development depending on their life history and environmental conditions, our study could be a starting point in understanding diapause development process in an economically important insect pest like PPM.

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Table 1: Temperature at which pupae were kept after collection and measurements were done. Source of pupae (treatment after collection) is also indicated.

Year	**Pupal condition (°C)**	**Measurement Temperature**	**Measurement and number of individuals**		
			Body temperature	*O_2 consumption*	*Weight loss*
2014	Lab High (20±1.2) Lab Low (10±1.8)	High (25.1±1.5)	Lab High (n=11) Lab Low (n=12)	×	×
2015	Field (12±4.4) Lab High (19.7±0.8) Lab Low (10±1.5)	High (23.9±0.8)	Field (n=7) Lab High (n=8) Lab Low (n=4) PD 2014 High (n=4)	×	×
2016	Lab High (20.2±0.9)	High (22.5±0.7)	Lab High (n=2) PD 2014 High (n=2) PD 2015 Lab High (n=1) PD 2015 Lab Low (n=3)	Lab High (n=12) PD 2015 Lab Low (n=4)	Lab High (n=12) PD 2015 Lab Low (n=4)
	Lab Medium (16.3±0.3	Medium (16.3±0.3)	Lab Medium (n=3) PD 2015 Lab Low (n=2)	Lab Medium (n=12) PD 2015 Lab Low (n=4)	Lab Medium (n=12) PD 2015 Lab Low (n=4)
	Lab Low (9.5±1.8)	Low (9.5±1.8)	Lab Low (n=2) PD 2015 Lab Low (n=2)	Lab Medium (n=12) PD 2015 Lab Low (n=4)	Lab Medium (n=12) PD 2015 Lab Low (n=4)
2017	Lab High (18.8±1.7)	High (18.8±1.7)	×	Lab High (n=38)	Lab High (n=38)
		Low (9.5±1.8)	×	PD 2015 Lab Low (n=4)	PD2015 Lab Low (n=4)

