How environmental conditions impact seed germination and consequently the invasion strategy of glossy buckthorn (Frangula alnus (Mill)) in Eastern Canada

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Research Article

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How environmental conditions impact seed germination and consequently the invasion strategy of glossy buckthorn (*Frangula alnus* (Mill)) in Eastern Canada

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ABSTRACT

Identifying the environmental factors that control germination is essential to understanding seed traits and their effect on fitness. Glossy buckthorn (*Frangula alnus*) is a shrub or small tree from Eurasia that has become invasive in North America, and which has negative impacts on plant communities and ecosystems. In this study, we analyzed the germination response of glossy buckthorn seeds to different temperatures, various stratification lengths and scarification conditions to measure the impact on breaking seed dormancy, as well as the effect of light in triggering germination. Survival analysis was performed utilizing a Kaplan-Meier estimator and Cox proportional hazard model. Glossy buckthorn seeds exhibited physiological dormancy and required cold stratification to germinate. Germination starts at the end of spring when temperatures reach 12°C and reach a peak later in the season with a germination rate of 70% at 20°C. At warmer temperatures, germination declines, and seeds are induced into dormancy again. Ungerminated seeds will form persistent seed banks that will ensure the survival of *F. alnus* over time and space as well as enhance its chances of a successful establishment and invasion. Due to climate change, increases in temperatures might impact seeds buried in the soil, shorten the length of stratification periods in some areas of North America and consequently affect the timing of germination resulting in a shift of its germination period.

Keywords: Invasive species, seed bank, survival analysis, climate change, alder buckthorn

INTRODUCTION

Seeds' primary functions are dispersion, colonization of new areas, and establishment of new plants. During germination, seeds transition from a heavily protected to a highly vulnerable phase. Biotic and abiotic factors have a substantial impact on the survival, growth, and establishment of seedlings (Fenner and Thompson 2005; Gioria and

Pyšek 2017). Germination also influences the traits that will be expressed throughout the plant's life (Donohue et al. 2010). Accordingly, germination timing is under high selective pressure, and many species have developed mechanisms to enhance their chances of survival (Gioria and Pyšek 2017). One important mechanism that plays a major role in delaying germination is seed dormancy (CC Baskin and Baskin 2014; Fenner and Thompson 2005).

Dormancy is a seed trait and can define the range of conditions in which a seed is able to germinate (Vleeshouwers et al. 1995). Nondormant seeds will germinate over the broadest set of conditions, whereas dormant seeds will only do so when a narrow set of conditions are met (CC Baskin and Baskin 2014; Walck et al. 2011). Dormancy prevents seeds from germinating despite environmental conditions being suitable to germination when such conditions are unlikely to persist (CC Baskin and Baskin 2014). Several environmental factors, such as light and water availability, play an integral role in seed germination. However, temperature is the most important factor that regulates the seasonal changes in dormancy and germination (CC Baskin and Baskin 2014; Benech-Arnold et al. 2000). Due to climate change, temperatures are projected to increase, and it may preclude, delay, or improve germination. Furthermore, these alterations will impact organisms, populations, and communities with very complex interactions and outcomes (Walck et al. 2011).

Frangula alnus Miller (Rhamnaceae), also known as glossy or alder buckthorn, is a shrub or small tree, native to Europe and Asia, that has become invasive and widespread in the United States and Canada (Catling and Porebski 1994; Converse 1984) impacting species and communities (Fiedler et al. 2012; Fiedler and Landis 2012), altering soil properties (Stokdyk and Herrman 2014, 2016), reducing the abundance of native herbs as well as inhibiting the regeneration of trees (Cunard and Lee 2009; Fagan and Peart 2004; Frappier et al. 2003), potentially reducing production of harvestable species as white pine (*Pinus strobus*) (Lanzer et al. 2017; Lee et al. 2017), red oak (*Quercus rubra* L.) and sugar maple (*Acer saccharum* Marsh.) (Hamelin et al. 2016).

Identifying the environmental factors that limit or promote the germination of invasive species can be essential towards understanding the traits that shape the species' capacity to invade new sites and determine when and where an invasion is likelier to occur. Consequently, its invasion strategy could be outlined helping the development of guidelines for its control (Sakai et al. 2001). The main goal of this study was to investigate the germination response of glossy buckthorn to environmental factors under laboratory conditions. In particular, the study aimed to (i) evaluate the effect of cold stratification and scarification on breaking dormancy of glossy buckthorn seeds, (ii) determine the optimum temperature for seed germination (iii) identify the effect of light on triggering germination. Finally, based on our results, we will discuss glossy buckthorn invasion strategy and how climate change might impact germination timing and its establishment success.

MATERIALS AND METHODS

Species and study area

Frangula alnus Miller blooms from May to early July and ripe fruits can be observed from the end of June to mid-August (Medan 1994). The drupaceous fruits are mainly dispersed by birds, rodents and/or simply falling to the ground where seedlings may establish. The seeds, usually two or three per fruit, can remain viable for at least three and a half years (Godwin 1943).

The study area was located in the regions of Ascot-Corner and Cookshire-Eaton, in southern Québec, Canada (45°27'11.4"N 71°41'01.2"W). With an annual average temperature of 5.6°C and total annual precipitation of 939 mm (as rain) and 206 cm (as snow) (Government of Canada 2021), this area was predominantly covered by forest stands, broadleaf deciduous, mixed wood and evergreen needleleaf, and regeneration stands (MRNFP 2004).

Seed collection and treatment

Ripened fruits of glossy buckthorn were hand-harvested in September 2018 from several plants located at the edges of planted and natural stands and along the sides of the roads. The fruits were placed into plastic bags and kept between 3° to 5°C for two days. Afterward, the seeds were washed and separated from the pulp. Immature or unfilled seeds were removed by the floating method (CC Baskin and Baskin 2014, p. 10), then clean seeds were air-dried in ambient conditions (approximately 20°C) for 68 hours. Dried seeds were then placed into sealed plastic bags and stored at 4°C. In order to avoid contamination, the seeds were sterilized by soaking them in a 0.5% sodium hypochlorite solution for 10 minutes and rinsed multiple times under a faucet before commencing the stratification process (CC Baskin and Baskin 2014, p. 19; Thomsen and Diklev 2000).

