1 **A tiger in the Upper Midwest: Surveillance and genetic data support the**
2 **introduction and establishment of *Aedes albopictus* in Iowa, USA**

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10 **Abstract**

11   *Aedes albopictus* is a competent vector of several arboviruses that has spread throughout

12   the United States over the last three decades after it was initially detected in Texas in

13   1985. With the emergence of Zika virus in the Americas in 2015-2016 and an increased

14   need to better understand the current distributions of *Ae. albopictus* in the US, we initiated

15   surveillance efforts to determine the abundance of invasive *Aedes* species in Iowa. Here,

16   we describe the resulting surveillance efforts from 2016-2020 in which we detect stable

17   and persistent populations of *Aedes albopictus* in three Iowa counties. Based on temporal

18   patterns in abundance and genetic analysis of mitochondrial DNA haplotypes between

19   years, our data support that populations of *Ae. albopictus* are overwintering and have

20   likely become established in the state. In addition, the localization of *Ae. albopictus*

21   predominantly in areas of urbanization and noticeable absence in rural areas suggests

22   that these ecological factors may represent potential barriers to their further spread and

23   contribute to overwintering success. Together, these data document the establishment of

24   *Ae. albopictus* in Iowa and their expansion into the Upper Midwest, where freezing winter

25   temperatures were previously believed to limit their spread. With increasing globalization,

26   urbanization, and rising temperatures associated with global warming, the range of

27   invasive arthropod vectors, such as *Ae. albopictus*, is expected to only further expand,

28   creating increased risks for vector-borne disease.

Introduction

Aedes albopictus is an invasive mosquito species that in recent decades has spread across multiple continents predominantly through global trade [1–4]. With the first report of its establishment in the United States (US) in Texas in 1985 [5], its range has continually expanded to more than 26 states, gradually spreading northward and westward across the US [6–8] with likely further expansion fueled by climate change and increasing urbanization [3]. With anthropophilic feeding behavior [4,9] and as a competent vector of at least 26 mosquito-borne arboviruses [10], the introduction of *Ae. albopictus* into new locations in the US raises a significant public health concern.

With *Ae. albopictus* as a competent vector of Zika virus (ZIKV) [11,12], the emergence of ZIKV in the Americas in 2015 and 2016 created a critical need to better understand the distributions of *Ae. albopictus* in the US in order to determine the potential risks for ZIKV transmission. Previous studies have described the detection of *Ae. albopictus* populations across the Midwest [7,8,13–17]. This includes Iowa, which has seen sporadic detections of *Ae. albopictus* that likely represent rare and unsuccessful introduction events [17]. However, with established populations of *Ae. albopictus* in the neighboring states of Missouri [7,8,13] and Illinois [7,8,16], there is a high likelihood of further *Ae. albopictus* introduction events and potential invasion with modeling suggesting that Iowa is within this species' predicted range [18]. Initial targeted surveillance in 2016 along the southern Iowa border failed to detect *Ae. albopictus* [19], yet with sampling only over a single year, there may not have been adequate trapping efforts to identify low-density populations.

In this study, we describe our continued monitoring of mosquito populations in Iowa through targeted surveillance efforts focusing on invasive *Aedes* species. Expanding on our initial efforts [19], we used a trapping network consisting of BG sentinel and Gravid *Aedes* traps from 2017 to 2020 to monitor mosquito populations in a total of 25 counties over a five year period (2016 to 2020). Through these efforts, we document the detection and likely establishment of *Ae. albopictus* in three Iowa counties. In addition, we provide evidence of their intra-county movement and the ecological variables that define their presence and absence, elucidating the potential ecological barriers that have thus far prevented their further spread to adjacent counties. Genetic analysis confirms the

59   subsistence of genetic haplotypes between years, supporting the establishment of *Ae.*

60   *albopictus* in each of the respective counties in which it has been detected, providing

61   insight into the origins of their introduction.

Methods

62

Mosquito Surveillance

63

64   Targeted mosquito trapping efforts were performed by Iowa State University personnel or

65   in collaboration with local public health departments between 2016 and 2020 from mid-

66   May through October when mosquitoes are most active in Iowa. While initial efforts in

67   2016 relied on the use of BG Sentinel and CDC light traps [19], trapping from 2017-2020

68   utilized BG Sentinel 2 (BG) traps and Gravid *Aedes* Traps (GATs) (Biogents,

69   Regensburg, Germany). These traps have proven to be highly effective in capturing

70   *Aedes albopictus* and other container-breeding *Stegomyia* specimens [20–24], with the

71   relatively low cost and little maintenance of the GATs enabling broader coverage with

72   each respective county. BG traps were used without a carbon dioxide source, relying on

73   human scent lures (BG-Lure, Biogents), while the GATs were equipped with sticky cards

74   to enable mosquito collections.

75   Following our efforts in 2016 which targeted 9 of the 10 counties along Iowa's southern

76   border [19], we expanded our trapping efforts in 2017 to incorporate all eastern border

77   counties along the Mississippi River and counties with more densely populated cities.

78   These counties were chosen based on their proximity to Missouri and Illinois which have

79   established populations of *Ae. albopictus* [7,8,13,16] and that have the highest potential

80   for introduction via shipping/transport into more densely populated areas. In subsequent

81   years (2018-2020), data from the previous trapping efforts allowed for more focused

82   surveillance, reducing the overall number of participating counties. Trapping efforts were

83   continued in each county where *Ae. albopictus* was detected, as well as in counties that

84   represented potential sites of introduction or spread from adjacent positive counties.

