Anther meiosis in sweet cherry is constrained by the chilling and forcing phases of dormancy

Running head: Dormancy and anther meiosis in sweet cherry

Erica Fadón, Sara Herrera, María Herrero and Javier Rodrigo

a INRES – Gartenbauwissenschaft, Universität Bonn, Bonn, Germany.
b Unidad de Hortofruticultura, Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA), Zaragoza, Spain.
c Departamento de Pomología, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas (EEAD – CSIC), Zaragoza, Spain.
d Instituto Agroalimentario de Aragón – IA2 (CITA – Universidad de Zaragoza), Zaragoza, Spain.

*Corresponding author: E-mail address: efadonad@uni-bonn.de (E. Fadón)

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Abstract

Anther meiosis in temperate fruit trees occurs once a year synchronized with the seasons. The alternation of dormant and growth cycles determines the optimum moment for the male gametophyte formation, a sensitive process to both cold and warm temperatures. This ensures pollen viability and subsequent reproduction success that guarantee fruit production. In this work, we explore how anther meiosis frame in seasonality in sweet cherry. For this purpose, the dormant phases, anther meiosis, and blooming dates were established in four cultivars with different flowering dates and chilling requirements over seven seasons. The chilling and heat requirements for each cultivar were empirically estimated, and chilling and heat temperatures were quantified according to the Dynamic and Growing Degree Hours (GDH) models respectively. Endodormancy was overcome approximately a fortnight earlier during the colder winters than during the milder winters. Against our initial hypothesis, these differences were not clearly reflected in the time of anther meiosis. The period between chilling fulfilment and meiosis lasted several weeks in which a high amount of GDH accumulated. Results showed that male meiosis is conditioned by endodormancy but especially by warm temperatures during the forcing period. This differs from that described in other related species and opens a frame for further studies to understand the strategies of synchronizing dormancy with seasons.
Introduction

Temperate fruit trees have evolved a perennial life strategy by synchronizing with the most suitable seasons for growth and becoming dormant in winter (Cooke et al. 2012; Rohde and Bhalerao 2007). Annual temperature variations and photoperiods regulate the dormancy and growth cycles (Heide and Prestrud 2005, Basler and Körner 2014) and influence the optimum moment for processes such as sexual reproduction and subsequent fruit development (Heide and Prestrud 2005; Kurokura et al. 2013). The response to temperature varies depending on the species and cultivar, and it affects adaptations to certain climatic areas, as well as the blooming and ripening periods (Horvath et al. 2003). Despite the importance of these features for fruit production, phenology prediction based on temperatures remains a challenge, as there are numerous uncertainties behind dormancy and temperature’s effect on phenology.

Dormancy presents three different phases: para-, endo- and eco-dormancy (Lang et al. 1987). In this work, we focused on endo- and eco-dormancy that prevent growth in winter, while paradormancy refers to growth suppression imposed in formed buds by other tree structures (e.g., apical dominance). During endodormancy, reproductive and vegetative meristems remain without apparent growth and protected inside the buds (Cooke et al. 2012; Hamilton et al. 2016; Rohde and Bhalerao 2007), and growth cannot be triggered by forcing temperatures (Lang et al. 1987). The recovery of growth capacity requires a certain period of time under low temperature conditions (Knight 1801), i.e., chilling (Coville 1920). A period of chilling does not cause an immediate resumption of growth, but a phase of ecodormancy when exposure to forcing temperatures is necessary for the initiation of blooming. Despite the importance of distinguishing these two phases, there is a lack of phenological or biological markers for the fulfilment of chilling. Dormancy is a complex
mechanism that results from the interaction of numerous physiological processes (Fadón et al. 2020a): hormonal regulation (Rinne et al. 1994), reserves (Fadón, Herrero, et al. 2018, Fernandez et al. 2019), cycles of communication at cellular (Rinne et al. 2001, 2011) and vessel levels (Evert and Derr 1967, Aloni and Peterson 1997), gene regulation (Ito et al. 2015; Saito et al. 2013; Yamane et al. 2011) and epigenetics (Rothkegel et al. 2017). However, further efforts are needed towards the finding of a reliable indicator for dormancy release (Fadón and Rodrigo 2018).

