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Genome evolution in an agricultural pest following adoption of transgenic crops

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26 **Significance Statement**

27 Evolution of pest resistance to management approaches in agricultural landscapes is common
28 and results in economic losses. Early detection of pest resistance evolution prior to significant crop
29 damage would benefit the agricultural community. It has been hypothesized that new genomic
30 approaches could track molecular signals of emerging resistance problems and trigger efforts to pre-
31 empt widespread damage. We tested this hypothesis by quantifying genomic changes in the pest
32 *Helicoverpa zea* over a 15 year period concurrent with commercialization and subsequent loss of
33 efficacy of transgenic Bt-expressing crops. Our results demonstrate the complex nature of evolution in
34 agricultural ecosystems and provide insight into the potential for and pitfalls associated with use of
35 genomic approaches for resistance monitoring. We discuss approaches for improvement.

Abstract

Replacement of synthetic insecticides with transgenic crops for pest management has been both economically and environmentally beneficial. These benefits have often eroded as pests evolved resistance to transgenic crops, but a broad understanding of the timing and complexity of adaptive changes which lead to field-evolved resistance in pest species is lacking. Wild populations of *Helicoverpa zea*, a major lepidopteran crop pest and the target of transgenic Cry toxin-expressing cotton and corn, have recently evolved widespread, damaging levels of resistance. Here, we quantified patterns of genomic change in wild *H. zea* collected between 2002 and 2017 when adoption rates of Cry-expressing crops expanded in North America. Using a combination of genomic approaches, we identified significant temporal changes in allele frequency throughout the genomes of field-collected *H. zea.* Many of these changes occurred concurrently with increasingly damaging levels of resistance to Cry toxins between 2012 and 2016, in a pattern consistent with polygenic selection. Surprisingly, none of the eleven previously described Cry resistance genes showed signatures of selection in wild *H. zea.* Furthermore, we observed evidence of a very strong selective sweep in one region of the *H. zea* genome, yet this strongest change was not additively associated with Cry resistance. This first, whole genome analysis of field-collected specimens to study evolution of Cry resistance demonstrates the potential and need for a more holistic approach to examining pest adaptation to changing agricultural practices.

54 **Introduction**

55 The study of rapid evolutionary responses to natural selection has gained momentum in the past
56 decade, and along with it recognition of the relevance of pest adaptation to control measures (Reznick
57 et al. 2019). Melander published the first observation of insecticide resistance evolution in 1914
58 (Melander 1914), and since then, pests and weeds have adapted to myriad chemical, biological, and
59 cultural control measures (Gould 1991, Palumbi 2001, Délye et al. 2013, Gould et al. 2018). While
60 there has been an emphasis on finding single genetic loci that confer resistance to pesticides,
61 recognition that surviving insecticide or herbicide applications could involve a suite of genomic
62 changes has increased (Baucom 2019). Yet a clear understanding of the timing and complexity of
63 changes associated with adaptation to agricultural ecosystems is lacking (Leon et al. 2020).

64 The bacterium, *Bacillus thuringiensis* (Bt), produces one or more crystalline (Cry) proteins that
65 are each toxic to a narrow range of insect pests when ingested. Genes encoding Cry toxins were
66 engineered into cultivated crops to reduce pest damage, producing so-called Bt crops, a major
67 innovation in agricultural pest management that has been adopted around the globe (NASEM 2016).
68 The economic and environmental benefits of this technology are significant: they can replace heavily
69 used synthetic insecticides, which have damaging off target effects on pollinators and natural enemies,
70 and have contributed to area wide pest suppression in non-Bt crops (Hutchison et al. 2010, NASEM
71 2016, Dively et al. 2018). A number of pest species have evolved resistance to one or more Cry toxins
72 and there is concern about the durability of these transgenic crops (Tabashnik and Carrière 2017).
73 Resistance management strategies have been implemented effectively for a few major pest species with
74 high susceptibility to the toxins (NASEM 2016). Unfortunately, these measures have been inadequate
75 for species where Cry toxins cause only moderate levels of mortality, providing opportunities for the
76 evolution of resistance (Tabashnik and Carrière 2017).

77 Discovery of resistance-conferring genes has been of long-standing interest, in part due to
78 expectations that this knowledge could lead to molecular diagnostic approaches that facilitate detection

79 of early resistance evolution in wild pest populations (US-EPA 1998, US-EPA 2018). Molecular
80 signals of resistance could then serve as triggers for regulatory actions that would minimize resistance
81 risk and preserve environmentally sustainable technologies, like Cry toxins (US-EPA 1998). Genes
82 discovered to have major impacts on Cry tolerance are being used to a limited extent for molecular
83 resistance monitoring (Morin et al. 2004, Gahan et al. 2007, Zhang et al. 2013, Jin et al. 2018). To
84 date, there are at least 11 such genes in the Lepidoptera (Wu 2014, Lin 2019), many of which were
85 discovered in laboratory-selected populations and from screening wild populations for single genes
86 with major effects on resistance (Morin et al. 2003, Gahan et al. 2001, Zhang et al. 2012, Jin et al.
87 2018). The question of whether molecular monitoring of these genes would effectively track resistance
88 evolution in wild populations remains unresolved.
89 *Helicoverpa zea* is a migratory pest species (Gould et al. 2002) which comprises a single
90 panmictic population in North America (Seymour et al. 2016). It has been controlled with Bt crops
91 since 1996, when corn and cotton expressing Cry1A became commercially available. In 2007, corn
92 and cotton cultivars producing both Cry1A and Cry2A were introduced. Since their
93 commercialization, North American adoption rates of Cry-expressing corn and cotton grew from 8 and
94 15% of corn and cotton acreage in 1997 to *ca.* 80% by 2012. This rapid expansion of Cry-expressing
95 crops placed significant selection pressure on their target pests, and widespread, damaging levels of
96 resistance to Cry-expressing crops by *H. zea* were documented by 2016 (Dively et al. 2016, Reisig et
97 al. 2018, Kaur et al. 2019, Yang et al. 2019). Existing Bt corn and cotton cultivars do not produce a
98 high dose of Cry toxin relative to what is required to kill heterozygote *H. zea* larvae with resistance
99 alleles (Luttrell and Jackson 2012). This likely contributed to their recent resistant status (Tabashnik
100 and Carrière 2017). Under these conditions, resistance to Cry toxins was predicted to be polygenic
101 (Gould 1998, Tabashnik et al. 2004) and to arise from standing genetic variation (Roush and McKenzie
102 1987).

103 Between 2002 and 2017, we saved *ca.* 1000 adult moths in ethanol each year, and we used
104 samples from these collections to track genomic changes in *H. zea* over time. Instead of simply
105 monitoring for changes in previously identified candidate genes, we took a gene agnostic approach and
106 scanned entire genomes for signatures of selection. Using two sequencing approaches, double-digest
107 Restriction-site Associated DNA sequencing (ddRAD-seq) and whole genome resequencing, we
108 identified multiple genomic regions with significant changes in allele frequency during a 15 year time
109 period. Shifts in allele frequency were used to calculate the strength of selection imposed on wild *H.*
110 *zea* in agricultural ecosystems. We also examined whether any of the 11 previously identified
111 candidate Cry resistance genes showed evidence of strong shifts in allele frequency over time.
112 Surprisingly, none of these genes implicated in Cry-toxin resistance underwent significant temporal
113 changes, nor did genes associated with detoxification of synthetic insecticides or plant defensive
114 compounds, as was observed in another species where Bt crops replaced insecticidal control (Fritz et al.
115 2018). Instead, we found multiple novel candidate genes within regions of greater than expected allele
116 frequency change, and quantified the effect size of the region showing the greatest temporal change on
117 Cry resistance.
118 This first whole genome analysis of field-collected specimens to study impacts of Bt crops
119 demonstrates the potential and need for a more holistic approach to examining pest adaptation to
120 changing agricultural practices. While monitoring previously identified candidate genes may prove
121 useful in some cases, our results indicate that such an approach is likely to miss critical evolutionary
122 events. Analysis of patterns of genomic changes in pest populations following the introduction of a
123 new control tactic or tool could prove useful, but requires follow-up experimental confirmation to
124 demonstrate clear, causal relationships among genes and adaptive phenotypes.
125
126

