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Cold tolerance and winter survival of seasonally-acclimatised second-instar larvae of the spruce budworm, *Choristoneura fumiferana*

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Abstract

1. Field-acclimatised spruce budworm larvae, *Choristoneura fumiferana*, supercool to as low as −41.6°C in winter months. Yet the extent to which they can withstand exposures to temperatures slightly above their supercooling point has never been investigated. In both January and February 2018, we tested various combinations of sub-zero temperatures (−37 to −42°C) and exposure durations (0.75–12 h) to estimate the combinations of temperature and exposure durations required to kill half the population (LTT_{50}). At −37 or −38°C, the estimated emergence probability was about 0.80 at all exposure durations. In contrast, the LTT_{50} was reached after 11.4 h at −39°C, 9.4 h at −40°C, and 3 h at −41°C. A temperature of −42°C was fatal to most larvae.
2. During the winters of 2017, 2018 and 2019, survival experiments were conducted at three latitudes (46–48°N) in Québec. Regardless of the year or latitude, none of the daily minimum temperatures recorded in January or February were cold enough to reach the LTT_{50}. However, the sudden drops in temperature that occurred after the winter thaw of March and in early December 2018 were likely responsible for the low proportions of emerged larvae observed.
3. Hence, despite the high capacity of spruce budworm larvae to withstand very low sub-zero temperatures in winter months, they remain highly vulnerable to cold spells during their early diapause or post-diapause development. Such climatic disturbances deserve more attention, as they may increase under climate change.

KEYWORDS
latitudes, lethal temperature–time of exposure, outdoor-acclimatisation, Tortricidae, winter survival

INTRODUCTION

Since the end of the 20th Century, the Arctic pole has warmed more than twice as fast as the average global temperature; a phenomenon called Arctic amplification (Screen & Simmonds, 2010; Serreze & Francis, 2006). Given this enhanced arctic warming, the intensity and frequency of various extreme climatic events, such as cold spells, have increased at mid-latitudes across the Northern Hemisphere (Cohen et al., 2012; Francis & Vavrus, 2012; Overland & Wang, 2015). As the Arctic amplification is expected to continue over the coming decades (Cohen et al., 2014, 2018), exposure of small terrestrial ectotherms, such as insects, to severe winters, may represent a real threat for native or introduced species living in or invading Northern countries. For instance, the cold snap that hit northeastern North America in January 2014 was so intense (−39 to −40°C for several hours) that it caused the collapse of a population of the hemlock looper, *Lambdina fiscellaria* (Guenée), in the Laurentian mountains of Québec, Canada (Delisle et al., 2019). Very similar cold air temperatures were recorded

in northern and southern regions of Québec in the winter of 2009, thereby limiting the long-term establishment of *L. fiscellaria* populations in these areas (Delisle et al., 2013). More recently, Jones et al. (2017) reported high overwintering mortality of the emerald ash borer, *Agrilus planipennis* (Fairmaire), in Syracuse, New York, United States of America, following the very cold front that moved across the entire state in February 2016.

To survive low winter temperatures, insects have developed two main strategies: they can either tolerate freezing or avoid it, depending on their ability to withstand extracellular ice formation. Freeze-tolerant species can synthesise ice-nucleating agents that trigger the nucleation of ice in safe extracellular spaces of their bodies. In contrast, freeze-avoiding or freeze-intolerant species prevent ice formation by supercooling, and thus maintain their body fluids in a liquid state at temperatures well below their melting point but die if freezing occurs (Lee Jr., 2010). The freeze-avoidance strategy is common among insects that overwinter in cold temperate climates of the Northern Hemisphere (Leather et al., 1993). However, this does not imply that all freeze-avoiding species will only die when they reach their freezing temperature or supercooling point (SCP). In fact, some insects may actually die at temperatures slightly above their SCP, if their exposure to such low sub-zero temperatures lasts long enough. Therefore, in addition to the SCP, the "lethal temperature-time of exposure" (LTT) may be an important complementary index of an insect's cold-hardiness, as demonstrated in several species (Delisle et al., 2013, 2019; Jing & Kang, 2003; Knight et al., 1986; Turnock & Fields, 2005).

The spruce budworm (SBW), *Choristoneura fumiferana* (Clem.) is one of the most destructive defoliators of coniferous forests in North America. This species feeds preferentially on balsam fir, *Abies balsamea* (L.) Mill. trees, but also attacks white spruce, *Picea glauca* (Moench) Voss, red spruce, *P. rubens* Sargent and, to a lesser degree, black spruce (*P. mariana*) Mill., Briton, Sterns & Poggenburg (Blais, 1958; Hennigar et al., 2008). In eastern Canada, massive SBW outbreaks have occurred almost every 30 to 40 years for at least the past 3–4 centuries (Blais, 1965; Boulanger et al., 2012; Boulanger & Arsenault, 2004; Krause, 1997; Royama, 1984; Simard et al., 2011). Together, the severity of the defoliation, as well as the length of the epidemic cycle, make the SBW the most threatening insect pest for the Canadian forest industry (MacLean, 2016).

A freeze-avoiding species, the SBW has developed several behavioural, morphological, and physiological adaptations to escape the rigours of winter temperatures (Han & Bauce, 1995a; Marshall & Roe, 2021). For instance, non-feeding first-instar larvae (L1) first find a suitable overwintering site preferentially at mid-crown on the bark of tree trunks or branches, or in flower scars of *A. balsamea*. Then, they spin a silk hibernaculum (Harvey, 1957) to protect themselves against extreme elements or external ice inoculation through the cuticle (Duman et al., 2002; Olsen et al., 1998). However, whether or not the presence of snow on trees can provide additional thermal protection for larvae overwintering in their hibernaculum remains to be seen. Once established in their shelter, L1 larvae remove the green material from their digestive tract (Han et al., 2000; Han & Bauce, 1993), thereby preventing freezing by potential ice nucleators (Duman et al., 1995). The excretion of this green material triggers a change in larval colour (from green to yellow), which coincides with a significant drop in the SCP of field-acclimatised L1's, going from -22.9 to -28.9°C in just a few days (Han & Bauce, 1993). Shortly after, SBW neonates moult into second-instars before entering an obligatory diapause that lasts about 6 months (from mid-August to mid-February) (Régnière, 1990). Meanwhile, field-acclimatised second-instar larvae (L2), reduce their SCP from -34°C in the fall to -42°C in the winter followed by a re-increase to -34°C as mean daily temperature rises in the spring (Han & Bauce, 1995a). Concomitant with these seasonal variations in SCP values, glycerol, a low molecular weight cryoprotectant derived from glycogen (Han & Bauce, 1998), increases ten-fold from early fall to mid-winter after which it progressively disappears in early spring. Such temporal variations in SCP values and glycerol content were not observed in SBW L2's maintained at a constant temperature of 2°C throughout their diapause; consequently, their survival following subsequent exposure to low sub-zero temperatures (-23°C) was considerably reduced (10% survived after 5 days) compared with field-acclimatised larvae (80% survived after at least 15 days) (Han & Bauce, 1995a). These results suggest that field-acclimatisation should be favoured over acclimation at low positive constant temperature (2°C) to accurately assess the thermal limits of SBW larvae, a proposition which Marshall and Roe (2021) supported in their recent detailed review of the overwintering physiology of this species.

