

# Animal Migration

## Wing morphology in migratory North American monarchs: characterizing sources of variation and understanding changes through time

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# 1 Wing morphology in migratory North American monarchs: characterizing sources of 2 variation and understanding changes through time

3

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8

9 **Abstract**

Monarch butterfly wing morphology varies substantially throughout their global range, both between resident and migratory populations and also within the migratory North American population. Here, we use a dataset comprising more than 1800 North American individuals collected between 1878-2017 to characterize the factors shaping continent-wide patterns of wing morphological variation. North American overwintering butterflies have forewings that are approximately 4.4% larger than those collected in summer breeding areas. Monarchs overwintering in Mexico have forewings that are approximately 1.8% larger than monarchs overwintering in California, conducive to the idea that migration distance is positively correlated with wing area. We find evidence for a latitudinal cline within North America, such that butterflies collected at higher latitudes have significantly larger and more elongated forewings. We also find a significant increase of approximately 4.9% in forewing area between 1878-2017, but no difference through time in wing elongation. This result is corroborated by a reanalysis of a recently published dataset of more than 600 butterflies from Mexican overwintering sites. We discuss possible

24 reasons for this increase in wing size through time, including northward shifts in the  
25 monarch's breeding range and changes in relative abundance of milkweed hosts, and  
26 present experimental data addressing the influence of larval host plant on adult wing  
27 morphology. Our analysis suggests that (1) migration is indeed an important selective force  
28 for monarch wing morphology; (2) wing size has increased through time in North America;  
29 (3) factors such as host plant identity must be considered to fully understand monarch  
30 wing morphological variation.

31

32 **Keywords:** monarch butterfly, migration, morphology, evolution, museum collections

33

34 **Introduction**

35 Migration has evolved across the tree of life as a way for organisms to exploit  
36 ephemeral or only seasonally available resources, often over enormous spatial scales. In  
37 organisms that migrate long distances, traits conducive to efficient movement are  
38 imperative. This can entail certain behavioral and physiological adaptations, such as  
39 periods of reproductive dormancy and lipid accumulation prior to or during migration  
40 [1,2], as well as morphological features that aid in long-distance movements. In the latter  
41 case, selection has favored certain body shapes in migratory fishes [3,4], wing loading  
42 patterns in migratory birds [5,6], and wing sizes and shapes in migratory insects [7,8].

43 Perhaps the best-known migratory insect is the monarch butterfly, whose seasonal  
44 migration spans thousands of kilometers across the North American continent. Past  
45 research has shown that migratory and non-migratory populations of monarch butterfly  
46 have highly divergent wing morphologies, with migrants typically having larger, more

47 elongated wings than non-migrants [8-11]. This pattern has been interpreted as natural  
48 selection operating on a tradeoff between long-distance flight in migrants and short-  
49 distance flights in summer breeding and non-migratory populations. Large, elongated  
50 wings are thought to aid in gliding flight and facilitate long-distance movements in  
51 migratory monarchs [8], whereas smaller, rounder wings are associated with  
52 maneuverability that may be more important for summer-breeding or non-migratory  
53 monarchs [10].

54 In addition to wing morphological differences between residents and migrants,  
55 studies have shown correlations between timing of migration, wing coloration, and wing  
56 size in North American migrants [12-15]. Likewise, two recently published studies using  
57 stable isotope data have shown that butterflies that migrate longer distances to  
58 overwintering sites have larger forewings [16,17]. An earlier study by Altizer and Davis  
59 (2010) also found differences in forewing area between eastern and western North  
60 American monarchs, which they attribute to differences in migration distance [8]. These  
61 results have been interpreted as migration acting as a selective episode on wing  
62 morphology, such that the largest and most capable long-distance migrants  
63 disproportionately reach overwintering grounds.

64 A number of factors are clearly important determinants of wing morphology in  
65 monarchs: for example, males are consistently larger than females, and as noted above,  
66 migration distance to overwintering sites seems to be positively correlated with forewing  
67 area. While these findings provide a useful starting point for understanding wing  
68 morphological variation among North American migrants, a number of other potentially  
69 important factors have yet to be fully considered. For example, the extent to which wing

70 morphology is phenotypically plastic is not well-studied, and few studies have considered  
71 how factors such as larval photoperiod and host plant identity might impact adult wing  
72 morphology (but see refs. 18,19).

73 One approach to understanding this variation is by conducting experimental  
74 manipulations to test specific hypotheses about how certain factors influence monarch  
75 wing morphology. Another approach is to take advantage of the extensive museum  
76 collections of North American monarchs and their associated metadata to build a  
77 comprehensive model that allows for testing multiple hypotheses about wing  
78 morphological variation. While the latter approach has the obvious shortcoming of  
79 integrating over many sources of uncertainty, an advantage is that it enables the evaluation  
80 of certain hypotheses that could not otherwise be tested, such as possible changes through  
81 time, both within and across years.

82 In this study, we combine data from museum specimens and contemporary  
83 collections of monarch butterflies to create a database of more than 1800 North American  
84 individuals. We then use linear mixed effects models to understand sources of variation in  
85 monarch forewing morphology. Specifically, we evaluate the effects of butterfly sex,  
86 overwintering status, latitude of collection, year of collection, photoperiod at time of  
87 collection, and membership in the eastern or western migratory population as predictors  
88 of forewing size and shape. We then compare a subset of our data to a recently published  
89 dataset of Mexican overwintering monarchs [17] to compare trends through time. Finally,  
90 we conduct a common garden rearing experiment using a split brood design to understand  
91 the impacts of larval host plant on adult wing morphological variation.

92

93

## **Methods**

94

### *Data Collection*

95 Specimens included in this study are primarily from museum collections, with some  
96 butterflies coming from previously published papers and others from contemporary  
97 collections of North American individuals (Table 1). For museum specimens, butterflies  
98 were photographed using either a Panasonic Lumix FZ80 (Panasonic Corp., Osaka, Japan)  
99 or Nikon D7100 (Nikon Corp., Tokyo, Japan) camera mounted on a camera stand or tripod.  
100 All images included a scale bar, and butterflies were placed so that forewings were in the  
101 same horizontal plane as this scale bar (Figure 1). For some individuals, wings were not  
102 spread evenly during pinning; in these cases, we angled the butterfly such that only one  
103 pair of wings was planar with the scale bar. Butterflies were photographed individually and  
104 were positioned to be at the center of the image to minimize possible effects of radial  
105 distortion. For contemporary butterfly collections, wings were clipped at the attachment  
106 point to the thorax and imaged using a flatbed scanner (Canon LiDE 120, Canon U.S.A., Inc.,  
107 Melville, NY) with a scale bar. In total, we included 1804 North American butterflies from  
108 16 museum and private collections (Figure 2, Table 1).

109 All images were measured using the image processing software ImageJ [20]. Briefly,  
110 images were scale calibrated, and then measurements were taken for both left and right  
111 forewings. We measured forewing length and width in the same way as previously  
112 published studies [8,16,17]. We manually defined the outline of monarch forewings by  
113 tracing a thin white line around the forewing margin in areas where forewing and  
114 hindwing overlapped. We then converted photos into 8-bit black/white images and used  
115 the wand tool to select the outline of each forewing. Perimeter was measured either by

116 fitting a cubic spline to the edge of this shape using the ‘Spline Fit’ feature, or by using the  
117 ‘Interpolate’ feature and selecting an interval of 25-40 pixels depending on the pixel  
118 density of the image; in both cases, this was done to minimize noise associated with the  
119 delineation of the wing outline. Forewing area was calculated as the area within this  
120 smoothed outline. In some cases where monarch forewings had minor damage that caused  
121 discontinuities in the outline, we manually corrected the outline of the wing. In cases where  
122 damage was more severe and the wing outline would have required more extensive  
123 interpolation, we omitted wings from analysis. Processing of scanned wings was performed  
124 in the same way as described for photographs. In most cases, both left and right forewings  
125 were measured for length, width, perimeter, and area. These values were used to calculate  
126 forewing aspect ratio and roundness as described in Altizer and Davis (2010) [8]. Briefly,  
127 higher values of roundness correspond to less elongated, more circular wing shapes, and  
128 we refer to wing shape from hereon in terms of wing elongation. Left and right wing  
129 measurements were then averaged into a single mean value for each butterfly.

130 For each specimen, we recorded the sex of the butterfly and all relevant locality  
131 information as well as the date of collection. This locality information was used to generate  
132 the latitude and longitude of collection for each individual using the geocode function in the  
133 ggmap package [21]. We did not record the elevation of collection, as this was not recorded  
134 for most specimens. Likewise, only a small number of specimens included information on  
135 possible larval host plant species. Specimens labeled as ‘ex-ovum’ or ‘ex-pupa’ were  
136 omitted from analysis. For overwintering status, we recorded butterflies as belonging to an  
137 overwintering population if they were collected from a known overwintering site between  
138 November 1 – February 15, or if the specimen label explicitly indicated membership in an

139 overwintering population. Butterflies were recorded as belonging to eastern or western  
140 North America based on the longitude of collection, with all individuals from west of  
141 110°W treated as western North American (see Figure 2).

142 For collection dates, we included the year, month, and day of collection when  
143 available. When month and day of collection were available, we calculated the Julian date of  
144 collection. Using the Julian date of collection, we created an index for photoperiod at the  
145 time of collection (hereby daylength index), with the minimum and maximum values at the  
146 winter and summer solstices, respectively. We included this term to account for the  
147 possibility that larval photoperiod might influence adult wing morphology [19], though we  
148 acknowledge that there may be substantial time lags between larval development and date  
149 of collection for adults. For a distribution of specimens across Julian dates and a visual  
150 representation of the daylength index, see Figure S1.