127 **Results**

128 *Strong temporal ddRAD-seq allele frequency shifts*

129 *H. zea* males were collected from five pheromone baited traps (Hartstack 1979) in Bossier
130 Parish, LA (SI Appendix, Table S1), and pooled by collection date in the months of May through
131 October, 2002 through 2017. Randomly sampled moths from a subset of years were selected for
132 genetic analysis; 2002, 2007, 2012, as well as in 2016, when strong phenotypic resistance to Cry1Ab
133 and increasing resistance to Cry2Ab was being documented (Dively et al. 2016). Two hundred and
134 sixty-five specimens were prepared into ddRAD-seq libraries and sequenced according to Fritz *et al.*
135 (2016, 2018). Following filtering and alignment to the 341 Mb *H. zea* genome, we identified 14,398
136 single nucleotide polymorphisms (SNPs) from 259 individuals with an average read count of 404,003
137 (SI Appendix, Figure S1). Nucleotide diversity (π), and heterozygosity (F) did not change between
138 years (SI Appendix, Table S2), and overall genomic divergence was low according to Weir and
139 Cockerham's F_{ST} ($F_{ST} < 0.004$; SI Appendix, Table S3). This low divergence was also supported by a
140 sequential k-means analysis, which showed that a single genetic cluster or population (k = 1) best
141 described our ddRAD-seq genotyped *H. zea* (SI Appendix, Figure S2). Interestingly, genetic
142 divergence that occurred between the 2012-2016 collection periods was at least 3 times higher (F_{ST} =
143 0.0025) than for any other sequentially sampled periods. Pairwise F_{ST} values were 0.0009 and 0.0002
144 for the 2002-2007 and 2007-2012 collection periods, respectively (SI Appendix,Table S3).

145 We used a discriminant analysis of principal components (DAPC) to further examine temporal
146 changes in the genomic structure of wild *H. zea*. Initially, we identified ddRAD SNP combinations
147 that clustered individuals into groups (k = 2-4) by maximizing between group variation and minimizing
148 within group variation. Using these SNP combinations, we estimated posterior membership
149 probabilities for each individual from our dataset from each collection year. When k was equal to 2,
150 samples within each of the collection years 2002, 2007, and 2012 were assigned to both genetic
151 clusters. In contrast, samples collected in 2016 were strongly biased toward a single genetic cluster

152 (Figure 1), and this trend persisted regardless of the k-value used (SI Appendix, Figure S3).).
153 Interestingly, the genotypic shift between 2012 and 2016 corresponded to significant increases in the
154 mean area consumed/ear of Cry1Ab + Cry2Ab expressing sweet corn (from near 0 to *ca.* 3cm^2 between
155 2010-2016) and the proportion of two-toxin expressing ears containing late instars (from near 0 to >
156 75%; Dively et al. 2016). We sequentially removed the top 1%, 2.5% and 5% of ddRAD SNPs that
157 had the greatest influence on genetic clustering, and re-evaluated population membership probability
158 (assuming k=2). From this we determined that up to 5% (n = 720) of ddRAD SNPs were responsible
159 for this observed genotypic shift (Figure S4).
160 To identify which of the 14,398 ddRAD SNPs experienced greater than expected shifts in allele
161 frequency over time, we conducted an F_{ST} outlier analysis (Whitlock and Lotterhos 2015). When we
162 calculated genetic divergence (F_{ST}) for each SNP across all 4 collection years, 53 had higher than
163 expected divergence (Figure 2, File S1). Twenty-two of those 53 were among the top variance
164 contributing SNPs that strongly influenced genetic clustering in our DAPC analysis. The 53 ddRAD
165 SNP outliers were spread across 47 (of 2,975) unique scaffolds, and their among-year F_{ST} values
166 ranged from 0.068 to 0.278.
167
168 *No significant allelic changes within candidate Bt resistance genes*
169 We found ddRAD-seq markers within or near (< 56kb) 10 of the 11 previously identified
170 candidate genes for Bt resistance, but none corresponded to outliers found over the time course of the
171 collections (Table 1). To verify this result we conducted Illumina whole genome sequencing (WGS) of
172 35 *H. zea* males collected from the same location in LA in 2002 (n = 13), 2012 (n = 11), and 2017 (n =
173 11) (SI Appendix, Table S4). After genome alignment and filtering, this approach yielded 4,999,128
174 SNPs, and 2,622 fell within the 11 candidate Cry resistance genes. We used a 10kb sliding window-
175 averaged Weir and Cockerham's F_{ST} analysis and observed that F_{ST} values calculated for each gene
176 ranged from -0.018 to 0.056 (Table 1; SI Appendix, Figure S5). The empirical threshold for

177 statistically significant genetic divergence, generated according to Rubin et al. (2010), was 6 standard
178 deviations ($ZF_{ST} > 6$) from the mean pairwise F_{ST} value in a window. None of the eleven candidate
179 genes contained windows that reached our threshold for statistical significance of $F_{ST} = 0.079$ ($ZF_{ST} >$
180 6) per 10kb window. Taken together, these results indicated that the Cry resistance in *H. zea* was not
181 caused by changes in the coding regions of any of these eleven candidate genes or their flanking cis-
182 regulatory sequences.
183
184 *Selection led to significant temporal divergence in novel genomic regions*
185 Using our full WGS SNP dataset, we conducted pairwise analyses of genomic divergence
186 between populations collected from 2002, 2012, and 2017 using 5, 10, 20 and 40 kb sliding window-
187 averaged Weir and Cockerham's F_{ST} along the length of the genome (SI Appendix, Figure S6). We
188 report on the 40 kb sliding window analysis because it captured the major genomic changes that
189 occurred for all sliding window sizes, but reduced the numbers of windows that appeared highly
190 divergent due to few SNPs in the window. Thirty-six genomic windows on 11 different scaffolds had
191 F_{ST} values that exceeded our empirical threshold for statistical significance of $F_{ST} = 0.047$ per 40 kb
192 when 2002 and 2017 populations were compared (Figure 3). For paired comparisons of populations
193 collected in 2002 and 2012, and 2012 and 2017 there were 55 and 30 genomic windows on 16 and 10
194 scaffolds with statistically significant genomic divergence, respectively (Figure 3; File S2).
195 Both genetic drift and selection influence the landscape of genomic divergence between
196 populations. Therefore, we quantified the frequency with which our empirical threshold of $F_{ST} =$
197 0.047, calculated from the pairwise comparison of samples from 2002 and 2017, could be reached in a
198 40kb genomic window due to drift alone (Hudson 2002). Genotypic simulations were conducted for a
199 single population sampled at two timepoints, but in the absence of selection. Allele frequency changes
200 within 40kb windows were examined for multiple population sizes (N = 21,000 - 140,000). The
201 proportion of genotypic simulations (of 20,000) that produced $F_{ST} > 0.047$ was the probability that our

202 empirical threshold of F_{ST} = 0.047 could be exceeded due to genetic drift. Results from our simulations
203 demonstrated this probability was very low (p = 0.0189 - 0) and negatively correlated with present day
204 population size (SI Appendix, Figure S7 & Table S5).

205 Because the population sizes used in the simulations are likely underestimates of the effective
206 population size of this migratory pest (Jones et al. 2019), it is highly improbable that our empirical F_{ST}
207 threshold could have been exceeded by the action of drift. As a demonstration of its large effective
208 population size, we collected fifth and sixth instar *H. zea* from ears of Obsession I (n = 8) and Cry-
209 expressing Obsession II (n = 8) sweet corn grown in Central Maryland Research and Education Center
210 (CMREC) demonstration plots. The genomes of these individuals, collected in September of 2017,
211 were sequenced with Illumina short reads, and their genotypes compared to samples collected in
212 Bossier Parish, LA, in that same year. After genome alignment and filtering for quality and linkage
213 disequilibrium, we examined 14,697 SNPs in the dataset, which yielded a genome-wide F_{ST} value of
214 0.0004. The bootstrapped 95% confidence intervals were (-0.0002, 0.0008), and their overlap with
215 zero indicated that allele frequencies in the LA and MD populations did not significantly differ from
216 one another.