Mosquito Sample Processing and Identification

85

86   Mosquito samples were collected three times a week from BG Sentinel traps, while GATs

87   were collected on a weekly basis. Samples were either transported directly or shipped to

88   Iowa State where mosquitoes were identified to species using morphological

89 characteristics and taxonomic keys [25]. Corresponding data were recorded according to

90 date, trap location, and trap type. *Ae. albopictus* samples were separated into site/date-

91 specific vials and stored in an ultra-low temperature freezer at -80°C for later genetic

92 analysis.

Geographic and Land Cover Analysis

94 The latitude and longitude coordinates of all trap locations were recorded and utilized to

95 plot trapping locations using QGIS version 3.14.1. A Landsat-based, 30-meter resolution

96 land cover layer, clipped to reflect our study area, was obtained from the Multi-Resolution

97 Land Characteristics Consortium (MRLC) National Land Cover Database (NLCD) [26]

98 which served as the base map and the source of all land cover output. Land cover

99 analysis was performed for the two counties in southeast Iowa (Lee and Des Moines

100 counties) which displayed widespread presence of *Ae. albopictus*. Trapping site locations

101 within these counties were examined in QGIS using a 500 meters radius around each

102 surveillance site, with the zonal histogram tool to list pixel counts of each unique land

103 cover value within that radius (buffer layer). This distance was chosen based on the

104 limited flight range of *Ae. albopictus,* which typically does not travel far from its site of

105 origin with a maximum flight range of ~500m [27–29]. Based on pixel numbers

106 corresponding to each land cover feature, the percent land cover was calculated for each

107 site and used to compare between locations where *Ae. albopictus* was present or absent.

Genetic Analysis

109 *Ae. albopictus* samples collected from site locations between 2017 and 2018 in each of

110 the three positive Iowa counties were examined by genetic analysis. A total of 165

111 samples were processed using the Marriott DNA extraction procedure [30–32] to isolate

112 genomic DNA which was used as a template for PCR genotyping. Similar to other studies

113 that have examined *Ae. albopictus* genetic haplotypes [16,33], a fragment of the

114 mitochondrial cytochrome c oxidase subunit 1 was targeted with the following primers:

115 2027F (5'-CCC GTA TTA GCC GGA GCT AT-3') and 2886R (5'-ATG GGG AAA GAA

116 GGA GTT CG-3'). PCR was performed using DreamTaq Green DNA Polymerase

117 (Thermo Fisher Scientific) under the following conditions: initial denaturation 94°C, 3 min;

118 denaturation 94°C, 30 sec; annealing 55°C, 30 sec; extension 72°C, 1 min for 35 cycles;

119 and a final extension 72°C, 6 min. PCR products were examined by electrophoresis on a

120 1% agarose gel, excised, and recovered using a Zymoclean Gel DNA Recovery Kit (Zymo

121 Research). Resulting DNA was cloned into a pJET 1.2/blunt cloning vector using the

122 CloneJET PCR Cloning Kit (Thermo Fisher Scientific), and subsequently transformed into

123 DH5-alpha competent *E. coli* (New England Biolabs). Bacteria were plated on LB agar

124 plates with a 100 µg/ml ampicillin concentration and incubated overnight at 37°C to select

125 for successfully transformed colonies. Individual colonies were randomly chosen from the

126 selection plates, suspended in 3 ml of LB broth and cultured overnight in a 37°C shaker

127 at 215 RPM. Plasmid DNA was isolated using the GeneJet Plasmid Miniprep Kit (Thermo

128 Fisher Scientific), with the presence of an insert validated by *Bgl*II digests and gel

129 electrophoresis. Sanger sequencing of the resulting samples was conducted by the Iowa

130 State University DNA Facility.

131 DNA sequencing data was aligned and edited manually using BioEdit version 7.0.5.3. To

132 minimize the possibility of polymerase error, at least 3 sequences from each individual

133 sample were combined to create a consensus sequence for each sample. Any unique

134 sequences were confirmed by the additional amplification using Phusion High-Fidelity

135 DNA Polymerase (Thermo Fisher Scientific) followed by cloning and sequencing using

136 the above methods. DNA from individual mosquito samples were grouped into haplotypes

137 where each haplotype represents a unique sequence, and the number of polymorphic

138 sites, haplotype diversity (*Hd*), and nucleotide diversity (π) were calculated using DnaSP

139 (version 6.12.03) [16,33,34]. A haplotype network was created in PopART [35] using the

140 median–joining network method [36] to visualize genetic relationships between

141 haplotypes and to display differences in population structure between sites [16].

Results

Mosquito surveillance and detection of *Ae. albopictus* in Iowa

144 To determine if the invasive mosquito species, *Ae. aegypti* and *Ae. albopictus*, could be

145 found in the state of Iowa, we performed targeted mosquito surveillance in a total of 25

146 counties from 2016 to 2020 (Figure 1A, Table S1, Table S2). After initial surveillance

147 efforts along the southern border of the state in 2016 [19], we extended our trapping

148 efforts from 2017-2020 to more densely populated counties and to those bordering the

149　Mississippi River (Figure 1A, Table S1, Table S2). Although *Ae. aegypti* and *Ae.*

150　*albopictus* were not detected in 2016 [19], a total of 432 *Ae. albopictus* were collected in

151　2017 from three Iowa counties (Polk, Lee, and Des Moines) (Figure 1A and 1B). In

152　subsequent years (2018 to 2020), *Ae. albopictus* were similarly detected in each of the

153　same three counties in increased numbers, reaching a high of 1,315 *Ae. albopictus*

154　detected in 2020 (Figure 1B). From 2017 to 2020, a total of 3,700 *Ae. albopictus* were

155　collected, with Lee County displaying the highest total of *Ae. albopictus* amongst the three

156　counties (Figure 1C) and consistently producing the highest number of *Ae. albopictus*

157　between years (Figure 1D). Together, these data suggest that in recent years *Ae.*

158　*albopictus* have been introduced into the state, and have potentially become established

159　in three Iowa counties.

