Semi-field and surveillance data define the natural diapause timeline for *Culex pipiens* across the United States

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Abstract

Culex pipiens is a globally-distributed mosquito of medical and veterinary importance, serving as a primary vector of West Nile virus (WNV). To survive winter, female *Cx. pipiens* mosquitoes undergo adult reproductive diapause initiated by photoperiod and temperature cues. While well-studied under laboratory conditions, the environmental signals that promote *Cx. pipiens* diapause induction in natural settings are less understood. Here, we evaluate *Cx. pipiens* in laboratory and semi-field studies to examine diapause induction, defining an approximate timeline beginning in late-August where mosquitoes become receptive to diapause. Using gravid (reproductive) mosquito surveillance data as a proxy for adult diapause incidence for locations across the United States (California, Colorado, Connecticut, Illinois, Iowa, Minnesota, Pennsylvania, and Virginia), we demonstrate consistent population declines coinciding with periods of diapause receptivity except in hybridization zones where *Cx. quinquefasciatus* is present, suggesting that *Culex* population genetics can significantly impact end-season population trends. Together, this study defines a window for diapause induction across the United States, shaped by temperature, latitude, elevation, and mosquito population genetics. Coinciding with the cessation of WNV activity, these data can have important implications for mosquito control, where targeted efforts prior to diapause induction can decrease mosquito populations and WNV overwintering to reduce mosquito-borne disease incidence the following season.

Introduction

Insects are one of the most diverse lifeforms on the planet, relying on a myriad of evolutionary adaptations to survive adverse ecological environments and climate conditions. This includes a state of dormancy known as diapause that is used to facilitate overwintering survival in temperate regions (1, 2), which depending on species can occur during the egg, larval, or adult stages (3).

The northern house mosquito, *Culex pipiens*, is an important vector of mosquito-borne pathogens such as West Nile virus (WNV) and Saint Louis encephalitis virus (SLEV), and serves as an important model to understand diapause physiology in insects (3–5). Evidence suggests that the immature life stages (larvae and pupae) respond to photoperiod and temperature cues to promote facultative diapause in adult female mosquitoes following eclosion (6–8), where the resulting females forego blood-feeding (9, 10) and remain in an arrested reproductive state characterized by small primary ovarian follicles (3, 11, 12). Additional physiological changes to the cuticle (13, 14) and reduced diuresis (15) protect against desiccation, while alterations to host metabolism increase lipid and glycogen storage (16–20) to help sustain overwintering survival.

Photoperiod and temperature are long-established components of mosquito facultative diapause induction, where short day lengths and cool temperatures promote this physiological state (6, 8). Previous studies with *Cx. pipiens* suggest that day lengths under 15 hours can elicit the diapause under laboratory conditions using cool temperatures (18-22°C) (6, 8). Evidence suggests that diapause induction is stronger with shorter photophases and lower temperatures, where ~100% of a colony can be induced under 12 hours of day light and at 18°C (6). However, higher temperatures can revert diapausing individuals or suppress diapause entry, even at lower photophases (5, 7, 21), demonstrating the combined importance of photoperiod and temperature in defining the diapause state.

Laboratory experiments to induce diapause using a short photoperiod (9:15) and cool temperature (19°C) have produced significant insights into the hormonal regulation (4, 18, 22, 23) and molecular physiology of *Cx. pipiens* diapause (17, 24–26), yet these

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70   experimental conditions in the laboratory do not capture the natural fluctuations in daily

71   temperature that encompass the realistic end-season conditions that promote diapause

72   induction. Limited studies have addressed the diapause induction timeline in natural

73   populations of *Cx. pipiens*. Semi-field experiments in Ontario, Canada, recorded diapause

74   incidence as early as July, with peak rates in mid-August (21), while field-collected

75   mosquito samples in Boston, USA revealed low levels of diapause incidence beginning

76   in mid-August, with peak incidence in late September/early October (27). In addition,

77   evidence suggests that there is annual variation in diapause induction, with peak periods

78   of induction varying by approximately two weeks between years (27). Together, this

79   geographic and yearly variability in diapause incidence highlight our limited understanding

80   of *Cx. pipiens* diapause under natural conditions.

81   Herein, we perform laboratory and semi-field studies to examine diapause induction in a

82   laboratory population of *Cx. pipiens*, confirming the requirements of both photoperiod and

83   temperature on diapause induction, as well as provide temporal evidence of the natural

84   seasonal conditions that promote diapause in central Iowa, USA. To place these data in

85   the context of diapause incidence in natural mosquito populations, we leverage gravid

86   *Cx. pipiens* population data from Iowa and multiple locations across the United States to

87   serve as a proxy for diapause incidence. These data suggest that temperature, latitude,

88   elevation, and *Culex* population genetics significantly impact natural diapause ecology.

89   Together, our data provide significant new insight into the complexity of *Cx. pipiens*

90   diapause induction and its influence in end-of-season mosquito population trends. These

91   results have important public health implications for mosquito-borne disease

92   transmission, and increase our understanding of how a globally changing climate may

93   extend mosquito activity and influence mosquito overwintering.

Results

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Diapause induction requires both short day lengths and cool temperatures

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96   With the intention to use a laboratory colony of *Culex pipiens* originally isolated from

97   Ames, Iowa and maintained for ~16 years in the laboratory (without selection for the

98   diapause state)*,* we first wanted to demonstrate diapause induction in this *Cx. pipiens*

99   population through laboratory experiments. To approach this question, we reared *Cx.*

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100   *pipiens* from first instar larvae to adults under different laboratory conditions (**Figure 1A**)

101   to examine the influence of temperature alone (cold; 16:8 L:D, 19°C), photoperiod alone

102   (dark; 9:15 L:D, 25°C), and the combined effects of temperature and photoperiod

103   (diapause; 9:15 L:D, 19°C) to promote adult diapause (10, 18, 28).

104   Using arrested ovarian development as a proxy for diapause induction (29), we examined

105   ovarian follicle length in individual female adult mosquitoes 6-8 days post-eclosion (6, 7)

106   from each experimental condition (**Figure 1B**). Interestingly, autogenous mosquitoes

107   (displaying follicle maturation without a blood meal) were detected in all experimental

108   conditions (**Figure S1**), suggesting that low rates of autogeny exist in our laboratory

109   colony of *Cx. pipiens*. Across experimental conditions, average follicle size was

110   comparable under the cold and dark conditions with standard laboratory rearing

111   conditions (control), yet were significantly reduced under diapause conditions (**Figure**

112   **1B**). Using a strict follicle size cutoff of ≤50 μm to designate individuals in diapause (29),

113   only the cool temperatures and short photoperiod of the "diapause condition" produced

114   individuals in the diapause state (**Figure 1B**), as demonstrated by the arrested follicle

115   morphology in diapausing individuals (**Figure 1C**). This confirms that diapause induction

116   in our laboratory colony of *Cx. pipiens* requires both short photoperiod and cool

117   temperatures similar to previous studies (6, 11).

118   Additional previously defined physiological features of diapause (10, 18, 30) were also

119   explored to further validate diapause induction in our laboratory colony. This includes

120   increased lipid accumulation (**Figure 1D**) (18, 31), a larger body size (**Figure 1E**) (30) ,

121   and reduced blood-feeding behavior (**Figure 1F**) (10), which together confirm the

122   diapause state. Moreover, we demonstrate that our non-diapause rearing conditions can

123   have additional influence on mosquito physiology, most notably the independent effects

124   of temperature and short photoperiod on body size (**Figure 1E**), and decreased feeding

125   behavior under cool temperature conditions (**Figure 1F**).

