



Prepupal diapause and instar IV developmental rates of the spruce beetle, *Dendroctonus rufipennis* (Coleoptera: Curculionidae, Scolytinae)

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ARTICLE INFO

Article history:

Received 3 May 2011

Received in revised form 21 June 2011

Accepted 22 June 2011

Available online 29 June 2011

Keywords:

Diapause

Respirometry

Metabolism

Voltinism

Phenology model

Bark beetle

ABSTRACT

The spruce beetle, *Dendroctonus rufipennis* (Kirby), is an important mortality agent of native spruces throughout North America. The life-cycle duration of this species varies from 1 to 3 years depending temperature. The univoltine cycle (one generation per year) is thought to maximize outbreak risk and accelerate host mortality in established outbreaks. Prepupal diapause is associated with the semivoltine cycle (one generation per 2 years) and we investigated thermal conditions that result in diapause induction. Preliminary experiments used respirometry in an attempt to distinguish the diapause state of experimental insects but the technique was apparently confounded by low respiration before and during pupation, regardless of diapause status. Therefore, diapause induction was deduced using developmental delays. The observed developmental response was not a “switch”, with developmental delay either present or absent, but instead varied continuously. We found that temperatures <15 °C from instar III through mid-instar IV were associated with developmental delays beyond that expected from cool temperatures. Moreover, the duration of exposure to cool temperatures was important in determining the degree of developmental delay. Small, if any, delays were observed if the cumulative exposure to <15 °C was <20 d whereas >40 d cumulative exposure was associated with distinct developmental suppression. Intermediate exposure to cool temperatures resulted in minor developmental delays. We used our results to parameterize a maximum likelihood estimation model of temperature-dependent instar IV developmental rates, including the effect of diapause. This model can be included as part of a spruce beetle phenology model for predicting population dynamics.

Published by Elsevier Ltd.

1. Introduction

Insect life-cycles are shaped by the seasonality of their environment. In response to seasonally predictable, yet unfavorable environmental conditions, many insect species have evolved a diapause syndrome wherein physiological, behavioral, and/or morphological adaptations enable survival during adverse conditions and facilitate ecologically appropriate timing of life-cycle events (Tauber et al., 1986). To survive adverse conditions, insects must predict, rather than monitor, seasonal changes using signals that are reliable, frequent, and recognizable (Danks, 1987). Photoperiod, or the length of day, is invariant from year to year and is often used by insects as a cue to adjust life-cycle events in advance of adverse conditions (Danilevsky, 1965). Other signals, including temperature, moisture, and food are used by some species either alone or

in conjunction with day length to keep track of seasonal time, especially in environments where day length changes are not detectable (Danks, 2007). There are some insect species where diapause induction is a response to daily temperature cycles (i.e., thermoperiods) in constant darkness (Beck, 1980). Because diapause can alter life-cycle timing and duration, knowledge of the conditions that induce or avert diapause is imperative in models developed for predicting population-level dynamics of economically and ecologically important insects.

The spruce beetle, *Dendroctonus rufipennis* (Kirby) (Coleoptera: Curculionidae, Scolytinae), is an important mortality agent of spruces, *Picea* spp. (Pinaceae), throughout its hosts' ranges in North America. Wide-spread and severe outbreaks have occurred in the recent past in Alaska (Holsten et al., 1999), Utah (Dymerski et al., 2001), and the Yukon Territory (Parks Canada, 2009). The spruce beetle has a highly variable life-cycle, with durations of 1–3 years although the semivoltine cycle is most common (Knight, 1961). Summer temperature has repeatedly been shown to be strongly correlated with spruce beetle life-cycle duration (Massey and Wygant, 1954; Dyer, 1969; Werner and Holsten, 1985; Hansen

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et al., 2001a). Outbreak potential is heightened when univoltine broods are produced (Reynolds and Holsten, 1994; Hansen and Bentz, 2003; Berg et al., 2006).

Spruce beetles emerge from host trees in late spring to infest new host trees, mate, and oviposit eggs in the phloem layer beneath the bark where larval development through four instars occurs throughout the summer. Relatively warm summer temperatures increase the probability that development will progress to the pupal and adult stages prior to winter, resulting in the univoltine life-cycle. If sufficient development does not occur prior to the beginning of reduced temperatures in the fall, spruce beetles enter a prepupal diapause for the winter, resulting in the semivoltine cycle. This diapause was first demonstrated by Dyer (1970). Larvae reared in relatively cool thermoperiod regimes experienced developmental delay during instar IV compared to insects reared at a constant 21.1 °C; the degree of delay was considerably longer than was expected from, simply, the lower temperatures. Dyer and Hall (1977) found additional evidence for diapause and proposed that temperatures below 15.1 °C during instar III will induce diapause during instar IV. Hansen et al. (2001b) found little evidence of diapause induction during instar III, but did find two lines of diapause evidence in a series of constant-temperature rearing experiments. First, the temperature-dependent developmental rate of instar IV was not well represented by a continuous function, as with other lifestages, having a notable disconnect between rates above and below 15 °C. Second, there was a dramatic difference in instar IV developmental times at 15 °C depending on whether larvae were reared at constant 15 °C from the egg stage or pre-conditioned at 21 °C into instar III. Namely, two of 15 larvae reared at 15 °C beginning as eggs developed similarly as the 21 °C pre-conditioned group (pupating within 29 days) whereas the other 13 failed to pupate after as long as 142 days when the experiment was ended. From their results, Hansen et al. (2001b) hypothesized that diapause is induced later in the life-cycle than instar III, possibly during the late instar IV or early prepupal phase.

Both univoltine and semivoltine spruce beetle have been found developing in the same host tree (Hansen et al., 2001a), indicating phenotypic plasticity for the trait and suggesting that microhabitat temperature plays a large role in diapause induction. Because the spruce beetle spends all but a few days of its life-cycle beneath the bark of a host tree, photoperiod may not be a major controller of diapause in this insect, although photoperiod has never been explicitly tested independent of temperature cues. Temperature cues, however, have been shown to induce diapause independent of photoperiod (Dyer, 1970; Dyer and Hall, 1977; Hansen et al., 2001b).

Our objective was to describe and model the lifestage-dependent conditions associated with prepupal diapause induction in spruce beetle. This information can ultimately be used in a lifestage-specific spruce beetle phenology model allowing for detailed predictions of population response to thermal conditions. Specifically, we investigated: (1) whether prepupal diapause induction is influenced by photoperiod; (2) the effect of instar IV maturity on diapause induction; (3) thermocycle (i.e., temperature-only aspect of a thermoperiod, with thermophase and cryophase durations held constant) conditions associated with diapause induction focusing on cryophase temperatures; and (4) diapause expression as a function of interactions among temperature, lifestage during low temperature exposure, and duration of low temperature exposure. We conducted five experiments, the first three to determine basic parameters associated with diapause induction and, based on the results thereof, two additional experiments to investigate interactions of temperature, larval maturity, and duration of exposure to low temperatures. We also developed a model for estimating instar IV temperature-dependent developmental

rates as a function of diapause inducing conditions during instars III and IV.