Temperate fruit trees, such as Prunus sp., are good models for dormancy studies because their clonal origin ensures consistent genetic differences between cultivars. Numerous studies have taken advantage of this characteristic, which allows the study of identical genotypes within different places and years. Fruit growers and scientist face the lack of a reliable indicator for dormancy with the establishment of the temperature requirements. These allows the prediction of the adaptation of a given cultivar to a certain growing area. For this purpose, the dormant and forcing periods are determined experimentally by evaluating bud growth in shoots collected periodically throughout winter and transferred to forcing chambers with warmer temperatures. Knowing the date that chilling fulfilment is achieved allows the quantification of chilling and heating temperatures over the dormant and forcing periods.

Three temperature models are commonly used to quantify chilling during endodormancy. The Chilling Hours model considers that temperatures between 0 and 7.2 °C contribute to endodormancy release (Weinberger 1950). A step forward, the Utah model weights different ranges of temperatures based on their effect on endodormancy completion (Richardson et al. 1974). Finally, the Dynamic model simulates a process-based dormancy model, although the biological process behind has not been yet identified, and only based
on temperature data (Erez and Couvillon 1987, Fishman et al. 1987, Erez et al. 1990). After chilling fulfilment, forcing temperatures are usually quantified during ecodormancy by using the Growing Degree Hours model (Richardson et al. 1975). This combination of chilling and heat temperature models has been largely applied to different fruit tree species (Fadón et al. 2020b). However, in most cases they result in low accuracy with the tree behaviour, due to different factors, such as the interaction between the chilling and heat accumulation that is usually not considered, the models are not species-specific, and the results obtained with currently available methodologies under particular conditions should be taken with caution when applied to different climatic regions (Luedeling and Brown 2011).

The temperature requirements of particular cultivars are cumbersome to calculate and result in variable data depending on the location of the experiment (Measham et al. 2017) and the model used (Seeley 1994). This leads to a scarcity of reliable information on the optimum temperatures for blooming of commercial cultivars (Fadón et al. 2020b). The increasing number of new cultivars from fruit breeding programs, the expansion of fruit tree cultivation to new areas, and the changeable climate conditions caused by global warming are increasing the demand for a better comprehension of dormancy.

In most temperate fruit trees, flowering only lasts a few days in spring, but it is the result of a continuous process over several months, beginning at the end of summer, halting during winter once flower verticiles differentiate, then resuming growth and anthesis in spring (Fadón, Rodrigo et al. 2018; Saito et al. 2015). Recent studies in Prunus sp. explored how stamen development fits with seasonality, offering milestones that mark developmental processes and dormancy (Fadón et al. 2019; Julian et al. 2011). Pollen mother cells are present during dormancy in apricot (Prunus armeniaca L.) (Julian et al.
2011), sour cherry (*Prunus cerasus* L.) (Felker et al. 1983), peach (*Prunus persica* L.) (Reinoso et al. 2002), and sweet cherry (*Prunus avium* L.) (Fadón et al. 2019). Subsequent anther meiosis has been identified as one of the first processes after chilling fulfilment (Fadón et al. 2019; Julian et al. 2011). In peach, gene expression of microsporogenesis and pollen maturation are associated with dormancy release (Ríos et al. 2013). Then, the subsequent microsporogenesis phases coordinate with flower bud phenological stages until pollen grains mature to match with anthesis, producing blooming (Fadón et al. 2019). Male gametophyte formation is sensitive to both cold and warm temperatures, which highlights the importance of the process initiating at the optimal moment (Hedhly 2011). However, how temperatures influence and regulate stamen development in temperate fruit trees remain unclear.

In this work, we thoroughly examined anther meiosis in relation to endodormancy completion and blooming dates in four sweet cherry cultivars over seven years with different winter temperature regimes. The range of cultivars with different blooming periods and the large time-scale of experiments offer an assortment of conditions to answer the following research questions: (i) what are the chilling and forcing requirements for meiosis and blooming completion in sweet cherry? (ii) How does previous winter temperature affect the anther meiosis date? (iii) How does anther meiosis fit within the dormant and forcing periods? We offer a contextualized overview of the anther meiosis over the dormant phases. Integrating the previous information, we hypothesised that anther meiosis is influenced by seasonal temperatures and could provide key information on the dormancy stage and flowering dates.