160　**_Ae. albopictus_ population dynamics support their ability to overwinter in Iowa**

161　To provide further support that *Ae. albopictus* have become established in each of the

162　three counties, we examined *Ae. albopictus* weekly numbers and overall contributions to

163　trapping yields within each of the mosquito trapping seasons from 2016-2020 in counties

164　for which *Ae. albopictus* were detected (Figure 2). Across counties, *Ae. albopictus*

165　populations reached peak abundance in late summer (late August, early September;

166　approximately weeks 35 and 36), followed by sharp declines in abundance by early

167　October (week 40) (Figure 2). For Polk County in central Iowa, *Ae. albopictus* was first

168　detected in week 31 (early August) in 2017, yet in subsequent years (2018-2020) were

169　consistently identified in mid-June (weeks 24 and 25; Figure 2A). Moreover, *Ae.*

170　*albopictus* represented a much larger percentage of overall trap yields between 2018-

171　2020 when compared to 2017 (Figure 2B), suggesting that their earlier abundance and

172　higher proportion in the collected samples are indicative of their potential establishment.

173　Similar patterns of *Ae. albopictus* abundance were also recorded in the southeastern

174　portion of the state in Des Moines and Lee counties (Figure 2). For Des Moines County,

175　the first detection of *Ae. albopictus* in 2017 occurred in week 30 (late July), while they

176　were regularly detected in June (weeks 23-26) in subsequent years (2017-2020; Figure

177　2C). Although sites in Des Moines County displayed some variability between trap types,

178　*Ae. albopictus* comprised between ~10-40% of the overall number of mosquitoes

179 collected in the county (Figure 2D). Lee County recorded the earliest detection of *Ae.*
180 *albopictus* in 2017, with the first samples identified in week 28 (mid-July). In the years
181 following (2018-2020), *Ae. albopictus* were found as early as week 21 (late-May; Figure
182 2E). Aside from 2016 when *Ae. albopictus* were not detected in our initial trapping efforts
183 [19], *Ae. albopictus* represented ~34% of the total trap yields when averaged across years
184 and trap types (Figure 2F). While we account for some yearly variation in occurrence and
185 overall abundance, these data provide further support for the overwintering and
186 establishment of *Ae. albopictus* in multiple Iowa counties.

187 **Influence of landscape ecology on *Ae. albopictus* abundance**
188 To determine if landscape ecology influences the presence of *Ae. albopictus* in Iowa, we
189 looked to more closely examine the trapping site locations in each of the *Ae. albopictus*-
190 positive counties. Trapping efforts in Polk County consisted of only a single site in close
191 proximity to a facility involved in tire transport, with surrounding areas serving as an ideal
192 habitat for *Ae. albopictus* (abundant breeding sites, tree cover, access to diverse hosts;
193 Figure S1). Additional focused trapping efforts (BGs, GATs) were not performed at other
194 locations in Polk County during this study. However, non-targeted surveillance involved
195 with our West Nile virus surveillance program using other trap types (New Jersey Light
196 Traps and Frommer Updraft Gravid Traps) have detected low numbers of *Ae. albopictus*
197 in 2019 and 2020 at nearby locations in Polk County (Figure S2). The detection of *Ae.*
198 *albopictus* in these suburban environments at locations that have been continuously
199 trapped since 2016, suggests that *Ae. albopictus* have likely dispersed greater than 3
200 miles from their presumed point of introduction in recent years, providing further support
201 for the ability of *Ae. albopictus* to overwinter in Polk County.

202 In contrast to the likely introduction of *Ae. albopictus* in Polk County associated with tire
203 transport, there were no obvious mechanisms for the introduction of *Ae. albopictus* in Des
204 Moines and Lee counties. As a result, the multiple trapping locations in Des Moines and
205 Lee counties provided a better opportunity to determine the influence of landscape
206 ecology on the presence or absence of *Ae. albopictus* (Figure 3). To address this
207 question, we performed comparative land cover analysis on a total of 37 trapping site
208 locations for which we defined each site for the presence/absence of *Ae. albopictus*

209 (Figure 3, Table S3). A total of 22 sites where *Ae. albopictus* were detected every year

210 were considered "positive", while the six sites for which *Ae. albopictus* were never found

211 were considered "negative" (Table S3). Nine other sites where *Ae. albopictus* were

212 identified but not collected every year were defined as "detected" (Table S3), suggesting

213 that these sites represent new introductions that may or may not support established

214 populations. Each of these trapping sites were mapped to their respective locations in

215 Des Moines and Lee counties (Figure 3A), and the landscape ecology of "positive",

216 "negative", and "detected" sites were compared (Figure 3B). We identified that areas of

217 low-density development were significantly correlated with the presence of *Ae. albopictus*,

218 while the percentage of agricultural areas were negatively associated with the presence

219 of *Ae. albopictus* (Figure 3B). These data are supported by the spatial locations of the

220 trapping site locations, where sites within urbanized areas were predominantly positive,

221 while those located in more rural areas were typically negative (Figure 3A). This

222 corresponds with the preferred habitat of *Ae. albopictus* which is most commonly

223 associated with urban and suburban environments [37,38].

