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Termination of pupal diapause in the pine processionary moth *Thaumetopoea pityocampa*

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Abstract. Diapause development is a complex process involving several eco-physiological phases. Understanding these phases, especially diapause termination, is vital for interpreting the life history of many insect species and for developing suitable predictive models of population dynamics. The pine processionary moth is a major defoliator of pine and a vertebrate health hazard in the Mediterranean region. This species can display either univoltine or semivoltine development, with a pupal diapause extending from a few months to several years, respectively. Although the ecological and applied importance of diapause is acknowledged, its physiological regulation in either case remains obscure. In the present study, we characterize pre-termination, termination and post-termination phases of pupae developing as univoltine or remaining in prolonged diapause. Changes in metabolic activity are monitored continuously using thermocouples, comprising a novel method based on direct calorimetry, and periodically by use of O_2 respirometry. The two methods clearly detect diapause termination in both types of pupae before any visible morphological or behavioural changes can be observed. Univoltine individuals are characterized by an increase in metabolic activity from pre-termination through to termination and post-termination, ultimately resulting in emergence. Remarkably, a synchronous termination is observed in individuals that enter prolonged diapause instead of emerging; however, in these pupae, the increased metabolic activity is only transient. The present study represents a starting point toward understanding the eco-physiology of diapause development processes in the pupae of the pine processionary moth.

Key words. Lepidoptera, Notodontidae, prolonged diapause, respiration, thermocouple.

Introduction

Diapause comprises a generic resistance form found in many arthropods in response to indirect cues predicting adverse conditions (Danks, 1987; Hodek, 2002). This dynamic process temporarily pauses morphogenesis and decreases metabolic activity (Tauber *et al.*, 1986**;** Danks, 1987). Historically, the phases of this process are defined subjectively, leading to ambiguity (Koštàl, 2006). However, attempts to standardize the terminology with respect to the phases of diapause have been made (Hodek, 1996**,** 1999) and Koštàl (2006) characterized three main sub-phases of diapause, defined as initiation, maintenance and termination.

The initiation phase of diapause is characterized by the cessation of direct development and a regulated suppression of metabolic rate. In the maintenance phase, metabolic rate is low and constant. The intensity of diapause decreases with time, whereas sensitivity to diapause terminating conditions increases. Abiotic factors such as temperature, humidity or presence of liquid water may regulate this phase. Diapause termination may be spontaneous or, more commonly, may require specific environmental conditions (Tauber & Tauber, 1976; Koštàl, 2006).

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Adverse conditions (e.g. cold or drought) may act as a reliable token signal and prevent an untimely termination of diapause (Lehmann *et al.*, 2017). Therefore, termination as an eco-physiological phase can be characterized by a decrease of diapause intensity to its minimum level. In addition to environmental factors, genetic factors may regulate this process (e.g. expression of diapause-upregulated genes rapidly declines and that of diapause-downregulated genes increases) (Danks, 1987; Denlinger & Armbruster, 2014).

Resumption of the potential to develop is the principal feature of diapause termination. Because diapause is a dynamic process, characterizing the precise termination point on an individual level is challenging and often arbitrary (but see Koštál *et al.*, 2017). Visually determining the timing of diapause termination is difficult or impossible. Therefore, investigating the physiological processes throughout diapause could repreent a pragmatic approach for determining and understanding its termination in insects (Soula & Menu, 2005; Sgolastra *et al.*, 2010; Lehmann *et al.*, 2018).

The pine processionary moth *Thaumetopoea pityocampa* (Denis & Schiffermüller) is an economically important gregarious pest of pines and cedars in the Mediterranean region and comprises a nuisance to humans and warm-blooded animals as a result of its urticating setae (Battisti *et al.*, 2017). After completion of larval development in silk tents, larvae leave the host tree, usually during spring, in a typical head-to-tail procession in search of pupation sites, where they bury themselves to a depth of 5–20 cm in the soil within individual cocoons (Démolin, 1971). After a period of prepupal diapause (Salman *et al.*, 2018), they develop into pupae and enter pupal diapause. The length of the pupal diapause is short (e.g. 1 month) at high elevation/latitude and long (e.g. up to 5 months) at low elevation/latitude, matching the local conditions favourable for development (Démolin, 1969). A variable proportion of the pupae however, enter prolonged diapause so that moth emergence is postponed to subsequent years (Démolin, 1990), with a maximum observed duration of 8 years (Salman *et al.*, 2016). The mechanisms of prolonged diapause induction and maintenance are unknown and the failure to predict these long cycles presents serious problems to pest managers. For example, Li *et al.* (2015) report testing cycles of pine processionary moth outbreaks in France using 32-year time series data from permanent plots. Although many monitoring plots over different eco-climatic regions of France show a cyclic outbreak of the pine processionary moth, anomalies are found in a large spatial scale. Amongst other factors, such as host tree quality and natural enemies, prolonged diapause is invoked as a factor interfering with or facilitating the cyclic dynamics of the pine processionary moth over different spatial scales, resulting in a lower reliability of outbreak predictions.