**Material and methods**
Plant material

Four sweet cherry cultivars were selected according to their blooming dates: ‘Cristobalina’ (extra-early), ‘Bing’ (medium), ‘Burlat’ (medium), and ‘Hedelfinger’ (late) (Gella et al. 2001). Three trees per cultivar were used from a cultivar collection located at “Centro de Investigación y Tecnología Agroalimentaria de Aragón” (CITA) in Zaragoza (Spain) at 41°44’30" N, 0°47’00" W and 220 m above sea level.

Determination of endodormancy

To estimate the date at which endodormancy was broken, three shoots (15–30 cm in length and 5 mm in diameter) with at least 10 flower buds were randomly sampled every week, starting in autumn on the 30th November until the onset of budbreak at the end of February or early March, depending on years. Shoots were placed on water-soaked florist foam and maintained in a growth chamber at 22 ± 1 °C with a 12-hour light photoperiod for 7 days (Fadón et al., 2018). The experiments were performed for the four cultivars over two winter seasons (2016–2017, 2017–2018), except for ‘Hedelfinger’ with only one season available. To determine differences in bud growth, 10 flower buds were randomly picked and weighed on the first and last day in the growth chamber. The end date of endodormancy was considered to be when the flower bud weight increased by at least 30% (Supplementary Fig. 1) (Brown and Kotob 1957; Fadón and Rodrigo 2019). As shoots were sampled weekly, an interval of three days before and after the sampling date was established, quantifying chilling accumulation during this period. Chilling requirements of each cultivar was considered as the average value over the two years studied.

Estimation of chilling and forcing requirements
Temperatures were recorded hourly at a meteorological station located in the experimental orchard over seven years. Chilling was quantified from the first of September to the date of endodormancy completion, according to the Dynamic Model, that proposed the accumulation of Chilling Portions (CP) (Fishman et al. 1987) and is suitable for this area (Luedeling and Brown 2011). After chilling fulfilment, forcing requirements were quantified until anthesis using the Growing Degree Hours (GDH) model (Richardson et al. 1975).

**Anther meiosis characterisation**

Anther meiosis was characterised under the microscope for each cultivar over seven years (2011–2015 and 2017–2018). Ten flower buds per cultivar were randomly collected weekly in the field from early-January to mid-February, and then every two days up to anthesis. The flower buds were individually weighed and fixed in ethanol:acetic acid 3:1 (v/v). To examine meiosis, anthers were removed from three flower buds per collecting day with a scalpel. To identify the different phases of pollen meiosis, a new staining method was designed to simultaneously observe the chromatin structure and the layer of callose surrounding the pollen mother cells, by combining 0.25 µg/ml of DAPI in 0.05 M TRIS buffer (pH 7.2) to observe nuclei (Williams et al. 1999), and 0.1% aniline blue in 0.1 N K₃PO₄ to stain callose (Currier 1957). The anthers were stained with this solution during 3-5 min., mounted by squashing and observed under a UV epifluorescence DM2500 microscope (Leica Microsystems, Cambridge, UK) with a 340–380 bandpass and 425 longpass filter, equipped with a Leica DFC-310 digital camera (Leica Microsystems, Cambridge, UK).
Phenology characterisation

Flowering dates of the four cultivars were recorded over the seven years according to the BBCH phenological scale: stage 60 (first flowers open), stage 65 (full bloom) and stage 69 (end of flowering) (Fadón et al. 2015).

Statistical analyses

One-way ANOVA were performed for the weekly flower bud weight per cultivar and year. When ANOVA generated a significant $F$-value, mean separations were determined by Duncan’s multiple-range test. These analyses were performed using SPSS 12.0 statistical software (SPSS Inc., Chicago, IL, United States). Dates of chilling fulfilment, pollen meiosis, and full bloom in the four sweet cherry cultivars over seven years were analysed by Pearson’s correlation coefficients using the R programming environment (R Development Core and Team, 2018; version 3.5.1).

Results

Endodormancy determination and estimation of chilling requirements.

