Variation in thermal sensitivity of diapause development among individuals and over time drives life history timing patterns in an insect pest

Short title: Thermal sensitivity of insect diapause

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Key words: physiological time, thermal performance curve, dormancy, winter, *Rhagoletis pomonella*, apple maggot

Abstract

Physiological time is important for understanding the development and seasonal timing of ectothermic animals, but has largely been applied to developmental processes that occur during spring and summer such as morphogenesis. There is a substantial knowledge gap in the relationship between temperature and development during winter, a season that is increasingly impacted by climate change. Most temperate insects overwinter in diapause, a developmental process with little obvious morphological change. We used principles from the physiological time literature to measure and model the thermal sensitivity of diapause development rate in the apple maggot fly *Rhagoletis pomonella*, a univoltine fly whose diapause duration varies substantially within and among populations. We showed that diapause duration could be modelled with simple linear relationships between diapause development rate and temperature. Low temperatures were necessary for most individuals to complete diapause, similar to many insects. However, we also found evidence for an ontogenetic shift in the thermal sensitivity of diapause: diapause development proceeded more quickly at high temperatures later in diapause and in the absence of quiescence, a phenomenon not previously reported. Increasingly warmer temperatures during and after winter may impact the phenology of this and other insects with temperature-sensitive diapause, impacting their viability.

Introduction

Physiological time is an important concept for understanding the development and seasonal timing of ectothermic animals (Taylor 1981; Buckley 2022). The cellular and physiological processes that underlie morphogenesis (development of an animal's body size, shape and structure) are temperature-dependent (Kipyatkov and Lopatina 2010; Shi et al. 2011; Damos and Savopoulou-Soultani 2012). The vast majority of animals on our planet are ectothermic, so the chronological time (i.e., number of hours/days) required to reach a developmental landmark depends on environmental temperature. Most natural environments exhibit substantial temperature variation; chronological time is thus a poor metric of life history timing (van Straalen 1983; Trudgill et al. 2005; Rebaudo and Rabhi 2018). Rather, ectothermic development is usually described in physiological time, conceptualized by the relationship between development rate (governed by physiological processes) and environmental temperature (Taylor 1981; van Straalen 1983). Environmental temperature variability, duration of temperature exposure, and ontogeny can complicate the calculation of physiological time (Kingsolver and Woods 2016; Sinclair et al. 2016; Buckley 2022), but physiological time models have been well-characterized in insects and can facilitate predictions of population dynamics during the growing season – i.e., during spring, summer, and fall (Powell and Bentz 2009; Régnière et al. 2012; Sridhar and Reddy 2013; Scranton and Amarasekare 2017). However, we have comparatively little insight into whether and how the same concept of physiological time applies to developmental processes that occur during winter. The relative dearth of information on the relationship between temperature and development during winter represents a substantial knowledge gap, especially if we want to understand the consequences of climate warming on insect phenology (Marshall et al. 2020; Buckley 2022).

Most temperate insects overwinter in diapause (Fig. 1 – green and yellow), a dormant, stress-tolerant state that delays growth, morphogenesis, and reproduction until conditions become permissive in spring or summer (Tauber and Tauber 1976; Hand et al. 2016; Wilsterman et al. 2021). Many insects spend the bulk of their life in diapause, either the majority of a calendar year (Fig. 1A) or sometimes multiple years (Tauber et al. 1986; Hanski 1988; Hahn and Denlinger 2011; Moraiti et al. 2014; Dupuis et al. 2016). If diapause ends during winter, the insect often enters another form of dormancy called 'quiescence' – a lack of morphogenesis due to non-permissive environmental conditions (Fig. 1B – dark blue; Jenkins et al. 2001; Hodek 2002; Koštál 2006). Unlike diapause, where dormancy is maintained during transient or early exposure to permissive environments, quiescent individuals are not recalcitrant and will immediately resume post-diapause processes such as growth and morphogenesis when conditions become permissive. Diapause and post-diapause quiescence can synchronize individuals to have similar life history timing, facilitating mate finding and supporting reproductive output (Tauber and Tauber 1976; Hand et al. 2016; Wilsterman et al. 2021). Given the prevalence of diapause in overwintering insects, any attempts to apply physiological time to overwintering development must consider this important process.

To expand the concept of physiological time to diapause, we must first establish that diapause is indeed a form of development. In contrast to growth and morphogenesis (Fig. 1 - gray) during warmer or more permissive periods of the year, there is often no obvious morphological change (morphogenesis) during diapause (but see Shingleton et al. 2003), leading some authors to prefer alternatives to the term 'diapause development' (Hodek 1996). However,

diapause is clearly a dynamic developmental process, where ‘development’ may primarily consist of progressive or cyclic changes in the molecular cellular milieu (Andrewartha 1952; Hodek 1996; Koštál 2006), including differential expression of often thousands of genes over time (Koštál et al. 2017; Dowle et al. 2020; Pruisscher et al. 2022). Indeed, diapause can have distinct phases (e.g., initiation, maintenance, termination; cf. Koštál 2006), each of which must complete before post-diapause processes such as morphogenesis can resume.

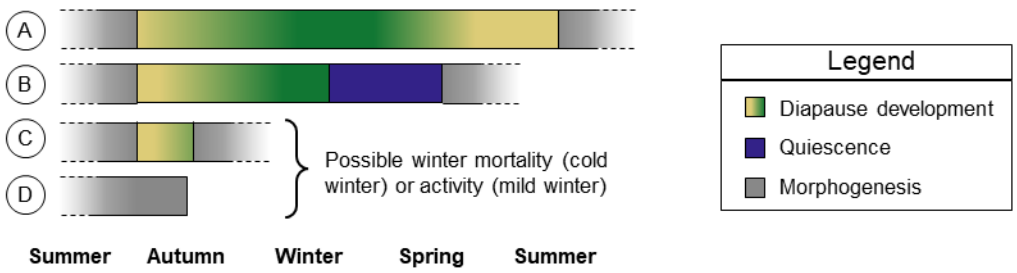

Figure 1. Example durations of diapause for insects that typically enter diapause in late summer or early autumn. (A) Some insects remain in diapause under warm (yellow) and cool (green) conditions for most of the year. **(B)** Many insects complete diapause during winter when it is cool (green), and enter a post-diapause dormancy called quiescence (blue), resuming post-diapause development (morphogenesis; gray) immediately when temperatures become permissive in the spring or early summer. **(C)** Warm summer or autumn conditions can cause individuals to complete diapause rapidly, referred to as ‘weak diapause’ or ‘shallow diapause’ in some species **(D)** Non-diapause individuals never enter diapause and may complete morphogenesis prior to winter.

Temperature has some well-described effects on diapause, but few studies have systematically investigated thermal sensitivity of diapause development. Chilling at low temperatures appears to be required for completion of the diapause program in many temperate species (Denlinger 2002), although there are many exceptions to this ‘rule’ (Hodek 2002; Zhu et al. 2009; Chen et al. 2014). In addition, warm temperatures can cause some insects to complete diapause relatively quickly (Fig. 1C) or never enter diapause (Fig. 1D; Denlinger 2002; Koštál 2006; Dambroski and Feder 2007; Toxopeus et al. 2021; Calvert et al. 2022). A common

experimental approach examines the impact of different chilling or winter durations to find the chilling threshold time required for diapause termination (e.g., Feder et al. 1997; Sgolastra et al. 2010; Higaki and Toyama 2012; Moraiti et al. 2014; Lehmann et al. 2017). However, experiments that manipulate only chilling duration do not provide much insight into the underlying thermal sensitivity of the diapause development process, and the potential impact of warming autumn and winter conditions.

Models of physiological time that measure morphogenesis during warm conditions are well-described, and can inform our approach to modelling diapause development. Nonlinear, hump-shaped functions relating environmental temperature to morphogenic development rate generally fit empirical data quite well (Kipyatkov and Lopatina 2010; Shi et al. 2011; Damos and Savopoulou-Soultani 2012; Kingsolver and Woods 2016; Sinclair et al. 2016). These models, a class of thermal performance curves, predict zero development below or above the low and high temperature limits, respectively. Between these lower and upper thermal limits, as temperatures increases morphogenic development rate increases to its highest value at the thermal maximum (c. 20 – 40 °C for many insects; Taylor 1981), and then sharply decreases back to a rate of zero as temperatures become too warm. The time to complete a developmental process at a particular temperature is the inverse of the development rate at that temperature (Kipyatkov and Lopatina 2010; Damos and Savopoulou-Soultani 2012), so development duration is shortest at the thermal maximum. Simpler slope plus intercept (straight line) models may also reasonably approximate the relationship between morphogenic development rate and temperature between the lower thermal limit and the thermal maximum (Trudgill et al. 2005; Kipyatkov and Lopatina 2010). Ontogenetic variation in thermal sensitivity of development is another important source of

variation in development times/rates that is well described and often incorporated into predictive models (Yurk and Powell 2010; Lopatina et al. 2014; Sinclair et al. 2016; Kutcherov 2020). Finally, genetic variation within populations may also explain a substantial portion of remaining, interindividual variation in development time that is often observed even under highly controlled laboratory conditions (Kingsolver et al. 2004)

We propose that diapause duration can be modelled such that physiological time serves as the diapause timer (cf. Hodek 2002). To do this, we can use a thermal performance curve of diapause development rate. Given the well-known chilling relationships described above, these diapause curves likely have very different shapes compared to functions describing morphogenic (non-diapause) development with maximum rates at high temperatures (cf. Hilbert et al. 1985). To our knowledge, there are no existing empirical estimates of thermal performance curves for diapause development, so we start with simple, hypothetical models that cover the most plausible forms of the relationship (Fig. 2 insets). Diapause phenology experiments often involve incubating insects at two different, consecutive temperatures (chilling then warming; e.g., Rull et al. 2016; Lindestad et al. 2020; Toxopeus et al. 2021), so we present the predicted diapause and total development times associated with these simple thermal sensitivity experiments (Fig. 2), as expanded upon below.