Among other cold-adaptations, SBW larvae produce antifreeze proteins (AFPs) or thermal hysteresis proteins (THPs) which prevent seeding ice crystals from growing larger by binding to particular facets of the ice crystals (Graether et al., 2000; Pertaya et al., 2008; Tyshenko et al., 1997). As demonstrated in several species, this ice growth inhibition causes a thermal hysteresis, a difference between the freezing and melting points, which further lowers the SCP and stabilises the metastable supercooled state of the insect (Duman et al., 1995; Zachariassen & Husby, 1982). However, according to Lee Jr. (2010) and Zachariassen et al. (2010), the biological capacity of AFPs to prevent ice from growing may be somewhat limited, especially if the insect supercooled intensely.

SBW populations are expected to migrate towards the Arctic pole and to higher elevations with climate change (Gray, 2008; Régnière et al., 2010). It is thus possible that substantial fractions of such populations die at temperatures well above their mean SCP, depending on the duration of very cold spells (<-39, -40°C, for a few hours or minutes). Furthermore, recent genomic analysis has revealed the existence of three spatially distinct SBW subpopulations (clusters) across its vast distribution area in the boreal forest of North America: (i) Western (Alaska, Yukon), (ii) Central (southeastern Yukon to the Manitoba-Ontario border) and (iii) Eastern (Manitoba-Ontario border to the Atlantic) (Lumley et al., 2020). Cold hardiness experiments conducted with two populations from the Central cluster (Inuvik, North-west Territories and High Level, Alberta) and two from the Eastern cluster (Manic-Cinq, Québec and Campbellton, New-Brunswick) showed evidence of local adaptation: populations from

higher latitudes exhibited greater phenotypic plasticity in response to temperature fluctuations than those from lower latitudes (Butterson et al., 2021).

The present study has two main objectives. The first is to assess the limits of SBW cold tolerance using outdoor-acclimatised L2s obtained from the rearing facility of Insect Production and Quarantine Laboratories (the IPQL, Natural Resources Canada, Great Lakes Forestry Centre, Sault Ste. Marie, Ontario, Canada). Cold tolerance was measured as the temperature and exposure duration required to kill 50% (LTT_{50}) of the L2 populations following exposure to various sub-zero temperatures for selected durations in mid-January and mid-February, the two coldest months of the year in Canada. The second objective is to validate the accuracy and reliability of these thermal limits by testing the ability of the L2s to overwinter successfully along with a latitudinal range of climatic regions of the province of Québec over several consecutive years.

When SBW diapause is completed by mid-February (Régnière, 1990), larvae enter a state of quiescence during which their morphogenesis, still arrested by the cold, can be shortly resumed following exposure to more favourable environments. During this quiescent post-diapause period, SBW larvae gradually lose their cold-hardiness (deacclimation) (Han & Bauce, 1995a) and may become highly vulnerable to repeated freeze–thaw cycles that often occur in early spring (Marshall & Sinclair, 2015). Alternatively, when the L2s are not sufficiently cold-hardy in late fall or early winter, a substantial drop in temperature may cause premature death (Han & Bauce, 1995b). In the course of our field experiments, attention was paid to the possible impact of such climatic hazards on SBW overwintering survival.

MATERIALS AND METHODS

Insects

All insects used in our experiments were produced by the IPQL rearing facility of the Canadian Forest Service. As reported by Roe et al. (2018), the IPQL colony is an outbred population composed of 16 families originating from various regions of Ontario, Canada. As opposed to an inbred population with homozygous genetic background, all matings in this heterozygous outbred population are performed among members of the same colony and new genetic material is not introduced from generation to generation to ensure a stable genetic composition. Each new generation is started by "chance mating" using large polyethylene bags (mating chambers) that contain 100 males and 100 females randomly chosen among all members of a family. There are 10 mating chambers per family, with 16 families per generation. The overall population of these 16 families is apparently large enough to avoid a high risk of inbreeding (Roe et al., 2018).

Each year, from 2017 to 2019, SBW egg masses (ca. 15,000, each containing 30–40 eggs), were shipped to our laboratory in Quebec City in mid- to late August to obtain diapausing L2s required for the experiments described below. Their arrival was synchronised with the oviposition period in the field (Régnière, 1983). Two or three egg masses were immediately placed in individual clear plastic cups (175 ml) (Dixie Consumer Products LLC, Brampton, Ontario, Canada) containing one balsam fir three-branch twig, bearing >100 flower scars to serve as overwintering sites for the newly hatched larvae (Figure 1a). Each cup, covered with a translucent lid, was then transferred to a growth chamber (Environmental Growth Chambers, model GC-15, Winnipeg, MA) maintained at 16 h light: 8 h dark photoperiod, $20°C \pm 1°C$, and $65\% \pm 5\%$ relative humidity for 2 weeks. This allowed enough time for eggs to hatch and neonates to spin up their hibernaculum, preferentially into the flower scars, before moulting to L2 and entering diapause (Figure 1b). To maintain diapausing L2s under optimal laboratory conditions, the temperature in the growth chamber was decreased from 20 to 15°C for the next 2 weeks and further down to 13°C for three more weeks. By late October, when ambient air temperatures were around 10–12°C, all cups were stored in an outdoor insectary to overwinter.

Experiments

Cold-tolerance assay of acclimatised L2s

For the laboratory cold-tolerance assay, a small balsam fir twig (single branch), bearing at least 10 visible hibernacula, was placed in a 1-dram glass vial covered with a small foam plug. In the fall of 2017 preceding the assays, all vials containing diapausing L2s were stored in the outdoor insectary for acclimatisation until further use.

The main objective of the laboratory cold-tolerance assay was to estimate the duration of exposure to constant sub-zero temperatures above the mean SCP required to kill 50% of the L2 population (LTT_{50}). After preliminary tests to determine the ranges of temperatures and exposure durations, all combinations of six constant sub-zero temperatures (-37, -38, -39, -40, -41, and $-42°C$; $\pm 0.05°C$) and eight exposure durations (0, 0.75, 1.5, 2.25, 3, 6, 9, and 12 h) were tested in random order in both mid-January and mid-February 2018. During preliminary cold tests, none of the L2s exposed to $-43°C$ survived after 0.8 h (48 min) of exposure, so this sub-zero temperature was excluded from the experiment. For each combination of temperature and exposure duration, four vials, each containing a single-branch twig with an average of 37 diapausing L2s (minimum: 10, maximum: 90), were put into an ultra-low temperature freezer (Cryo-fridge Revco [Baxter] model SSC750ABA, Asherville NC), with a lower temperature limit of $-50°C$. Exceptions to this procedure were the 0- and 3-h durations for which there were eight replicates per temperature and exposure duration. To verify that the freezer reached the stated temperatures of -37 to $-42°C$ and record temperatures, a Hobo data logger (Hobo Prov2, Hoskin Scientific, Montréal, Québec) was placed inside the cryo-fridge Revco near the vials in each assay.