224 Furthermore, our trapping data provide support for the expansion of *Ae. albopictus* in both

225 Des Moines and Lee counties. The presence of several sites for which *Ae. albopictus*,

226 were detected, but not necessarily established, supports the movement of this mosquito

227 species into new areas. This is further evidenced by the presence of *Ae. albopictus* in

228 Keokuk (Lee County; Figure 3A) in 2018, where previous trapping efforts in 2016 [19] and

229 2017 suggested that these mosquito species were noticeably absent. This contrasts

230 surveillance in Ft. Madison (Lee County; Figure 3A) where *Ae. albopictus* have been

231 detected at every site since 2017 (Figure 3A, Table S3). With these two cities separated

232 by ~16 miles, these data imply that the distribution of *Ae. albopictus* in Lee County may

233 be continually expanding into new locations.

234 **Genetic haplotype analysis identifies likely sources of introduction and supports**

235 **overwintering of *Ae. albopictus* in Iowa**

236 To better understand the origins of the *Ae. albopictus* collected in each of the three

237 positive counties, DNA was isolated from a total of 165 individual samples and sequences

238 of the mitochondrial CO1 gene were analyzed similarly to previous studies [16,33].

239 Sequence analysis resulted in the identification of 8 genetic haplotypes (Figure 4A, Table
240 S4), distinguished by single nucleotide polymorphisms ranging between one to three
241 nucleotides (Figure 4A, Table S5). The most abundant haplotypes, *hap_1* and *hap_3*, are
242 distinguished by two nucleotides and were found in each of the three *Ae. albopictus*
243 positive counties (Figure 4A). Both haplotypes represent common DNA haplotypes
244 detected in other locations in the United States [16,33], southeast Asia [33,39], and
245 Europe [33] (Table S4). Moreover, *hap_2* and *hap_3* were previously detected in Illinois
246 [16] (Table S4), which due to its close proximity suggests that Illinois may serve as the
247 likely origin and source for the introduction of *Ae. albopictus* in Iowa.

248 For each of the Ae. albopictus counties, three or more haplotypes were detected, with
249 both Des Moines and Lee counties displaying a total of five haplotypes (Figure 4B). While
250 the majority of samples in Polk and Lee counties represented a single haplotype, Des
251 Moines County displayed the most diverse population with *hap_1*, *2*, and *3* comprising
252 the majority of samples (Figure 4B). When samples were examined between years (2017
253 and 2018), the predominant haplotype(s) were consistent between years in each *Ae.*
254 *albopictus* positive county (Figure 4C), providing further support for the establishment of
255 these populations in each of the respective counties.

256 **Ae. albopictus overwintering in below freezing winter isotherms**
257 Based on our surveillance data (Figure 2) and genetic analysis of DNA haplotypes (Figure
258 4), our results provide a strong argument for the introduction and establishment of *Ae.*
259 *albopictus* in Iowa. Since overwintering temperatures have largely been attributed to
260 limiting the spread of *Ae. albopictus* in North America [40], we examined the average
261 winter temperature (December, January, February) isotherms for Iowa (1981-2010). Each
262 of the *Ae. albopictus*-positive counties have average winter temperatures below freezing,
263 with Des Moines and Lee counties in the -3 to -4°C isotherm, and Polk County in the -4
264 to -5°C isotherm (Figure 5A). While January temperatures (typically the coldest month)
265 vary between years, temperatures ranged between -1 to -6°C in the *Ae. albopictus*-
266 positive counties during our study period (Figure 5B). These low temperatures are
267 traditionally considered to be not conducive to *Ae. albopictus* overwintering [18,40],
268 suggesting that the *Ae. albopictus* populations in Iowa have been able to adapt to these

269   freezing temperatures or have found adequate insulated environments to survive the

270   winter. With the potential that global warming may have promoted elevated winter

271   temperatures that may increase the chances of *Ae. albopictus* overwintering in the state,

272   we examined the 30-year average January temperatures from 1981-2010 and 1991-2020

273   (Figure 5C). While Polk County displayed slightly warmer temperatures in recent years,

274   temperatures in both Des Moines and Lee counties were ~0.5°C cooler (Figure 5C),

275   arguing that the recent expansion of Ae. albopictus into these areas is not the result of

276   warmer winter temperatures.

Discussion

278   While limited detections of *Ae. albopictus* in Iowa (12 total from 1999-2016) have

279   previously been described [17,41], these rare incidents were likely the result of isolated

280   introduction events. However, with the introduction of Zika virus in the Americas in 2015-

281   2016, there was an increased need to define the range of competent *Aedes* vectors

282   throughout the US. Although our initial efforts in 2016 along the southern Iowa border did

283   not detect *Ae. albopictus* [19], the results of our expanded surveillance efforts from 2017-

284   2020 presented here describe the detection of more than 3,700 *Ae. albopictus* samples

285   from three Iowa counties.

286   From these data, several lines of evidence support the establishment of *Ae. albopictus* in

287   Iowa. This includes the consistent, early-season detection of *Ae. albopictus* in May and

288   June, as well as the high percentage of the overall trapping yields for each of the three

289   *Ae. albopictus*-positive counties. This is further validated by the occurrence of consistent

290   mtDNA haplotypes between years, indicative of stable, genetic populations of *Ae.*

291   *albopictus* that support mosquito overwintering.

292   While we cannot fully eliminate the possibility that new, yearly introductions may also

293   contribute to the *Ae. albopictus* samples that were collected, the high number of individual

294   mosquito samples collected for each *Ae. albopictus*-positive county makes this possibility

295   unlikely. Although the primary site examined in Polk County is associated with tire

296   transport, the surrounding areas are ideal *Ae. albopictus* habitat, with adequate tree

297   cover, the presence of human and mammalian hosts, and an abundance of human-

298   derived containers/tires that can serve as sites for oviposition that would support *Ae.*

299   *albopictus* in much greater density. Although no obvious mechanisms of introduction by
300   tire transport have been determined for Des Moines and Lee counties, the proximity to
301   the Mississippi River and interstate highways that support human-associated transport
302   likely account for the large number of *Ae. albopictus* collected in both locations. However,
303   the consistency between years and the representation of similar *Ae. albopictus* mtDNA
304   haplotypes across each county suggest that these are stable populations, supporting that
305   the overwintering and establishment of *Ae. albopictus* as the most likely cause for the
306   mosquitoes collected during our study period.