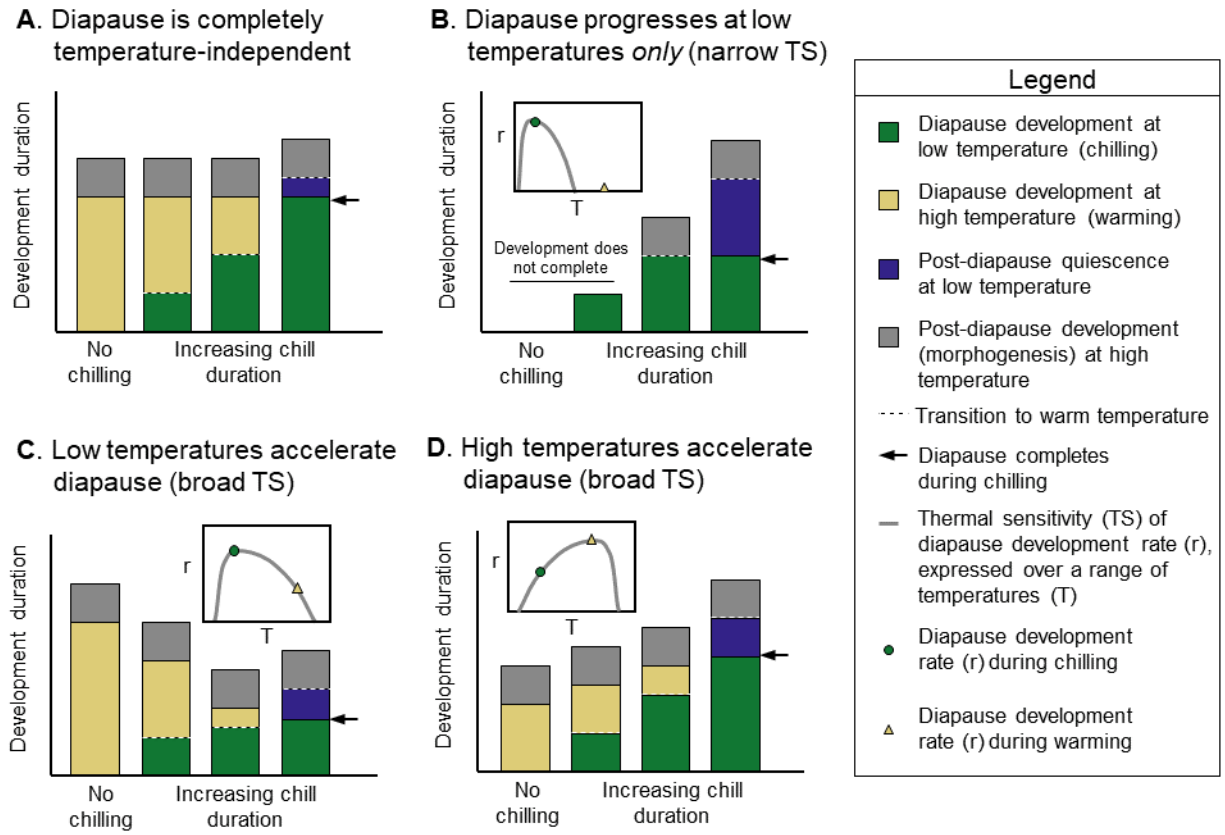

Figure 2. Hypothesized effect of a single (A) chronological or (B – D) physiological diapause timer on development duration under different combinations of chilling and warming. Transition from cold to warm temperature indicated by dashed line. Development duration includes diapause development under cold (green) and warm (yellow) conditions, and post-diapause (e.g., morphogenic) development (gray) that can only occur under warm conditions. If individuals remain chilled after diapause completes (arrows), cold-induced post-diapause quiescence (dark blue) occurs until animals are returned to warm temperatures. Insets: relationship between temperature (T) and diapause development rate (r) at a low (chilling) and high (warming) temperatures based on an example thermal sensitivity (TS) of the diapause timer. The equations that explain the relationships between development rates, chill duration, and development time are fully explained in Appendix A. Multiple diapause timers may exist (e.g., for different stages of diapause), which would result in more complex relationships between chilling duration and development duration (not shown).

Our models make several simplifying assumptions about the environmental sensitivity of diapause and about how an observer measures diapause duration. We do not make any assumptions about how diapause is induced, but do assume that temperature alone (and not photoperiod or other cues) influences the duration of diapause. This assumption applies

reasonably well to many insects that complete diapause without additional environmental cues regardless of whether diapause was induced by a non-temperature cue (Tauber and Tauber 1976; Hodek 1996; Denlinger 2022). Directly observing the precise timing of the end of diapause is often not practical. This is because overt, morphological changes (morphogenesis) associated with resumption of post-diapause growth and development often occur well after hormonal and metabolic pulses that delineate the true physio-developmental transition (Hodek 1996, 2002; Denlinger 2002). Thus, we further assume that a measurement proxy for diapause duration also includes some amount of post-diapause development (morphogenesis; Fig. 2 - gray), culminating in a clear phenotypic marker such as a life stage transition (e.g., adult emergence of a pupal diapausing insect) or a behavior (e.g., emergence from hibernacula). Finally, we assume that though diapause may progress at relatively cold temperatures, post-diapause development only proceeds at relatively warm temperatures. If diapause ends during low temperature exposure, quiescence occurs (Fig. 2 – dark blue), which delays post-diapause development because low temperatures impede morphogenesis (Jenkins et al. 2001; Hodek 2002; Koštál 2006).

Consider a naïve model in which diapause development progresses at a constant rate over time, independent of temperature. Under this chronological timer, diapause duration is approximately the same regardless of thermal environment (Fig. 2A) – some proportion of diapause development (and post-diapause development) occurs during chilling and the rest of diapause development occurs during warming. The only treatment that can increase overall development duration is chilling the insects for longer than their diapause duration (e.g., Fig. 2A; longest chill duration), which delays resumption of post-diapause development while insects are in chilling-induced quiescence. More nuanced chronological diapause timers are possible, e.g.,

chronological timers that are “started” by chilling or warming, but we focus here on the simplest model as a point of comparison to models that incorporate physiological time (Fig. 2B-D).

Realistically, we expect temperature to influence diapause development in some way. First, consider the classic “chill-dependent” diapause (or chilling threshold) model (cf. Dambroski and Feder 2007), where diapause can only progress at low temperatures. In this case, we predict that the physiological diapause timer has a fairly narrow thermal sensitivity, such that the rate of diapause development (*r*) is high at low temperatures, but zero at high temperatures (Fig. 2B inset). Individuals must be chilled for a sufficient time (chilling threshold) to complete diapause, and those that experience no or short chilling durations never complete 100% of the diapause program, and therefore cannot proceed to post-diapause development when warmed (Fig. 2B). Alternatively, the physiological diapause timer might have a broad thermal sensitivity, with a thermal maximum for diapause development rate at low (Fig. 2C inset) or high (Fig. 2D inset) temperatures. If low temperatures accelerate the diapause timer, diapause duration and chilling duration have an inverse relationship: long chilling durations result in short development times (Fig. 2C). Under this diapause timer, the longer the individual is in a cold environment, the greater the proportion of diapause completed prior to warming, so less time is needed at warm temperatures to complete diapause. If high temperatures accelerate the diapause timer, we expect a positive correlation between chill duration and development time, with the shortest diapause duration in individuals that are only exposed to warm temperatures due to high rates of diapause development at these temperatures (Fig. 2D).

Just as thermal sensitivity may vary among individuals and over time during morphogenic development (Yurk and Powell 2010; Lopatina et al. 2014; Sinclair et al. 2016; Kutcherov 2020), diapause development performance curves may differ among individuals and over time. For example, early diapause (e.g., initiation, maintenance) may have a different thermal sensitivity than late diapause (e.g., termination), which we have not captured in our simple models (Fig. 2). However, each thermal sensitivity model in Figure 2 can be applied to a distinct stage of diapause rather than diapause as a whole, if needed. To avoid confusion between development rate and changes in development rate across diapause development, we refer to the latter as changes or variation across ontogeny.

In this study we fit these naïve and physiological time models (Fig. 2) to measurements of diapause duration across simple cold/warm temperature treatments in the univoltine apple maggot fly *Rhagoletis pomonella* (Diptera; Tephritidae). *Rhagoletis pomonella* diapause as pupae under the soil for most of the year (Fig. 1A), and this diapause duration is not highly photosensitive (Prokopy 1968; Feder et al. 1997), making *R. pomonella* a good model to examine the impact of temperature on diapause development. In addition, intra/inter-population variation in diapause related to seasonality has been intensively studied in *R. pomonella* (Dambroski and Feder 2007; Lyons-Sobaski and Berlocher 2009; Powell et al. 2020; Calvert et al. 2022). Our study demonstrates that diapause duration can be modelled using the principles of physiological time, with implications for the effect of changing climates on the life history timing of many insect species. Some elements of the models may be somewhat idiosyncratic to *R. pomonella*, but we argue that accounting for ontogenetic and interindividual variation will likely be similarly critical in other species.

Materials and Methods

Rhagoletis pomonella as a diapause model

Rhagoletis pomonella has recently evolved to be an agricultural pest, laying its eggs in fruits of apple trees (*Malus domesticus*) that were introduced to North America several hundred years ago. It natively infests hawthorn (*Crataegus* spp.) trees throughout North America, and both apple- and haw-infesting populations mate and oviposit in the late summer/early autumn (Dean and Chapman 1973). Larvae develop within fruit, burrow into the soil to pupate, and then most pupae enter a long diapause and spend up to 10 months in the soil, eclosing as adults the following summer (e.g., Fig. 1A; Dean and Chapman 1973). Differences in life history timing (e.g., between apple- and hawthorn-infesting *R. pomonella*; (Bush 1969; Feder et al. 1993) are driven by differences in diapause duration; the duration of post-diapause morphogenesis leading to adult eclosion in *R. pomonella* varies little among individuals at a given temperature (Powell et al. 2020). The lower temperature limit for this pupal morphogenesis is c. 6°C, and above this the rate of morphogenic development increases approximately linearly with temperature up to 26°C (Reissig et al. 1979). If *R. pomonella* pupae complete diapause at low temperatures (< 6°C) they will enter post-diapause quiescence until temperatures become permissive (> 6°C) for post-diapause development (morphogenesis).

There are three well-described, discrete diapause phenotypic classes in *R. pomonella* that vary in diapause intensity, or recalcitrance to develop despite permissive temperatures (Dambroski and Feder 2007; Calvert et al. 2022). In the absence of low temperatures, a small proportion (<10 %) of *R. pomonella* pupae avert diapause completely (non-diapause phenotype; ND), developing directly into adults without ever entering dormancy (e.g., Fig. 1D; Prokopy

1968; Dambroski and Feder 2007; Calvert et al. 2022). Of the remaining pupae that enter diapause (as determined by a dramatic suppression of metabolic rate; cf. Ragland et al. 2009), most exhibit the prolonged chill-dependent diapause (CD) phenotype and remain in diapause for an extended period of several months (e.g., Fig. 1A; Feder et al. 1997; Dambroski and Feder 2007; Calvert et al. 2022). A small portion (< 20 %) of pupae exhibit the weak diapause phenotype (WD; cf. Toxopeus et al. 2021), also termed shallow diapause (Dambroski and Feder 2007; Calvert et al. 2022); they enter diapause, but will terminate diapause (increase metabolic rate) after only a few weeks and develop into adults if held at warm temperatures (e.g., Fig. 1C). These three diapause intensity phenotypes are relatively discrete, with multimodal distributions of eclosion and even distinct allele frequencies (Feder et al. 1997; Dambroski and Feder 2007; Calvert et al. 2022).

Intra- and inter-population variation in diapause development is pervasive and well-characterized in nature, including variation in diapause intensity (Tauber and Tauber 1972; Dingle et al. 1990; Masaki 2002; Chen et al. 2013; Geisert and Meinke 2013; Papanastasiou and Papadopoulos 2014). Thus, we argue that the relative complexity that we observe *R. pomonella* diapause is important for understanding the fairly general diversity in diapause phenotypes across temperate insects. In *R. pomonella* we also have the advantage of a large body of literature on diapause variation that allows us to realistically parameterize our models.

Insect collection and diapause incidence

We collected hawthorn fruits (*Crataegus* spp.) infested with *R. pomonella* in Denver CO during August 2017 and 2018, brought them to the lab, and collected emerging larvae and pupae

as previously described (Toxopeus et al. 2021). Briefly, we placed fruits in wire mesh baskets suspended over plastic trays in an environmentally-controlled room (c. 22°C). We collected newly formed pupae three times per week, and transferred them to a Percival DR-36VL incubator (Percival Scientific, Perry IA) set to 21°C, 14:10 L:D and 80% R.H. for 10 days. Diapause *R. pomonella* pupae suppress their metabolic rate during these 10 days, after which metabolic rate stabilizes (Ragland et al. 2009). We defined 10 d post-pupariation as "time zero" in our experiments.