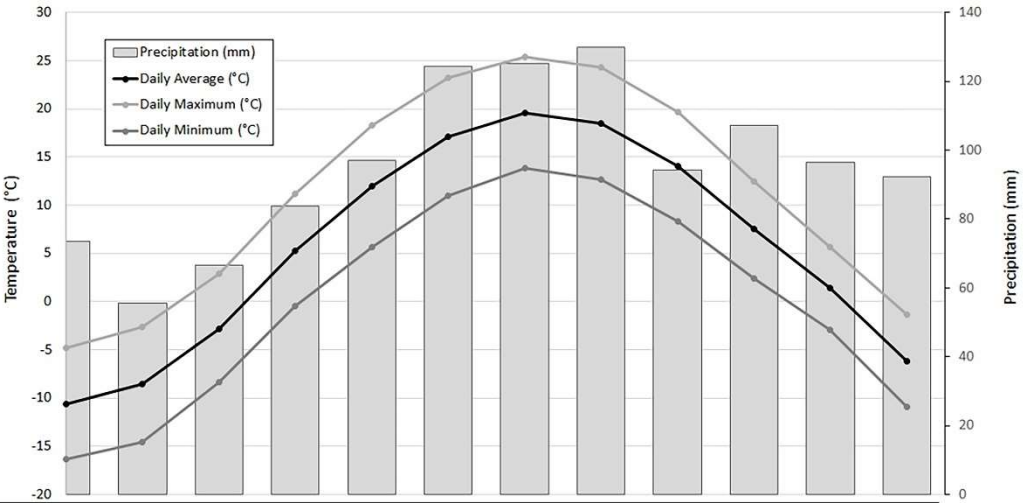
Stratification

The stratification process consisted of humidifying the seeds and placing them into low-temperature as part of dormancy-breaking process (CC Baskin and Baskin 2014; Cavieres and Arroyo 2000). To evaluate the impact of stratification on germination, seeds were placed into sealed plastic bags containing sterile sand which was humidified with distilled water and kept in a cold chamber (3 to 5°C) for different periods (0, 4, 8, 12, 16, and 20 weeks). After the corresponding stratification period, 100 seeds were then placed into 9 cm petri dishes (10 seeds per petri dish), between two layers of humidified filter paper (Whatman Grade 1-90mm), sealed with Para-Film, and placed into a growth chamber. At 0 weeks of stratification, the seeds were placed directly into the growth chamber - without undergoing any dormancy-breaking treatment. In the growth chamber, the seeds were stored at 20°C and exposed to a 12-hour photoperiod.

Germination in a range of temperature

After an optimal stratification length was identified (*see results*), the effect of temperature on germination was assessed by placing stratified seeds (12, 14, 16 weeks) into the growth chamber with a 12-hour photoperiod at 12, 14, 16, 18,

and 20°C. This set of temperatures simulated the monthly mean temperatures registered during the growing season (from May to September) (Government of Canada 2021). Moreover, to determine the impact of higher temperatures on germination, which are common during the summer, glossy buckthorn seeds were also placed at 24 and 28°C. Climate data used in this study was obtained from the Bromptonville meteorological station located approximately 21 km away of the study area (45°29'00.000" N; 71°57'00.000" W, an altitude of 130 m a. s. l). Mean monthly temperature and snow values were provided based in records from 1981 to 2010 for all months of the year (Fig.1) (Government of Canada 2021).



	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Snowfall (cm) *	49.4	39.9	35.8	9.8	0.1	0	0	0	0	2.2	17.3	52.2
Average Snow Depth (cm) **	16	20	14	2	0	0	0	0	0	0	1	10
Snow Depth at Month-end (cm)	18	18	5	0	0	0	0	0	0	0	2	14

*depth of newly fallen snow
**depth of accumulated snow on the ground

Figure 1. Daily maximum, average and minimum temperature, precipitation, and average snowfall and depth by month of the year registered for 1981 to 2010 at the Bromptonville station (ID station: 7020860), in Québec, Canada. Data and calculated values were provided by *Environment and Climate Change Canada* (graph modified from Canadian Climate Normals, (Government of Canada 2021)).

Darkness and scarification

To evaluate the effect of light and darkness on germination, petri dishes were kept at 20°, 24° and 28°C, wrapped in aluminum foil and were examined at dark under a green-colored light to avoid seed stimulation (CC Baskin and Baskin 2014, p. 26). To evaluate the impact of scarification on germination, the seed coats were partially cut. Seeds were then submitted to a 12-hour photoperiod at 12, 16, 20, 24, and 28°C.

All germination experiments were performed at Université du Québec à Montréal, Canada, from November 2018 to June 2019. Seeds were kept in growth chambers for 30 days and examined every 48 hours for any germinated seeds

that were then counted and removed. Seeds were considered "*germinated*" when exhibiting a radicle length of 2 mm or more (Côme 1982).

Viability of non-germinated seeds

After 30 days, non-germinated seeds were gently pinched with forceps and dead seeds collapsed. The firm ones were then soaked for 24h, cut and had their seed coat removed. The seeds were then re-soaked in a solution of 2,3,5-triphenyl tetrazolium chloride (TTC 0.1%) for 12 hours at 25°C. Seeds were analyzed under a stereoscopic microscope and red or pink uniformly stained seeds (cotyledons and embryo) were classified as viable (Ronnie 2003).

Statistical analysis

Our germination experiments generated an interval dataset. Life-table estimators were designed for this type of data but according to McNair et al. (2012), germination experiments are interval type and statistical methods designed for exact data should be used instead, with the condition that very few seeds (less than 5%) were lost during the experiments. For these reasons, the Kaplan-Meier estimator and Cox proportional hazard model were used. Seed status were coded "1" for germinated and seeds that did not germinate by the end of the experiment were coded "0" (right-censored observation). Rotten seeds were also coded "0" and their removal time was recorded. All the variables were considered as categorical covariates with dark and scarified treatments being coded "1" and light and non-scarified treatments being classified as "0". The germination data was submitted to a survival analysis (or time-to-event analysis) using the statistical software NCSS 12 Data Analysis (NCSS, LLC, Kaysville, UT, USA).