2. Materials and methods

2.1. Phloem sandwiches

For all experiments, larvae were reared in 15 × 15 cm phloem sandwiches which enable visual inspection of lifestage change on a daily basis (Bentz et al., 1991; Hansen et al., 2001b). Parent spruce beetles came from an infested Engelmann spruce (*Picea engelmannii* Parry ex. Engelm.), cut from the Fishlake National Forest, Utah (38° 37.424' N; 111° 59.188' W), 27 September 2006. Infested bolts were stored at 0 °C until use. An uninfested Engelmann spruce was cut from the Cache National Forest, Utah (41° 37.243' N; 111° 26.002' W), 17 October 2006. As needed, infested bolts were placed in rearing cans at room temperature, emerged adults collected, and female–male adult pairs were manually inserted into bolts from the uninfested spruce. After 7–10 days, eggs were collected from the distal portion of each gallery in an effort to obtain relatively fresh eggs of similar age. Twelve eggs were inserted into separate niches of each phloem sandwich. Sandwiches were held in constant humidity desiccators which were kept in environmental chambers (Percival Scientific, Inc., Perry, Iowa; Gray et al., 1998). Environmental chambers were calibrated to within 0.1 °C of the target temperature as monitored by an independent datalogger (Campbell Scientific, Inc., Logan, Utah). Larval instar was distinguished based on head-capsule width (Hall and Dyer, 1974) and the transition between instars was marked by cast head-capsules following molting. Some larvae were occasionally not visible as they submerged into the phloem, and observations were omitted if the specific date of molting or pupation could not be determined.

2.2. Evaluation of the diapause state

Reduced respiration is often used as an indication of the diapause state (Danks, 1987; Tauber et al., 1986). To evaluate the use of respiration for detecting prepupal diapause in the spruce beetle, we conducted two preliminary laboratory experiments. In the first experiment, larvae were reared in a thermocycle regime that simulated phloem temperatures recorded at a field site where the semivoltine life-cycle was observed (Hansen et al., 2001a), i.e., conditions associated with prepupal diapause. Instar IV larvae and prepupae were periodically sampled from thermocycle-treated bolts and allowed to equilibrate for 1 h at 20 °C before conducting constant volume respirometry (PTC-1 temperature cabinet and PELT-3 controller, Sable Systems International, Las Vegas, Nevada). Larvae and prepupae were placed individually into glass respirometry chambers attached to a CO₂ analyzer (Sable CA-1B, Sable Systems International, Las Vegas, Nevada). Respiration, expressed as CO₂ output, was measured at 20 °C after an additional 60 min. Data acquisition and constant volume calculations were made, respectively, with DATACAN and CONVOL software (Sable Systems International, Las Vegas, Nevada). Prepupae were weighed (±0.001 mg; Cahn C-33, Thermo Orion, Beverly, MA) to calculate fresh weight standardized expression of respiration, ml CO₂ g⁻¹ h⁻¹. Results showed markedly reduced respiration as the prepupal stage was attained during September and respiration remained low through June; a U-shaped pattern over time consistent with other diapausing insects (Danks, 1987). We did not, however, compare rates to a reference treatment without other evidence of diapause, i.e., one wherein developmental delays were not observed.

A second experiment was conducted using larvae reared at either constant 12 or 18 °C. Respiration rates of prepupae were measured as described above. We found no significant respirometry

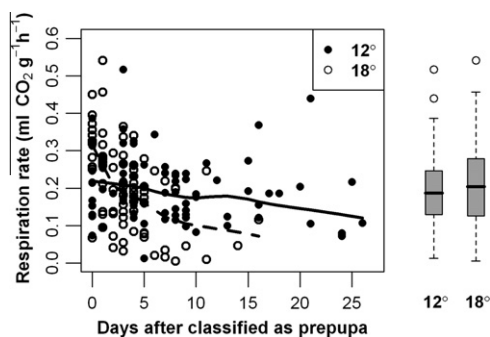


Fig. 1. Scatterplot (left) of prepupal respiration rates beginning when individuals were classified as prepupae and boxplots (right) of prepupae treated at 12 and 18 °C; the former group failed to pupate by the end of the experiment, after 100 days, whereas the latter group pupated within 21–35 d (included time as instar III). The solid line is the loess smooth fit for 12 °C treated prepupae and the dashed line is the loess smooth fit for the 18 °C treated prepupae. For each boxplot, the horizontal line is the median, the box shows the 25th and 75th percentiles, whiskers show 10th and 90th percentiles, and circles show outliers.

differences (PROC MIXED, SAS Institute, Inc., Cary, NC, USA; $F_{1,139} = 0.16$; $p = 0.6865$; Fig. 1) among 12 and 18 °C treated prepupae despite that the group held at 12 °C failed to pupate within 100 d while the 18 °C group pupated within 21–35 d (time in the treatment included instar III for both groups). Prepupal respiration was similarly reduced in all individuals regardless of treatment, despite obvious differences among development times. Histolysis, the dissolution of organic tissue, occurs during the prepupal phase and can also result in reduced respiration (Denlinger et al., 1972). Because we interpreted the 18 °C treated group as having averted diapause and the 12 °C treated group as having been diapause induced, we concluded that respirometry was inadequate for distinguishing spruce beetle prepupal diapause.

Another hallmark of diapause is developmental suppression in the diapausing lifestage even under favorable temperatures (Tauber et al., 1986; Danks, 1987; Zaslavski, 1988). Because our interest in diapause stems from the association among developmental delays and the resulting semivoltine life-cycle, measurement of developmental suppression directly addresses our objectives. Following diapause induction, the magnitude of developmental suppression can be variable among species and individuals within a species (Danks, 1987). Moreover, developmental suppression can appear as a continuous response rather than a discrete reaction. Developmental delays of only a few days are within the range of possible diapause responses (Danks, 1987; Zaslavski, 1988), and these short delays are distinguished from aseasonal quiescence in that, in the case of the latter, development *immediately* resumes following the return of favorable temperatures (Tauber et al., 1986). Danks (1987) suggested that diapause definitions be expanded to include developmental suppression that lasts longer than the adverse conditions.

2.3. Experiment I: photoperiodic diapause induction

We tested for photoperiodic diapause induction in a two by two factorial experiment by comparing short and long day treatments in diapause inducing and averting constant temperature regimes; constant temperatures above about 15 °C will avert diapause whereas cooler temperatures will induce diapause (Dyer and Hall, 1977; Hansen et al., 2001b):

- (a) short day (8 h photophase: 16 h scotophase), diapause inducing (12 °C);
- (b) short day (8 h photophase: 16 h scotophase), diapause averting (18 °C);

- (c) long day (16 h photophase: 8 h scotophase), diapause inducing (12 °C);
- (d) long day (16 h photophase: 8 h scotophase), diapause averting (18 °C).

Sixteen phloem sandwiches were held at 21 °C for 19 days in constant darkness until the larvae therein attained instar II or III. Sandwiches were then randomly assigned to one of the four treatments. Full spectrum lighting was used in the environmental chambers. Pupation of individuals was monitored for up to 100 days after being placed in the treatment.

2.4. Experiment II: effect of instar IV maturity on diapause induction

Based on earlier results suggesting diapause induction just prior to the prepupal stage (Hansen et al., 2001b), we tested for timing of induction sensitivity within instar IV. This and the remaining experiments were conducted in constant darkness. Larvae were initially reared in phloem sandwiches at constant 21 °C. After all larvae within the sandwich attained instar IV, sandwiches were maintained at 21 °C for various durations (0–15 days) such that there was a range of instar IV maturity represented before being placed into a diapause inducing temperature (12 °C). The experiment was repeated with an initial rearing temperature of 18 °C rather than 21 °C. The duration at this temperature after the instar IV molt ranged from 4 to 17 days before being placed at 12 °C. The experiment was conducted a third time with a rearing temperature of 15 °C. Because this temperature might induce diapause if larvae are exposed to it before instar IV (Hansen et al., 2001b), sandwiches were initially maintained at 18 °C until all individuals attained instar III at which time the sandwiches were transferred to 15 °C. The duration at 15 °C ranged from 0 to 16 days after the instar IV molt before transfer to 12 °C. Pupation during the experiment was considered evidence of diapause aversion and no pupation at the end of the experiment was considered evidence of diapause induction.