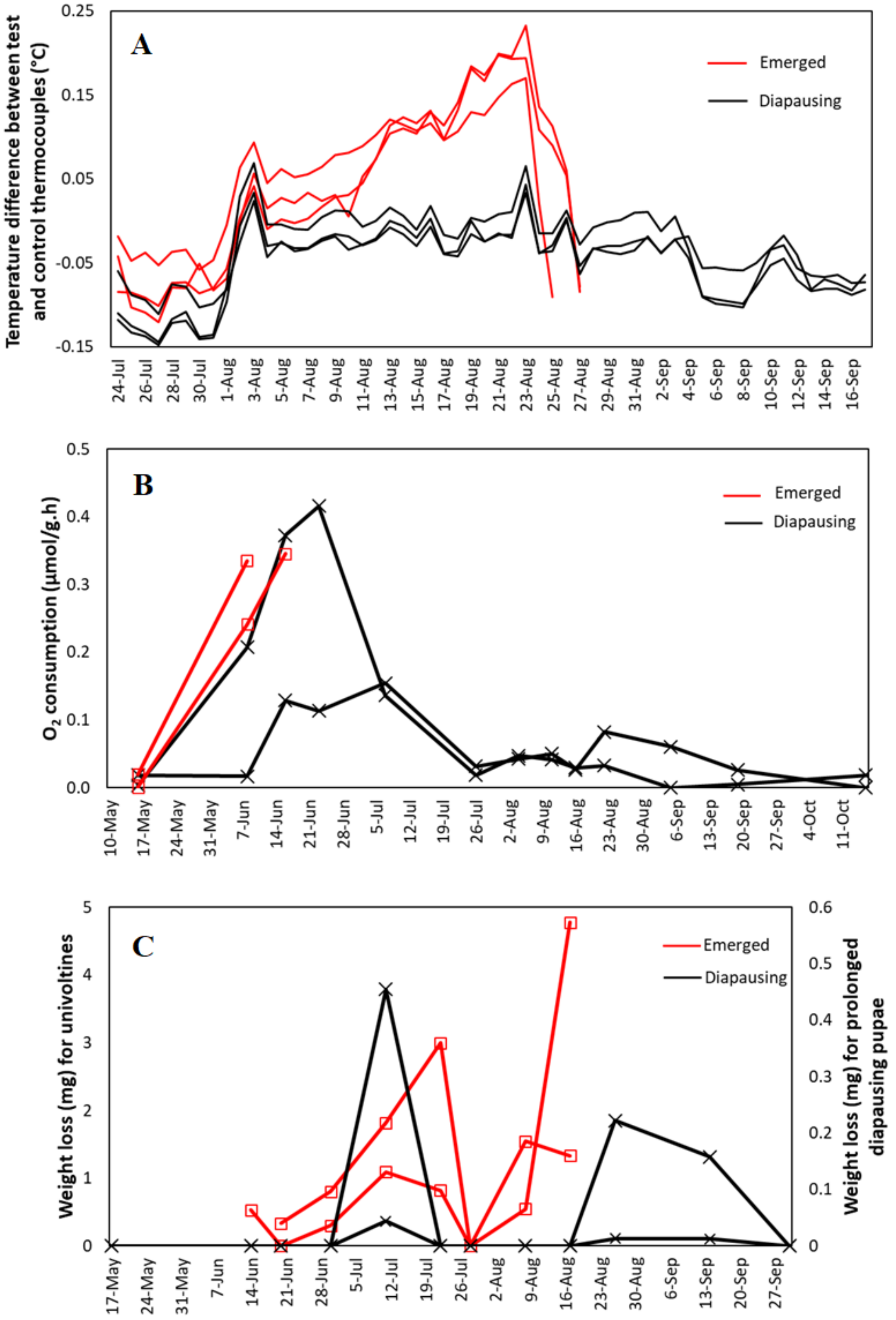

Figure 1: Body surface temperature (A), O_2 consumption (B), and body weight loss (C) in representative emerged (red) and prolonged diapausing (black) pupae of pine processionary moth.