307   The presumed establishment of *Ae. albopictus* in Iowa challenges previous studies that
308   have suggested that *Ae. albopictus* populations rarely extend north of 40°N latitude [7,18],
309   presumably due to freezing winter temperatures that limit the ability of *Ae. albopictus* to
310   overwinter in North America [40,42]. With average winter temperatures below 0°C which
311   have traditionally limited overwintering and the expansion of *Ae. albopictus* [42], the
312   freezing winter conditions in Iowa have traditionally been viewed as a major limitation to
313   sustaining overwintering populations [18,40]. However, the detection of stable
314   populations of *Ae. albopictus* in regions of Iowa with average winter temperature ranging
315   from -3 to -5°C argue that these mosquito populations have potentially adapted to survive
316   these harsh winter conditions. This also raises questions if winter temperatures alone are
317   responsible for this climactic barrier, where the presence of snow cover may also help
318   insulate overwintering *Ae. albopictus* eggs to enhance their survival [43]. Therefore, the
319   microclimate of overwintering eggs may have a larger influence on the overwintering
320   survival of *Ae. albopictus*.

321   An additional consideration of winter temperatures is the importance of urban heat islands
322   [44–46], which may provide differences in microclimate across an urban and suburban
323   landscape that results in warmer winter temperatures [45,46], potentially improving *Ae.*
324   *albopictus* overwintering survival [47]. This is supported by the importance of urbanized
325   development on the consistent detection of *Ae. albopictus* in Des Moines and Lee
326   counties, where the presence of more rural, agricultural environments had significant
327   influence on the presence/absence of *Ae. albopictus*. Furthermore, our site in Polk County
328   also resides in an urbanized environment. Therefore, these urban microenvironments

329   may provide increased "insulation" from the harsh winter temperatures that have been

330   traditionally believed to serve as an ecological barrier for *Ae. albopictus* expansion. As a

331   result, this would explain the higher density of *Ae. albopictus* collected in urban

332   environments, and the low density or absence of *Ae. albopictus* collected in more natural

333   or agricultural areas.

334   At present, it is unclear as to the exact timing of when *Ae. albopictus* were introduced into

335   the state. Prior to 2016, mosquito surveillance in Iowa predominantly focused on West

336   Nile virus [31], utilizing a trap network that was not ideal for the collection of invasive

337   *Aedes* species and did not extend into areas that would most likely serve as points of

338   introduction. In 2016, our initial surveillance efforts along Iowa's southern border failed to

339   detect *Ae. albopictus* [19], yet in hindsight, these predominantly agricultural and less

340   densely populated areas did not represent ideal *Ae. albopictus* habitats. However, our

341   trapping efforts in 2016 did include Lee County [19], where our more focused efforts

342   described in this study from 2017-2020 did result in the detection of established

343   populations of *Ae. albopictus*. This discrepancy is most likely due to differences in trap

344   locations in Lee County when compared between 2016 and 2017-2020, which for 2016

345   included only sites near Keokuk, while in 2017-2020 included both Keokuk and Ft.

346   Madison. It is therefore of note that beginning in 2017 and in subsequent years, each of

347   the trapping sites in Ft. Madison were positive for *Ae. albopictus*. Yet, for Keokuk which

348   is ~16 miles away, the first detection of *Ae. albopictus* wasn't until 2018. This includes at

349   least one site with continual trapping efforts in 2016 [19] and 2017 for which *Ae. albopictus*

350   was later detected in 2018. These intra-county differences suggest that the introduction

351   of *Ae. albopictus* in Lee County likely occurred prior to 2017 for Ft. Madison, while the

352   introduction into Keokuk is more recent, potentially even during the years of our study

353   (2017-2020) and suggests that their distribution is continuing to expand in the county.

354   Similar to Ft. Madison (Lee County), we believe that the detection of *Ae. albopictus* in

355   Burlington (Des Moines County) occurred prior to our trapping efforts in 2017 based on

356   their abundance and distribution throughout the city. However, previous surveillance

357   efforts in Des Moines County have been limited, preventing the determination of a

358   definitive timeline for their introduction. Based on the prevalence of multiple mtDNA

359   haplotypes in Des Moines County, multiple invasion events may have contributed to the

360 established *Ae. albopictus* populations in the county. For both Des Moines and Lee
361 counties, the proximity of the Mississippi River and interstate highways to the urban areas
362 of both counties likely represents the most feasible route of introduction through freight
363 and shipping along the waterway or from interstate transport from neighboring Illinois.

364 In contrast, the introduction of *Ae. albopictus* into Polk County, are inextricably tied to the
365 tire transport industry. Due to the potential for yearly infestations, which may be
366 responsible for previous detections of *Ae. albopictus* in the county [7,8,17], is difficult to
367 definitively demonstrate that the populations of *Ae. albopictus* identified in Polk County
368 are of established populations and not an annual infestation. Yet, during the course of our
369 study (2017-2020), there is strong evidence that we may have captured a local infestation
370 that was able to overwinter and establish in the area. Support for this includes an ~10-
371 fold increase in the number of *Ae. albopictus* collected between 2017 and 2018 (35 and
372 321 respectively), a dramatic increase in the percentage of *Ae. albopictus* in overall gravid
373 *Aedes* trap yields (21% in 2017 compared to an average of 62% from 2018-2020), and
374 the consistent detection of two predominant mtDNA haplotypes (*hap_1* and *hap_3*)
375 between 2017 and 2018. Moreover, the recent detection of *Ae. albopictus* at low densities
376 at other non-focused trapping sites in close proximity to the tire facility support their
377 potential expansion and establishment in the area.