2.5. Experiment III: determination of cryophase threshold

Using a diapause-averting thermophase (18 °C; 12 h duration), we tested a range of cryophase temperatures (0, 3, 6, 9, 12, and 15 °C; 12 h duration) for evidence of diapause induction. Larvae were initially reared at 21 °C until they were instar II or III, and then placed into the experimental temperature regimes. Pupation during the experiment was considered evidence of diapause aversion and no pupation, when the experiment was ended after 131 days, was considered evidence of diapause induction.

2.6. Experiment IV: interactions among thermocycle, instar IV maturity and duration in cold

We examined diapause induction using four thermocycle treatments near or below the induction threshold identified in Experiment III. The thermocycle treatments were as follows, with thermophase (T) and cryophase (Cr) durations of 12 h each: 18T:12Cr, 18T:9Cr, 15T:12Cr, and 15T:9Cr. Before placement into one of the thermocycle treatments, larvae were reared at 21 °C through 0–9 days past the instar IV molt. Because of intra-phloem sandwich variability in development times, some larvae were instar III when the sandwiches were transferred to the thermocycle treatment. For these individuals, we recorded the number of days to complete instar III during the treatment. Larvae were maintained in the thermocycle regimes for 5, 15, 25, or 35 days before being returned to 21 °C. The number of days to complete instar IV (i.e., pupation) was recorded for each individual. Because developmental delays were not pronounced as with the previous

experiments, we made no attempt to classify the diapause status of experimental organisms. Additionally, the thermocycle data were overly complicated for use in the instar IV developmental rate model (described in Section 2.9). Instead, the results were used to validate that model.

2.7. Experiment V: interactions among lifestage, temperature, and duration in cold

Results from Experiments I–IV suggested that there might not be any discrete diapause threshold or lifestage, rather a complex continuum of conditions affecting diapause induction. For example, diapause may be relatively difficult to induce in immature (instar I) and mature (advanced instar IV) larvae but easier to induce in an intermediate lifestage. Moreover, the influence of low temperature and duration of exposure to low temperature may not be discrete but, instead, vary continuously through multiple lifestages.

This experiment was similar to Experiment IV except that we used an array of constant temperatures (6, 9, and 12 °C) rather than thermocycle regimes and examined the response of instars II, III, and IV rather than focusing on instar IV. Larvae within phloem sandwiches were reared at 21 °C to the target lifestage then placed in the experimental temperature for 7, 21, or 35 d before being placed at 18 °C to monitor pupation. Number of days to complete instar IV (i.e., pupation) was recorded for each individual.

2.8. Statistical analyses

For Experiments I and II, statistical analyses were made using generalized linear mixed models (PROC GLIMMIX, SAS Institute, Cary, North Carolina, USA; Littell et al., 2006). Ratios of generalized chi-square to degrees of freedom were used to check for overdispersion. Denominator degrees of freedom were specified as Kenward–Roger type. For Experiment I, development times were compared among long- and short-day photoperiods; error distribution was specified as Poisson. For Experiment II, we tested the rearing temperature, days after the instar IV molt before being placed at 12 °C, and their interaction for predicting diapause induction. In this case the response variable was binary (0 = pupation, 1 = no pupation), and we used a binomial error distribution. Graphical analyses were used to interpret developmental delays due to diapause induction of individuals in Experiment III. In Experiments IV and V, developmental delays of individuals among treatments were minimal and statistical models were insufficient for interpreting results from these experiments.

2.9. Instar IV developmental rate model

Data from Experiments I, II, and V were used to parameterize a model describing instar IV developmental rates, including diapause (measured as developmental delay), as a function of temperature. The thermocycle data from Experiments III and IV were overly complicated for this purpose but we did include the development time data reported in Hansen et al. (2001b). Our modeling approach includes three submodels which were parameterized simultaneously using a maximum likelihood estimation (MLE) procedure and all available data. First, a “diapause induction” submodel calculates an index of the amount of time, during instars III and IV, below a diapause induction threshold temperature. Based on our observations suggesting a continuous response to diapause-inducing conditions, we assumed a logistic function:

$$I_D = \frac{1}{1 + e^{\alpha(D_0 - D)}}, \quad (1)$$

where I_D is the index of diapause inducing conditions, α is the maximal rate of transition from non-diapause to diapause response

(i.e., the maximal steepness of a logistic curve ranging from no diapause inducing conditions to maximal diapause inducing conditions), D_0 is the number of days below the threshold temperature required to give an index of 50%, and D is the cumulative number of days spent by an individual below the threshold temperature during instars III and IV. D is calculated as:

$$D = \sum_{i3, i4} \text{Days with } T \leq T_D, \quad (2)$$

where $i3$ and $i4$ indicate that the summation is applied to instars III and IV, T is the temperature experienced by the larva; and T_D is the diapause induction threshold temperature.

Second, a “diapause-related developmental delay” factor submodel estimates the degree of developmental delay as a function of I_D :

$$F = 1 + (t_D - 1) \times I_D = \frac{t_D + e^{\alpha(D_0 - D)}}{1 + e^{\alpha(D_0 - D)}}, \quad (3)$$

where F is the diapause-related developmental delay factor applied to the non-diapausing developmental rate and t_D is the maximum diapause-related developmental delay factor. The inclusion of t_D reflects the observation that diapause-inducing temperatures may result in substantial developmental delays but they do not indefinitely prevent pupation (Hansen et al., 2001b). The diapause-related developmental delay factor (F) acts to continuously adjust the instar IV developmental rate. When D is small, F is close to 1 and the developmental rate is close to the non-diapausing developmental rate. When D is large, F is close to t_D and the developmental rate is maximally suppressed.

The third submodel estimates the non-diapausing, temperature-dependent instar IV developmental rate. The model's form is based on the rate function described by Logan et al. (1976). For purposes of fitting, rate curves were converted into predicted development times and fitted to observed development times using MLE. This has the advantage of not altering the variance structure by transforming observations from times (days to complete lifestage) to rates (fraction of lifestage completed each day) (Bentz et al., 1991; Yurk and Powell, 2010). The equation, expressed as a rate, is:

$$r(T) = \begin{cases} 0, & T < p_1, \\ p_2 [(e^{p_3(T-p_1)} - 1)(e^{p_3(p_5-p_1)} - 1)e^{(t-p_5)/p_4}], & p_1 < T < p_5, \\ 0, & p_5 < T, \end{cases} \quad (4)$$

where $r(T)$ is the temperature-dependent developmental rate (without diapause); p_1 is the lower developmental threshold; p_2 is the peak rate control parameter; p_3 is the low temperature acceleration of rates; p_4 is the width of upper thermal boundary layer; and p_5 is the upper developmental threshold.

