

Embryonic stage of obligatory diapause and effects of abiotic conditions on egg hatching in the balsam twig aphid, *Mindarus abietinus*

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Abstract

Diapause-mediated dormancy in overwintering insect eggs has rarely been studied with regard to the ecological factors controlling postdiapause development. In insects of temperate latitudes, water availability at the end of winter, in interaction with temperature, could control the resumption of development for insect stages in postdiapause quiescence. The balsam twig aphid, *Mindarus abietinus* Koch (Hemiptera: Aphididae), overwinters as eggs in southern Québec, Canada, on balsam fir, *Abies balsamea* (L.) Miller (Pinaceae), in Christmas tree plantations, where it is known as a pest. Previous work has shown that eggs of this aphid maintain low water content during winter, presumably to survive sub-zero temperatures. Conversely, in late winter and early spring, they passively or actively absorb surrounding moisture, which is accompanied by notable changes in size, shape, and fresh mass. The primary objective here was to determine the embryonic stage at which winter diapause starts and is maintained in *M. abietinus*, a relatively primitive aphid. Secondly, we tested the hypothesis that free water availability to postdiapause eggs, in combination with temperatures above developmental threshold, is essential for embryonic development and hatching, by experimentally soaking field-collected eggs in water at controlled frequencies. We observed that embryogenesis starts at the time of egg laying and stops after a few days, before the anatrepsis stage of blastokinesis is complete, when the germ band has not yet entirely immersed itself into the yolk. We also found that water surrounding overwintered eggs on fir shoots, in interaction with temperature regime, significantly increases *M. abietinus* egg hatching rates. Potential impacts of environmental factors such as precipitation are discussed in relation to *M. abietinus* egg hatching rates and potential for population growth in spring.

Introduction

Insects from temperate latitudes show great diversity in seasonal adaptations to adverse environmental conditions (Danks, 2007). For northern insects of the boreal forest, diapause-mediated dormancy, wherein development is restricted by external and/or internal controls, is a common survival strategy against harsh winter conditions (Tauber et al., 1986; Leather et al., 1993; Denlinger, 2002; Hahn & Denlinger, 2011). The life stage in which an insect enters this state of metabolic depression and developmental arrest varies greatly between species, ranging from early embryonic stages within the egg to fully mature adults

(Denlinger, 2002). In certain insect species, diapause seems to be genetically determined and occurs at a particular stage, even in the absence of typical diapause-inducing stimuli such as temperature and photoperiod (i.e., obligatory diapause) (Košťál, 2006).

This is probably the case for the balsam twig aphid, *Mindarus abietinus* Koch (Hemiptera: Aphididae), an insect known as a pest for the aesthetic damage it causes to growing fir shoots in spring on balsam fir, *Abies balsamea* (L.) Miller (Pinaceae), in commercial Christmas tree plantations (Kleintjes et al., 1999; Fondren & McCullough, 2003). Overwintering eggs, usually located near the base of fir needles, are deposited by sexually-reproducing females in early July. These eggs survive the winter in obligatory diapause and complete embryogenesis to hatch only the following year in early

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May in southern Québec, Canada (Deland et al., 1998; Doherty et al., 2017).

As for most terrestrial arthropods in northern North America (Sømme, 1982), *M. abietinus* overwintering eggs are most likely freeze intolerant (Turnock & Fields, 2005; Doherty et al., 2018a), a strategy in which the supercooling point of the egg is lowered in order to avoid spontaneous ice formation at sub-zero temperatures (Bale, 1987; Bale & Hayward, 2010). In diapausing eggs, partial desiccation may substantially decrease their supercooling point by concentrating egg fluid solutes like polyols, which have a cryoprotective effect at high concentrations (Zachariassen, 1985; Gehrken & Sømme, 1987; Danks, 2000; Holmstrup et al., 2010).

Based on recent observations (Doherty et al., 2018a), we know that *M. abietinus* overwintering eggs partly desiccate after being laid on fir shoots, presumably to avoid freezing, by maintaining a relatively low water content during winter diapause. Eggs can thus be visually separated into three categories: completely flat and non-viable (C1), semi-flat with a visible depression on their top surface (C2), and round and turgid (C3). After several months in diapause, they absorb surrounding moisture (i.e., liquid free water and/or ambient air humidity) in late winter and early spring, thus visibly increasing in size, shape, and mass, from C2 to C3 (Doherty et al., 2018a). Water runoff temporarily retained by fir shoots during snowmelt and/or rainfall could be important sources for such noticeable changes in postdiapause egg shape and fresh mass. However, it is unknown how water presence, be it liquid or vapour, affects egg diapause, resumption of embryonic development in the spring, and subsequent hatching rates.

Interactions between temperature, water access, and relative humidity have only rarely been studied in regard to postdiapause development in insect eggs (e.g., Hand, 1983; Bethke & Redak, 1996; Pires et al., 2000). For *M. abietinus* overwintering eggs, surrounding water during snowmelt and ambient relative humidity may both play a role in diapause termination and/or postdiapause quiescence (Doherty et al., 2018a), once diapause development is complete. If moisture influences postdiapause egg development (Tauber et al., 1998), direct or indirect contact with water may be critical to successful hatching of *M. abietinus* eggs (Hodek, 2003).

As diapause termination is a complex phenomenon involving both internal and external cues, our current understanding is far from complete (Košťál, 2006). For insects in northern climates, diapause termination can occur as early as January when the organism enters a state of postdiapause quiescence. At this stage, development rate is reduced by limiting environmental conditions, especially temperature below the lower developmental

threshold, and dryness (Denlinger, 2002; Hodek, 2002). Theoretically, development for an insect in postdiapause quiescence should respond directly to temperature and possibly moisture, once favourable conditions are met, usually during the seasonal rise of ambient temperature (Danks, 1987).