151

#### 152 *Data Analysis*

153 All data were analyzed using linear mixed models in the lme4 package [22] in R  
154 version 3.4.4 [23]. We used wing area as the response variable for analyses addressing  
155 forewing size, as this value was almost perfectly correlated with wing length ( $R^2 = 0.925$ )  
156 and width ( $R^2 = 0.945$ ) (see Figure S2). For analyses of wing shape, we used roundness as  
157 our response variable. All continuous predictor variables were centered and scaled. Since  
158 we were primarily interested in variation within North American migrants, we omitted  
159 non-migratory butterflies from Florida by excluding Florida specimens collected further  
160 south than 28°N (see Figure 2).

161 In all models, we included collection ID as a random intercept to account for  
162 possible discrepancies between collections in image generation or scale calibration. We  
163 also included a random intercept term for the state of collection (e.g. Alabama vs. Arkansas  
164 vs. California, etc.) to partially account for spatial non-independence of sampling in our  
165 dataset. For fixed effects, we included butterfly sex, latitude of collection, overwintering  
166 status, membership in eastern versus western North America, and daylength index. We  
167 also include an interaction term between overwintering status and eastern versus western  
168 North America, based on results from previous studies about migration distance acting as a  
169 selective filter on wing morphology.

170 We compared our data to the recently published dataset in Flockhart *et al.* (2017)  
171 [17]. Their dataset comprised 613 overwintering butterflies from a number of Mexican  
172 overwintering sites, spanning the years 1974-2014. Using their online supplementary  
173 information, we re-analyzed their data with a simple linear model that evaluated wing area  
174 as a function of migration distance (using the distance to centroid measure), sex, and year  
175 of collection. For comparison between datasets, we restricted our results to the same time  
176 period (1974-2014) and included only non-overwintering individuals ( $n = 493$ ) to  
177 determine whether wing morphology for overwintering and summer-breeding butterflies  
178 showed similar patterns over this time period.

179

180 *Host plant experiment*

181 As part of a different experiment, we evaluated the contribution of larval host plant  
182 to adult forewing morphology. Milkweed species were chosen as part of an experiment  
183 designed to investigate patterns of local adaptation (e.g. monarch population x milkweed

184 species interactions) to host plants across the monarch's global range (Freedman *et al.*, in  
185 prep). As such, only two of the milkweed species included here are commonly encountered  
186 by monarchs in North America. We grew *Asclepias syriaca* as our eastern North American  
187 host plant, *A. fascicularis* as our western North American host plant, *A. curassavica* (Guam:  
188 Mariana Islands), *Gomphocarpus physocarpus* (Maui: Hawaiian Islands), and *G. fruticosus*  
189 (Queensland: Australia). For the purposes of analysis, we combine results from the two  
190 *Gomphocarpus* species, as they are close relatives that potentially hybridize in their native  
191 and introduced ranges [24,25], and are virtually indistinguishable outside of minor  
192 differences in fruit and flower morphology [25]. For a summary of monarch populations,  
193 number of maternal families, and host plant species tested, see Table S1.

194 We reared approximately 225 caterpillars from 25 maternal families in mesh bags  
195 on live host plants (*i.e.* not clipped leaves) grown from seed in a greenhouse. We then  
196 collected adult butterflies that eclosed from each host plant and recorded their wing  
197 morphology in the same way as described above.

198 To evaluate these data, we used a linear mixed model with wing area or elongation  
199 as our response variables. We treated maternal family as well as greenhouse and  
200 greenhouse block as random intercept terms, with fixed effects for monarch population  
201 and host plant species. Comparisons within factor levels (*i.e.* pairwise differences between  
202 host plants and monarch populations) were made using Tukey HSD tests.

203

## 204 Results

205 As in previously published studies, male monarchs had forewings that were 1.8%  
206 larger than females ( $t = 6.41$ ,  $p < 0.001$ ) (Table 2), corresponding to an absolute difference

207 of  $15.9 \pm 2.5$  mm<sup>2</sup>. Overwintering butterflies were 4.4% larger than non-overwintering  
208 butterflies ( $t = 5.06$ ,  $p < 0.001$ ), and there was a significant interaction between  
209 overwintering status and presence in eastern versus western North America driven by  
210 discrepancies in the size of overwintering individuals ( $t = 2.24$ ,  $p = 0.029$ , Figure 3). Wing  
211 area increased with latitude ( $t = 2.80$ ,  $p = 0.008$ , Figure 4), such that monarchs collected  
212 from the northernmost extent of the migratory range were approximately 6% larger than  
213 monarchs from non-overwintering areas in the southernmost areas of the monarch's range.  
214 Butterflies also become larger through time ( $t = 2.23$ ,  $p = 0.026$ ), with wing area increasing  
215 by approximately 4.9% (an absolute difference of  $43.5 \pm 19.5$  mm<sup>2</sup>) between 1878-2017  
216 (Figure 5a).

217 In contrast to wing area, the only significant predictor for wing elongation was  
218 latitude, with butterflies collected at higher latitudes having significantly more elongated  
219 wings ( $t = 2.16$ ,  $p = 0.035$ ) (Table 2). Sex was a marginally significant predictor of wing  
220 elongation, such that males had slightly more elongated wings than females ( $t = 1.92$ ,  $p =$   
221 0.055). Butterflies collected during shorter daylengths had slightly more elongated wings ( $t$   
222 = 1.86,  $p = 0.063$ ) (Table 2).

223 When we reanalyzed the data in Flockhart *et al.* (2017) [17], we found an increase of  
224 1.9% for wing area between 1974-2014 ( $t = 2.06$ ,  $p = 0.040$ ) and 1.4% for wing length ( $t =$   
225 2.74,  $p = 0.006$ ) (Figure 5b), even after accounting for inferred migration distance. We  
226 found the same pattern when we restricted our main dataset to the same time period and  
227 included only non-overwintering butterflies, with a 5.5% increase through time for wing  
228 area between 1974-2014 ( $t = 2.26$ ,  $p = 0.026$ ) and a 2.9% increase for wing length ( $t =$   
229 2.18,  $p = 0.032$ ) (Figure 5c).

230 We found strong effects of larval host plant identity on adult wing size (Table 3).  
231 Specifically, monarchs had the largest forewings on *Asclepias syriaca* compared to other  
232 tested species, with forewings that were 8.7% larger than monarchs reared on *A.  
233 fascicularis* ( $z = 3.943$ ,  $p < 0.001$ ), 6.2% larger than monarchs reared on *A. curassavica* ( $z =$   
234 3.090,  $p = 0.010$ ), and 4.1% larger than monarchs reared on *Gomphocarpus spp.* ( $z = 2.374$ ,  
235  $p = 0.080$ ) (Figure 6). By contrast, there were no host plant effects on wing elongation  
236 (Table 3). Wing area and body mass were highly correlated (Figure S3).

237

## 238 Discussion

239 Migratory North American monarch butterflies show substantial variation in their  
240 wing morphology, although the factors contributing to this variation remain relatively  
241 poorly characterized. Here, we use a large dataset and show that sex, overwintering status,  
242 and latitude of collection all explain a substantial proportion of the observed variation in  
243 monarch forewing size. Additionally, we find a significant increase in the size of North  
244 American monarch forewings through time, a pattern that has not been observed before  
245 and that was corroborated by re-analysis of the data from Flockhart *et al.* (2017) [17].  
246 Finally, we show that larval host plant identity can have strong impacts on monarch wing  
247 morphology, with certain milkweed species such as *A. syriaca* supporting especially large  
248 butterflies.

249 The most consistent source of variation in our dataset was sex, with male butterflies  
250 having forewings that were approximately 1.8% larger and marginally more elongated  
251 than female butterflies. Sexual dimorphism in forewing size has been documented in  
252 numerous studies of monarchs [9] and has generally been interpreted as the result of

253 sexual selection favoring larger males, possibly due to increased ability to overcome female  
254 resistance to mating, female preference, or sperm competition [26,27]. Larger size in males  
255 could also be related to monarch mating behavior, which involves a lengthy copulation and  
256 post-nuptial flight period of up to 16 hours in which males fly with females attached [28].  
257 Male forewings were slightly more elongated than female forewings, a pattern that has not  
258 been shown before. This result seems to be partly driven by the inherent correlation  
259 between forewing area and forewing roundness ( $R^2 = 0.25$ , Figure S2), whereby larger  
260 forewings also tend to be more elongated.

261 One possible implication of the discrepancy in size between males and females is  
262 differing migration capabilities based on sex. This discrepancy in wing size could  
263 contribute to differing migration success between sexes, with males potentially better  
264 suited for long-distance migration. Although neither Yang *et al.* (2016) [16] nor Flockhart  
265 *et al.* (2017) [17] found sex-based differences in inferred migration distance, the  
266 increasingly male-biased sex ratio at overwintering sites in Mexico could potentially reflect  
267 sex-based differences in migration ability and/or distance [26]. However, this runs counter  
268 to tagging data from Steffy (2015) [29], which indicated that female monarchs have higher  
269 success in reaching Mexico than males.