To predict instar IV temperature-dependent development times as a function of diapause-inducing conditions, the number of days (D) during instars III and IV spent below the threshold (T_D) is calculated, giving the individual's diapause-related developmental delay factor (F). Development time (t_{Apred}) is then predicted by integrating the rate curve over the individual's temperature exposure, prorated by the diapause-related developmental delay factor:

$$1 = \int_0^{t_{Apred}} \frac{1}{F} r(T(t)) dt, \quad (5)$$

where r is the instar IV developmental rate; T is temperature (°C); and t is time (days). That is, the non-diapause developmental rate is suppressed by a factor of $1/F$. These equations were used to make predictions for all input developmental data, assuming log-normal distribution of errors. For each individual (j) we calculated:

$$\delta_j \left(\frac{t_{4pred}}{t_{4obs}} \right). \quad (6)$$

Parameters were then chosen to minimize the negative-log-likelihood (NLL):

$$NLL = \sum_j \left[\frac{(\ln(\delta_j) + \frac{\sigma^2}{2})^2}{2\sigma^2} + \frac{1}{2} \ln(2\pi\sigma^2\delta_j^2) \right]. \quad (7)$$

There were 10 parameters to be estimated (nine plus the variance, σ^2), three of which were manually chosen to ensure that values were within known biological limits: (1) the upper developmental threshold, which is $\geq 21^\circ\text{C}$ (Hansen et al., 2001b) and $< 27^\circ\text{C}$ (Hansen, unpublished data); (2) the lower developmental threshold, which is $\leq 5.5^\circ\text{C}$ (Hansen et al., 2001b) but assumed not $< 0^\circ\text{C}$; and (3) the thermal threshold for diapause induction, in the range 12 – 16°C (Dyer and Hall, 1977; Hansen et al., 2001b; results from Experiment III). NLL values were compared among iterations of models with different manually-selected parameter estimates (lower NLL values are better). Plots of predicted against observed instar IV development times were also subjectively compared based on how well the data fit within the 95% uncertainty cone, defined as the 2.5% and 97.5% quantiles of the log-normal distributions. The final model, with manually-chosen and MLE-optimized parameter estimates, was selected based on low NLL value and how well the uncertainty cone encompassed observations.

3. Results

3.1. Experiment I: photoperiodic diapause induction

Total time to pupation was compared among treatments. Treatments began 19 days after the phloem sandwiches were constructed, having been held at 21°C during that initial interval. At 18°C , all individuals pupated. Long- (mean = 26.4 days, including time as instar III; standard deviation = 5.7, $n = 8$) and short-day treated (mean = 28.2 days, including time as instar III; standard deviation = 5.8, $n = 6$) larvae were not significantly different in development times ($F_{1,12} = 0.24$; $p = 0.6341$). At 12°C , no pupation was observed in either photoperiod treatment after 100 days when the experiment was ended. In contrast, development time of non-diapausing instar IV is predicted to require about 29 days at 12°C (see Section 3.6). Regardless of the photoperiod treatment, 18°C treated prepupae did not exhibit evidence of diapause while 12°C treated prepupae did.

3.2. Experiment II: effect of instar IV maturity on diapause induction

The majority of larvae that were moved from 18 or 21 – 12°C within 10 days after the instar IV molt failed to pupate after an additional 41–78 days when the experiment was ended, an indication that diapause was induced (Fig. 2). Instar IV larvae that were more mature (i.e., > 10 d past the instar IV molt) when placed at 12°C , however, pupated within 10–34 days despite the cool temperature. None of the larvae pre-treated at 15°C pupated after 53–78 days at 12°C , regardless of instar IV age when placed at 12°C . Diapause induction (i.e., failure to pupate) was significantly affected by the number of days after the instar IV molt before being placed at 12°C ($F_{1,30} = 8.44$; $p = 0.0068$). The effect of pre-treatment temperature (15, 18, or 21°C) was marginally significant in explaining diapause induction ($F_{1,30} = 3.78$; $p = 0.0613$).

3.3. Experiment III: determination of cryophase threshold

Thirteen of 14 surviving larvae in the warmest cryophase treatment (15°C) pupated (mean time = 39.6 d after the treatment started including time as instar III, range 27–61 d). The 12°C

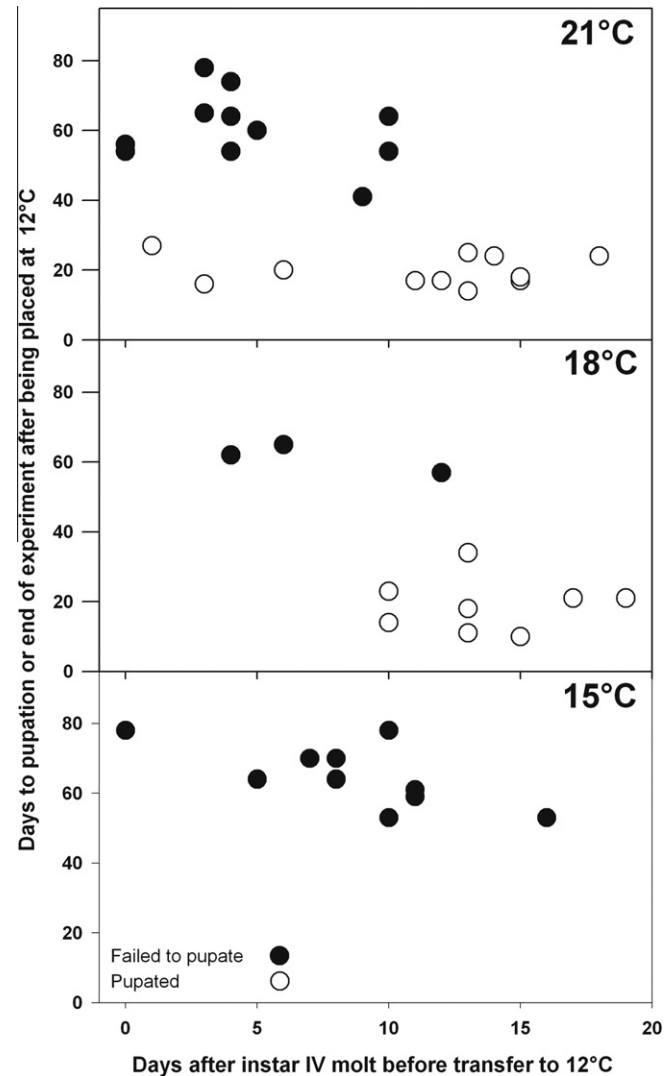


Fig. 2. Incidence of pupation as a function of instar IV maturity in a range of pre-treatment temperatures (15, 18, or 21°C) before being placed at 12°C (Experiment II). Zero days on the x-axis marks the instar IV molt. Zero days on the y-axis indicates the transfer to 12°C .

cryophase treatment also had 14 surviving larvae but only two pupated (mean time = 41.0 d after the treatment started including time as instar III, range 34–48 d). When the experiment was ended 131 days after starting the treatment, the other 12 individuals remained in the prepupal stage. No pupation was observed in any of the colder cryophase treatments (Fig. 3). Linear interpolation of these results indicates that a cryophase threshold of about 13.6°C should be associated with 50% incidence of diapause induction.

At the end of the experiment, insects failing to pupate were placed at 21°C to monitor any additional development. All larvae had developed to the prepupal phase well before this time. Pupation was observed for these insects 8–41 days after being placed at 21°C , with a mean time of 20.1 days. By comparison, the entire prepupal phase of non-diapausing larvae requires, on average, 5.3 days at 21°C (Hansen et al., 2001b).