126   **Evaluating natural diapause induction in semi-field experiments**

127   While laboratory studies are required to understand the physiological aspects of mosquito

128   diapause, the environmental conditions used to promote diapause induction in the

129 laboratory do not accurately depict the natural end-of-season conditions in temperate
130 climates where temperatures are variable and photoperiods are less extreme than the 9-
131 hour photoperiod typically used in laboratory studies of mosquito diapause (**Figure 2A**).
132 In an effort to better understand the natural diapause conditions for *Cx. pipiens*, we
133 performed a two-year semi-field study in Ames, Iowa, USA (**Figure 2B**) enabling a
134 structured approach to examine the life history conditions that result in diapause under
135 natural conditions. To approach this question, we reared *Cx. pipiens* from our laboratory
136 colony at semi-field locations (**Figure S2**) from first instar larvae to adults at different
137 timepoints according to epidemiological week from July through late-September (**Figure
138 2B**). Initially in 2020, groups of lab-reared first instar *Cx. pipiens* larvae were placed
139 outside every three weeks from week 30 to week 39 (July to late-September; **Figure 2B**),
140 with each group representing an approximate one-hour difference in photoperiod (**Figure
141 S3**) ranging from ~15 to 12 hours of daylight at the onset of larval development. A similar
142 approach was employed in 2021, with experimental groups deployed at weeks 30, 33,
143 34, 37, 38, and 39 (**Figure 2B, Figure S3**) to provide further resolution into the natural
144 diapause induction timeline.

145 To determine which mosquitoes had entered diapause, we examined ovarian follicle size
146 as in **Figure 1**. In 2020, a total of 193 adult female mosquitoes were examined from four
147 experimental groups, of which diapause was detected in mosquitoes with a rearing onset
148 initiated during weeks 36 and 39 (early- and late-September; **Figure 2C, Table S1**).
149 Similar results were obtained in 2021, where 115 mosquitoes across six experimental
150 groups were examined, with diapause detected in groups with a rearing onset as early as
151 week 34 (late August) and increasing in intensity through the remainder of the
152 experimental timepoints initiated in September (**Figure 2D, Table S1**). For both years,
153 diapause induction was strongest (≥50% of mosquitoes in diapause) after week 38 (late-
154 September; **Figure 2E**), when immature mosquito development occurred with ~12 hours
155 of daylight and an average temperature of 15°C (**Figure 2F**), yet was readily detected in
156 groups reared under 13.5 hours of daylight and ~20°C (**Figure 2F**).

157 To further examine this receptive period able to promote diapause induction, we placed
158 lab-reared pupae under semi-field conditions each week from weeks 36-40 (September

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159 to early-October) and evaluated their ability undergo reproductive diapause. While the

160 transfer of lab-reared pupae to artificial diapause conditions (9:15 L:D, 19°C) results in

161 nearly 100% diapause induction (6), under our semi-field conditions diapause induction

162 was inefficient, with diapause only detected in low frequency (6-17%) from pupae placed

163 outside in weeks 39 and 40 (**Figure S4**).This corresponds to the approximate

164 environmental conditions in mid-to late-September that result in ≥50% diapause induction

165 in our larval groups (**Figure 2E**), suggesting that the environmental signals during late-

166 September may be driving diapause induction in our semi-field conditions. In addition, the

167 low frequency of diapause induction at the pupal stages is suggestive that the cumulative

168 exposure over immature developmental stages may enhance diapause induction as

169 previously suggested (7).

170 Based on our results (**Figure 1**) and other previous studies (6, 11), temperature is an

171 important, yet complex variable in the context of diapause induction. When examined

172 between years, there is slight variation in diapause induction (**Figure 2E**), likely the result

173 of temperature differences shifting the timing of diapause induction between years

174 (**Figures S3 and S5**, **Table S1**). In addition, our data highlight the potential importance

175 of diurnal fluctuations in temperature that occur during periods of diapause receptivity

176 (**Figure S6)**, where daily low temperatures may be able to sustain diapause induction

177 signals even during the exposure to high daily temperatures (>30 °C) in our semi-field

178 studies that would typically "break" diapause induction (6). Temperature also had a

179 significant influence on mosquito development and survival as temperatures declined in

180 late-summer/early-fall. As the season progressed, our experimental groups displayed

181 increased developmental times, most notably slowing larval and pupal development,

182 ultimately influencing adult eclosion (**Figure S3**). Moreover, in both years of our semi-

183 field study, larvae placed outside in late September (weeks 38 and 39) experienced

184 significant mortality in immature stages due to suboptimal temperatures for mosquito

185 development and survival, resulting in the low numbers of individual mosquitoes that

186 contributed to our analysis of reproductive diapause (**Figure 2C** and **Figure 2D**).

187 **Mosquito surveillance data inform diapause induction in Iowa field populations**

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188 While our semi-field study provides valuable new insight into the conditions and timing of

189 natural diapause induction (**Figure 2**), these experiments were performed using a

190 laboratory-derived population of *Cx. pipiens* and may not fully capture diapause induction,

191 such that natural field populations of mosquitoes may be more receptive to photoperiod

192 and temperature. To measure the natural diapause timeline in populations of *Cx. pipiens*,

193 we utilized long-term mosquito surveillance data from central Iowa to capture adult *Cx.*

194 *pipiens* population trends in natural field settings.

195 Using different trap types to estimate general mosquito abundance (New Jersey light trap;

196 NJLT) or blood-fed/reproductive mosquito populations (grass infusion-baited gravid traps;

197 gravid), we examined Cx. *pipiens* population dynamics in central Iowa (**Figure S7**) from

198 2016-2021 as a proxy for diapause induction in natural field populations. NJLT data

199 demonstrate early-season peaks (May/June) in *Culex pipiens* group (32–34) abundance,

200 which taper mid-summer (July) before a late-season rise in September (**Figure 3A**). In

201 contrast, gravid *Cx. pipiens* populations peak mid-summer (week 32), then decline by

202 61% by week 40 (**Figure 3B**). Comparisons of NJLT and gravid population trends using

203 a linear regression of annual slope values over weeks 30-40 were significant (P=0.0079),

204 supporting that only reproductive gravid *Cx. pipiens* populations are declining during the

205 late-summer (**Figure S8**). When placed in the context of diapause induction established

206 in our semi-field experiments (**Figure 2**), gravid adult field populations experience

207 significant declines during September, corresponding with the approximate

208 environmental signals able to promote diapause in our semi-field studies. Moreover, the

209 cessation of mosquito surveillance after week 40 (October) tightly corresponds with the

210 high rates of diapause in our semi-field groups suggesting that after the beginning of

211 October most emerging females will be in diapause (**Figure 3B**).

212 ***Cx. pipiens* diapause incidence shares similar timelines across the United States**

213 Based on our observations of *Cx. pipiens* diapause induction in semi-field (**Figure 2**) and

214 natural field conditions in Iowa (**Figure 3**), we wanted to similarly examine potential

215 diapause timelines across the United States. To approach this question, mosquito

216 surveillance data was collected from across the country (California, Colorado,

217　　Connecticut, Illinois, Minnesota, Pennsylvania, and Virginia) to examine adult *Cx. pipiens*

218　　population dynamics, similar to that described in Iowa (**Figure 3**).