270 The next largest source of variation was overwintering status, with monarchs  
271 collected from overwintering sites being significantly larger than those away from  
272 overwintering sites. This result is consistent with previous findings and supports the idea  
273 that long-distance migration to overwintering locations may act as a selective episode on  
274 wing morphology, such that only the most capable migrants eventually reach these sites  
275 [16,17]. An alternative explanation for this finding is that summer breeding butterflies that

276 develop under warmer temperatures and longer photoperiod might be smaller as a result  
277 of phenotypic plasticity. Temperature and photoperiod effects on adult size and wing  
278 morphology have been documented in monarchs [19,31]. However, the daylength index  
279 was only a modest predictor of wing size in our dataset (Table 2) and in the opposite  
280 direction predicted, with forewing size being slightly larger for butterflies collected during  
281 longer days, suggesting that photoperiod *per se* does not strongly drive patterns of wing  
282 morphological variation. Likewise, Flockhart *et al.* (2017) [17] did not find strong support  
283 for a statistical model that included temperature in the natal range as a predictor of adult  
284 wing morphology.

285 We found a significant interaction between overwintering status and membership in  
286 the eastern versus western North America population of butterflies. This pattern was  
287 driven by larger overwintering butterflies in eastern compared to western North America;  
288 by contrast, there were no apparent differences in wing morphology between eastern  
289 versus western summer breeding butterflies (Figure 3). Furthermore, when monarchs  
290 from eastern North America and California were reared together on the same host plants  
291 and in the same environment, we did not find any differences between butterflies from east  
292 versus west ( $z = 0.21$ ,  $p > 0.8$ ). This result makes sense in light of the recent insight that all  
293 North American monarchs form a randomly mating panmictic population, with enough  
294 exchange of migrants at Mexican overwintering sites to collapse any pattern of genetic  
295 differentiation [32,33]. These results suggest that it is the nearly ten-fold difference in  
296 average migration distance between eastern and western overwintering monarchs that  
297 drives differences in wing area [8], rather than any genetic differentiation between  
298 populations. Note, however, that all western overwintering butterflies included in our

299 study were collected in California, even though some portion of western summer breeders  
300 apparently migrate to Mexico in the autumn [34].

301 We also found a significant effect of latitude of collection for both wing area and  
302 elongation. This result contrasts with those of Li *et al.* (2016) [11], who did not find  
303 evidence for a latitudinal cline in wing morphology and instead suggested that migratory  
304 status rather than latitude drives patterns of monarch wing morphology. Latitudinal clines  
305 in body size related traits are ubiquitous across both endo- and ectotherms [35,36], with  
306 size typically increasing with latitude as we saw in our data. However, it is important to  
307 note that latitude of collection for adult monarchs may be a poor proxy for the latitude at  
308 which larvae developed, which is when adult wing morphology is determined. This may be  
309 especially true for butterflies collected during the spring and fall migration periods, as  
310 individuals collected during these intervals are more likely to have originated from distant  
311 locations.

312 There are multiple possible reasons for the latitudinal cline in wing morphology.  
313 The first and most parsimonious explanation is that migration acts as a selective filter  
314 during the spring re-migration away from overwintering sites, such that only the most  
315 capable migrants reach more northerly latitudes. A second hypothesis relates to  
316 development rate and temperature, whereby caterpillars that develop under cooler  
317 temperatures such as those that might be encountered at higher latitudes are more likely to  
318 grow large [35,36]; however, we again note that Flockhart *et al.* (2017) [17] found no  
319 relationship between temperature in the inferred natal range and wing area for Mexican  
320 overwintering butterflies. A final possible explanation is that host plants that occur further

321 north in the monarch's North American range support especially large butterflies, an  
322 explanation that we discuss later.

323 The final predictor that explained a substantial portion of the variation in North  
324 American monarch wing morphology was the year of collection, with butterflies increasing  
325 significantly in size over our sampling window. This pattern is intriguing and has not been  
326 documented for monarch butterflies before. This result was further corroborated by  
327 reanalyzing the data from Flockhart *et al.*'s (2017) [17] Mexican overwintering butterflies,  
328 which also show a significant increase in wing size through time. While the Flockhart paper  
329 chose to include year as a random effect to account for stochastic year-to-year variability  
330 (e.g. annual differences in climate suitability of breeding grounds), our overall dataset  
331 includes observations from 119 unique years, which should integrate over the impacts of  
332 any stochastic year-to-year variation. Both datasets indicate a significant size increase  
333 through time, although the magnitude of this increase seems to be greater in the summer  
334 breeding ( $1.18 \pm 0.54 \text{ mm}^2/\text{year}$ ) compared to overwintering butterflies ( $0.44 \pm 0.21$   
335  $\text{mm}^2/\text{year}$ ). In contrast, neither our data nor the Flockhart data suggest a change in  
336 forewing elongation through time. Combining our results with those of Flockhart *et al.*  
337 provides compelling evidence that the observed increase in size through time is not simply  
338 an artifact of small sample sizes or stochastic year-to-year variability.

339 In assessing the result of increased size through time, it is important to keep two  
340 things in mind: the first is that the absolute change in wing area over the sampling window  
341 is still relatively modest, corresponding to an overall increase of approximately 4.9% (43.6  
342  $\text{mm}^2$ ) over 140 years. By comparison, the difference in any given year between  
343 overwintering and summer-breeding butterflies is 4.4% (38.8  $\text{mm}^2$ ), and the average size

344 difference between a monarch collected in southern Texas versus southern Manitoba is  
345 4.7% ( $41.1 \text{ mm}^2$ ). More functional studies linking wing morphology and flight performance  
346 [e.g. 37,38] are needed to fully understand how biologically meaningful the increase in  
347 wing area may be. Second, there are a number of non-mutually exclusive explanations for  
348 the observed pattern of increased forewing size through time, and we do not claim to  
349 understand exactly why we see this pattern.

350 One possible explanation is that there is ongoing directional selection for increased  
351 forewing size, whereby monarchs that are larger have a relative fitness advantage and are  
352 therefore shifting the overall mean phenotypic value towards larger wing sizes. However,  
353 monarchs have been migratory over large spatial scales within North America for an  
354 estimated 20,000 years (and probably longer) [39,40], so it is not immediately clear why  
355 they would not have already been near their optimum wing morphological phenotype.  
356 Another possible explanation is that the increased size through time reflects a change in  
357 the natal origins of monarchs over the sampling window, such that monarchs reaching the  
358 overwintering grounds and subsequently recolonizing North America are increasingly  
359 derived from more distant locations. Flockhart et al. (2017b) [41] found no evidence for the  
360 hypothesis that natal origins changed during the time period between 1974-2014.  
361 However, other studies have suggested that the monarch's breeding range may be shifting  
362 northward [42], and there is evidence that the pace of the monarch's southward migration  
363 is becoming faster, potentially as a consequence of a northward range shift [43].

364 Yet another possible reason for the increase in size through time is that the  
365 assemblage of milkweed host plants in North America has changed over the sampling  
366 window. While the absolute abundance of milkweeds has likely decreased precipitously

367 throughout part of the monarch's breeding range in the past two to three decades [44,45],  
368 less is known about the relative abundance of individual milkweed species. If species such  
369 as *A. syriaca* that support especially large adult butterflies have become relatively more  
370 frequent over the sampling window, this could yield an apparent increase in size for the  
371 overall population. This hypothesis is supported by a recent analysis of herbarium records  
372 for ten common North American milkweeds, including more than 3000 records of *A. syriaca*  
373 from 1900-present [46]. While overall milkweed abundance declined over the sampling  
374 window (especially since 1940), the relative abundance of *A. syriaca* increased during this  
375 time.

376 Lastly, the observed increase in monarch size may also be attributable to climate  
377 change, which could influence monarch forewing size in at least two ways. First, climate  
378 change may make areas of the southern and central U.S. unsuitable for summer breeding  
379 monarchs due to thermal constraints on caterpillar development [42,47-49], thereby  
380 forcing monarchs further north during the summer and leading to fall migrants that must  
381 migrate longer distances. Second, climate change may be expanding the northern range  
382 edge of some North American milkweeds such as *A. syriaca* and by extension expanding the  
383 northern extent of the monarch's North American range [42]. However, this latter  
384 hypothesis seems unlikely to generate a discernible change in monarch wing morphology  
385 given the relative rate at which milkweed species' ranges may be expanding compared to  
386 the overall distance covered by migratory monarchs. Comparison of contemporary records  
387 of *Asclepias* with those listed in Woodson (1954) [50] and those compiled by Boyle *et al.*  
388 [46] would be useful to understand how climate change may be shifting the northern range  
389 limit of certain milkweed species.

390 Our rearing experiment showed that larval host plant has a strong influence on  
391 adult wing morphology. This pattern has been documented in one other study [18], which  
392 showed that adult forewing length varies by up to 4.5 mm on co-occurring *Asclepias* species  
393 in Iowa. A difference of this magnitude is comparable or even larger than that observed  
394 between migratory and non-migratory populations of butterflies [8,11], suggesting a strong  
395 environmental component for variation in wing size. Host plant effects may also explain  
396 why there is a slight excess of small butterflies in the overall distribution of forewing areas  
397 for wild caught butterflies (Figure 3, Figure S2), as these individuals could reflect  
398 butterflies that developed on poor-quality milkweed species or caterpillars that pupated  
399 prematurely, potentially due to food limitation [51]. By contrast, we saw no impact of host  
400 plant identity on wing elongation, suggesting a smaller contribution of environmental  
401 influences for monarch wing shape measurements. The sequential nature of the monarch's  
402 spring re-migration brings it into contact with distinct assemblages of milkweed species as  
403 it moves north [42,52], and understanding how each of these species influences adult size  
404 would be a useful avenue for future research into wing morphological variation. Because  
405 our host plant experiment only included two North American milkweed species, evaluating  
406 the influence of milkweed species on migratory wing morphology will require more  
407 thorough investigation.

