Title: Autumn larval cold tolerance does not predict the northern range limit of a widespread butterfly species

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Author's Contributions

PT and HMK: conceived the ideas, designed the methodology and wrote the paper. PT collected data and performed analyses. HM: helped design the methodology and edit the paper. All authors contributed critically to the drafts and gave final approval for publication.

Data availability

Should the manuscript be accepted, the data supporting the results will be archived in an appropriate public repository (Dryad or Figshare) and the data DOI will be included at the end of the article.

Abstract

Climate change is driving range shifts, and a lack of cold tolerance is hypothesized to constrain insect range expansion at poleward latitudes. However, few, if any, studies have tested this hypothesis during autumn when organisms are subjected to sporadic low temperature exposure but may not have become cold tolerant yet. In this study, we integrated organismal thermal tolerance measures into species distribution models for larvae of the Giant Swallowtail butterfly, *Papilio cresphontes,* living at the northern edge of its actively expanding range. Cold hardiness of field-collected larvae was determined using three common metrics of cold-induced physiological thresholds: the supercooling point (SCP), critical thermal minimum (CT_{min}), and survival following cold exposure. *P. cresphontes* larvae in autumn have a CT_{min} of 2.14°C, and were determined to be tolerant of chilling. These larvae have a SCP of -6.6°C and can survive prolonged exposure to -2°C. They generally die, however, at temperatures below their SCP (-8°C), suggesting they are chill tolerant or modestly freeze avoidant. Using this information, we examined the importance of low temperatures at a broad scale, by comparing species distribution models of *P. cresphontes* based only on environmental data derived from other sources to models that also included the cold tolerance parameters generated experimentally. Our modelling revealed that growing degree-days and precipitation best predicted the distribution of *P. cresphontes*, while the cold tolerance variables did not explain much variation in habitat suitability. As such, the modelling results were consistent with our experimental results: low temperatures in autumn are unlikely to limit the distribution of *P. cresphontes*. Further investigation into the ecological relevance of the physiological thresholds determined here will help determine how climate limits the distribution of *P. cresphontes*. Understanding the factors that limit species distributions is key to predicting how climate change will drive species range shifts.

Keywords: mechanistic species distribution model, survival assays, Maxent, global warming, cold distribution limits, insect, low temperature limit

Introduction

Over the past few decades, range shifts due to climate and land use changes have been reported in both plants and animals. Many are experiencing a poleward and/or upward shift in distribution, pushing their northern and upper range limit to higher latitudes and altitudes (Chen et al., 2011; VanDerWal et al., 2013; but see Kerr et al. 2015). However, there has been substantial variation in the degree and direction of range shift across taxonomic groups (Chen et al., 2011; Lenoir & Svenning 2015). For example, some insect taxa (i.e., dragonflies, spiders)

have shifted northwards to a much greater extent than mammals and birds, whereas other groups have expanded southwards. This variation in range shifts remains difficult to explain, and thus necessitates a better understanding of the factors influencing species' distributions (Buckley & Kingsolver, 2012; Sunday et al. 2012; Sgrò et al., 2016).

A climate change-driven range shift in insects is often attributed to the relaxation of a harsher poleward climate since temperature acts as a physiological limitation and as a phenological cue (Logan & Bentz, 1999; Root et al., 2003; Elkinton et al., 2008). For those species with northern range edges associated with temperature clines, climatic warming has frequently resulted in the colonization of suitable areas in more northern latitudes (Parmesan, 1996; Parmesan & Yohe, 2003). For example, the northern range expansion of the deer fly (*Lipoptena cervi*) in Finland is due to warmer temperatures during the summer (Härkönen et al., 2010). However, for many other species, it remains unclear how climate change has led to range shifts (Chen et al., 2011; Sunday et al., 2012).

Low temperatures throughout the year can constrain the ability of insects to persist at northern range limits as they can influence any life cycle stage (Ungerer et al., 1999). Overwinter survival has been shown to be a key factor limiting the ranges of insects (Crozier, 2003). For example, the northern range edges of the Southern pine beetle (*Dendroctonus frontalis*) and Sachem skipper butterfly (*Atalopedes campestris*) are limited by temperatures below -16°C (Lesk et al., 2017) and the -4°C January isotherm (Crozier, 2003), respectively. Overwintering insects can adopt one of multiple physiological strategies for surviving winter (Williams et al., 2015); some insects can survive internal ice formation and are termed freeze tolerant, some physiologically supress the temperature at which their body fluids spontaneously freeze (the supercooling point; SCP) and are termed freeze avoiding, while others remain susceptible to chilling (termed chill susceptible), and seek relatively warm microhabitats to overwinter (Sinclair, 1999; Denlinger & Lee, 2010; Overgaard & MacMillan, 2017).

While winter represents a considerable challenge (Robinet & Roques, 2010; Williams et al., 2015), insects are also likely to be particularly vulnerable to low temperatures during autumn. Autumn is when most insects are acquiring cold tolerance through physiological adjustments (acclimatization) and have therefore not reached their peak cold hardiness (i.e., the capacity to tolerate intensity and duration of cold exposure; Tauber et al., 1986). For example, monarch butterflies, *Danaus plexippus,* that were not yet acclimated had a mortality rate of 60% when exposed to low temperatures in September and October compared to 20% for acclimated ones (Larsen & Lee, 1994). There is also a much higher frequency of cold temperature events in autumn than in the summer (Danks, 1978). Yet, no study, to our knowledge, has considered low temperatures during autumn as a limiting factor on the geographic distributions of insects.

Regardless of season, cold exposure can cause insects to cross physiological thresholds leading to sublethal effects. At a species-specific low temperature, most insects lose the ability for coordinated movement (a critical thermal minimum; CT_{min}) after which they enter a state of complete neuromuscular silence termed chill coma (Gibert & Huey, 2001; MacMillan & Sinclair, 2011; Oyen & Dillon, 2018). Many insects can recover from this state with no immediate evidence of injury; but prolonged or severe cold exposure can cause behavioural defects, or slow or halt development (Asahina, 1970; Rojas & Leopold, 1996; Overgaard & MacMillan, 2017). Chill susceptible insects suffer from a loss of homeostasis at low temperatures well above their SCP, while those more tolerant to chilling survive such exposures (Overgaard & MacMillan, 2017). While freeze tolerant insects can survive freezing of the extracellular fluid at their SCP, they can suffer from ice-related injuries below this temperature. By contrast, freeze avoidant insects cannot survive at temperatures below their SCP. Thus, the relationship between the SCP and injury/mortality is critical to determining the cold tolerance of these insects (Sinclair et al., 2015).