In the present study, in an attempt to understand the diapause mechanism of pine processionary moth pupae, we characterize pre-termination (comprising so-called diapause initiation and maintenance), termination and post-termination (resulting in either emergence or prolonged diapause). Two nondestructive methods for detecting changes in metabolic activity in pupae are used: continuous direct calorimetry with thermocouples and periodic O_2 respirometry. Other methods for assessing metabolic activity were considered but not used because they are either complementary (CO_2 production), less precise (weight loss) or destructive (metabolite analysis). We hypothesized that (i) the spike of metabolic activity associated with termination of diapause can be detected using both methods and (ii) intrinsic metabolic activity differs among the pre-termination, termination and post-termination phases. Clarification would allow a better understanding of the factors involved in diapause regulation and its significance in population dynamics.

Materials and methods

Insect collection and maintenance

Insects were collected from a population that was investigated previously for the occurrence of prolonged diapause in the Southern Alps, Vinschgau, Schlanders Vetzan, Italy (coordinate intervals 46°37′40″ to 46°37′56″N, 10°47′30″ to 10°48′06″E; 835–1047 m a.s.l.) (Salman *et al.*, 2016), between March and June from 2014 to 2017. To intercept larvae when they start their pupation procession, 33 pine trees carrying tents were selected haphazardly in winter each year and an Ecopiège trap (www.ecopiege-boutique.com/15_collier-ecopiege; La Mesange Verte, France; accessed on 13 September 2018) was deployed on each tree. Each trap consisted of a collar around the trunk that leads to either a pot on the ground or a plastic bag hung to the trunk, both containing soil. Traps were visited during and after pupation processions, and containers with insects in soil were taken to the laboratory and kept at a mean ± SD temperature of 21 ± 1.2 °C. Cocoons and their content were checked every 4–5 days under a fume hood to assess development. When pupae formed, all the cocoons were removed from the containers, and then pupae were extracted from the cocoons and placed individually in transparent plastic vials (5 mL). Holes (diameter 2.5 mm) were drilled in the stoppers of the vials for ventilation. Vials were kept vertically in 96-well polystyrene trays (21.8 × 21.8 cm; wells of 1.7 cm in diameter and 2.2 cm in depth) at constant temperature in darkness. Because trees hosted more than one winter tent and a winter tent may host more than one family as a result of the merging of two or more initial colonies (Roques, 2015), pupae obtained from all trees were pooled and the individuals used in the experiments were chosen haphazardly. Thus, the results provide a general reference for the population used in the present study and do not account for potential effects associated with the specific traits of each colony.

Direct calorimetry with thermocouples

Body heat production reflects metabolic changes in ectotherms and can be measured using thermocouples (direct calorimetry; Lighton, 2008). The body temperature of pupae was monitored using Teflon-insulated, fine-wire E-type thermocouples (Omega Engineering, Norwalk, Connecticut) with a diameter of 0.0762 mm. Twenty-five thermocouples were sequentially connected to an AM2 5 T 25-channel solid state multiplexer connected to a CR23X datalogger (Campbell Scientific Inc. Logan,

Utah), achieving an overall resolution better than 0.01 °C. The datalogger was programmed to measure body temperature every 10 s and to record average values every 15 min. The tip of each thermocouple probe was positioned in contact with the dorsal abdomen of the pupa with a paper-tape strip (width 1.5 cm), which was wrapped twice around a pupa. A plastic microtube (1.5 mL) filled with water was used as a control, and one thermocouple probe was positioned in contact in a similar manner. Emergence was checked and recorded twice a week. At the end of each experiment, pupae were checked and their status (dead or in prolonged diapause) recorded.