Non-parametric and semi-parametric methods of survival analysis are based on the distribution of germination times of each seed and are principally modeled using two related probability functions: survivor function and hazard function (McNair e al. 2012; Romano and Stevanato 2020). When analyzing germination data, the survival function *S(t)* is the probability that the germination time is greater than the given time (*t)*, and the hazard function *h(t)* is the probability that a non-germinated seed at time *t* will germinate in the next instant. Survival indicates the cumulative non-germination whereas the hazard function indicates the chance of experiencing germination with dimensions of 1/time. An increase in the hazard rate will decrease the survivor function (Klein and Moeschberger 2003). The non-parametric Kaplan-Meier method estimates the survivor function, without assuming a specific functional form according to the following formula (Kaplan and Meier 1958):

$$\hat{S}_t = \prod_{t_i \leq t} \left[1 - \frac{d_i}{ni}\right]$$

Where t_i is the duration of the study at point i, d_i is the number of germinated seeds up to point i and ni is number of seeds at risk of germination immediately prior to t_i.

Stratification and temperature - Kaplan-Meier estimates of survivor functions were generated for each stratification period (4, 8, 12, 16, and 20 weeks) and for each temperature (12, 14, 16, 18, and 20°C). To analyze the impact of colder and warmer temperatures on germination, the data was split into three groups*: (1)* mean temperatures for summer months (18 and 20°C), *(2)* coldest temperatures (12 and 14°C) and, *(3)* higher temperatures (24 and 28°C). Survival curves were built plotting the survival probability against time and then the median survival time was calculated. As seeds germinate independently of one another, the probability of germination was multiplied together using the Nelson-Aelen estimator to obtain the cumulative hazard function. Greenwood's formula was used to calculate the linear pointwise confidence intervals for the K-M survival and cumulative hazard. Differences between treatments were assessed by comparing the survival curves, including the hazard rates of the groups (hazard ratio). Log-rank tests were performed to test the null hypothesis that the hazard rates of all treatments were equal at all times. Most of these tests are performed by comparing the actual hazard rate against the expected hazard rate. The various hazard rates may be weighted equally through time or differently with some tests applying heavier weight to early or later germination (Klein and Moeschberger 2003). The hypothesis of hazard rate equality is rejected if this value was less than 0.05.

Darkness and scarification – K-M survival functions were also calculated for seeds that were submitted to continuous darkness and scarification treatments. Survival curves were built as described above and were compared to the ones from the control group (seeds germinated at light – 12 hours photoperiod; and non scarified seeds, respectively). The null hypothesis that the survivor curves did not differ from each other was tested using a set of log-rank tests. To analyze the potential effects of multiple covariates (dark/light, scarification, and temperature) on germination simultaneously, Cox's Proportional Hazards regression was used. Prior to testing, violations of the proportional hazards assumptions in Cox regression were assessed by plotting the log of the cumulative hazard functions against time and checking for parallelism (Hess 1995; Romano and Stevanato 2020). Cox's proportional hazard model calculates the hazard rate (chance of germination) and can be written as:

$$h\{(t), (Z_1, Z_2, \ldots, Z_m)\} = h_0(t) \exp(\beta_1 z_1 + \beta_2 z_2 + \ldots + \beta_m z_m)$$

Where *h(t...)* indicates the hazard, given the values of the *m* covariates (predictors or explanatory variables) for the respective cases (Z_1, Z_2, …, Z_m), and *t* is the respective survival time. The term $h_0(t)$ is the baseline hazard and constitutes the hazard for the respective individual when all independent variable values are equal to zero. And $\beta_1 + \beta_2 + \ldots + \beta_m$ are regression coefficients capturing information about the relationship between the covariates and the hazard function (Hill et al. 2006; McNair et al. 2012; Romano and Stevanato 2020). In the Cox model, the goal is to estimate the β_m and then test to see whether they are significantly different from 0. A process called partial likelihood provide the estimation of β_m, and the Efron method was used to accommodate tied event times that are very common in germination data (McNair et al. 2012).

The different treatments (dark/light, scarified/non-scarified seeds by temperature) were compared using a log-rank test. The ratio of the total observed germinated seeds versus the expected ones was calculated. The hazard ratio for one group is relative to the other, and it is constant with respect to time. The Wald test was performed to test the significance of the individual regression coefficients. Although NCSS does not support random effects (commonly referred to as frailty effects) functionality in its Cox modeling, petri dish locations were rotated every two days in order to minimize differences in germination conditions. (Romano and Stevanato 2020).

RESULTS

Seed viability

A total of 2,100 glossy buckthorn seeds were used in this study, 1,042 germinated, 9 were lost (rotten), and 1,049 seeds did not germinate. The latter were tested for viability and only 4 were non-viable (2 seeds from the stratification experiment: 4 and 8 weeks; and 2 seeds from the 20°C and 28°C, 12h light). Glossy buckthorn seeds presented a viability of 99.8% (n=2100 seeds).

Stratification

Glossy buckthorn seeds were unable to germinate without undergoing a period of stratification. Kaplan-Meier curves show the probability of not germinating for each stratification treatment per interval (in days) (Figure 2). The value of *S(t)* is constant during an interval (i.e., at the beginning and throughout the interval). The vertical lines in the curves represent the occurrence of germination and the vertical distances between intervals illustrate the change in the cumulative probability of not germinating in a specific time. The length of horizontal lines indicate how long the survival lasts and determine the curve steepness (Romano and Stevanato 2020). At time 0, the survival rate for this interval is 1 (100%), which means no seeds have germinated. In the next intervals, seeds begin germinating and then the chance of not germinating drops for the ones left over the course of the experiment. Seeds stratified for 4 weeks have the highest probability of not germinating (*S(t)*= 0.89 ± 0.03 SE), followed by 8-week stratification with *S(t)*= 0.59 ± 0.04 SE. In other words, only 11% of the seeds germinated (1- 0.89 = 11%) when stratified for 4 weeks. For seeds submitted to 8 weeks of stratification, the germination increased to 40 %. Longer stratification periods (12, 16 and 20 weeks) decrease the probability of not germinating to 0.29, 0.31 and, 0.27 (± 0.04 SE), resulting in 71%, 69%, and 72% germination, respectively. The median germination time occurred at 14 to 16-day interval. When comparing all the stratification periods, our results from the Mantel-Haenszel log-rank test (Table 1, left side) shows that survival curves of 4 and 8 weeks are significantly different from each other and from all the other stratification periods ($p<0.001$). The 12, 16, and 20-week stratification periods did not differ from each other ($p>0.1$) indicating that 12 weeks is the minimum stratification time for optimal germination, and if the seeds stay longer, there is no recognizable impact on final germination outcomes.