217

218 *Identification of novel genes under selection*

219 We examined the genes near regions of greatest temporal genomic divergence to determine
220 whether any were associated with detoxification of plant-produced or human-applied toxicants. Our
221 list of candidates included those from families of genes associated with Cry resistance, but also
222 resistance to synthetic insecticides and xenobiotics. Eleven genes, including 3 peptidases, 4
223 choline/carboxylesterases, 2 cytochrome p450s, 1 ABC transporter, and 1 tetraspanin, were near or
224 within regions of significant temporal genetic divergence. No annotated proteases, proteinases,
225 cadherins, voltage-gated channels, alkaline phosphatases, or glutathione-S-transferases were found
226 within 50 kb of regions with elevated divergence. Evidence from a recent study suggests that

227 increasing adoption of Bt crops led to a reduction in the frequency of synthetic insecticide resistance-
228 conferring alleles, likely due to a reduction in synthetic insecticide applications (Fritz et al. 2018).
229 Therefore, we specifically examined two genes known to be involved in pyrethroid resistance. Neither
230 the voltage-gated sodium channel gene (*vgsc*), nor a cytochrome p450 (*cyp337b3*) which underlie
231 pyrethroid resistance in the close *H. zea* relatives, *Chloridea virescens* (previously *Heliothis virescens*;
232 Taylor et al. 1993) and *Helicoverpa armigera* (Joußen et al. 2012), respectively, were near regions of
233 elevated genomic divergence. All genes found within 50kb of genomic windows with higher than
234 expected divergence can be found in File S3.

235 We reasoned that increasing adoption of Bt crops over time would result in strong, sustained
236 selection, leading to significant allele frequency changes across multiple collection years. Therefore,
237 we identified scaffolds with elevated temporal genomic divergence in more than one pairwise
238 comparison. Five scaffolds showed signs of significant allele frequency change in at least two pairwise
239 comparisons. These included scaffold 173, which contained a *venom dipeptidyl peptidase 4* - like gene
240 (HzOG204148), and scaffold 569, which contained two *carboxypeptidase Q (cpq)* -like genes
241 (HzOG204151 and HzOG204153). Scaffold 1964 contained an RNA-directed DNA polymerase
242 (HzOG206500), and scaffold 65 had one genomic window of elevated divergence in multiple pairwise
243 comparisons which was near 7 genes, including *tetraspanin* (*tspan*) *68C* (HzOG211510) and a
244 *krueppel*-like gene (HzOG211515). Finally, scaffold 1612 contained 2 genomic windows with
245 elevated divergence across all three by-year comparisons, which overlapped with *cyp333b3*
246 (HzOG200024). Of these five scaffolds, 569 and 1612, had levels of genetic divergence that greatly
247 exceeded all others between 2002 and 2017 ($F_{ST} > 0.089$), and such divergence was never observed for
248 genetic simulations in the absence of selection (SI Appendix, Table S5).

249 The genome assembly of *H. zea* is fragmented into 2,975 genomic scaffolds, which makes
250 detecting the extent of a selective sweep challenging. Under conditions of strong selection, the
251 footprint in the genome should be broad, and we reasoned there was potential for some of these

252 temporally divergent scaffolds to be physically linked. We used tblastn to align the genes from the 5
253 scaffolds showing strong temporal divergence in multiple by-year comparisons to the *Bombyx mori*
254 genome (ASM15162v1), and identified their relative positions assuming conserved macrosynteny
255 among Lepidoptera (d'Alençon et al. 2010). Four of these five scaffolds were adjacent to one another
256 and syntenic to *B. mori* chromosome 13, indicating that the breadth of the selective sweep extended for
257 *ca.* 3 Mb (SI Appendix, Figure S8). Linkage disequilibrium within the putative sweep region decayed
258 more slowly than both genome-wide average rates of decay, as well as decay along single, similarly-
259 sized scaffolds (> 1Mb) without evidence of genomic divergence (SI Appendix, Figure S9). This
260 region of the *B. mori* genome contained 134 unique genes, none of which were previously known to be
261 involved in Cry resistance (File S4). Interestingly, we re-examined our ddRAD-seq dataset and
262 detected two ddRAD-seq markers found within this 3 Mb genomic region (on scaffold 569), which
263 were outliers according to OutFLANK. This further confirmed the significance of changes occurring
264 in this genomic region, but with a much larger sample size (n > 45 per year).
265 The 3 Mb putative sweep region experienced the greatest temporal change in frequency
266 according to our whole genome scan. Yet our DAPC analysis identified 720 ddRAD SNPs on 505
267 unique scaffolds which caused a major architectural shift in the genomes of *H. zea* collected between
268 2012 and 2016 (Figure 1; SI Appendix Figures S3 & S4). Of those 720 ddRAD SNPs, only 5 aligned
269 to this 3 Mb region. This pattern, consistent with polygenic selection on standing variation, indicated
270 that allele frequency changes at many loci are involved in *H. zea* adaptation over the study period. To
271 test how and whether strong temporal genomic changes should be used for genomic monitoring, our
272 further analyses focused on the 3 Mb putative sweep region due to the breadth and magnitude of the
273 observed allele frequency changes.
274
275
276

Temporal shifts in the frequency of non-synonymous mutations

Given the significant levels of genomic divergence near *cyp333b3* and the two *carboxypeptidase Q-like (*hereafter *cpq*) genes detected by ddRAD-seq and WGS datasets, as well as the putative functional roles of these genes in insect metabolism and detoxification, we further examined protein-coding changes at these genes over time. Based upon our temporal analysis, genomic divergence was strongest at the 3' end of all three genes (SI Appendix, Figure S10). While we detected 50 and 40 SNPs within *cpq1* and *cpq2*, respectively, only 3 were associated with putative changes in amino acid sequence (Table 3). One transition from cytosine to thymine caused a putative alanine to valine substitution in *cpq1*, and over time, the cytosine declined significantly from 0.73 in 2002, to 0.32 and 0.14 in 2012 and 2017, respectively (Freeman-Halton Fisher's exact $p = 0.0001$; Table 2). Two SNPs in *cpq2* showed modest changes in allele frequency over time, although not statistically significant when a Bonferroni-corrected α value was used to account for multiple comparisons (Table 2). One cytosine to thymine transition encoded an alanine to valine amino acid substitution at 152,595 bp, and a second occurred at 152,183 bp, encoding a threonine to isoleucine substitution. Finally, there were 28 SNPs present in the protein-coding sequence of *cyp333b3*, 10 of which produced putative amino acid changes. Five of these non-synonymous mutations underwent statistically significant changes over time ($p < 0.003$; Table 2).

We also compared the frequencies of these non-synonymous mutations in the LA and MD populations collected in 2017, and for all 8 SNPs, there was no statistically significant difference in allele frequency based on collection location (SI Appendix, Table S7). Therefore, if allele frequency changes in these genomic regions were adaptive, they are not locally isolated to LA, but instead, present throughout the Eastern United States. These results and the low overall F_{ST} value for the two-population comparison (reported above) support previous findings that *H. zea* is highly migratory with little population structure.

302 *Strength of selection experienced by wild* H. zea

303 Using the temporal changes in allele frequency, we calculated the strength of selection required
304 to produce these statistically significant protein-coding changes over time. The selection coefficient(*s*)
305 gave the strength of selection against *q*, the declining allele, examined under 3 different dominance
306 scenarios of *p:* complete dominance, no dominance, and recessiveness. For 5 of the 8 non-synonymous
307 mutations (Table 2), *q* was the allele present in the reference genome, and in the remaining cases, *q* was
308 the alternate allele. We calculated mean values of *s* from allele frequency changes which occurred
309 during the 2002-2012 collection period, as well as the 2012-2017 collection period. Values of s varied
310 according to selection period and dominance scenario (Figure 4), but most fell within the range of 0.03-
311 0.17. When *p* was assumed to be recessive, stronger selection was required to produce the observed
312 changes in allele frequency in the 2002-2012 collection period due to its low frequency (*ca.* 0.25 - 0.3).
313 Yet when *p* was dominant, stronger selection was required to produce the observed changes in allele
314 frequency between 2012-2017, due to its relatively high starting frequency (*ca.* 0.65-0.75). In the case
315 of the SNP at 36,959 bp on scaffold 1612, it's frequency (*p*) was already 0.95 in 2012. Assuming
316 dominance of *p*, the strength of selection that would have been required to increase its frequency to 1
317 by 2017 was very high ($s = 0.70$), which increased the variance around the mean value of *s* (Figure 4).
318