116 Cold tolerance traits, like the CT_{min}, SCP, or survival following a cold stress frequently
117 correlate strongly with insect distribution (Bozinovic et al. 2011; Gouveia et al. 2014; Andersen
118 et al. 2015). For example, the CT_{min} and CT_{max} of *Drosophila* species have been used to
119 accurately predict their current distributions(Overgaard et al., 2014). Similarly, the SCPs and
120 potential for mortality at more mild temperatures were used to understand and predict the
121 potential invasion limits of pine beetles in North America (Ungerer et al., 1999; Bleiker et al.,
122 2019). However, given the limited number of taxa in which these relationships have been
123 explored, we have little to no ability to generalize predictions about how climate influences
124 species' distributions via low temperatures to other taxa (Ouimette 2018).
125 Species distribution modelling is a commonly used approach to evaluate recent range
126 shifts and forecast future shifts due to climate change. For example, they have been used to
127 predict invasive species' distributions (Jiménez-Valverde et al., 2011), define species' ranges
128 and predict impacts of climate change on available habitat (Araujo et al. 2005). Using
129 correlations between georeferenced occurrence records and a set of environmental variables
130 with geospatial data, these models predict a species' suitable habitat (Elith et al., 2011). While
131 the approach is useful for many applications, these models often violate key assumptions, such
132 as predicting habitat suitability in novel conditions and omitting key biotic variables known to
133 influence geographic distributions such as species interactions (Tingley et al. 2014; Briscoe et
134 al. 2019).
135 One way that has been demonstrated to improve the accuracy of these models is to
136 incorporate physiological variables or stress tolerance thresholds (e.g., heat tolerance,
137 metabolic needs, thermal limits; (Kearney & Porter, 2009; Overgaard et al. 2014). By using
138 variables derived directly from these physiological traits, the underlying processes explaining
139 the species' distribution are thought to be better incorporated into the model (Kearney & Porter,
140 2009). For example, Elith et al. (2010) built a distribution model for the cane toad (*Bufo marinus*)

141 based on the impact of topography on the above-ground activity of adults and the temperature
142 requirement of larval growth and survival. Models produced in such a way are called
143 mechanistic or process-based models. They calculate the likelihood of presence by eliminating
144 regions where the conditions are detrimental to species persistence (Buckley et al., 2010). In
145 some contexts, these models have been shown to be more accurate than (Peterson, 2011), or
146 complement weaknesses of (Martinez 2015), correlative models when modelling the
147 fundamental niche and can strengthen predictions about future distributions (Buckley et al.,
148 2011; Martinez 2015; Evans et al. 2016; Kotta et al. 2019). The main limitation with this
149 approach is that experimentally derived variables can be time-consuming to develop and thus
150 not feasible to do for large assemblages of species or over broad ranges (Peterson 2011; Evans
151 et al. 2016). It can also be difficult to find traits that are predictive and functional in the species'
152 environment (Kearney & Porter, 2009).

153 Here, we use a widespread butterfly, the Giant Swallowtail, *Papilio cresphontes*, as a case
154 study to test the hypothesis that low temperatures during autumn are the limiting factor of the
155 northern edges of insect species' distributions. Specifically, the two main objectives are to: i) find
156 relevant cold tolerance thresholds of *P. cresphontes* collected in autumn; and ii) determine the
157 relative importance of these thresholds on the distribution of *P. cresphontes* at the landscape
158 level. Simultaneously, we aim to determine the cold tolerance strategy of larvae at the northern
159 range limit. Determining the species' cold tolerance strategy improves our knowledge about the
160 importance of low temperatures on *P. cresphontes* survival and helps identify which low
161 temperature thresholds are likely to be most relevant at the species' northern range. In this
162 study, we focus on the pre-overwinter life stage (i.e., larval stage), which is in contrast to most
163 other studies which have determined the cold tolerance strategy of the life stage that
164 overwinters (Radchuk et al., 2013). To determine the importance of low temperatures at a broad

scale, we model the geographic distribution of *P. cresphontes* with the cold tolerance parameters we generated experimentally.

P. cresphontes has undergone a rapid expansion over the past decade and now occurs as far north as Ottawa, Ontario, Canada. Finkbeiner (2011) hypothesized that the range expansion into New York state from 2000 to 2010 was due to the disappearance of frost (as defined meteorologically) in September. In spite of this, they still showed frost resistance in larvae and that individuals could survive exposure to -3.9°C. However, these observations were based on field observations of only a few specimens and the cold tolerance strategy was not determined. Therefore, a more in-depth study of the impact of low temperature on *P. cresphontes* is warranted.

To establish the larval cold tolerance strategy, we measured three common cold-induced physiological thresholds: SCP, CT_{min}, and survival following cold exposure. To gain a better estimate of the cold tolerance strategy, thresholds (SCP and low temperature survival) were measured for two generations and multiple sites across a latitudinal gradient at the northern range limit. Latitude has the potential to affect cold tolerance due to its correlation with climate (e.g., photoperiod, temperature; Sømme, 1982; Tanaka, 1996) but see Yoshio & Ishii, 2001). Therefore, latitude could affect when and to what extent organisms are cold hardy. Combined, these experiments were used to identify a potentially relevant low temperature threshold of larvae collected at the northern range edge. We examined the importance of low temperatures at a broad scale by comparing species distribution models of *P. cresphontes* based only on environmental data derived from other sources to models that also included the cold tolerance parameters generated experimentally.

188 **Materials and methods**

189 1. Study system

190 The Giant Swallowtail (*Papilio cresphontes*) butterfly is a member of the Papilionidae
191 family. The species' range extends from Costa Rica throughout the North American continent.
192 The bulk of the population is concentrated in the Eastern United States. It ranges as far north as
193 the Ottawa region, Ontario, Canada. (Figure 1)

194 *P. cresphontes* larvae undergo five instars before entering the chrysalis stage (Bullock,
195 1991). In Ontario, there are two generations with flights occurring from May to July and again
196 from late July to late September (Layberry, Hall & Lafontaine, 1998). The larval stage lasts from
197 3-4 weeks and pupae formed in the summer emerge after 10-12 days, but those that develop in
198 autumn will remain as pupae until spring. The overwintering pupae undergoes winter diapause,
199 a state of lowered metabolism, with reduced respiration, and no feeding or growth occurs (Scott,
200 1997). Once eclosed, adults live for 6-14 days (Layberry, Hall & Lafontaine, 1998).

201 *P. cresphontes* is known to use plants of the Rutaceae family as their primary food
202 source in the larval stage. In Ontario, those plants consist mainly of Northern Prickly Ash
203 (*Zanthoxylum americanum*) and Hoptree (*Ptelea trifoliata*). The adults are generalists and will
204 gather nectar from most flowering plants, for example, goldenrod (*Solidago*), swamp milkweed
205 (*Asclepias incarnata*) and azalea (*Rhododendron azaleastrum*)(McAuslane, 2004).