To estimate the proportion of *R. pomonella* pupae in each diapause phenotypic class (ND, WD, CD), we measured CO_2 production in a subset of 2018 pupae at 10 d and 45 d post-pupariation at 21°C using stop-flow respirometry as previously described (Ragland et al. 2009; Toxopeus et al. 2021). A small number (6 out of 200) with intermediate metabolic rates were excluded because they could not be unambiguously assigned to a diapause phenotype. Because we wanted to focus our analysis on diapause development, we excluded ND pupae from our subsequent analyses, either based on metabolic rate, or very short (< 40 days; cf. Feder et al. 1997; Toxopeus et al. 2021) times to complete development to adulthood after chilling treatments (see below).

Temperature treatments and development tracking

<u>Experiment 1: Different durations of chilling</u>

To determine whether diapause was governed by physiological timers, and whether diapause development was accelerate at low or high temperatures, we compared the relationship between chilling duration and diapause duration in *R. pomonella* to the predictions in Figure 2.

At time zero (10 d post-pupariation), pupae were separated into groups of c. 100 individuals. One group was kept at 21°C for the duration of the experiment (no chilling). All other groups were transferred directly to simulated winter (chilling at 4 °C and c. 85% R.H) for one of several chilling durations ranging from 2 to 29 weeks. Following chilling, pupae were transferred back to 21°C. Each experimental group was checked three times per week at 21°C to determine when adults eclosed from the puparia, the marker that diapause and post-diapause development (morphogenesis) were complete. For each treatment group, we calculated the mean developmental duration (including time at 4°C and 21°C), the coefficient of variation (CoV) of mean developmental duration, and the proportion of pupae that eclosed as adults. Summaries of development duration only included individuals that eclosed as adults.

Mean diapause duration and the incidence of quiescence in *R. pomonella* was determined in each temperature treatment group based on two assumptions. 1) At 4°C, pupal morphogenesis (i.e. post-diapause development) cannot occur (Reissig et al. 1979), but diapause development can occur, based on the observation that pupae can complete development following prolonged chilling (Feder et al. 1997; Powell et al. 2020; Toxopeus et al. 2021). 2) At 21°C, time to complete post-diapause pupal morphogenesis is relatively invariant among individuals, so the time to complete development is largely driven by the time it takes to complete diapause (Feder et al. 1997; Powell et al. 2020). If post-chill developmental duration is approximately equal to the time to complete morphogenesis (c. 30 days), we can infer that the diapause process completed at 4°C and post-diapause quiescence may have occurred (e.g., longest chill duration bars in Fig. 2). However, if the post-chill developmental duration is greater than that required to complete morphogenesis, we can infer that only a portion of the diapause process was completed

at 4°C and the remaining proportion of diapause development occurred at 21°C prior to post-diapause morphogenesis (e.g., all chill durations except the longest in Fig. 2C, 2D). In this latter case, cold-induced quiescence does not occur, so diapause duration (e.g., green + yellow bars in Fig. 2) is the difference between age at eclosion (e.g., total height of bars in Fig. 2) and the duration of morphogenesis (e.g., gray bars in Fig. 2).

Experiment 2: Chilling for the same duration but at different ages

To detect ontogenetic shifts in the thermal sensitivity of diapause development, we compared developmental duration of *R. pomonella* pupae exposed to the same chilling durations but at different chronological ages. We included additional temperature treatments in which groups of c. 100 pupae were kept at 21°C for a warming period (2 to 6 weeks beyond time zero) prior to chilling (4°C) for 2, 3, 6, or 9 weeks. Pupae were then transferred back to 21°C and eclosion was tracked as described above. We compared developmental duration in these treatments to pupae that were chilled at time zero (10 d post-pupariation) for 2, 3, 6, or 9 weeks. If chilling at different chronological ages resulted in a decrease or increase in total development time, this would suggest that the thermal sensitivity of the diapause timer can vary with ontogeny, i.e., it can change over the course of diapause development.

Diapause timer computational simulations

Initial inspection of our empirical data suggested the results could not be explained by one of the simple relationships between diapause development rate and temperature (Fig. 2; see Results for additional details). Indeed, due to variation in the proportion of individuals that eclosed (completed diapause and post-diapause development) following each temperature

treatment in Experiment 1 and the presence of WD and CD diapause phenotypes, it was clear that we needed to account for interindividual and ontogenetic variation in thermal sensitivity of diapause development. To do this, we developed a simulation model that incorporated these factors. The full derivation of the model equations and an explanation of model assumptions (summarized in Table A1) are available in Appendix A, and briefly described below. All simulations and analyses were conducted in R v4.1.0 (R Core Team 2022), and our code is available at https://anonymous.4open.science/r/diapause-thermal-sensitivity-0DDC/.

Based on estimated proportions of diapause intensity phenotypes (see Results), we simulated a population that included 150 WD and 850 CD individuals, each with its own diapause timer(s) that specified diapause development rate at 4°C and 21°C. We modeled one diapause timer for each WD individual, and two diapause timers (early and late diapause development) for each CD individual, accounting for ontogenetic variation in diapause development in CD individuals. Within each diapause phenotype (WD, CD), diapause development rates varied among simulated individuals. In addition, we incorporated some chilling-related mortality for both diapause phenotypes based on empirical observations. Total development duration (diapause and post-diapause development) was calculated from development rates (Eq'ns 1.1 – 2.4) for each simulated individual that was 'exposed' to 4°C (chilling) at time zero for between 0 and 400 days, followed by warming at 21°C, similar to Experiment 1 above.

Total development time (diapause and post-diapause development) of each WD individual as a function of time at 4°C was simulated with the following equations that included a single diapause timer:

$$Y = \frac{r-R}{r}X + \frac{1}{r} + \frac{1}{r_g} \text{ when } 0 \leq X < \frac{1}{R} \qquad \text{(Eq'n 1.1)}$$

$$Y = X + \frac{1}{r_g} \text{ when } \frac{1}{R} \leq X < X_z \qquad \text{(Eq'n 1.2)}$$

$$Y = 0 \text{ when } X \geq X_z \qquad \text{(Eq'n 1.3)}$$

where Y is the time to complete development since time zero, X is the duration of chilling (d), R is the rate of diapause development at 4°C (d^{-1}; e.g., green circle in Fig. 2 insets), r is the rate of diapause development at 21°C (d^{-1}; e.g., yellow triangle in Fig. 2 insets), r_g is the rate of post-diapause development (morphogenesis) at 21°C (d^{-1}), and X_z is the duration of chilling (d) that causes mortality in WD individuals. The inverse of each rate is the time to complete that process (diapause or morphogenesis) at the respective temperature. When $Y = 0$ (e.g., Eq'n 1.3), that indicates that an individual was unable to complete development and adult eclosion never occurred, so development time is effectively 0 d. We use $Y = 0$ rather than recording simulated individuals as dead because (as described below for CD individuals) some individuals fail to complete development because they are alive but temperatures are not permissive for development (e.g., no chilling and shortest chilling treatment in Fig. 2B).

To model total development time (diapause and post-diapause development) of each CD individual, we included two diapause timers – one each for early (e) and late (l) diapause development – in the following equations:

$$Y = 0 \text{ when } X < \frac{1}{R_e} \qquad \text{(Eq'n 2.1)}$$

$$Y = \frac{r_l - R_l}{r_l} X + \frac{R_l + R_e}{R_e r_l} + \frac{1}{r_g} \text{ when } \frac{1}{R_e} \leq X < \frac{1}{R_e} + \frac{1}{R_l} \qquad \text{(Eq'n 2.2)}$$

$$Y = X + \frac{1}{r_g} \text{ when } \frac{1}{R_e} + \frac{1}{R_l} \leq X < X_z \qquad \text{(Eq'n 2.3)}$$

$$Y = 0 \text{ when } X \geq X_z \qquad \text{(Eq'n 2.4)}$$

where R_e is the rate of early diapause development at 4°C (d^{-1}), R_l is the rate of late diapause development at 4°C (d^{-1}), r_l is the rate of late diapause development at 21°C (d^{-1}), X_z is the duration of chilling (d) that causes mortality in CD individuals, and all other symbols are as described for the WD individuals. Early diapause must complete before late diapause can begin. We assumed that early diapause development did not proceed at warm temperatures (21°C), i.e. chilling was required for completion of early diapause (cf. Fig. 2B), and so individuals did not complete development and eclose ($Y = 0$) if chilling duration was insufficient (less than $1/R_e$) for individuals to complete early diapause (Eq'n 2.1).

Our simulations used the equations above and accounted for interindividual variation in diapause development rates among simulated individuals in the following way. The rates of diapause development for each of the simulated WD individuals (R and r in Eq'ns 1.1 – 1.2) and CD individuals (r_l, R_e, R_l in Eq'ns 2.1 – 2.3) were randomly sampled from inverse Gaussian distributions of rates with the parameters described in Table A2. We then calculated the same summary metrics (mean and CoV development duration, proportion eclosion) for our simulated population as for our empirical population for each chill duration used in Experiment 1, including only eclosed ($Y > 0$) individuals from summaries of development duration (as we did for our empirical population). We compared the fit of our simulation summary metrics to the empirical summary metrics by calculating the sum of squared residuals (SSR) of mean development duration and the χ^2 of proportion eclosion between the two datasets. We used

multiple iterations of these simulations to numerically solve for the parameters of our rate distributions (μ, λ in Table A2) that minimized the SSR and χ^2 values.

While the assumptions in our final simulation model were justified by empirical data (see Appendix A), we also tested the sensitivity of the model to these assumptions. To do this, we either modified or removed parameters from Eq'ns 1.1 – 2.4, ran our simulation, calculated the SSR and χ^2 parameters as described above, and visually compared the fit of the simulation summary metrics to the empirical summary metrics. The alternative simulations we ran included: 1) only one diapause phenotype (Eq'ns 2.1 – 2.4) instead of distinct WD and CD phenotypes; 2) both WD and CD phenotypes, but no ontogenetic variation in diapause development rates in CD individuals; 3) mortality due to chilling in only one of or neither WD and CD phenotypes. All alternative models are summarized in Supplementary Information and a subset of the most relevant alternative simulations are included in the main text.

Results

The results detailed below broadly support the hypothesis that insect development during winter-like conditions can be explained by timers reflecting thermally sensitive, physiological time. The thermal sensitivity of these timers differ between diapause intensity phenotypes, among individuals, and across ontogeny. While there are multiple potential interpretations of the empirical data, below we focus on interpretations that were supported by our subsequent simulation models.

Diapause timers vary among diapause intensity phenotypes

Based on the proportion of individuals that completed development under different chilling and warming treatments in Experiment 1, our data suggested that weak diapause (WD) and chill-dependent diapause (CD) phenotypes have different diapause timers. Consistent with a number of previous observations (Dambroski and Feder 2007; Toxopeus et al. 2021; Calvert et al. 2022), we confirmed the presence of ND (non-diapause), WD, and CD *R. pomonella* pupae reared under constant warm conditions using metabolic rate measurements (Fig. 3A). We note that these diapause phenotypes are relatively distinct rather representing a continuous distribution of diapause intensities. Under constant warmth, all ND flies completed their eclosion before WD flies began eclosing, and most CD flies remained in diapause under these conditions (did not eclose at all; Fig. S1, S2). CD pupae were most abundant (82%), followed by WD (15%), and ND (3%; Fig. 3B), the latter of which was excluded from our analyses (see Methods for details).