In all, 480 vials were tested: 2 months $\times$ (6 temperatures $\times$ 6 exposure durations $\times$ 4 vials $+6$ temperatures $\times$ 2 exposure durations $\times$ 8 vials). Exposure duration treatments of 0 h from all test temperatures were considered as a single control with 96 replicates. Prior to each temperature assay, vials containing diapausing L2s (including the control

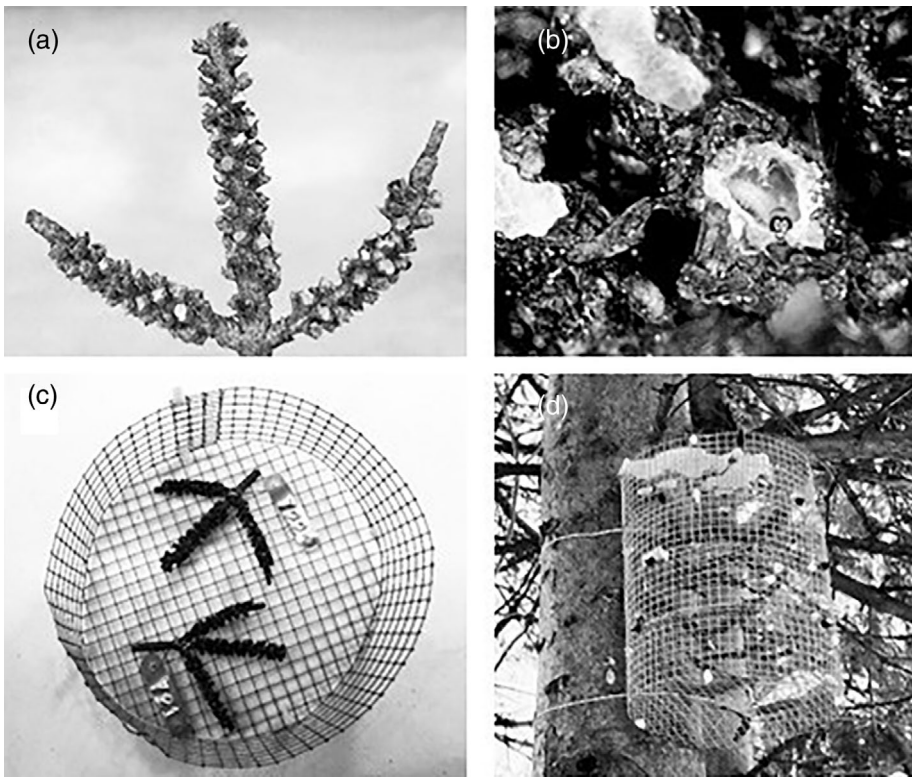

FIGURE 1 SBW hibernacula spun inside the floral scars of a three-branch balsam fir twig (a), second-instar of *Choristoneura fumiferana* larvae (L2) emerging from its hibernaculum (b), two balsam fir three-branch twigs tied to the base of a circular wire-mesh cage (c), four cages attached together to form a tube fixed to a tree trunk (d)

vials) were transferred from the outdoor insectary to a freezer set at −15°C (Kenmore, model 675-83,680-OX, USA), at 07:00 in the morning, for pre-conditioning. Twelve hours later, the L2s were gradually acclimated at −35°C by placing all vials in a double-styrofoam box that was immediately transferred to an ultra-low freezer (Thermo Scientific Revco Ultima Plus, model 5308, Marietta, OH), with a lower temperature limit of −40°C. Using a Hobo data logger similar to that described above, the decrease from −15 to −35°C was reached after 5 h, at a rate of 4°C per hour. The next morning (07:00), at the beginning of each temperature assay, control vials were put in a small styrofoam box and returned to −15°C until the freezing test was completed. After each non-zero exposure duration to any given temperature, experimental vials were returned to −15°C, as were the controls. The rewarming rate was obtained following the opposite procedure used for the cooling rate. When the last exposure duration was completed (19:00) all vials were stored in the outdoor insectary until the next spring.

Two weeks prior to incubation, all vials were transferred from the outdoor insectary to a cool room maintained at 2°C ± 0.5°C, 65% ± 5% relative humidity, under a 16 h light: 8 h dark photoperiod. On April 24, half the vials containing a single-branch twig from each temperature and exposure duration combination, including the controls, were placed in clear plastic Solo cups (29.5 ml) (Solo®, Lake Forest, IL). They were equally distributed among four growth chambers (described above) for incubation at 16 h light: 8 h dark photoperiod, 15°C ± 0.5°C and 65% ± 5% relative humidity (standard conditions). Due to the lack of space in growth chambers and the limited time allocated for counting the emerged larvae (2 h/day from 10:00 to 12:00), the remaining vials were kept for an additional week at 2°C before their incubation on April 30. Throughout the incubation period, the number of larvae that emerged was counted daily. When the emergence period was completed, each twig was dissected to determine the number of larvae that did not emerge.

Overwintering survival of L2s

From 2017 to 2019, winter survival experiments of the L2s were conducted at six experimental sites located at three latitudes (46, 47 and 48°N), with two sites per latitude. The sites were: Armagh (46°45′N, 70°39′W, 271 m), Sainte-Foy (about 5 m from our outdoor insectary) (46°48′N, 71°17′W, 95 m), Épaule (47°15′N, 71°11′W, 778 m), Étape (47°33′N, 71°13′W, 797 m), La Doré (48°43′N, 72°43′W, 190 m) and Albanel (48°55′N, 72°23′W, 161 m), all located in the province of Québec, Canada (Figure 2). The overwintering experiment started on the 1st week of November in all experimental sites. Throughout the season, air temperatures were recorded every 15 min with a shielded datalogger (Pro v2, Hoskin Scientific, Montréal, QC; Solar radiation shield, model 4115-A- MAN-RS1, Onset Computer Corporation, Bourne, MA) attached to the trunk of a tree 3 m above the ground, in each site. On all trees, cages and dataloggers were placed under the branches to protect them from solar radiation and faced north.

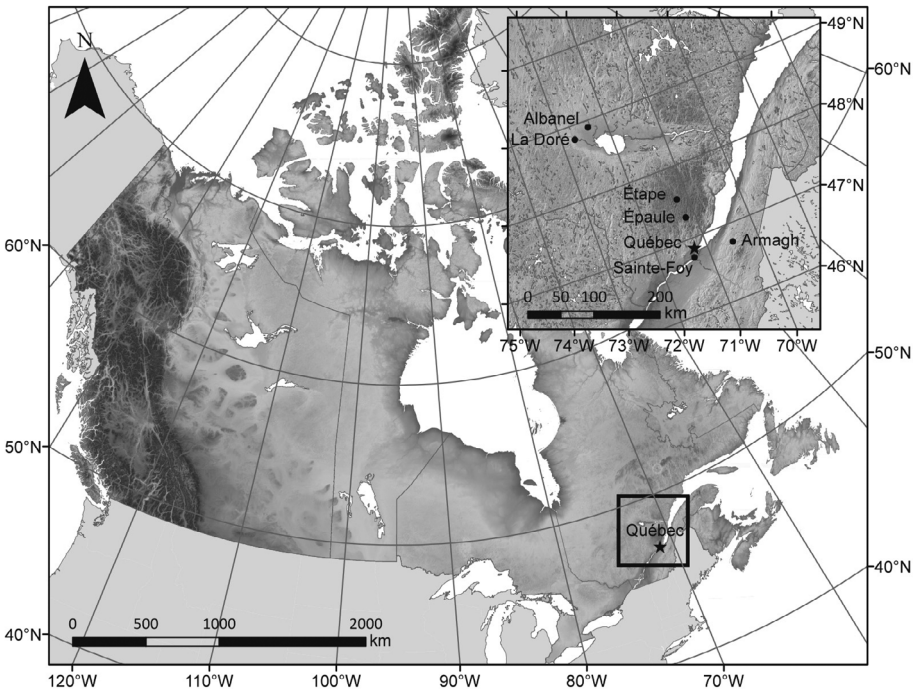

FIGURE 2 Map showing the six experimental sites located in the province of Québec, Canada, at three latitudes: 46°N = Sainte-Foy and Armagh; 47°N = Épaule and Étape; 48°N = La Doré and Albanel