Endodormancy was experimentally determined for four sweet cherry cultivars over two consecutive winters (Supplemental Fig. 1). The quantification of CP until the end date of endodormancy allowed us to estimate the chilling requirements of each sweet cherry cultivar, which showed large differences: the extra-early blooming cultivar ‘Cristobalina’ required $32 \pm 4$ CP, the medium blooming cultivars ‘Burlat’ and ‘Bing’ required $38 \pm 4$ CP and $43 \pm 3$ CP, respectively, and the late blooming cultivar ‘Hedelfinger’ had $49 \pm 2$ CP requirement.
Temperatures were highly variable between years during dormancy: years 2011-12 and 2014-15 showed higher average temperatures, and 2012-13 showed lower average temperatures than the other years (Fig. 1 A). The quantification of chilling according to the Dynamic model, that considers the effective temperatures for chilling during endodormancy, allowed to detect two patterns of chilling accumulation (Fig. 1 B). Chilling portions rapidly accumulated over three years (2010–11, 2012–13 and 2016–17), which were considered cold winters; while the accumulation of CP occurred later in autumn and reached lower values for each date in the other four years (2011–12, 2013–14, 2014–15 and 2017–18), which were considered mild winters (Fig. 1 B). During the cold winters, ‘Cristobalina’ fulfilled its chilling requirements in early-December, ‘Burlat’ in mid-December, ‘Bing’ around the third week of December, and ‘Hedelfinger’ in early-January (Fig. 1 B). During the mild winters, chilling requirements were fulfilled in the third week of December for ‘Cristobalina’, in late-December for ‘Burlat’, in early-January for ‘Bing’, and in mid-January for ‘Hedelfinger’ (Fig. 1 B). Therefore, chilling fulfilment was achieved with a time lapse of approximately a fortnight between cold and mild winters for each cultivar (Fig. 1 B). These marked conditions throughout the years provided a good opportunity to explore anther meiosis and flowering dates according to chilling accumulation.

The triggering of anther meiosis in cold and mild winters

The first clear sign of the onset of anther meiosis was the callose layering around the pollen mother cells with a dense nucleus (Fig. 2 A). Then, chromatin changes associated with meiosis occurred inside the callose layer: cromatine condensed for chromosome formation during prophase I (Fig. 2 B), there was subsequent chromosome
alignment at metaphase I (Fig. 2 C) and the first meiotic division with the presence of two nuclei at telophase I (Fig. 2 D). DNA duplicated during prophase II (Fig. 2 E), and a second division occurred until telophase II with four nuclei (Fig. 2 F). Cytoplasm distribution occurred with the formation of inner callose layers until the characteristic tetrad formed (Fig. 2 G). Then callose was progressively degraded (Fig. 2 H) until microspore release (Fig. 2 I). From these observations, three main stages of development were chosen to compare pollen development between cultivars and years during the forcing period (Fig. 3):

1. The sporogenous tissue, representing the stage prior to meiosis (Fig. 2 A);
2. The presence of pollen mother cells surrounded by callose, indicating the occurrence of anther meiosis (Fig. 2 B-F);
3. The microgametogenesis that begins after meiosis, resulting in young microspores and the subsequent stages of pollen formation prior to anthesis (Fig. 2 I).

The sporogenous tissue was observed from early-January to February or March, depending on cultivars and years (Fig. 3). The cultivars with low chilling requirements underwent meiosis earlier than those with high chilling requirements. The dates of meiosis ranged widely from the end of January to mid-February for the cultivar ‘Cristobalina’ (Fig. 3 A) and were less variable between years for the other cultivars: ‘Burlat’ (Fig. 3 B), ‘Bing’ (Fig. 3 C) and ‘Hedelfinger’ (Fig. 3 D). The blooming dates occurred in March for ‘Cristobalina’ (Fig. 3 A), between the mid-March and mid-April for ‘Burlat’ (Fig. 3 B) and ‘Bing’ (Fig. 3 C), and during the first half of April for ‘Hedelfinger’ (Fig. 3 D).

The variation in meiosis dates between years appeared to be more related to flowering (Pearson’s $r = 0.92$, $P$-value $< 0.01$) than with chilling fulfilment dates (Pearson’s $r = 0.75$, $P$-value $< 0.01$) when data from all cultivars were considered. However, the dates of chilling fulfilment, meiosis and flowering did not correlate when the
analysis was performed per cultivar (Table 1). The long time lapse and the mismatch observed between endodormancy completion and anther meiosis prompted us to explore the forcing conditions after chilling accumulation.