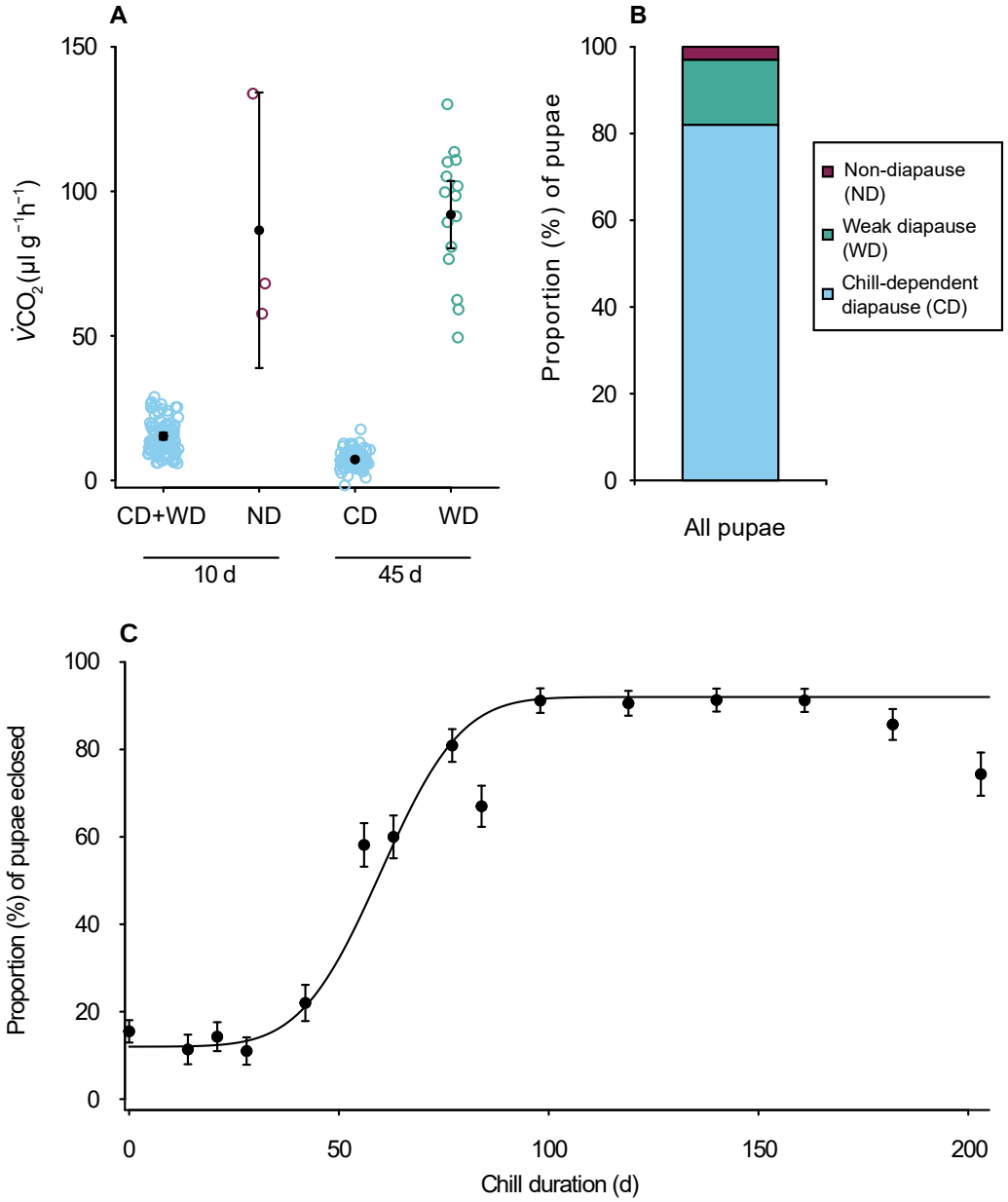

Figure 3. Diapause incidence and the effect of chilling on diapause completion in *Rhagoletis pomonella*. (A) Mass-specific metabolic rate after 10 or 45 days of constant warmth post-pupariation was used to determine diapause phenotype: non-diapause (ND), weak diapause (WD), and chill-dependent diapause (CD). Each open circle represents one individual. Filled black circles represent the mean of each group. Error bars represent 95% confidence intervals; small error bars are obscured by symbols. **(B)** The proportion of ND, WD, and CD pupae inferred from the metabolic rate data in **(A)** and duration of total development (Fig. S2) in groups that were not metabolically-phenotyped. **(C)** Each point represents the proportion of c. 100 pupae that completed diapause and post-diapause development (eclosed as adults) following exposure to chilling (4°C) for a specific duration at time zero (10 d post-pupariation). Error bars represent the standard error of proportion. Black line is a cumulative distribution fit with a mean ± standard deviation of 60 ± 15 d chilling.

The two types of diapause timers in this population included a rarer one that did not require chilling, and a common one that required moderate to prolonged chilling. As previously documented (Feder et al. 1997; Dambroski and Feder 2007; Toxopeus et al. 2021), some diapause *R. pomonella* pupae were able to complete diapause and eclose as adults following a range of temperature treatments, from no chilling (warming only) to prolonged chilling (>200 days) at 4°C (Fig. 3C). Approximately 15% of our population eclosed with little to no chilling (0 – 4 weeks at 4°C; Fig. 3C), which is similar to the proportion of WD pupae in our population (Fig. 3B). We therefore inferred that the WD pupae had a diapause timer that did not require chilling to complete the diapause program. However, as chill duration increased, the proportion eclosion increased in a sigmoidal pattern to a maximum of 91% (Fig. 3C), suggesting that the remaining CD pupae had a diapause timer that required a threshold cold exposure (chilling) to successfully complete the diapause program. We confirmed that the majority of uneclosed pupae following short chill treatments appeared to be alive and undeveloped (Fig. S1), suggesting that they were unable to complete diapause, rather than dead, or developed but unable to leave the puparium.

Variation in diapause timers among individuals

The relationship between chill duration and proportion eclosion in Experiment 1 (Fig. 3C) also suggested that there was substantial interindividual variation in diapause timers among individuals within each diapause phenotype. This is best illustrated here in CD pupae, but our simulation models (see below) also confirm interindividual variation in WD diapause timers. If we assumed that 15% of eclosed *R. pomonella* are WD individuals (see Fig. 3B), any proportion eclosion above 15% indicated completion of development by CD individuals. Thus, the increase in proportion eclosion following between c. 50 and 100 days of chilling (Fig. 3C) represented an increase in the number of CD individuals that had experienced sufficient chilling to complete the diapause program. This chilling threshold was quite variable among individuals in the population; 60 days of chilling was required for 50% of flies to eclose, and more than 90 days of chilling was required for maximum (91%) proportion eclosion (Fig. 3C). This sigmoidal pattern in proportion eclosion can be explained by continuous variation among CD individuals in rate of diapause development at 4°C (Fig. 3C). Those that eclosed after shorter chill durations were likely able to complete a larger proportion of the diapause program at 4°C than other individuals due to higher diapause development rates at 4°C, resulting in less time at 21°C required to complete the diapause program.

Diapause is governed by physiological timers

When *R. pomonella* were exposed to increasing chill durations in Experiment 1, the mean total development time increased (Fig. 4), suggesting that the diapause timers were temperature-sensitive, as expected for physiological timers. However, low temperatures did not universally accelerate development (decrease mean development time), as the classic chilling model (Fig.

2B) would predict. Indeed, at first glance, the positive relationship between chilling duration and mean total development time (Fig. 4) is broadly consistent with a diapause timer that is accelerated by high temperatures and has a broad thermal sensitivity (Fig. 2D). However, as discussed above, the proportion of eclosed individuals varied with chill time due to the requirement for chilling in most (likely CD) individuals, which is partially consistent with the predictions of the classic, chilling only model (Fig. 2B). So, it seems that none of the simple models individually explain all of the observations, especially given that WD and CD pupae respond differently to chilling.

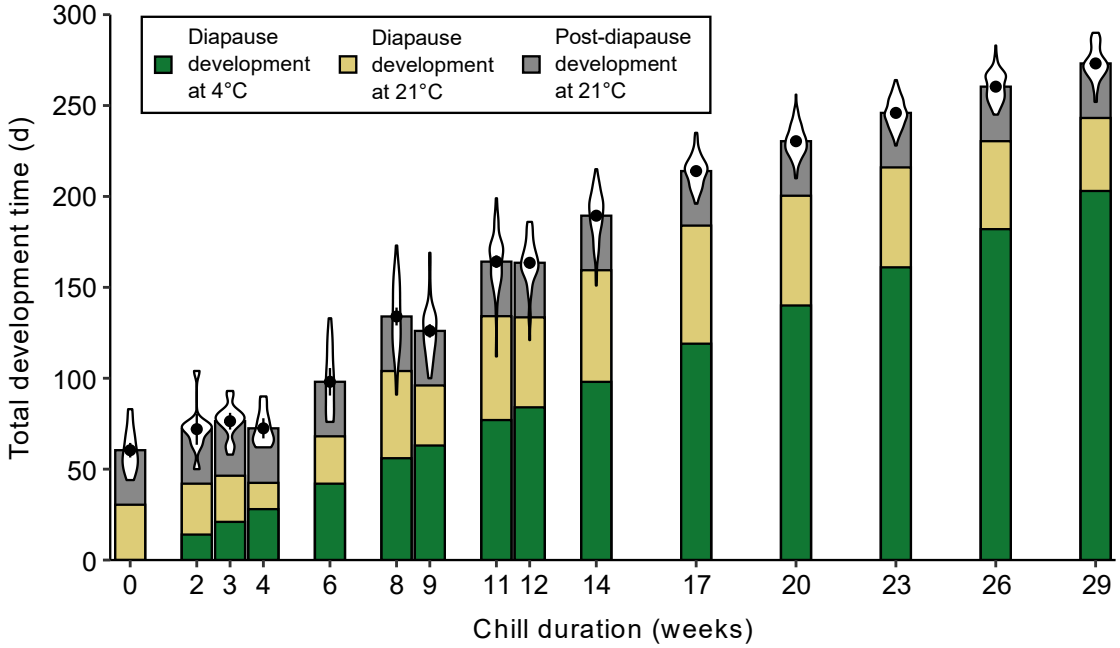

Figure 4. The effect of chilling on the time to complete diapause and post-diapause development in *Rhagoletis pomonella*. Pupae were exposed to chilling (4°C; green) for a specific duration at time zero (10 d post-pupariation) followed by warming (21°C). Each violin plot represents the distribution of times to complete development from 10 d post-pupariation to adult eclosion, with means represented by points, and error bars representing 95% confidence intervals. Small error bars are obscured by symbols. Bars below the violin plots show the mean duration of diapause development at 4°C (green – dictated by the chilling time) and 21°C (yellow - estimated). We assumed a mean value of 30 days for post-diapause development (morphogenesis; gray) at 21°C; gray bars are thus invariant across treatments.