Moreover, little is known about the actual stage during which aphid egg diapause begins and embryonic development is suspended. To our knowledge, the only detailed study on this subject is that by Behrendt (1963), who determined that eggs of the black bean aphid, *Aphis fabae* Scopoli (Hemiptera: Aphididae), enter diapause during the anatrepsis phase of embryogenesis. This early stage of blastokinesis involves immersion of the embryo into the yolk at the posterior pole of the egg, to form the germ band and the amnion, thus inverting the anterior-posterior and dorsal-ventral axes of the embryo (Heming, 2003; Miura et al., 2003; Panfilio, 2008). However, nothing is currently known about the stage at which diapause is initiated and embryonic development stops in primitive, conifer-feeding aphids like *M. abietinus*.

The main objectives of this study are two-fold. First, we wish to characterise the stage of *M. abietinus* diapause through microscopic photography of freshly collected eggs from the field, from the time of deposition by sexual females in early July to completion of embryogenesis in March and April of the next year. We aimed to determine at which point embryonic development is halted, indicating diapause initiation, and whether the normal response to favourable conditions and postdiapause development has returned by midwinter. We hypothesise that, like its distant aphid cousin *A. fabae*, diapause for *M. abietinus* eggs could be initiated during the early stages of blastokinesis. Second, we aimed to test the effects of temperature in interaction with water access (i.e., temporary contact by immersion in water) and air moisture on the hatching rate of postdiapause eggs. We tested the hypotheses that higher temperatures in the favourable range should decrease development time, and that moisture determines hatching rates, either by an increase in relative air humidity or by direct contact with surrounding water. This study should help understand how the early seasonal interactions between temperature and moisture affect postdiapause embryonic development in overwintering insect eggs.

Materials and methods

Diapause termination and postdiapause egg development

The objective here was to estimate the time of diapause termination from hatching rates measured under controlled conditions and to observe postdiapause egg development during the critical period in late winter and early spring.

From 18 February to 24 April 2017, fir shoots carrying overwintering eggs were collected weekly from a balsam fir plantation (45.905°N, 71.036°W) near the municipality of Courcelles in southern Québec, Canada. In order to avoid premature development, samples were kept at 2 °C until eggs were counted. When an egg was located, the fir shoot was cut down to 1 cm in length with a razorblade. Two eggs were randomly distributed per 2-ml microtube (Starstedt, Nümbrecht, Germany) modified with holes pierced through the lid for air circulation. These were placed in a growth chamber set to provide a sinusoidal temperature regime of daily fluctuation between 7 and 13 °C, averaging 10 °C, in a PGR15 growth chamber (Controlled Environments, Winnipeg, MB, Canada) set to a L16:D8 photoperiod and 65% r.h., which are likely to be favourable conditions for *M. abietinus* postdiapause egg development during springtime. Eggs were monitored daily for hatching and temperatures were recorded hourly with an integrating HOBO data logger (Onset, Cape Cod, MA, USA) to estimate remaining physiological time before hatching (see below). For data analytical purposes, hatching was assumed to have occurred at midpoint between two consecutive observations (i.e., a precision of ± 12 h). We used a lower development threshold of 5.4 °C, as previously determined (Doherty et al., 2018b), in order to regularly estimate the remaining physiological time for completion of embryonic development of *M. abietinus* eggs collected weekly (converted to growing degree days above development threshold, $GDD_{5.4}$). Hourly temperatures were also recorded in the plantation with a HOBO Pro v2 data logger (Onset) installed in the middle height section of a tree, ca. 1 m aboveground and shielded from direct exposure to sunlight, in order to relate the accumulation of physiological time in $GDD_{5.4}$ in the growth chamber to natural development and hatching in the field. $GDD_{5.4}$ accumulation in the plantation was calculated using method 2 in McMaster & Wilhelm (1997), which has proved to be useful for springtime temperatures (Doherty et al., 2018b). To confirm that development was complete in the field, hatching rates were observed in the experimental plantation and also in two other plantations located nearby on 28 April 2017.

Observing embryogenesis of eggs before and after diapause

Every week during postdiapause development in March and April 2017, around 10 freshly-collected eggs were carefully removed from the shoot under a stereomicroscope. Individual eggs were directly placed on a microscope slide, submerged in water, and lightly pressed under a cover glass to be observed and photographed under a microscope at 200 \times with a C-mount camera, in order to follow natural embryonic development

during the season. Pictures that clearly portrayed key steps of embryogenesis were matched with the daily accumulation of $GDD_{5.4}$ during late winter and early spring 2017 (see above). In order to determine whether embryogenesis begins before diapause is initiated after egg laying in summer, eggs, which were laid in the 1st week of July 2017 based on field observations, were regularly collected and similarly observed and photographed during July and August 2017.