206

207 2. Cold tolerance experiments

208 *i) Experimental overview*

209 To determine the impact of low temperatures on *P. cresphontes* larval survival and
210 developmental success, three experiments were conducted (Figure 2; Figure S1). The first
211 experiment was done to determine the cold tolerance strategy of the larvae by measuring the
212 SCP in relation to cold survival. Additionally, since the results from this initial experiment were

213 not enough to clearly define the cold tolerance strategy, further low-temperature survival assays
214 (i.e. the ambient exposure of larvae to low temperatures for an extended period of time) were
215 conducted to better determine the impacts of prolonged low temperature exposure on survival
216 and developmental success. A third experiment was conducted to measure the CT_{min}.
217 The SCP and low-temperature survival assays were measured for both generations and
218 multiple sites (n=4) across a latitudinal gradient at the northern range limit (Figure 1, 2).
219
220 *(ii) Field sampling*
221 To gain a better understanding of the cold tolerance strategy, larval sampling occurred
222 during the two *P. cresphontes* generations: July and August of 2018. A total of 117 larvae were
223 collected from four sites around Ottawa, Ontario, Canada (Mud Lake, Shirley's Bay, Brockville
224 and Queens University Biological Station (QUBS)) spanning a 100 km latitudinal gradient at the
225 northern range limit of *P. cresphontes* (Figure 1). These four sites were accessible and had a
226 high abundance of larvae.
227 Until the start of the experiments, captured individuals in July were provided with a fresh
228 supply of *Z. americanum* and kept in an LTCP-19 Biochamber at the University of Ottawa,
229 Canada (45.423325, -75.683177) on a 21°C/25°C 15h: 9h L:D cycle with light intensity peaking
230 at noon. These conditions were chosen to match the average environmental conditions in July
231 for the Ottawa region. To replicate the conditions required for larvae to prepare to overwinter,
232 the chamber parameters were modified weekly for the August generation to match the
233 conditions from August to October (meteomedia.ca; Figure S1). Over the time spent in the
234 chamber, peak daily temperatures reached a maximum 25 °C and a minimum of 15°C overnight
235 at the beginning and by the end, daytime temperatures only reached 10°C and a minimum of
236 6°C overnight (Figure S1). Likewise, photoperiod fell from 14.5h to 11h. To validate our

treatments, we recorded the realized climate regime in the environmental chamber using a temperature logger (HOBO Pendant, Onset Computer Corporation, Bourne, MA).

(iii) Experimental details

Experiment 1: Supercooling point (SCP) test

To identify the cold tolerance strategy of *P. cresphontes* larvae, we quantified the SCP and survival following a freezing event in the first generation (i.e. the July generation; n=27 from QUBS; Figure 2; Figure S1). The SCP test was done following the recommended methodology of Sinclair (2015) and details can be found in Appendix S1.

To get a more accurate estimate of SCP, another SCP test was done on the second generation (i.e. the August generation; n=29; Figure 2, Table S1; Figure S1). The individuals from the August generation were subjected to the same cooling protocol as described above but were kept submerged until all of the larvae had frozen. This was done to obtain the full distribution of SCP values.

Experiment 2: Low-temperature survival assays

To identify the consequences of chronic low temperature likely to be experienced by the larvae before pupation, three low-temperature survival assays were conducted (Figure 2; Figure S1). The duration and temperature of the assays were chosen based on the SCP determined in the first experiment and on meteorological data in the Ottawa area representing the developmental period before pupation. In the Ottawa region, this period corresponds to the month of October. We considered temperatures from 2012 to 2017, which correspond with the timing of the recent range expansion of *P. cresphontes* into the Ottawa area (Ontario Butterfly Atlas: www.ontarioinsects.org/atlas_online.htm) and used the 'OTTAWA CDA RCS' weather station (45.38, -75.71). For this timeframe, the mean and lowest temperatures recorded during

frost events (as determined meteorologically: the span of time in which the temperature is below 0°C for more than one hour) were -1.33°C and -6.3°C, respectively, and the average frost time was 6.24 hours. Consequently, and in conjunction with the measured SCP of -5.6°C determined in the first experiment, trials were run at -2°C, -6°C, -8°C for 7 hours each.

To determine whether larvae were chill susceptible, the first assay was done at -2°C (Figure 2). Mortality at this temperature (i.e., above the SCP; see results) would indicate this species is chill susceptible whereas survival would confirm the ability of larvae to tolerate exposure to low temperatures above their SCP, thus making them chill tolerant or freeze avoidant. The second test was done at -6°C to distinguish between freeze avoidant and freeze tolerant strategies (Figure 2). Considerable mortality near the mean SCP would mean larvae are freeze avoidant whereas high survival would mean they are more likely freeze tolerant. Finally, to find a potentially ecologically relevant low temperature threshold causing high mortality (i.e. a potential lower lethal limit), we also tested larval survival at the lowest recorded temperature for October at this weather station from 2012-2017 (i.e. -8°C). Exposure at this temperature tests the maximum larval cold tolerance and their resistance to freezing since it is well below the mean SCP obtained in the previous experiment (i.e., -5.77 °C; Figure 2).

The low-temperature assays were done in an environmental growth chamber (Biochambers model LTCB-19) for both larval generations and across multiple sites (Figure 2; Table S1). The -2°C test was not repeated in August because i) there was high survival with the July individuals (see results); ii) a low number of specimens were collected in the field, and iii) this temperature was unlikely to be a limiting factor for larval survival and thus was not a priority to test. Larvae were weighed and measured before being placed in individual containers inside the chamber. The chamber temperature was brought down from 21°C to the predetermined test temperature at a rate of 0.1°C per minute. Once the desired minimum temperature was reached, the temperature remained constant for seven hours before being increased back to

287 21°C at the same rate. Larvae were then left to recuperate. They were allowed to feed on
288 freshly harvested *Z. americanum* leaves during the trial and were checked daily for mortality.
289 Death was determined when larvae did not react to physical stimuli: larvae were poked with a
290 stick, sprayed with water and then shaken. All larvae that survived to pupation were kept until
291 emergence.
292
293 Experiment 3: Critical thermal minimum (CT_{min})
294 To identify the temperature at which larvae lose coordination, a CT_{min} test was done on
295 twenty larvae were caught in July from the QUBS site, following the method of Andersen et al
296 (2014)(see Appendix S1 for details). The CT_{min} test was only done on July individuals since it
297 required a higher number of specimens than the other tests and our sample size was limited.
298
299 *iv) Statistical analysis*
300 To compare survival rates of larvae of the July generation that froze to those that did not
301 freeze during the SCP experiment, a $\chi 2$ test was used. The rate above and below the SCP for
302 the August generation could not be compared since all larvae were exposed to low
303 temperatures until SCP was reached (i.e. no survival rate was calculated above SCP). To test
304 whether mean SCP differed across sites for the August generation, an ANOVA was run. Since
305 there was no significant difference in mean SCP across sites (df=3, F-value=0.94 $p = 0.44$), site-
306 level data were pooled. The assumptions of normality and homogeneity were evaluated using
307 the Shapiro-Wilk test and the Brown-Forsythe for homogeneity of variance test, respectively.
308 To compare the larval survival rate and the rates of pupation and adult emergence
309 between generations and sites for the low-temperature assays, a $\chi 2$ test was used. There was
310 no impact of site on survival rate or developmental success in any assay (Table S2), so site-
311 level data were pooled. All analyses were conducted in R (version 3.4.1).