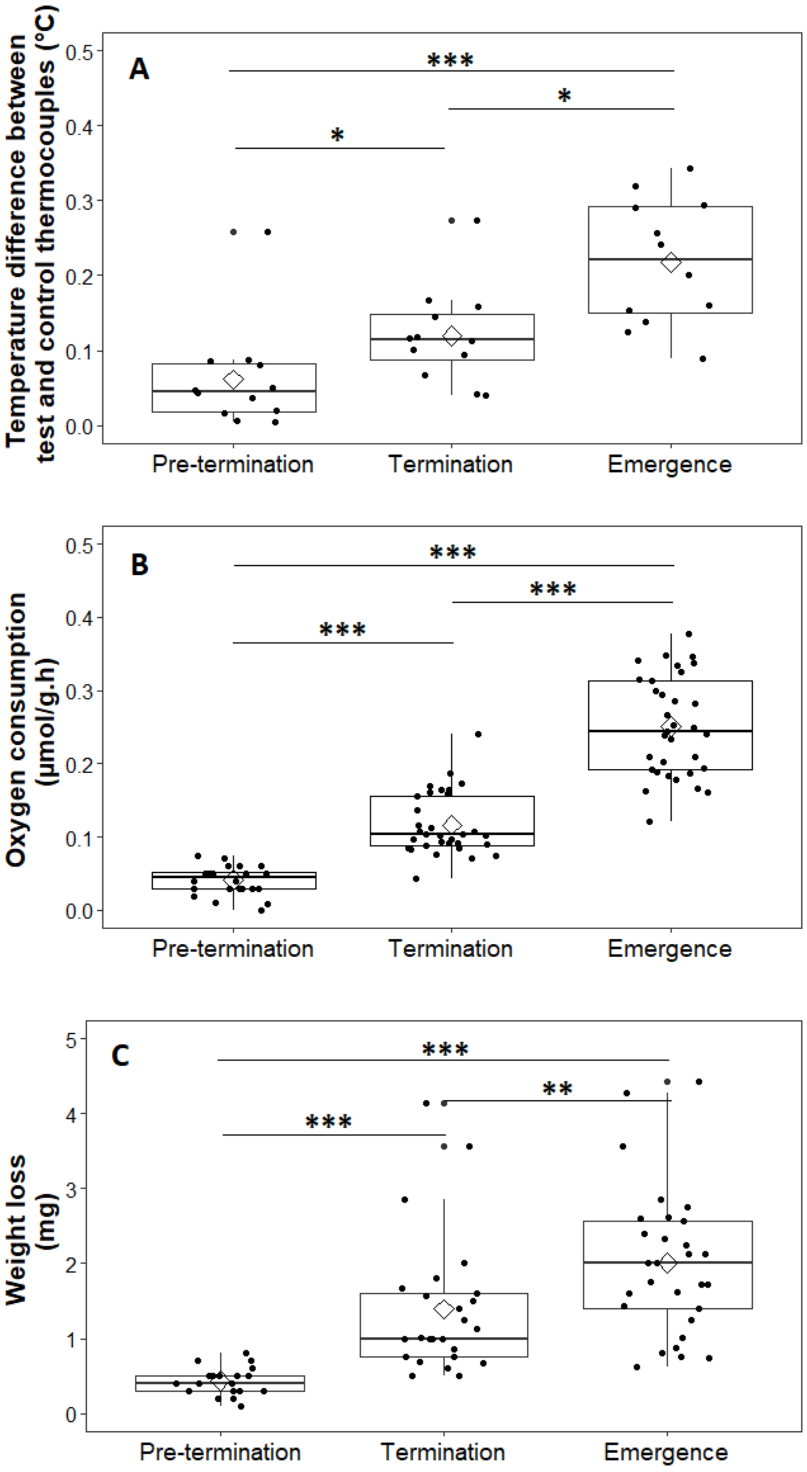

Figure 2: Body surface temperature (A), O2 consumption (B), and body weight loss (C) during pre-termination, termination, and emergence phases for univoltine pupae. Phases were determined based on figure 1. Boxplots represent minimum, 1st quartile, median, 3rd quartile, and maximum values. Open diamond shapes on boxplots delineate mean of observed values represented as jittered black dots. Asterisks representing significance levels in post-hoc test are: (***) = P<0.001; (**) = 0.001<P>0.01; (*) = 0.01<P>0.05; (ns) = P>0.05.

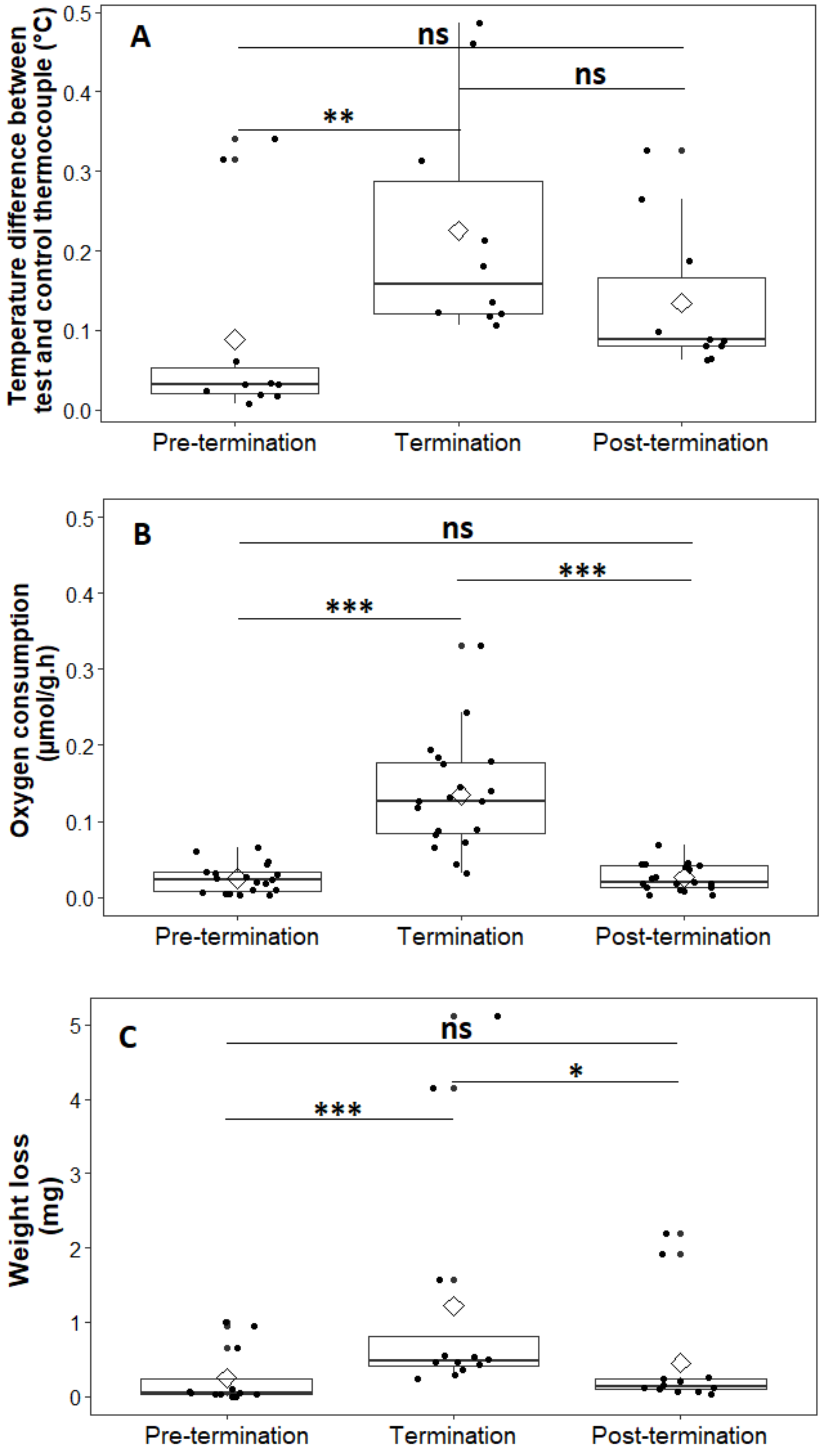

Figure 3: Body surface temperature (A), O_2 consumption (B), and body weight loss (C) during pre-termination, termination, and post-termination phases for prolonged diapausing individuals. For asterisks representing significance levels, see the caption of Figure 1.