Diapause timers can have broad thermal sensitivity

Although chilling was required for completion of diapause in most of our population, the diapause program had a fairly broad thermal sensitivity for both WD and CD pupae. First, we reasoned that diapause could progress at the two temperatures we used in Experiment 1: 4°C and 21°C, as explained below. Second, we also found diapause could complete after "chilling" at the relatively warm temperature of 15°C (Fig. S3). We assumed the time to complete post-diapause development was c. 30 days at 21°C for all individuals because this morphogenic process is largely invariant in *R. pomonella* (Powell et al. 2020). Under this assumption, we inferred that the average diapause program completed at 21°C (post-chill) for all chill treatments in Experiment 1 because the mean time to eclose following chilling (yellow + gray bars; Fig. 4) was always greater than the mean time to complete post-diapause morphogenesis at 21°C (gray bars; Fig. 4). Therefore, pupae were able to complete part of the diapause program at 4°C and the remaining proportion at 21°C (green and yellow bars; Fig. 4). This was likely true for WD pupae (all or most of the eclosed individuals in the 2 – 4 weeks chilling treatments) and CD pupae (all or most of the eclosed individuals after prolonged chilling; Fig. 4). Consistent with this finding, none of our treatments included a clear period of cold-induced post-diapause quiescence (cf. longest chill treatments in Fig. 2). Indeed, data collected by Feder et al. (1997) suggested that more than 40 weeks of chilling are required for CD *R. pomonella* from hawthorn fruits to complete diapause at low temperatures and enter quiescence, which is longer than the maximum chilling duration (29 weeks) we used in our study.

Thermal sensitivity of the diapause timer can change during ontogeny

While early chilling seemed to be required for completion of diapause in CD pupae (Fig. 3C), prolonged cold exposure slowed down diapause development (Fig. 4). This suggested that CD pupae had at least two diapause timers: one that governed early diapause development and was accelerated by low temperatures (chilling), and another that governed late diapause development and was accelerated by high temperatures (warming). Though a similar pattern is expected when comparing diapause to post-diapause development in most insects (Jenkins et al. 2001; Hodek 2002), we emphasize that this ontogenetic shift in thermal sensitivity of development rate clearly occurs during diapause in CD *R. pomonella* because our chill durations were too short to induce quiescence.

Indeed, when we exposed groups of *R. pomonella* pupae to identical cold treatments at different ages during Experiment 2, cold treatments later in development increased diapause duration (slowed development) compared to cold treatments initiated at time zero (Fig. 5). Because the age at chill exposure affected total development time, this suggested that the rate of diapause development at low temperatures could change during diapause ontogeny. However, there were multiple factors at play that could influence mean development time in these treatments (Fig. 5), including differences in the proportion of individuals that eclosed (Fig. S1) and how many of those individuals were CD vs. WD. Disentangling these effects empirically was challenging because it is only possible to metabolically phenotype CD and WD accurately when these individuals are held at warm temperatures for at least 45 days pre-chill (Dambroski and Feder 2007; Toxopeus et al. 2021; Calvert et al. 2022), which we could not do in all treatments. To address this issue, we developed simulation models to investigate whether

interindividual and temporal variation in diapause timer thermal sensitivity could explain the trends we observed in our empirical diapause development data.

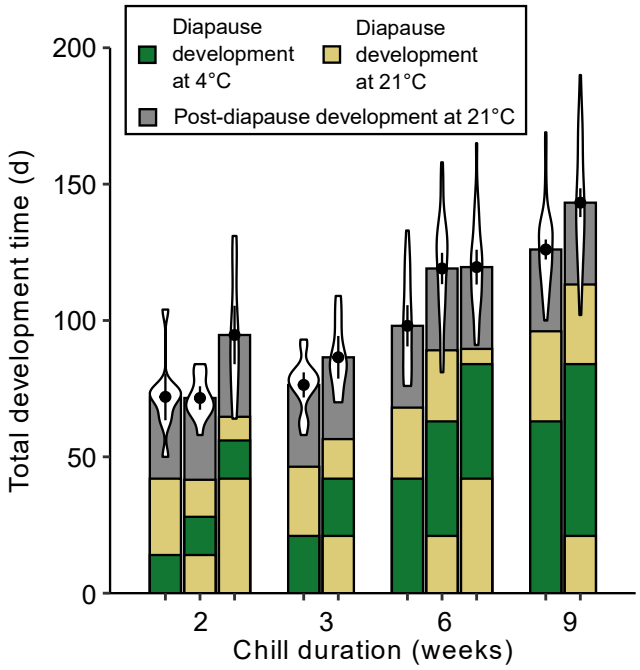

Figure 5. The effect of chilling at different ages on the on the time to complete diapause and post-diapause development in *Rhagoletis pomonella*. Pupae were exposed to chilling (4°C) for a specific duration at time zero (10 d post-pupariation), or 2 – 6 weeks after time zero followed by warming (21°C). Each violin plot represents the distribution of times to complete from 10 d post-pupariation to adult eclosion, with means represented by points, and error bars representing 95% confidence intervals. Small error bars are obscured by symbols. Bars below the violin plots show the mean duration of diapause development at 4°C (green – dictated by chilling time) and 21°C (yellow - estimated), and assumed duration of morphogenesis at 21°C (gray).

Accurate models must incorporate variation in diapause timers between diapause phenotypes, among individuals, and across ontogeny of diapause development

A simulation model incorporating interindividual and ontogenetic variation in diapause development rates, as well as differences between diapause phenotypes, provided an exceptional fit to our empirical observations. We simulated a population of *R. pomonella* consisting of two groups (WD and CD) with different diapause timers (Fig. 6A, B), and additional assumptions described in Table A1. Our simulated population had approximately the same proportion of WD

(15%) and CD (85%) pupae as our empirical population (Fig. 3B). In the simulated population, each WD pupa had one diapause timer with a broad thermal sensitivity (Fig. 6A), while each CD pupa had two diapause timers: one for early diapause development (pre-threshold) progressing only at low temperatures, and one for late diapause development with a broad thermal sensitivity (Fig. 6B). Both the thermal sensitivities of WD and late CD development overlapped with the thermal sensitivity of post-diapause development (Reissig et al. 1979). This allowed completion of the diapause program at warm temperatures, followed immediately by post-diapause development.

We modelled mean and interindividual variation in diapause development rate at two temperatures (4°C and 21°C). Development time had a Gaussian distribution (Fig. S2), so we modelled interindividual variation in development rates with an inverse Gaussian distribution. However, the final simulation model fit to empirical data did not differ substantially if we modelled rates with a Gaussian distribution instead (Fig. S4). Interindividual variation in development rate was higher at the temperatures that were closer to the thermal optima for both WD and CD populations, and was high early in diapause development for the CD population (Fig. 6A, B). The final simulation model fit to empirical data was sensitive to both these assumptions (Figs. S5, S6). We also assumed that development rates within an individual were correlated across temperature (and time, in the case of CD pupae). That is, a simulated individual with a development rate higher than the mean at 4°C would also have a development rate higher than the mean at 21°C. However, the final simulation model fit to empirical data did not differ substantially if we violated this assumption (Figs. S5, S6).

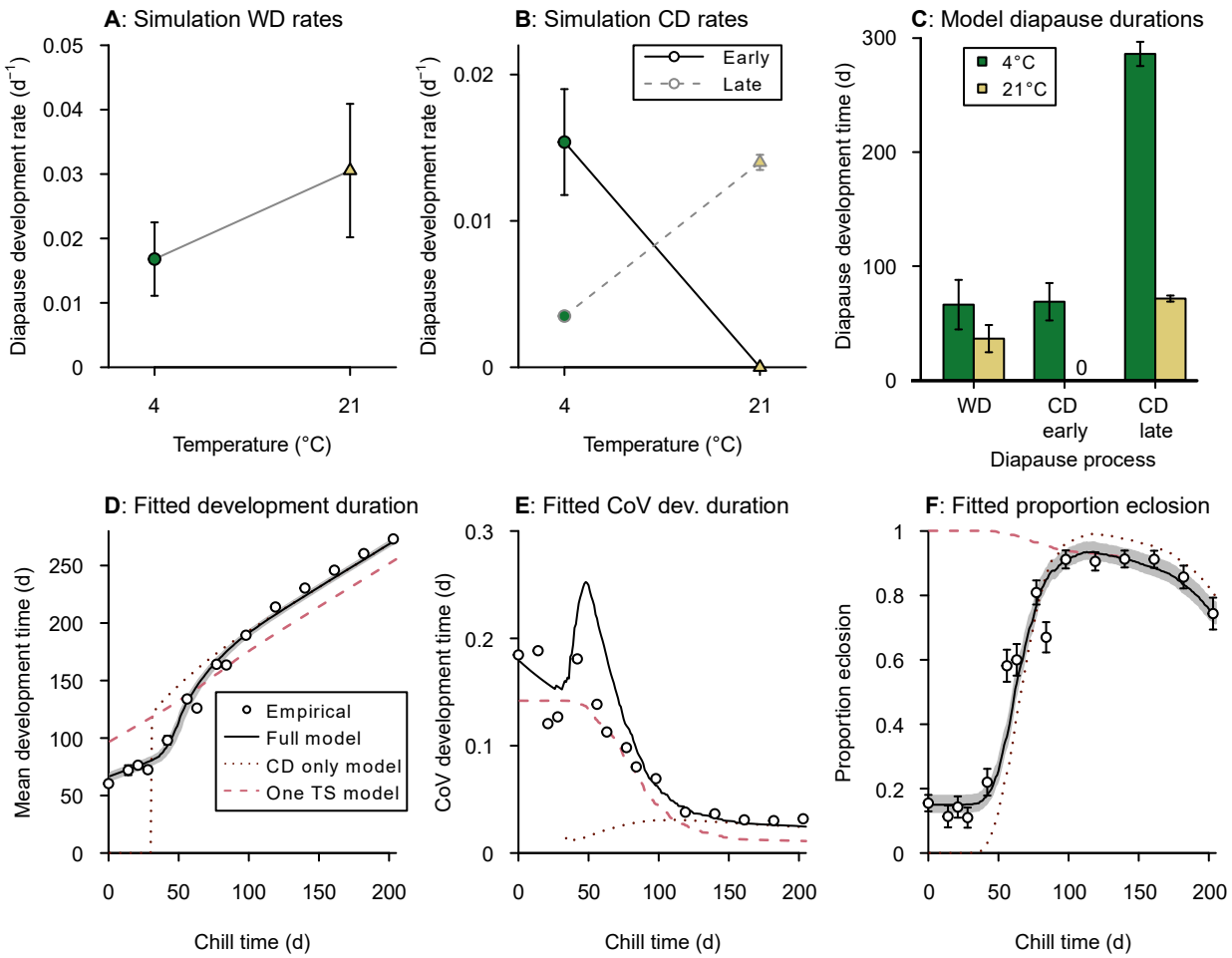

Figure 6. Summary of simulated diapause development in *Rhagoletis pomonella*. Mean and standard deviation of diapause development rates in a simulated population of **(A)** 150 weak diapause (WD) pupae and **(B)** 850 chill-dependent diapause (CD) pupae under cold (green) and warm (yellow) conditions during early and late diapause development. **(C)** Mean and standard deviation of time to complete diapause in simulated WD pupae and early and late diapause in simulated CD pupae at 4°C or 21°C. 0 indicates failure to complete the developmental process. **(D-F)** Summary metrics of development time and proportion eclosion phenotypes versus chill time for empirical data (symbols) and fitted simulation models (lines). The full simulation model (black line, Eq'ns 1.1 – 2.4) is compared to models that only included a single diapause phenotype (purple dotted line) or did not allow for ontogenetic shifts in thermal sensitivity (TS) of diapause development rates (pink dashed line). Gray shading indicates the 95% confidence interval of the simulation summary metrics in (D) and (F). Error bars represent standard error of the (D) mean or (F) proportion in empirical data. Small error bars are obscured by symbols. CoV, coefficient of variation of mean development time.