3.4. Experiment IV: interactions among thermocycle, instar IV maturity, and duration in cold

None of the experimental insects exhibited developmental delays as discernible as those in the preceding experiments

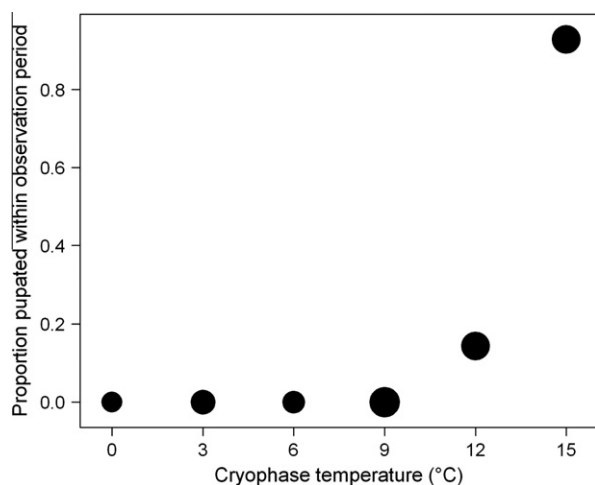


Fig. 3. Proportion of pupated spruce beetles within the observation period (131 d) in thermocycle regimes with an 18 °C thermophase (12 h) and cryophase temperatures varying from 0 to 15 °C (Experiment III). Larvae were instar II and III when the experiment was started and beetles that pupated did so within 27–61 d. Circle size is proportional to number of observations at each coordinate.

(Fig. 4). Development time appreciably beyond that expected given the temperatures (based on the non-diapausing temperature-dependent rate model described in Section 3.6 and the variance reported in Hansen et al., 2001b) was observed in only 15 individuals. Most of those 15 insects had been in their respective thermocycle regimes for 25 or 35 days. Statistical analysis was not possible given that we could not distinguish the diapause state of these prepupae with only minor developmental delays. Two treatments (18T:12C and 18T:9C) were identical in temperature regime to two treatments in Experiment III, wherein substantial

developmental delays were observed. In this experiment, most larvae had molted to instar IV before placement in the thermocycle, whereas larvae were instar II or III when the treatments were started in Experiment III. Moreover, Experiment IV had a 35 d maximum in the thermocycle treatment whereas time in the thermocycle treatment was 131 d in Experiment III. The relatively “normal” development despite exposure to cool temperatures early during instar IV appears contrary to results from Experiment II. In Experiment II, however, exposure to cool temperatures was ongoing. These results indicate that diapause-related developmental delays requires exposure to at least 25 days of cool temperatures and that increasingly cooler temperatures do not increase the likelihood of developmental delays. Results from this experiment were used to evaluate the instar IV developmental rate model (see Section 3.7).

3.5. Experiment V: interactions among lifestage, temperature, and duration in cold

As with Experiment IV, developmental delays varied continuously and we did not conduct statistical analyses for testing diapause induction. Again, only about 15 individuals exhibited development times appreciably longer than expected given the temperatures (Fig. 5). Using estimated diapause-independent developmental rates in preliminary data exploration (based on the non-diapausing temperature-dependent rate model described in Section 3.6), treatment temperature (6, 9, or 12 °C) appeared not related to unexpected developmental delays. Treatment duration, however, appeared correlated with developmental delays beyond that expected under diapause-independent development. More specifically, prolonged exposure to cool temperatures (i.e., <15 °C) during instar IV or instars III and IV combined were associated with developmental delays. Exposure to cool temperatures during instars II, III, or II and III combined were not linked with unexpected development delays.

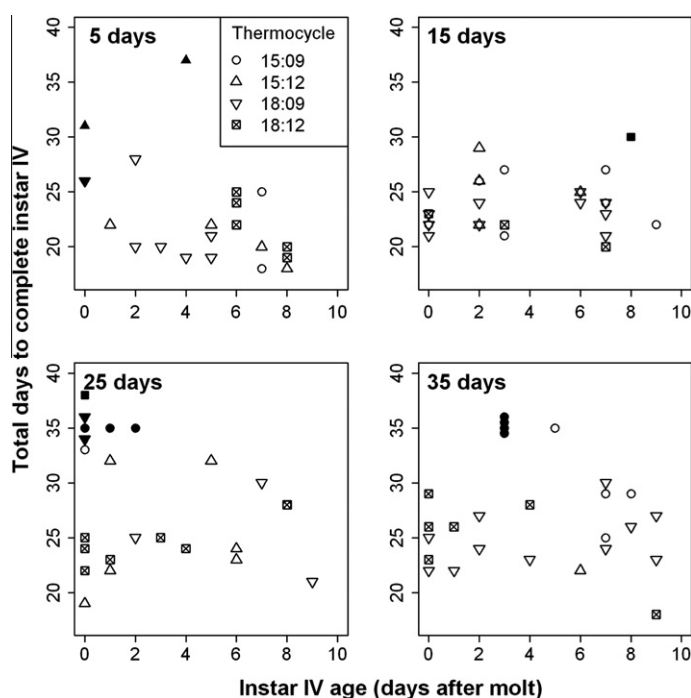


Fig. 4. Time to complete instar IV as a function of instar IV maturity (days past the instar IV molt at 21 °C when placed in a thermocycle treatment). Each panel depicts the response according to the total duration (5, 15, 25, or 35 d) in the thermocycle before being returned to 21 °C. Open symbols are observations with development times within expected ranges, based on the non-diapausing temperature-dependent rate model described in Section 3.6 and the variance reported in Hansen et al. (2001b). Solid symbols are observations with longer than expected development times.

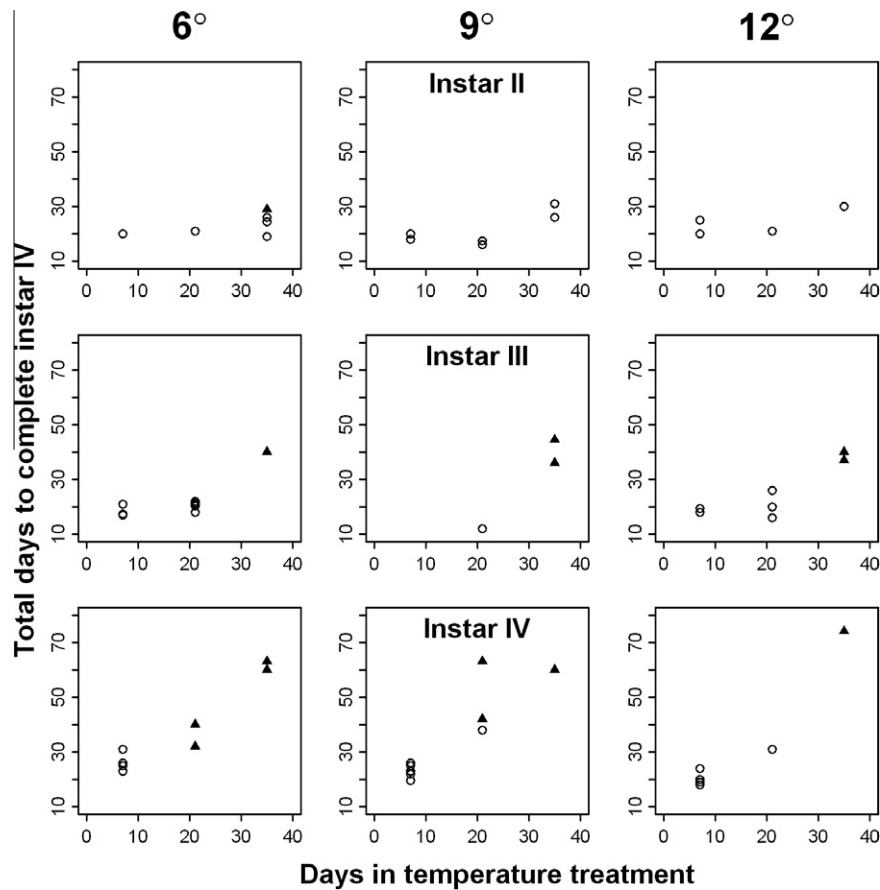


Fig. 5. Total days to complete instar IV as a function of days (7, 21, or 35) in a cool constant temperature treatment (left column 6 °C; middle column 9 °C; right column 12 °C) by instar at the beginning of the cool temperature treatment (top row instar II; middle row instar III; bottom row instar IV). Larvae were reared at 21 °C before the treatment started, then placed at 18 °C after the allotted duration in the cool temperature. Solid triangles are observations with longer than expected development times, based on the non-diapausing temperature-dependent rate model described in Section 3.6 and the variance reported in Hansen et al. (2001b). Open circles are observations with development times within expected ranges.