408 Our paper highlights the need for more careful consideration of the full range of  
409 factors that may contribute to variation in monarch butterfly wing morphology as well as  
410 the utility of using large datasets to test hypotheses. For example, our results contrast with  
411 previous published works suggesting that there are no latitudinal clines in North America  
412 [11] and that there are inherent size differences between eastern and western North

413 American butterflies [8]. Future studies that focus on naturally occurring patterns of  
414 variation in monarch wing morphology should be mindful of the myriad environmental  
415 influences (natal host plant species, developmental photoperiod and diapause status,  
416 developmental temperature) that may contribute to this variation. Finally, we highlight the  
417 tremendous utility of using museum collections [53,54], without which we could not have  
418 detected changes through time or achieved such a wide geographical range of samples.

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436

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Collection	Count	Collection Year Range
American Museum of Natural History	88	1908-1985
Bishop Museum of Hawaii	4	1953-1953
Bohart Museum (UC Davis)	199	1918-1999
California Academy of Sciences	256	1891-2001
Miscellaneous contemporary specimens	19	2016-2017
Cornell University Insect Collection	154	1915-1996
Mexican individuals - David M.	66	1978-2012
Essig Museum (UC Berkeley)	116	1919-2002
Harvard Museum of Comparative Zoology	73	1878-1984
Los Angeles County Museum of Natural History	190	1902-1996
Yang <i>et al.</i> (2016), <i>Ecography</i>	182	2011-2012
Li <i>et al.</i> (2016), <i>Animal Migration</i>	57	2007-2012
McGuire Center (University of Florida)	83	1928-2012
Personal Collection: Myron Zalucki	11	2009
Peabody Museum (Yale University)	19	1949-1978
UC Riverside Insect Collection	74	1917-2004
Smithsonian	109	1885-1989
<b>Cumulative total</b>	<b>1804</b>	<b>1878-2017</b>

**Table 1** – Summary of collections used in the current study. Butterflies from Yang *et al.* (2016) and Li *et al.* (2016) were the same images used in those studies, but re-measured according to our measurement protocols. Totals do not include butterflies from Florida collected at locations below 28 degrees of latitude (n = 56), as these are likely year-round breeding individuals.

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**(a). Response variable: Forewing area**

Predictor	DF	$\chi^2$	p
<b>Sex</b>	<b>1</b>	<b>41.02</b>	<b>&lt;0.001***</b>
<b>Year</b>	<b>1</b>	<b>4.95</b>	<b>0.026*</b>
<b>Overwintering status</b>	<b>1</b>	<b>25.65</b>	<b>&lt;0.001***</b>
<b>East vs. west</b>	<b>1</b>	<b>3.60</b>	<b>0.058</b>
<b>Latitude</b>	<b>1</b>	<b>7.82</b>	<b>0.005**</b>
<b>Daylength index</b>	<b>1</b>	<b>3.63</b>	<b>0.057</b>
<b>Overwintering status * East vs. west</b>	<b>1</b>	<b>5.01</b>	<b>0.025*</b>

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**(b). Response variable: Forewing elongation**

Predictor	DF	$\chi^2$	p
<b>Sex</b>	<b>1</b>	<b>3.68</b>	<b>0.055</b>
Year	1	1.83	0.176
Overwintering status	1	1.60	0.205
East vs. west	1	1.43	0.232
<b>Latitude</b>	<b>1</b>	<b>5.32</b>	<b>&lt;0.021*</b>
<b>Daylength index</b>	<b>1</b>	<b>3.47</b>	<b>0.062</b>
Overwintering status * East vs. west	1	0.97	0.325

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**Table 2** – ANOVA results for models assessing (a) forewing area and (b) elongation. Predictors with p<0.1 are shown in bold, with asterisks corresponding to levels of significance. Results were calculated using type III sums of squares in the car package [55].

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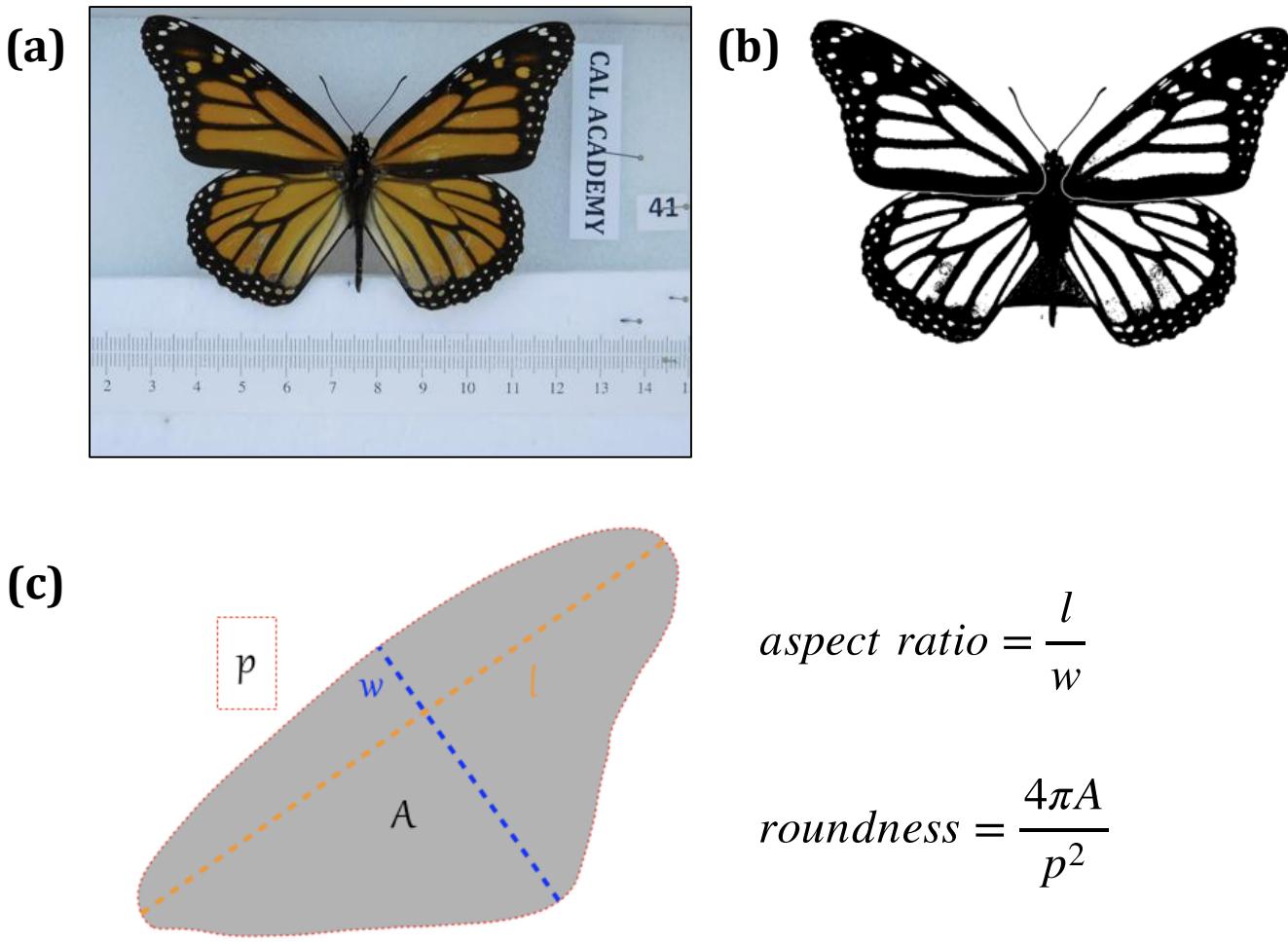
**(a). Response variable: Forewing area**

Predictor	DF	$\chi^2$	p
Milkweed species	3	<b>17.76</b>	<b>&lt;0.001***</b>
Sex	1	2.95	0.086
Monarch population	3	5.10	0.164