Logged data were downloaded using software provided by Campbell Scientific Inc. (https://www.campbellsci.com/pc200w). To obtain biologically relevant data, daily mean temperature was calculated for both test and control thermocouples. The difference between test and control thermocouples was used as a proxy of metabolic activity.

O_2 consumption

A four-channel fibre-optic O_2 meter connected to a computer running FIRESTINGO$_2$ software (Pyro Science GmbH, Germany) was used to measure O_2 consumption rate in a stop-flow experiment, aiming to quantify the aerobic metabolic rate of pupae. The measuring principle comprises red light excitation and detection in the near infrared spectrum using luminescent O_2 indicators. Four 2.5-mL plastic vials were used as respirometry chambers. A circular cut on the lid was made to fit a green sensor spot. A protective plastic tube was then protracted from the cut and glued at the base. An O_2 probe (type OXROB3; Pyro Science GmbH) containing the O_2 sensitive part at the terminal point was entered through a protective plastic tube to touch the sensor spot. During measurement, the respirometry chambers were immersed in water to improve thermal stability over time. A glass laboratory thermometer was used to record the water temperature to be set in FIRESTINGO$_2$. Before each measurement, the assay vials were left to stabilize at water temperature for 3 min. The O_2 data were recorded every 1 s for 30 min. After every measurement, the measured individuals were weighed to the nearest 0.1 mg (AT460; Mettler-Toledo, Switzerland).

To calculate O_2 consumption rate, we used:

$$\text{O}_2 \text{ consumption rate } \left(\mu\text{mol O}_2\ \text{g}^{-1}\ \text{h}^{-1}\right) = a\ \left(V_{\text{resp}}\ w^{-1}\right)$$

where a is slope, V_{resp} is volume of the respirometry chamber minus the volume of the insect, and w is the weight of the insect (g). The package 'respirometry' (Birk, 2018) in R (R Foundation for Statistical Computing, Austria) was used for importing raw O_2 data and converting imported data from Torr to μmol L^{-1}. A linear model was used for each channel to obtain the slope of the linear equation between values of O_2 (μmol L^{-1}) and the assay duration (h) (for R code, see Supporting information, File S1). Pupal volume was calculated by multiplying the individual fresh mass by the slope of a standard regression calculated from other pupae ($n = 20$) between pupal fresh mass and pupal volume. The volume for the corresponding subsample was obtained using the water displacement method. Air volume surrounding the insect inside a respirometry chamber was obtained by subtracting the calculated insect volume from the chamber volume. Final O_2 consumption rate was calculated using the equation above.

Measurements were performed on average once a week for each individual, starting from pupal formation until moth emergence for univoltine individuals and until 3 months later for prolonged diapause individuals. Values of O_2 consumption during pre-termination, termination and post-termination phases were averaged to calculate the metabolic activity. Metabolic activity during the post-termination emergence phase for univoltine individuals comprised the last measurement before emergence.

Statistical analysis

One-way analysis of variance (ANOVA) was used to test the significance of difference among phases for each type of development (univoltine and prolonged diapause) and for each method (direct calorimetry and O_2 consumption). For O_2 consumption, individuals measured in at least three sessions were included in the analyses. Data were log- or square root-transformed before the analyses if the data were not normally distributed and homoscedastic. All statistical analyses were performed using R, version 3.3.0 (R Core Team, 2017).

Results

Temporal variation of body temperature and oxygen consumption was obtained for univoltine individuals (body temperature, $n = 12$; O_2 consumption, $n = 30$), as well as for prolonged diapause individuals (body temperature, $n = 8$; O_2 consumption, $n = 19$), from pupation until 2 months after the emergence of univoltine individuals.

Univoltine individuals showed a synchronous spike in body temperature approximately 20 days before emergence, which was considered as the termination point of diapause (examples shown as dashed lines in Fig. 1A). The spike was followed by a progressive increase in body temperature, which was higher than that of prolonged diapause individuals until 1–2 days before emergence and, subsequently, the body temperature suddenly dropped. Individuals that did not emerge in the year of pupation (i.e. prolonged diapause) spiked synchronously with univoltine individuals, although their body temperature decreased progressively, returning to values slightly higher than those of the pre-termination phase. Minor synchronous spikes were observed during the post-termination period.