3.6. Instar IV developmental rate model

After comparing MLE models estimated using a range of chosen values for T_D (Eq. (2)), p_1 (Eq. (4)), and p_5 (Eq. (4)), we selected a model with the best uncertainty cone fit (Fig. 6) although the NLL value of this model was not the lowest. The model with the lowest NLL value had a worse uncertainty cone fit and a p_1 estimate of 0 °C, a value we presume is unrealistically low. Our assumption of log-normal errors was supported by a histogram of the error distribution (not shown).

Although the model includes a manually-chosen T_D value of 13.5 °C (Table 1), model fit was similar using threshold temperatures slightly higher and lower than 13.5 °C; this is because no experiments included temperatures between 12 and 15 °C. The maximal rate of diapause response transition (α) occurs at about 39 d (D_0 ; Table 1). This reflects Experiment V results wherein 35 d of cool temperature exposure resulted in minor developmental delays while considerably longer exposure to cool temperatures in Experiments I and II resulted in pronounced developmental delay.

The index of diapause inducing conditions (I_D) reflects experimental results correlated with prepupal diapause induction, namely, cumulative time below a threshold temperature during instars III and IV. Increasing values of I_D increase the likelihood of diapause induction. But the observed developmental response to diapause induction is not simply a switch wherein diapause is either expressed or not. Instead, increasing time below T_D results in increasingly delayed development (F; Fig. 7). Minimal developmental suppression is estimated to occur if D is less than

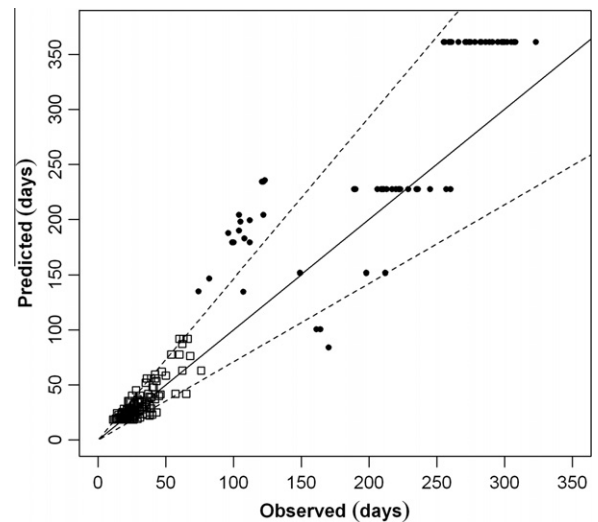


Fig. 6. Predicted against observed number of days to complete instar IV. Predicted times were estimated using the MLE model (Eqs. (1)–(4); Table 1). Solid circles represent data with calculated $I_D \geq 0.5$ and hollow squares represent data with $I_D < 0.5$. These distinctions are arbitrary and are only displayed for the purpose of comparing responses of larvae with high and low I_D values. The solid line represents the 1:1 line and the dashed lines represent the 95% uncertainty cone (2.5 and 97.5% quantiles of the log-normal distributions).

about 20 d while pronounced, near maximal developmental delays should result from more than about 60 d of cool temperatures.

Table 1
Definitions and parameter estimates for Eqs. (1)–(4) using a maximum likelihood estimation (MLE) procedure. Estimates for parameter values in bold were manually chosen, i.e., external to MLE optimization, to ensure values within known biological limits.

Parameter	Interpretation	Units	MLE Estimate
T_D	Diapause induction threshold	°C	13.5
α	Maximal rate of diapause response transition	Days ⁻¹	0.0939
D_0	Cumulative number of days below T_D when α occurs	Days	39.013
t_D	Maximum developmental suppression factor	None	3.5254
p_1	Lower developmental threshold	°C	2.9
p_2	Peak rate control parameter	Days ⁻¹	0.42133
p_3	Low temperature acceleration of rates	°C ⁻¹	0.008805
p_4	Width of upper thermal boundary layer	°C	1.7847
p_5	Upper developmental threshold	°C	24.0

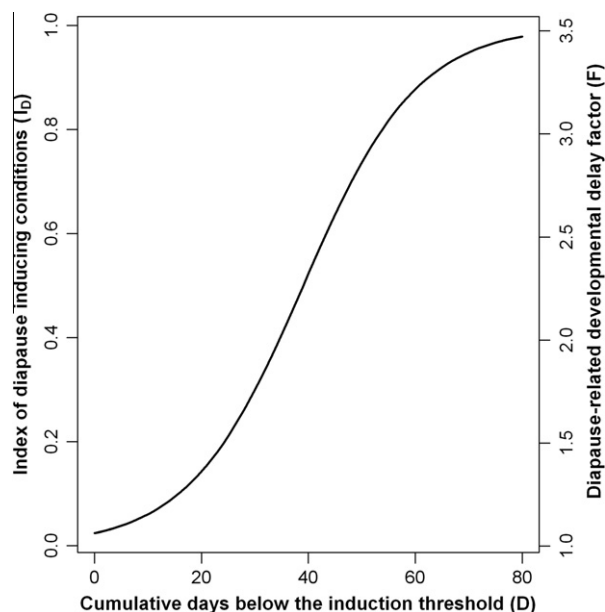


Fig. 7. Index of diapause inducing conditions (I_D ; Eq. (1)) and diapause-related developmental delay factor (F ; Eq. (3)) as a function of cumulative days (D ; Eq. (2)) below the induction threshold (T_D ; 13.5 °C) during instars III and IV. Increasing I_D increases the likelihood of diapause induction, resulting in increasingly reduced developmental rates.

MLE-predicted developmental rates approximate developmental rates without diapause when temperature is above T_D . A steep decrease in developmental rate occurs at the diapause induction threshold, T_D (Fig. 8). The MLE model (Eqs. (1)–(4); Table 1) allows for near non-diapause development if D is small (e.g., <20) and substantially delayed development if D is large (e.g., >60). Minor or intermediate developmental delays result from intermediate values of D . Thus, the MLE model is appropriate given our observation that instar IV development varies continuously in response to diapause-inducing conditions.