319 *Frequency of non-synonymous substitutions prior to 2002*

320 The intermediate frequency of the non-synonymous polymorphisms in 2002 compelled us to
321 examine the frequencies of those polymorphisms in *H. zea* collected from our LA trapping sites in
322 1998, shortly after the release of Cry-expressing crops in 1996. Moths from 1998 had DNA quality
323 that was too low for use in ddRAD-seq and WGS analyses, but short fragments could be PCR
324 amplified. We therefore amplified and Sanger sequenced *ca.* 200bp DNA fragments spanning one
325 non-synonymous polymorphism in each of *cpq1* and *cyp333b3* from 20-26 *H. zea* collected in 1998.
326 The respective frequencies of the reference alleles for *cpq1* and *cyp333b3* were 0.93 and 0.90 in 1998

327 compared with 0.73 and 0.65 in 2002, and 0.14 and 0.18 in 2017 (Table 2) showing further evidence of
328 genomic divergence at these sites. The change in allele frequency between 1998 and 2002 occurred
329 prior to introduction of varieties with Cry2 genes.
330
331 *Linkage of Cry resistance to* H. zea *Chr13*

332 To test whether this 3 Mb region with significant temporal genetic divergence was linked to Cry
333 resistance, we bioassayed the progeny of two F_2 genetic crosses between recently field-collected Cry
334 resistant grandparents and Cry susceptible grandparents (Figure 5A & B). Cry resistant larval growth
335 was 6 and 14 times greater than that of susceptibles when fed a diagnostic dose of diet containing
336 Cry1Ab and Cry1A.105 + Cry2Ab2 sweetcorn leaf tissue, respectively, and these differences were
337 statistically significant (Cry1Ab: df = 1, $X^2 = 314.57$, $p < 0.0001$; Cry1A.105 + Cry2Ab2: df = 1, $X^2 =$
338 409.97, $p < 0.0001$).

339 Following 7 days of exposure to untreated, Cry1Ab, or Cry1A.105 + Cry2Ab2 treated diet, F_2
340 larval growth phenotypes from each family were measured. Bioassayed individuals were genotyped by
341 PCR and Sanger sequencing of fragments spanning two non-synonymous mutations in the *cyp333b3*
342 and *cpq1* genes. Genotypes at these two loci were highly correlated within individuals due to physical
343 linkage (Kendall's tau = 0.989, z = 12.1, $p < 0.0001$), so further analysis focused only on *cpq1*. We
344 postulated that individuals with the derived *cpq1* allele, which caused a Valine (V) substitution, should
345 grow larger when placed on diet containing Cry-treated leaf tissue. In general, heterozygous F2s
346 tended to grow larger on Cry1Ab and Cry1A.105 + Cry2Ab2 treated diet than did homozygotes (Figure
347 5D & E), and this trend was marginally significant for the F2s fed on two toxin diet (df = 2, $\chi^2 = 5.03$, p
348 = 0.081). Surprisingly, F2 larvae fed upon a diet containing untreated leaf tissue grew significantly
349 larger when they were homozygous for the derived allele (df = 2, $\chi^2 = 23.64$, $p < 0.0001$; Figure 5C).
350 While the rapid allele frequency changes on chromosome 13 are fascinating, the environmental

351 conditions that could have selected for both greater larval growth of homozygotes on untreated leaf
352 tissue and marginally significant increases in growth of heterozygotes on treated diet, remain
353 unresolved.

354

355 **Discussion**

356 In this study, we compared the genomes of *H. zea* collected between 2002 and 2017 to quantify
357 changes that occurred during a period of rapid adoption of a major agricultural innovation, Cry toxin-
358 expressing crops, in the United States. We found substantial changes in the frequencies of SNPs
359 throughout the *H. zea* genome that occurred in concert with the evolution of resistance to Cry toxins in
360 field populations of *H. zea*. A major finding of our work is that none of the previously identified
361 candidate Cry resistance genes in Lepidoptera showed signatures of selection in the field-collected *H.*
362 *zea*, nor were genes in the strongest selective sweep region associated with resistance in an additive
363 way. Instead, the timing and complexity of the genomic changes that occurred in *H. zea* were
364 consistent with the predicted polygenic nature of Cry resistance in this species, and provide insight into
365 how, when and whether genomic approaches could be used for resistance risk monitoring.

366 Over the past four decades there has been an emphasis on the search for single genes that confer
367 resistance to control measures in insect pests and weeds, and we now have a catalogue of such genes
368 (Ffrench-Constant 2013, Feyereisen et al. 2015, Délye et al. 2013, Heckel 2020). There are cases
369 where a single gene diagnostic (Donnelly et al. 2016) or a genomic diagnostic targeted at multiple
370 previously established candidate genes (Weetman et al. 2018) has been useful in monitoring for
371 resistance, but recently there has been concern that these focused approaches could lead to
372 complacency, as resistance evolves based on unexpected genes (Heckel 2020) or based on multiple
373 genes (Délye et al. 2013, Van Leeuwen et al. 2020, Leon et al. 2020) that are already at low
374 frequencies in pest or weed populations. Our work demonstrates that these concerns are appropriate. If

375 any or all of the previously identified candidate genes had been used in a diagnostic for Cry resistance
376 in *H. zea*, the diagnostic would have missed the coming problem.

377 While functional roles of specific genes that we identified have not yet been demonstrated,
378 multiple *H. zea* genomic regions have changed dramatically since the introduction of Cry toxin-
379 producing cotton and corn. The most compelling changes were found within a broad selective sweep
380 on the *H. zea* homolog of *B. mori* chromosome 13. WGS and ddRAD-seq datasets both showed
381 stronger than expected temporal genomic changes in a roughly 3 Mb region over the 15 year sampling
382 period. When the results of the genome analyses were combined with PCR analysis of SNPs within
383 candidate genes from 1998 samples from the same location, allele frequency changes of up to 0.79
384 occurred in these genes with metabolic functions or that are associated with xenobiotic resistance
385 (Ward 1976, Guzov et al. 1998, Bown and Gatehouse 2004). Interestingly, two of these genes (*cpq1*
386 and *cpq2*) were from families capable of binding Cry toxins in *H. armigera* (Da Silva et al. 2018).

387 Analysis of genetic crosses between resistant and susceptible strains found that this genomic
388 region was not linked to Cry resistance in mendelian, or even additive way (Figure 5). Progeny on the
389 diagnostic dose rarely reached the weights of their resistant parents (145-150mg), indicating that *H. zea*
390 resistance is quantitative in nature. Yet those that did had at least one copy of the derived allele at
391 *cpq1*. Furthermore, heterozygotes with one copy of the derived *cpq1* allele had marginally significant
392 increases in larval growth on diet containing Cry1A.105 + Cry2Ab2 expressing leaf tissue, indicating a
393 small, non-additive effect on resistance. Interestingly, individuals that were homozygous for the
394 derived allele also had significantly greater larval growth on diet containing untreated leaf tissue,
395 suggesting the benefits of genetic changes at this locus are not restricted to Cry resistance. This likely
396 genotype by environment interaction may explain how the derived allele could reach high frequency in
397 a way that selection for overdominance by Cry toxins alone could not. If the benefits of this allele
398 were realized only in heterozygotes exposed to Cry toxins, selection should have maintained this allele

399 at intermediate frequency. The functions of genes in the 3 Mb sweep region are not clear, particularly
400 how or why they promote increased larval growth on non-expressing plant tissue. Yet the
401 heterogeneous North American environment, with selective pressures from both Cry-expressing and
402 non-expressing host plants of *H. zea,* provides a landscape with capacity to drive the derived allele to a
403 frequency greater than 0.5. This finding highlights both the complex nature of Cry resistance in *H. zea*,
404 as well as risks associated with genomic monitoring. Strong selective sweeps do not necessarily point
405 to resistance genes with large effect sizes, and future studies should be directed at understanding what
406 sweeps in regions with complex genotype by environment interactions can tell us about resistance risk.

407 We emphasize that we detected multiple regions of the genome that changed during the course
408 of our sampling, a pattern consistent with polygenic adaptation from standing genetic variation. Our
409 findings are consistent with a recent study of crosses of Cry2 resistant *H. zea* to a susceptible strain that
410 found phenotypic variance fitting a polygenic model (Yang et al. 2020). In our study, strong polygenic
411 change was most apparent between the years 2012 and 2016 (Figure 1) when widespread and damaging
412 levels of resistance to Cry toxins were documented (Dively et al. 2016). Ongoing, experimental,
413 quantitative genomic studies will shed light on which of these genomic regions contribute most
414 substantially to Cry tolerance in field-collected larvae.