O_2 consumption, although discontinuous, varied greatly during the phases (Fig. 1B). When measurements started, oxygen consumption was very low and gradually increased until a sudden change of slope, around June 10, for both univoltine and prolonged diapause individuals. After the emergence of univoltine individuals, the prolonged diapause pupae decreased their O_2 consumption to the level observed during the pre-termination phase. When the prolonged diapause individuals emerged in the subsequent year(s), their values of body temperature and oxygen consumption were similar to those of univoltine individuals (data not shown).

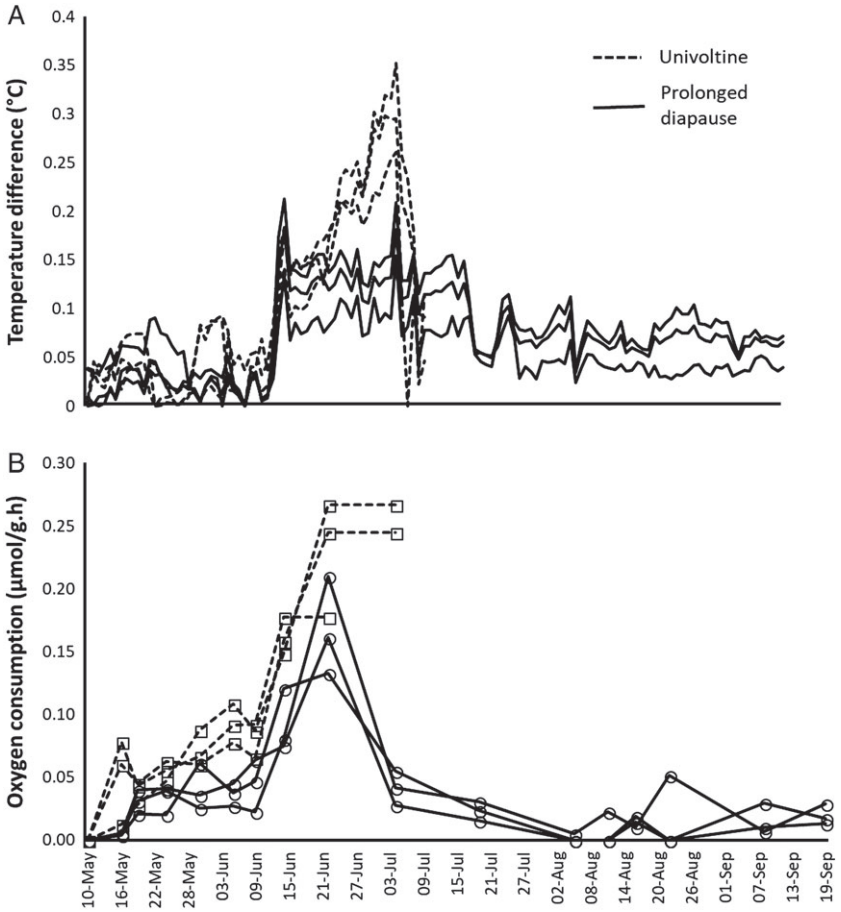

Fig. 1. Variation of body temperature (A) and O_2 consumption (B) in univoltine (dashed) and prolonged diapause (solid) example individuals.

Identification of phases for each individual allowed a comparison of the values of metabolic proxies for univoltine and prolonged diapause individuals. In univoltine individuals (Fig. 2), body temperature and O_2 consumption increased significantly from pre-termination to termination and post-termination, resulting in moth emergence (ANOVA, $F_{2,33} = 16.35$, $P < 0.001$ and $F_{2,87} = 106.50$, $P < 0.001$, respectively). In prolonged diapause individuals (Fig. 3), both body temperature and O_2 consumption increased significantly from pre-termination to termination but then declined in a different manner (ANOVA, $F_{2,21} = 28.07$, $P < 0.001$ and $F_{2,54} = 47.56$, $P < 0.001$, respectively). Body temperature remained at an intermediate level between those observed in pre-termination and termination, whereas oxygen consumption returned to the values observed in the pre-termination period.

Discussion

By adopting a novel approach for measuring pupal metabolic activity continuously via thermocouple-based direct calorimetry, the present study identifies the termination phase of diapause in both univoltine and prolonged diapause individuals, as confirmed by oxygen consumption. The discovery of a distinct termination phase concurs with the study by Démolin (1990), who refers to this as the 'key period' based on a destructive method of dissecting prolonged diapause individuals, as well as the finding of the corpus luteum or yellow body in the ovaries, as a likely remnant of the attempt to resume development (Biliotti *et al.*, 1964). Unfortunately, no precise documentation of those observations is available. A nondestructive, continuous method of monitoring metabolic activity is preferable to confirm the phases of diapause development because dissection cannot determine the future fate of dissected individuals that otherwise could have stayed in prolonged diapause.