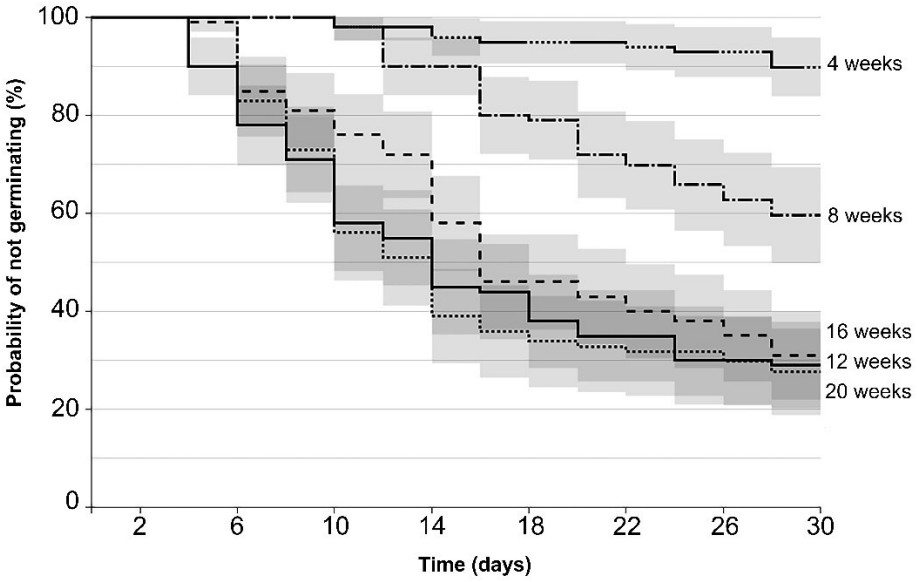


Figure 2. Kaplan-Meier estimates of survivor functions for *Frangula alnus* seeds submitted to different stratification periods (in weeks). The Y-axis reflects the cumulative probability and the time in days is indicated along the X-axis. The curves represent the probability of not germinating at the beginning and throughout the interval until the beginning of the next interval (95% confidence intervals).

Table 1. Results of the Mantel-Haenszel Log-rank test. The hazard ratios were calculated comparing the actual hazard rate to the expected one for each treatment; hazard ratio = 1). Comparisons between different stratification periods (in weeks) are shown in the left side of the table, and temperatures (ºC) are in the right side of the table. Statistical significance (p<0.05).

Mantel-Haenszel Log-rank Test							
Stratification (in weeks)	**Hazard Ratio**	**Chi-Square**	**Prob Level**	**Temperature (ºC)**	**Hazard Ratio**	**Chi-Square**	**Prob Level**
4/8	0.24	24.081	<0.0001	12/14	0.37	11.233	<0.0001
4/12	0.11	86.076	<0.0001	12/16	0.27	24.269	<0.0001
4/16	0.12	78.235	<0.0001	12/18	0.23	34.155	<0.0001
4/20	0.10	89.914	<0.0001	12/20	0.14	71.335	<0.0001
8/4	4.13	24.081	<0.0001	14/12	2.68	11.233	<0.0001
8/12	0.32	31.496	<0.0001	14/16	0.67	2.84	0.0919
8/16	0.38	22.809	<0.0001	14/18	0.56	6.955	0.0084
8/20	0.29	35.92	<0.0001	14/20	0.31	31.929	<0.0001
12/4	8.99	86.076	<0.0001	16/12	3.75	24.269	<0.0001
12/8	3.11	31.496	<0.0001	16/14	1.48	2.84	0.0919
12/16	1.23	1.339	0.2471	16/18	0.82	0.924	0.3364
12/20	0.98	0.016	0.9002	16/20	0.44	17.173	<0.0001
16/4	8.24	78.235	<0.0001	18/12	4.36	34.155	<0.0001
16/8	2.63	22.809	<0.0001	18/14	1.79	6.955	0.0084
16/12	0.81	1.339	0.2471	18/16	1.22	0.924	0.3364
16/20	0.77	2.091	0.1481	18/20	0.54	10.451	0.0012
20/4	9.57	89.914	<0.0001	20/12	7.09	71.335	<0.0001
20/8	3.40	35.92	<0.0001	20/14	3.23	31.929	<0.0001
20/12	1.02	0.016	0.9002	20/16	2.27	17.173	<0.0001
20/16	1.30	2.091	0.1481	20/18	1.85	10.451	0.0012

Temperature

Seeds at 20°C had the lowest chance of not germinating (0.30 ± 0.04 SE), resulting in 70% germination. At 18 and 16°C, seeds reached 53 and 45% germination with *S(t)*= 0.47 ± 0.04 SE and 0.55 ± 0.04 SE, respectively. At the coldest temperature (12°C), germination occurred in 14% of the seeds and rose to 34% at 14°C (*S(t)*= 0.86 ± 0.03 SE and 0.66 ± 0.04 SE, respectively). Results of the Mantel-Haenszel log-rank test (hazard ratios) (Table 1, right side), show the survival functions of seeds at 12°C are significantly different from all the other temperatures ($p<0.0001$). When comparing germination at 16°C, to 14°C and 18°C, no significant difference was found. However, germination at 14°C is significantly different from germination at 18°C. At warmer temperatures (24 and 28°C), germination reached 31% and 29%, respectively. Kaplan-Meier curve from seeds at 20°C was significantly different from 24 and 28°C. However, 24 and 28°C were not significantly different from each other (Figure 3, b). The temperatures (18/20°C) that resulted in the higher germination results were then grouped and compared to germination from the coldest temperatures (12/14°C) and the warmest ones (24/28°C) (K-M curves Figure 3, c). Warmer and colder temperatures increase the probability of not germinating (0.70 ± 0.03 SE and, 0.76 ± 0.03 SE), reducing germination of glossy buckthorn from 61.5% at 18/20°C to 30% at 24/28°, and to 24% at 12/14°C. However, higher temperatures did not delay germination that started at 4-6 days interval. When comparing the warmest temperatures to the coldest ones (12/14°C), it is possible to see a delay in germination that starts on the 10 to 12-day interval. The M-H log-rank test shows no significant difference between the warmest and coldest temperatures, however, tests that place more weight on hazards at the beginning of the study show a *p*-value at the limit of significance (Gehan-Wilcoxon: $p=0.0562$; Peto-Peto and mod. Peto-Peto: $p=0.0524$) (Table 2).