In our simulation, only the WD pupae could complete diapause development at low or high temperatures, while CD pupae required at least some chilling to complete diapause development. The WD rate of diapause development was approximately twice as high at 21°C than 4°C (Fig. 6A), resulting in faster completion of diapause (shorter development times) at 21°C (Fig. 6C). We note that WD pupae could have more than one diapause timer (e.g., different timers in early vs. late development), but this added complexity was not necessary to explain the general trends in our empirical data. For each simulated CD pupa, the early diapause timer only allowed diapause to progress at low temperatures, and after some threshold chill duration (68.2 ± 16.4 d; Fig. 6C), a diapause timer with a different thermal sensitivity allowed CD pupae to complete late diapause development at 4°C or 21°C (Fig. 6B). In late diapause, diapause development rates were approximately four times higher at the warmer temperature (Fig. 6B), resulting in faster completion of late diapause at 21°C than 4°C (Fig. 6C). We acknowledge that a small proportion of CD pupae can likely complete pre-threshold diapause development at 21°C in actual *R. pomonella* populations (Dambroski and Feder 2007), but that this pre-threshold development is so slow that individuals did not complete development within the measurement period of our experiments.

Using the full model described in the methods, our simulated population (Fig. 6A, B) produced a close fit to the empirical data for mean development time (Fig. 6D), variation in development time (Fig. 6E), and proportion eclosion (Fig. 6F) following different chilling durations. If we only used one diapause phenotype (CD), or used two phenotypes (WD and CD) but did not allow for ontogenetic variation in rates for CD pupae, the simulation produced a considerably poorer fit to the empirical data (Fig. 6D-F). This reinforced the conclusions from

our empirical dataset that WD and CD are distinct phenotypes, rather than different ends of a continuum of diapause intensity. Our full simulation model captured the prominent discontinuity/transition in development time (Fig. 6D) and proportion eclosion (Fig. 6F) at ~60 days chilling caused by a transition in high proportion of WD to high proportion of CD phenotypes among eclosed individuals. It likewise captured the decline in proportion eclosion at long chill times (Fig. 6F) driven by overwinter mortality. The simulated variation in development time (Fig. 6E) was over-estimated for moderate chill treatments (6 – 14 weeks). Attempts to modify the distributions of diapause development rates (including pre-threshold diapause development at 21°C) to improve the fit for development time metrics resulted in poor fit for proportion eclosion or CoV metrics (see Fig. S7-9 for alternative simulations that modify these parameters). A more complex model (e.g., more than two diapause timers) could potentially improve the fit, but would not change the core principles of our simulation: diapause is a temperature-sensitive process that varies among individuals, across diapause phenotypes, and – in the case of CD pupae – across ontogeny.

Discussion

Our study demonstrates that principles of physiological time that predict developmental duration during the growing season in ectotherms can also predict diapause developmental duration under various simulated winter conditions. A few studies have shown that different chilling temperatures impact diapause duration, with the fastest diapause development (shortest diapause duration) at temperatures between 0°C and 7°C (Irwin et al. 2001; Sgolastra et al. 2010; Xiao et al. 2013; Lehmann et al. 2017). Others have suggested that the impact of chilling changes over time during diapause, with late chilling causing an increase in diapause duration (Nomura and Ishikawa 2000; Xiao et al. 2013). Here we use data from *R. pomonella* to parameterize well-fitting models that incorporate similar principles. Our experimental design exposing each group of *R. pomonella* to two temperatures (chilling and warming) for different durations and at different ages facilitated detection of at least two distinct phases of diapause development, each with its own thermal sensitivity of diapause development rate. It likely would have been difficult to detect this ontogenetic shift in thermal sensitivity of diapause development in a classic thermal performance curve experiment, where multiple temperatures treatments were used but each group of insects was exposed to a single temperature (e.g., Irwin et al. 2001; Sgolastra et al. 2010; Xiao et al. 2013; Lehmann et al. 2017). Although we do not know the exact shape of the diapause development rate thermal performance curves in *R. pomonella*, our simple linear models are sufficient to explain most of the impacts of chilling and warming on two diapause phenotypes in our populations under laboratory conditions.

Our experiments on thermal sensitivity of development in chill-dependent diapause revealed only two distinct stages of diapause (early and late) rather than the three stages

(initiation, maintenance, termination) conceptualized in a broadly accepted ecophysiological model of diapause development (Koštál 2006; Koštál et al. 2017). This is most likely because diapause initiation in *R. pomonella* is relatively short; it takes less than 10 days post-pupariation for pupae to suppress their metabolic rate (Ragland et al. 2009) and subsequently enter the maintenance stage. Because we started our experiments 10 days post-pupariation (after initiation), the 'early diapause' stage we describe in CD pupae likely represents maintenance, while the 'late diapause' stage likely represents a long and gradual process of diapause termination, also termed horotely (Hodek 1996, 2002). Additional work is needed to determine whether this pattern (different stages of diapause development have different thermal sensitivities) applies broadly to other insects, especially those that experience a broad range of temperatures during diapause or do not routinely experience quiescence.

Increasing environmental temperatures will likely affect insect diapause development across multiple seasons, with implications for life history timing. It is already known that warm pre-winter conditions can negatively impact some insects by causing aversion or early termination of diapause (Denlinger 2002; Koštál 2006; Toxopeus et al. 2021). In addition, warm temperatures can cause energy drain that leads to mortality (Sinclair 2015; Roberts et al. 2021; Nielsen et al. 2022). As autumn temperatures increase and winter warm spells become more frequent (Williams et al. 2015; Marshall et al. 2020), early diapause development in chill-dependent *R. pomonella* will likely slow down, resulting in an overall delay in the resumption of post-diapause morphogenesis in the following summer or fall, and a possible mismatch in adult eclosion and host fruit availability. Conversely, the predicted global increase in spring temperatures (Williams et al. 2015; Marshall et al. 2020) may shorten diapause duration in

R. pomonella because this warming is likely to coincide with the later diapause development process that is accelerated at high temperatures. Thus, any models that attempt to predict insect phenology based on rates of post-diapause morphogenesis (e.g., Reissig et al. 1979) should take diapause termination timing into account, especially for species with a temperature-dependent diapause termination.

The physiological or cellular processes that determine diapause duration still remain elusive (Hand et al. 2016). There are well-characterized hormonal responses that govern the transition from diapause to post-diapause development in photoperiodically- or pharmacologically-terminated diapause (Denlinger 2002; Denlinger et al. 2012). However, if diapause termination is a gradual, horotelic process, diapause duration is likely governed by some kind of diapause timer that is temperature-sensitive. For example, in a chill-dependent diapause (or stage of diapause), the time to complete diapause (or a stage of diapause) may depend on a particular physiological or cellular process that is active at low temperatures. There could be a single candidate molecule that must be accumulated or degraded over time, such as a particular gene product (e.g., *aldose reductase* and *sorbitol dehydrogenase* in *Pyrrhocoris apterus*; Koštál et al. 2008), protein (e.g., p26 in *Artemia franciscana*; King and MacRae 2012) or metabolite (e.g., alanine in *Pieris napi*; Lehmann et al. 2018); sorbitol in *Bombyx mori*, (Chino 1958). Conversely, there could be a suite of genes or molecules involved in regulating diapause duration. For example, multiple studies have shown that gene expression changes over time in diapause (Robich and Denlinger 2005; Kim et al. 2006; Rinehart et al. 2007; Reynolds and Hand 2009; Emerson et al. 2010; Ragland et al. 2010; Urbanski et al. 2010; Kankare et al. 2016; Green

and Kronforst 2019), although few have studied this process at low temperatures (Salminen et al. 2015; Koštál et al. 2017; Dowle et al. 2020; Pruisscher et al. 2022).

Our simulation and empirical datasets, and the transcriptomic studies cited above are both highly consistent with temperature-modulated developmental processes dictating the duration of diapause. Many of the temperature-sensitive genes in late diapause development of *R. pomonella* are related to cell cycle and developmental progression (Meyers et al. 2016), although there is no obvious morphological change during diapause. Indeed, genetic variants associated with diapause duration in *R. pomonella* are enriched for genes also associated with egg-to-adult developmental duration in *Drosophila melanogaster* (Dowle et al. 2020). In addition, neural differentiation and remodeling (brain development) might occur during 6 months (42 weeks) of chilling (Kharva et al. 2022), further suggesting that many developmental regulatory pathways govern diapause duration just as they govern 'typical' (non-diapause) developmental duration. We suggest that this may be a general phenomenon. Diapause has evolved rapidly and repeatedly across the arthropod phylogeny (Ragland and Keep 2017; Denlinger 2022), suggesting that diapausing clades likely coopt existing developmental machinery from non-diapausing ancestors to initiate and regulate diapause development. This evolutionary hypothesis would also predict that models of physiological time would fit developmental data from both diapause and non-diapause development.

Conclusions

Applying the principles of physiological time to overwintering *R. pomonella* revealed that diapause is temperature-sensitive, thermal sensitivity of diapause development rate can

change over time, and substantial portions of the diapause program progress more quickly at warm temperatures. We also showed that the combination of two diapause timers (early and late diapause) can account for interindividual synchronization of post-diapause development, even in the absence of quiescence. Understanding the thermal sensitivity of overwintering development (even if that development occurs in a “dormant” state) is important for predicting how warming temperatures will impact the phenology of ectothermic animals. Additional work to elucidate the cellular and physiological basis for diapause timers is still needed, with implications for understanding how diapause has evolved.

Acknowledgements

The authors would like to thank M. Calvert, M. Davenport, N. Sanaei, E. Rader, and M. Haigh for assistance with collecting processing *R. pomonella* in the lab. We would also like to thank J. Kingsolver for comments on an earlier version of this manuscript. This work was funded by National Science Foundation IOS 1700773 and DEB 1638951 grants to GJR.

Data and Code Accessibility

All analysis code and data referenced in this manuscript is available at https://anonymous.4open.science/r/diapause-thermal-sensitivity-0DDC/.

Author Contributions Statement

JT, EJD, and GJR conceptualized the experiments. JT, EJD, and LA conducted the experiments. JT and EJD analyzed the data. JT and GJR drafted the manuscript. All authors contributed to editing and approving the final manuscript.

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Figure Legends

Figure 1. Example durations of diapause for insects that typically enter diapause in late summer or early autumn. (A) Some insects remain in diapause under warm (yellow) and cool (green) conditions for most of the year. **(B)** Many insects complete diapause during winter when it is cool (green), and enter a post-diapause dormancy called quiescence (blue), resuming post-diapause development (morphogenesis; grey) immediately when temperatures become permissive in the spring or early summer. **(C)** Warm summer or autumn conditions can cause individuals to complete diapause rapidly, referred to as 'weak diapause' or 'shallow diapause' in some species **(D)** Non-diapause individuals never enter diapause and may complete morphogenesis prior to winter.

Figure 2. Hypothesized effect of a single (A) chronological or (B – D) physiological diapause timer on development duration under different combinations of chilling and warming. Transition from cold to warm temperature indicated by dashed line. Development duration includes diapause development under cold (green) and warm (yellow) conditions, and post-diapause (e.g., morphogenic) development (grey) that can only occur under warm conditions. If individuals remain chilled after diapause completes (arrows), cold-induced post-diapause quiescence (dark blue) occurs until animals are returned to warm temperatures. Insets: relationship between temperature (T) and diapause development rate (r) at a low (chilling) and high (warming) temperatures based on an example thermal sensitivity (TS) of the diapause timer. The equations that explain the relationships between development rates, chill duration, and development time are fully explained in Appendix A. Multiple diapause timers may exist (e.g.,

for different stages of diapause), which would result in more complex relationships between chilling duration and development duration (not shown).