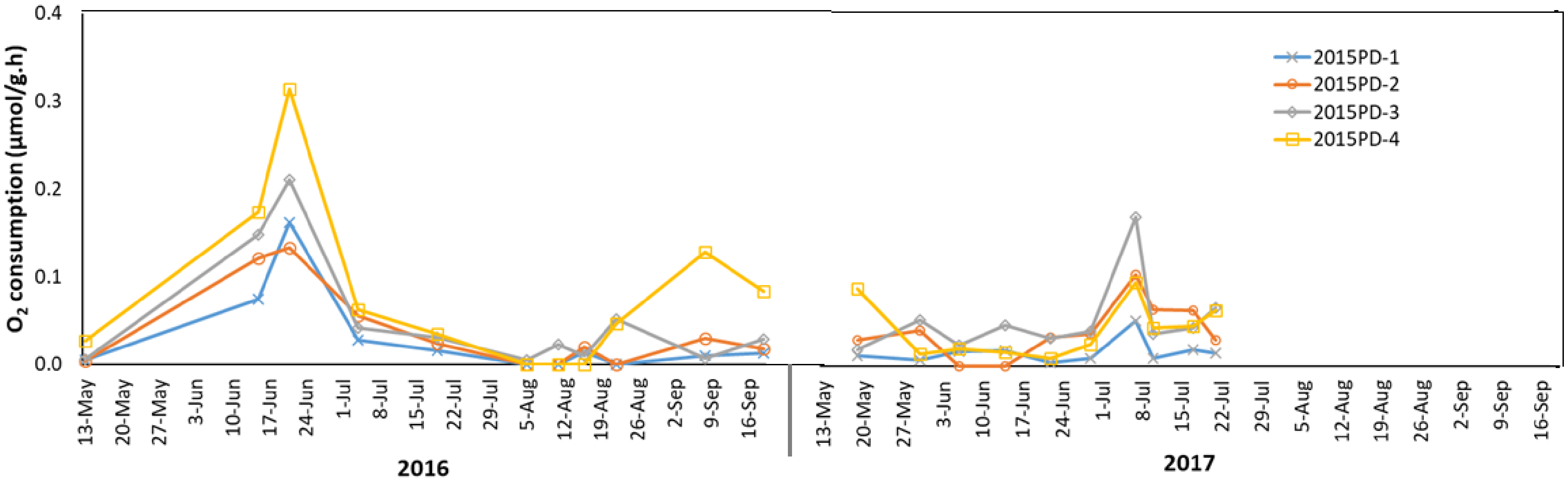

Figure 4: Repeated termination of four individuals at low temperature coming from 2015 cohort in 2016 and 2017.