219　　For locations with both NJLT and gravid trap types, linear regressions of annual slopes

220　　by trap type confirm that gravid trends were distinct from general population trends (NJLT)

221　　from weeks 30 to 40 at each location (Iowa, *P*<0.01; California, *P*<0.001; Colorado,

222　　*P*<0.05; Connecticut, *P*<0.0001) (**Figure S8**). When historical gravid trapping data were

223　　used to compare end-season (weeks 30-40) across the country, there was a consistent

224　　decline in *Cx. pipiens* gravid populations from mid-summer (July, week 30) to late-

225　　summer/early-fall across the United States (**Figure 4A**), with the exception of gravid

226　　populations from Suffolk, Virginia which displayed a slightly increased trend in *Cx. pipiens*

227　　abundance (**Figure 4A**). These observations coincide with the decline of gravid

228　　populations in Iowa over August and September (**Figure 3**), where gravid populations in

229　　most location undergo notable declines in gravid *Cx. pipiens* populations from week 30

230　　to 40 ranging from ~32 to 90% (**Figure 4B**).

231　　Lower latitude sites (Suffolk, Virginia and California) (**Figure S9**) had less pronounced

232　　population declines (and even increased *Cx. pipiens* gravid populations; **Figure 4B**),

233　　highlighting the significant influence (R^2=0.69, *P*=0.01) of latitude on end-of-season *Cx.*

234　　*pipiens* dynamics (**Figure 4B**). This coincides with similar effects of latitude and

235　　photoperiod on diapause induction in other insects (35, 36) and mosquito species (37,

236　　38). Moreover, in both Virginia and California, evidence suggests that these regions are

237　　within hybridization zones between *Cx. pipiens* and *Culex quinquefasciatus* (39–41)

238　　(**Figure 4C**), a morphologically identical species that does not undergo diapause (8, 30,

239　　42). As a result, the absence or low incidence of diapause in these locations may be due

240　　to the respective misidentification of *Cx. quinquefasciatus* as *Cx. pipiens,* the

241　　hybridization of these species resulting in intermediate diapause phenotypes (8, 30), or

242　　the potential that these could be populations of *Cx. pipiens* f. molestus that do not undergo

243　　reproductive diapause (43).

244　　To make comparisons of diapause-relevant environmental factors across sites, average

245　　weekly day length and average temperature values were compiled from each location (or

246   comparably close locations) (**Figure S10**). Temperatures across all study sites noticeably

247   declined over the 10-week period, with Minnesota (Minneapolis/St. Paul) and Colorado

248   (Larimer County) having the coldest average temperature (~10 °C) at week 40 (**Figure**

249   **S9)**. Connecticut, Illinois (Chicago), and Pennsylvania displayed comparable average

250   temperatures (~15 °C) at week 40 (**Figure S10)**, similar to the average temperatures in

251   Iowa at this timepoint (**Figure 2F**). Of note, the two locations (Northern California; Suffolk,

252   VA) with the smallest changes in gravid trap numbers (**Figure 4B**), which suggest little to

253   no diapause, had much higher temperatures across the entire 10-week period, with week

254   40 averages of ~20 °C (**Figure S10)**. Although average day lengths varied slightly at the

255   start (week 30), with the highest latitude location (Minnesota) having slightly longer day

256   lengths, all included locations converged to near identical levels (~12 hours) by the fall

257   equinox (week ~38).

258   Our nationwide gravid trap data also allude to the influence of elevation in *Cx. pipiens*

259   diapause induction, where surveillance data from Colorado display sharp declines in

260   gravid populations beginning in late-August (week 34) and the termination of surveillance

261   activities only weeks later (week 37; **Figure 4A**). This accelerated timeline suggests that

262   the high altitude of Larimer County, CO (**Figure S9**) enhanced the environmental signals

263   that promote diapause induction, similar to that described in other mosquito species (37,

264   44).

Discussion

266   Although diapause is a critical component to the success of *Culex pipiens* overwintering

267   survival in temperate regions, our understanding of the environmental signals that

268   promote diapause induction under natural conditions has thus far been limited. Using

269   laboratory and semi-field experiments to inform historical mosquito surveillance trends

270   from across the county, we provide a definitive diapause induction timeline broadly shared

271   in true *Cx. pipiens* populations across the United States. While temperature and

272   photoperiod are integral components of diapause induction in laboratory and field

273   conditions, our data suggest that latitude and elevation can further amplify the effects of

274   temperature and photoperiod under natural conditions, as well as highlight the importance

275   of the influence *Culex* population genetics in defining diapause incidence (**Figure 5**).

276   There are many physiological and behavioral changes associated with diapause (10, 11,
277   13, 31, 45), yet the key feature to confirm reproductive diapause in *Cx. pipiens* is an
278   arrested ovarian development phenotype (11, 46, 47). Morphologically this corresponds
279   to arrested ovarian follicle development, in which follicles lack yolk granulation and
280   become stunted in size (48). Arrested ovarian development has also been quantified
281   using either direct measurements of the primary follicle (5, 18, 25, 29, 30), or the size
282   ratio of the primary follicle to the secondary follicle (6, 42, 46, 49) to establish diapause.
283   In our experiments, we relied on measurements of the primary follicle to determine
284   reproductive diapause. Previous experiments have defined diapause using this
285   methodology with follicle lengths ranging from 30-70 μm (5, 18, 25, 29, 30, 49). However,
286   our *Cx. pipiens* colony produced primary follicle lengths of ~75 μm in under standard
287   rearing conditions. As a result, a more conservative cutoff of ≤50 μm was used to confirm
288   reproductive diapause in our lab colony at the risk of potentially excluding some individual
289   mosquitoes that did not meet these strict criteria in our laboratory and semi-field
290   experiments.

291   In laboratory experiments using an established colony of *Cx. pipiens*, we observed a
292   relatively low frequency of diapause induction when we applied our strict criteria for
293   ovarian arrest. Although diapause is genetically determined [reviewed in 48], our colony
294   of *Cx. pipiens* has been maintained in artificial laboratory conditions after its initial
295   colonization (~2005) without re-invigoration from wild-caught specimens or artificial
296   diapause cycling (29). As a result, our laboratory colony may have become desensitized
297   to the thermal and photoperiod cues required to promote diapause, similar to the influence
298   of artificial rearing conditions on diapause incidence in other insect species (51, 52). In
299   addition, our laboratory diapause induction experiments used a fluorescent light source,
300   which previous studies have suggested is less efficient than incandescent light at
301   promoting diapause in *Cx. pipiens* (8). While at present we cannot provide a clear
302   explanation for the low rates of diapause induction in our laboratory experiments, the
303   same lab colony was used in our semi-field experiments where diapause incidence
304   reached in excess of 50%, suggesting that the predisposition for diapause remains in our
305   *Cx. pipiens* colony. Factors such as the length of crepuscular periods, more extreme low

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306 daily temperatures, or diurnal temperature fluctuations may be important natural variables

307 that can overcome diapause de-sensitivity resulting from long-term colonization.

