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ZZ Top: faster and more adaptive Z chromosome evolution in two Lepidoptera

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1 ZZ Top: faster and more adaptive Z 2 chromosome evolution in two Lepidoptera 3

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10 Abstract

11 The rate of divergence for Z or X chromosomes is theoretically predicted to be greater than autosomes,
12 but the possible explanations for this pattern vary, as do empirical results from diverse taxa. Even
13 among moths and butterflies (Lepidoptera), which generally share a single-origin Z chromosome, the
14 handful of available studies give mixed support for faster or more adaptive evolution of the Z
15 chromosome, depending on the species assayed. Here, we examine the molecular evolution of Z
16 chromosomes in two additional lepidopteran species: the Carolina sphinx moth and the monarch
17 butterfly, the latter of which possesses a recent chromosomal fusion yielding a segment of newly Z-
18 linked DNA. We find evidence for both faster and more adaptive Z chromosome evolution in both
19 species, though this effect is strongest in the neo-Z portion of the monarch sex chromosome. The neo-Z
20 is less male-biased than expected of a Z chromosome, and unbiased and female-biased genes drive the
21 signal for adaptive evolution here. Together these results suggest that male-biased gene accumulation
22 and haploid selection have opposing effects on long-term rates of adaptation and may help explain the
23 discrepancies in previous findings as well as the repeated evolution of neo-sex chromosomes in
24 Lepidoptera.

25 Introduction

26 Explaining the patterns of genetic variation in natural populations is a foundational goal of population
27 genetics. In the most basic terms, variation is shaped by either selective or random (*i.e.* neutral)
28 processes. But beneath this simplicity, dynamics quickly become more complicated. For example, the
29 efficiency of selection relative to drift depends on the effective population size of the genes in question
30 (Ohta 1992). Simple census population size is often a poor proxy for the effective population size, as
31 historical population size changes have long-lasting effects (Tajima 1989). Also, different parts of the
32 genome may have different population sizes due to either differences in ploidy or conditional limitations
33 on expression. For organisms with chromosomal sex determination, the sex chromosomes present a
34 particularly complex confluence of the above processes (Wilson Sayres 2018).

35 Relative to the rest of the genome, sex chromosomes have smaller population sizes, occurring at either
36 half (in the case of the Y or W) or two thirds (in the case of the X or Z) the frequency of autosomes.
37 Evolution of the Y and W is thought to be driven mainly by the lack of recombination on these
38 chromosomes, leading to the degeneration of all but the most essential genes in many cases
39 (Charlesworth and Charlesworth 2000; Bachtrog 2013). X and Z chromosomes, however, maintain a
40 large set of functional genes in spite of their lower population size. This reduced population size should
41 decrease the efficiency of selection and increase genetic drift (Vicoso and Charlesworth 2009). However,
42 because the X/Z is haploid in one sex, new mutations may be more exposed to selection than on
43 autosomes, causing increased rates of adaptation (Rice 1984; Charlesworth et al. 1987). Both increased
44 drift and increased selection may lead to more rapid rates of molecular evolution on the X/Z relative to
45 autosomes, a phenomenon called “Faster-X” (or, likewise, “Faster-Z”). Although increased divergence of
46 sex chromosomes has been observed repeatedly in diverse taxa, discerning between drift or selection as
47 the primary cause of this Faster-X pattern remains an outstanding challenge, and potentially depends on
48 which sex is heterogametic (Baines et al. 2008; Meisel and Connallon 2013; Kousathanas et al. 2014;
49 Hayes et al. 2020).

50 A further complication for assessing Faster-X is sex-biased selection on the sex chromosomes. Because
51 of hemizyosity in males, the X spends more time in females than males (and vice versa for the Z). This
52 inequality is predicted to cause an accumulation of sex-biased genes on the sex chromosomes (Rice
53 1984; Chapman et al. 2003), a pattern that has been observed repeatedly for both male and female
54 heterogametic species (e.g. flies and mice: Meisel et al. 2012; birds: Wright et al. 2012; and butterflies
55 and moths: Mongue and Walters 2017). Genes with sex-biased expression carry their own evolutionary

56 complexities. Because selection can only act on expressed phenotypes, sex-limited genes should be
57 shielded from selection half the time, and thus experience increased divergence due to drift (Dapper
58 and Wade 2016). Sex-specific selection may also influence evolutionary rates and empirical studies
59 indicate sex-biased genes, particularly male-biased genes, evolve faster than unbiased genes, likely due
60 to sexual selection (Grath and Parsch 2016). Thus, an overabundance of sex-linked male-biased genes
61 could in itself cause faster-X, but this scenario is arguably more likely for faster-Z, because Z
62 chromosomes tend to be masculinized while X chromosomes are feminized (Walters and Hardcastle
63 2011; Meisel et al. 2012; Wright et al. 2012; Mank et al. 2014). However, if the faster-X effect primarily
64 reflects increased adaptation due to ploidy of expression, then it will be particularly pronounced for
65 genes expressed primarily in the heterogametic sex (Baines et al. 2008). Thus faster-Z adaptation should
66 be most apparent for female-biased genes (Parsch and Ellegren 2013; Sackton et al. 2014).

67 In the better-studied X chromosome systems, a faster-X effect is commonly found, and evidence for
68 more adaptive evolution tends to be associated with species with larger effective population sizes
69 (typically invertebrates, reviewed in Meisel and Connallon 2013). Z chromosome systems are less well-
70 studied, with available results coming mostly from birds. These studies indicate avian Z-linked genes
71 diverge faster primarily due to increased genetic drift, not adaptation (Mank et al. 2009; Wang et al.
72 2014; Wright et al. 2015; Xu et al. 2019; Hayes et al. 2020), though one study did show increased
73 adaptive divergence of gene expression on the Z (Dean et al. 2015). If larger effective population sizes
74 yield greater faster-X adaptation, then the strongest test for adaptive Z evolution may come from ZW
75 systems with large natural populations, like insects.