312

313 2. Distribution modelling

314 *i) Overall approach*

315 To determine the limiting factors of the current geographic distribution of *P. cresphontes*,
316 we modelled its range at two different spatial extents: northern range and the entire distribution
317 (Figure 1); and using two different modelling approaches. We wanted to determine whether the
318 relative importance of variables differs between the northern range and the entire distribution.
319 We defined the northern range as the area encompassing the upper 50th percentile of
320 occurrences based on both latitude and longitude in the northeastern part of the range (Figure
321 1). The 50th percentile represented a clear threshold in the latitudinal distribution of occurrences.
322 To assess if the physiological-based cold tolerance metrics were significant predictors of *P.*
323 *cresphontes'* distribution, the modelling was done using two approaches: correlative and
324 mechanistic. The correlative approach was based on environmental data derived from other
325 sources (e.g., remote sensing, weather stations) whereas the mechanistic approach additionally
326 included parameters derived from the cold tolerance experiments.

327 For both approaches, we tested the importance of the cold tolerance strategy
328 parameters by comparing i) model fit with and without them and ii) the proportion of suitability
329 explained among a number of climate-related factors hypothesized to constrain the range of *P.*
330 *cresphontes* at the landscape scale.

331

332 *ii) Data*

333 Models were built using all available occurrence records for North America from e-
334 butterfly (www.e-butterfly.org, accessed July 2018, 2255 records), the Global Biodiversity
335 Information Facility (GBIF; https://www.gbif.org, accessed September 2018, 3420 records) and
336 Moths and Butterflies of North America (www.butterfliesandmoth.org, accessed October 2018,

1206 records) from 1980 to 2018. This timeframe was chosen to match the timeframe of the available environmental variables. Records that were obviously inaccurate (e.g., in the middle of the ocean) or had no geographical coordinates were removed. To reduce the likelihood of an occurrence being based on the same individual being observed on multiple occasions, duplicate records across data sources were also removed. To reduce sampling bias, we used a low-end estimate of dispersal ability (1 km) and randomly selected a single occurrence across data sources per 1 km^2. In total, 3510 and 1457 occurrences were used for the North American and the northern range extent, respectively. Occurrences were then mapped onto a North America Lambert Conical Conic projection.

Sixteen environmental variables were initially considered for the model building based on previous work done on butterflies (Appendix S1; Table S3). The variables included represented both the direct and indirect effects (i.e. via a species' interaction) climate can have on a species' range (Appendix S1). Variables were created for both range extents and then screened for collinearity (Appendix S1; Table S4). All climatic data were downloaded at a 1 km resolution and restricted to 1980-2010. All data was downloaded at a North American extent, with the exception of Normalized Difference Vegetation Index (NDVI), which was global in extent.

For the mechanistic models, we created variables for both range extents based on the results from the cold tolerance experiments and minimum daily temperature data from Daymet (Table S3). Three variables were built for each extent by summing the number of days below the following thresholds: 2.14°C (i.e., CT_{min}), -6.6°C (i.e. the SCP from the August generation) and -8°C (i.e. potential lower lethal limit) for each year of the study period 1980-2010 (Table S3). The SCP from the August generation was chosen since it is the one most likely to match when larvae are more likely to experience low temperatures.

iii) Modelling

362 Models were built using Maxent (version 3.4.1, Philipps et al. 2006) with the BIOMOD2
363 package (Damien Georges) in R (version 3.41.1). Maxent is a commonly used species
364 distribution modelling technique for presence-only data (Merow et al., 2013). Maxent uses a
365 maximum-entropy approach to model species distributions. It relates the species' presences
366 and pseudo-absences to the environmental variables to predict where the habitat is suitable
367 across the mapped geographical space. Maxent is one of the more common species distribution
368 modelling software (Elith et al., 2011) and it performs very well compared to other modelling
369 techniques when dealing with presence-only data (Hernandez et al., 2006). Details on the
370 modelling are presented in Appendix S1.
371
372 *iv) Statistical analysis*
373 To determine the role of low temperature in limiting the distribution of *P. cresphontes*,
374 model accuracy was compared across the two extents and approaches using t-tests.
375 Comparisons were based on AUC, Kappa and TSS.
376
377
378 **Results**
379 *1. Cold tolerance experiments*
380 Experiment 1: Supercooling point (SCP) test
381 The results from the July and August SCP experiments provide support for a chill
382 susceptible strategy in *P. cresphontes*. The mean SCP for July was -5.8°C (0.2SE; n=14). There
383 was no significant difference in survival for July larvae that froze compared those that did not
384 freeze during the SCP experiment (above: 77% (10/13) vs. below: 43% (6/14 larvae); $\chi 2$= 1.2, df
385 = 1, p-value = 0.2).

386 The mean SCP for all August larvae was -6.6°C (0.2SE; n=28) and for the upper 50% of
387 larvae (directly comparable to the July experiment), the mean SCP was -5.9°C (0.2SE; n=14).
388 The August larvae had a 3.5% (1/29) survival rate following freezing (i.e., exposure to
389 temperatures below the mean SCP). The low survival below SCP and apparent lack of change
390 in the SCP suggests that larvae are tolerant of moderate chilling, but intolerant of freezing, and
391 are not undergoing a change in SCP under simulated autumn conditions.
392
393 Experiment 2: Low-temperature survival assays
394 *i) Survival*
395 Our low-temperature survival assays further demonstrate that larvae are chill tolerant
396 or modestly freeze avoidant, as they are unable to survive exposure to temperatures below their
397 SCP. In the -2°C test, July larvae had a survival rate of 93% after 24 hours (Table 1). Therefore,
398 single ecologically-relevant exposures to temperatures above their SCP do not have a large
399 impact on larval survival. Moreover, while the survival rate of larvae in July was more affected
400 by exposure to -6°C than -2°C (Table 1), it was still rather high (70%). Therefore, larvae are able
401 to survive a 7 h cold exposure near their SCP and thus may be chill tolerant or freeze avoidant.
402 August larval survival was significantly higher than that of the July larvae following
403 exposure to -6°C (100%, 17/17; χ^2 -squared = 5.1, df = 1, p-value = 0.024; Table 1). This finding
404 provides further evidence in support of a modestly freeze avoidant strategy since the mean SCP
405 for the August generation (-6.6°C) was lower than the test temperature (-6°C) and survival was
406 still quite high.
407 In comparison, in the extreme low-temperature test (-8°C), larvae had marginal
408 survival in both generations (July: 12.5% (1/8); August: 10% (1/10)) and there was no
409 improvement in survival across generations (χ^2 = 3.03e-31, df = 1, p-value = 0.99; Table 1). This