Testing the effects of temperature and moisture on hatching rate

Overwintering eggs ($n = 260$) were collected in the plantation on 27 February 2017 and categorised for shape as described in Doherty et al. (2018a). Fir shoots bearing the eggs were randomly distributed equally among 12 assigned containers designed to control air moisture (ca. 21 eggs per container). Rearing containers consisted of two 19.4-l clear plastic boxes sealed together with tape, in which the fir shoots were kept hydrated in Aquapics stuck into foam sheets. Two desiccants were added to six of the rearing containers, in order to maintain relative humidity as low as possible (i.e., dry air treatment). Desiccants used were 99% purified silica gel (Laboratoire MAT, Québec City, QC, Canada) and Drierite (VWR International, Mont-Royal, QC, Canada) which were replaced as needed when indicated by colour change due to high moisture content, usually every 2–3 days. These two desiccants were used jointly in order to better maximise dehumidification in the dry air containers. The six other rearing containers were used for the high air moisture treatment, by keeping water levels constant at the bottom of containers, below the foam sheets. This assured that the air was saturated around shoots bearing eggs. Container replicates of both air moisture treatments were then equally allocated to two groups of six, which were randomly assigned to a growth chamber set to provide one of two temperature regime treatments: a ‘warm’ regime fluctuating between 7 and 13 °C (as described above) and a ‘cold’ regime fluctuating from 2 to 8 °C, both with the same controlled photoperiod and relative humidity conditions. In each temperature treatment (i.e., growth chamber), the six containers thus formed three replicate pairs of a dry air and a humid air treatment. Each pair was then randomly assigned to one of three soaking treatments. Soaking consisted of immersing the entire shoot in water for ca. 2 s, which was assumed to mimic frequent shoot drenching in water during springtime snowmelt, as previously observed (Doherty et al., 2018a). Shoots were either soaked daily, weekly, or never (control). To assure control for slight

fluctuations in actual abiotic conditions in containers, relative humidity and ambient temperature inside all rearing containers were monitored for at least a week with a HOBO Pro v2 data logger. Eggs were observed daily before soaking and any change in shape was noted, until hatching. Unhatched eggs at the end of the experiment were observed under a microscope to determine whether any development had occurred.

Statistical analysis

Experimental data were analysed with SAS v.9.4 (SAS Institute, Cary, NC, USA) using Proc LOGISTIC for the binary outcome of egg hatch. Fixed effects were temperature (cold or warm daily fluctuating regime), relative humidity (dry or humid), soaking frequency (never, daily, or weekly), and their interactions. Since the model consisted of class variables, Wald χ^2 tests were performed with significance level set to $\alpha = 0.01$ to reduce the chances of committing a type I error (Daniel & Cross, 2013), for there was 'pseudoreplication' of rearing containers in a same growth chamber, which could slightly underestimate the within-container variance. Least square means for the proportion of egg hatching per rearing container were estimated along with their 95% confidence intervals (95%

CI). Firth's penalised maximum likelihood estimation was also applied to the analysis, for there was 100% hatching in one of the rearing containers (Firth, 1993).

Results

Diapause termination during late winter and early spring egg development

Only 17.6% of *M. abietinus* field-collected eggs on 18 February 2017 hatched when placed in the fluctuating temperature regime, the rest were nonviable (Figure 1). At this time, 48.6–67.3% (95% CI based on the modified Wald method for binomial proportions) of collected eggs were categorised as C2 (visibly depressed). However, 80.0% of eggs collected 9 days later on 27 February hatched, when 71.9–82.1% of eggs were categorised as C3 (round and turgid) and only 6.6–14.0% were C2. A period of abnormally high winter temperatures was recorded in the plantation between 18 and 27 February 2017 (Figure 1A). Afterwards, most eggs were C3, the rest of them being completely flat and nonviable (C1 eggs). We noted that only C3 eggs hatched in the growth chamber. Hatching rates in the growth chamber varied between 65.0 and 92.5% for subsequent collection dates and the estimated

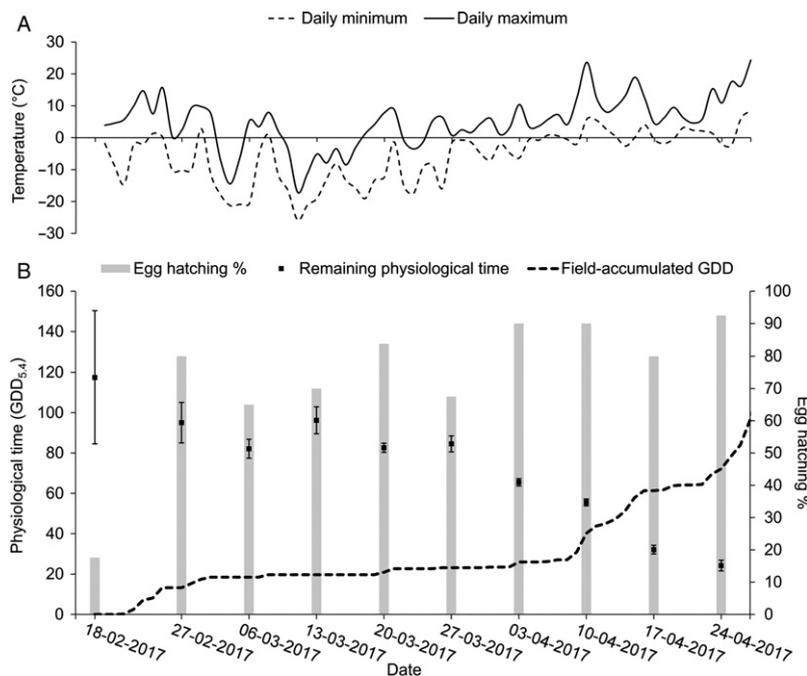


Figure 1 Accumulation of physiological time for postdiapause eggs of *Mindarus abietinus* in relation to field temperatures in Courcelles, Québec, Canada, in 2017. (A) Field daily maximum and minimum temperature fluctuations in late winter and early spring. (B) Hatching rate and mean (\pm 95% confidence interval) estimated remaining physiological time to stem mother hatching based on growing degree days above development threshold (GDD_{5.4}) during postdiapause development up to egg eclosion under controlled conditions in the growth chamber. Accumulated GDD_{5.4} is calculated based on daily field temperatures recorded in the balsam fir plantation.

remaining physiological development time above threshold ($GDD_{5.4}$) decreased as the season advanced (Figure 1B). As predicted, field-accumulated $GDD_{5.4}$ increased gradually as temperatures rose (Figure 1). Also, mean (\pm 95% CI) egg hatching in the region of Courcelles was estimated at $75.7 \pm 7.2\%$ on 28 April in plantations.