308 While short photoperiod (9:15 L:D) and cool temperatures (19°C) are traditionally used to

309 initiate diapause in the laboratory (26, 28, 53), these experimental conditions do not

310 accurately reflect the natural onset of diapause in the field. Photoperiod values only

311 vaguely provide a timeline of diapause potential between the summer solstice (15 hours

312 of light) where low-level diapause is possible (6, 21) and the winter solstice (9 hours of

313 light) where temperatures prove impossible for mosquito survival and development in

314 temperate climates. Similarly, as temperatures can subvert diapause induction (6), the

315 potential for natural fluctuations in the end-season may affect presumed diapause

316 timelines derived from stable lab combinations of photoperiod and temperature. However,

317 only a limited number of studies have examined *Cx. pipiens* diapause in field or semi-field

318 settings (21, 27, 49).

319 Through our semi-field studies conducted over a two-year period (2020-2021), we

320 systematically reared mosquitoes over weeks 30-40 to capture the natural conditions that

321 promote diapause induction in *Cx. pipiens*. From these experiments, we define a timeline

322 of brood receptivity to diapause beginning in late-August (week 34) when immature

323 mosquitoes have the potential to emerge in adult diapause. With increasing diapause

324 incidence as the season progresses, our data support that there is a critical field

325 photoperiod in mid-September (week 38) where ~50% of the immature *Cx. pipiens*

326 population reared at this time emerged in the adult diapause state. This closely coincides

327 with the results of a previous semi-field study in Boston, USA, where peak diapause

328 incidence occurred in late September and early October (27). While diapause can be

329 induced in pupae under artificial diapause conditions (6), the low occurrence of pupal

330 diapause in our semi-field study during these peak periods of diapause induction

331 suggests that cumulative environmental signals experienced over all immature stages

332 may enhance diapause incidence as previously proposed (7).

333 While our semi-field experiments are informative in exploring natural diapause induction,

334 factors such as the gaps between experimental cohorts and the reliance on a laboratory

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335 colony of *Cx. pipiens* are known limitations. To overcome this, we employed the use of

336 *Cx. pipiens* surveillance data as a proxy for adult diapause incidence (21). Through the

337 use of a multi-year data set that captured long-term weekly adult abundance, we

338 demonstrate that gravid female mosquito populations begin to decline in August until they

339 are effectively depleted by October. Although some of these trends will be in part from

340 encroaching cold temperatures that reduce blood-feeding behavior, as was demonstrated

341 in the laboratory, these observations closely align with the natural diapause conditions

342 defined in our semi-field study. Initially focused on surveillance data from Iowa, additional

343 data from across the country provided an opportunity to study diapause timelines in a

344 larger context, where surveillance data at the national scale revealed that latitude,

345 elevation, and *Culex* population genetics may influence *Culex* diapause incidence in

346 addition to temperature and photoperiod.

347 When our data are placed in the context of the wide geographic range within the United

348 States, the importance of latitudinal patterns on diapause incidence begin to emerge for

349 *Cx. pipiens*, where we identify a gradient in the effects of latitude and elevation on

350 diapause incidence, similar to that described in mosquito species (37, 44, 54, 55).

351 Moreover, our data suggest that *Culex* population genetics may also have significant

352 influence on diapause induction where *Cx. pipiens* hybridization with the morphologically

353 indistinguishable *Cx. quinquefasciatus* may result in intermediate diapause phenotypes

354 (30). Together with the potential of non-diapausing *Cx. pipiens* f. molestus populations,

355 the genetics of local *Culex pipiens* s.l. populations may contribute to the absence or low

356 incidence of diapause in a given location, where only genetically pure populations of *Cx.*

357 *pipiens pipiens* enter reproductive diapause (6, 49). However, only limited studies have

358 examined *Cx. pipiens* s.l. population genetics in the United States (39, 56–62),

359 highlighting the important need to better define these important vector species. Of note,

360 few pure *Cx. pipiens pipiens* were detected in northern California (57, 63), which may

361 account for the weak population declines observed in the gravid trap data from California.

362 This coincides with previous observations in northern California that *Cx. pipiens* s.l. do

363 not enter diapause (49). The population genetics of *Cx. pipiens* s.l. in Virginia have not

364 been previously examined, yet due to the geographic location, there is likely hybridization

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365 within the *Cx. pipiens* complex that may similarly partially account for the observations in
366 the gravid trap data.

367 Although temperature is considered an important signal for diapause induction, its
368 contributions to diapause have primarily been evaluated under stable conditions in the
369 laboratory, with little insight into weekly temperature variability and daily temperature
370 fluctuations that occur in nature. As a result, identifying periods of diapause-receptivity to
371 temperature alone is difficult, especially when temperature can influence *Culex* species
372 immature development times (64, 65), as well as adult survival, blood-feeding, and
373 fecundity (65). Similar to these experiments, we observed extended larval development
374 times with cooler average temperatures, which may allow for the increased accumulation
375 of lipids in subsequent diapausing adults (18). However, as temperatures continue to drop
376 in late-summer and early-fall when developing larvae can emerge in diapause, there is a
377 tight balance between accumulating enough resources to enter diapause and not being
378 able to survive temperatures that do not allow for further development. As such,
379 temperature can be a highly confounding variable, one that likely accounts for small
380 variations in the timing of diapause induction between years.

381 An additional, often overlooked aspect of temperature is the influence of daily temperature
382 fluctuations on mosquito physiology. In *Aedes aegypti*, large diurnal temperature ranges
383 negatively impact mosquito development (66), adult female fecundity (66), and vector
384 competence (67). Given that diurnal temperature ranges are largest in temperate climates
385 during the late-summer and early-fall when mosquitoes are receptive to diapause, we
386 hypothesize that these daily temperature fluctuations may similarly influence *Culex*
387 physiology and diapause induction. Moreover, daily temperature fluctuations may protect
388 the commitment to diapause if diapausing adults are exposed to higher temperatures that
389 would regularly break reproductive diapause under laboratory conditions (5).

390 Although long inferred, the relationship of diapause induction to the cessation of West
391 Nile virus (WNV) activity has yet to be fully explored. Our data provide strong support that
392 the diapause incidence in the late-summer/early-fall coincides with the dramatic decline
393 of human WNV cases and mosquito infection rates in late-September and early-October

394 in temperate regions of the United States (32, 68, 69). Moreover, since diapausing

395 females do not blood feed (10), it argues that the overwintering of WNV in *Cx. pipiens*

396 (70–73) occurs via vertical transmission as previously suggested (74, 75). Coincidently,

397 the period of diapause receptivity in the late-summer and early-fall also corresponds the

398 peak of WNV mosquito infection rates (32). Although vertical transmission is an inefficient

399 process (70–73), the increased prevalence of WNV infection in mosquito populations

400 during the approximate time when conditions are favorable for diapause induction may

401 enhance WNV overwintering in diapausing female mosquitoes. As a result, measures to

402 control *Culex* populations prior to diapause induction may not only reduce mosquito

403 populations in the following spring as previously suggested (76), but also limit WNV

404 overwintering and subsequent disease burdens in the following season.

405 In summary, our findings provide a definitive examination of diapause induction in *Cx.*

406 *pipiens* supported by laboratory, semi-field, and field-collected surveillance data from

407 across the United States. We demonstrate the dynamic nature of diapause ecology

408 influenced by yearly variation in temperature, as well as the effects of latitude, elevation,

409 and mosquito population genetics that ultimately determine the overall end-season

410 population structure of *Cx. pipiens* and its role in WNV transmission. Taken together,

411 these data demonstrate the importance of mosquito diapause in defining periods of

412 mosquito-borne disease transmission in the United States. With evidence suggesting that

413 rising global temperatures can alter diapause incidence (77), the effects of climate change

414 may extend transmission seasons and increase the incidence of mosquito-borne disease

415 in temperate regions throughout the world (77–79).