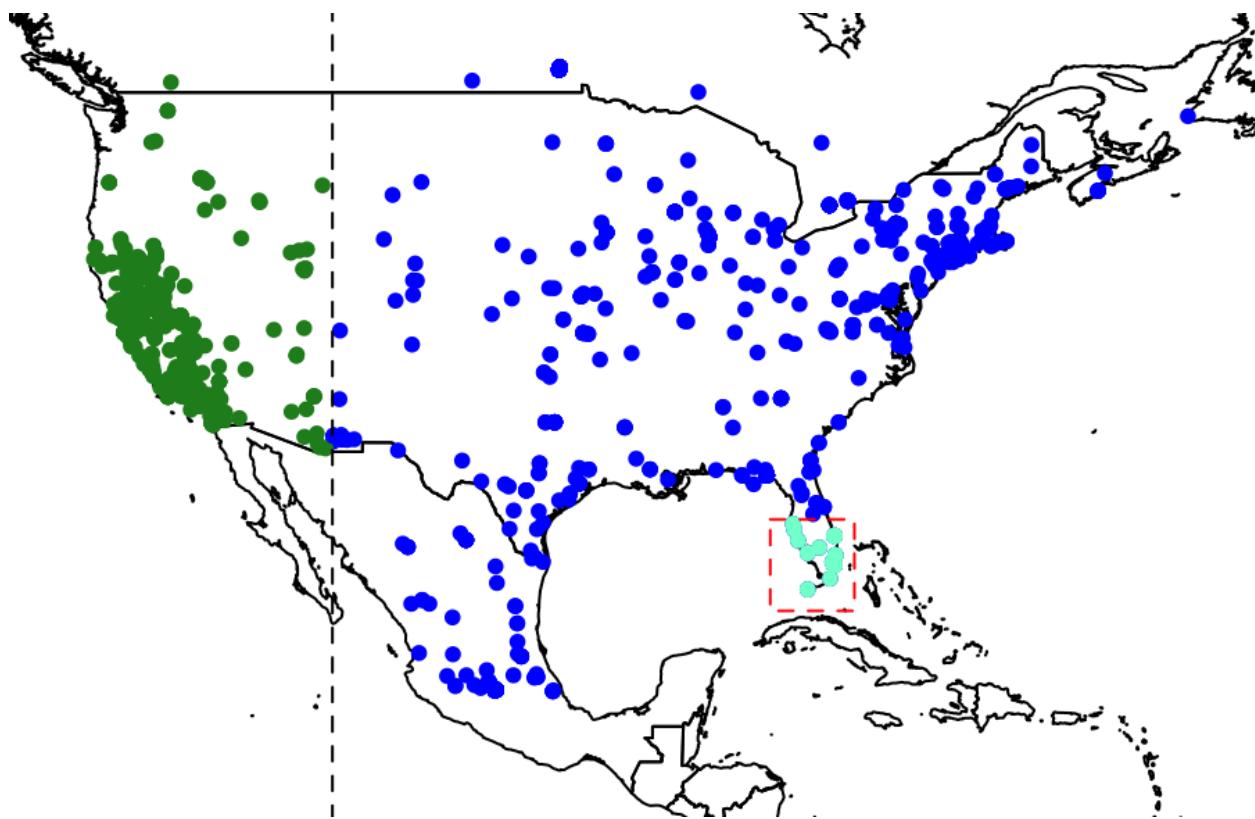
**(b). Response variable: Forewing elongation**

Predictor	DF	$\chi^2$	p
Milkweed species	3	5.23	0.155
Sex	1	<b>5.25</b>	<b>0.022*</b>
Monarch population	3	2.24	0.524

581  
582 **Table 3** – ANOVA results for experiment assessing the contribution of milkweed host plant, sex,  
and monarch population for (a) forewing size and (b) forewing roundness. Milkweed species  
contributes strongly to variation in forewing size but not roundness.

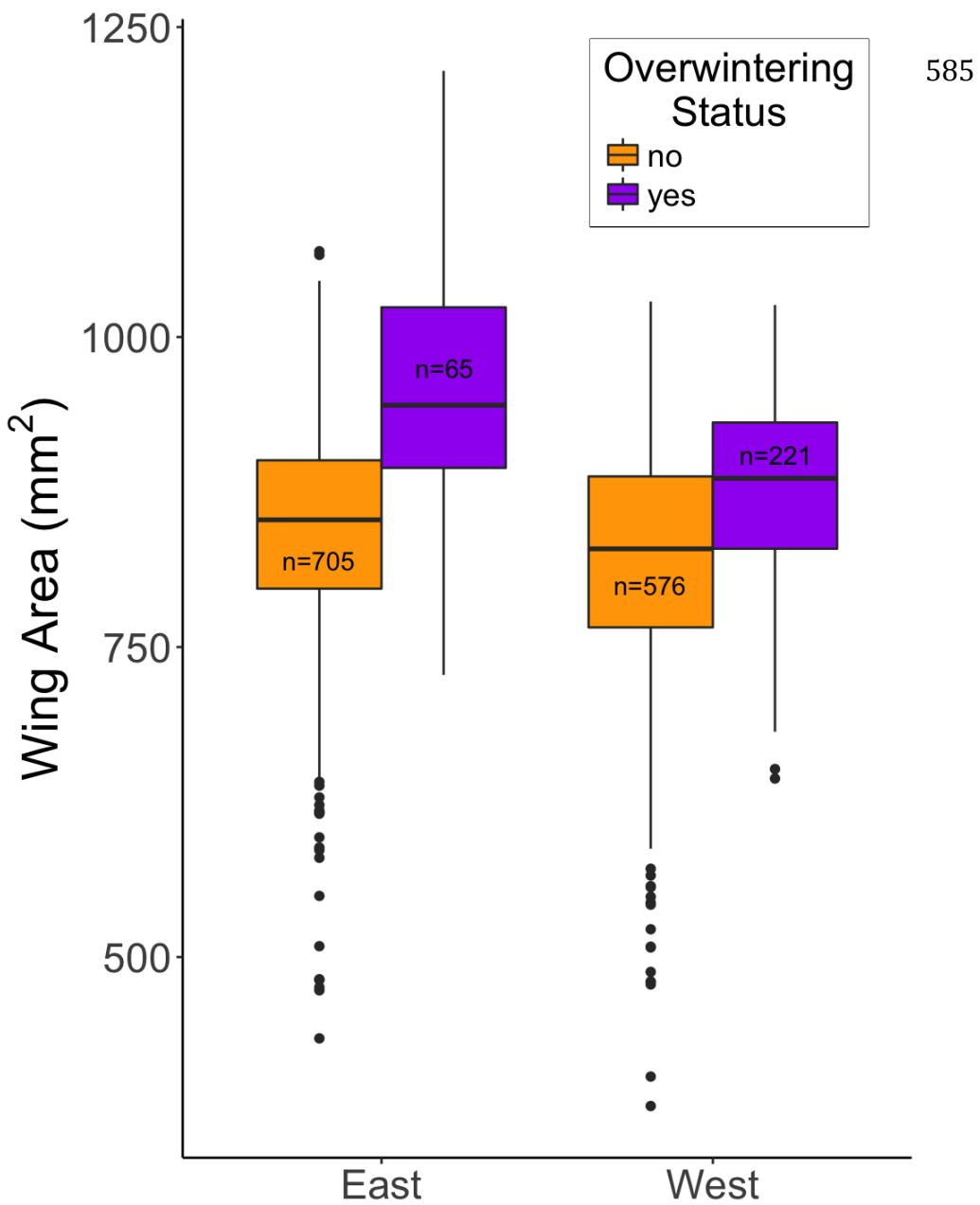


**Figure 1** – Example of image used for data generation. (a). Unaltered image, including scale bar, of a female monarch in the California Academy of Sciences collection. (b). 8-bit black and white image showing delineation of forewing outline. (c). Left forewing and the measurements taken from it. The red outline of the wing corresponds to a spline with 435 discrete points and was used to generate a smoothed estimate of wing perimeter. Length ( $l$ ), width ( $w$ ), perimeter ( $p$ ), and area ( $A$ ) were measured for both left and right forewings, with averages of these values used to generate measurements of aspect ratio and wing roundness for each individual butterfly. Throughout the text of this manuscript, we use roundness as our descriptor for forewing shape and use this term interchangeably with forewing elongation (greater forewing elongation corresponds to lower roundness).