Prediapause and postdiapause embryogenesis

The terminology for aphid embryogenesis used here is that of Blackman (1987) and Miura et al. (2003). When field-collected eggs ($n \approx 10$) of a particular date were observed under the microscope, most had advanced to a similar stage of embryonic development. Thus, a single representative picture for the selected collection dates is shown in Figure 2. Eggs collected on 5 July 2017 were only 1–2 days old, as mature oviparae were then observed walking on fir shoots and eggs were still bright yellow and had not yet blackened (i.e., serosal cuticle not yet produced). The bacterial mass (Figure 2A), in the posterior pole of the egg, appeared as a light brown zone clearly distinct from the

yolk, which filled the rest of the egg. The dorsal surface of the egg was covered in wax filaments produced by the ovipara during oviposition (Figure 2A). In eggs collected on 9 July, formation of the blastoderm on the inner periphery of the egg appeared with the synchronous formation of cell membranes around cleavage energids (Figure 2B). By this time, the eggshell had changed from bright yellow to dark green, but the chorion was still translucent enough for observation of egg content under the microscope. On 12 July, the bacterial mass had started to move from the posterior end of the egg towards the center, revealing the formation of the columnar embryonic primordium or germ band, which we could not clearly delimitate through the blackened serosal cuticle (Figure 2C). Following this date, the bacterial mass apparently stopped its migration from the posterior end of the egg, at approximately one third the length of the egg (Figure 2C). All eggs observed from mid-July until mid-August (four collection dates) were very similar in this respect. Therefore, we conclude that embryonic development had stopped, regardless of

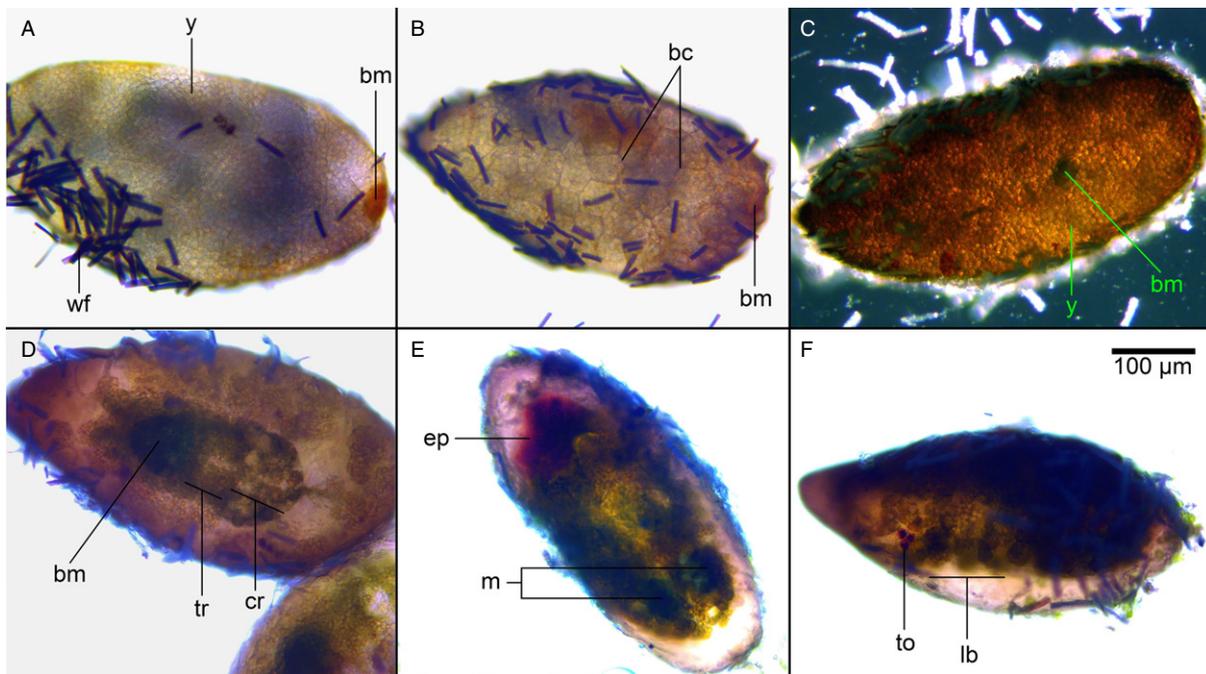


Figure 2 Stages of (A–C) prediapause and (D–F) postdiapause embryonic development of *Mindarus abietinus* eggs. Note that pre- and postdiapause eggs were all collected in 2017 and thus belong to successive generations of sexual reproduction. Anterior pole of the egg points to the left, and ventral surface of the egg faces upward, except in panel F which is a lateral view. (A) Recently deposited egg (5 July), the orange-coloured bacterial mass is visible at the posterior pole. (B) Cellularised blastoderm visible through the chorion (9 July). (C) Germ band sinking into the yolk during anatrepsis (2 August), the apparent stage of developmental arrest in early diapause. (D) Anatrepsis complete (23.1 $GDD_{5.4}$ on 27 March). (E) Katatrepsis complete (40.1 $GDD_{5.4}$ on 10 April). (F) Embryonic development nearly complete with stem mother eye formed (61.3 $GDD_{5.4}$ on 17 April). Abbreviations: bc, blastoderm cellular limits; bm, *Buchnera* bacterial mass; cr, cephalic region; ep, eye primordia; lb, limb buds; m, mycetome; to, triommatidium; tr, thoracic region; wf, wax filaments; y, yolk. [Colour figure can be viewed at wileyonlinelibrary.com]

temperature, just before complete immersion of the embryo into the yolk to complete anatrepsis (i.e., the first blastokinetic movement) (Miura et al., 2003).