Materials and methods

416

417 *Mosquito rearing*

418 A laboratory colony of *Cx. pipiens* mosquitoes originally isolated from field collections in

419 Ames, Iowa has been constantly maintained in Iowa State University's Insectary at 25 °C,

420 85% RH, and 16:8 (L:D) on 10% sucrose *ad libitum* since approximately 2005. Larvae

421 were fed using a 50/50 mix of crushed Milk-Bone® and Tetramin® fish food, while

422 commercial sheep blood (Hemostat Laboratories) was used for egg production.

15

423 *Laboratory diapause induction experiments*

424 Laboratory experiments to examine diapause induction were performed by placing newly

425 hatched first instar mosquitoes in Percival incubators where they were reared under

426 different experimental conditions (*Control*: 25°C, 16:8 (L:D); *Cold*: 19°C, 16:8 (L:D); *Dark*:

427 25°C, 9:15 (L:D); *Diapause*: 19°C, 9:15 (L:D)) to examine the independent and combined

428 influence of temperature and photoperiod on diapause induction. Experimental conditions

429 were selected based on previous studies (10, 26, 30) in which diapause was induced by

430 a short photoperiod (9:15) and cool temperatures (19°C).

431 *Confirmation of reproductive diapause*

432 Ovaries were dissected from females aged 6-8 days in 1% PBS solution and mounted

433 with Aqua-Poly/Mount (Polysciences Inc). To confirm reproductive diapause, primary

434 follicle lengths were measured under 200x magnification using an Olympus BX40

435 compound microscope according to ovary morphology as previously defined (28, 29). Ten

436 measurements per ovary were recorded to calculate an average follicle length per

437 individual. Individuals with average follicle size under 50 μm were defined as being in

438 diapause (29).

439 *Wing length measurements*

440 Measurements of wing length served as a proxy for mosquito body size (80, 81), where

441 the right wing was dissected and measured from the alula to the most distal tip of the wing

442 under 10x magnification using a dissecting microscope and Nikon imaging software (NIS

443 Elements D 3.2).

444 *Blood-feeding behavior*

445 Approximately 20-30 adult females (6-8 days post-eclosion) from each experimental

446 rearing condition as described above, were challenged with defibrinated sheep blood

447 (Hemostat Laboratories) using an artificial membrane system. After approximately one

448 hour, the number of mosquitoes with a visible blood meal were recorded (of the total) to

449 calculate the percentage of mosquitoes taking a blood meal. Experiments were performed

450 in three independent biological experiments.

451 *Lipid staining*

452 Nile Red (Thermo Fisher Scientific) was used to visualize differences in lipid stores of

453 adult females (6-9 days old) reared under diapause (19 °C, 9:15 L:D) and control

454 conditions (25 °C, 16:8 L:D). Fat bodies were dissected in 4% paraformaldehyde, and

455 stained using a 1:100 PBS dilution of 500 µg/ml Nile Red powder in acetone stock as

456 previously described (18, 31). After incubating tissues for 10 minutes, samples were

457 imaged using a Nikon 50i fluorescent microscope and processed with Nikon imaging

458 software (NIS Elements D 3.2).

459 *Semi-field studies of diapause induction*

460 To examine diapause induction in a natural setting, first instar larvae from our laboratory

461 colony were placed at three locations in Ames, Iowa in 2020 and two locations in 2021

462 (**Figure S2**) to mimic the emergence of mosquito populations at different timepoints

463 throughout the season. In 2020, batches were placed outside every three weeks

464 according to epidemiological week, from week 30 (July 19th) to week 39 (September

465 20th), approximately corresponding to a one-hour loss in daylight between each group.

466 In 2021, batches were again placed outside at weeks 30, 33, and 39 to replicate

467 experiments from the previous year. Unfortunately, larvae were not available to repeat

468 the week 36 timepoint in 2021, however additional groups were included from weeks 34,

469 37, and 38 to provide additional resolution to diapause induction. Larval density in each

470 batch was approximately 300-400 per tray in 1L of distilled water. Mosquitoes were fed

471 daily with 50 mg of a 50/50 mixture of Milk-Bone® mix and Tetramin® fish food. Upon

472 pupation, pupae were placed into mosquito breeder eclosion chambers (BioQuip), with

473 adults were provided with 10% sucrose *ad libitum* for 6-8 days before collections to

474 determine diapause incidence. In addition, lab-reared pupae were placed outside in

475 mosquito breeder eclosion chambers in 2021 from weeks 37-40 to compare diapause

476 induction rates between mosquito life stages.

477 *Mosquito population trends in Iowa*

478 Mosquito surveillance was performed in two central Iowa counties (Polk, Story) by Iowa

479 State University personnel or local public health partners from mid-May (week 20) through

480 the first week of October (week 40). Mosquito collections were performed using infusion-

481 baited Frommer Updraft Gravid Traps (John W. Hock Company) targeting gravid adult

482   female mosquitoes at 16 sites over a six-year period (2016-2021; **Figure S6**). In addition,

483   a total of 14 New Jersey light traps (NJLTs) were used to measure mosquito abundance

484   (2016-2021). Trapping sites (gravid or NJLT) with less than three years of mosquito data

485   were excluded from the study. All mosquito samples were identified using morphological

486   keys (40, 82) where possible. Due to damage to morphological features that help to define

487   *Culex* species (83), mosquito identifications of *Culex pipiens* and *Culex restuans* from

488   NJLT traps were defined as *Culex pipiens* group as previously (32, 34). Gravid trap

489   specimens were identified to species (*Culex pipiens*) (82). To normalize trapping efforts,

490   raw   mosquito   counts   were   converted   to   a   trap   index   (defined   as

491   the number of mosquitoes collected / number of trapping nights) and then averaged by

492   week.

493   *Iowa Climate and Photoperiod data*

494   Daily temperature data (°C) was collected and averaged into weekly values by year using

495   the Iowa Environmental Mesonet (https://mesonet.agron.iastate.edu) for the Southeast

496   Ames station (IA0203). Photoperiod data for the duration of the study period was collected

497   as hours of daylight for Des Moines, Iowa from daylight tables provided from an online

498   Sunrise and Sunset table (www.timeanddate.com).

499   *National trends in diapause induction, temperature, and Culex species hybridization*

500   Mosquito surveillance data were provided from additional locations across the United

501   States (California; https://vectorsurv.org/, Colorado, Connecticut, Illinois, Minnesota,

502   Pennsylvania, and Virginia) representing the temperate range of *Cx. pipiens* in the United

503   States (40). The years included from each data set are as follows: California (2005-2020),

504   Colorado (2015-2020), Connecticut (2006-2020), Illinois (2016-2020), Minnesota (2016-

505   2020), Pennsylvania (2007-2017), and Virginia (2010-2020). To reflect the end-of-season

506   population trends, data was trimmed to reflect weeks 30-40 where applicable (Colorado

507   ends at week 37, Chicago ended most weeks at 39), with raw mosquito counts normalized

508   using trap index averages to account for differences in trapping efforts (number of

509   mosquitoes / numbers of trapping nights) as previously performed for the Iowa dataset.

510   Data from Illinois and Minnesota represents *Culex pipiens* group (a combination of the

511 morphologically similar *Culex restuans* and *Cx. pipiens* species) (32, 33), while records

512 from other states were morphologically identified as *Cx. pipiens*.