To assess the winter survival of SBW L2s, two three-branch balsam fir twigs, each bearing an average of 99.1 L2s in 2017, 96.1 in 2018, and 102.8 in 2019, were secured to the base of a homemade circular wire-mesh cage (25 cm in diameter × 3 cm in height) with thin copper wire (Figure 1c). Four of these cages were attached together to form a tube. The tube was fixed to the trunk of a tree 3 m above the ground (Figure 1d). There were three tubes in each site standing 3 m apart. Overall, 144 twigs were tested each year (2 twigs/cage x 4 cages/tube × 3 tubes/site × 6 sites). Each year, at the end of March, all tubes were brought back to the laboratory and stored in the outdoor insectary until mid-April. To prevent L2s from emerging precociously at this time of year, all tubes were transferred from the outdoor insectary to a cold room at −5°C ± 1°C, 65% ± 5% relative humidity, under a 16 h light: 8 h dark photoperiod. A week later, the temperature in the cold room was increased to 4°C with half the tubes remaining at this temperature for one more week and the other half for two more weeks. Prior to incubation in a growth chamber maintained at standard conditions, the two twigs from each cage in each tube were re-arranged as follows.

In 2017, a twig from each of cages 1 and 2 in each tube was placed in an individual Tissue-Tek® polyethylene cup (250 ml) (Miles, Etobicoke, ON) for incubation in growth chamber 2 on 24 April; the other twig from each of these two cages were incubated a week later in the same growth chamber. Similarly, one twig from each of cages 3 and 4 was allocated to growth chamber 4 on 24 April; the second twig from the same cages was put in growth chamber 4 on 30 April. In 2018, the allocation of twigs to growth chambers was very similar except that the two growth chambers used for the second incubation date were not the same as those used for the first. In 2019, the scheme was exactly as in 2017 except that growth chamber 2 was replaced by growth chamber 3. Growth chambers 2, 3 and 4 were of the same type; the four growth chambers used in 2018 were all of the same types and they were smaller than those used in 2017 and 2019.

The twig in each cup was examined daily to determine the number of larvae that successfully emerged. If required, the internal surface of the cup was sprayed lightly with demineralized water to prevent twig desiccation. Two weeks after the end of the emergence period, each twig was dissected to determine the number of L2s that did not emerge.

Statistical analysis

Cold-tolerance assay

In both January and February of 2018, the proportion of larvae that emerged in the control vials suggested that even under our favourable standard environmental conditions, a fraction C of overwintering L2s die naturally. The number r of larvae that emerged out of n in each vial was assumed to follow a binomial distribution with probability of emergence $\pi = (1 - C)\theta$ where θ is the (conditional) probability of emergence given that the L2 is viable. This non-linear model must combine two parts: one for the controls where $\text{logit}(1 - C) = \log[(1 - C) / C]$ depends linearly on an intercept, possibly temperature, and some random effects, and one for the rest of the data where $\pi = (1 - C)\theta$ and $\text{logit}(\theta)$ is a linear form that includes effects of temperature, exposure duration, and their interaction, plus random effects. Two separate preliminary generalised linear mixed models

were fit to r (Agresti, 2013). In the control group, $\theta = 1$ and the logit $(\pi) = \text{logit}(1 - C)$. Including temperature effects in this preliminary model provided a test that the natural emergence probability was constant over the course of the experiment. Temporarily assuming $C = 0$, the second preliminary binomial model for r in treated vials had logit (θ) defined as in the joint model. In both preliminary models, potential random effects involved months, incubation dates, growth chambers, and vials as dictated by the experimental design. The random parts of the preliminary models were reduced based on Wald tests of their variance components. Preliminary models also provided reasonable initial values of the parameters in the iterative estimation process used to fit the joint model.

To express π as a function of temperature and exposure duration among treated vials, their effects on logit(θ) were modelled as linear functions of 4 and 3 cubic spline bases functions, respectively, and the effect of their interaction, as linear functions of the tensor product of the two sets of basis functions (Wood, 2017). Expected probabilities of emergence $\widehat{\pi}$, were computed from the final joint model over a fine grid of temperatures and exposure durations from which points where $\widehat{\pi} = 0.5$ were extracted to obtain an estimate of the LTT_{50}. Ninety-five-percent confidence intervals (CI) around the estimated LTT_{50} at the six experimental temperatures were obtained by a bootstrap method (Wicklin, 2018) with 5000 bootstrap samples. The combined analysis was performed with the NLMIXED procedure of SAS® 9.4 (SAS, Institute Inc, Cary, NC). Its GENMOD and GLIMMIX procedures were used in the preliminary analyses. The basis function values for temperature and exposure durations were obtained from the smoothCon function of the mgcv R package, and those for the grid to predict π, from its PredictMat function (Wood, 2021).

Winter survival

The winter survival experiment of the overwintering L2s was conducted in six experimental sites, each year from 2017 to 2019. For each three-branch twig bearing n larvae, the number r of larvae that emerged the next spring was assumed to follow a binomial distribution with probability π of emerging. In the initial generalised mixed binomial model for r, the logit of the probability of emergence was assumed to depend linearly on year, site, and their interaction, plus several random effects consistent with the experimental design (Agresti, 2013). The mean logit of the probability of emergence per site was analysed through a set of five orthogonal contrasts: two orthogonal polynomials for the linear and quadratic components of the effect of latitude, and one contrast between the two sites at each latitude. In the presence of a year × site interaction, the p-values (P_{adj}) of 15 contrasts, 5 per year, were adjusted for multiplicity by the Holm-simulation method (Westfall et al., 2011). These tests were conducted at the $\alpha = 0.05$ level. Approximate 95% CI that accompany each mean estimate were computed on the logit scale and back-transformed for presentation in the figure or in the text (these intervals were not adjusted for multiplicity). The model was fit with the GLIMMIX procedure of SAS® 9.4.

RESULTS

Cold-tolerance assay

Preliminary analyses suggested that in either sub-model, the only random effects with a substantial variance component were those associated with vials. Data from the control vials supported the idea that temperature had little effect on the probability of emergence ($F_{5,90} = 0.48$, $p = 0.79$). The final joint model for r is described in Appendix S1.

Under standard conditions, the probability of emergence among the controls, $1 - C$, was estimated as 0.936 (CI: 0.924, 0.946) from the joint model. As a function of exposure duration at each of the experimental temperatures from −37 to −42°C, the estimated probability of emergence, $\widehat{\pi}$, was approximately constant between 2 and 8 h of exposure (Figure 3; Appendix S2). As expected at −37°C and −38°C, more than 50% of larvae (∼80%) emerged at all experimental exposure durations. At −39°C, at least 50% of larvae or more emerged after exposures of 11.4 h or less (CI: 10.6, 11.9). At −40°C, the LTT_{50} was reached after about 9.4 h (CI: 8.4, 9.9). At −41°C, 50% emergence was attained after about 3.0 h (CI: 1.9, 7.6). A temperature of −42°C was fatal to more than 50% of larvae (∼70%) at most exposure durations. This was consistent with the fact that no larva emerged at −43°C (data not shown), a temperature level included in our preliminary experiment, but excluded from the final experimental design for that reason. The estimated LTT_{50} is the contour line of the estimated response surface $\widehat{\pi}$ at $\widehat{\pi} = 0.50$ (Figure 4; Appendix S3).