410 low survival further suggests larvae may use a freeze avoidant strategy since this temperature is
411 below the mean SCP measured in the August generation.
412
413 *ii) Developmental success*
414 Overall, low temperature exposure impacted pupation and adult eclosion. The
415 percentage of larvae that successfully pupated and eclosed generally decreased as the
416 temperatures got lower (Table 1). The percentage of larvae that successfully pupated at -8°C
417 was low (July: 12.5%; August: 10%). Therefore, although larval survival can be high after
418 exposure to low temperatures, chilling also had effect on developmental success, which is
419 indicative of sub-lethal chilling injury occurring, even in the absence of freezing.
420
421 Experiment 3: CT_{min}
422 The average CT_{min} of larvae was 2.14°C (0.26SE; n=20).
423
424
425 2. Distribution modelling
426 *i) Model performance*
427 Model predictive ability varied depending on the metric of accuracy used. All models
428 were considered good based on TSS (i.e. between 0.4-0.75) and AUC (>0.8), whereas based
429 on Kappa, the northern range models were weak (<0.4) and the full range models were fair
430 (>0.5; Figure 3).
431 Statistically, the mechanistic models were significantly more accurate than the
432 correlative models in some but not all cases (Figure 3; Table S5). However, in the select cases
433 where there was a significant difference between modeling approaches, the effect size was
434 small, implying the difference is unlikely to be biologically significant (e.g., AUC difference for

435 northern range comparison: 0.011; Figure 3, Table S5). Therefore, it is unlikely the inclusion of
436 cold tolerance metrics significantly improves the prediction of habitat suitability of *P.*
437 *cresphontes*.

438 The accuracy of the full range models was significantly higher than the accuracy of the
439 northern range models for all evaluation metrics and approaches (Figure 3; Table S6). Although
440 the effect size is also not large, the significant difference in accuracy was consistent across all
441 comparisons (Figure 3; Table S6). Therefore, the habitat suitability of *P. cresphontes* is
442 predicted more accurately across its full range than its northern range.

443

444 *ii) Variable importance*

445 Growing degree-days was the most important factor explaining the habitat suitability of
446 *P. cresphontes* across all model extents and approaches, explaining between 27-35% of the
447 variation in habitat suitability (Table 3). Precipitation was the second most important factor
448 across both extents and approaches. Across the full and northern range extents, GDD and
449 precipitation combined explained the majority of the variation in habitat suitability (~50%; Table
450 3). The physiologically derived variables included in the final model (i.e., CT_{min} and the potential
451 lower lethal limit) only explained a small part of variation in habitat suitability (~7.5%) and ranked
452 5th and 6th in model contribution (Table 3).

453

454 **DISCUSSION**

455 Although insects are likely to be particularly vulnerable to low temperatures during
456 autumn, no study, to our knowledge, has considered low temperatures during autumn as a
457 limiting factor on the geographic distributions of insects. Here, we test this hypothesis at the
458 northern edge of the distribution of the widespread butterfly, the Giant Swallowtail,
459 *P. cresphontes*. Our study contributes three main findings that lead to a rejection of this

460 hypothesis. First, given the survival of larvae to prolonged exposures to temperatures close to
461 but not below their SCP, they are chill tolerant or modestly freeze avoidant at their northern
462 range limit at this time of year. Their low survival below the SCP provides support that this
463 species is unable to handle freezing before pupation and that they may use a freeze avoidant
464 strategy. However, further testing for seasonal plasticity in the SCP is required to confirm a
465 freeze avoidant strategy as we had limited power and were unable to directly compare the
466 change in SCP between the two generations. An active depression of the SCP over the season
467 would indicate that larvae are accumulating cryoprotectants and that the larval cold tolerance
468 could be increasing, thus supporting a freeze avoidant strategy.

469 Our results demonstrate that ecologically relevant exposures to temperatures above the
470 SCP are not lethal, but below the SCP are; thus providing a clear picture of the lower end of
471 thermal tolerance for this species. This means that for *P. cresphontes* larvae, a single overnight
472 exposure to temperatures near -8°C will impede their survival and could represent a potential
473 lower lethal limit. Consequently, areas with early autumn temperatures that reach -8°C should
474 be inhospitable for *P. cresphontes*. However, in the Ottawa region, which is at the northern
475 range limit, there are only 0-3 frost episodes on average from September to October. Therefore,
476 low temperatures (i.e. <-7°C) during early autumn are unlikely to be experienced by the larvae
477 there or anywhere in its current range. This suggests that a single exposure to low temperatures
478 in early autumn is unlikely to limit the current northern range of *P. cresphontes* through cold-
479 induced mortality. However, there may be sublethal effects of chilling (e.g., effects on
480 developmental success), which could still impact overall insect fitness.

481 Second, we found that exposure to normal frost temperatures for this time of year in this
482 area (i.e., -1°C to -2°C), which are above the SCP, did not affect larval survival. Therefore, it is
483 also unlikely that frost in early autumn is a limiting factor of *P. cresphontes'* northern range. This
484 is in concordance with the field observations from Finkbeiner (2011), which showed that

485 *P. cresphontes* larvae can survive frost events. Since larvae had no problem reaching pupation
486 after exposure to -2°C, the most common frost temperature, and the majority of larvae still
487 successfully pupated (60%) after the low frost temperature test (i.e., -6°C), the cold tolerance of
488 larvae at the current northern range limit seems sufficient to cope with the climate they
489 experience. However, further testing in ambient conditions and with a proper control is needed
490 to determine whether cold exposure on larvae have significant sub-lethal effects on eclosion as
491 rates of eclosion in this experiment were less than 30% but a control group was not included
492 due to sample size constraints. It has been demonstrated elsewhere that the success of each
493 life stage matters in the response of butterfly species to climate change (Radchuk et al. 2012).
494 Third, the species distribution modelling results were consistent with the experimental
495 results: cold related variables did not explain the distribution of *P. cresphontes* at a broad scale.
496 Together, these results provide strong support that exposure to temperatures above -6.6°C
497 during autumn does not limit the northern range of *P. cresphontes*. Instead, growing degree-
498 days and precipitation are the most important predictors, of those tested here, on the distribution
499 of *P. cresphontes* at a broad scale. While other factors were not tested here, for example, biotic
500 interactions (e.g., host plant occurrence), our results suggest that *P. cresphontes* depends on
501 specific heat accumulation and water availability to complete its life cycle. These factors have
502 also been identified as important in predicting the range of other butterflies (Luoto et al., 2006;
503 Eskildsen et al., 2013). Evidence suggests there is a strong relationship between the number of
504 growing degree-days and the growth rate of larvae, and the foraging activities of adults (Ritland
505 & Scriber, 1985; Kukal & Dawson, 1989; Schneider & Root, 2002). Precipitation can limit the
506 range of insects directly due to dehydration or indirectly by imposing limitations on primary
507 productivity, which in return transposes to higher resource availability.
508 The full range model was more accurate at predicting the distribution of *P. cresphontes*
509 than the northern range model. Similar results have been found for other species, with model

accuracy and performance increasing with larger study extents (VanDerWal et al., 2009; Connor et al., 2019). This is likely a result of smaller environmental gradients in the northern range, which causes the model to have a greater difficulty detecting differences between presences and absences, and in turn leads to difficulties predicting presences and absences in novel areas (Smith & Santos, 2019). Prevalence can also decrease with larger study extents (Barbet-Massin et al., 2012). However, the accuracy of the two models was still different using TSS, a metric that is not sensitive to prevalence (Table S4; Figure 3). Nevertheless, even if model performance was lower in the northern range relative to the full range, the model was still valid and performed adequately across all metrics.