Meiosis and flowering related to forcing temperatures

The forcing period, from chilling fulfilment to bloom, lasted from 85 ± 7 days for ‘Hedelfinger’ to 97 ± 8 days for ‘Burlat’ (Table 1). The quantification of GDH from the end date of endodormancy to full bloom allowed us to estimate the forcing requirements of the cultivars, which were 6954 ± 675 GDH for ‘Cristobalina’, 9345 ± 834 GDH for ‘Burlat’, 7153 ± 915 GDH for ‘Bing’ and 7762 ± 882 GDH for ‘Hedelfinger’ (Table 2). These values presented no differences considering cold and mild years, however the dispersion of these data was mainly caused by the cold years that showed a higher variance (7 – 16 %) that the mild years with lower values (3 – 7 %) (Table 2). The forcing period (days) and requirements (GDH) were similar among these cultivars despite its different chilling requirements and blooming dates (Tables 1 and 2).

The GDH accumulated at different rates depending on the year and cultivar (Fig. 4). These differences were more accentuated when early chilling fulfilment occurred, i.e. during the cold winters (Fig. 4 A, C, E and G) and for cv. ‘Cristobalina’, which showed early dates of chilling fulfilment in both cold and mild winters (Fig. 4 A and B). In these cases, resulted in greater variability in GDH accumulation until meiosis and flowering dates within each cultivar (Fig. 4 A, B, C, E and G). In contrast, GDH accumulation after milder winters showed less variability between years, resulting in similar dates of meiosis and blooming, as occurred with mid-chilling cultivars ‘Burlat’ and ‘Bing’ and the high-chilling cultivar ‘Hedelfinger’ (Fig. 4 D, F and H).
The comparison of the GDH accumulation after chilling fulfilment (Fig. 4) with the pattern of bud growth (Fig. 5) shows that the flower buds maintained a constant weight (about 0.04 g/bud) despite the forcing temperature accumulation in this phase. This period slightly varied, depending on cultivars and years, ranging from between 56 ± 9 and 72 ± 7 days (for ‘Cristobalina’ and ‘Burlat’, respectively) (Table 1), differences were not associated with the flowering dates of the cultivar, i.e. ‘Cristobalina’ (extra-early flowering blooming period) and ‘Hedelfinger’ (late blooming period) showed similar periods (56±9 and 63± day respectively). Once anther meiosis occurred, the flower buds resumed growth as GDH accumulated. Flower bud weight increased significantly coinciding with anther meiosis or up to two weeks later (Fig. 5). After meiosis, a period ranging from 31 ± 6 to 23 ± 2 days (for ‘Cristobalina’ and ‘Hedelfinger’, respectively) occurred until full bloom (Table 1), during which the buds phenologically grew and developed.

Discussion

Chilling and forcing requirements in sweet cherry

The combination of shoot experiments with chilling quantification have been widely used for endodormancy determination in temperate fruit trees (Fadón and Rodrigo 2018, Fadón et al. 2020b). The use of this approach for this study produced a range of chilling requirements between 32 ± 4 and 49 ± 2 CP, for ‘Cristobalina’ and ‘Hedelfinger’ respectively. These results agree with previous studies in sweet cherry (Alburquerque et al. 2008; Fadón, Herrero et al. 2018, Fadón, Rodrigo et al. 2018; Campoy et al. 2019) and are similar to those reported in apricot (Campoy et al. 2012; Ruiz and Egea 2007) and are similar to those reported in apricot (Campoy et al. 2012; Ruiz and Egea 2007) and are similar to those reported in apricot (Campoy et al. 2012; Ruiz and Egea 2007) and are similar to those reported in apricot (Campoy et al. 2012; Ruiz and Egea 2007) and are similar to those reported in apricot (Campoy et al. 2012; Ruiz and Egea 2007). Although, other Prunus sp., such as almond (Prunus dulcis (Mill.) D.A. Webb) (Sánchez-Pérez et al.

Once the chilling requirements were fulfilled, the forcing requirements ranged between 6969 ± 728 and 9464 ± 894 GDH for ‘Cristobalina’ and ‘Burlat’ respectively. Heat requirements in sweet cherry are much higher than those reported for peach (Richardson et al. 1975), apricot (Campoy et al. 2012; Ruiz et al. 2007), or almond (Egea et al. 2003). The fact that sweet cherry bloomed significantly later than other related species is, probably, mainly due to the differences in the forcing requirements rather than in the chilling requirements.