Overwintering survival

Fixed effects of year, site and their interaction on the probability of emergence all appeared important ($F_{2,17.7} = 76.8$, $p < 0.0001$ for year; $F_{5,17.7} = 30.5$, $p < 0.0001$ for site; $F_{10,17.7} = 9.89$, $p < 0.0001$ for their interaction). In 2017, the estimated mean probability of emergence decreased gradually as latitude increased ($F_{1,18.1} = 13.9$, $p_{adj} = 0.017$ for the linear component of the effect of main latitude on $\log[r/(n-r)]$, and $F_{1,18.1} = 0.17$, $p_{adj} = 0.95$ for its quadratic component, Figure 5a, Appendix S4). At each main latitude (46, 47 and 48°N), there was no indication that the probability of emergence differed between the two sites ($F_{1,18.6} = 4.01$, $p_{adj} = 0.39$ for Armagh vs Sainte-Foy; $F_{1,18.1} = 1.12$, $p_{adj} = 0.81$ for Épaule vs Étape; $F_{1,17.6} = 0.22$, $p_{adj} = 0.95$ for Albanel vs La Doré).

In 2018, the estimated mean probabilities of emergence were 0.790 and 0.846 at the sites located near 48°N and 46°N, respectively, but at the main latitude of 47°N, they dropped to an average of 0.585 (Figure 5b, Appendix S4). There was no evidence that the mean emergence probability differed between the main latitudes of 48°N and 46°N ($F_{1,17.6} = 2.33$, $p_{adj} = 0.62$ for the linear component of the effect of main latitude). However, the data suggest that the drop in the emergence probability at 47°N was substantial ($F_{1,17.2} = 30.2$, $p_{adj} = 0.0005$ for the quadratic effect of latitude). There was no indication that the probability of emergence differed between the two

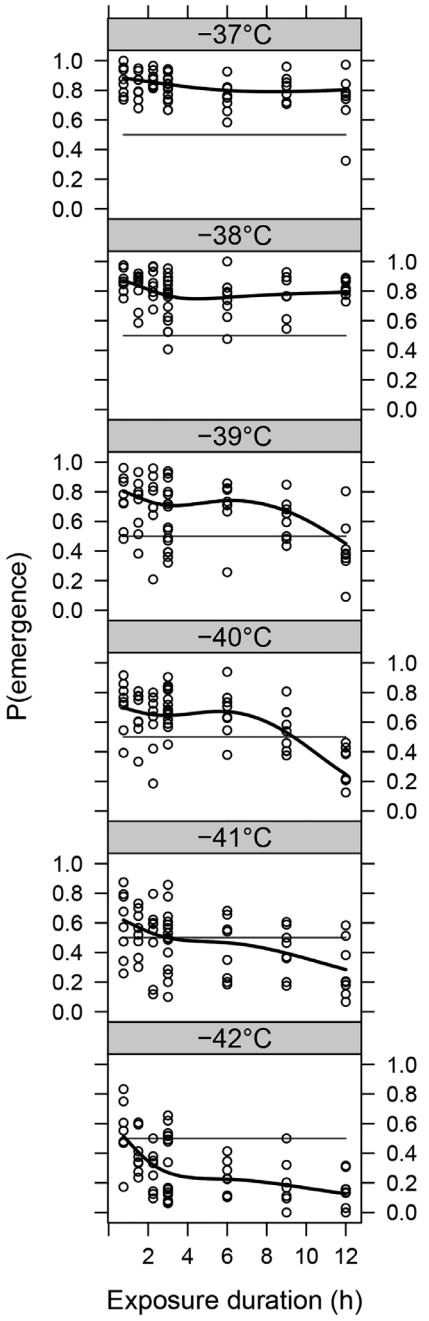

FIGURE 3 Observed (full circles) and predicted (thick lines) mean probability of emergence of *Choristoneura fumiferana* second-instar larvae as a function of exposure duration per experimental temperature with thin grey horizontal lines at 0.50 probability of emergence

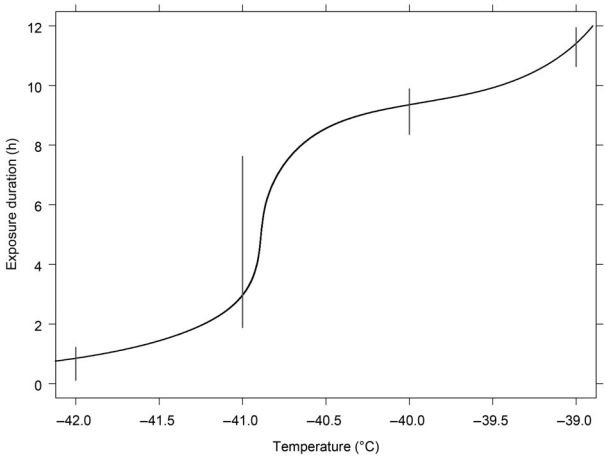

FIGURE 4 LTT_{50} curve from the estimated response surface (thick line) and approximate 95% confidence intervals (CI) at experimental temperatures (vertical grey lines)

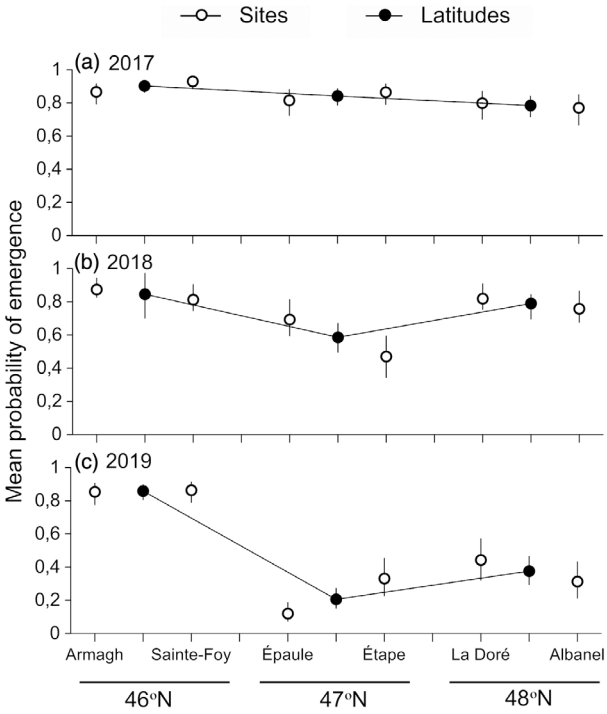

FIGURE 5 Mean estimated probability of emergence of *Choristoneura fumiferana* second-instar larvae in the spring after they overwintered at six sites in the province of Québec and their 95% confidence intervals (CI = vertical lines) per year (a: 2017; b: 2018; c: 2019), per site (empty circles) and per main latitude (full circles)

sites within each main latitude ($F_{1,17.2} = 1.14$, $p_{adj} = 0.81$ for Albanel vs La Doré; $F_{1,17.0} = 7.26$, $p_{adj} = 0.13$ for Épaule vs Étape; $F_{1,17.9} = 1.77$, $p_{adj} = 0.70$ for Armagh vs Sainte-Foy).