Supplementary material

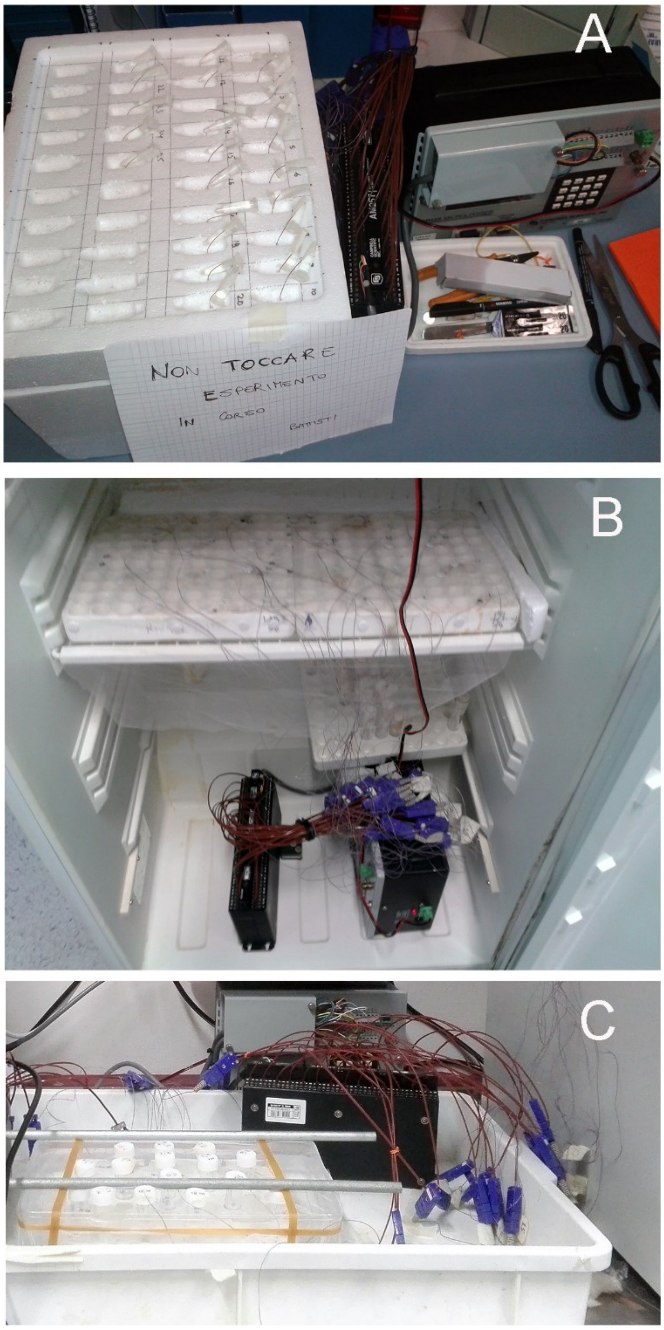

Figure S1: Body temperature monitoring with thermocouples. A) polystyrene box used in 2014. The lid contained with wells is positioned on top of the box during the photograph was taken. Generally, the box was mounted on top of the lid. System was being tested with microtubes contained with water at the image. B) Two 96-well polystyrene was used in 2015. C) experiment at high temperature in 2016.