Although the use of thermocouples in different biological fields is well established (Kuusik *et al.*, 1994; Harak *et al.*, 1998; Crosthwaite *et al.*, 2011; Hanson & Venette, 2013), a method for detecting phases of diapause development with thermocouples is currently unavailable. However, Jõgar *et al.* (2005) report the use of thermocouples in a study investigating the physiological characteristics of the pupae of *Pieris brassicae* during the initial diapause stage characterized by a maximum suppression of metabolic rate. The measuring principle of direct calorimetry with thermocouples is straightforward. Furthermore, it takes both aerobic and anaerobic metabolism into account (Lighton, 2008), differently from O_2 measurement. In the present study,

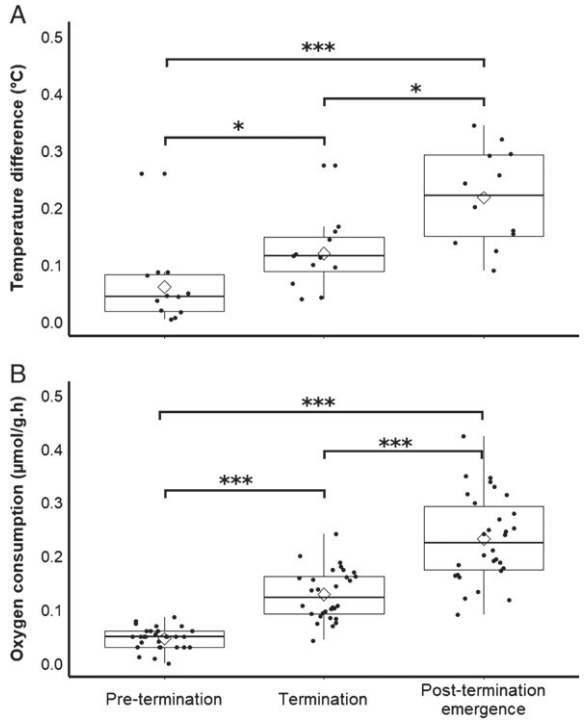

Fig. 2. Body temperature (A) and O_2 consumption (B) of univoltine pupae during the pre-termination, termination and post-termination (emergence) phases. Boxplots represent minimum, first quartile, median, third quartile and maximum values. Open diamond shapes in boxplots indicate the mean of observed values represented by jittered black dots. Asterisks representing significance levels in the post-hoc test: ***$P < 0.001$; *$0.01 < P < 0.05$.

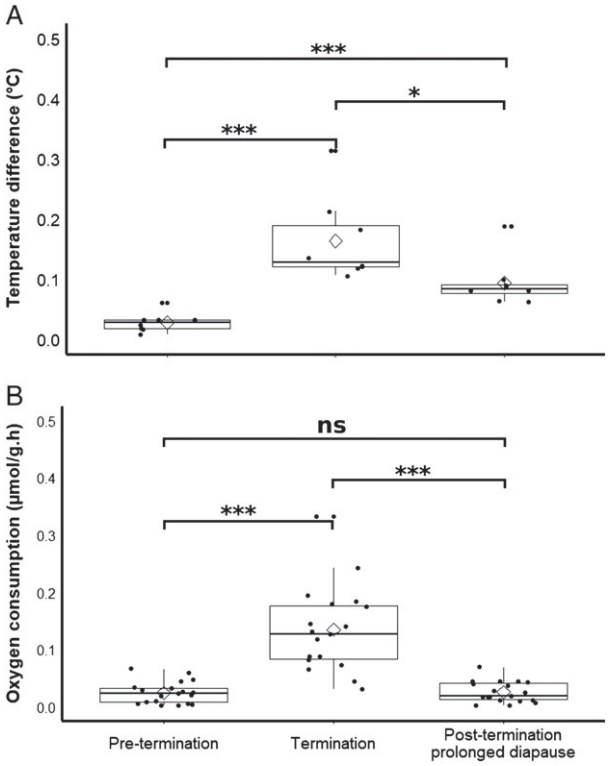

Fig. 3. Body temperature (A) and O_2 consumption (B) of pupae in prolonged diapause during the pre-termination, termination and post-termination (prolonged diapause) phases. Boxplots represent minimum, first quartile, median, third quartile and maximum values. Open diamond shapes in boxplots delineate the mean of observed values represented by jittered black dots. Asterisks representing significance levels in the post-hoc test: ***$P < 0.001$; *$0.01 < P < 0.05$; not significant (ns) $P > 0.05$.

thermocouples also detect the precise timing of emergence, as shown by the typical drop in body temperature that occurs 1–2 days before emergence, probably as a result of of water evaporation associated with the breaking of the pupal case and consequent psychrometric depression.