In 2019, estimated emergence probabilities were low at the main latitudes of 48 and 47°N, and high at the two southern sites ($F_{1,17.4} = 88.0$, $p_{adj} < 0.0001$ for the linear component; $F_{1,17.6} = 87.2$, $p_{adj} < 0.0001$ for the quadratic component) (Figure 5c, Appendix S4). The data suggest that the probability of emergence was higher at Étape than at Épaule ($F_{1,17.7} = 13.6$, $p_{adj} = 0.018$) but there was no evidence that this probability differed among the two sites at the main latitudes of 48°N ($F_{1,17.3} = 2.58$, $p_{adj} = 0.61$ for Albanel vs La Doré), or 46°N ($F_{1,17.6} = 0.06$, $p_{adj} = 0.95$ for Armagh vs Sainte-Foy). At Étape, the estimated odds of emergence were 3.64 times (CI: 1.74, 7.59) greater than at Épaule.

Average daily minimum temperatures recorded each year at the two sites per latitude are shown in Figure 6 (Appendix S5).

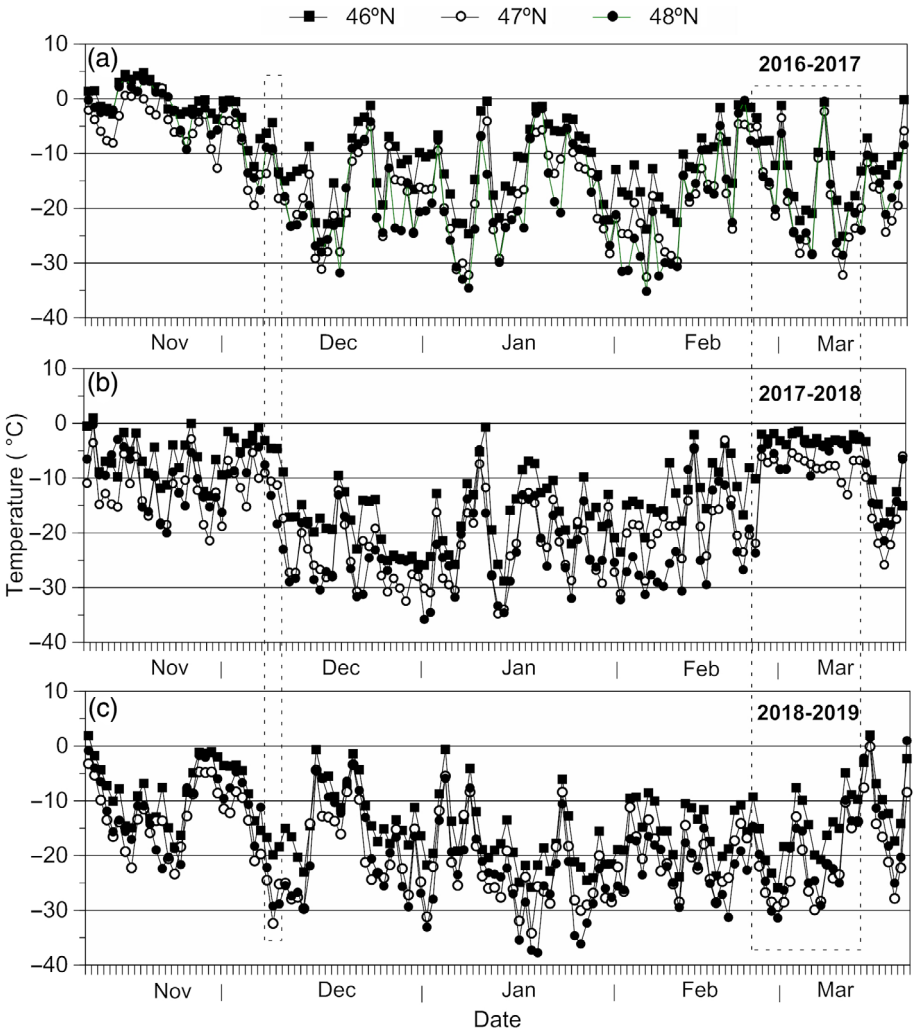

FIGURE 6 Average daily minimum temperatures recorded at the two sites per latitude (46, 47 and 48°N), from November 8 to March 21 of the years 2016–2017 (a), 2017–2018 (b) and 2018–2019 (c). For clarity, confidence intervals (CI) associated with mean daily minimum temperatures recorded throughout the experimental period are not shown. Dashed lines highlight two specific periods in the spring of 2017–2018 (b) and the late fall of 2018–2019 (c)

The winter of 2017 was particularly cold at the main latitude of 48°N (Figure 6a). For instance, in early January and early February, the temperature at this latitude declined as low as −36°C, for nearly 2 h. A somewhat similar drop in temperature also occurred in early February at the latitude of 47°N, but this cold temperature only lasted 45 min. In contrast, average daily minimum temperatures at the southern sites were consistently above −30°C.

The winter of 2018 was quite similar to that of 2017 (Figure 6b). First, temperatures as low as −36/−37°C were recorded for at least 1–4 h at the main latitudes of 47°N (Étape) and 48°N (Albanel), respectively. Second, the average temperature never fell below −30°C at the two southern sites. However, unlike the winter of 2017, temperatures at the end of February 2018 suddenly increased from −25/−20°C to −10/−2°C in all sites and remained stable at this high level for the next 17 days, clearly indicating that a prolonged late winter thaw had swept across the whole experimental area. However, on March 18, the temperature quickly dropped to −26°C at the main latitude of 47°N compared with −21°C/−16°C at the other two main latitudes.

Overall, the winter of 2019 was much colder than those of the two previous years. This was particularly the case at the highest latitude where temperatures from mid-to-late January fluctuated between −35 to −39°C for several hours (Figure 6c). Furthermore, the beginning of December 2018 was exceptionally cold with the temperature falling as low as −29 and −32°C, at the respective latitudes of 48 and 47°N. Although it only dropped to −20°C at the two southern sites, this temperature was much cooler than those recorded during the same period in 2017 or 2018 (∼ −5°C) (Figure 6a,b).

DISCUSSION

The cold tolerance assays conducted in mid-January and mid-February 2018 with second-instar SBW larvae, acclimatised to

outdoor conditions since October 2017, showed that ≤50% of the larvae emerged after 48 min (0.80 h) at −42°C or after 3 h at −41°C (Figures 3 and 4). This behaviour of the LTT curve was somewhat expected given that, for the two winter months combined, the mean SCP should be about −41.6°C (approximate CI: −42.9, −40.4), as estimated from the reconstruction of the analysis of variance from the mean SCP's, their standard errors, and sample sizes reported in figure 2c of Han and Bauce (1995a) for January and February. In addition, larvae were not expected to survive exposures to a sub-zero temperature of −43°C. As SBW is a freeze-avoiding species, these results strongly suggest that the large majority of the L2s submitted to −41 or −42°C died from freezing injuries (i.e., reached their SCP) at almost all exposure durations tested.