76 Butterflies and moths (Lepidoptera) are the one of the oldest and most diverse female-heterogametic
77 groups and lepidopteran species are routinely observed to have effective population sizes orders of
78 magnitude larger than most vertebrates (Mongue et al. 2019). In spite of these large population sizes,
79 evidence for a lepidopteran faster-Z effect is mixed, with one study finding faster rates of evolution on
80 the Z (Sackton et al. 2014) and two others not (Rousselle et al. 2016; Pinharanda et al. 2019). Likewise,
81 evidence for more adaptation is conflicting, with two of the previous studies finding more adaptation on
82 the Z (Sackton et al. 2014; Pinharanda et al. 2019) and the third finding the opposite: an increase in
83 purifying selection (Rousselle et al. 2016). These contradictory results are particularly baffling given that
84 all Lepidoptera are thought to share a single-origin Z chromosome (Fraïsse et al. 2017) and high levels of
85 synteny (*i.e.* conserved gene order) across their phylogeny (Ahola et al. 2014; Davey et al. 2016; Kanost
86 et al. 2016). In other words, the Z chromosome is substantially conserved across taxa; thus, differences

87 in observed molecular evolution may be attributable to a mixture of methodology and lineage-specific
88 effects (*e.g.* mating systems skewing effective population sizes). Here, we combine genome-wide
89 polymorphism and divergence data with gene expression analysis in a pair of distantly related
90 Lepidoptera to better understand whether and why the Z chromosome evolves faster than autosomes.
91 We take advantage of robust sequencing data in the Carolina sphinx moth, *Manduca sexta*, and the
92 recent discovery of a neo-Z chromosome in the monarch butterfly, *Danaus plexippus* (Mongue et al.
93 2017), to examine how newly sex-linked sequence evolves.

94 Methods

95 Data sources

96 We intersected population genomic data with sex-specific expression data using published datasets for
97 two Lepidoptera: the Carolina sphinx moth, *Manduca sexta*, and the monarch butterfly, *Danaus*
98 *plexippus*.

99 For *M. sexta*, counts of polymorphisms came from whole-genome resequencing of 12 North Carolinian
100 males and divergence came from one sequenced *Manduca quinquemaculata* male (Mongue et al. 2019)
101 aligned to the *M. sexta* reference genome (Kanost et al. 2016). Gene expression levels were obtained
102 from a large RNA-seq dataset that contains numerous stage- and tissue-specific samples (Cao and Jiang
103 2017). However, sex was not recorded for many of these samples, so we limited our analysis to tissues
104 where comparable male and female data were available: adult heads and antennae, as well as adult and
105 pupal testes and ovaries.

106 For the monarch butterfly, polymorphisms came from a large-scale resequencing project (Zhan et al.
107 2014), from which we selected 12 males from the North American migratory population; divergence
108 data came from sequencing of a *Danaus gilippus* male from the same dataset. RNA-seq data came from
109 Illumina sequencing of transcripts from male and female adult butterfly heads, midguts, thoraces, and
110 gonads (ovaries and testes), each sampled in triplicate. Read counts were initially quantified with RSEM
111 (Li and Dewey 2011), then normalized to FPKMs with Trinity using a TMM scaling factor (Grabherr et al.
112 2011). The three replicates were averaged to give a single expression value per tissue and sex.

113 For each gene in the genome, we used the SNP-calling pipeline described in Mongue *et al.* (2019).
114 Briefly, we took Illumina resequencing data through the *Genome Analysis Toolkit* best practices pipeline
115 for SNP-calling (McKenna et al. 2010) to generate a set of high-quality variants. We classified each single
116 nucleotide variant as synonymous or non-synonymous using custom databases in *SNPeff* (Cingolani et al.

117 2012) and normalized variant counts by the number of non-synonymous or synonymous sites in each
118 gene, using custom R scripts to annotate and sum the degeneracy of each amino acid coding site (R Core
119 Team 2017).

120 [Assignment of sex linkage](#)

121 Z-linkage in *D. plexippus*, including the presence of a neo-Z segment, was previously characterized using
122 a combination of synteny with other Lepidoptera and differential coverage between male and female
123 sequencing data (Mongue et al. 2017). Z-linkage in *M. sexta* has also been previously assessed, though
124 only via synteny with *Bombyx mori* (Kanost et al. 2016). To directly assess Z-linkage via sex-specific
125 sequencing depth, we generated ~16x coverage Illumina sequencing from a female *M. sexta* and
126 compared coverage with one of the male samples (S35) having a comparable sequencing depth. For
127 each sample, we used *BEDtools* to calculate per-base coverage across the genome and also the median
128 coverage for each scaffold (Quinlan and Hall 2010). Scaffold medians were normalized by dividing by the
129 mean of all medians for that sample. Then, we assessed Z linkage of each scaffold by taking the \log_2 of
130 the ratio of male:female normalized coverage for each scaffold. Under this metric, autosomal scaffolds
131 cluster around 0 while Z linked scaffolds group around 1.

132 [Assessment of Sex-bias](#)

133 To evaluate sex-bias in expression, we calculated the specificity metric (SPM) for male versus female
134 expression for each annotated gene in the genome (Kryuchkova-Mostacci & Robinson-Rechavi 2017).
135 We first summed FPKM (fragments per kilobase of transcript per million mapped reads) in each sex and
136 divided by the number of replicates for that tissue in that sex to obtain a mean value for each sex and
137 tissue combination. With these values we calculated SPM as the square of expression in one sex divided
138 by the sum of squared expression in both sexes. This calculation gave us a specificity value ranging from
139 0 to 1, inclusive, indicating what proportion of a given gene's expression was unique to one sex. As
140 implemented here, an SPM = 1 indicates completely female-specific expression, SPM = 0 indicates male-
141 specific expression, and SPM = 0.5 reflects equal expression between the sexes.