The extra-early blooming cultivar ‘Cristobalina’ provides a special focus for comparison. This cultivar has lower chilling requirements than the other sweet cherry cultivars, although showing similar heat requirements. It requires a chilling accumulation similar to that of the apricot cultivar ‘Currot’, which blooms about a month earlier due to its low forcing requirements (Ruiz et al. 2007). However, ‘Cristobalina’ blooms on dates similar to apricot cultivar ‘Murciana’, which requires higher chilling and lower GDH accumulation than ‘Cristobalina’ (Ruiz et al. 2007). These comparisons highlight the long forcing periods required for sweet cherry to bloom, that are similar for both early and late blooming cultivars. In other *Prunus* species, such as apricot (Julian et al. 2014; Ruiz et al. 2007) and almond, the dormant phase has been reported to be the main factor determining flowering time (Egea et al. 2003). While in sweet cherry blooming dates should be determined by the chilling requirements and a long period of heat accumulation.

The procedure followed in this study has been widely used to calculate the temperature requirements, however it is currently under question. Light variations in the methodology to establish endo- and eco-dormancy periods may lead to very different
results (Campoy et al. 2019; Fadón et al. 2020b), and temperature models present low adaptability to the range of climates for temperate fruit production (Luedeling et al. 2011).

Meiosis in anthers and winter temperatures

Endodormancy and anther meiosis were separated by a period of about a month and a half in the four cultivars studied, even the extra-early blooming cultivar ‘Cristobalina’. Meiosis has been reported to be one of the first events occurring after endodormancy (Fadón et al. 2019; Julian et al. 2011), but results herein highlight the fact that significant forcing temperature accumulation also occurred before growth restoration in sweet cherry. Far from having a standard pattern, the triggering of anther meiosis differs among species, occurring prior to dormancy establishment in species such as hazelnut (*Corylus avellana* L.) (Tiyayon and Azarenko 2005), *Rhododendron* sp. (Mirgorodskaya et al. 2015) and *Camellia japonica* (Zhang et al. 2017) and closely after dormancy in apricot (Julian et al. 2011) and peach (Ríos et al. 2013). Our results indicate that anther meiosis in sweet cherry occurs well after dormancy.

The influence of the chilling accumulation on the triggering of anther meiosis was evaluated under mild and cold winter conditions. While a low variation in GDH accumulation was found after the mild winters, in cold winter years, in which chilling requirements were fulfilled early, wider variations were found, which can be explained by the unsuitability of the GDH model to predict the forcing phase or the counterbalance between the chilling and heat temperatures (Fernandez et al. 2020). The high heat requirements reveal a greater influence of the forcing conditions than described for apricot, where anther meiosis is mostly determined by the date of chilling fulfilment (Julian et al. 2014). The high amount of GDH quantified between the end of endodormancy and meiosis
highlights the importance of this phase in sweet cherry, although the lack of studies in other species hampers the completion of a comparative framework.

Anther meiosis splits the forcing period into two phases

Results herein frame anther meiosis by establishing two different phases during the forcing period. The first phase occurs from chilling fulfilment until anther meiosis, which corresponds to quiescence (Considine and Considine 2016) or ecodormancy (Lang et al. 1987), in which the flowers buds do not grow despite the GDH accumulation and still are in the phenological stage BBCH 50, dormant buds (Fadón et al. 2015). In this stage, the flower primordia are present inside the buds with all verticiles differentiated (Fadón, Rodrigo, et al. 2018), including anthers showing sporogenous tissue (Fadón et al. 2019). Anther meiosis occurs just prior to the flower bud weight increment (phenological stage BBCH 51), preluding the second phase, in which the buds show active growth until full bloom (phenological stage BBCH 59) (Fadón et al. 2015), while microgametogenesis occurs inside the anthers (Fadón et al. 2019).

These two states within the forcing phase represent different sensitivities to freezing temperatures. During the ecodormancy or quiescence phase, the buds are acclimated to resist low temperatures. Subsequent active growth resumption implies deacclimation and frost sensitivity (Julian et al. 2007; Yamane et al. 2006). This work showed that GDH accumulation between chilling fulfilment and meiosis resulted in highly variable ecodormancy or quiescence phases between the cold and milder winters, in contrast to the consistent GDH accumulation that resulted from anther meiosis to full bloom. The early concept of heat units was originally designed for growing green plants (Reaumur 1735); however, it is commonly applied along the entire forcing period, even though the trees do
not show growth during the ecodormancy or quiescence phase (Richardson et al. 1975). Further studies are needed to explore the responses to temperatures that occur in temperate trees during the forcing period.