3.7. Model evaluation

Data from Experiment IV, which were not used in model parameterization, were used to evaluate the instar IV developmental rate model. Comparing MLE predictions to Experiment IV observations, the majority of data fell within the 95% uncertainty cone with few outlying observations (Fig. 9). Data from Experiment III, which likewise were not used in model parameterization, were not included in the evaluation because none had accurate observations of both instar IV molting and pupation dates. Nonetheless, the model substantially under-predicts time to complete instar IV under the thermal conditions in Experiment III. Recall that most

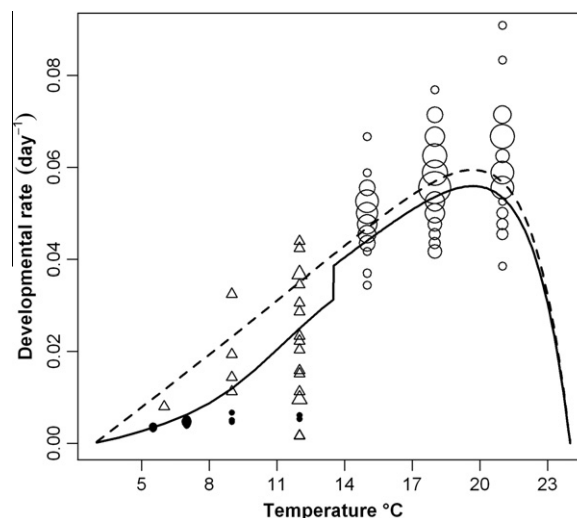


Fig. 8. Instar IV developmental rate as predicted by the MLE model that includes prepupal diapause (solid line; Eqs. (1)–(4); Table 1) overlaid with the non-diapause developmental rate (dashed line; Eq. (4) only). Circles are constant temperature data from Hansen et al. (2001b); hollow circles represent observations without evidence of diapause and solid circles represent observations apparently including diapause. Hollow triangles are “pulse” data from the current study wherein individuals passed portions of instar IV at temperatures below and above the diapause induction threshold (Experiments II and V). Symbol size is proportional to number of observations at each coordinate (because of very small differences among rates at cooler temperatures, there are more observations than apparent in this figure at 5.5, 7, and 9 °C). Note that depicted developmental rates are for constant temperature with the larva being exposed to that temperature until instar IV is completed. Under field conditions (i.e., with varying thermoperiods), increasing values of D will increasingly suppress the developmental rate even for temperatures $>T_D$.

insects had not pupated after 131 days (of which an estimated 6–12 d were passed as instar III), yet the model predicts instar IV completion after 34 d in 18T:12Cr, 43 d in 18T:9Cr, 58 d in 18T:6Cr, 82 d in 18T:3Cr, and 82 d in 18T:0Cr.

4. Discussion

Relative metabolism, measured as O_2 consumption or CO_2 production, has been widely used as an indicator of the insect diapause state (Tauber et al., 1986 and references therein; Danks, 1987 and references therein). Our results suggest that the occurrence of histolysis during the prepupal phase, in preparation for pupation, masks reduced respiration associated with diapause in this species. Denlinger et al. (1972), for example, found markedly lower O_2 consumption among non-diapausing *Sarcophaga* flesh flies from puparium formation through adult eclosion although rates dropped even lower for diapausing pupae. In an apparently non-diapausing flea, *Xenopsylla conformis*, CO_2 production was

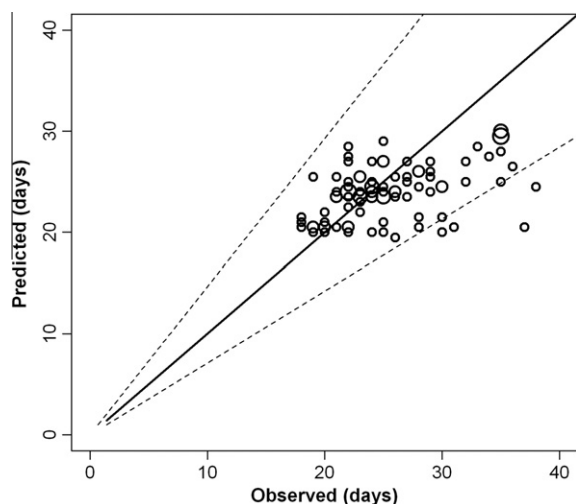


Fig. 9. Predicted against observed number of days to complete instar IV using observations from Experiment IV. Each 12 h cryophase was counted as 0.5 *D* (Eq. (1)). These data were *not* used to parameterize the MLE model (Eqs. (1)–(4); Table 1). The solid line represents the 1:1 line while the dashed lines represent the 95% uncertainty cone. Circle size is proportional to number of observations at each coordinate.

lowest in cocooned stages (includes prepupa, late and early stage pupa, and pre-emerged adult) compared to larval and adult stages (Fielden et al., 2001); among the cocooned stages, rates were lowest among prepupae although these were not significantly different than those of early pupae, late pupae, or pre-emerged adults. Although comparatively low respiration rates during the prepupal stage are not universal among holometabolous insects (e.g., Gromysz-Kalkowska and Hubicka, 1988; Takahashi-Del-Bianco et al., 1998), our results indicate that spruce beetle prepupal respirometry is not indicative of developmental delays, i.e., diapause. All spruce beetle prepupae exhibited similarly reduced respiration regardless of whether they pupated soon after reaching the prepupal stage or were developmentally-arrested for multiple weeks in the prepupal stage (Fig. 1). Although these results may suggest that *all* spruce beetles undergo prepupal diapause, with diapause development proceeding relatively quickly if temperatures are continuously $>15^{\circ}\text{C}$, this hypothesis is not supported by supplemental Experiment III results in that pupation was further delayed despite rearing at constant 21°C after 131 d in the thermocycle treatments. Previous studies have used respiration rates to investigate diapause in the prepupal stage of other species (e.g., Schneiderman and Williams, 1953; Takeda, 1978; Kemp et al., 2004) but these did not include comparison to non-diapausing prepupae, so it is unclear if our results are specific to the spruce beetle.

Another feature of diapause is a developmental delay in the diapausing lifestage (Tauber et al., 1986; Danks, 1987; Zaslavski, 1988) and modeling the conditions that result in developmental delay was our motivation for this work. Because respirometry was not indicative of developmental delays, direct observation of morphological development was a more relevant metric for assessing spruce beetle prepupal diapause status. Our experiments found evidence of developmental delays that cannot be explained simply by relatively low temperatures, and we were able to describe conditions associated with diapause induction. Among Experiments I–III, pupation was suppressed for many weeks and months in some treatments and we presumed these insects were in diapause. Among Experiments IV and V, however, observed developmental delays were much shorter, on the order of a few days to a few weeks. Developmental delays of only a few days are within the range of possible diapause responses (Danks, 1987; Zaslavski,

1988) and developmental delays on the order of 10 days have been used for classification of prepupal diapause status among two hymenopterans (Rabb and Thurston, 1969; Brodeur and McNeil, 1989). Nevertheless, we were not confident classifying the diapause state of spruce beetles with minor developmental delays given the variance in developmental rates across a range of temperatures (Fig. 8). Rather than assign diapause status to insects with minor developmental delays, we used the observations in a model of instar IV developmental rates that includes the continuous effects of diapause inducing conditions.

At this point, the reader may ask whether prepupal diapause has been adequately demonstrated for spruce beetle. For example, a relatively high threshold for pupation, such as has been proposed for mountain pine beetle (*Dendroctonus ponderosae* Hopk.; Logan and Bentz, 1999), could explain some of our results. The lower developmental threshold for pupation (i.e., completion of instar IV), however, is no higher than for any other spruce beetle lifestage (Hansen et al., 2001b), albeit developmental rates at temperatures $<15^{\circ}\text{C}$ are very slow (Fig. 8). Another alternative explanation is that cool-treated prepupae lack the necessary momentum, as a consequence of reduced feeding and metabolism, to pupate at cool temperatures. Under this hypothesis it is sensible to expect, simply, longer times to complete instar IV as temperatures cool and there should be some simple lower threshold for pupation. Indeed, a continuous relationship between developmental rates and temperature was observed for other spruce beetle lifestages including the pupal lifestage which also requires metamorphosis to complete (Hansen et al., 2001b). But spruce beetle instar IV had a disconnected, rather than continual, response when reared at constant temperatures (Fig. 8). This suggests that two developmental processes are at play, one at temperatures $>15^{\circ}\text{C}$ and another at temperatures $<15^{\circ}\text{C}$. In the latter case, we presume that simultaneous morphological and diapause development are resulting in unexpectedly long development times. Additionally, Experiment III results provide compelling evidence that the phenomenon we observed is diapause rather than a simple quiescence, lack of momentum, or phenomenon related to pupation thresholds. Despite a favorable thermophase temperature of 18°C , most individuals failed to pupate within 131 d when the cryophase was $\leq 12^{\circ}\text{C}$. Even if development were *completely* suspended during the cryophase, development in the absence of diapause during the 18°C thermophase should have resulted in pupation within about 35 d, twice the mean expected time at a constant 18°C (17.6 d; Hansen et al., 2001b).