142 We sought to make our methodology comparable to existing faster-Z studies, which have used fold-
143 change in expression to delineate sex-biased genes. In those analyses, sex-bias cut-offs are typically 1.5x
144 difference in expression between males and females (e.g. in Pinharanda et al. 2019). This difference
145 corresponds to a 70-30 bias in SPM. Thus, we took female-biased genes to be those with SPM > 0.7 in
146 females, male-biased genes with SPM < 0.3 in females, and unbiased genes to be those that fell within

147 (0.3, 0.7). We further verified that our results were robust to cutoff thresholds by re-analyzing the sex-
148 bias data using stricter 85-15 bias. As these results were qualitatively the same as the 70-30 cutoffs, we
149 only present the former in the main text and the latter in the supplement.

150 We have shown previously that both the *D. plexippus* and *M. sexta* Z chromosomes are masculinized
151 based on distributions of genes encoding sperm proteins (Mongue and Walters 2017), but this RNA-seq
152 expression dataset affords the opportunity to validate those results with a more complete set of sex-
153 biased genes. As such, after classification of sex-biased genes, we used a set of X^2 tests of independence
154 to assess whether or not the proportion of sex-biased genes differed between the autosomes and
155 (neo-)Z chromosomes.

156 Finally, as Z chromosomes spend more time in males than females and males are often thought to have
157 a higher variance in reproductive success than females, it is possible that the effective population size of
158 the Z is substantially smaller than its census size (Vicoso and Charlesworth 2009). To investigate this
159 possibility, we compared neutral variation between the Z and autosomes in our two study species. In
160 brief, we used a series of custom R scripts to parse out putatively neutral (four-fold degenerate) sites
161 across the genome, and used the population genomics tool ANGSD (Korneliussen et al. 2014) to
162 estimate heterozygosity (Watterson's Θ) separately for all four-fold degenerate sites on the Z and
163 autosomes. We then took the ratio of the mean per-site heterozygosity of the two regions as the
164 difference in effective population size.

165

166 [Statistical analysis of molecular evolution](#)

167 Divergence and polymorphism rates are compound ratios with asymmetric bounds (i.e. both range from
168 0 to infinity) and are thus not normally distributed. As such, we analyzed molecular evolution with a
169 series of non-parametric tests. Initially, we tested for a faster-Z effect by comparing the scaled rate of
170 divergence (dN/dS) of autosomal and Z-linked genes using Kruskal-Wallis tests with either 1 degree of
171 freedom in *M. sexta* or 2 degrees of freedom in *D. plexippus* to account for 3 potential classes of linkage
172 (autosomal, ancestral Z, and neo-Z). With this distinction established, we assessed the effect of sex-
173 biased gene expression on rates of evolution with another set of Kruskal-Wallis tests to determine if
174 there was an effect of sex-bias generally. In the case of significant results, pair-wise post-hoc differences
175 were investigated with a Nemenyi test. Equivalent tests were performed for polymorphism data as well.

176 Finally, we synthesized the polymorphism and divergence data to calculate α , the proportion of
177 substitutions driven by adaptive evolution. Specifically, we used a modified calculation of the neutrality
178 index (NI) to correct for the bias inherent in a ratio of ratios (Stoletzki and Eyre-Walker 2011) for each
179 class of genes to give us a point-estimate of α ($= 1 - NI$) summed across genes within a bias class and
180 linkage group. We assessed significance via a permutation test framework, as in Mongue *et al.* (2019).
181 That is, we compared evolution of two gene classes, calculated the point-estimate α for each, then took
182 the absolute value of the difference of these estimates as our permutation test statistic. Next, we
183 combined the two gene sets and randomly drew two permuted classes of sizes equal to the true classes
184 without replacement. We calculated the absolute difference in α for these two random gene sets and
185 repeated this for 10,000 permutations. In doing so, we built a distribution of differences in point
186 estimates of α that could be expected by chance alone. We then compared our true value to this
187 distribution and took the p-value to be the proportion of times we observed a greater value in the
188 permuted distribution than the true value. All of these analyses were completed with custom R scripts in
189 R version 3.4 (R Core Team 2017).

190 Results

191 Assignment of sex-linkage in *Manduca sexta*

192 Based on synteny in previous analyses, 27 scaffolds were already annotated as Z-linked in the *M. sexta*
193 assembly (Kanost et al. 2016). By using new resequencing data from a male and female, we validated
194 and updated our knowledge of sex-linkage in this moth. We restricted our coverage analysis to scaffold
195 above the genome N90 (45Kb) to avoid coverage differences that could arise by chance on short
196 sequences. Using this metric, we have recovered all previously annotated 27 scaffolds as male-biased in
197 coverage and thus Z-linked. Additionally, we identified another 9 scaffolds as Z-linked, totaling 2.1Mb
198 and containing 43 annotated genes. Seven of these scaffolds were previously unassigned (due to unclear
199 orthology); the other two had previously been assigned to autosomes but are clearly Z-linked by
200 coverage. The updated linkage information is included as a supplementary datatable.

201 Sex-bias on the Z chromosomes

202 Based on the assignment of sex-biased genes from the RNA-sequencing data, the gene-content differs
203 between the Z and autosomes in both the Carolina sphinx moth ($\chi^2_2 = 47.37$, $p = 5.2 \cdot 10^{-11}$) and monarch
204 butterfly ($\chi^2_2 = 30.04$, $p = 3.0 \cdot 10^{-7}$). In both species, this difference comes from an excess of male-biased
205 genes on the Z chromosome, as well as a paucity of female-biased genes on the *Manduca* Z and

206 unbiased genes on the *Danaus* ancestral Z (Table 1). These results hold for both these cutoffs for sex
207 bias and for stricter criteria (85-15 SPM, see supplement). It is worth noting that the male bias in
208 expression on the Z chromosome is not the result of dosage effects, as both *M. sexta* and *D. plexippus*
209 have been shown generally to have sex-balanced expression on the Z chromosome (Smith et al. 2014;
210 Gu and Walters 2017).