378 Together, our results provide strong evidence for the presence and establishment of *Ae.*
379 *albopictus* populations in Iowa, demonstrating the further expansion of *Ae. albopictus* into
380 the Upper Midwest region of the United States. Importantly, with consistent winter
381 temperatures in Iowa that are below freezing, this challenges existing beliefs that these
382 winter temperature extremes serve as the primary boundary for *Ae. albopictus*
383 overwintering and expansion [18,39,40,42]. With the additional recent detection of *Ae.*
384 *albopictus* in Wisconsin [15], this raises an increased need for continual surveillance to
385 monitor the further spread and expansion of *Ae. albopictus* in the Upper Midwest and
386 other regions of the world on the northern range of the expansion of *Ae. albopictus.*
387 Through increased urbanization and predicted climate change, the distribution of *Ae.*
388 *albopictus* is only expected to further spread [3], highlighting the increased risk of
389 mosquito-borne disease transmission in new regions of the world.

Acknowledgements

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536 **Figures**
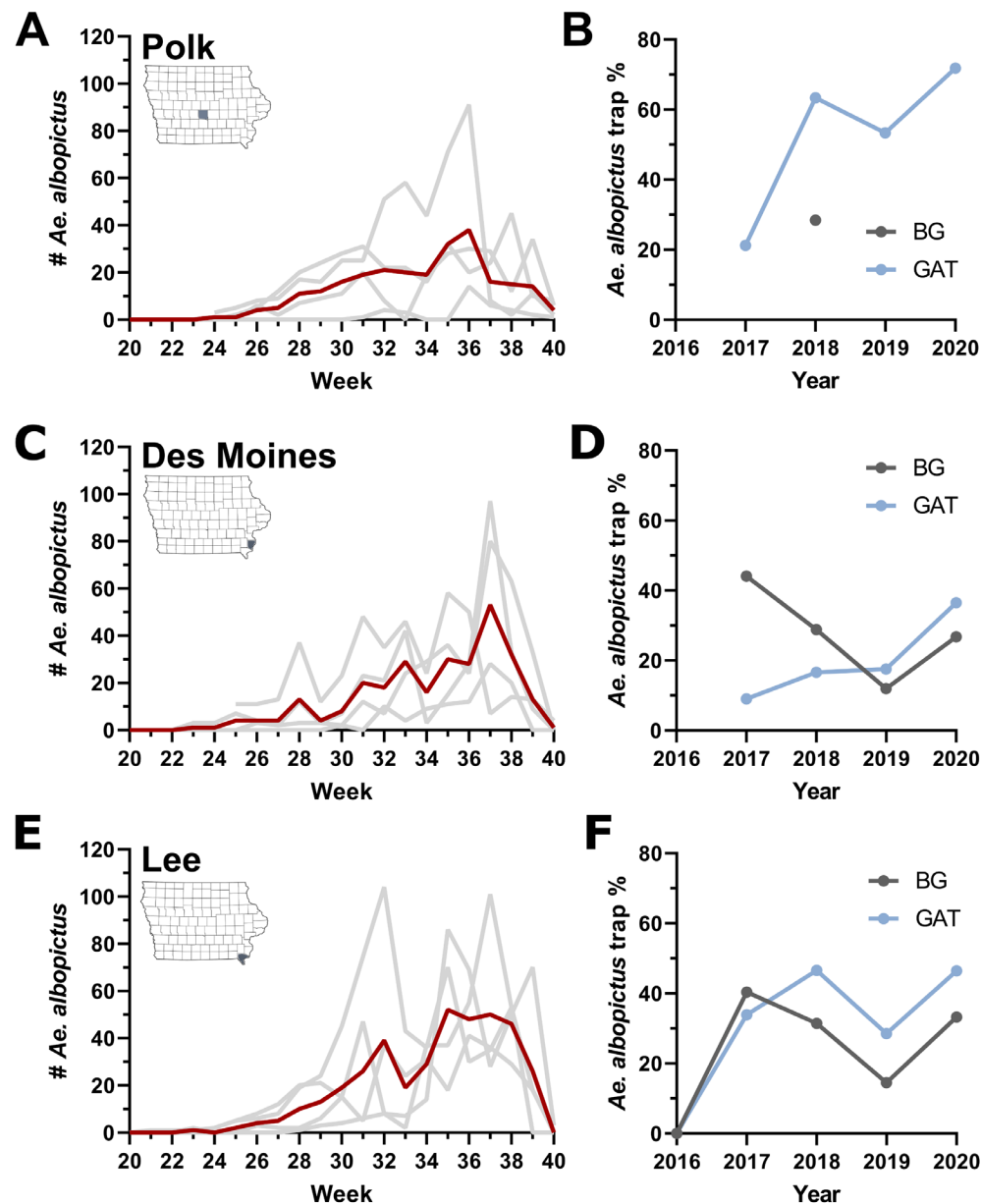