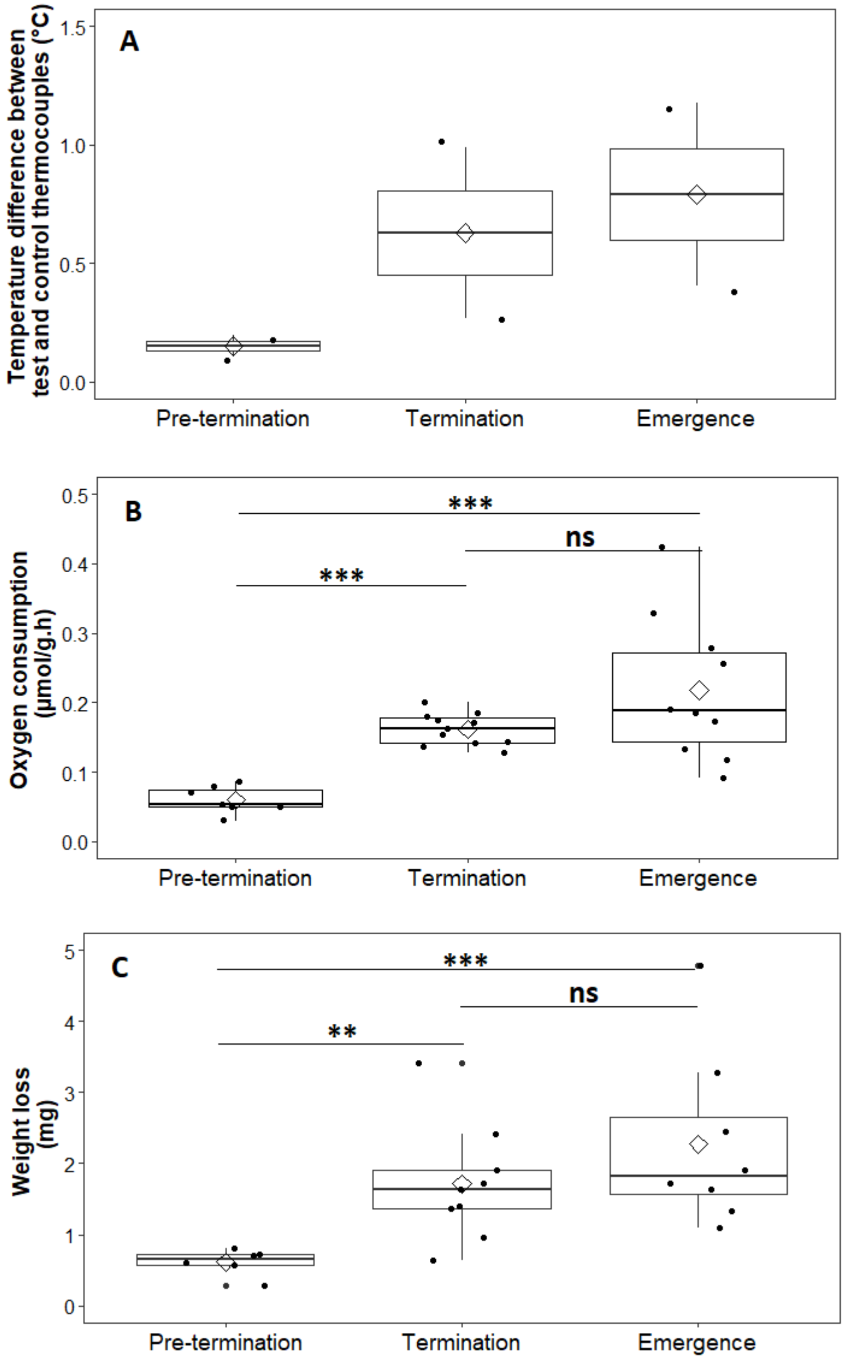

Figure S2: Body surface temperature (A), O_2 consumption (B), and body weight loss (C) during pre-termination, termination, and emergence phases for univoltine pupae at the medium temperature. Phases were determined based on figure 1. Boxplots represent minimum, 1st quartile, median, 3rd quartile, and maximum values. Open diamond shapes on boxplots delineate mean of observed values represented as jittered black dots. Asterisks representing significance levels (Only for B and C) in post-hoc test are: (***) = *P*<0.001; (**) = 0.001<*P*>0.01; (*) = 0.01<*P*>0.05; (ns) = *P*>0.05. Note only two data point for body temperature (A).

Table S1: Fate of cohorts collected from Venosta at the end of moth emergence in the year of collection.

Cohort	Treatment	Initial number of pupae	Individuals		
			Emerged (%)	*Dead (%)*	*Prolonged (%)*
2014	High	313	68	30	2
	Medium	621	57	24	19
	Low	311	13	63	24
2015	High	1661	60.7	38.4	0.9
	Low	279	27.2	31.2	41.6
2016	High	69	94.2	4.3	1.4
	Medium	72	91.7	8.3	0.0
	Low	74	17.6	67.6	14.9
2017	High	110	59.1	40.9	0.0

Source code S1: R code for analysis of slope factor

```
rm(list=ls())  # Clear memory
install.packages("respirometry")  # Install package “respirometry”
install.packages("dplyr")  # Install package “dplyr”
library(respirometry)  # Load package “respirometry”
library(dplyr)  # Load package “dplyr”
options(scipen = 999)  # Disable scientific notation in R

# Import firesting txt file. Be careful about the date format. It should be adapted with the computer
# in use.
mydata <- import_firesting("myfile.txt", o2_unit = "umol_per_l", date = "%m/%d/%Y %X",
            overwrite_sal = NULL, keep_metadata = FALSE, drop_channels = TRUE,
            split_channels = FALSE)

attach(mydata)

# Mutate a variable named HOUR to convert variable TIME in the unit of second to the unit of Hour.
# It is important to put "unclass" before the variable TIME to make the variable numeric for analysis.
# Assign the mutated code line into a variable for further analysis

mut.mydata <-  mutate(mydata, HOUR = unclass(TIME)/3600)
attach(mut.mydata)

# Subset top 1650 rows to represent first 30 minutes of measurement in case the date was recorded
# for more than 30 minutes.
sub.mut.mydata <- mut.mydata[1:1650,]
attach(sub.mut.mydata)

# Build linear model for each channel
m1 <- lm(CH_1_O2 ~ unclass(HOUR))
m2 <- lm(CH_2_O2 ~ unclass(HOUR))
m3 <- lm(CH_3_O2 ~ unclass(HOUR))
m4 <- lm(CH_4_O2 ~ unclass(HOUR))
# Get slope factor of the regression equation
```

```
m1$coefficients
m2$coefficients
m3$coefficients
m4$coefficients
```

CONCLUSION

With increasing human-mediated climate warming, biological entities are expanding their range into places that were previously physiologically impossible (Parmesan 2006). Range expansion of this sort becomes a concern when a pest is involved. Pine processionary moth (PPM) is an active range expanding pest species (Battisti et al. 2005) having significant economic impact in terms of tree growth (Jacquet et al. 2013) and human health (Battisti et al. 2011, 2017). Therefore, it became a major species of attraction for many entomologists in the Mediterranean region. Consequently, we already know a lot about many aspects of its natural history, genetic diversity and structure, spatio-temporal dynamics, interaction with trees, responses to natural enemies, medical and veterinary impacts (Roques 2015). Nevertheless, one important aspect of its life history, diapause, has always been neglected in spite of its acknowledged importance in pest establishment and dynamics (chapter 2).