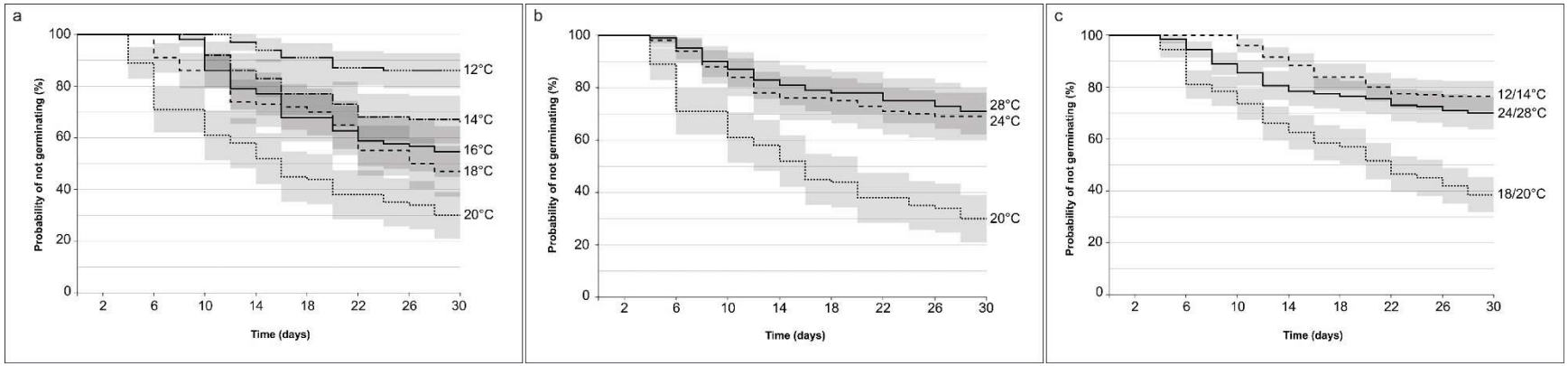


Figure 3. Kaplan-Meier estimates of survivor functions of *Frangula alnus* seeds germinated at (a) 12, 14, 16, 18, and 20ºC; (b) 20, 24, and 28ºC; (c) optimal temperature (18/20ºC), coldest temperature (12/14ºC), and warmest temperatures (24/28ºC). All curves with 95% confidence intervals.

Table 2. Results of the comparison tests for survival functions between the warmest (24/28ºC) and coldest temperatures (12/14ºC). The asterisk indicates a *p*-values at the limit of statistical significance ($p < 0.05$).

Test	*X2*	df	*p*-value	*z*-value	Across Time
M-H Log-rank	2.688	1	0.1011	±8.384	Equal weights across all times
Gehan-Wilcoxon	3.646	1	*0.0562	±3447	Places very heavy weight on hazards at the beginning of the study
Tarone-Ware	3.148	1	0.076	±170.119	Places heavy weight on hazards at the beginning of the study.
Peto-Peto	3.761	1	*0.0524	±8.474	Places a little more weight on hazards at the beginning of the study
Mod. Peto-Peto	3.764	1	*0.0524	±8.453	Places a little more weight on hazards at the beginning of the study
Fleming-Harrington (0.5, 0.5)	0.021	1	0.8847	±0.235	Places a little more weight on hazards at the end of the study
Fleming-Harrington (1, 1)	0.104	1	0.7467	±0.192	Places heavy weight on hazards at the end of the study.

Dark and light

Darkness had a negative impact on germination as it increased the probability of not germinating for all tested temperatures (20, 24, and 28°C) (K-M curves are shown in Figure 4). Germination at 20°C went down from 70% (at 12h light) to 54% (at dark), from 31% to 15% at 24°C, and from 29% to only 7% at 28°C. A 2-day delay in germination can also be observed for seeds that germinated at dark for all temperatures. The results of the log-rank tests demonstrated significant difference between all treatments (Table 3). However, test that placed higher weight on hazards at the end of the germination period (Fleming-Harrington), indicated that the survival curves for germination in light and dark at 20°C are equal. The potential effects of light/dark and temperature (as covariates), were tested by Cox proportional hazard model considering 20°C and the 12-hour photoperiod as reference groups. These results are shown in table 4. All regression coefficients (β_i) are negative indicating that the seeds tested in darkness and at warmer temperatures had a lower probability of germination than the ones in light at 20°C. Hazard ratios are represented by exponentiated coefficients (Exp β_i) that present the effect size of covariates. A hazard ratio equal to 0.5827 (<1) indicates that the variable "darkness" alone will decrease the hazard function by 41.7%. Warmer temperatures (exp β_i =0.3096 for 24°C and 0.2788 for 28°C) and darkness decreased the hazard function even further by 69% and 72%, respectively. Moreover, the factor of light can be calculated by the reverse 1/ exp β_i (dark) and is equal to 1.71, indicating that the probability of germination increases by a factor of 1.71 due to light (p=0.0029) when temperature is constant. The *p*-value (<0.0029) further suggests a strong relationship between the covariates (darkness and temperature) and reduced chance of germination. Moreover, when the 95% confidence intervals do not overlap with the values of 1, it is further indicative of a strong relationship (Romano and Stevanato 2020).