Figure 2. Abundance of *Ae. albopictus* in each positive Iowa county. The temporal abundance and percentage of *Ae. albopictus* in overall trap yields is displayed for Polk (**A, B**), Des Moines (**C, D**) and Lee County (**E, F**). Temporal abundance for each county (**A, C, E**) is displayed by epidemiological week, with the average abundance (2017-2020) displayed in red, while individual years are denoted by light gray lines. The percentage of *Ae. albopictus* collected of the total trap yields (**B, D, F**) are displayed for BG Sentinel (BG) and Gravid *Aedes* traps (GAT) for each year.
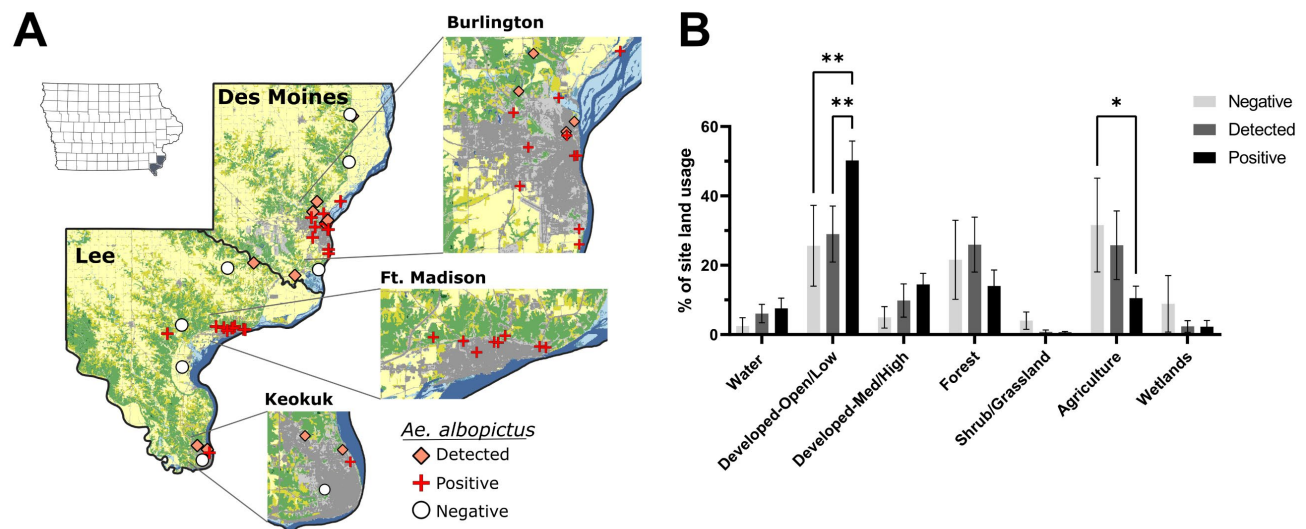
Figure 3. Landscape ecology influences *Ae. albopictus* abundance. Trapping site locations for both Des Moines and Lee counties display the presence/absence of *Ae. albopictus* as either positive (detected every year; red cross), detected (detected in some years; orange diamond), or negative (never detected; white circle) (**A**). Insets display expanded views for the most populous cities in Des Moines (Burlington) and Lee County (Ft. Madison and Keokuk). To better understand differences in the ecology of sites where *Ae. albopictus* were positive, detected, or negative, land use/land cover analysis was performed using 500m radius around each trapping location and displayed for different land use/land cover classifications (**B**). Statistical analysis was performed using a 2-way ANOVA and a Tukey's multiple comparison test using GraphPad Prism software. Asterisks denote significance (*p < 0.05; **p < 0.01).
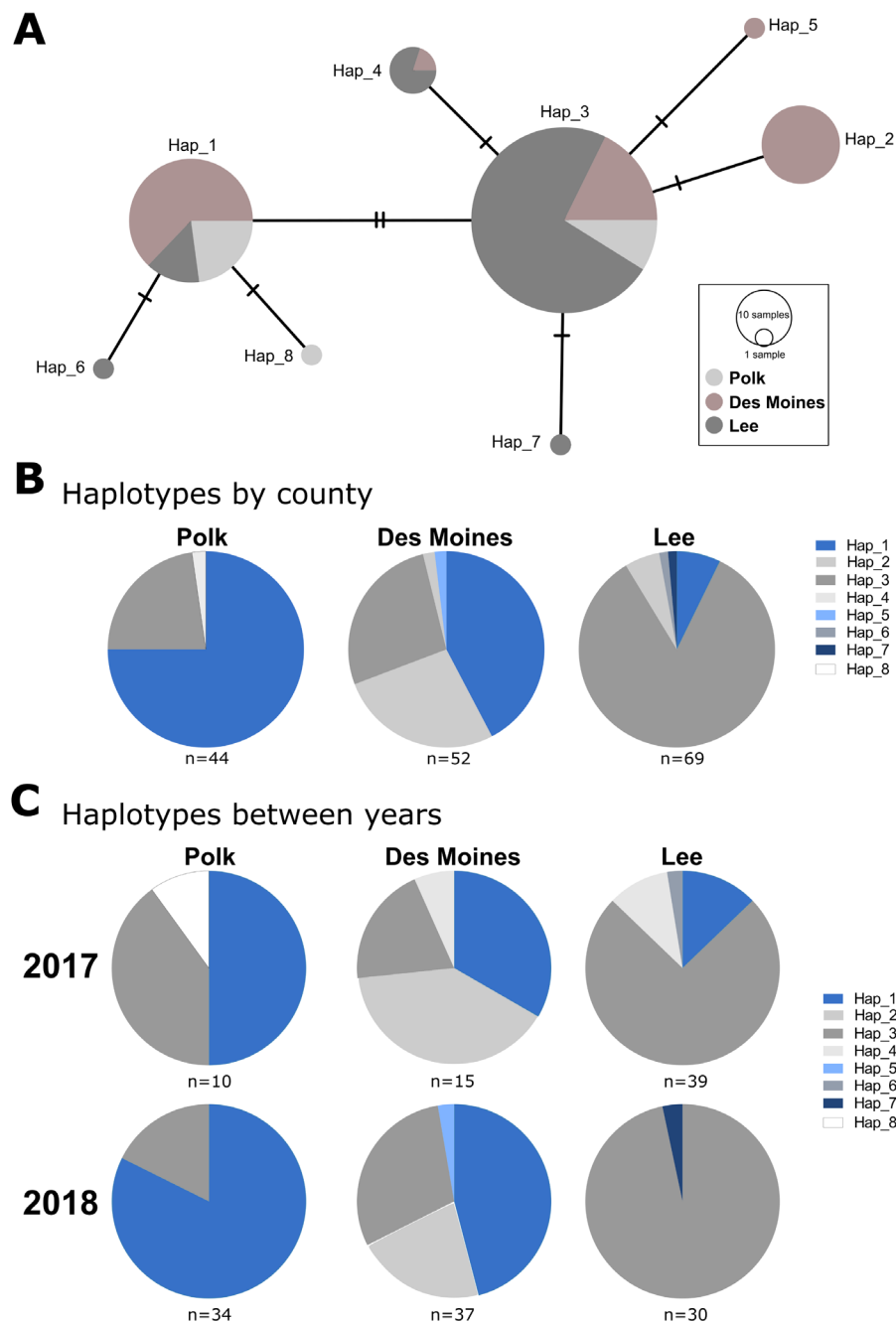