For the eggs collected in late winter 2017 (18 February), the bacterial mass was still at the same position as observed for eggs collected in mid-August. However, significant changes had occurred for eggs collected on 27 March 2017, when the embryo had clearly immersed itself into the yolk, thus completing anatrepsis. The posterior end of the embryo, along with the bacterial mass, now faced the anterior pole of the egg (Figure 2D). Katatrepsis, the second embryonic movement involving exit from the yolk mass, was complete for eggs collected on 10 April (Figure 2E), as the cephalic region of the embryo then faced the anterior pole of the egg, as indicated by the reddish eye primordia. The formation and bilateral splitting of the dark green mycetome along the midline, and the presence of eye primordia indicate that development had well advanced by 10 April. Eggs collected ca. 10 days before hatching on 17 April had a complete triommatidium and three limb buds, presumably for each thoracic segment (Figure 2F). Estimates of field-accumulated GDD_{5.4} for egg eclosion were matched to early spring embryogenesis stages for panels D, E, and F in Figure 2.

Interactions between temperature and moisture affecting hatching rate

Mindarus abietinus eggs hatched after 14.7 ± 2.3 (mean \pm 95% CI) days of incubation in the warm treatment and 41.4 ± 6.5 days in the cold treatment. Only the C3 eggs, which represented the majority, hatched and thus the C1 and C2 eggs were excluded from the analysis (Table 1). Recorded average relative humidity for all treatments is presented in Table 2. Air relative humidity in containers and its interactions with temperature and/or soaking frequency had no significant effect on hatching rate, nor did the interaction temperature*soaking

Table 1 Analysis of variance for fixed effects of temperature regime, ambient air relative humidity, soaking frequency, and their interactions, on hatching rates of *Mindarus abietinus* post-diapause eggs, based on Wald χ^2 tests ($\alpha = 0.01$)

Effect	d.f.	Wald statistic	P
Temperature (T)	1	8.8878	0.0029
Air moisture (M)	1	0.3520	0.55
T*M	1	0.8166	0.37
Soaking (S)	2	13.7717	0.0010
T*S	2	9.1233	0.010
M*S	2	1.5701	0.46
T*M*S	2	5.1199	0.077

Bold values indicate significance $P < 0.01$.

frequency, although marginally so in this case (Table 1). However, soaking frequency and temperature regime both had a significant direct effect on hatching rate (Figure 3). When least square means were sliced for temperature regime, soaking frequency became highly significant ($\chi^2 = 18.73$, d.f. = 2, $P < 0.0001$) for the warm treatment, with both weekly and daily soaking significantly increasing hatching rate (Figure 3). Soaking did not have a significant effect on hatching rate when sliced for temperature regime in the cold treatment ($\chi^2 = 0.63$, d.f. = 2, $P = 0.73$), which is not surprising.

No significant changes in egg shape were noted, except in the warm dry air and no soaking treatment, where 44.4% apparently lost water and visibly changed from C3 to C2. Some of the unhatched eggs from each treatment were observed under the microscope to determine whether

Table 2 Mean (\pm 95% CI) relative humidity (%) in 12 rearing containers ($n = 735$ readings, once every 15 min)

Air moisture treatment	Soaking treatment	Temperature treatment	
		Cold	Warm
Dry	Never	44.2 \pm 1.3	50.7 \pm 1.8
	Weekly	75.9 \pm 1.3	36.2 \pm 0.9
	Daily	69.8 \pm 1.7	48.5 \pm 2.6
Humid	Never	96.4 \pm 0.2	95.1 \pm 0.3
	Weekly	99.8 \pm 0.1	96.9 \pm 0.2
	Daily	99.7 \pm 0.1	98.0 \pm 0.4

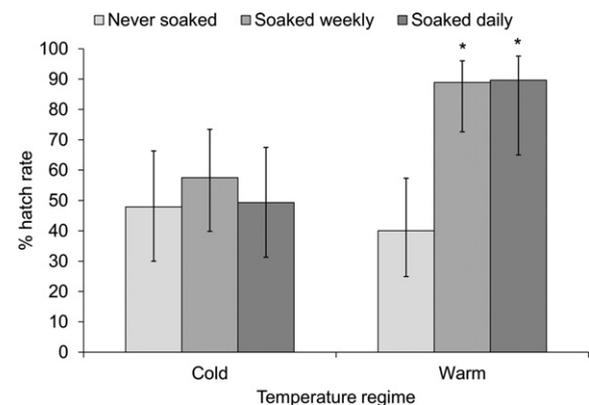


Figure 3 Least square mean estimates (\pm 95% confidence intervals) of *Mindarus abietinus* postdiapause egg hatching rates (%) provided by the Wald χ^2 tests, according to temperature regime and soaking frequency. Most unhatched eggs in the low-temperature regime (2–8 °C) contained a fully-developed stem mother nymph, but no apparent development was observed in unhatched eggs from the high-temperature regime (7–13 °C). The asterisk indicates a highly significant effect of soaking frequency compared to the 'no soaking' control ($*P < 0.0001$).

any embryogenesis had occurred. The unhatched eggs in the high-temperature regime contained no visible embryonic structures, indicating that no development had occurred and the eggs were probably nonviable. However, the majority of unhatched eggs in the low-temperature regime contained a near to fully-developed nymph that had apparently died within the egg, possibly because hatching movements were inhibited. No eclosion was noted in these eggs, kept under observation for over 2 weeks in that regime after mean hatching was observed.