211 Rates of divergence

212 We found that the Z chromosome has higher scaled divergence than the autosomes in both species, the
213 Carolina sphinx moth ($X^2_1 = 6.89$, $p = 0.009$, Figure 1A) and the monarch butterfly ($X^2_2 = 9.72$, $p = 0.008$).
214 For monarchs, we further classified the Z into the ancestral- (*i.e.* long-term sex-linked) and neo-Z (the Z
215 sequence resulting from a milkweed-butterfly-specific autosomal fusion). Based on the significant
216 chromosomal linkage effect, we conducted post-hoc testing and found that the signal for faster-Z
217 evolution comes primarily from the neo-Z, which diverges distinctly faster than the autosomes ($p =$
218 0.006 , Figure 1D) and marginally faster than the ancestral Z ($p = 0.048$). The ancestral Z was not faster
219 evolving than the autosomes ($p = 0.99$).

220 In Carolina sphinx moths, sex-biased expression did not impact divergence rates on the Z chromosome
221 ($X^2_2 = 1.12$, $p = 0.571$, Figure 1C). On the autosomes however, there was a clear effect of sex-biased
222 expression ($X^2_2 = 26.26$, $p = 1.98 \times 10^{-6}$). Post-hoc testing revealed this to be driven largely by male-biased
223 genes, which had higher divergence rates than unbiased ($p = 8.1 \times 10^{-6}$) or female-biased genes ($p =$
224 4.2×10^{-5}). Female-biased genes do not evolve at a different rate than unbiased genes ($p = 0.63$).

225 In monarchs, like sphinx moth, sex-biased expression affected evolutionary rates of autosomal loci ($X^2_2 =$
226 249 , $p < 1.0 \times 10^{-10}$, Figure 1F). Unlike sphinx moths however, the effect of sex-bias did not differ
227 between sexes. Both male-biased ($p < 1.0 \times 10^{-10}$) and female-biased genes ($p < 1.0 \times 10^{-10}$) evolve faster
228 than unbiased genes according to post-hoc testing, though male-biased and female-biased genes did not
229 evolve differently from each other ($p = 0.75$).

230 Considering the monarch Z chromosome, both the ancestral ($X^2_2 = 9.99$, $p = 0.007$) and neo ($X^2_2 = 11.85$,
231 $p = 0.003$) segments showed a sex-bias effect. For the ancestral Z, this difference is driven solely by
232 faster evolution of male-biased genes compared to unbiased genes ($p = 0.005$); evolutionary rates of
233 female biased genes did not differ significantly from the unbiased nor male-biased genes on the
234 ancestral Z. On the neo-Z, female-biased genes evolve faster than both male-biased ($p = 0.044$) and
235 unbiased genes ($p = 0.002$); divergence of male-biased genes did not differ from unbiased on the neo-Z.

236 Rates of polymorphism

237 Unlike with divergence, we found that that sex-linkage did not strongly impact rates of polymorphism in
238 *M. sexta* ($X^2_1 = 2.57$, $p = 0.110$) However, sex-biased expression did impact rates of polymorphism ($X^2_2 =$
239 43.45 , $p = 3.7 * 10^{-10}$). Here again, male-biased genes showed increased variation compared to unbiased
240 genes ($p = 1.4 * 10^{-10}$) and female-biased genes ($p = 0.002$). Female-biased and unbiased genes did not
241 significantly differ from each other ($p = 0.14$).

242 In monarchs, sex linkage strongly impacted polymorphism ($X^2_2 = 34.18$, $p = 38 * 10^{-8}$). Both the ancestral
243 Z ($p = 3.9 * 10^{-7}$) and neo-Z ($p = 0.02$) had lower rates of polymorphism than the autosomes, but the two
244 portions of the Z did not differ from each other ($p = 0.27$). Sex-biased expression did not significantly
245 affect polymorphism on either part of the Z (ancestral: $X^2_2 = 2.70$, $p = 0.259$; neo: $X^2_2 = 5.75$, $p = 0.06$). In
246 contrast, autosomal genes did show an effect of sex-bias, with female-biased genes showing the highest
247 rates of polymorphism, higher than male-biased ($p = 1.8 * 10^{-10}$) or unbiased genes ($p < 1.0 * 10^{-10}$); male-
248 biased genes had elevated rates of polymorphism compared to unbiased genes ($p < 1.0 * 10^{-10}$).

249 Rates of adaptive evolution

250 The variable patterns of divergence and polymorphism observed for sex-linked and sex-biased loci may
251 reflect differing rates of adaptive evolution among these groups of genes. To examine this, we estimated
252 the proportion of adaptive substitutions (α) for each gene-class, first contrasting the Z versus autosomes
253 as a whole, and subsequently further partitioning loci by sex-biased expression.

254 In *M. sexta*, the Z overall showed more adaptive evolution than the autosomes ($p = 0.039$). Adaptation
255 of male-biased ($p = 0.340$) and female-biased genes ($p = 0.812$) did not differ based on genomic location,
256 but genes with unbiased expression showed higher rates of adaptive evolution (α) on the Z chromosome
257 than the autosomes ($p = 0.007$; Figure 2A).