While growing degree-days and precipitation explain a lot of the variation in habitat suitability for *P. cresphontes*, we may have underestimated the role of cold tolerance at the northern range edge for three reasons. First, low temperature thresholds, like the CT_{min}, although not lethal, could still impact *P. cresphontes'* survival in natural conditions due to the enhanced risk of predation or starvation linked with the loss in mobility at temperatures below 2°C. While a possibility, this hypothesis depends on a high likelihood of predation, which is unknown across *P. cresphontes'* range (Hazel et al., 1998; McAuslane, 2009). Low temperature may also lead to sublethal effects on developmental success, energy status and behaviour. Second, it is also possible that more prolonged or repeated exposure to low temperatures could impact *P. cresphontes* larval survival or have sublethal effects at the northern range edge because we did not test for the effects of repeated cold injury or chilling. In *Drosophila*, multiple cold events could cause an accumulation of cold injuries resulting in larval death or in sub-lethal effects such as decreased feeding, fecundity and dispersal ability (Marshall & Sinclair, 2012). Third, the SCP estimated here is effectively a theoretical one. Since we could not collect and test the last emerging larvae of the season due to the difficulty in finding them, they might not have reached their peak cold tolerance and therefore *in situ,* SCP could be lower (i.e., their cold

535 hardiness may have been underestimated). On the other hand, the *in situ* SCP may be higher
536 since environmental factors in natural conditions, such as air moisture or exposure to ice
537 nucleators, may further affect survival at temperatures above SCP. Nevertheless, because the
538 low temperature thresholds derived experimentally in this study could be an underestimate, the
539 mechanistic models may not have been optimized perfectly, thus underestimating the role of
540 cold tolerance metrics at a broad scale. However, since temperatures below the SCP (i.e.
541 <-7°C) are very unlikely to occur multiple times before pupation, if at all, low temperatures during
542 autumn remain unlikely to limit the northern range of *P. cresphontes*.

543 Given our lack of knowledge about the overwintering ecology and physiology of this
544 species in its northern range, pupal overwinter survival may be a more important limiting factor
545 for the species' northern range limit. Unfortunately, it is unknown whether they overwinter in the
546 leaf litter on the ground or tree branches above the ground. If on the ground, they would likely be
547 covered in snow (average annual snowfall in Ottawa is 224 cm (Environment and Climate
548 Change Canada)) and therefore buffered against low temperatures. The temperature 30 cm
549 below snow cover is usually -4°C (Flin 2008). This behaviour would increase their chances of
550 surviving the winter. Indeed, West (1996) showed that in Virginia, *P. cresphontes* pupate 3-25
551 cm off the ground on dead branches. In the northern range, it is likely that even at these heights,
552 chrysalids would be covered by snow. While ontogenetic variation in thermal tolerance is
553 present in other species (Terblanche et al. 2006; Marais et al. 2009; MacLean et al., 2016; but
554 see Ouimette, 2018) and only larvae were tested in this study, *P. cresphontes* larvae purge their
555 food and liquids before pupation, thus they are likely to be even more cold tolerant at the pupal
556 stage. Other members of *Papilio* are more cold tolerant than what we determined for the larval
557 stage of *P. cresphontes. P.canadensis* and *P.glaucus,* are considered to be freeze tolerant at
558 the pupal stage and have an SCP of -23°C to -27°C (Kukal et al., 1991). *P. xuthus* is freeze
559 avoidant with an SCP of -25°C (Shimada 1988). Therefore, the pupae of *P. cresphontes* are

likely to have even greater cold hardiness than the larvae and thus be able to survive the winter at the northern range if they pupate at similar heights as the populations in Virginia.

Conclusions

Our results demonstrate that the cold tolerance of *P. cresphontes* larvae is well matched to its environment in the northern part of its range and that it is unlikely that low temperatures in autumn are limiting its range. This suggests that the warming of autumn temperatures over the past decade is unlikely to have been the factor that led to the recent range expansion of *P.cresphones*, as originally hypothesized by Finkbeiner (2011). Instead, the warming of winter temperatures could have ameliorated overwintering survival, leading to an improvement in habitat suitability. Further study on determining the key factor(s) limiting the northern range of *P. cresphontes* should focus on defining the cold hardiness of the pupal stage, the overwintering behaviour and the ecological relevance of the physiological thresholds found in this study. Determining the ultimate factors that limit species' distributions will be critical in accurately predicting species' range shifts in response to future climate change.

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Tables

Table 1. Survival rates and developmental success for the low-temperature assays. Test temperature and generation are shown. The percentage of initial larvae that survived the first 24 hours after the experiment, successfully pupated and eclosed as adults is alos shown. All percentages are based on the number of larva that began the experiment.

Test	Generation	Number of larvae	Larval survival (%)	Pupation (%)	Eclosion (%)
-2°C	July	23	93	78.5	52
-6°C	July	10	70	60	30
	August	17	100	100	0
-8°C	July	8	12.5	12.5	12.5
	August	10	10	10	0

820 Table 2. Contribution of the environmental variables in explaining habitat suitability across different model extents and approaches.
821 Shown is the rank order of variable importance and mean (± S.E) proportion of variance explained. In bold is the variable that
822 explains the most amount of variation for each model type.

Variables	North America				Northern range			
	Correlative		Mechanistic		Correlative		Mechanistic	
	Rank order	Variation explained (SE)	Rank order	Variation explained (SE)	Rank order	Variation explained (SE)	Rank order	Variation explained (SE)
Growing degree-days	**1**	**31.33 (0.19)**	**1**	**27.34 (0.23)**	**1**	**37.17 (0.08)**	**1**	**35.03 (0.06)**
Precipitation	2	24.87 (0.08)	2	21.70 (0.085)	2	23.94 (0.08)	2	22.47 (0.07)
Extreme maximum temperature	3	17.54 (0.06)	3	16.22 (0.06)	6	2.38 (0.02)	7	2.41 (0.02)
Normalized Difference Vegetation Index	4	15.45 (0.10)	4	15.87 (0.11)	4	16.77 (0.11)	4	14.89 (0.11)
Mean temperature of the coldest month	NA	NA	NA	NA	3	16.9 (0.06)	3	15.57 (0.05)
Precipitation as snow	5	10.80 (0.07)	5	10.05 (0.07)	5	2.84 (0.02)	6	3.46 (0.02)
CT_{min}*	NA	NA	NA	NA	NA	NA	5	5.97 (0.03)
Potential lower lethal temperature*	NA	NA	6	8.80 (0.04)	NA	NA	8	0.16 (0.004)