Discussion

A key finding of this study is prediapause embryogenesis in *M. abietinus* overwintering eggs. Incipient development was not only visible from the exterior colour change of fertilised eggs, from bright yellow to black (Blackman, 1987), but also from microscopic imagery taken at regular intervals during July and August. Blastoderm formation in field-collected eggs was associated with eggshell darkening, probably caused by the tanning of a cuticle deposited by the serosa (Miura et al., 2003). By mid-July, the early formation of the column-like embryonic primordium was indicated by the migration of the darker bacterial mass towards the center of the egg. However, when the bacterial mass stopped its migration towards the center of the egg once it reached approximately a third of the length, no further morphological progress was noted between mid-July and mid-August. Presumably, the apparent developmental arrest of this early-stage embryo is mostly independent of temperature and/or other summer conditions and is thus internally controlled, which is characteristic of obligatory diapause (Tauber et al., 1986; Denlinger, 2002).

Our observations indicate that *M. abietinus* embryogenesis stops before complete immersion of the germ band into the yolk, just before anatrepsis is completed. This would place the *M. abietinus* diapause initiation slightly earlier than observed in *A. fabae* by Behrendt (1963), who reported that egg diapause occurs at the end of anatrepsis, after complete germ band immersion into the yolk, which would have been observed here if this was the case for *M. abietinus*. The simple microscopic preparation used here may have partly limited detailed observation of germ band formation through the darkened serosal cuticle and the yolk. Nevertheless, our observations are clearly consistent with those of Behrendt (1963) and, among others, for aphids overwintering as eggs in temperate latitudes (Blackman, 1987). This is unlike eggs of the pea aphid, *Acyrtosiphon pisum* Harris, whose diapause is not a complete cessation of development, but most likely a very slow developmental pathway taken by sexually produced embryos during winter (Shingleton et al., 2003).

Mindarus abietinus eggs are laid in early July and apparently stop developing in mid-July, regardless of environmental cues, especially photoperiod, which is relatively stable shortly after the summer equinox in late June, but diminishes rapidly in late summer. This endogenous interruption of egg development in *M. abietinus* follows the production of the sexuales (i.e., males and oviparae) by the winged, parthenogenetic viviparous females, the sexuparae, which mature and disperse by flight in late June in Québec (Doherty et al., 2017). Hypothetically, the sexuparae could be the sensitive stage to the diapause-inducing stimuli suggested by Tauber et al. (1986), known as grandparental control. In this scenario, the abiotic conditions surrounding the development of sexuparae would determine the fate of *M. abietinus* overwintering eggs, although we cannot exclude genetic control. However, few studies have looked at these effects on the production of sexuales by sexuparae in aphids (e.g., Blackman, 1975; Margari-topoulos & Tsitsipis, 2002).

Partial prediapause embryogenesis in *M. abietinus* in the preceding summer may help explain their early hatching in May (Deland et al., 1998; Doherty et al., 2017), weeks before the spruce spider mite, *Oligonychus ununguis* (Jacobi), and the pine needle scale, *Chionaspis pinifoliae* (Fitch), two other known Christmas tree pests overwintering as eggs in southern Québec (Doherty et al., 2018b). Prediapause development possibly is a specific adaptation of *M. abietinus* to its main host, the balsam fir. Hatching nymphs need to settle onto fir needles very early in the spring in order for the maturing stem mothers to be ready to migrate onto new-year foliage when bud break occurs, to finally mature and found a colony on the burgeoning shoot (Fondren & McCullough, 2003). Partial embryonic development in the previous year would thus hasten early spring postdiapause development when temperature and, apparently, moisture requirements are met.

Data from our experiment suggest that postdiapause development is possible in the field only once free water intake can occur, most likely by absorption through the serosal cuticle, as indicated by change in egg shape and moisture content from C2 to C3, which was observed during late February 2017 and was matched with an increase in hatching rate. The long period of relatively high temperatures between 18 and 27 February 2017 likely provided the conditions of snowmelt on fir shoots, which is consistent with what had been observed in 2016 (Doherty et al., 2018a). Therefore, we suggest that the interactions between temperature, snowmelt, and possibly rainfall trigger water intake in postdiapause *M. abietinus* eggs, which could be tested on C2 eggs collected in January and experimentally exposed to soaking treatments similar to what was done here. It is probable that eggs enter postdiapause

quiescence by midwinter, which is indistinguishable from the actual diapausing state when abiotic conditions directly inhibit development (Tauber et al., 1986; Denlinger, 2002).

The data support our hypothesis that contact with moisture improves hatching rates, but only for the high-temperature regime which fluctuated daily between 7 and 13 °C. Hatching rates in the low-temperature regime (2–8 °C) did not vary significantly between soaking treatments, which can be explained by frequently observed condensation on fir shoots under these conditions. This occurred mainly in the weekly and daily soaking treatments, and only occasionally in the humid air container where no soaking occurred. The lower temperatures apparently did not allow for complete evaporation on soaked fir shoots and, as a result, some of the soaked eggs remained continuously submerged in water. As eggs are usually located at the base of fir needles, water frequently forms a meniscus between the needle and the shoot (J-F Doherty, pers. field obs.), submerging any egg present. Unhatched eggs from the low-temperature regime most often contained a nearly or fully developed first-instar nymph, which died inside the egg probably due to the hypoxic conditions underwater. Also, it is possible that a lack of energy required to hatch from eggs at temperatures close to 2 °C, experienced daily, may partly explain why nymphs could not break from the eggshell and died as unhatched eggs. Additionally, temperatures under the 5.4 °C threshold could have negatively impacted overall development in the low-temperature regime.