258 In monarchs, the Z also exhibited increased rates of adaptation compared to autosomes ($p = 0.0004$;
259 Figure 2B, left). Considered separately, both the ancestral and neo-Z segments evolved more adaptively
260 than the autosomes (ancestral-Z vs. autosomes: $p = 0.0338$, neo-Z vs. autosomes: $p = 0.0005$). The neo-Z
261 segment trended towards more adaptive evolution than the ancestral Z, but not strongly ($p = 0.079$).
262 Turning to sex-bias, we found that male-biased genes did not evolve differently across the genome
263 (autosomal vs. neo-Z $p = 0.318$, autosomal vs. ancestral Z $p = 0.092$, ancestral vs neo-Z $p = 0.500$). In
264 contrast, female-biased genes evolved more adaptively on the neo-Z than the autosomes ($p = 0.0474$) or
265 ancestral Z ($p = 0.008$). Additionally, ancestrally Z-linked female-biased genes did not evolve differently

266 than their autosomal counterparts ($p = 0.539$). Furthermore, unbiased genes on the neo-Z showed
267 greater rates of adaptation than unbiased genes on the autosomes ($p = 0.018$) or ancestral Z ($p = 0.048$).

268 The effective population size of the Z chromosome

269 Under the simplest biological conditions, we expect the ratio of population sizes of Z:Autosomes to be
270 0.75 (because males and females each make up half of the population (Wilson Sayres 2018)). We found
271 for *Manduca sexta* that in practice this ratio is much smaller $Ne_Z:Ne_A = 0.44$. For *Danaus plexippus*, the
272 difference in population sizes is less skewed, $Ne_Z:Ne_A = 0.66$. Intriguingly, this difference is not uniform
273 across the monarch Z. The ancestral portion of the Z has a lower population size, $Ne_{Z_Anc}:Ne_A = 0.58$, but
274 the neo-Z holds essentially as much diversity as the autosomes, $Ne_{Z_Neo}:Ne_A = 0.98$.

275 Discussion

276 New evidence for a faster-Z

277 While previous evidence for faster-Z evolution in Lepidoptera has been mixed, we found that the Z
278 chromosome is faster evolving (*i.e.* has a higher dN/dS ratio) than the autosomes in both *Manduca sexta*
279 and *Danaus plexippus*. At first pass, our results seemingly align with a report of faster-Z evolution in
280 silkmoths (Sackton et al. 2014), but are contrasting with other studies in butterflies (Rousselle et al.
281 2016; Pinharanda et al. 2019). However, a more nuanced consideration indicates some congruence with
282 both sets of studies. Monarchs show an overall fast Z, but this result is driven by the newly sex-linked
283 neo-Z portion of the monarch evolving faster than the autosomes. Considering only the ancestral
284 portion, which is homologous to the Z of the butterflies previously studied, there is no evidence for a
285 faster ancestral-Z in monarchs. However, evidence for higher rates of adaptive evolution (α) on the Z is
286 less ambiguous in our insects; both *Manduca* and *Danaus* showed overall more adaptation for Z-linked
287 genes, as reported in *Bombyx*.

288 Beginning with the simpler case of *Manduca*, we found that increased adaptation on the Z chromosome
289 is driven by genes with unbiased (*i.e.* equal) expression in the two sexes. These genes will be expressed
290 in the haploid state in females and thus should experience more efficient selection than unbiased genes
291 on the autosome (which are always diploid expressed). Female-biased genes should theoretically follow
292 this pattern as well, but the lack of a clear signal might be partially attributable to the relatively small
293 number of female-biased genes on the Z, which reduces power to detect differences in adaptive
294 evolution. Moreover, the effective population size of the *Manduca* Z compared to the autosomes is
295 much lower than the neutral expectation (0.44, as opposed to 0.75). With such a decrease in the

296 population of Z chromosomes, selection is predicted to be less efficient (Vicoso and Charlesworth 2009)
297 and may further limit the adaptive evolution of female-biased genes.

298 *Danaus* presents a more complicated case, owing to a Z-autosomal fusion in this genus (Mongue et al.
299 2017). Intriguingly, it is the neo-Z that best fits with theoretical predictions for adaptive Z evolution.
300 Increased adaption (compared to the autosomes) is concentrated in unbiased and female-biased genes
301 as expected. It also is worth noting that the neo-Z has an inferred effective population size nearly equal
302 to that of the autosomes ($Ne_{Z_neo}:Ne_{Autos} = 0.98$). This is a perplexing result that cannot be attributed to
303 sequence homology with a neo-W, which if exists at all must be highly divergent from the neo-Z,
304 (Mongue et al. 2017; Gu et al. 2019). Parity in effective population size of the sex chromosomes and
305 autosomes has been attributed to biased sex ratios and/or higher variance in the reproductive success
306 of the heterogametic sex in other taxa (Hedrick 2007; Ellegren 2009). A skewed sex ratio seems unlikely,
307 as only a male-biased population could restore parity to the Z:A ratio. No such dynamics have been
308 observed (on the contrary, another *Danaus* species is known to have male-killing genetic elements
309 (Smith et al. 2016)), and more to the point, this dynamic should affect the ancestral and neo-Z equally. A
310 high variance in female reproductive success could also generate roughly equal effective population
311 sizes of the Z and autosomes and should be more apparent in regions with fewer male-biased genes (*i.e.*
312 the neo-Z), but there is currently little ecological data with which to assess this possibility. Whatever the
313 cause of this parity in variation, this observation itself points to different evolutionary dynamics
314 between the ancestral and neo-Z, and implies that selection to remove deleterious variation should be
315 more efficient than on the autosomes for all dominance coefficients of deleterious mutations (Vicoso
316 and Charlesworth 2009).

317 In contrast to the neo-Z, the *Danaus* ancestral Z has a much lower effective population size compared to
318 the autosomes (0.58), yet it evolves more adaptively than the autosomes overall. This result is driven by
319 increased adaptation among Z-linked male-biased genes. As discussed above, this result points to a
320 difference in evolutionary dynamics between the neo- and ancestral segments. Moreover, this suggests
321 that, in terms of genetic diversity, the difference in census size between the autosomes and sex
322 chromosomes is relatively unimportant in this butterfly. One possible explanation is the unusually strong
323 sexual selection in this species. Female monarchs are highly polyandrous in nature (Pliske 1975), which
324 has been implicated in the elevated adaptation of sperm protein coding genes on the autosomes
325 (Mongue et al. 2019). As the ancestral Z is highly masculinized in gene content, it stands to reason that
326 similarly strong selection may apply to these male-biased genes. Circumstantial evidence for this can be

327 seen in the effective population size ratios of the ancestral Z segment compared to the non-
328 masculinized neo-Z segment.