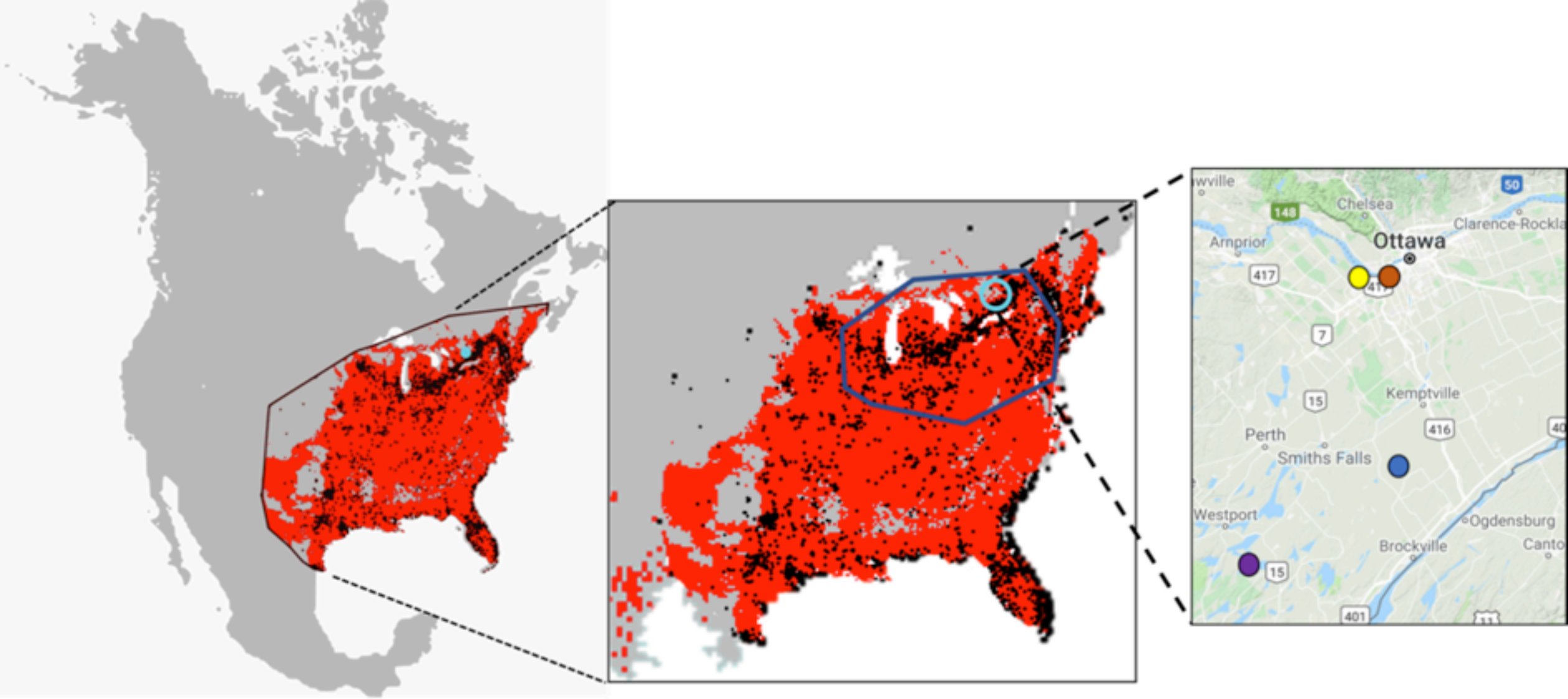
823 * variable derived experimentally

Figures

Figure 1. The geographic distribution and northern range of *Papilio cresphontes*. The first panel shows the entire range of *P. cresphontes* encompassed by the minimum convex polygon (thin black line). The second panel shows the position of the northern range, denoted by the solid blue line, relative to the entire range. The blue circle indicates the locations of the field sites. In the first two panels, the occurrence points (black) are shown against the predicted habitat suitability (red). The third panel shows the field sites where larvae were collected at the northern range limit: the blue dot indicates the Brockville site (44.84952, -75.75226), red indicates the Queen's University Biological Station site (44.56747, -76.32454), brown indicates the Mud lake site (5.37192, -75.79451) and yellow indicates the Shirley's Bay site (45.36546, -75.88302).

Figure 2. Overview of cold tolerance experiments conducted in this study. Shown are the supercooling point (SCP), low temperature survival assays, and critical thermal minimum experiments (CT_{min}). Sites included in the experiments are: Brockville, Queen's University Biological Station (QUBS), Mud lake and Shirley's Bay. For number of larvae in each experiment, please refer to Table S1.