Our cold-tolerance assays also revealed that SBW L2s were able to sustain prolonged exposures to temperatures slightly above their mean SCP, as estimated from the literature. For instance, the estimated probability of emergence remained fairly constant at approximately 0.70 following 2–8 h of exposure to sub-zero temperatures ≥−40°C; the LTT_{50} was reached after 11.4 h (CI: 10.6, 11.9) at −39°C or 9.4 h (CI.: 8.3, 9.9) at −40°C (Figures 3 and 4). This high degree of cold tolerance may reflect the fundamental role of the AFPs in halting the growth of embryonic ice crystals, especially at temperatures close to the homogenous ice nucleation temperature of pure water (−38.5°C). However, as pointed out by Lee Jr. (2010) and Zachariassen et al. (2010), internal ice will form eventually despite the innate propensity of insects to supercool intensely, as appears to be the case in SBW L2s.

Insects may freeze at temperatures slightly above their own SCP. This has been demonstrated in several species including larvae of the wheat stem sawfly, *Cephus conctus* Norton (Salt, 1950), pharate first instar of the gypsy moth, *Lymantria dispar* (L.) (Waggoner, 1985), quiescent eggs of the Mexican pine white butterfly, *Neophasia terlooii* (Behr) (Halbritter et al., 2017) as well as overwintering eggs of the hemlock looper, *L. fiscellaria* (Delisle et al., 2013). In the latter species, the authors demonstrated that among eggs that did not hatch following 16 h of exposure to −37 or −35°C in mid-January or mid-February, 69% died from freezing even though the mean SCP of the population was estimated as −40.1°C (CI: −40.7, −39.6) at that time of year. Furthermore, death due to freezing injury (internal ice formation with a latent heat release signal) was time-dependent with eggs succumbing sooner at −37°C (2.3 h) than at −35°C (3.9 h). Interestingly, in adults of the bark beetle, *Ips acuminatus* (Gyll.), the probability of freezing at temperatures slightly above the mean SCP was also observed (Gerken, 1989). Unlike the other species mentioned above, *I. acuminatus* produced, in addition to ethylene glycol, an AFP that exhibits a thermal hysteresis activity of ∼4°C, a value quite similar to that obtained in cold-acclimated (4°C) SBW larvae (Tyshenko et al., 1997). Hence it appears that AFP production with high hysteresis activity does not fully protect the insect from ice nucleation at sub-zero temperatures slightly above its SCP.

Although we did not record larval body temperature during the cold-tolerance assay, we propose that the LTT_{50} we obtained at −40 or −39°C and corresponding exposure durations of 9.4 and 11.4 h, respectively, are likely necessary to cause freezing injury among the larvae that died between this critical point (LTT_{50}) and the end of the cold test. In contrast, larvae that succumbed before reaching the LTT_{50} at these two sub-zero temperatures (i.e. prior to 9.4 h or 11.4 h) probably died from chilling injury or other causes such as disease. Similarly, the fact that the LTT_{50} was not reached when larvae were submitted to −37 or −38°C, even after 12 h of exposure, suggests that freezing could hardly occur at these two sub-zero temperatures. However, the estimated probability of emergence at −37 or −38°C was not as high (0.80) as in the control groups (0.94), indicating that chilling injury was likely the major cause of death at these two sub-zero temperatures. This does not exclude the possibility that a small fraction of SBW L2 samples froze at −37 or −38°C, as previously demonstrated in the hemlock looper when eggs were subjected to temperatures 10–15°C above their mean SCP (Delisle et al., 2013). Along with this argument, it is worth noting that under very cold temperatures (< −35°C), two naturally occurring AFPs, one from an Arctic fish (type-III AFP from ocean pout) and one from a beetle (*Tm*AFP from yellow mealworm, -*Tenebrio molitor* (L.)) have been shown to exhibit two contrasting behaviours in aqueous solutions. In addition to their role of inhibiting ice crystal growth, both AFPs may also promote ice nucleation at temperatures 2–3°C above the homogeneous freezing temperature (−38.5°C) (Eickhoff et al., 2019). However, to prove that the LTT_{50} is a reliable indicator that freezing has occurred in SBW larvae, the cold-tolerance assay would need to be repeated while concomitantly monitoring the body temperature of each larva to confirm freezing with a latent heat release signal.

Overwintering survival experiments conducted in the winter of 2017 showed that SBW larvae were less likely to emerge as latitude increased. Indeed, the probability of emergence declined from 0.902 at the main latitude of 46°N to 0.784 at the main latitude of 48°N (Figure 5a). Daily minimum temperatures recorded throughout the winter of 2017 followed a similar latitudinal trend, with temperatures decreasing as low as −35/−36°C at the highest latitude (La Doré, Albanel) but not lower than −25/−30°C at the lowest latitude (Armagh, Sainte-Foy) (Figure 6a). Despite this substantial linear decline, the emergence probability obtained at the highest latitude was quite high (>0.784) and consistent with our cold-tolerance assays which showed that the likelihood of surviving at −37 or −38°C was close to 0.80 at almost all exposure durations tested (Figure 3).

Unlike the previous year, the probability that SBW L2s emerged in the winter of 2018 did not decrease linearly with latitude but it followed a curvilinear trend, with fewer larvae emerging at the intermediate latitude (0.586) than at the highest (0.790) or lowest (0.846) latitudes (Figure 5b). Although temperatures recorded in January and February 2018 declined as low as −36/−37°C at 47°N (Étape and Épaule), it does not explain the low emergence probability obtained at this intermediate latitude given that for similar sub-zero temperatures recorded at the highest latitude, the emergence probability was much higher (0.846). However, a closer examination of the daily minimum temperatures recorded immediately after the prolonged winter thaw of March 2018 revealed that temperature suddenly dropped to −26°C at the 47°N sites but fell to only −21/−16°C at the two other

latitudes. At first sight, it may seem peculiar that SBW larvae were not sufficiently cold hardy in mid-March to withstand a temperature of −26°C given that Han and Bauce (1995a) demonstrated that they can supercool to as low as −38/−36°C at that time of year. On the other hand, post-diapausing larvae may have de-acclimated following exposure to an extended warm spell as has been shown in several other insect species (Fields et al., 1998; Sobek-Swant et al., 2012; Williams et al., 2014; Zeng et al., 2008), with no ability to regain their cold-hardiness following subsequent cold exposure (re-acclimation) (Sobek-Swant et al., 2012). Further investigations on the physiological regulation of these two processes (de-acclimation and re-acclimation) would be required to substantiate their impact on SBW cold-hardiness.