415 The use of genomic approaches to study rapid evolution in natural populations has gained
416 momentum (*e.g.* Crossley et al. 2017, Fritz et al. 2018, Nosil et al. 2018, Weetman et al. 2018, Bi et al.
417 2019, Dayan et al. 2019, Laurentino et al. 2020). Yet their use for studying pest adaptation to
418 agricultural systems has been limited to a few cases (reviewed in Pélissié et al. 2018). As we attempt to
419 apply these approaches to a variety of agricultural systems, system- and method-specific issues will
420 need to be addressed to ensure accuracy. Results from our experiments indicate that genomic
421 approaches may hold promise for monitoring resistance risk in pest populations exposed to novel
422 management practices, with several caveats for how we interpret our results.

423 One concern is that major genomic changes identified through genomic monitoring might not
424 be due to the control tool being studied. Deployment of pest management innovations do not take
425 place in the absence of other existing selection pressures in the environment. When transgenic cotton
426 and corn were introduced into North American agricultural systems, the use of synthetic insecticides
427 were reduced, though certainly not eliminated. In a previous study using ddRAD-seq, we found that
428 another pest of cotton in Louisiana, *Chloridea virescens*, did not experience changes in the frequency
429 of Bt resistance candidate genes. Rather, changes to the frequency of an allele that conferred resistance
430 to pyrethroid insecticides were observed over a 15 year period (Fritz et al. 2018). Indeed, our data
431 suggest that the greatest allele frequency changes in the *H. zea* genome were not solely associated with
432 Cry resistance. Therefore, confirming the strength of association between any major genomic change
433 with a resistance phenotype is critical.

434 This confirmation testing can be conducted using the types of simple crosses and PCR-based
435 assays that we carried out in our study. Such assays can determine the importance of rapidly changing
436 loci to resistance phenotypes and alleviate concerns regarding resistance risk. Conversely, knowledge
437 of allele frequency changes and confirmation of their impact on field resistance could then be used to
438 make predictions for future changes in resistance in field populations of pests. In cases similar to *H.*
439 *zea* on Cry-expressing crops where the control tactic does not kill all pest individuals or has sublethal
440 effects, collections from paired sentinel plots could also point to genomic regions associated with
441 resistance. Such paired sentinel plots are already being used for determining the current extent of
442 resistance, but typically monitor damage to the crop rather than the genetics of the pest (Dively et al.
443 2016, 2020). Focusing on damage and the economic impact of resistance does little to predict risk of
444 resistance and then mitigate it. Once widespread damage has occurred, resistance alleles have likely
445 already spread widely in the landscape. Instead, sampling and sequencing early instar larvae found in

446 treated and untreated sentinel plots would allow researchers to quantify genomic change associated
447 with the pest management tool compared to the population at large.

448 A second concern is that population demographic processes, including migration patterns and
449 population expansion can result in the appearance of genome-wide shifts in allele frequency, which are
450 not due to selection. When this occurs, the risk for genomic monitoring would be to falsely implicate a
451 region of the genome in evolving resistance. We were fortunate to be studying a highly migratory
452 species (Hartstack et al. 1982, Gould et al. 2002, Seymour et al. 2016) with little population structure
453 over large geographic areas (Perera and Blanco 2011, Seymour et al. 2016). Our finding of negligible
454 genomic differences between samples collected in 2017 from Louisiana and Maryland are consistent
455 with results from these previous studies. Additionally, *H. zea* feeds on a number of host plants, and this
456 seems to have resulted in the population densities remaining similar to those prior to the use of
457 transgenic cotton and corn (Micinski et al. 2008). It is not surprising that with this species, measured
458 overall genomic diversity remained constant. For a species that interbreeds over such a large area,
459 population demographic processes are to result in detection of false positives. Had we been studying
460 an insect or weed with strong local population structure, or one in which the control measure caused
461 severe reduction and resurgence of the pest, our experimental approaches would have required
462 additional careful consideration.

463 Finally, many so-called "mega-pest" lineages are thought to have genomes that already contain
464 variants, which prime them for the evolution of resistance to novel pest control measures (Crossley et
465 al. 2017, Pélissié et al. 2018). When selection is applied to these populations, they experience soft
466 selective sweeps at genomic regions that are typically spread throughout the genome. Such signatures
467 of polygenic selection in field-collected samples can be difficult to bioinformatically tease apart from
468 the impacts of drift. Fortunately, new computational approaches which can better differentiate soft
469 selective sweeps from background selection or other population demographic processes are becoming

470 available (Schrider and Kern 2016), and some even detect the proportion of genome-wide changes in
471 populations due to selection using temporal datasets (Buffalo and Coop 2019). Further development of
472 bioinformatic approaches specifically aimed at detecting temporal genomic changes at both single loci
473 and those spread throughout the genome will provide critical tools for resistance risk monitoring in
474 agricultural systems.

475 The benefits of genomic monitoring of pest species go beyond identifying the mechanisms of
476 resistance and applications for resistance risk mitigation. Adoption of genomic approaches for
477 resistance monitoring will be crucial for addressing the decades-long debate on exactly how pest
478 species rapidly adapt to management practices (Crow 1957). Even today, the extent to which a species
479 response to selection is mono- or polygenic, from standing genetic variation or novel mutation remains
480 unclear. Here we present evidence on the complex nature of genomic change during expansion of a
481 major agricultural innovation, Cry-expressing crops, in one important pest species. Extension of
482 genomic monitoring programs, accompanied by targeted experimental confirmation, to additional pest
483 species on broad spatial and temporal scales could provide critical insight into the nature of adaptation
484 by these species to human-imposed selection.

485

486 **Data Availability**

487 Data for this study are available at: to be completed after manuscript is accepted for publication.
488 All scripts and barcodes files used to perform these analyses can be found at
489 https://github.com/mcadamme/FieldHz_Pop_Genomics.

490

491 **Competing Interests**

492 The authors declare they have no competing interests.

493

494

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499

500 **Methods**

501 *Insect collections for ddRAD-seq and WGS analyses*

502 *H. zea* adults were collected by pheromone-baited trap bi-weekly from May through September
503 in Bossier Parish, Louisiana (SI Appendix, Table S1) in 2002, 2007, 2012, 2016, and 2017. Late instar
504 (5-6th) *H. zea* were also collected in 2017 at the University of Maryland CMREC farms in Prince
505 George's county, MD, and reared to adulthood prior to genomic analysis.

506

507 *DdRAD-seq library preparation*

508 Specimens from the years 2002, 2007, 2012, and 2016 were prepared into double-digest RAD-
509 seq (ddRAD-seq) libraries for population genomic analysis according to Fritz *et al*. (2016, 2018), and
510 sequenced on 8 runs of an Illumina MiSeq.

511

512 *DdRAD-seq Bioinformatic Analysis*

513 Merged, filter-trimmed reads were aligned to the *H. zea* genome (v. 1.0, NCBI Bioproject
514 PRJNA378438; Pearce et al., 2017) with Bowtie2 (Langmead and Salzberg 2012). Genotypes were
515 called with BCFtools (Danecek et al. 2016) and filtered to include SNPs that i) had a depth of coverage
516 > 3, ii) were present in at least 75% of the 259 individuals in our filtered dataset, iii) had a minor allele
517 frequency of 0.05, iv) had a maximum of 2 alleles, and v) were thinned to 1 per 200 bp to reduce the
518 degree of linkage disequilibrium between SNPs on the same ddRAD-seq marker.