Figure 3. A comparison of model accuracy for the two model extents (northern and full range) and approaches (correlative and mechanistic) using all three evaluation metrics (AUC (Area under the receiver operating characteristic curve), Kappa, TSS (True skills test). Mean accuracy scores and standard error bars across 100 iterations are shown with the y-axis cropped at 0.2. Grey and black bars represent the mechanistic and correlative models, respectively. Letters indicate that mean scores are significantly different from each other ($p<0.05$).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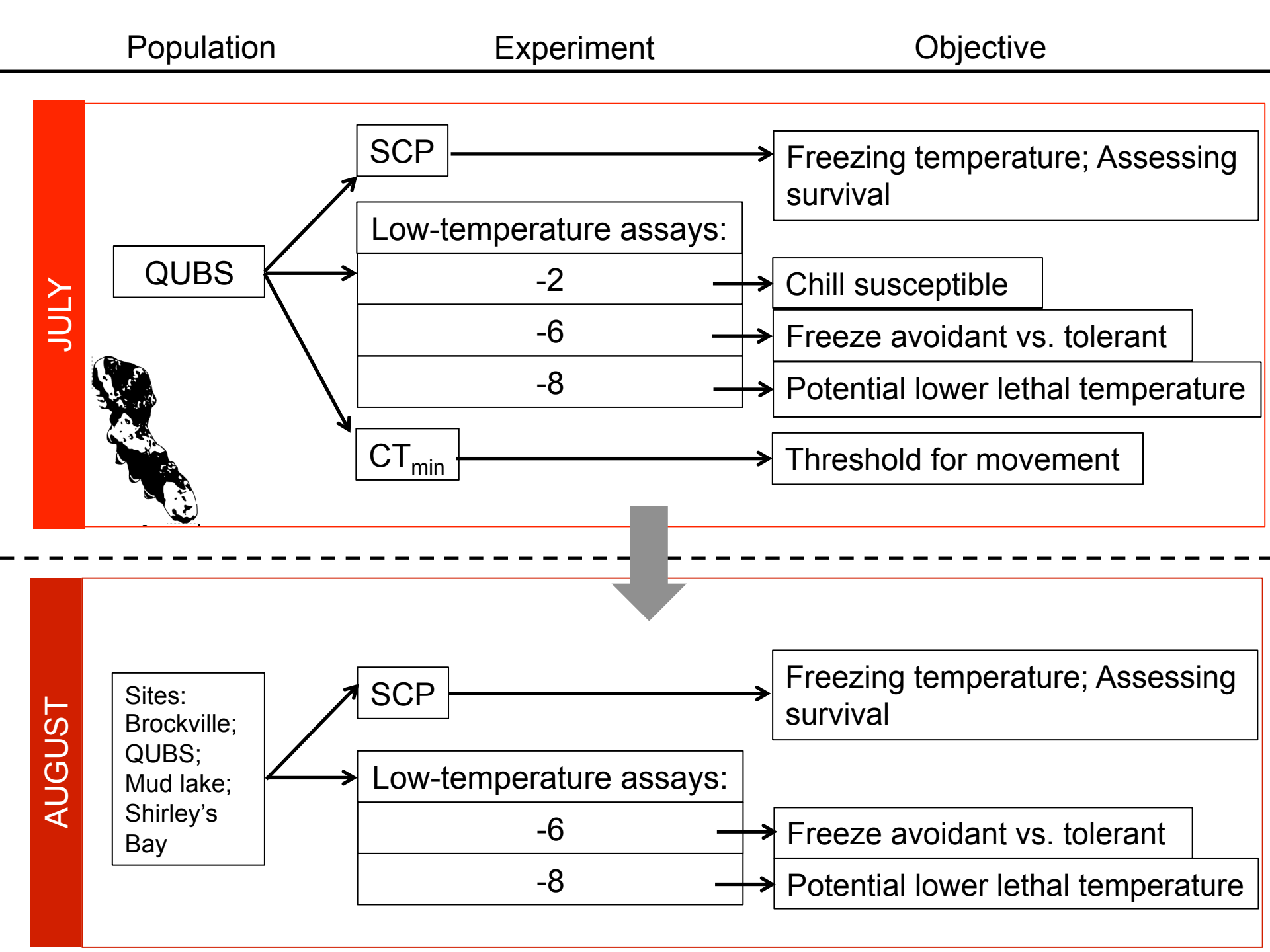
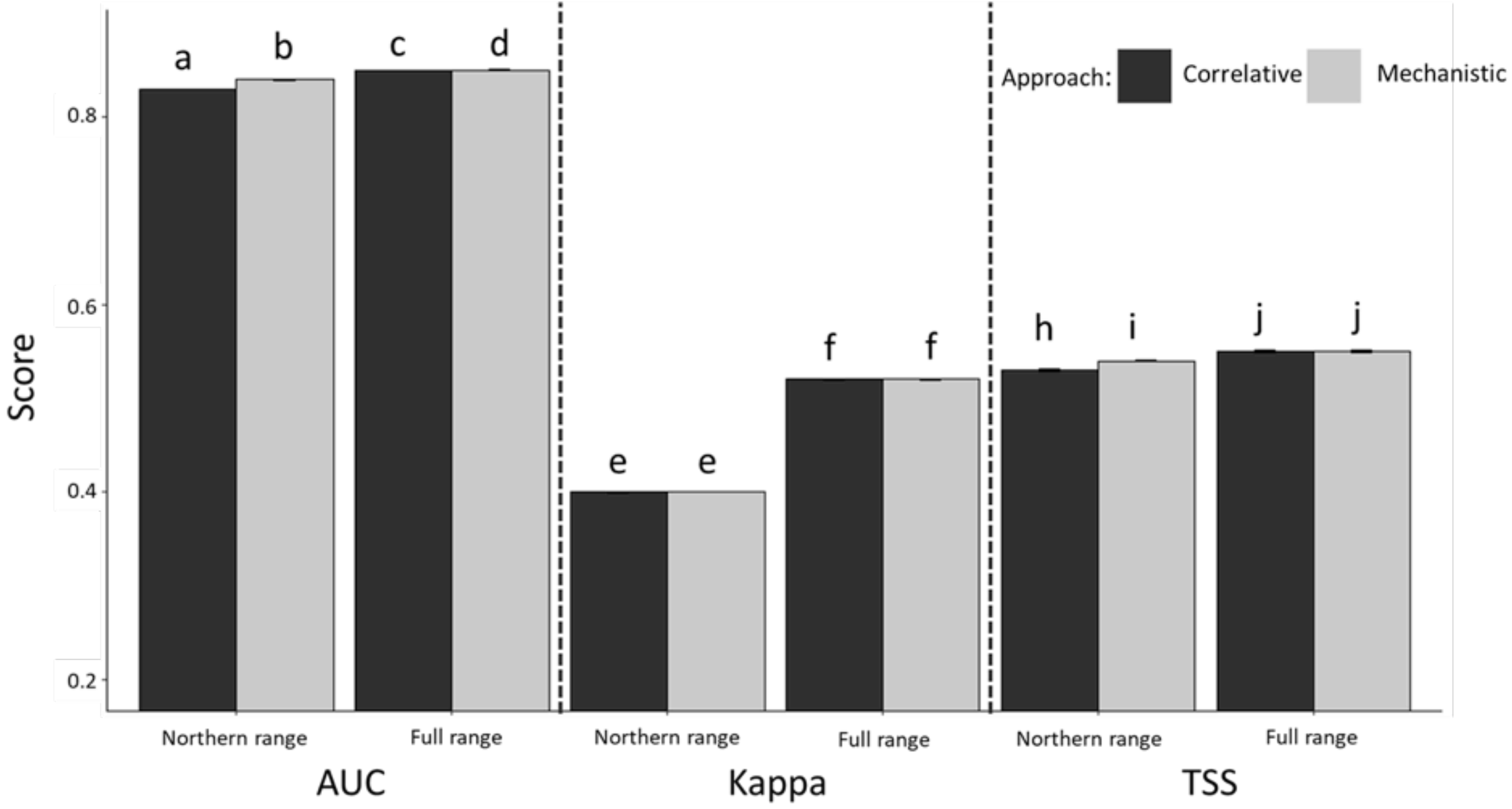

Population
Experiment
Objective
JULY
QUBS
SCP
Freezing temperature; Assessing survival
Low-temperature assays:
-2
Chill susceptible
-6
Freeze avoidant vs. tolerant
-8
Potential lower lethal temperature
CTmin
Threshold for movement
AUGUST
Sites: Brockville; QUBS; Mud lake; Shirley's Bay
SCP
Freezing temperature; Assessing survival
Low-temperature assays:
-6
Freeze avoidant vs. tolerant
-8
Potential lower lethal temperature


Approach:
Correlative
Mechanistic
Score
0.8
0.6
0.4
0.2
a
b
c
d
e
e
f
f
h
i
j
j
Northern range
Full range
Northern range
Full range
Northern range
Full range
AUC
Kappa
TSS