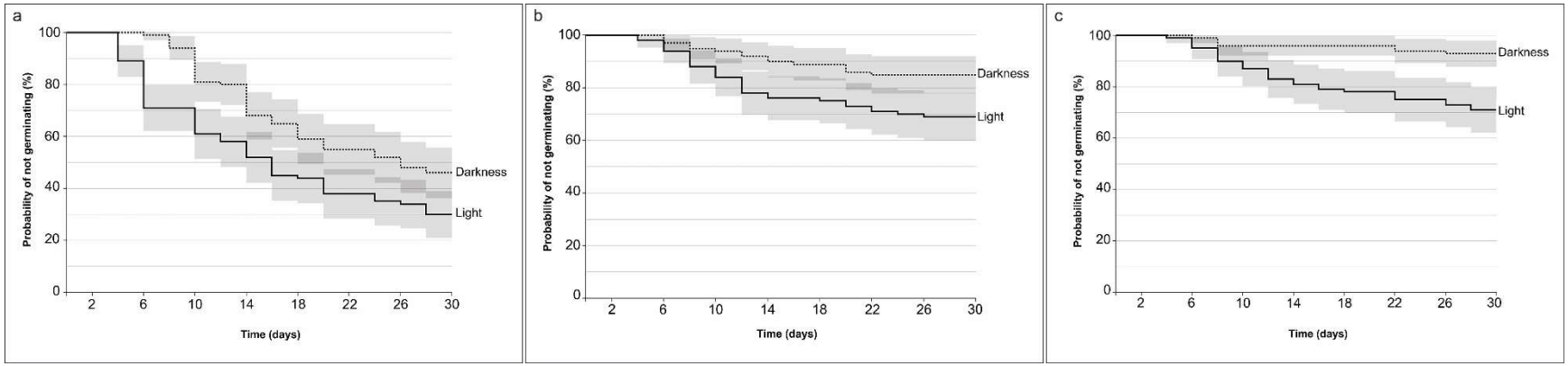


Figure 4. Kaplan-Meier survival curves of *Frangula alnus* seeds germinated at light and dark conditions at (a) 20°C; (b) 24°C and (c) 28°C (95% Confidence intervals).

Table 3. Log-rank test results for survival functions between light and dark treatments at each temperature (20, 24 and 28ºC).

	Temperature 20ºC			**Temperature 24ºC**			**Temperature 28ºC**		
Test	***X2***	***p*-value**	***z*-value**	***X2***	***p*-value**	***z*-value**	***X2***	***p*-value**	***z*-value**
M-H Log-rank	9.071	0.0026	±15.864	7.239	0.0071	±8.994	16.069	<0.0001	±11.889
Gehan-Wilcoxon	12.765	0.0004	±2819.000	7.341	0.0067	±1626.000	15.791	<0.0001	±2162.000
Tarone-Ware	10.961	0.0009	±209.973	7.301	0.0069	±120.836	15.946	<0.0001	±160.249
Peto-Peto	12.993	0.0003	±12.912	7.362	0.0067	±7.920	15.957	<0.0001	±10.664
Mod. Peto-Peto	13.015	0.0003	±12.840	7.363	0.0067	±7.877	15.955	<0.0001	±10.606
Fleming-Harrington (0.5, 0.5)	1.944	**0.1632	±2.974	5.390	0.0203	±2.280	14.821	<0.0001	±3.019
Fleming-Harrington (1, 1)	1.078	**0.2993	±1.012	4.304	0.0380	±0.709	13.138	0.0003	±0.899

** *p*-values indicates a non statistical significance ($p < 0.05$

Table 4. Summary table of the Cox's PH model for *Frangula alnus* germination data. Darkness and temperature were selected as covariates. Seeds that germinated in light at 20ºC were selected as reference group.

Variables	βi	**SE of** βi	**Exp** βi	**Wald z-Value**	**p-Value**	**Exp** βi **95% CI**
Darkness only	-0.54006	0.181218	0.5827	-2.9802	0.0029	0.4085-0.8312
Darkness at 24ºC	-1.172558	0.216176	0.3096	-5.4241	<0.0001	0.2027-0.4729
Darkness at 28ºC	-1.277308	0.221334	0.2788	-5.771	<0.0001	0.1807-0.4302

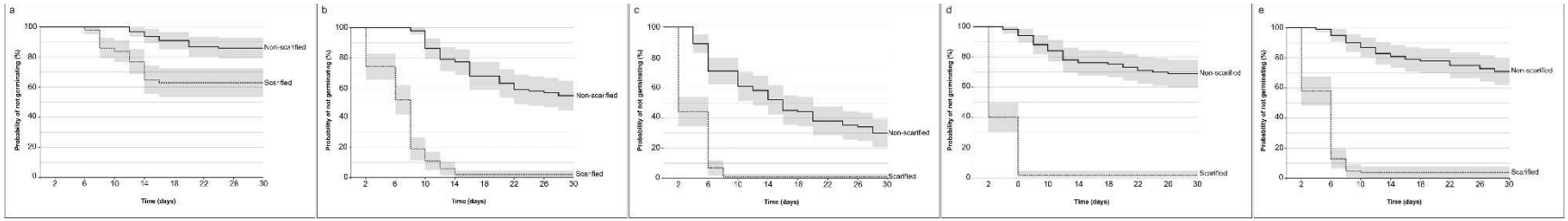


Figure 5. Kaplan-Meier survival curves of *Frangula alnus* seeds that were scarified and non-scarified at different temperatures: (a)12 ºC, (b) 16 ºC, (c) 20 ºC, (d) 24 ºC, and (e) 28ºC). 95% confidence intervals.

Scarification

Scarification decreased the probability of not germinating at all tested temperatures (K-M curves are shown in Figure 5). At 12°C, germination increased from 14% ($S(t)$= 0.86 ± 0.03 SE) to 37% ($S(t)$= 0.63 ± 0.04 SE), when seeds were scarified. At warmer temperatures (16 to 28°C), germination occurred in 96 to 99% of scarified seeds. Log-rank tests show a significant difference between scarified and non-scarified seeds at all temperatures. However, when using tests that apply heavier weighting to hazards at the end of the germination period, it seems that there is no difference between scarified and non-scarified seeds at 12°C (Table 5).