Figure 3. Diapause incidence and the effect of chilling on diapause completion in *Rhagoletis pomonella*. (A) Mass-specific metabolic rate after 10 or 45 days of constant warmth post-pupariation was used to determine diapause phenotype: non-diapause (ND), weak diapause (WD), and chill-dependent diapause (CD). Each open circle represents one individual. Filled black circles represent the mean of each group. Error bars represent 95% confidence intervals; small error bars are obscured by symbols. **(B)** The proportion of ND, WD, and CD pupae inferred from the metabolic rate data in **(A)** and duration of total development (Fig. S2) in groups that were not metabolically-phenotyped. **(C)** Each point represents the proportion of c. 100 pupae that completed diapause and post-diapause development (eclosed as adults) following exposure to chilling (4°C) for a specific duration at time zero (10 d post-pupariation). Error bars represent the standard error of proportion. Black line is a cumulative distribution fit with a mean ± standard deviation of 60 ± 15 d chilling.

Figure 4. The effect of chilling on the time to complete diapause and post-diapause development in *Rhagoletis pomonella*. Pupae were exposed to chilling (4°C; green) for a specific duration at time zero (10 d post-pupariation) followed by warming (21°C). Each violin plot represents the distribution of times to complete development from 10 d post-pupariation to adult eclosion, with means represented by points, and error bars representing 95% confidence intervals. Small error bars are obscured by symbols. Bars below the violin plots show the mean duration of diapause development at 4°C (green – dictated by the chilling time) and 21°C

(yellow - estimated). We assumed a mean value of 30 days for post-diapause development (morphogenesis; grey) at 21°C; grey bars are thus invariant across treatments.

Figure 5. The effect of chilling at different ages on the on the time to complete diapause and post-diapause development in *Rhagoletis pomonella*. Pupae were exposed to chilling (4°C) for a specific duration at time zero (10 d post-pupariation), or 2 – 6 weeks after time zero followed by warming (21°C). Each violin plot represents the distribution of times to complete from 10 d post-pupariation to adult eclosion, with means represented by points, and error bars representing 95% confidence intervals. Small error bars are obscured by symbols. Bars below the violin plots show the mean duration of diapause development at 4°C (green – dictated by chilling time) and 21°C (yellow - estimated), and assumed duration of morphogenesis at 21°C (grey).

Figure 6. Summary of simulated diapause development in *Rhagoletis pomonella*. Mean and standard deviation of diapause development rates in a simulated population of **(A)** 150 weak diapause (WD) pupae and **(B)** 850 chill-dependent diapause (CD) pupae under cold (green) and warm (yellow) conditions during early and late diapause development. **(C)** Mean and standard deviation of time to complete diapause in simulated WD pupae and early and late diapause in simulated CD pupae at 4°C or 21°C. 0 indicates failure to complete the developmental process. **(D-F)** Summary metrics of development time and proportion eclosion phenotypes versus chill time for empirical data (symbols) and fitted simulation models (lines). The full simulation model (black line, Eq'ns 1.1 – 2.4) is compared to models that only included a single diapause phenotype (purple dotted line) or did not allow for ontogenetic shifts in thermal sensitivity (TS) of diapause development rates (pink dashed line). Gray shading indicates the

95% confidence interval of the simulation summary metrics in (D) and (F). Error bars represent standard error of the (D) mean or (F) proportion in empirical data. Small error bars are obscured by symbols. CoV, coefficient of variation of mean development time.

Appendix A: Simulation Model Building

Relationships between diapause development rates, temperature, and development duration

Development rates (R) are often expressed as proportion development per day (d^{-1}), and the time (d) to complete development (Y) is simply equal to the inverse of that development rate ($Y = 1/R$) (Kipyatkov and Lopatina 2010; Shi et al. 2011; Damos and Savopoulou-Soultani 2012). The classic method to determine thermal sensitivity of a development rate is to rear groups of individuals at different temperatures and calculate development rate at each temperature as the inverse of developmental duration (Kipyatkov and Lopatina 2010). However, we can assess the relative breadth of thermal sensitivity of diapause development, as well as shifts in thermal sensitivity over time by using the following experimental design. Groups of diapausing individuals are exposed to a cold temperature (e.g., 4°C) for different lengths of time (X), followed by exposure to a warm temperature (e.g., 21°C) until evidence of post-diapause morphogenesis is observed. These temperatures must be selected such that post-diapause morphogenesis can only occur at the warm temperature (rate of morphogenesis at the cold temperature = 0). We can represent R as the rate of diapause development at the cold temperature, r as the rate of diapause development at the warm temperature, and r_g as the rate of post-diapause morphogenesis at the warm temperature. Total time to complete development following a range of chilling durations can then be modelled in several ways, depending on the thermal sensitivity of diapause development.

<u>A single diapause timer: narrow vs. broad thermal sensitivity</u>

If diapause development rate has a narrow thermal sensitivity such that diapause only progresses at low temperatures (e.g., Fig. 2B), the rate of diapause development at the warm

temperature is zero, i.e. $r = 0$. Therefore, any cold exposure duration that is less than the time required to complete diapause at 4°C ($1/R$) will result in a total development time (Y) of zero because diapause is unable to complete (and no post-diapause morphogenesis is observed) at the warm temperature:

$$Y = 0 \text{ when } X < \frac{1}{R} \qquad \text{(Eq'n A1.1)}$$

If an individual is kept at the cold temperature for a sufficient time to complete diapause ($1/R$), post-diapause morphogenesis will resume when transferred to the permissive warm temperature, and total development time will be:

$$Y = X + \frac{1}{r_g} \text{ when } X \geq \frac{1}{R} \qquad \text{(Eq'n A1.2)}$$

where $1/r_g$ is the time to complete post-diapause morphogenesis at the warm temperature. If an individual is chilled for longer than is needed to complete diapause ($> 1/R$), that individual will enter post-diapause quiescence, and any additional chilling will increase total development time with a slope of 1.

Conversely, we can model a broad thermal sensitivity of diapause development rate, i.e. diapause can progress at both cold and warm temperatures (e.g., Fig. 2C and D). In this case, if the individual is never chilled:

$$Y = \frac{1}{r} + \frac{1}{r_g} \text{ when } X = 0 \qquad \text{(Eq'n A2.1)}$$

where $1/r$ is the time to complete diapause at the warm temperature. Conversely, if the individual is chilled for sufficient time to complete diapause development:

$$Y = X + \frac{1}{r_g} \text{ when } X \geq \frac{1}{R} \qquad \text{(Eq'n A2.2)}$$

If an individual is chilled for longer than is needed to complete diapause, that individual will enter post-diapause quiescence, which will increase total development time with a slope of 1. If the individual is chilled at the cold temperature for a duration < 1/*R*, some proportion of diapause development ($X \times R$) completes in the cold, and the remaining proportion of diapause development ($1 - X \times R$) completes during post-chill warming. The time to complete diapause development is therefore:

$$Y = X + \frac{1-(X \times \mathrm{R})}{r} + \frac{1}{r_g} \text{ when } 0 \leq X < \frac{1}{R} \qquad \text{(Eq'n A2.3)}$$

which simplifies to a classic linear relationship ($y = mx + b$) between X and Y:

$$Y = \frac{r-R}{r} X + \frac{1}{r} + \frac{1}{r_g} \text{ when } 0 \leq X < \frac{1}{R} \qquad \text{(Eq'n A2.4)}$$

Using this equation, we see that the slope will be negative if chilling accelerates diapause development ($R > r$; Fig. 2C), and positive if warming accelerates diapause development ($r > R$; Fig. 2D).

<u>Two diapause timers: incorporating a shift in thermal sensitivity over time</u>

If thermal sensitivity of development can change during ontogeny, we must introduce additional diapause development rates. R_e and r_e are the rates of diapause development at cold and warm temperatures (respectively) early in diapause devleopment, and R_l and r_l are the rates of diapause development at cold and warm temperatures (respectively) late in diapause development. The relationship between chill time (X) and total development time (Y) in this more complicated scenario can be broken down into early and late diapause sections.

First, let's consider a narrow thermal sensitivity for our early diapause timer, such that diapause only progresses at low temperatures. Any cold exposure duration that is less than the

time required to complete early diapause at 4°C ($1/R_e$) will result in a total development time (Y) of zero because early diapause is unable to complete at the warm temperature (preventing any further development), similar to Equation A1.1:

$$Y = 0 \text{ when } X < \frac{1}{R_e} \qquad \text{(Eq'n A3.1)}$$

Conversely, we can consider a broad thermal sensitivity for our early diapause timer. For chill times that are insufficient to complete early diapause, some proportion of early diapause development ($X \times R_e$) completes in the cold, and the remaining proportion of early diapause development ($1 - X \times R_e$) completes during post-chill warming. In this case, the total time to complete diapause is:

$$Y = X + \frac{1-(X \times R_e)}{r_e} + \frac{1}{r_l} + \frac{1}{r_g} \text{ when } X < \frac{1}{R_e} \qquad \text{(Eq'n A3.2)}$$

where X is the duration of partial early diapause development in the cold, $(1 - X \times R_e)/r_e$ is the time to complete the rest of early diapause at the warm temperature, and $1/r_l$ is the time to completed late diapause development at the warm temperature. This simplifies to a classic linear relationship ($y = mx + b$) between X and Y:

$$Y = \frac{r_e - R_e}{r_e} X + \frac{1}{r_e} + \frac{1}{r_l} + \frac{1}{r_g} \text{ when } X < \frac{1}{R_e} \qquad \text{(Eq'n A3.3)}$$

Using this equation, we see that the slope will be negative if chilling accelerates diapause development ($R_e > r_e$; Fig. 2C), and positive if warming accelerates diapause development ($r_e > R_e$; Fig. 2D).

For the late diapause timer, we can consider the same two scenarios as for our early diapause timer. If the late diapause timer has a narrow thermal sensitivity (diapause only progresses at low temperatures), any cold exposure duration that is less than the time required to

complete early *and* late diapause at 4°C ($1/R_e + 1/R_l$) will result in a total development time (Y) of zero because diapause is unable to complete at the warm temperature, similar to Equation A1.1:

$$Y = 0 \text{ when } X < \frac{1}{R_e} + \frac{1}{R_l} \qquad \text{(Eq'n A3.4)}$$

This would result in a development time pattern that is indistinguishable from a single diapause timer that also has a cold-optimized narrow thermal sensitivity. However, if the late diapause timer has a broad thermal sensitivity, chilling that is sufficient for completion of early diapause but too short for completion of late diapause will result in the following:

$$Y = X + \frac{1-([X-\frac{1}{R_e}] \times R_l)}{r_l} + \frac{1}{r_g} \text{ when } \frac{1}{R_e} \leq X < \frac{1}{R_e} + \frac{1}{R_l} \qquad \text{(Eq'n A3.5)}$$

where X is the duration of early diapause and partial late diapause development in the cold, and $(1 - (X - 1/R_e) \times R_l)/r_l$ is the time to complete the rest of late diapause at the warm temperature. This simplifies to a classic linear relationship ($y = mx + b$) between X and Y:

$$Y = \frac{r_l - R_l}{r_l} X + \frac{R_l + R_e}{R_e r_l} + \frac{1}{r_g} \text{ when } \frac{1}{R_e} \leq X < \frac{1}{R_e} + \frac{1}{R_l} \qquad \text{(Eq'n A3.6)}$$

Using this equation, we see that the slope will be negative if chilling accelerates late diapause development ($R_l > r_l$; Fig. 2C), and positive if warming accelerates diapause development ($r_l > R_l$; Fig. 2D).