519 We calculated average nucleotide diversity (π) and heterozygosity (the inbreeding coefficient
520 F) values for populations collected in each year, and examined genome-wide divergence for each pair
521 of years using pairwise F_{ST} (Weir and Cockerham 1984). A k-means analysis in R (R core team 2008)
522 modeled the number of putative populations or clusters (k = 1:10) into which the LA *H. zea* fit
523 according to genotype (adegenet v. 2.1.1, Jombart et al. 2010, Jombart and Ahmed 2011). Using the
524 three best-fitting numbers of clusters (k = 2:4) as priors, we applied a Discriminant Analysis of
525 Principal Components (DAPC) in adegenet to identify groups of genetically similar *H. zea* individuals.
526 Weir and Cockerham's F_{ST} was calculated for each SNP to quantify genetic divergence across years
527 and identify those with higher than expected divergence over time (OutFLANK v. 0.2, Whitlock and
528 Lotterhos 2015). Locations of genes already implicated in Bt resistance in the *H. zea* genome were
529 identified by blast, and we used custom scripts to identify the nearest ddRAD-seq marker to each
530 (Table 1).
531
532 *WGS library preparation*
533 Genomic DNAs from additional adults collected in 2002 (n= 13), 2012 (n = 11), and 2017 (n =
534 11) were prepared into two Illumina TruSeq LT libraries (Illumina, Inc. San Diego, CA), and
535 sequenced on two Illumina NextSeq500 runs.
536
537 *WGS bioinformatic analysis*
538 Following filter-trimming and reference genome alignment, called SNP genotypes were filtered
539 to removed SNPs i) with a coverage depth < 3, ii) absent from 8 or more individuals sequenced, iii)
540 with minor allele frequency of < 0.05, and iv) with > 2 alleles per locus. Temporal change at Bt
541 resistance candidate genes was measured by pairwise F_{ST} averaged across SNPs found within each
542 gene. We applied a sliding window analysis, where windows with an average F_{ST} value greater than 6

543 standard deviations from the mean ($ZF_{ST} > 6$) were considered to have undergone statistically
544 significant divergence (Rubin et al. 2010).
545 Sliding window averaged F_{ST} values were also used to identify novel signatures of strong
546 selection across the genome using 40kb windows with a 10kb step. Additional sliding window and
547 step sizes were also examined (SI Appendix, Figure S2). Genotype simulations under neutral
548 expectations using realistic population demographic conditions generated a distribution of F_{ST} values,
549 which were compared against empirical F_{ST} values from our genome scan as a secondary approach to
550 examining statistical significance (Hudson 2002; SI Appendix, Supplemental Methods). Results of our
551 whole genome scan were compared to results from our ddRAD-enabled outlier analysis to determine
552 which regions were detected in both datasets. We queried lists of genes within 50kb of each novel
553 genomic window that underwent statistically significant temporal divergence to identify those in
554 families associated with metabolism and detoxification (SI Appendix, Supplemental Methods). For the
555 5 most divergent scaffolds, we determined scaffold order and proximity by tblastn alignment of
556 protein-coding regions to the *B. mori* genome, assuming conservation of macrosynteny among
557 Lepidopteran genomes (d'Alençon et al. 2010).
558
559 *Genome similarity between* H. zea *collected from MD and LA in 2017*
560 A global F_{ST} analysis compared *H. zea* collected at CMREC in Prince George's county, MD, in
561 2017 to those collected in Bossier Parish, LA, in 2017, and overlap of the bootstrapped 95% confidence
562 intervals with zero determined statistical significance.
563
564 *Gene-specific analyses*
565 Temporal and spatial differences in the frequencies of non-synonymous substitutions within
566 *cpq1*, *cpq2* and *cyp333b3* were analyzed with a Bonferroni-corrected two-sided Fisher's exact test.
567

568 *Strength of selection*

569 We calculated strength of selection (*s*) imposed upon *cpq1*, *cpq2*, and *cyp333b3* non-
570 synonymous substitutions under 3 different dominance scenarios: dominance of the allele that was
571 increasing in frequency, *p*, no dominance of *p*, and recessiveness of *p* (Falconer and Mackay 1996).
572 Our model assumed 5 generations per year (Reay-Jones 2019) for each selection period: 2002-2012
573 and 2012-2017.

574

575 *Frequency of non-synonymous substitutions prior to 2002*

576 Genomic DNA was isolated from *H. zea* collected in 1998 from Hartstack traps in Bossier
577 Parish, LA, PCR-amplified using primers flanking non-synonymous mutations in *cpq1* and *cyp333b3*
578 (SI Appendix, Table S8), and Sanger sequenced, allowing us to determine their frequency prior to
579 2002.

580

581 *Test for linkage*

582 Using F2 progeny from crosses between Cry-tolerant field-collected and Cry susceptible
583 laboratory-reared *H. zea* (Benzon Research Inc., Carlisle, PA), we measured 7-day larval growth
584 phenotypes following exposure to Cry-treated and untreated diet (Dively et al. 2016; SI Appendix,
585 Supplemental Methods). All F2s were genotyped at non-synonymous mutations in the *cyp333b3* and
586 *cpq1* genes by PCR and Sanger sequencing, but due to linkage disequilibrium between markers, only
587 *cpq1* was further analyzed. We used linear models to quantify the effect of *cpq1* genotype on 7 day
588 larval weight following exposure to treated or untreated diets. Statistical significance was determined
589 using model comparison by likelihood ratio test (SI Appendix, Supplemental methods).

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806 **Tables**

807

808 **Table 1** - Candidate genes previously documented as being involved in Bt resistance in lab or field-
809 collected Lepidopteran species, their best alignments to the *H. zea* genome. We calculated the average
810 Weir and Cockerham's F_{ST} value for SNPs within the gene-containing region of each scaffold. The
811 number of SNPs used to calculate the average F_{ST} value can be found in parentheses. Whole genome
812 sequencing samples from the years 2002 and 2017 were used for the comparison. We also report
813 where the nearest SNP marker produced by ddRAD sequencing was found relative to each candidate
814 gene and its F_{ST} value. When ddRAD SNPs were found within the gene, the average F_{ST} value across
815 SNPs within the gene are reported. When the ddRAD SNP was found outside of the gene, the F_{ST}
816 value for the nearest SNP is reported.

817

Candidate Gene	**Uniprot/ Genbank ID**	**Citation**	***H. zea* scaffold**	**Gene Position (bp)**	**Avg F_{ST} (Num SNPs)**	**Nearest ddRAD SNP (bp)**	**ddRAD Avg F_{ST} (Num SNPs)**
alp	ACP39715.1	Jurat-Fuentes and Adang 2004	KZ117832.1	1,946 - 16,347	-0.011 (171)	NA	NA
apn1	Q11000.1	Gill *et al.* 1995	KZ118301.1	140,429 - 161,526	-0.005 (353)	in gene	-0.003 (3)
apn4	AF378666.1	Banks *et al.* 2001	KZ118301.1	168,017 - 174,495	0.056 (170)	22,238	-0.005 (1)
abcA2	KP259911.1	Tay *et al.* 2015	KZ118207.1	171,103 - 192,554	0.002 (217)	in gene	0.006 (2)

abcC2	GQ332571.1	Gahan *et al.* 2010	KZ118297.1	98,882 - 114,139	-0.004 (100)	3,178	-0.004 (1)
abcG1 (white)	KM667971.1	Guo *et al.* 2015a	KZ118424.1	2,680 - 24,508	0.015 (58)	12,964	0.001 (1)
calp	AAK85198.1	Gahan *et al.* 2001	KZ117563.1	271,234 - 288,320	0.015 (201)	56,926	-0.007 (1)
cad2	AGG36450.2	Zhang *et al.* 2017	KZ118195.1	87,164 - 95,177	-0.003 (44)	20,535	-0.003 (1)
cad-86C	*H. zea* assembly v. 1.0 ID 537580	Fritz *et al*. 2020	KZ117463.1	550,935-603,715	-0.008 (1079)	in gene	-0.004 (2)
map4K4	AKC96184.1	Guo *et al.* 2015b	KZ118817.1	92,551 - 104,845	-0.018 (65)	35,125	0.005 (1)
tspan1	AYM54687.1	Jin *et al*. 2018	KZ118424.1	106,132 - 117,025	-0.008 (164)	27,211	0.003 (1)

818

Table 2 - Putative amino acid substitutions (AA Sub) for two *carboxypeptidase Q* genes (*cpq1* and *cpq2*) and *cyp333b3*. We quantified frequency changes in the codon present in the reference genome (Ref Codon) for *H. zea* populations collected in 1998 (n chr = 40-52), 2002 (n chr = 26), 2012 (n chr = 22), and 2017 (n chr = 22). A Freeman-Halton extension of a Fisher's exact test with a bonferroni correction (α = 0.006) determined statistical significance (*). *Cpq1* extended from 8583 to 10,070 bp on scaffold 569, and *cpq2* extended from 151,736 to 160,706 bp, while cyp333b3 extended from 32,819 to 39,355 bp on scaffold 1612. All three genes are transcribed from the reverse strand.