A few questions have been answered in this PhD project. Although the existence of prolonged diapause (PD) was reported long ago, no study provided its maximum duration was available. The second chapter of the thesis provided evidence of existence of at least 7 years of PD in one mountain population of PPM. Using dataset of 18-year surveillance with four large cages and 15 pheromone-baited traps in an outbreak population in the southern-central Alps of northern Italy, we established the importance of PD individuals in the sustenance of population density. In addition, we found that individuals with PD tend to emerge earlier than their current-year counterparts. Moreover, we found an elevational gradient of the duration of PD and retrieval rate of moths. This study underscores the importance of long-term surveillance of population for understanding the dynamics of PD.

Although several studies have been conducted on the incidence of PD in PPM across it natural range, they were often published as forest service or forest health reports and not available to the large public. A collection of these reports, their validation and comparison with data from papers allowed to make a synthesis that included most of the pest range, including the sibling species *Thaumetopoea wilkinsoni* in the Near East. How individuals in a population determine whether to emerge as univoltine or prolonged their diapause for one or more additional years remains a long-unanswered puzzle. The third chapter provides some of the first information suggesting that winter conditions at the larval stage influence this decision.Data analysis suggests a strong U-shaped relationship between the incidence of PD and average winter temperature and a linear positive relationship between mortality and proportion of individuals in PD. The result contributes to the advancement of knowledge on the ecology, population dynamics and distribution of the PPM. Such data can guide further studies and may constitute important tools to inform decisions aiming at control of this pest.

As said above, much of the natural history of PPM has been studied, however, we know little about the contribution of its dormant stages to the success of this species. Earlier, Demolin (1969) produced a conceptual model of its life-cycle in different localities to predict its phenology. However, the model lacks the description of dormant prepupal stage and its significance in, e.g., synchronization of emergence. Besides, no study reported on the occurrence of diapause in PPM in a stage other than pupa. The fourth chapter discusses how with a weekly sampling during the two-month long procession period of pine processionary prepupae, we discovered that prepupae differentially regulate their development time in such a way that moth emergence of short-lived adults resulted to be concentrated and synchronized in less than one month. We found that early descending individuals don’t pupate immediately, rather wait as diapausing prepupae for their late counterparts, whereas late-descending prepupae develop into pupae much faster. The finding of prepupal diapause and its significance in synchronization of emergence may improve the existing model of phenology and provide managers with a new tool to handle this pest.

The last chapter mainly focused on the physiological characterization of diapause termination in both univoltine and prolonged diapausing individuals. Understanding successive eco-physiological phases of diapause development is important for practical purpose (Danks 1987; Koštál 2006). No such study is available for PPM. Although Demolin (1990) hypothesized about existence of a key period in the univoltine and prolonged diapausing individuals, his method and conclusions were not clear. Despite that, his study was a good point to start our investigation. The last chapter of this thesis elaborate on the process of diapause development in PPM, identifying the existence of pre-termination (comprising both initiation and maintenance), termination and post-termination (post diapause). We used body temperature, O_2 consumption and weight loss as proxy of metabolic activity. The study confirmed the hypothesis of existence of key period (termination) in univoltine PPM. Most exciting was the finding of repeated termination of diapause in prolonged diapausing individuals. This study is a starting point for the study of diapause development in PPM from an ecological point of view.

Compared to simple phenomenon of diapause, field of prolonged diapause in insects per se is not vast. A lot of questions regarding the regulation of prolonged diapause are still unresolved. When we consider PPM, the gap of knowledge in this regard is gigantic. Being an important pest of Mediterranean forests, PPM deploys diapause for its success. We know almost nothing about the regulation of diapause in this species. Despite that, this thesis attempted to start the effort of answering a few of thousands of questions on the regulation of diapause in PPM. Overall, the existence of a 7-year long PD and its role in population dynamics has been demonstrated. A significant role of winter temperature as a factor regulating PD is clear now. In addition, existence of diapause in the prepupal stage and its important role in emergence synchronization has been discovered for the first time. And finally, the eco-physiological phases of diapause development in the univoltine and repeated termination mechanism in prolonged diapausing individuals have been confirmed physiologically.