329 [Reconciling existing investigations of lepidopteran Z chromosome evolution](#)

330 Our results most strongly agree with existing work from the silkworm genus *Bombyx* (Sackton et al.
331 2014), which found both fast and adaptive Z effects. Efforts in other butterflies have found no fast Z
332 effect. In the case of satyr butterflies, this negative result may be attributable to “noisy” sequence data
333 (*de novo* transcriptome assemblies were used) and potential uncertainty in Z-linkage (which was
334 inferred from sequence homology to another butterfly species) (Rousselle et al. 2016). Separately, in the
335 case of *Heliconius* butterflies, it is worth noting that point estimates for α and dN/dS largely fit
336 predictions for a fast and adaptive Z, but results did not differ significantly between the Z and
337 autosomes thanks to high variance in these estimates, especially on the Z chromosomes (Pinharanda et
338 al. 2019). In this case, the use of a relatively small RNA-sequencing dataset created a smaller dataset of
339 sex-biased genes with which to work, and only 200 of about 700 total Z-linked genes were analyzed.

340 Nonetheless, this collection of lepidopteran faster-Z studies suggests a phylogenetic signal for Z
341 chromosome evolution. *Bombyx* and *Manduca* are species from sister families of moths (Kawahara and
342 Breinholt 2014) and share patterns of faster and more adaptive Z evolution. Satyrs, *Heliconius*, and
343 *Danaus* butterflies all fall within the family Nymphalidae and show mixed to negative evidence for
344 increased divergence and adaptation on the (ancestral) Z. In other words, there is more agreement for Z
345 chromosome evolution for more closely related species (though insects in general and Lepidoptera
346 specifically are an ancient group, so none of these species are “closely-related” by vertebrate-centric
347 expectations). These observations demonstrate that sex-linkage *per se* does not lead to consistent
348 evolutionary outcomes for the genes involved. Instead faster-Z evolution is likely to depend on the
349 demographic history or degree of sex-bias of the Z chromosomes examined. This is illustrated by the
350 relatively young neo-Z in monarchs, which is not masculinized like the ancestral lepidopteran Z
351 sequences and instead appears comparable to autosomes in the proportion of unbiased and female-
352 biased genes (Mongue and Walters 2017). Intriguingly, the monarch neo-Z fits completely within the
353 theoretical prediction for adaptive faster-Z evolution. It appears to be faster evolving due to increased
354 adaptation of unbiased and female-biased genes that are subject to haploid selection (Charlesworth et
355 al. 1987). These observations present the possibility that faster-Z dynamics may be transient rather than
356 perpetual.

357 Indeed, two prominent hypotheses for sex chromosome evolution combine to suggest this transient
358 faster-Z dynamic. Adaptive evolution of the sex chromosomes is thought to be driven by the hemizygous
359 expression of some genes in one sex (Charlesworth et al. 1987), but depending on the dominance of
360 gene expression, genes benefitting the opposite sex are predicted to accumulate on that sex
361 chromosome (Rice 1984). As such, if the sex chromosomes change composition over evolutionary time,
362 they may bias towards alleles benefitting the homogametic sex (e.g. male-benefitting, male-biased
363 genes on the Z). Genes with haploid expression (e.g. unbiased or female-biased genes in ZZ/ZW
364 systems), will become less abundant and thus less important to the overall evolution of the
365 chromosome. Moreover, if sexual selection produces high variance in male reproductive success, the
366 effective population size of Z chromosomes can be substantially depressed below the census size,
367 further limiting the role of positive selection on the few unbiased or female-biased left on the Z.
368 Particularly old sex chromosomes should be more likely to experience these effects.

369 To take this thread of logic to its end, this scenario may also explain the relative abundance of neo-Z
370 chromosomes in Lepidoptera (Nguyen et al. 2013; Nguyen and Paladino 2016; Mongue et al. 2017). The
371 strongly conserved synteny across species implies that small-scale gene trafficking events are rare (but
372 evidence is somewhat contradictory here as well, see: Touns et al. 2011; Wang et al. 2012) and fusion-
373 fission events may be the key source of linkage shuffling in Lepidoptera. For a highly masculinized Z
374 chromosome, a sudden influx of unbiased and female-biased genes onto the Z can create strong positive
375 selection and favor these fused chromosomes, even at initially low frequencies, helping them to escape
376 elimination by drift. Under this paradigm, even the seemingly contradictory findings on Z chromosome
377 evolution can be reconciled as being the product of lineage-specific differences in sex-biased gene
378 content and chromosomal history. If this line of reasoning is accurate, it should be borne out in other
379 Lepidoptera with neo-Z chromosomes. More comparisons of independent neo-Z chromosomes will be
380 needed for this validation.

381 Data accessibility

382 *Manduca sexta* whole genome resequencing data can be found on NCBI's Sequence Read Archive with
383 the following accessions: SRP144217, PRJNA639154. *Danaus plexippus* RNA sequencing can be found
384 with PRJNA522622. The *M. sexta* expression data can be found as a supplementary table in Cao and
385 Jiang (2017), <https://doi.org/10.1186/s12864-017-4147-y>. The *D. plexippus* sequencing data can be
386 found in Zhan et al. (2014), <https://doi.org/10.1038/nature13812>. Please see the supplement to this
387 manuscript for specific samples used here.

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543 **Table 1.** Sex bias of the Z chromosomes in the two species studied with raw counts and proportions in
544 parentheses. In both species, composition of the Z differs from composition of the autosomes due to an
545 increased proportion of male-biased Z-linked genes (based on X^2 p-values $< 1.0 \cdot 10^{-6}$; note that this
546 significant result holds in monarchs whether the Z is considered as one category or two (i.e. neo and
547 ancestral)). The sphinx moth Z is depleted for female-biased genes, while the monarch (ancestral-)Z is
548 depleted for unbiased genes.