Scaffold	**Gene**	**SNP Pos (bp)**	**AA Sub**	**Ref Codon**	**2002 freq**	**2012 freq**	**2017 freq**	**p-val**
KZ118395.1	*cpq1*	9,409	A → V	GCT	0.73	0.32	0.14	0.0001*
KZ118395.1	*cpq2*	152,183	T → I	ATA	0.83	0.73	1	0.022
KZ118395.1	*cpq2*	152,595	A → V	GTA	0.84	0.77	1	0.049
KZ117131.1	*cyp333b3*	35,685	S → T	AGC	0.65	0.32	0.18	0.0001*
KZ117131.1	*cyp333b3*	36,339	K → N	TTT	0.69	0.27	0.09	0.0003*
KZ117131.1	*cyp333b3*	36,579	G → V	GGA	0.69	0.27	0.09	< 0.0001*
KZ117131.1	*cyp333b3*	36,959	T → A	GCC	0.62	0.95	1	< 0.0001*
KZ117131.1	*cyp333b3*	37,490	S → N	AGC	0.73	0.32	0.09	0.003*

Figures

Figure 1 - Posterior membership probabilities for *H. zea* individuals collected in Bossier Parish, LA, in 2002, 2007, 2012, and 2016. Genotypic clustering used ddRAD-seq generated SNP data and assumed a prior number of clusters (k) equal to two.

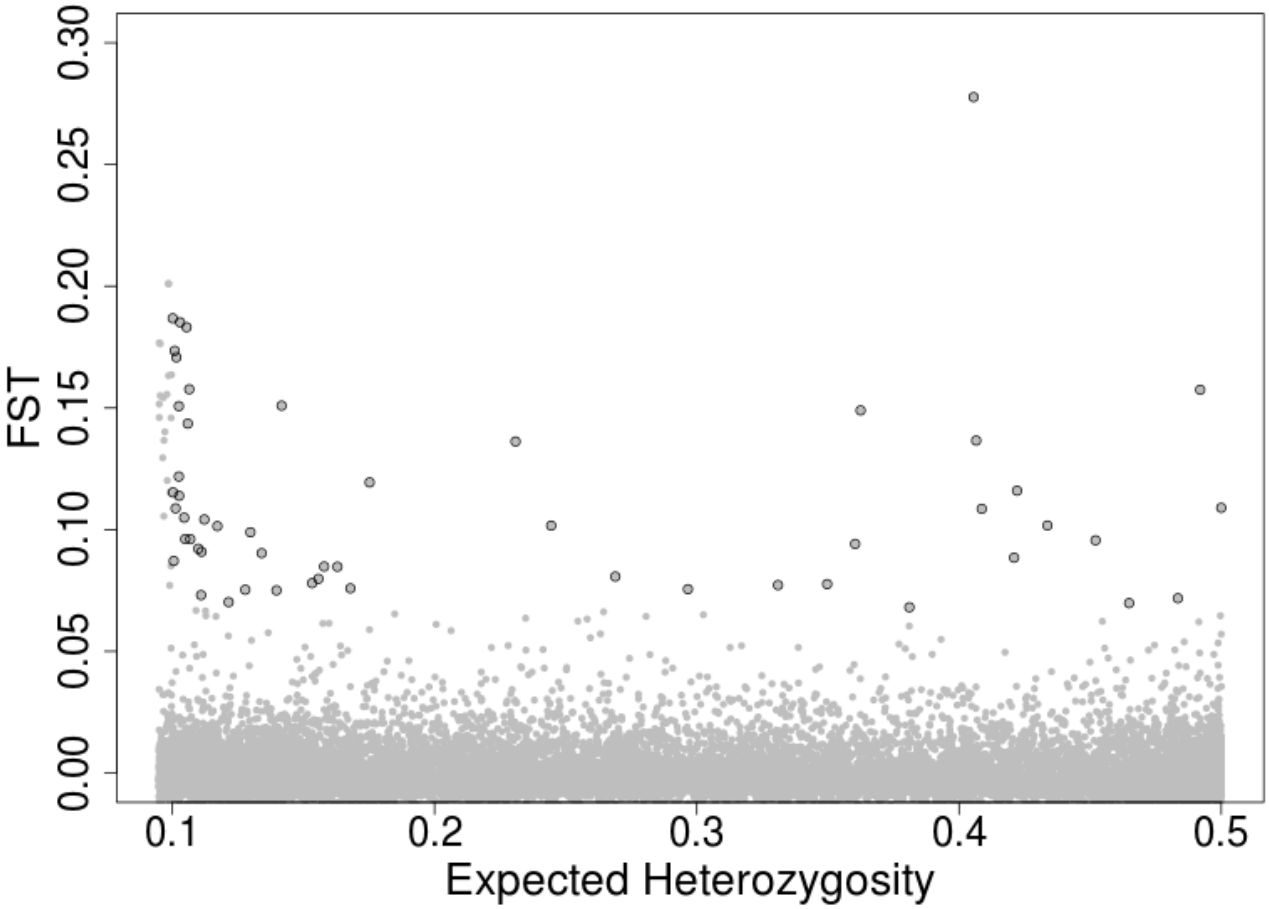

Figure 2 - F_{ST} outlier analysis of *H. zea* collected from Bossier Parish, LA in the years 2002, 2007, 2012, and 2016. Each grey point represents one of the 14,398 SNPs examined in our total dataset. Those circled in black represent SNPs that were identified as outliers and indicate they were under directional selection.

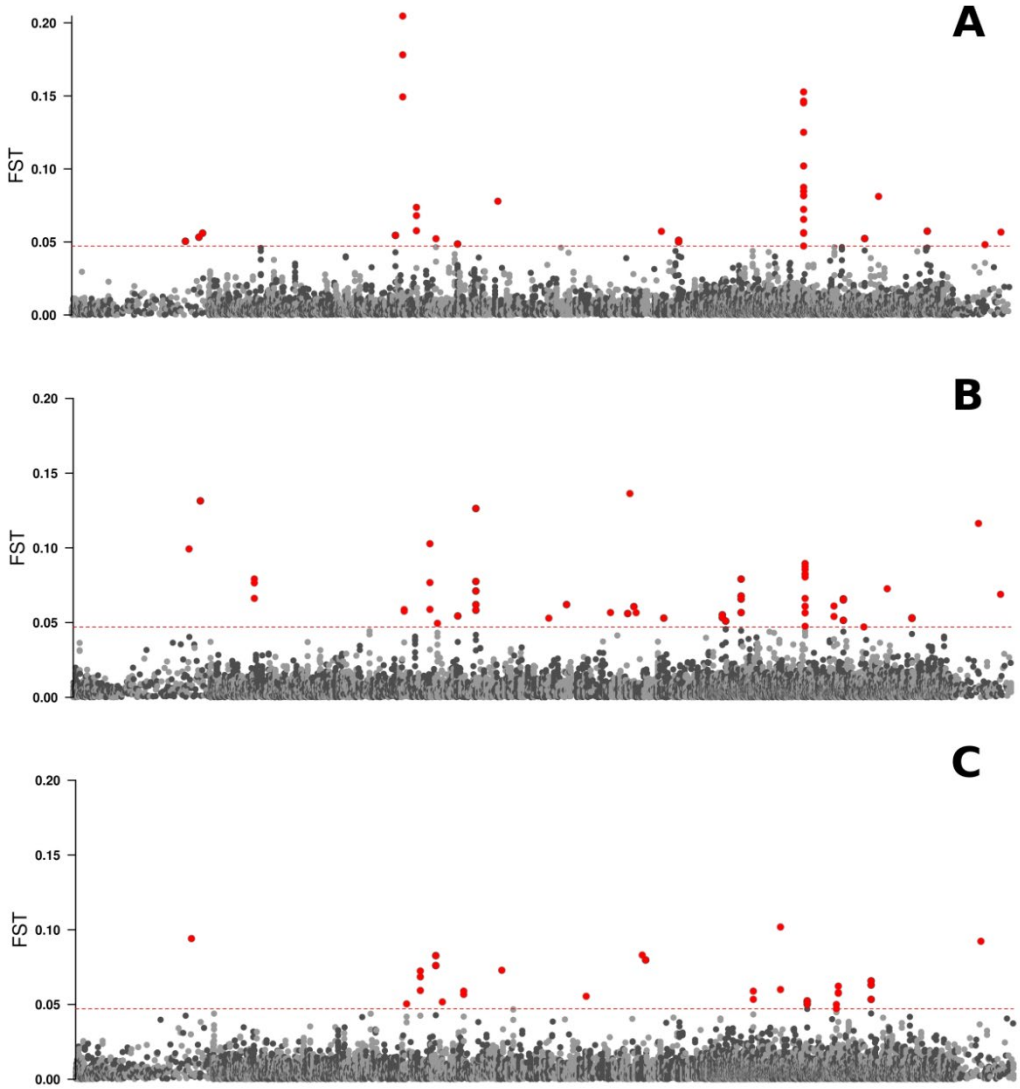

Figure 3 - Manhattan plots of genomic divergence for individuals collected from pheromone-baited traps in Bossier Parish, LA in A) 2002 and 2017, B) 2002 and 2012, and C) 2012 and 2017. Alternating light and dark grey points demarcate averaged F_{ST} values for 40kb windows with a 10kb step for each of the 2,975 genomic scaffolds. Red points represent statistically significant genetic divergence.