In the high-temperature regime, water excess on soaked shoots usually dried up within a day or two, which can explain substantially increased hatching rates in the weekly and daily soaking treatments. For both temperature regimes, however, relative air humidity did not appear to have any effect on hatching rate. Note that controlling ambient air relative humidity for the dry air treatment was not complete. The desiccants did lower relative humidity considerably, but there was variation between the dry air treatments, partly because soaking the shoots temporarily increased air humidity. Nevertheless, relative humidity did not seem to have any detectable effect even in the control treatment (no soaking). Control shoots were not manipulated and were kept in the relatively stable conditions of the rearing container, either in dry or humid air treatments. Also, in the 'warm dry air, no soaking' treatment, almost half the eggs apparently lost water and strongly resembled the C2 eggs, which characterise the diapause and postdiapause periods of winter. This could be a physiological response to the adverse conditions of desiccation in the rearing container, maybe even a return to quiescence, but further study is needed.

This experiment helped us understand the optimal conditions in which *M. abietinus* postdiapause egg development and fundatrix hatching is possible in the field. If frequently submerged in water for prolonged periods during postdiapause, it is likely that development will occur, but too much may be deadly if condensation persists and water is excessive in the vicinity of eggs. Our results also suggest that temperatures close to 2 °C may not provide enough energy for normal embryonic movements and for developed nymphs to break the eggshell in order to hatch successfully. What clearly favoured hatching in *M. abietinus* eggs in these experiments was daily or weekly soaking in a relatively warm environment, which allowed for temporary direct contact with water before its evaporation from the soaked shoot. In the absence of soaking, only 40% of eggs hatched in the high-temperature regime, which is less than half of those that hatched in the weekly and daily soaking treatments. Therefore, moisture surrounding eggs was temporarily required for increased hatching rates in C3 eggs. As eggs may have absorbed water in the field before they were collected, it is probable that additional submersion is necessary for optimal development. Little snowfall on shoots and low precipitation in spring during postdiapause development of *M. abietinus* eggs may result in low hatching rates, ultimately affecting summer population densities.

Photoperiod and temperature are usually the main focus in studies on insect seasonal ecology and rarely has moisture been regarded as a vital factor in diapause regulation (Tauber et al., 1998). However, water has been shown to be an important environmental cue before, during, and after diapause across several orders of insects and for all developmental stages (for examples, see review by Hodek, 2003). In temperate climates where cold hardiness may depend on dehydration, water intake or absorption may be necessary to allow proper diapause development (Danks, 1987). When moisture is reported as a crucial factor in postdiapause resumption of development (e.g., *Apolygus lucorum* Meyer-Dür in northern China; Jin et al., 2016), an unbalance between water and temperature may ultimately decrease hatching rates, as observed here for *M. abietinus* in the low-temperature regime. As with *M. abietinus*, egg size increase under high-moisture treatment was necessary for successful hatching in *Trirhabda geminata* Horn, a species of southwestern USA (Bethke & Redak, 1996).

In summary, our study has demonstrated that partial prediapause embryogenesis in *M. abietinus* overwintering eggs is required and that diapause is initiated a few days after egg laying, during the formation of the embryonic germ band, apparently soon after anatrepsis commences. The possibility for completion of embryogenesis returns in

the late winter, during postdiapause quiescence. In the seasonal transition period between winter and spring, when temperatures above the lower developmental threshold (5.4 °C) and moisture requirements are met, eggs absorb water and change in size, which occurred in late February in this study. Our laboratory experiment, where access to water and temperature were controlled, supports the hypothesis that an optimal water balance is required for optimal hatching rates of overwintered eggs of *M. abietinus*.