One of the most important implications of the lack of a rapid response to GDH accumulation after endodormancy, is the complexity of managing sweet cherry orchards in comparison with other fruit species. This is especially true in new cultivating regions that lack historical information (George and Erez 2000), since it is impossible to establish whether enough chilling was accumulated until several weeks later.

Conclusion

This study shows that the anther meiosis and blooming dates do not directly reflect the annual variability of endodormancy completion, since they are highly influenced by temperatures during the forcing period. The forcing period is longer and plays a more decisive role in blooming occurrence in sweet cherry than in other cultivated Prunus sp. By relating the dates of the anther meiosis with the heat accumulation and the pattern of bud growth, the existence of two different phases was evidenced: cold-acclimated buds do not grow until anther meiosis occurrence, then buds grow and deacclimate, becoming sensitive to frosts. This raises the question of the suitability of the GDH model in these two different periods, and invites further research to deepen in this topic. This work offers a comprehensive partitioning of dormancy, proposing the anther meiosis as an important developmental milestone in the forcing period, which would be worth to explore this fact in other species and environmental conditions. On that basis, further work on biology and modelling would be needed to build knowledge on dormancy.
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Figure legends

Figure 1. Temperature trends and chilling accumulation over seven years. (A) Weekly average temperature (thin line) and trendline (second order polynomial; thick line), from October to January. (B) Chilling portion (CP) accumulation and dates of chilling fulfilment (CF) for each year in four sweet cherry cultivars.

Figure 2. Microsporogenesis in sweet cherry. (A) Early-stage pollen mother cells surrounded by callose layering. (B) Prophase I, (C) Metaphase I, (D) Telophase I, (E) Prophase II, (F) Telophase II, (G) Tetrad. (H) Callose degradation, early microspore released. (I) Microspores released. Squashed anthers double stained with Aniline Blue and DAPI. Scale bars: 20 µm. ca callose, chr chromatine, mi microspores, and arrows indicate the nuclei.

Figure 3. Stages of pollen development during the forcing period in four sweet cherry cultivars over three cold years and four mild years. (A) ‘Cristobalina’, (B) ‘Burlat’, (C) ‘Bing’, and (D) ‘Hedelfinger’. CF: chilling fulfilment.

Figure 4. Growing Degree Hours (GDH) accumulation from chilling fulfilment (CF) to anther meiosis and blooming over three cold winters and four mild winters for four sweet cherry cultivars. ‘Cristobalina’, (A) cold and (B) mild winters. ‘Burlat’, (C) cold and (D) mild winters. ‘Bing’, (E) cold and (F) mild winters. ‘Hedelfinger’, (G) cold and (H) mild winters.

Figure 5. Flower bud growth from dormancy to bud burst over three cold winters and four mild winters in four sweet cherry cultivars. ‘Cristobalina’, (A) cold and (B) mild winters. ‘Burlat’, (C) cold and (D) mild winters. ‘Bing’, (E) cold and (F) mild winters. ‘Hedelfinger’, (G) cold, and (H) mild winters.
Supplementary Figure 1. Dormancy estimation in four sweet cherry cultivars over two winters: 2016 – 2017 (grey) and 2017 – 2018 (black). Chilling fulfilment (snow flakes) considered as an increment on flower bud weight of at least 30% (horizontal line) after 7 days in the growth chamber. (A) ‘Cristobalina’, (B) ‘Burlat’, (C) ‘Bing’, and (D) ‘Hedelfinger’.

Table 1. Pearson’s correlation coefficients and time intervals (days) between the dates of chilling fulfilment, meiosis, full bloom, and budburst in four sweet cherry cultivars over seven years.