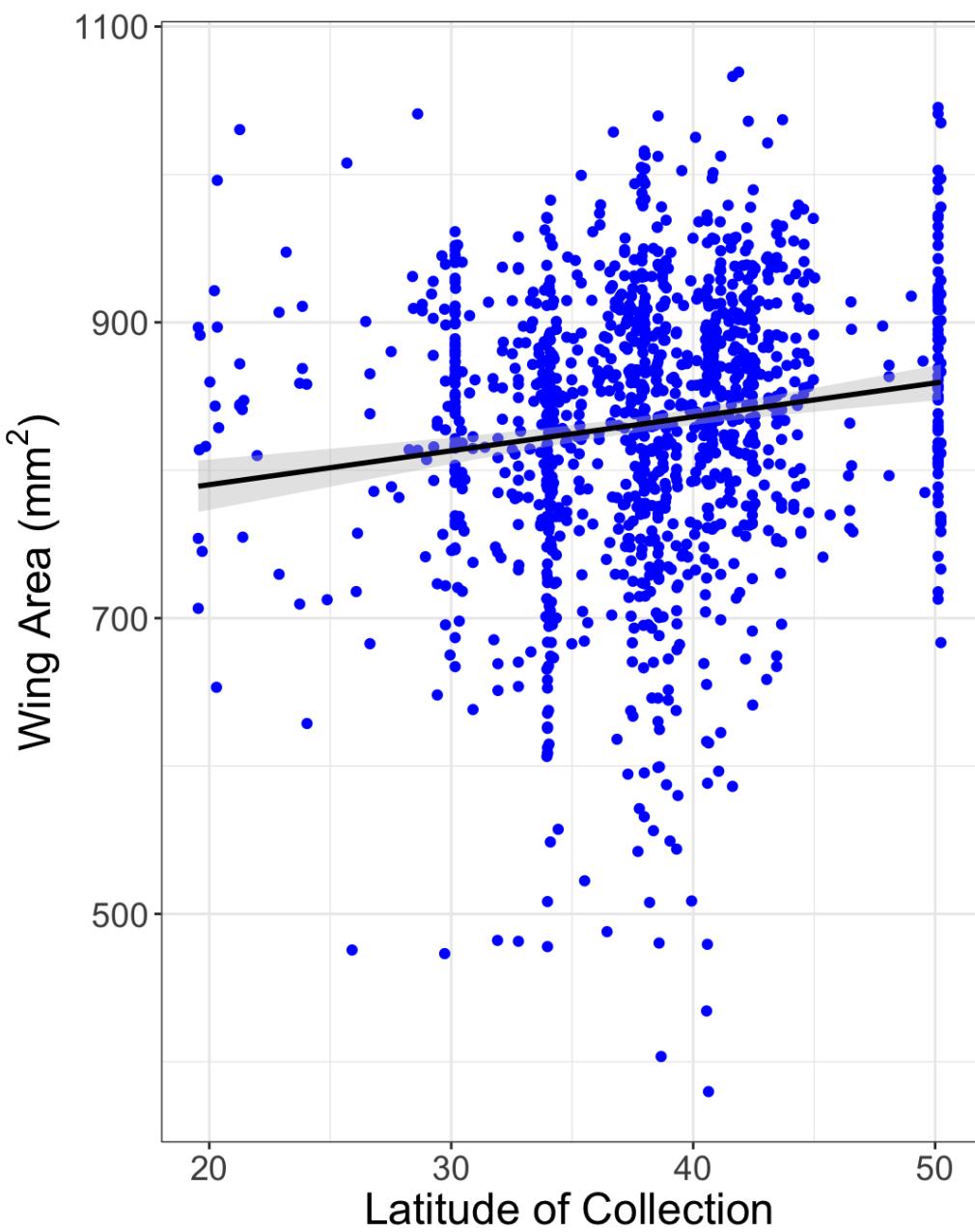


**Figure 2** – Map of sampling locations for butterflies included in the present study.

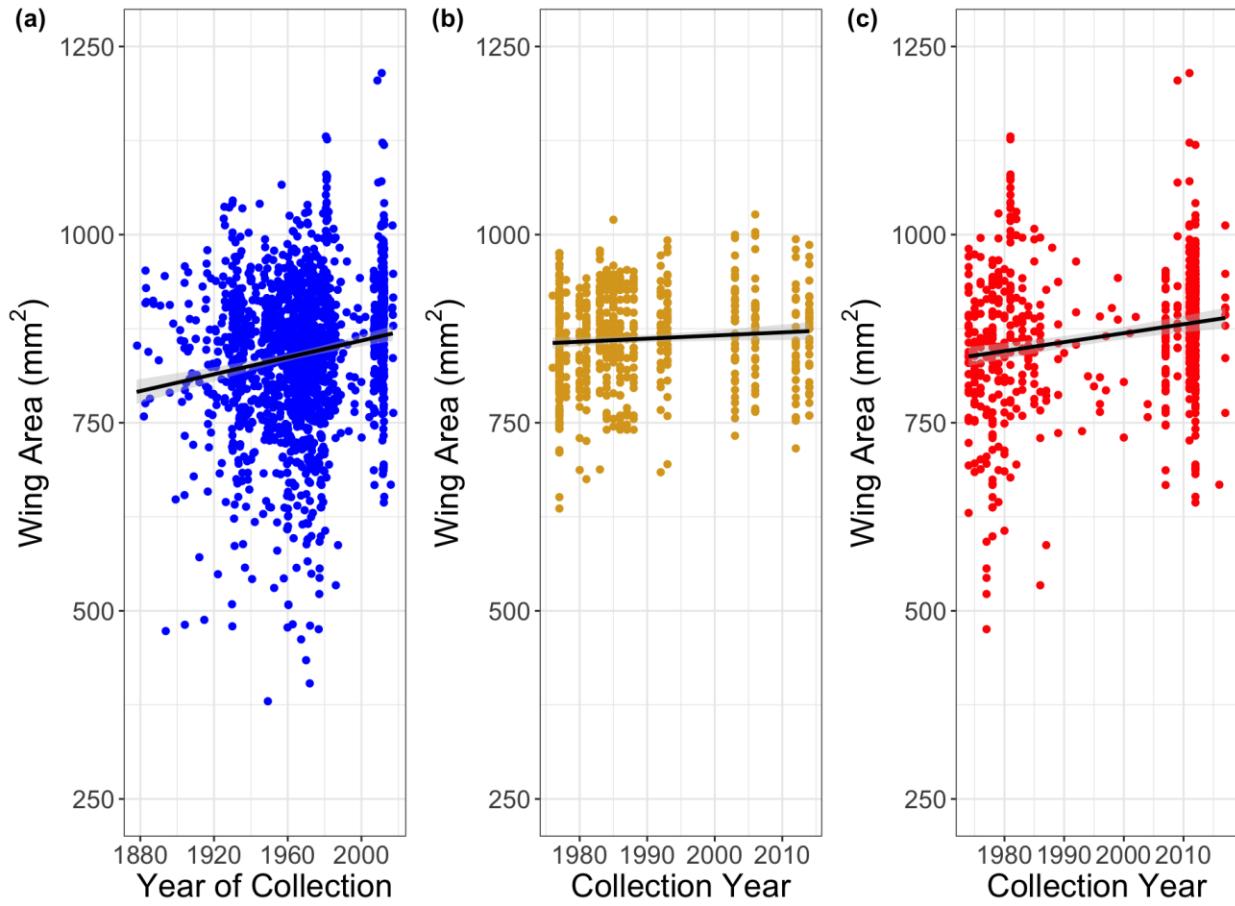
Eastern North American butterflies are colored in blue, while western butterflies are colored in dark green; the divide between them is shown as a dashed vertical line at 110°W. Non-migratory monarchs from south Florida are shown in aquamarine and were not included in analyses of continent-wide patterns.



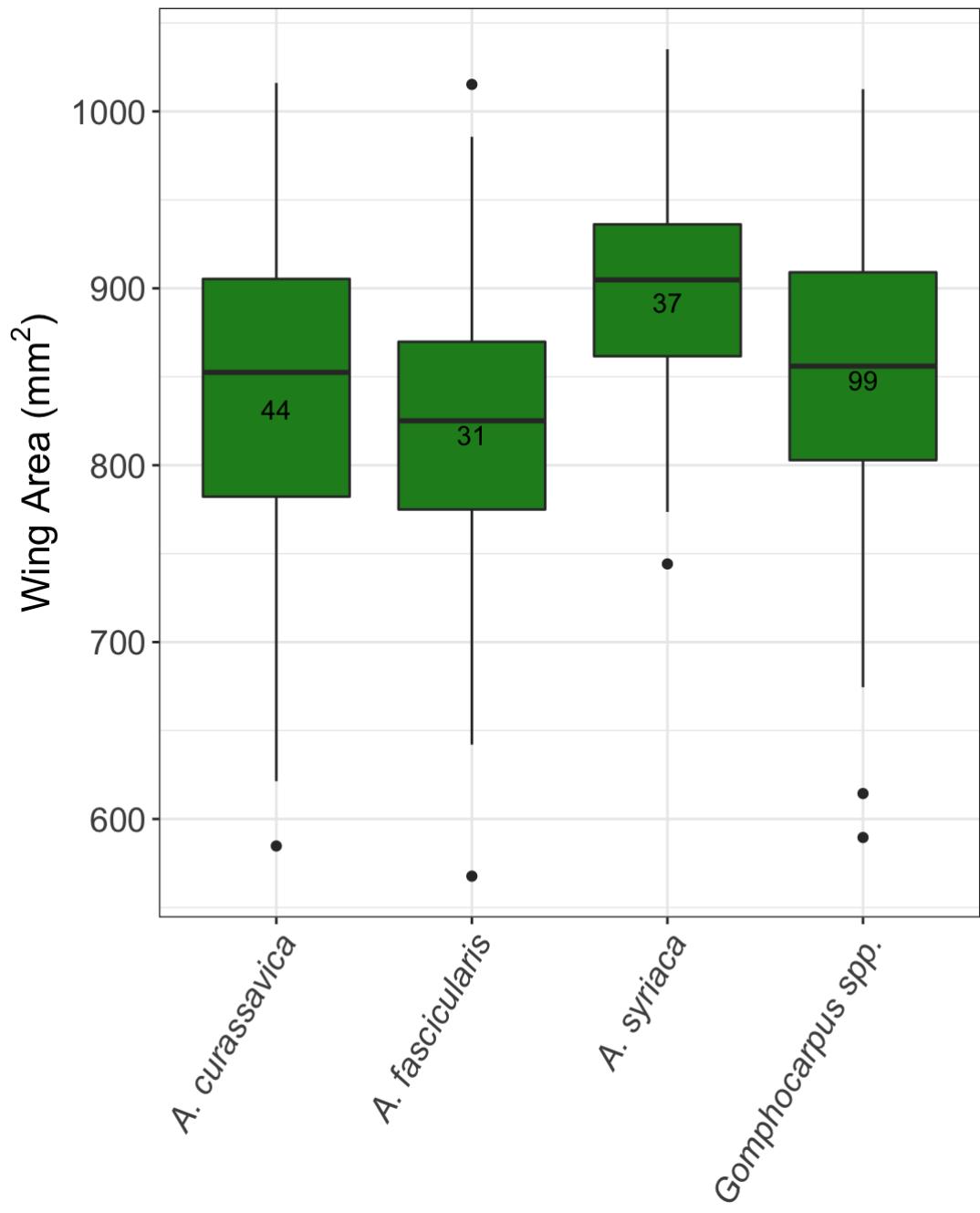
**Figure 3** – Overwintering individuals are significantly larger than non-overwintering individuals across the dataset. There was a significant interaction between overwintering status and membership in eastern versus western North America, such that summer breeding butterflies are approximately the same in both locations, but butterflies overwintering in Mexico are larger than butterflies overwintering in California.



**Figure 4** – Monarch forewing area increases significantly with latitude of collection. Points shown here do not include overwintering individuals. Shaded areas around trend lines correspond to 95% confidence intervals.