	Carolina sphinx moth		Monarch butterfly		
	Autosomes	Z	Autosomes	Ancestral Z	Neo-Z
Male-biased	2477 (0.21)	177 (0.34)	4721 (0.35)	279 (0.47)	184 (0.39)
Unbiased	7219 (0.63)	295 (0.56)	7529 (0.56)	278 (0.46)	243 (0.52)
Female-biased	1856 (0.16)	55 (0.10)	1248 (0.09)	44 (0.07)	41 (0.09)

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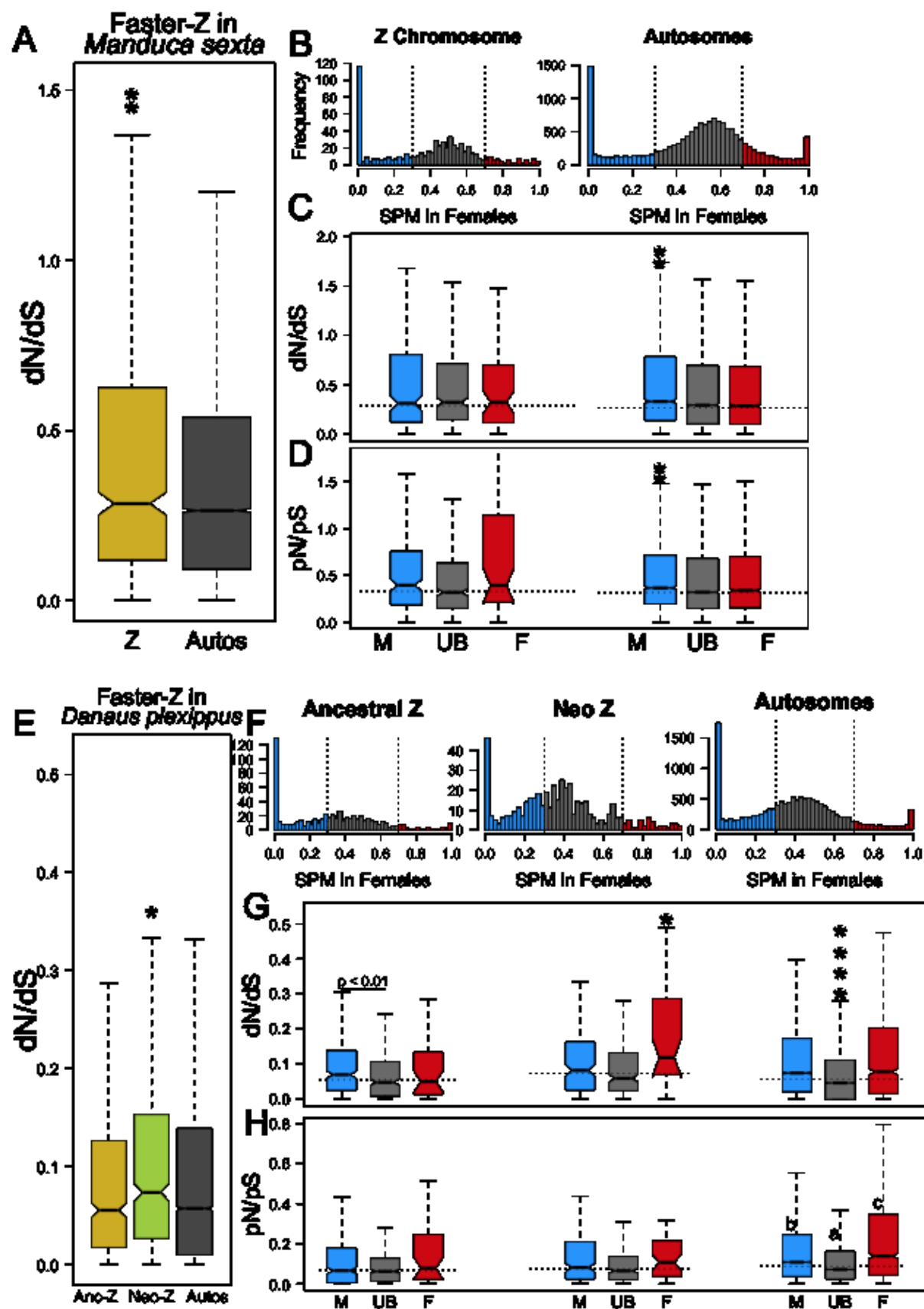
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559 **Figure 1.** Faster-Z evolution in Carolina sphinx moths and monarchs. Throughout, asterisks represent
560 statistical differences of one group from all others to which it is compared, with the number of asterisks
561 indicating the level of significance (* < 0.05, ** < 0.01, *** < 0.001, etc.). Horizontal lines with significance
562 annotations are given for significant pairwise differences. **A.** The Z evolves faster than the autosomes in
563 *Manduca sexta*. **B.** The distributions of sex-bias for both z-linked (left) and autosomal (right) genes are
564 plotted with dashed lines to indicate the traditional cutoff points for sex-bias analysis. Bias is plotted
565 such that higher SPM values are more female biased in expression, while values closer to 0 are male-
566 biased. **C.** Rates of divergence for genes in each sex-bias class (M: male-biased, UB: unbiased, F: female-
567 biased). In sphinx moths, only autosomal genes show differences between rates of evolution of genes
568 with different sex-bias. **D.** Likewise, male-biased genes have higher pN/pS than on other bias classes, but
569 only on the autosomes. **E.** The neo-Z is the source of a faster-Z signal in monarch butterflies. **F.** Again we
570 plot distributions of sex-bias categories for genes on the ancestral Z (left), neo-Z (middle), and
571 autosomes (right). **G.** In monarchs, male-biased genes evolve more quickly on the ancestral Z. Female
572 biased genes evolve more quickly on the neo-Z, and unbiased genes evolve more slowly on the
573 autosomes. **H.** Finally, sex-biased genes hold different levels of polymorphism on the autosomes, with
574 unbiased genes having the lowest pN/pS, followed by male-biased, then female-biased with the highest
575 (graphically represented as $a < b < c$).