Results from the Cox proportional model are shown in Table 6. Scarification had a regression coefficient of βi 2.184 indicating that scarified seeds had a higher probability of germination than non-scarified ones. The regression coefficient βi for temperatures both colder and warmer than 20°C was negative, representing a decrease in germination associated with this covariate of non-scarified seeds. The negative effect of temperature was offset by scarification which increased the hazard function. However, colder temperatures were still able to decrease germination at 12 and 16ºC. On the other hand, at warmer temperatures, scarification obscured the effect of temperature and resulted in an overall increase in germination. In the non-scarified seed trials, temperature decreased the hazard rate by 87% at 12°C. When scarified seeds were submitted to the same temperature, a reduction of 64% was observed. At warmer temperatures, scarification increased the hazard function throughout the study period by 218.5% for seeds at 24°C, and by 121.8% for seeds tested at 28°C.

Table 5. Results of log-rank tests for survival functions of *Frangula alnus* between scarified and non-scarified seeds at different temperatures (16, 20, 24, and 28ºC).

	12ºC			**16ºC**		**20ºC**		**24ºC**		**28ºC**	
Test	***X2***	***p*-value**	***z*-value**	***X2***	***z*-value**	***X2***	***z*-value**	***X2***	***z*-value**	***X2***	***z*-value**
M-H Log-rank	16.006	0.0001	±13.832	161.643	±57.996	112.018	±48.110	170.058	±54.665	156.755	±53.994
Gehan-Wilcoxon	18.193	0.0000	±2659.000	150.400	±9161.000	109.967	±8002.000	161.982	±9434.000	148.234	±9071.000
Tarone-Ware	17.134	0.0000	±191.839	157.764	±724.55	112.335	±615.078	167.813	±715.467	153.987	±696.995
Peto-Peto	17.815	0.0000	±12.430	147.166	±38.374	98.938	±27.148	156.836	±32.873	142.576	±34.032
Mod. Peto-Peto	17.827	0.0000	±12.365	147.050	±38.132	98.892	±26.986	156.784	±32.684	142.501	±33.831
Fleming-Harrington (0.5, 0.5)	5.876	0.0154	±2.453	132.220	±18.855	41.124	±9.687	85.620	±11.392	105.207	±14.127
Fleming-Harrington (1, 1)	2.273	0.1316	±0.540	125.769	±8.057	43.762	±4.662	84.446	±5.261	102.722	±6.089

p-values for comparisons from 16ºC to 20ºC were <0.0001, to avoid repetition they were omitted.

Table 6. Cox's PH model results showing the effects of covariates scarification and temperature on seed germination of *Frangula alnus*. Temperatures were 12, 16, 24 and 28ºC. Non-scarified seeds at 20ºC were used as refence group.

	Variables	βi	**SE of** βi	**Exp** βi	**Wald *z*-Value**	***p*-Value**	**Exp** βi **95%CI**
	Scarification	2.184117	0.16691	8.8828	13.0856	<0.0001	6.4044-12.3204
	Temperature:						
No-scarified	12°C	-2.04755	0.293012	0.1291	-6.9879	<0.0001	0.0727-0.2292
	16°C	-0.7432	0.191143	0.4756	-3.8882	0.0001	0.327-0.6917
	24°C	-1.11704	0.215928	0.3272	-5.1732	<0.0001	0.2143-0.4997
	28°C	-1.2169	0.221057	0.2961	-5.5049	<0.0001	0.192-0.4567
Scarified	12°C	-1.03846	0.354765	0.354	-2.9272	0.0034	0.1766-0.7095
	16°C	-0.1734	0.240298	0.8408	-0.7216	0.4705	0.525-1.3466
	24°C	1.158736	0.258686	3.1859	4.4793	<0.0001	1.9188-5.2897
	28°C	0.796751	0.263518	2.2183	3.0235	0.0025	1.3235-3.7182

DISCUSSION

Breaking dormancy and germination pattern

Glossy buckthorn seeds exhibit physiological dormancy (PD) and do not germinate immediately after being dispersed as they require a minimum period of cold stratification to break dormancy. In the study area, stratification of glossy buckthorn seeds may start as early as mid-November, when the daily maximum temperature drops and remains below 5ºC. In the following months, temperatures decrease further resulting in ideal conditions for glossy buckthorn seeds to stratify and for dormancy to be broken. According to our findings, glossy buckthorn seeds require relatively higher temperatures to germinate (only 14% germination was observed at 12ºC), which may inhibit seedling emergence in the early spring, such as in April (daily average of 5,3 ºC (±2,9 SD)). The transition from winter to spring in Québec is characterized by unstable conditions with frequent snowfall (Gouvernement du Québec 2021). As a consequence, this delay in beginning germination may increase the chance of survival of glossy buckthorn seedlings giving the species a competitive advantage over earlier germinating species.

Based on our results, glossy buckthorn seeds begin germinating at the very end of spring when temperatures reach 12ºC (May). At the beginning and throughout the summer, germination will increase and speed up as the temperature rises (14, 16 and 18ºC) reaching the highest germination rate (70%) at 20ºC. At warmer temperatures (24 and 28ºC), a decrease in germination was observed suggesting that higher temperatures experienced in mid-summer prevent most seeds from germinating and/or induce them into dormancy again (secondary dormancy). This may be a reason that the glossy buckthorn seeds do not germinate in early autumn when temperatures are very similar to the ones experienced in spring/early summer as the seedlings would not have enough time to grow before winter (registered daily average temperature of 14.0 ºC (±1.3 SD) in September and 7.5 ºC (±1.3 SD) in October) (Fig. 1). A similar germination timing was observed by (Eisenhaure et al. 2021), while studying impacts of different substrates on glossy buckthorn seedlings' emergence in New Hampshire, USA. Mean temperatures from May to August in their study areas are 0.7 to 1.9ºC warmer than the ones registered for our study site (NOAA 2021). The authors recorded the greatest seedling emergence at the beginning of June, with a sharp decline occurring in early July and complete cessation by mid-August and September (Eisenhaure et al. 2021). Their findings corroborated by ours help to identify glossy buckthorn's response to temperatures and outline its germination pattern. This timing allows glossy buckthorn seeds to germinate during a window of time that promotes seedling survival by germinating during more stable environments and taking advantage of most of the growing season. By the end of summer, seeds that did not germinate will be incorporated into the seed bank and need to undergo another period of cold stratification to break dormancy (CC Baskin and Baskin 2014; Moravcová et al. 2006).