**Figure 5 –** (a) Our dataset, including all observations, shows a significant increase through time in wing area. (b) The Flockhart data using Mexican overwintering individuals also shows a significant increase in wing area through time. (c). Our data from summer breeding butterflies, restricted to the same time interval as the Flockhart data, also shows a significant increase through time. Points shown in (a) and (c) do not include overwintering individuals. Shaded areas around trend lines correspond to 95% confidence intervals.



**Figure 6** – Monarch forewing area varies substantially based on larval host plant. Adults reared on *A. syriaca* were significantly larger than adults reared on *A. curassavica* ( $p = 0.009$ ) and *A. fascicularis* ( $p < 0.001$ ), and marginally larger than adults reared on *Gomphocarpus* ( $p = 0.076$ ). Numbers inside bars correspond to pooled sample sizes for any particular host species. *Gomphocarpus spp.* includes both *Gomphocarpus fruticosus* and *G. physocarpus*.

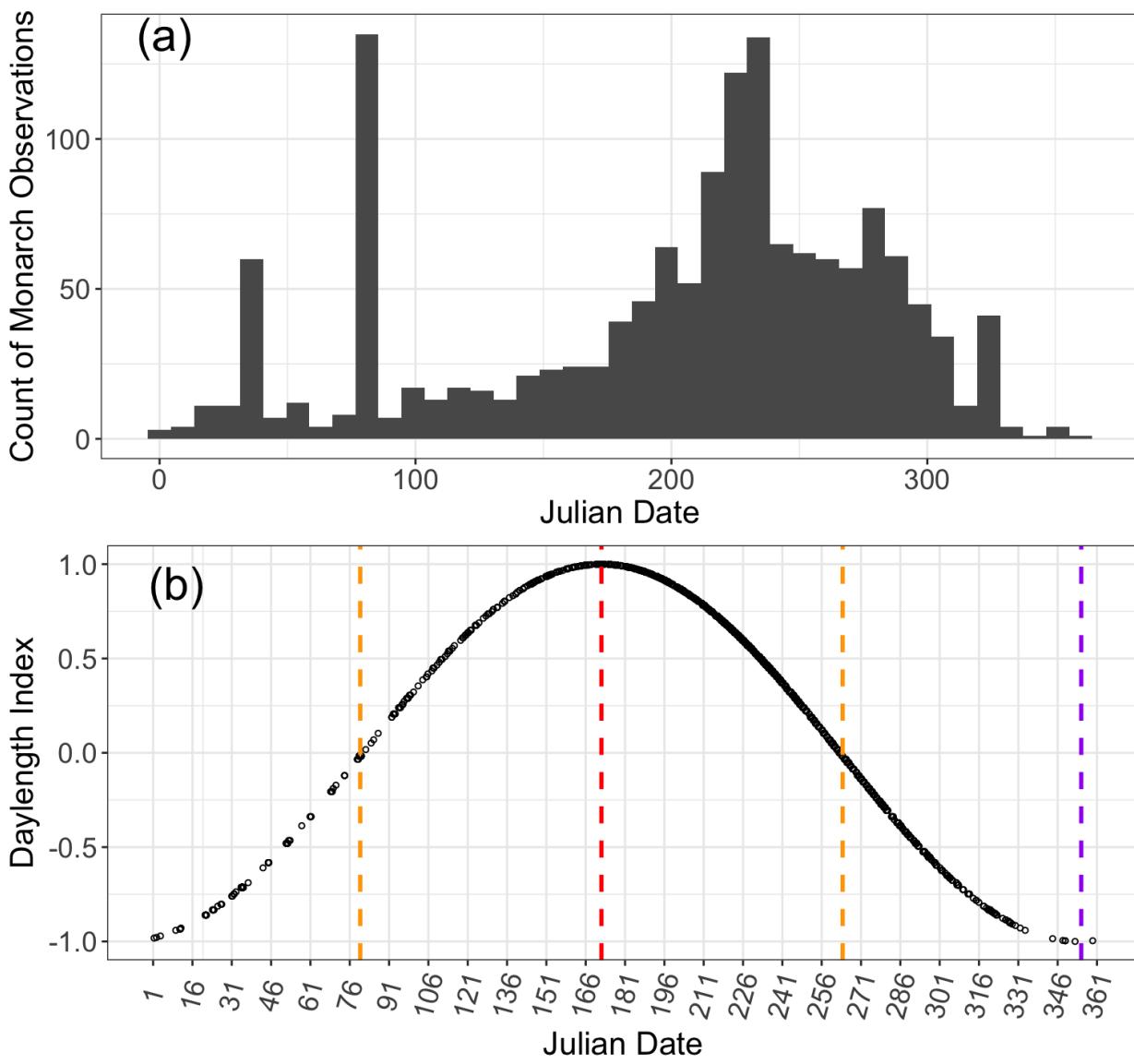
Monarch Population	Maternal Families	Larval Host Species			
		<i>Asclepias syriaca</i>	<i>Asclepias fascicularis</i>	<i>Asclepias curassavica</i>	<i>Gomphocarpus spp.</i>
<b>Eastern North America (IA, MI)</b>	7	10	8	12	23
<b>Western North America (CA)</b>	10	12	11	13	24
<b>Hawaii (Maui)</b>	7	7	6	13	25
<b>Australia (QLD)</b>	1	8	7	7	27
<b>Cumulative</b>	<b>25</b>	<b>37</b>	<b>32</b>	<b>45</b>	<b>99</b>

**Table S1** – Summary of host plant experiment. Monarch population refers to the source of collection for each population of monarchs tested. Maternal families refers to the number of maternal families tested for each monarch population. Numbers below each milkweed species correspond to the number of butterflies measured from each monarch population on each host plant. For *Gomphocarpus spp.*, we combine data from *Gomphocarpus* sourced from Australia and Hawaii, which likely represents a mix of *G. physocarpus* and *G. fruticosus*.

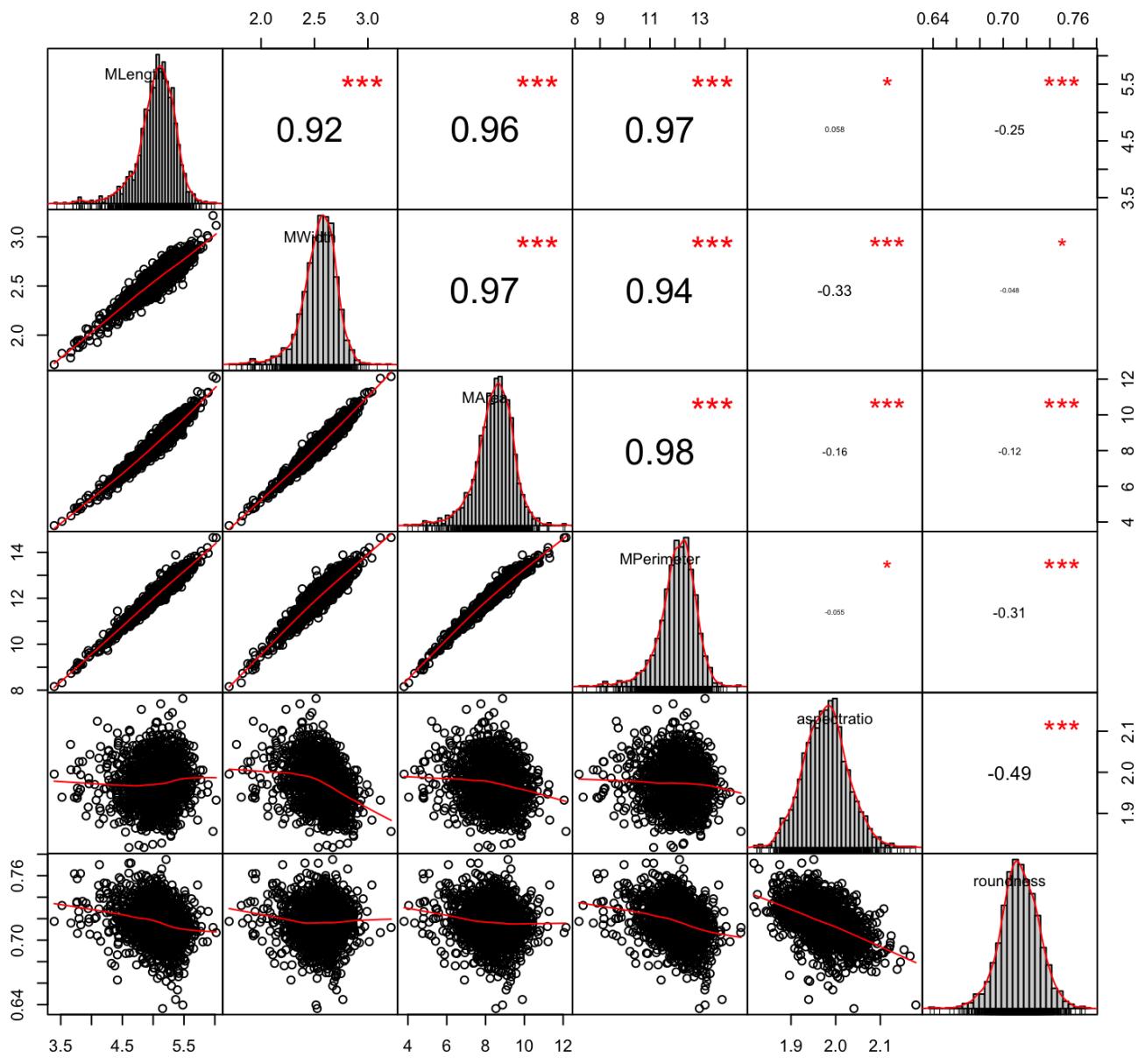
Wing size effects consistent with selection	Evidence for Effect
<p><b>Migratory filtering</b> – Long-distance migration acts as a selective episode that favors individuals with larger wings</p>	<ul style="list-style-type: none"> <li>• Migratory butterflies have larger wings than non-migratory butterflies [8-11].</li> <li>• Stable isotope data show a correlation between migration distance and forewing size [16,17].</li> <li>• Overwintering monarchs are larger than summer-breeding monarchs; Mexican overwintering monarchs larger than California overwintering monarchs (Fig. 3).</li> <li>• Butterflies from higher latitudes have larger wings (Fig. 4), possibly due to selection acting during spring re-migration.</li> </ul>
<p><b>Sexual selection</b> – Larger males may mate more and sire more offspring, either due to overcoming female resistance, female choice, or sperm competition</p>	<ul style="list-style-type: none"> <li>• Larger males mated more than smaller males in a cage experiment [26].</li> <li>• Sperm from larger males may have a fertilization advantage in multiply-mated females [27].</li> </ul>
Wing size effects consistent with phenotypic plasticity	Evidence for Effect
<p><b>Host plant effects</b> – Butterflies may attain different sizes depending on larval host plant (identity, quantity, quality)</p>	<ul style="list-style-type: none"> <li>• Adult forewing size varies as a function of larval host plant species (Fig. 6). Common milkweed (<i>A. syriaca</i>), which is the predominant host for monarchs that reach Mexican overwintering sites, produces large adult butterflies [18].</li> <li>• Food limitation may cause premature pupation and lead to small adults [50].</li> </ul>
<p><b>Temperature effects</b> – Butterflies that develop at lower temperatures are expected to have larger body size</p>	<ul style="list-style-type: none"> <li>• Limited evidence in monarchs (though see ref 30), but many other Lepidopterans show negative correlations between larval development temperature and adult body size.</li> </ul>
<p><b>Photoperiod effects</b> – Decreasing photoperiod leads to reproductive diapause and other migration-associated phenotypes</p>	<ul style="list-style-type: none"> <li>• Monarchs reared under decreasing photoperiod have larger wings and higher body mass than monarchs reared under constant photoperiod [19].</li> </ul>