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References

- Bale JS (1987) Insect cold hardiness: freezing and supercooling – an ecophysiological perspective. *Journal of Insect Physiology* 33: 899–908.
- Bale JS & Hayward SAL (2010) Insect overwintering in a changing climate. *Journal of Experimental Biology* 213: 980–994.
- Behrendt K (1963) Über die Eidiapause von *Aphis fabae* Scop. (Homoptera, Aphididae). *Zoologische Jahrbücher* 70: 309–398.
- Bethke JA & Redak RA (1996) Temperature and moisture effects on the success of egg hatch in *Trirhabda geminata* (Coleoptera: Chrysomelidae). *Annals of the Entomological Society of America* 89: 661–666.
- Blackman RL (1975) Photoperiodic determination of the male and female sexual morphs of *Myzus persicae*. *Journal of Insect Physiology* 21: 435–453.
- Blackman RL (1987) Reproduction, cytogenetics and development. *Aphids: Their Biology, Natural Enemies and Control*, Vol. 2A (ed. by AK Minks & P Harrewijn), pp. 163–196. Elsevier, Amsterdam, The Netherlands.
- Daniel WW & Cross CL (2013) *Biostatistics: A Foundation for Analysis in the Health Sciences*, 10th edn. John Wiley and Sons, Hoboken, NJ, USA.
- Danks HV (1987) *Insect Dormancy: An Ecological Perspective*. Biological Survey of Canada (Terrestrial Arthropods), Ottawa, ON, Canada.
- Danks HV (2000) Dehydration in dormant insects. *Journal of Insect Physiology* 46: 837–852.
- Danks HV (2007) The elements of seasonal adaptations in insects. *Canadian Entomologist* 139: 1–44.
- Deland J-P, Berthiaume R, Hébert C & Cloutier C (1998) Programme alternatif de protection du sapin de Noël contre le puceron des pousses dans le contexte d'une saine gestion des ressources environnementales. Ministère de l'Environnement et de la Faune, Québec, QC, Canada.
- Denlinger DL (2002) Regulation of diapause. *Annual Review of Entomology* 47: 93–122.
- Doherty J-F, Guay J-F & Cloutier C (2017) Temperature-manipulated dynamics and phenology of *Mindarus abietinus* (Hemiptera: Aphididae) in commercial Christmas tree plantations in Québec, Canada. *Canadian Entomologist* 149: 801–812.
- Doherty J-F, Guay J-F & Cloutier C (2018a) Early springtime water absorption by overwintering eggs of *Mindarus abietinus* (Hemiptera: Aphididae): possible implications for cold hardiness and diapause termination. *Canadian Entomologist* 150: 174–179.
- Doherty J-F, Guay J-F & Cloutier C (2018b) Novel temperature-dependent development rate models for postdiapause egg eclosion of three important arthropod pests found in commercial Christmas tree plantations of southern Québec, Canada. *Environmental Entomology* 47: 715–724.
- Firth D (1993) Bias reduction of maximum likelihood estimates. *Biometrika* 80: 27–38.
- Fondren KM & McCullough DG (2003) Phenology and density of balsam twig aphid, *Mindarus abietinus* Koch (Homoptera: Aphididae) in relation to bud break, shoot damage, and value of fir Christmas trees. *Journal of Economic Entomology* 96: 1760–1769.
- Gehrken U & Sømme L (1987) Increased cold hardiness in eggs of *Arcynopteryx compacta* (Plecoptera) by dehydration. *Journal of Insect Physiology* 33: 987–991.
- Hahn DA & Denlinger DL (2011) Energetics of insect diapause. *Annual Review of Entomology* 56: 103–121.
- Hand SC (1983) The effect of temperature and humidity on the duration of development and hatching success of eggs of the aphid, *Sitobion avenae*. *Entomologia Experimentalis et Applicata* 33: 220–222.
- Heming BS (2003) *Insect Development and Evolution*. Cornell University Press, Ithaca, NY, USA.
- Hodek I (2002) Controversial aspects of diapause development. *European Journal of Entomology* 99: 163–173.
- Hodek I (2003) Role of water and moisture in diapause development (a review). *European Journal of Entomology* 100: 223–232.
- Holmstrup M, Bayley M, Pedersen SA & Zachariassen KE (2010) Interactions between cold, desiccation and environmental toxins. *Low Temperature Biology of Insects* (ed. by DL Denlinger & RE Lee Jr), pp. 166–187. Cambridge University Press, Cambridge, UK.
- Jin Y, Chen P, Zhang Y, Tian G, Feng H & Hou Y (2016) Effect of water on survival and development of diapausing eggs of *Apolygus lucorum* (Hemiptera: Miridae). *PLoS ONE* 11: e0161657.
- Kleintjes PK, Lemoine EE, Schroeder J & Solensky MJ (1999) Comparison of methods for monitoring *Mindarus abietinus* (Homoptera: Aphididae) and their potential damage in Christmas tree plantations. *Journal of Economic Entomology* 92: 638–643.
- Košťál V (2006) Eco-physiological phases of insect diapause. *Journal of Insect Physiology* 52: 113–127.

- Leather SR, Walters KFA & Bale JS (1993) *The Ecology of Insect Overwintering*. Cambridge University Press, Cambridge, UK.
- Margaritopoulos JT & Tsitsipis JA (2002) Production of sexual morphs by apterous virginoparae of *Myzus persicae* (Hemiptera: Aphididae) in relation to pre- and postnatal exposure to short day conditions. *Bulletin of Entomological Research* 92: 321–330.
- McMaster GS & Wilhelm WW (1997) Growing degree-days: one equation, two interpretations. *Agricultural and Forest Meteorology* 87: 291–300.
- Miura T, Braendle C, Shingleton A, Sisk G, Kambhampati S & Stern DL (2003) A comparison of parthenogenetic and sexual embryogenesis of the pea aphid *Acyrtosiphon pisum* (Hemiptera: Aphidoidea). *Journal of Experimental Zoology* 295B: 59–81.
- Panfilio KA (2008) Extraembryonic development in insects and the acrobatics of blastokinesis. *Developmental Biology* 313: 471–491.
- Pires CSS, Sujii ER, Fontes EMG, Tauber CA & Tauber MJ (2000) Dry-season embryonic dormancy in *Deois flavopicta* (Homoptera: Cercopidae): roles of temperature and moisture in nature. *Environmental Entomology* 29: 714–720.
- Shingleton AW, Sisk GC & Stern DL (2003) Diapause in the pea aphid (*Acyrtosiphon pisum*) is a slowing but not a cessation of development. *BMC Developmental Biology* 3: 7–18.
- Sømme L (1982) Supercooling and winter survival in terrestrial arthropods. *Comparative Biochemistry and Physiology* 73: 519–543.
- Tauber MJ, Tauber CA & Masaki S (1986) *Seasonal Adaptations of Insects*. Oxford University Press, New York, NY, USA.
- Tauber MJ, Tauber CA, Nyrop JP & Villani MG (1998) Moisture, a vital but neglected factor in the seasonal ecology of insects: hypotheses and tests of mechanisms. *Environmental Entomology* 27: 523–530.
- Turnock WJ & Fields PG (2005) Winter climates and coldhardiness in terrestrial insects. *European Journal of Entomology* 102: 561–576.
- Zachariassen KE (1985) Physiology of cold tolerance in insects. *American Journal of Physiology* 65: 799–832.