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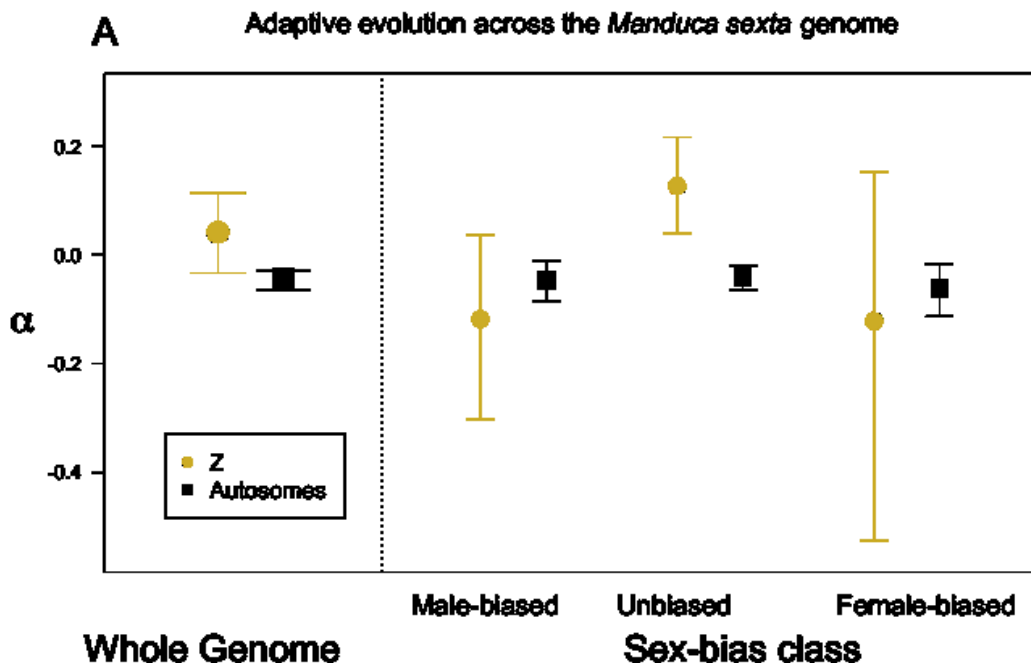
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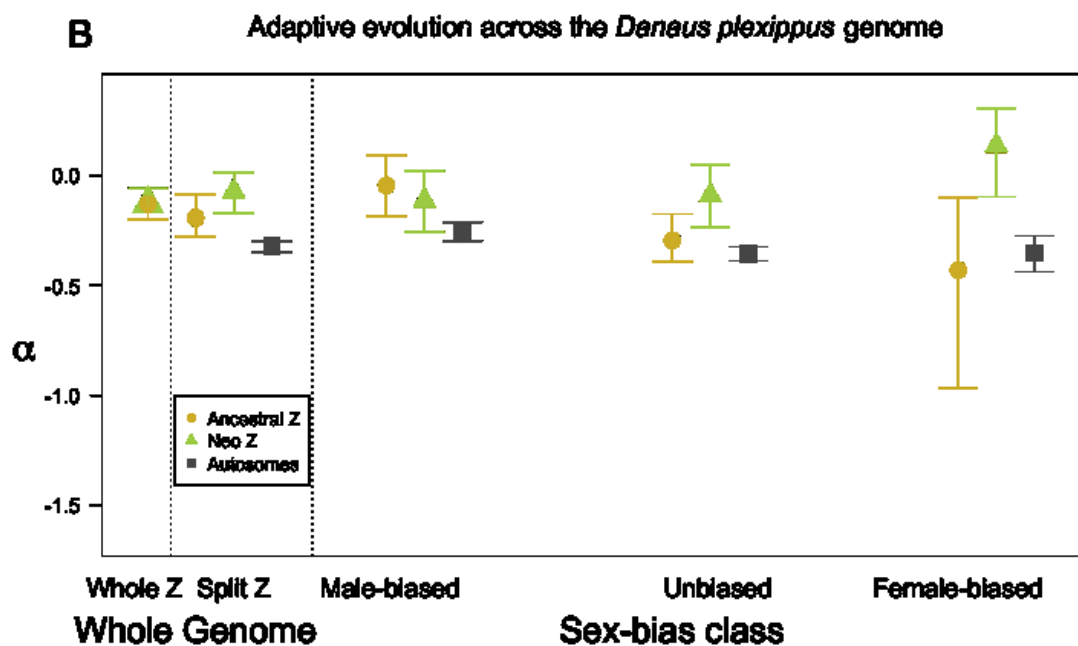
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590 **Figure 2.** Adaptive evolution across the genomes of the two Lepidoptera considered in this study. In
591 each panel coarse-scale comparison of the Z chromosome to autosomes are plotted left of the dotted
592 lines. Points are the point estimate of the α statistic and error bars represent 95% confidence intervals
593 for each point estimate obtained by parametric bootstrapping. In the Carolina sphinx moth (**A**), the Z
594 evolves more adaptively than the autosomes overall (left of dash). This pattern appears to be driven by
595 unbiased genes (right of dash). In the monarch butterfly (**B**), the whole Z is more adaptively evolving
596 than the autosomes (leftmost), and both the ancestral and neo- segments show elevated α compared to

597 the autosomes (middle). For the ancestral Z, male-biased genes drive the increase in adaptation; in
598 contrast, unbiased and female-biased genes are more adaptively evolving on the neo-Z.