Effects of light and scarification on germination

Light is another important factor that can prompt or prevent seed germination (CC Baskin and Baskin 2014; JM Baskin et al. 1989; Fenner and Thompson 2005; Yin et al. 2009). *Frangula alnus* seeds can germinate in both light and darkness, although with less success for the latter. Early in the spring, more light can reach the soil in temperate

forests when deciduous leaves are not open. However, light can also reach the soil late in fall or during the growing season when canopy gaps are created due to removal of vegetation or other disturbance (Fenner and Thompson 2005, p. 164). According to our findings, glossy buckthorn seeds in well-lit habitats at mild temperatures (20ºC) will germinate more frequently than the ones at warmer temperatures and in shaded areas.

Scarification has different effects on the germination of several species, including changes in light and/or darkness requirements (Takaki & Gama, 1998), and dormancy levels (Matus-Cádiz and Hucl 2005). In glossy buckthorn, scarified seeds were able to overcome limiting temperatures and germinated two times more often at colder temperatures (12 ºC) and more than three times as often when submitted to warmer temperatures (24 and 28ºC) compared to non-scarified seeds. The speed of germination was also higher in scarified seeds as germination began 2 to 8 days earlier than non-scarified seeds (see K-M germination curves for comparisons). However, to further understand the effect of scarification on glossy buckthorn seeds, studies comparing natural scarification caused by the seed's passage through a frugivore's gut (often birds) and the effect of burial on germination must be conducted as we are not aware of any study on these subjects.

Spread and invasion strategy

According to Pyšek and Richardson (2007), invasive species germinate earlier, quicker, and/or over a wider range of environmental conditions than native or non-invasive ones which provide a competitive advantage (Gioria and Osborne 2014; Gioria and Pyšek 2016). However, in volatile environments, forming a seed reservoir may be a better strategy for invasive species as the buried seeds are able to ensure the persistence of the species while mitigating competition (Gioria et al. 2012; Gioria and Pyšek 2017; Thompson and Grime 1979). Glossy buckthorn seeds require cold stratification, germinate better in light, and achieve greater germination rates at mild temperatures which are known to delay germination and promote seed burial (CC Baskin and Baskin 2014; Gioria and Pyšek 2017; Grime et al. 1981). Glossy buckthorn seeds are known to form seed banks that can remain viable in the soil for up to three years (Godwin 1943). Our research suggests that the formation of seed banks is the main strategy used by glossy buckthorn to invade and establish in novel habitats.

The timing and rate of germination affect the relationship between invasive and native species, as these interactions influence the ability of each species to compete for space and resources (Benech-Arnold et al. 2000; Gioria and Osborne 2014). The fruits produced and released at the end of the growing season are dormant and will spend the least favorable season buried in soil. As most of the seeds require higher temperatures and light to germinate, germination will occur later when environmental conditions are more stable and when competition with other plants may be reduced (e.g., in open areas or recently formed canopy gaps), which can potentially increase its fitness (Gioria and Pyšek 2017; Harper 1977). Invasive species with light-sensitive seeds (photoblastic) may have an advantage during the spreading and establishment processes as most seeds will not germinate when deeply buried (CC Baskin and Baskin 2014; Gioria and Pyšek 2017). Suitable temperatures, disturbances resulting in the canopy opening, or otherwise exposure of the seeds in the soil may promote germination during most of the growing season which would

allow seedlings to grow and become established before the temperature drops below 0ºC. When germination does not occur, the seeds are incorporated into seed banks.

As an invasive species, glossy buckthorn capitalizes on these seed traits by ensuring germination at "safer" moments and spaces or otherwise amassing a persistent seed bank that promotes maintenance of the species in case of unfavorable conditions. When applying control methods, managers and researchers must take into consideration the germination mechanisms of glossy buckthorn, its response to environmental conditions, and the resulting formation of seed banks in invaded areas.

Climate change and future research directions

By 2100, eastern Canada will face warmer annual temperatures (3 to 5ºC) leading to longer growing seasons while precipitation will decrease and increase during the summer and winter, respectively (Bush and Lemmen 2019; Dukes et al. 2009). Shorter winters may diminish stratification periods and decrease the number of seeds germinating. However, as a 12-week stratification period may successfully break dormancy, it is unlikely that glossy buckthorn will be affected in Québec, but warmer regions in the United States may be impacted. A temperature increase will result in glossy buckthorn seeds germinating earlier, such as, near the beginning of spring, granting the seeds access to a longer growing season. However, germination may also be ending earlier due to the high-temperature peaks during the summer, shifting the overall germination period. Beyond the impacts on stratification and germination, it is difficult to predict how glossy buckthorn will be affected by climate change, as the climate alters resource availability, landscapes, and impacts the dynamics among species.

CONCLUSION

This study provides us with a stronger understanding of how temperature, light, stratification, and scarification affect glossy buckthorn seeds as well as its seasonal germination pattern in North America. Glossy buckthorn seeds require a cold stratification to germinate and achieve higher germination rates at moderate temperatures and in well-lit conditions and relies in the formation of persistent seed banks as a strategy to colonize new habitats. Climate change will increase temperatures which may impact the seeds buried within the soil, including the length of stratification periods in some areas of North America and the timing of germination. Warmer temperatures in the spring and peaks earlier in the summer may also shift the germination period of glossy buckthorn. The experimental conditions of constant moist substrate and temperatures applied in this study are simple when compared to the complexity of reactions triggered by moisture stress and fluctuating temperatures in natural environments. For this reason, we recommend further studies and recognize the need for caution when interpreting our findings. Despite this limitation, the data shows several coherent patterns that can be used to better predict the impact of climate change on glossy buckthorn germination.

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