513 Daily high and low temperatures for all site locations were compiled using the Iowa

514 Environmental Mesonet (https://mesonet.agron.iastate.edu). All locations reflect 10-year

515 averages (2010-2020) where environmental data were paired to trapping locations as

516 follows: California (Sacramento, CATSAC), Connecticut (statewide average, CT0000),

517 Colorado (east Fort Collins, CO3006), Illinois (Chicago O'Hare International airport,

518 ILTORD), Minnesota (Minneapolis-St. Paul, MNTMSP), Pennsylvania (southeastern PA,

519 PAC003), and Virginia (Suffolk, VA8192). Photoperiod data was collected from an online

520 Sunrise and Sunset table (www.timeanddate.com) for field locations in California

521 (Sacramento), Colorado (Fort Collins), Connecticut (New Haven), Illinois (Chicago),

522 Minnesota (Minneapolis), Pennsylvania (Philadelphia), and Virginia (Chesapeake) for

523 2021.

524 Elevation data for all site locations are provided as a county-level average value collected

525 using https://en-gb.topographic-map.com/.

526 The proposed ranges of *Cx. pipiens and Culex quinquefasciatus,* as well as proposed

527 areas of hybridization, were created in ArcGIS, adapted from Darsie and Ward (40).

528 *Data and Statistical Analyses*

529 Laboratory comparisons comparing ovarian follicle size, wing length, and the percent

530 blood-feeding across experimental groups were analyzed using a one-way ANOVA with

531 a Tukey post-hoc analysis using GraphPad Prism 7.0. Average weekly temperature data

532 were visualized using loess smoothing in R (version 3.6.3). Gravid and NJLT population

533 trends for weeks 30-40 were examined for the Iowa dataset and other locations where

534 NJLT data was provided using yearly slope values and negative binomial regressions

535 with an unpaired *t*-test to demonstrate significant differences in the dynamics of gravid

536 populations. Mosquito population trends were also evaluated by calculating the difference

537 in the average trap index at weeks 30-33 and week 40, and displaying these trends as a

538 percent change ((week40−week 30-33) / week 30-33) ×100) value to demonstrate

19

539 changing temporal trends in gravid populations from mid-season to end-season time
540 points.

Acknowledgements

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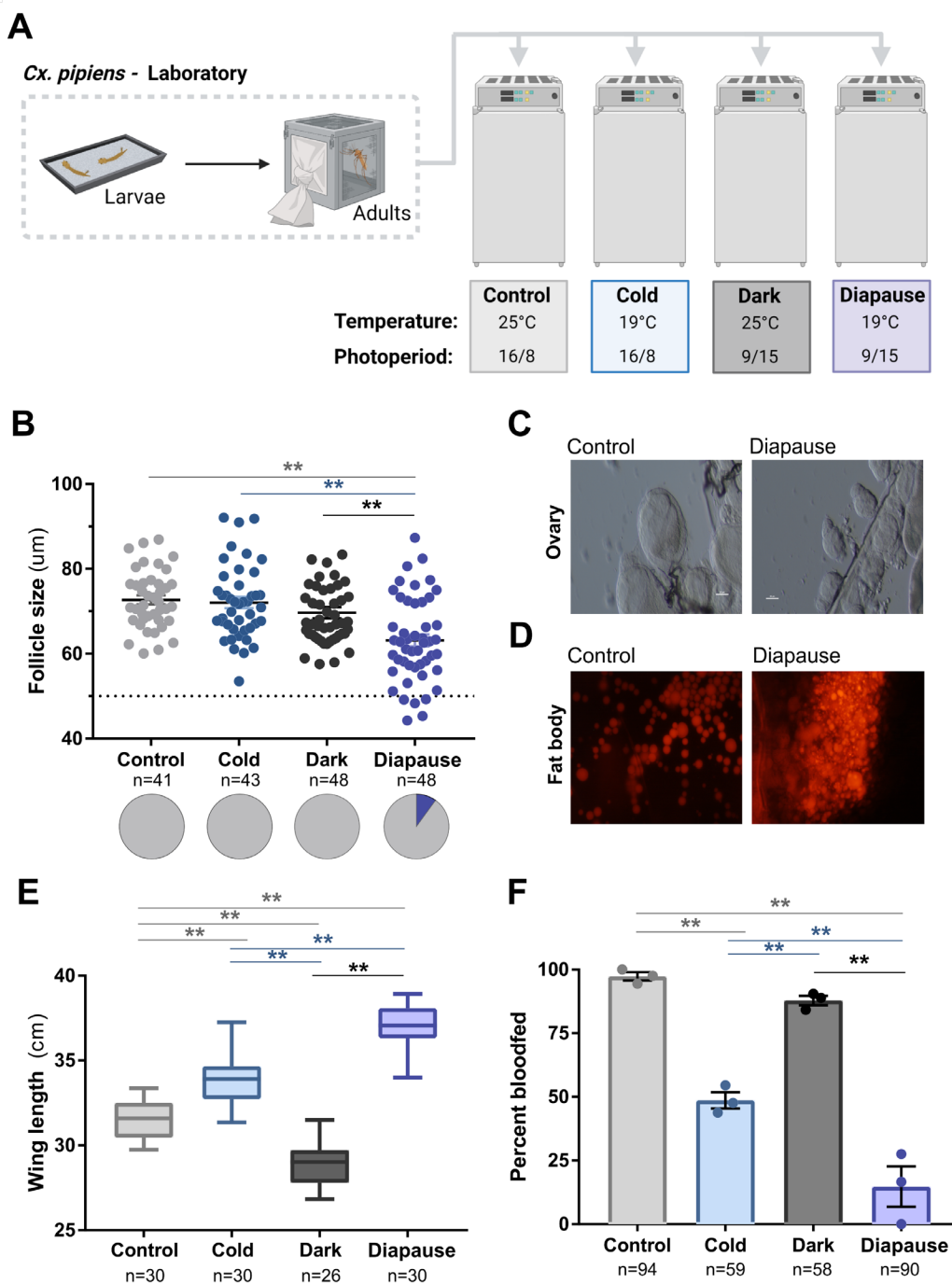
Figures

Figure 1. Laboratory conditions to explore *Culex* diapause induction. *Culex pipiens* first-instar larvae were either maintained under standard insectary conditions (control) or transferred to rearing conditions to examine the effects of cold temperature (cold), short photoperiod (dark), or known conditions to promote diapause (**A**). Adult females were collected from these respective conditions 6-8 days post-eclosion for downstream