Winter survival experiments conducted in 2019 differed markedly from those performed in the two previous years. First, even though the emergence probability at the main latitude of 46°N was as high as those observed the first years (>0.805) (Figure 5), those obtained at the two other latitudes (47 and 48°N) were well below 0.5, with the lowest value (0.120) being obtained at the Épaule site (Figure 5c). Second, despite the fact that the winter of 2019 was the coldest of the 3 years (Figure 6c), none of the sub-zero temperatures (−36/−39°C) recorded in January or February 2019 were low enough in intensity or long enough in duration to reduce the emergence probability below 0.5. We, therefore, suggest that the unusually cold weather that occurred on December 8, 2018 provides the best explanation for the lower emergence probabilities obtained at the 47°N (0.206) and 48°N (0.376). In fact, the temperature at Épaule dropped drastically to −32°C for at least 1 h on that day (Figure 6c). As mean SCPs of SBW larvae decreased from −34 to −36°C between mid-November and mid-December (Han & Bauce, 1995a), a temperature as low as −32°C in early December was probably too close to their SCP to protect larvae against freezing. Moreover, glycerol in early December is about half the amount normally produced in January (Han & Bauce, 1995a); this suggests that the cold-hardiness of the L2 was not optimal at that time of year. Although not statistically different, the emergence probabilities obtained at the other three sites (Étape: 0.331, Albanel: 0.313, and La Doré: 0.443) were slightly higher than those observed at Épaule (0.120), likely because the temperature in early December did not drop below −30°C at these three sites.

Interestingly, when diapause initiation in SBW larvae was intentionally delayed by several weeks relative to the normal situation, Han and Bauce (1995b) showed a strong correlation between the resulting high overwintering mortality and the failure of larvae to accumulate sufficient glycerol when they were exposed to lethal temperatures (−29.6°C) as early as December, 1993. In the present study, we provided further evidence that early winter frosts may represent a considerable challenge for the overwintering success of SBW larvae, without, however, modifying the normal course of diapause initiation. This aspect deserves more attention since the occurrence and severity of such climatic disturbances are likely to increase under future climate change scenarios (Kodra et al., 2011).

Overall, this study has shown that SBW L2s are quite well adapted to survive the harsh winter conditions that prevail in eastern Canadian boreal forests. Indeed, this species has the ability to withstand several hours of exposure to −39 or −40°C before reaching its LTT_{50}, but it required only a few hours or minutes to reach this point at −41 or −42°C, respectively (Figures 3 and 4). These two sub-zero temperatures are very close to the SBW L2s mean freezing point estimated as −41.6°C from Han and Bauce (1995a) who measured the SCP according to the standard method of Lee Jr. (2010). These two consistent results suggest that SBW larvae from the IPQL have maintained their genetic stability and integrity despite the long-term establishment of the colony. Furthermore, our estimated values of SBW cold tolerance are very consistent with our field data. At the Sainte-Foy site, in particular, temperatures never dropped below −30°C in either January or February, and larvae successfully survived for three consecutive winters in that area. This, however, does not imply that SBW larvae never meet harsher conditions in northern Québec: over the last 60 years, temperatures ≤ −41°C have been frequently recorded at latitudes similar to those tested in the present study or further north. At Chibougamau and Chapais (located 49°N) for instance, the temperature in winter months fell ≤−41°C once every 2 years on average over a 60-year period (Environment Canada, 2019). Moreover, during the third SBW outbreak that occurred over the last century in eastern Canada (1968–1988), endemic populations were detected for the first time north of the 50th parallel (Navarro et al., 2018). Tree-ring chronologies from this study revealed that the northern regions of eastern Canadian forests, largely dominated by black spruce, experienced weaker outbreaks than those occurring in the southern regions, mostly dominated by balsam fir and white spruce. According to Navarro et al. (2018), severe SBW outbreaks have been rare in the northern regions for at least three main reasons. First, black spruce is less vulnerable to SBW because its late budburst phenology increases larval mortality (Nealis & Régnière, 2004). Second, cold and short summers limit the establishment of endemic populations (Navarro et al., 2018). Third, early frosts may hamper egg hatching (Pureswaran et al., 2015). However, as SBW populations from the northern regions of Québec are more likely to face winter temperatures close to their thermal limits, we argue that cold weather was also crucial in preventing outbreaks of these populations.

As stated earlier, wild SBW populations from the Central genomic cluster (e.g. Inuvik, NWT) are apparently more cold-hardy than those from the Eastern genomic cluster (e.g. Manic-Cinq, QC; Campbellton, NB) (Butterson et al., 2021), but the overall capacity of these wild populations to supercool following acclimation at 2°C for at least 12 weeks into diapause (−32 to −37°C, see their Figure 3, late diapause group) was never as high as that of the IPQL larvae (∼ −42°C) acclimatised to outdoor conditions (Han & Bauce, 1995a). Even though the ultimate goal of Butterson et al. (2021) was to dissect the mechanisms of basal cold tolerance and plastic responses to fluctuating temperatures in these wild populations, their study also provided evidence that their larvae could hardly withstand the very low sub-zero temperatures (−37 to −42°C) to which our IPQL larvae were submitted in the course of this study. These two facts raise the following question: would these inter-regional differences in cold

resistance have been detected if the L2s had been acclimatised to field conditions before experiencing cold tests? For instance, based on our study, the L2s were able to tolerate temperatures as low as −41°C for 3 h before reaching their LTT_{50}. Therefore a population from Inuvik should be able to resist similar or even lower sub-zero temperatures, given that in the winter of 2021, the temperature in this subpolar region dropped at least five times to −41°C and remained stable at this low level for 3–10 consecutive hours (Environment Canada, 2021). Such climatic conditions are just as severe as those encountered in the northern regions of Québec. Hence we recommend that the cold hardiness and thermal tolerance of these wild populations be reassessed with particular attention to the acclimatisation process.

Most likely, the low capacity of the IPQL colony to withstand exposures to common winter temperatures reported by Butterson et al. (2021), Marshall & Sinclair (2015, see the SCP values in figure S2 of their Supporting information), Han & Bauce (1995a, acclimation at 2°C) relative to the cold tolerance exhibited by field-acclimatised larvae from the same colony in this study and in Han and Bauce (1995a) is due to the differential pre-conditioning of the larvae. Consequently, a fresh comparison of the cold tolerance of larvae from the old colony with that of larvae from the ones recently established at the IPQL facility (Perreault et al., 2021) seems warranted. It is our hope that despite these disparities, the knowledge acquired about the limits of SBW cold tolerance in the winter months will eventually serve to refine existing models aimed at predicting the abundance as well as the range expansion of this species, especially towards the Arctic pole.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest in the work reported in this paper.

DATA AVAILABILITY STATEMENT

Data available in article supplementary material. The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Appendix S1. Ancillary results for the cold tolerance assays

Appendix S2. Data Figure 3.xlsx: the data file to draw Figure 3. Its "typ" variable takes the values "hor" for grey horizontals at the probability of emergence $\pi = 0.50$, "obs" for observed proportion emerged per vial, and "prd" for predicted π at each temperature. Other variables are "temp" = Temperature, "duration" = exposure duration, "survival" = probability of emergence, observed or estimated, "temp_char" is a character version of the "temp" variable.

Appendix S3. Data Figure 4.xlsx: the data file to draw Figure 4. Its "grp" variable takes the values "LTT" for the estimated LTT_{50} curve at $\pi = 0.50$, and values "−42°C," "−41°C," "−40°C," "−39°C" for each 95% confidence interval among exposure durations at the LTT_{50}.

Appendix S4. Data Figure 5.xlsx: the data file to draw Figure 5. "Mean survival" for the mean estimated probability of emergence and their 95% confidence limits per main "latitude", per "site" and per "year."

Appendix S5. Data Figure 6.xlsx: the data file to draw Figure 6. "Mean min" for the average daily minimum temperatures recorded at the two sites per latitude (46, 47, and 48°N) from November 8 to March 21 of the years 2016–2017, 2017–2018 and 2018–2019.

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