This project has prepared the ground for several possible future works. Among them, some are:

- Testing the findings in other populations from both core and edge of the range to confirm ubiquity of the phenomena.
- Understanding genetic regulation of diapause and prolonged diapause using metabolomics and transcriptomics, now that the genome of PPM is available (Kerdelhué et al. submitted).
- More data are required to understand the importance of climate change in regulating diapause. Acquiring such data from long-term studies would be one of the next works. The study site of Venosta Italy is particularly important because there is a clear tendency to reduce the frequency and duration of PD with warmer winters observed in the last decade.
- Role of diapause and prolonged diapause in phenology switch should be made clear. For example, an unusual summer population has been discovered in Portugal recently (Santos et al. 2007). This summer-feeding population shifted its phenology compared to its winter-feeding counterparts, probably because of sudden phenological shifts of some individuals from sympatric winter population. Were those isolated individuals diapausing as pupae and terminated their diapause untimely for some unknown reason, resulting in a founder effect? Testing such kind of role in allochronic speciation would be a fantastic inclusion for the future tasks.
- Repeated termination of diapause in the pupal stage of PPM implies existence of an endogenous clock mechanism. Whether it is a circannual system regulated by any external cue or by hourglass or interaction of both needs future experiment. In addition, finding out the role of such mechanism in the termination of diapause would be a novel dimension of study.

- Based on the knowledge of diapause and prolonged diapause discussed in this thesis, an improved phenology model can be developed and used for predicting the effects of climate change under different scenarios.
- Lee et al. (2015) have shown the occurrence of cyclic periodicity of population outbreak of PPM. PD has been assumed to be one of the factors responsible for the cyclicity of the outbreaks. It is time to develop theoretical models for testing the importance of PD for the periodicity of PPM outbreaks.
- It is known that some natural enemies (parasitoids) of PPM respond to diapause and prolonged diapause by shifting their emergence, although mechanisms are unknown. Future study investigating this response would be useful to predict the role of enemies in the regulation of population with PD.
- We found that a high percentage of individuals entered prolonged diapause after a record outbreak. But recent years, characterized by lower density, showed much lower rates of PD. Future studies should investigate whether the incidence of prolonged diapause is density dependent or not.
- How the diapause and PD respond to extreme events of temperature and rainfall is also unknown and it could be a good source of future study for understanding evolutionary tactic in PPM.
- Future studies of prolonged diapause with PPM may finally contribute to the fascinating field of aging research.
- Last but not least, we should keep in mind that the wonderful projects with PPM have to deal with an urticating insect. Every person, including myself, gets easily sensitized to the setae and experience all sorts of problems that sometimes make it very difficult to continue in the planned work. Even if all the necessary precautions are taken, it is practically impossible to work under totally safe conditions. This problem, which represents a scientific challenge per se, is seriously limiting the amount of work that can be done with this insect, especially when larvae and pupae are concerned.

Some potential implications of the thesis outcomes for the management of PPM populations are as follows:

- Modelling outbreak cycle: Success of modelling efforts to predict outbreak pattern of PPM have been limited for the gap in biological knowledge on diapause, especially prolonged diapause (Li et al. 2015). This thesis has presented or confirmed some key preliminary information on the biology of diapause that has paved the way for its inclusion in the existing models.

- Temperature has been proven again to be an important extrinsic factor, in this case regulating incidence of prolonged diapause. Study in Venosta over the last 18 years has shown that winter temperature regulates the incidence of prolonged diapause. In other words, higher temperature reduces the incidence, as well as, possibly act for release of the individuals from the previous cohorts already in the soil. Confirmation of such role of temperature covering entire geographic range of PPM has been discussed in the third chapter. Take home message for the managers is that, by only measuring the winter temperature they would be able to prepare themselves with appropriate tools for managing PPM in the current and next year. In addition, the effect of global warming on the population dynamics can be better assessed once the effect of temperature on the induction and maintenance of PD is clarified.
- Manipulation of adults through mating disruption is a classic way of population management. Therefore, how the emergence timing is regulated is important to know. In the fourth chapter, we have identified the prepupal diapause as an important player in this case. Knowing the role of this so-far-unknown stage would definitely be an addition to the toolbox of the managers. Phenology model would be improved with this addition.
- Although not discussed in detail, parasitoids (especially hymenopterans) have been found to enter prolonged diapause along with their hosts. Such information may provide opportunity to strengthen biological control through natural enemies of PPM. However, further research on host-parasitoid relation is needed to wield such tools. Natural enemies are locally important for the regulation of the populations and the role of diapause can be important in the density-dependent relationships.
- Accurate prediction of seasonal activities of insect requires understanding diapause that comprises different eco-physiological phases. Detecting the phases using easy methods is a challenge. Fifth chapter of the thesis has described the use of temperature monitoring using thermocouples in detecting termination of diapausing individuals. Use of such easy tool would be handy for use in the field for directly understanding the physiological state of field populations. This sort of direct detection will give managers a tool for understanding the activities of insects in real-time.

- In addition to the detection of diapause termination, the thesis confirmed a repetitive termination in the prolonged diapausing individuals. This finding paves the way of further research for understanding the "decision" mechanism of termination, which will improve predicting the amount of putative emerged individuals diapausing in the soil.

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