When individuals are chilled for a sufficient time to complete diapause or for longer, total development time will be expressed similar to Equations A1.2 and A2.2:

$$Y = X + \frac{1}{r_g} \text{ when } X \geq \frac{1}{R_e} + \frac{1}{R_l} \qquad \text{(Eq'n A3.7)}$$

This relationship holds as long as both diapause processes can complete at 4°C, and does not depend on whether the early and late diapause timers have a narrow or broad thermal sensitivity.

More complicated models are also possible (e.g., 3 or more diapause timers, each with their own thermal sensitivity), but we were able to simulate a population closely predicting empirical observations in *R. pomonella* using a maximum of two diapause timers per diapause phenotype.

Simulation of Rhagoletis pomonella diapause development

We calculated total development time (since time zero) for each simulated individual (and whether that individual would complete development) under a range of chill durations using the assumptions in Table A1 and the equations in the main text. The rationale behind these equations and assumptions (as well as tests of those assumptions) is explained in the subsections below. Each individual in the population was simulated with its own diapause development rates at 4°C and 21°C, and these rates were sampled from simulated distributions of diapause development rates. The population distributions (mean and variation) of these simulated diapause rates are summarized in Table A2.

Table A1. Assumptions for development of simulated weak diapause and chill-dependent diapause pupae chilled at 4°C at time zero (10 d post-pupariation) for different durations, followed by warming at 21°C.

	Weak diapause	**Chill-dependent diapause**
Thermal sensitivity	Diapause progresses across a wide range of temperatures and is accelerated by warming.	Early diapause only progresses well at low temperatures, while late diapause progresses across a wide range of temperatures and is accelerated by warming.
Variation in development rates	Diapause development rates at 4°C (R) and 21°C (r) have an inverse-Gaussian distribution.	The rate of early diapause development at 4°C (R_e) has an inverse-Gaussian distribution. Early diapause development cannot progress at 21°C (r_e = 0, or very close to 0).
	The ratio of the rate at 4°C to the rate at 21°C does not vary among individuals.	The rates of late diapause development at 4 °C (R_l) and 21 °C (r_l) are correlated with the rate of early diapause development.
	The rate of post-diapause morphogenesis at 21°C (r_g) is invariant.	The rate of post-diapause morphogenesis at 21°C (r_g) is invariant.
Mortality due to chilling	Pupae die if chilled for X_z days.	Pupae die if chilled for X_z days.

Table A2. Model parameters of a simulated population of 150 weak diapause and 850 chill-dependent diapause pupae. Development rates were modelled with inverse Gaussian distributions with mean μ and shape λ (Fig. S4). Corresponding mean ($\bar{x}$) and standard deviation (*s*) of times to complete diapause or a component of diapause (early and late) at a single temperature (4°C and 21°C, respectively) are shown in the same row. The effect of increasing or decreasing these rates can be seen in Fig. S7.

	Development rates (d^{-1})		**Development times (d)**	
	Parameter	Values	Parameter	Values
Weak diapausers (*N* = 150)				
At 21 °C	r	$\mu = 0.030,\ \lambda = 0.25$	$\frac{1}{r}$	$\bar{x} = 35.9, s = 11.9$
At 4 °C	R	$\mu = 0.017, \lambda = 0.1$	$\frac{1}{R}$	$\bar{x} = 65.7, s = 21.7$
Chill-dependent diapausers (*N* = 850)				
At 21 °C	r_e	$\mu = 0$	$\frac{1}{r_e}$	0 (no development)
	r_l	$\mu = 0.014,\ \lambda = 10$	$\frac{1}{r_l}$	$\bar{x} = 71.0, s = 2.7$
At 4 °C	R_e	$\mu = 0.0155,\ \lambda = 0.3$	$\frac{1}{R_e}$	$\bar{x} = 68.2, s = 16.4$
	R_l	$\mu = 0.0035, \lambda = 3$	$\frac{1}{R_l}$	$\bar{x} = 285.6, s = 10.6$

Thermal sensitivity, mortality and equations for simulated weak diapause

We simulated our **weak diapause (WD)** individuals with a broad thermal sensitivity of diapause development (Table A1) for the following reasons. 1) Our empirical data (and that of others; Dambroski and Feder 2007; Calvert et al. 2022) shows that WD *R. pomonella* can eclose rapidly (35 – 65 days post-pupariation) with no chilling, suggesting diapause can complete at warm temperatures. 2) The proportion of pupae that eclosed with little to no chilling (0 – 4 weeks) was constant at approximately 15% (Fig. 3C), and approximately 15 % of our population consisted of WD pupae (Fig. 3B), suggesting that all flies that eclosed with 0 – 4 weeks chilling were WD individuals. 3) During these first 4 weeks of chilling, mean total development time

increased with chilling, but post-chill development time decreased (yellow + gray in Fig. 4), suggesting diapause could progress at 4°C as well as 21°C. Further, the positive slope of mean development time vs. chill time during these first 4 weeks of chilling (Fig. 4) suggested that WD pupal diapause was accelerated by warm temperatures (cf. Fig. 2D).

To model total development time in our simulated WD pupae, we used Equation A2.4 (above), and Equations A2.5 – A2.6 (below) [Equations 1.1 – 1.3 in the main text]. We know that WD pupae are killed by moderate chilling (Toxopeus et al. 2021), so we also incorporated a mortality term into our final model. Equation A2.5 is a slightly modified Equation A2.2. that incorporates this mortality:

$$Y = X + \frac{1}{r_g} \text{ when } \frac{1}{R} \leq X < X_z \qquad \text{(Eq'n A2.5)}$$

Where X_z is the chill time that causes mortality in that WD individual. Chill times longer than X_z resulted in a total development time of zero because no eclosion was observed:

$$Y = 0 \text{ when } X \geq X_z \qquad \text{(Eq'n A2.6)}$$

See Fig. S8 for the impacts of removing WD mortality from our models.

<u>Thermal sensitivity and equations for simulated chill-dependent diapause</u>

We simulated our **chill-dependent diapause (CD)** individuals with two diapause timers: an early diapause timer with a narrow thermal sensitivity and a late diapause timer with a broad thermal sensitivity of diapause development (Table A1). Our reasoning for this was as follows. 1) For moderate chill times (6 – 14 weeks), as chill time increased proportion eclosion increased in an approximately sigmoidal pattern (Fig. 3C). We moved forward with the likely assumption that this trend in proportion eclosion was driven by CD pupae, as WD pupae seemed to exhibit a

proportion eclosion pattern that did not depend on chilling. Therefore, CD pupae seemed to require chilling (e.g. due to a narrow thermal sensitivity of diapause development) early in development. 2) We saw no evidence for post-diapause quiescence in the chilling treatments we used in our study; even our longest chill treatment (29 weeks) resulted in post-chill development time that took longer than the mean time to complete morphogenesis at 21°C (Fig. 4). Therefore, although chilling was required early in diapause development, there seemed to be a later diapause process with a broad thermal sensitivity – one that could complete at either 4°C or 21°C. Further, the positive slope of mean development time vs. chill time following prolonged chilling (17 – 29 weeks) (Fig. 4) suggested that this late diapause process in CD pupae was accelerated by warm temperatures (cf. Fig. 2D).

To model total development time in our simulated CD pupae, we used Equations A3.1 and A3.6 (above), and Equations A3.8 – A3.9 (below) [Equations 2.1 – 2.4 in the main text]. We saw increased mortality after prolonged chilling (Fig. S1) that was likely due to death of CD pupae (because WD pupae likely die after moderate chilling). Equation A3.8 is a slightly modified Equation A3.7. that incorporates mortality:

$$Y = X + \frac{1}{r_g} \text{ when } \frac{1}{R_e} + \frac{1}{R_l} \leq X < X_z \qquad \text{(Eq'n 3.8)}$$

where X_z is the chill time that causes CD pupa mortality. Chill times longer than X_z resulted in a total development time of zero because no eclosion was observed:

$$Y = 0 \text{ when } X \geq X_z \qquad \text{(Eq'n 3.9)}$$

See Fig. S8 for the impacts of removing CD mortality from our models.

Variation in diapause development rates

Because development time generally had a Gaussian distribution (Fig. S2), we simulated variation in diapause development rates (r, R, R_s, r_d, R_d) among individuals with inverse Gaussian distributions (Table A1). Normal distributions of diapause development rates did not substantially improve the simulation model fit to empirical data (Fig. S4).

We assumed that different rates of diapause development (at 4°C vs. 21°C, pre- vs. post-threshold) were correlated within an individual (Table A1). This assumption is based on principles and examples from the morphogenesis development rate literature. For example, development times across life history stages can be correlated within individuals such that some individuals consistently develop faster than others at multiple temperatures. This can be seen in yellow dung fly larvae vs. pupae (Blanckenhorn 1997) and grasshopper eggs early and late in embryogenesis (Cherrill and Begon 1989). See Figs. S5 and S6 for the minimal impact of this assumption about rate correlation within an individual on model fit.

We allowed for a change in the magnitude of interindividual variation across temperatures (e.g., variation in *R* [4°C] and *r* [21°C]), and across ontogeny (e.g., variation in *Rs* [pre-threshold] and *Rd* [post-threshold] at 4°C). Variation in morphogenic development rates can change across temperature; for example several fruit fly species shown less variation in pupal development rate at high temperatures than low temperatures (Vargas et al. 1996). The morphogenic development rate/time literature contains several examples of changes in the variability of development rate across life stages, e.g., egg development time is less variable than pupal development time in Mountain Pine Beetle at a given temperature (Yurk and Powell

2010). See Figs. S5 and S6 for the substantial impact of these assumptions about variation in rates across temperature and ontogeny, respectively, on model fit.

We did not model interindividual variation in time to complete post-diapause morphogenesis (Table A1) because this post-diapause morphogenesis is minimally variant among individuals of *R. pomonella* at a given temperature, and variation in eclosion time is generally driven by variation in diapause duration in *R. pomonella* (Powell et al. 2020)

Alternative thermal sensitivity patterns

While the simulation model outlined in Tables A1 and A2 effectively captured the general trends in mean total development time, CoV development time, and proportion eclosion, we considered whether alternative thermal sensitivity patterns could improve model fit. A small proportion of CD *R. pomonella* can eclose without chilling, although this typically takes more than 150 d (Dambroski and Feder 2007). We therefore considered whether CD pupae could be modelled with a rate of pre-threshold diapause development at 21°C that was greater than zero ($r_e > 0$). When we calculated simulation summary metrics for our $r_e > 0$ model, the fit to empirical data was very poor (Fig. S9A vs. B). However, in our empirical data experiment, we did not track eclosion indefinitely, so we did not count any pupae that might have eclosed after a prolonged time (e.g., > 100 days) post-chill. If we incorporated a 'cutoff' of 115 days into our $r_e > 0$ model – i.e. if we did not 'count' any simulated eclosion that occurred after 115 days post-chill – the fit of this model (Fig. S9C) was similar but not a dramatic improvement relative to our base model ($r_e = 0$; Fig. S9A).