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789   experiments. (**B**) Ovary dissections were performed on adult mosquitoes from each
790   rearing condition to determine the average primary follicle size to confirm reproductive
791   diapause. Average primary follicle size is depicted for each individual mosquito, with an
792   average follicle size was <50 µm (dotted line) used to confirm reproductive diapause.
793   Solid black lines represent the median values for each experimental condition, while pie
794   charts display the percentage of mosquitoes in diapause under each condition. Additional
795   confirmations of mosquito diapause were performed by examining differences in ovarian
796   follicle morphology (**C**) and fat body lipid staining with Nile Red (**D**) between mosquitoes
797   reared under control or diapause conditions. (**E**) Wing length measurements on adult
798   females were performed as a proxy body size for each condition. (**F**) Blood-feeding
799   behavior was evaluated in adult female mosquitoes from each treatment by challenging
800   with an artificial membrane feeder containing sheep blood. Significance was determined
801   in follicle size, wing length, and blood-feeding experiments using a one-way ANOVA test
802   and Tukey post-hoc comparisons to compare between experimental treatments.
803   Asterisks denote significance (** P<0.01). n, number of individual mosquitoes examined.
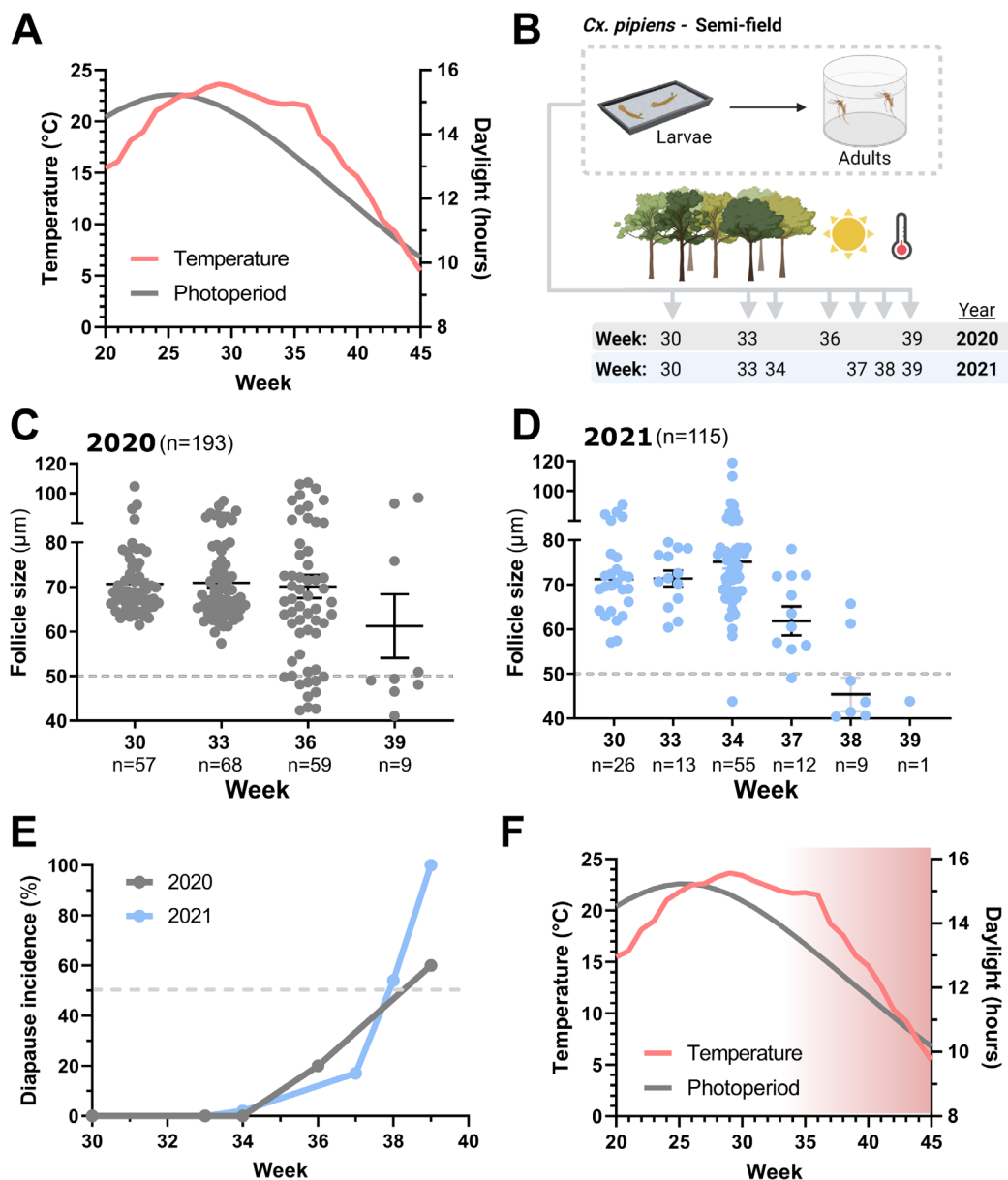
Figure 2. Examining the natural diapause timeline in semi-field experiments. (**A**) Temperature (°C) and photoperiod (daylight) averages (2009-2019) are displayed for the study site location (Ames, IA) over the mosquito season (weeks 20-45). (**B**) Overview of the semi-field experiments performed in 2020 and 2021 where first-instar larvae from a lab colony of *Cx. pipiens* were placed outside at weekly timepoints (between week 30 and 39). Larval groups were reared in a semi-field environment, with resulting adult females used for downstream experiments 6-8 days post-eclosion. A total of 193 adult female mosquitoes were collected in 2020 (**C**) and 115 adult females from 2021 (**D**) to determine if mosquitoes were in reproductive diapause. For **C** and **D**, each dot represents the average follicle size for an individual mosquito, with a 50 μm threshold (dotted line) used to determine individuals in the diapause state. The mean follicle size (+/- SEM) is displayed for each experimental cohort. n, number of individual mosquitoes examined.