Table 2. Heat accumulation (GDH) between the dates of chilling fulfilment, meiosis, and full bloom in four sweet cherry cultivars over seven years.
Cristobalina (32 ± 4 CP)

Burlat (38 ± 4 CP)

Bing (43 ± 3 CP)

Hedelfinger (49 ± 2 CP)

Δ weight (%)
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<th>Days correlations</th>
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<td>From CF to meiosis</td>
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<td>From meiosis to BG (interval)</td>
<td>- 0 – 11 days 0 - 11 days 5 – 11 days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>From CF to FB</td>
<td>0.33 n.s.</td>
<td>97±8 9 91±4 4</td>
<td>103±11 10 91±4 4</td>
<td></td>
</tr>
<tr>
<td>From CF to meiosis</td>
<td>0.45 n.s.</td>
<td>72±7 4 68±3 4</td>
<td>79±5 7 68±3 4</td>
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</tr>
<tr>
<td>From meiosis to FB</td>
<td>0.83*</td>
<td>25±4 17 26±4 15</td>
<td>24±6 23 26±4 15</td>
<td></td>
</tr>
<tr>
<td>From meiosis to BG (interval)</td>
<td>- 0 – 6 days 0 – 9 days 0 – 4 days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>From CF to FB</td>
<td>0.50 n.s.</td>
<td>90±7 8 86±3 3</td>
<td>95±8 9 86±3 3</td>
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<tr>
<td>From CF to meiosis</td>
<td>-0.44 n.s.</td>
<td>65±6 9 61±2 4</td>
<td>71±1 6 61±2 4</td>
<td></td>
</tr>
<tr>
<td>From meiosis to FB</td>
<td>-0.17 n.s.</td>
<td>24±5 19 25±4 16</td>
<td>25±4 26 25±4 16</td>
<td></td>
</tr>
<tr>
<td>From meiosis to BG (interval)</td>
<td>- 0 – 6 days 0 – 6 days 3 - 6 days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>From CF to FB</td>
<td>0.24 n.s.</td>
<td>85±7 8 82±3 3</td>
<td>95±1 1 82±3 3</td>
<td></td>
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<tr>
<td>From CF to meiosis</td>
<td>0.73 n.s.</td>
<td>63±6 9 59±1 2</td>
<td>71±1 1 59±1 2</td>
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<tr>
<td>From meiosis to FB</td>
<td>0.54 n.s.</td>
<td>23±2 11 23±3 12</td>
<td>25±4 14 23±3 12</td>
<td></td>
</tr>
<tr>
<td>From meiosis to BG (interval)</td>
<td>- 0 – 4 days 1 - 4 days 0 days</td>
<td></td>
<td></td>
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</tbody>
</table>

CF chilling fulfilment; FB full bloom; BG bud growth.
<table>
<thead>
<tr>
<th>GDH accumulation</th>
<th>all years</th>
<th>cold years</th>
<th>mild years</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>mean±sd</td>
<td>CV</td>
<td>mean±sd</td>
</tr>
<tr>
<td>CRIST. From CF to FB (Heat requirements)</td>
<td>6969±728</td>
<td>10</td>
<td>6891±1114</td>
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<tr>
<td>From CF to meiosis</td>
<td>3553±593</td>
<td>10</td>
<td>3709±902</td>
</tr>
<tr>
<td>From meiosis to FB</td>
<td>3416±340</td>
<td>10</td>
<td>3182±298</td>
</tr>
<tr>
<td>BURLAT From CF to FB (Heat requirements)</td>
<td>9464±894</td>
<td>9</td>
<td>9441±1501</td>
</tr>
<tr>
<td>From CF to meiosis</td>
<td>5599±734</td>
<td>13</td>
<td>5659±1242</td>
</tr>
<tr>
<td>From meiosis to FB</td>
<td>3865±437</td>
<td>11</td>
<td>3782±651</td>
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<tr>
<td>BING From CF to FB (Heat requirements)</td>
<td>8769±768</td>
<td>9</td>
<td>8759±1243</td>
</tr>
<tr>
<td>From CF to meiosis</td>
<td>5059±471</td>
<td>9</td>
<td>5078±614</td>
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<tr>
<td>From meiosis to FB</td>
<td>3710±436</td>
<td>12</td>
<td>3681±634</td>
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<tr>
<td>HEDEL From CF to FB (Heat requirements)</td>
<td>9528±402</td>
<td>4</td>
<td>9778±660</td>
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<tr>
<td>From CF to meiosis</td>
<td>5600±458</td>
<td>8</td>
<td>5618±807</td>
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<tr>
<td>From meiosis to FB</td>
<td>3928±235</td>
<td>6</td>
<td>4159±147</td>
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</tbody>
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CF chilling fulfilment; FB full bloom