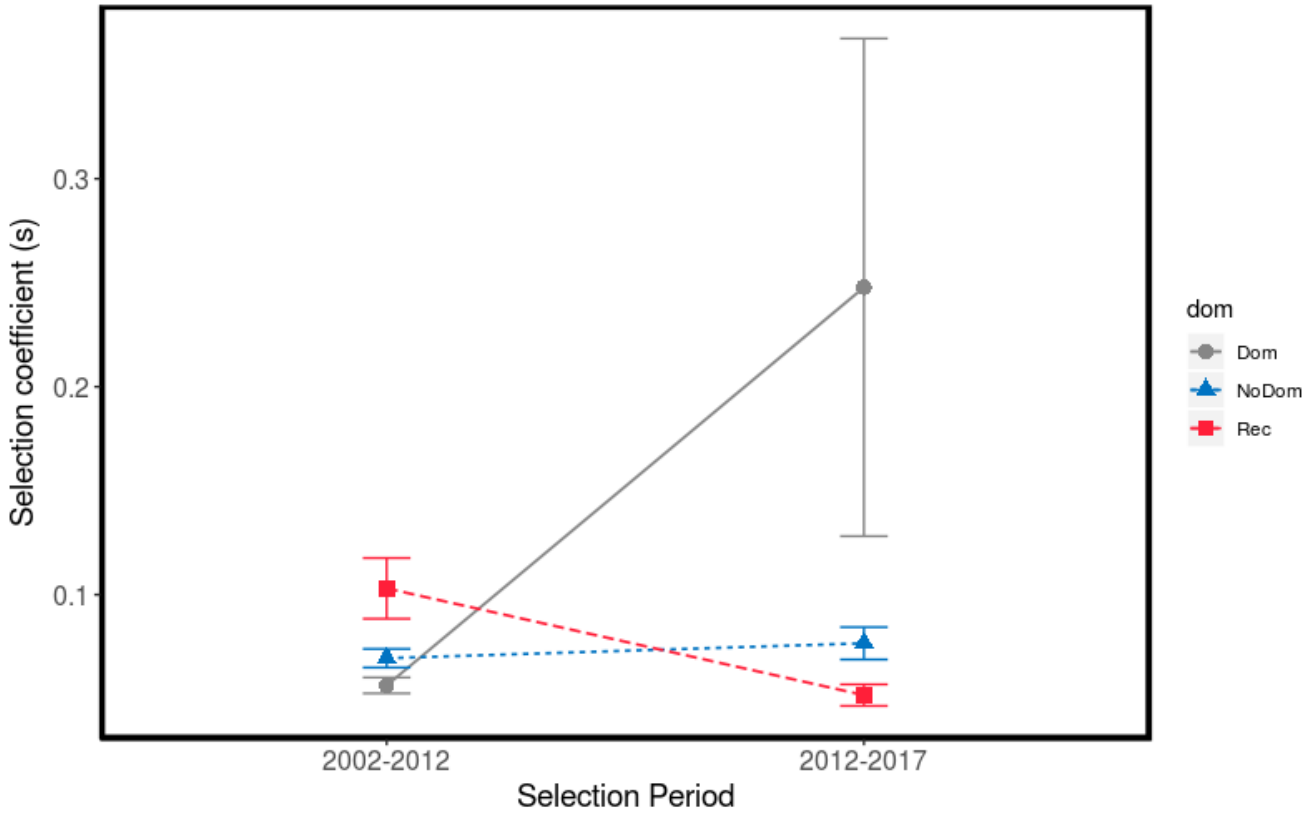

Figure 4 - Values of *s*, the selection coefficient, required to produce the observed allele frequency changes at SNPs within protein-coding regions in field-collected *H. zea* during two selection periods: 2002-2012 and 2012-2017. Points represent the mean values (n = 6 SNPs), and error bars represent standard error of the mean.

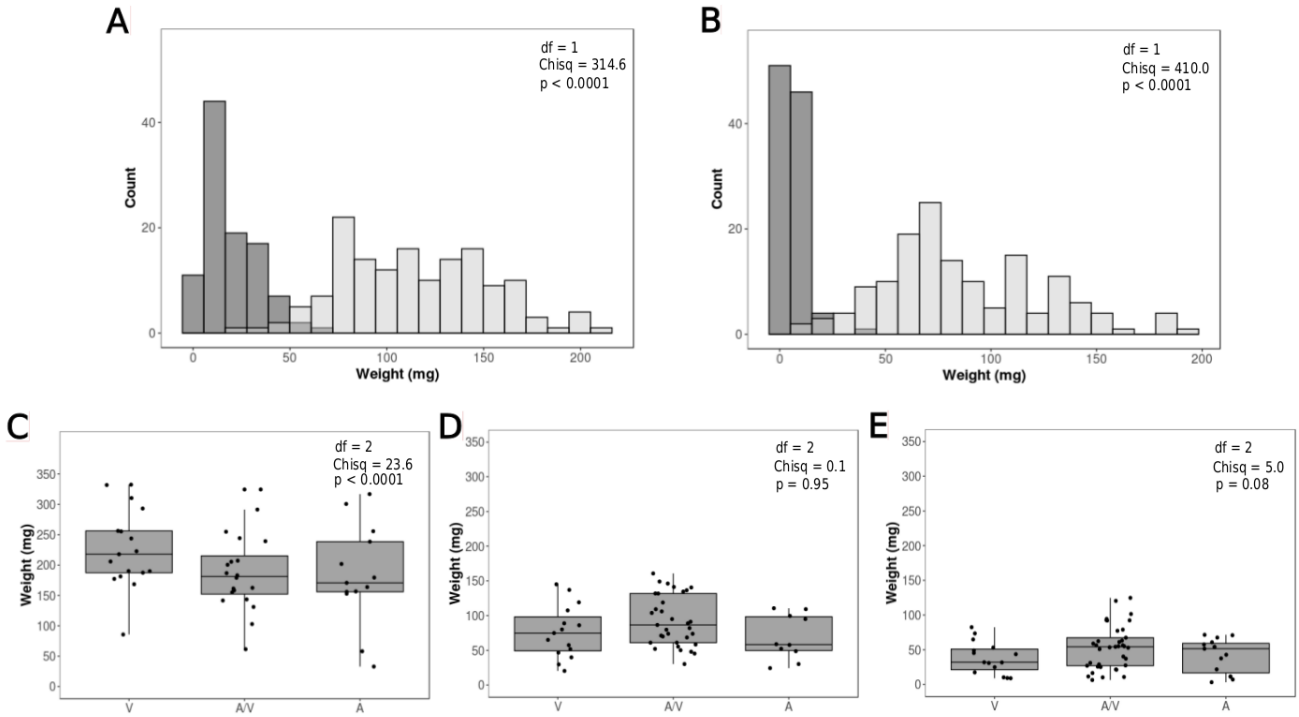

Figure 5 – Distribution of parental populations and F_2 larval growth phenotypes on Cry-treated diet. at a marker in the *cpq1* gene. Panels A and B show the distributions of larval weights for the susceptible (dark gray) and resistant (light gray) parental strains used for crosses after 7 days on diets containing Cry1Ab and Cry1A.105 + Cry2Ab2 expressing leaf tissue. Panels C, D, and E show the distributions of 7 day larval weights by *cpq1* marker genotype for progeny from two F2 mapping families. Grey boxes represent the upper and lower quartiles with the median (black horizontal line). Vertical lines show the minimum and maximum weights for each genotype, and points represent 7 day weights of each individual overlaid on the boxplots. V is the homozygous genotype putatively linked to Cry resistance, A/V represents heterozygotes, and A represents the homozygous genotype putatively linked to susceptibility.