Nevertheless, the results obtained by monitoring temperature with thermocouples are not always clear and measurements are sometimes variable. Pupae often wriggle their abdomen during diapause, resulting in a different position of contact between the thermocouple and the pupal integument during the measurement session. Coupled with this source of noise, the maintenance of stable surrounding temperatures is difficult because the differences associated with changes in the pupal metabolic activity are small (0.05–0.1 °C). If a homogeneous temperature could be maintained throughout the experimental set-up, the difference among phases would be more significant and clearly detectable. Improving this method or finding better solutions to reduce variability could potentially comprise a future project for use both in the laboratory and field.

Although the metabolic activity monitored by O_2 consumption was discontinuous, it confirms the results of direct calorimetry, especially for univoltine individuals and for prolonged diapause individuals during the pre-termination and termination phases. During post-termination, however, the temperature is higher than in pre-termination, whereas oxygen consumption is the same, indicating that pupae are releasing heat with an apparently low uptake of oxygen. A likely explanation for this inconsistency relies on the fact that temperature is measured continuously, whereas oxygen consumption is measured relatively punctually at intervals of 5–15 days. There is evidence that pupae of some Lepidoptera may show discontinuous gas-exchange cycles (Hetz, 2007), such as by opening the spiracles for gas exchange at large intervals of hours. Therefore, it cannot be excluded that, during our 30-min measurement, little or no O_2 is used from the chamber because the spiracles are closed and pupae are using the O_2 contained in their tracheal system. This can be considered as a limitation of our method, which could be overcome by more frequent O_2 measurement or longer assays to minimize the bias. Alternatively, real-time flow-through respirometry at high accuracy would be necessary to determine whether diapausing pupae of the pine processionary moth exhibit discontinuous gas-exchange cycles.

The maintenance of some metabolic activity during the post-termination phase of prolonged diapause individuals goes against the expectation of a return to values of the pre-termination period. Interestingly, maintenance of metabolic activity during post-termination is also observed under field conditions during autumn and winter (A. Battisti & M.H.R. Salman, unpublished data). Maintaining metabolism in the first

2–3 months after prolonged diapause has started could prove to be important for pupal performance because, during this period, they are exposed to the hot and dry conditions of the summer in the Mediterranean region (Halperin, 1969; Markalas, 1989; Halperin, 1990). Being metabolically active could allow pupae to be balanced in water retention and thermoregulation (Acar *et al.*, 2001) under harsh conditions.

Synchronization of diapause termination among individuals exposed to the same cabinet conditions, regardless of their cohort of origin, is observed for both univoltine and prolonged diapause individuals. Because the pine processionary moth is a gregarious insect, with individuals from one procession clustering within a pupation site in the soil (Roques, 2015), it could be speculated that a communication system operates at short range within the pupal stage. Our experimental set-up does not exclude this possibility because pupae could communicate through acoustic or chemical cues. Alternatively, it might be hypothesized that the response to external factors (e.g. fluctuation of temperature) is synchronous or that a cohort-specific clock drives the process. These alternatives need to be studied in further experiments.

In summary, in the present study, three phases of diapause development are identified in the pupae of the pine processionary moth. However, it might be possible to distinguish other sub-phases if more care is taken with the study design and data collection. The methods employed to detect metabolic activity in the present study could be coupled with modern technology, such as the use of microcomputed tomography, enabling the detection of morphological changes in the development of ovaria, as is reported by Lowe *et al.* (2013) in a lepidopteran pupa, or the use of a precision infra-red camera (Prashar & Jones, 2014). Although eco-physiological phases are not standardized throughout insect taxa, and different insects may show differences in diapause development depending on their life history and environmental conditions, the present study represents a starting point for understanding diapause development in the pupae of an economically important insect pest.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

File S1. The file Supplementary material.doc contains the R codes used for analysis of the slope for O_2 consumption.

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