Figure 4. Comparative analysis of *Ae. albopictus* mtDNA haplotypes identified in Iowa. Comparisons of *Ae. albopictus* mtDNA haplotypes identified in Iowa are displayed as circles, with dashes on connecting lines indicating the number of nucleotide differences between haplotypes (**A**). Circle size corresponds to the number of individual samples, with differences in color representing the proportion samples from each respective county. Additional pie charts display differences in haplotypes between each Ae. albopictus- positive county (**B**) and the persistence of haplotypes between years (**C**).
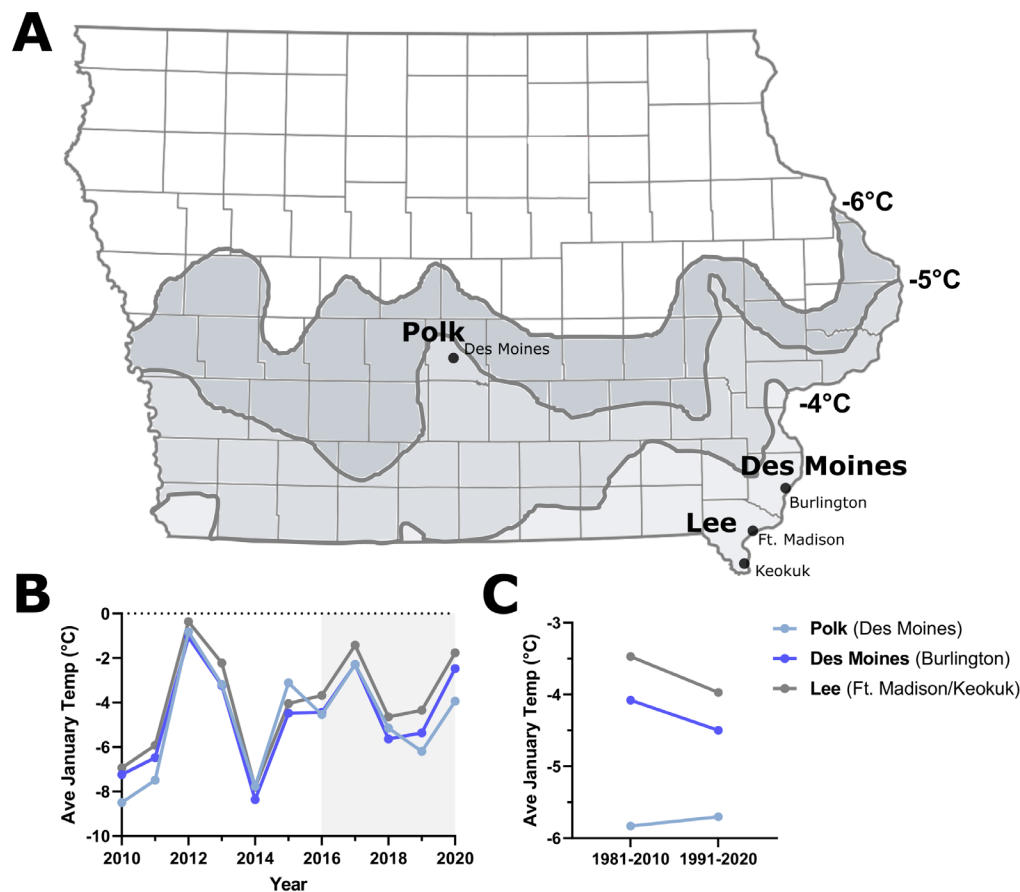
Figure 5. Overwintering temperatures in Iowa. Average winter (December, January, February) temperatures are displayed for Iowa (**A**). Shaded regions represent different temperature isotherms. Cities and counties where stable populations of *Ae. albopictus* have been detected are shown. Annual January temperatures are displayed from 2010-2020 to indicate differences in yearly temperatures for each respective *Ae. albopictus*-positive county (**B**). The shaded region from 2016-2020 represent the study period where targeted trapping efforts have focused on invasive *Aedes* species. Differences in January temperatures from the 30-year average from 1981-2010 and 1991-2020 examine the potential impacts of climate change on overwintering temperatures (**C**).