Our results suggest that temperature plays a role in spruce beetle diapause induction independent of photoperiod. Photoperiod alone was insufficient for diapause induction, a result consistent with other investigations (e.g., Beck, 1982; Bradford and Roff, 1997). We found that the temperature cue for diapause induction must be received early in instar IV, otherwise pupation proceeds despite relatively cool temperatures (Fig. 2). Extrapolating from data in Hansen et al. (2001b), the cue must begin no later than about mid-way through completion of instar IV. This result does not support Hansen's et al. (2001b) suggestion that the induction-sensitive phase is during late instar IV or early prepupa. A few observations suggest instar III may be part of the induction sensitive phase (Fig. 5), but all observations with developmental delays suggesting diapause included low temperature exposure during early instar IV.

The temperature threshold for diapause induction appears to have some variation, probably due to genetic differences as well as ongoing responses to conditions encountered, including differences related to thermocycle versus constant temperature treatments. Dyer and Hall's (1977) results indicated a threshold of 15.1°C and Hansen et al. (2001b) suggested a threshold near 15°C . Our results indicate a threshold of 13.6°C should result in diapause induction

for 50% of prepupae although 12 °C did not induce diapause in all experimental insects nor did 15 °C avert diapause for all experimental insects (Fig. 3). Temperatures increasingly cooler than 15 °C did not appear to increasingly affect the incidence or degree of developmental delays associated with spruce beetle prepupal diapause, although we found a marginal effect of pre-treatment (diapause averting) temperature in Experiment II.

The duration of exposure below the threshold was important for diapause induction. Almost none of the insects exposed to cool temperatures <21 d exhibited developmental delays and all insects with substantial delays were exposed to cool temperatures for >40 d. Insects with minor delays were exposed to cool temperatures for 21–35 d. Beck (1982) also found that time below a threshold temperature was important in diapause induction for a lepidopteran, *Ostrinia nubilalis*, and that the absolute temperature relative to the threshold made little, if any, difference. Time below a threshold temperature is analogous to short day lengths for insects responding to photoperiod (Danks, 1987).

In agreement with Danks (1987), our results suggest that spruce beetle prepupal diapause should be considered a continuous process, not an “all-or-none phenomenon”. We observed multiple insects wherein development was delayed in the prepupal stage beyond that explained by, simply, the temperature regime yet these delays were only on the order of days to a few weeks (Figs. 4 and 5). In all of these cases, larvae were exposed to temperatures <15 °C for 21–35 d including during early instar IV. When returned to a favorable temperature (18 or 21 °C), development resumed after the brief delay. And while pupation can occur at 12 °C or colder, prolonged developmental arrest seems to require ongoing temperatures <15 °C. Many authors have considered this type of response to indicate diapause “intensity” or “depth” (Tauber et al., 1986; Danks, 1987 and references therein) although we cannot assess intensity without first distinguishing and measuring the thermal response of this species’ diapause development (Gray et al., 2001).

Our primary motivation for this work was to understand diapause-related developmental delays for inclusion in a phenology model. To that end, we developed a temperature-dependent developmental rate model for spruce beetle instar IV that includes diapause as a continuous process. Using MLE optimization, the model estimates developmental rates and these are continuously modified by diapause inducing conditions. The temperature-dependent developmental rate model (without diapause; Eq. (4)) was developed using techniques successfully applied to other insects including mountain pine beetle (Logan et al., 1976; Bentz et al., 1991; Powell and Logan, 2005). The effect of diapause considers all elements we found essential for diapause induction (Eqs. (1)–(3)). Cumulative exposure to diapause inducing temperatures during instars III and IV continuously modifies the degree of suppression applied to the developmental rate curve (Figs. 7 and 8). Also, because cool temperatures do not indefinitely prevent pupation (Hansen et al., 2001b), the model includes a parameter that limits the duration of developmental suppression (t_D ; Eq. (3)). The model well-predicts an independent data set (Experiment IV; Fig. 9) although it substantially under-predicts observations from Experiment III. Under field conditions, the model will apply some amount of developmental suppression to relatively cool temperature regimes resulting in predicted developmental delay. This diapause-related delay should take the modeled insect into the onset of winter, at which point cold temperatures (<2.9 °C; Table 1) will suppress further development. That is, we expect the model will successfully distinguish univoltine and semivoltine cycles given a range of thermal regimes from subalpine environments.

Although the physiological mechanism for spruce beetle prepupal diapause is unknown, our results suggest that a molting inhibitor may be either maintained or temporarily added with

continued exposure to cool temperatures. For example, cool temperatures could elicit the production of juvenile hormone (JH), in turn preventing the release of ecdysteroids needed for pupation (Takeda, 1978; Chippendale, 1983; Raabe, 1989). Hypothetically, the spruce beetle larva’s brain could be controlling JH titers in a dynamic manner such that pupation is merely delayed, rather than arrested, upon the return of favorable temperatures. This hypothetical mechanism is not in accord with classical descriptions of the diapause response (Tauber et al., 1986) but it does fit our observations that pupation can be delayed for short or long intervals depending on the thermal regime encountered.

The continuous diapause response model is ecologically satisfying. Spruce beetle host trees grow in subalpine climates, which generally have short growing seasons but are also unpredictable in that relatively warm conditions are possible. Prepupal diapause acts to reduce the likelihood of overwintering in the pupal stage which is thought to be a cold-intolerant lifestage for the mountain pine beetle (Amman, 1973; in dozens of mid-winter field samples, we have not observed spruce beetle pupae, only prepupae and teneral adults, Hansen and Bentz unpublished data). Therefore, a semivoltine life-cycle ensures adaptive seasonality (Logan and Bentz, 1999) under typical subalpine conditions. A univoltine cycle, however, confers two considerable benefits: (1) reduced exposure to woodpecker predation and lethal winter temperatures (Knight, 1961); and (2) greater overall population success (Amman, 1973; Hansen and Bentz, 2003). A continuously-varying diapause response affords spruce beetle maximal opportunity to develop on a univoltine cycle, should favorable conditions prevail, while bestowing adaptive seasonality should cool conditions prevail. Thus, the spruce beetle’s ongoing responses to environmental conditions acts to select the most advantageous pathway given the conditions encountered (Danks, 1991). Our model describes the continuous response of spruce beetle instar IV larvae to temperature and includes the effect of diapause on temperature-dependent developmental rates. This model can be included in a lifestage-specific phenology model for predicting spruce beetle voltinism and, ultimately, population success under a changing climate.

Acknowledgements

We thank Ryan Bracewell for assisting with phloem sandwich observations and Dr. David Denlinger for help interpreting the impact of metamorphosis on insect metabolism. Dr. David Turner reviewed the statistical methods. We are grateful to two anonymous reviewers for their constructive comments. This research was funded by the Rocky Mountain Research Station, U.S. Forest Service. The use of trade or firm names in this paper is for reader information only and does not imply endorsement by the U.S. Department of Agriculture of any product or service.

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