817   (**E**) The percentage of adult diapause incidence recorded by larval onset week is
818   summarized for 2020 and 2021. The dashed line corresponds to when 50% of the
819   mosquitoes in a given larval onset week result in reproductive diapause. From our
820   experiments, we define an approximate period of diapause receptivity (**F**), where larval
821   development under temperature and photoperiod conditions of 13.5 hours of daylight and
822   average temperatures under 20 °C may give rise to adult reproductive arrest, resulting in
823   increasing diapause incidence (red gradient) as these environmental conditions continue
824   to decrease over the course of the season.
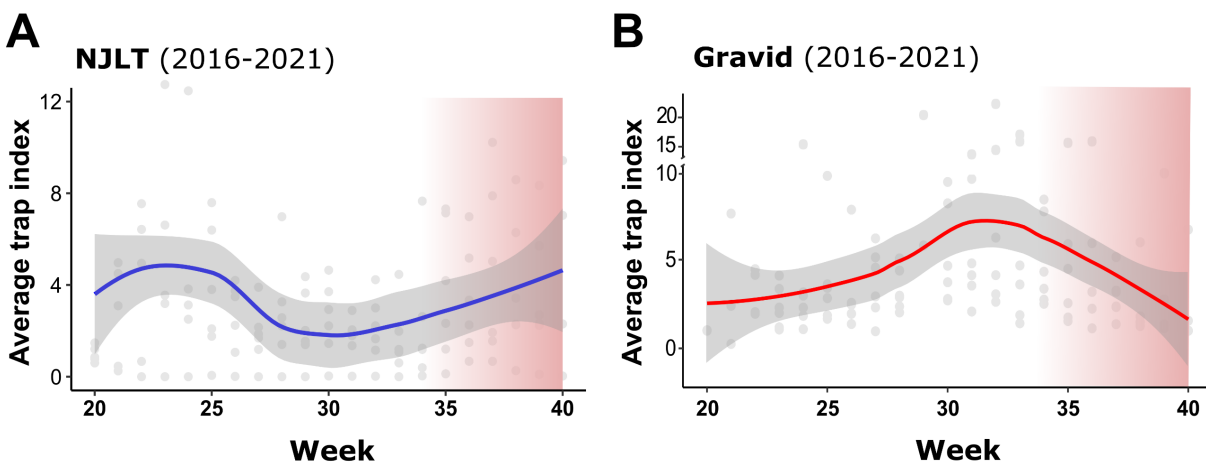
Figure 3. Iowa mosquito surveillance data confirms the natural diapause timeline. Mosquito surveillance from central Iowa display mosquito populations trends that examine general population abundance (**A**) or represent only reproductive populations (**B**). Data in **A** represent *Cx. pipiens* group abundance measured using New Jersey Light Traps (NJLT) from 14 sites in central Iowa, while data in **B** display Gravid trap data from 16 sites in central Iowa that monitor reproductive female *Cx. pipiens* populations. For both **A** and **B**, collections were performed from 2016-2021 from weeks 20 to 40. Individual grey dots represent yearly trap index averages by week, with the blue (NJLT) or red (Gravid) lines representing the loess-smoothed mean and 95% confidence intervals (grey shaded area). The approximate period of diapause receptivity (as defined by our semi-field experiments) is displayed by the red gradient.
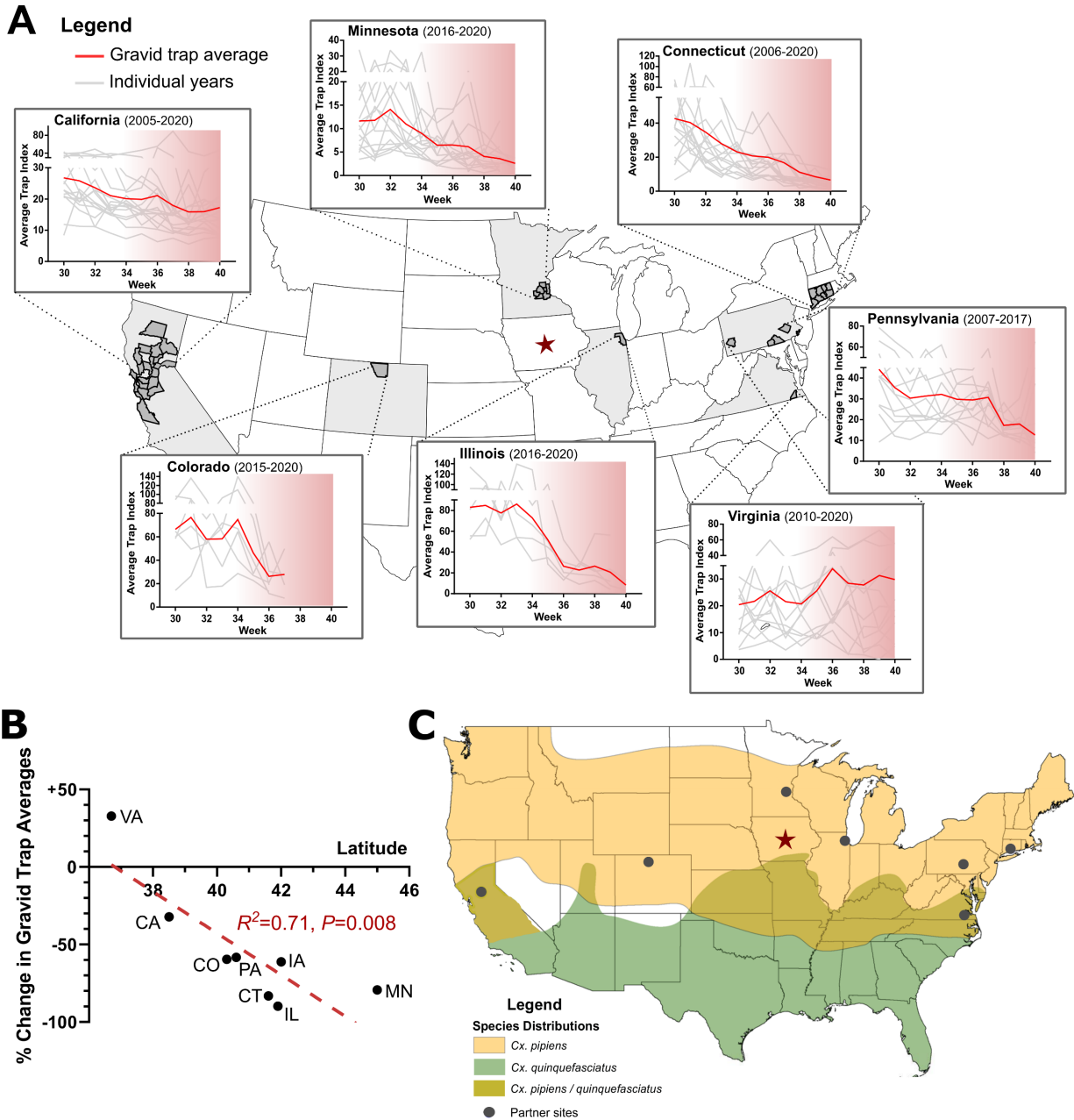
**Figure 4. Gravid population trends from across the United States provide insight into the biotic and abiotic factors that influence *Cx. pipiens* diapause.** (**A**) End-of-season *Cx. pipiens* gravid trap data (weeks 30-40) provided by locations across the United States. States included in our analysis are denoted in light grey, with individual county-level data included in the statewide analysis shown in dark grey. Iowa is denoted by the red star. For each state, red lines represent the loess-smoothed mean of the gravid (reproductive) population abundance, while grey lines display data from an individual year included in our analysis. The approximate period of diapause receptivity (as defined by our semi-filed experiments) is displayed by the red gradient. (**B**) Sites at higher latitudes

847   demonstrated more pronounced declines in gravid trap averages from the weeks 30-33
848   to week 40. (**C**) Map of the reported distributions of *Cx. pipiens* (yellow), *Cx.*
849   *quinquefasciatus* (green), and areas of potential genetic hybridization between these
850   species (olive). Site locations providing gravid trap data are shown by the grey dots, while
851   Iowa is denoted by the red star.

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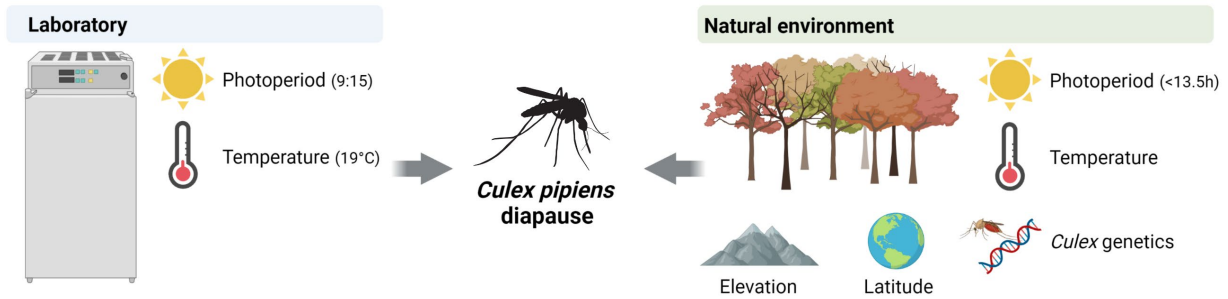
Figure 5. Overview of the factors that influence *Cx. pipiens* diapause in the laboratory and under natural conditions. The combination of short-day lengths and low temperatures form the basis for diapause induction in laboratory studies of *Cx. pipiens*, yet additional ecological factors (latitude, elevation, population genetics) influence diapause dynamics in natural settings.