593

**Table S2** – Summary of sources of monarch wing morphological variation.

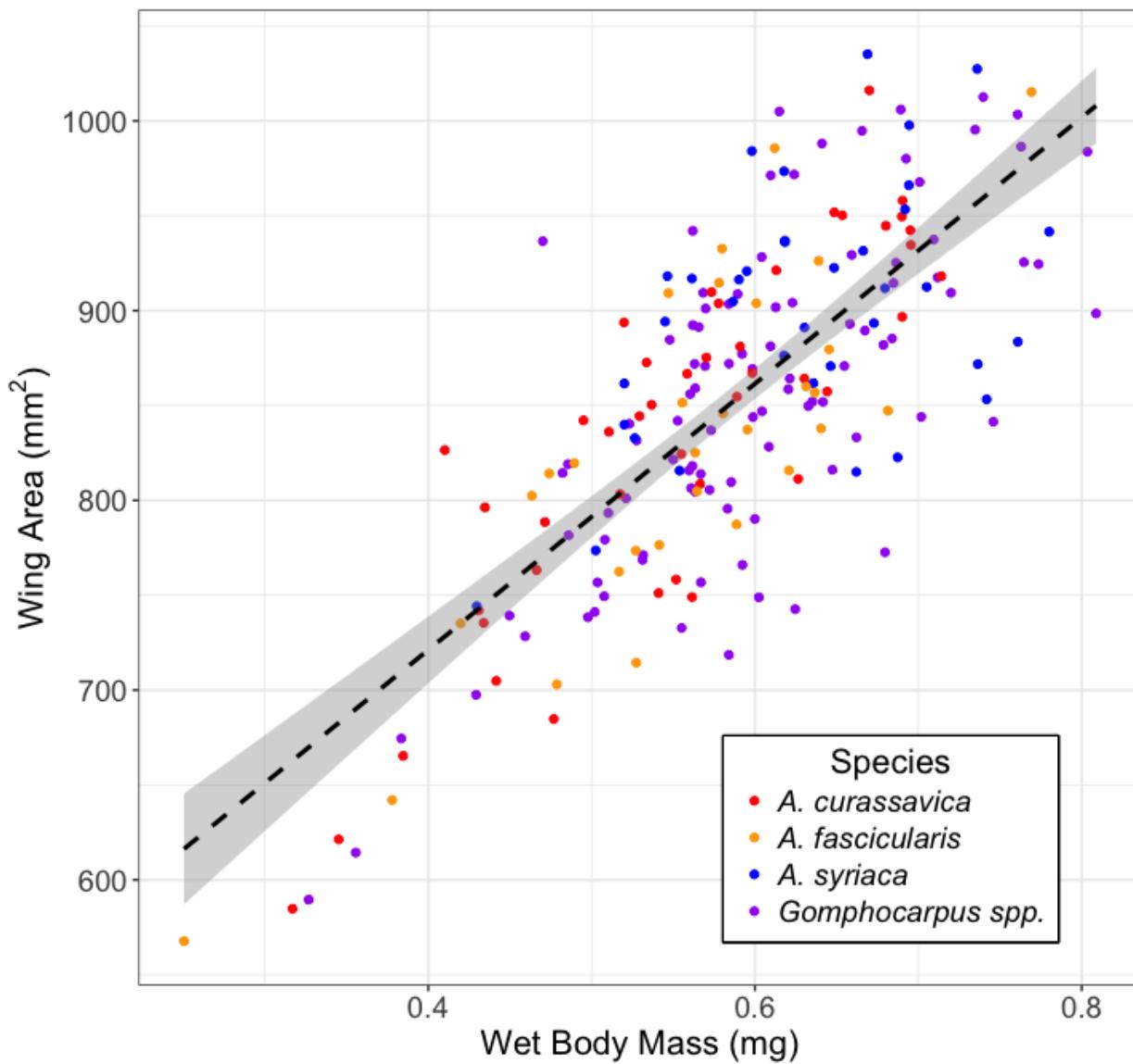


594



**Figure S2** – Correlations between all measured wing values. The diagonal of the matrix shows the distribution of values for each forewing measurement (mean of left and right forewings), in order from top left: length, width, area, perimeter, aspect ratio, roundness. Off-diagonal elements in the upper right show the  $R^2$  value for a simple linear regression between all pairwise combinations of measurements. When forewing area is included as a predictor in a model with wing roundness as the response variable, forewing area is indeed a significant predictor and reapportions variance previously associated with sex.

596



597

**Figure S3** – Wing area and body mass were highly correlated ( $R^2 = 0.57$ ,  $p < 0.001$ ) for butterflies reared on various host plant species, suggesting that wing area may be an appropriate proxy for wing loading.

Table 1

<b>Collection</b>	<b>Count</b>	<b>Collection Year Range</b>
<b>American Museum of Natural History</b>	88	1908-1985
<b>Bishop Museum of Hawaii</b>	4	1953-1953
<b>Bohart Museum (UC Davis)</b>	199	1918-1999
<b>California Academy of Sciences</b>	256	1891-2001
<b>Miscellaneous contemporary specimens</b>	19	2016-2017
<b>Cornell University Insect Collection</b>	154	1915-1996
<b>Mexican individuals - David M.</b>	66	1978-2012
<b>Essig Museum (UC Berkeley)</b>	116	1919-2002
<b>Harvard Museum of Comparative Zoology</b>	73	1878-1984
<b>Los Angeles County Museum of Natural History</b>	190	1902-1996
<b>Yang <i>et al.</i> (2016), Ecography</b>	182	2011-2012
<b>Li <i>et al.</i> (2016), Animal Migration</b>	57	2007-2012
<b>McGuire Center (University of Florida)</b>	83	1928-2012
<b>Personal Collection: Myron Zalucki</b>	11	2009
<b>Peabody Museum (Yale University)</b>	19	1949-1978
<b>UC Riverside Insect Collection</b>	74	1917-2004
<b>Smithsonian</b>	109	1885-1989
<b>Cumulative total</b>	<b>1804</b>	<b>1878-2017</b>

Table 2

<i>(a). Response variable: Forewing area</i>			
Predictor	DF	$\chi^2$	p
<b>Sex</b>	<b>1</b>	<b>41.02</b>	<b>&lt;0.001***</b>
<b>Year</b>	<b>1</b>	<b>4.95</b>	<b>0.026*</b>
<b>Overwintering status</b>	<b>1</b>	<b>25.65</b>	<b>&lt;0.001***</b>
<b>East vs. west</b>	<b>1</b>	<b>3.60</b>	<b>0.058</b>
<b>Latitude</b>	<b>1</b>	<b>7.82</b>	<b>0.005**</b>
<b>Daylength index</b>	<b>1</b>	<b>3.63</b>	<b>0.057</b>
<b>Overwintering status * East vs. west</b>	<b>1</b>	<b>5.01</b>	<b>0.025*</b>

<i>(b). Response variable: Forewing elongation</i>			
Predictor	DF	$\chi^2$	p
<b>Sex</b>	<b>1</b>	<b>3.68</b>	<b>0.055</b>
Year	1	1.83	0.176
Overwintering status	1	1.60	0.205
East vs. west	1	1.43	0.232
<b>Latitude</b>	<b>1</b>	<b>5.32</b>	<b>&lt;0.021*</b>
<b>Daylength index</b>	<b>1</b>	<b>3.47</b>	<b>0.062</b>
Overwintering status * East vs. west	1	0.97	0.325

Table 3

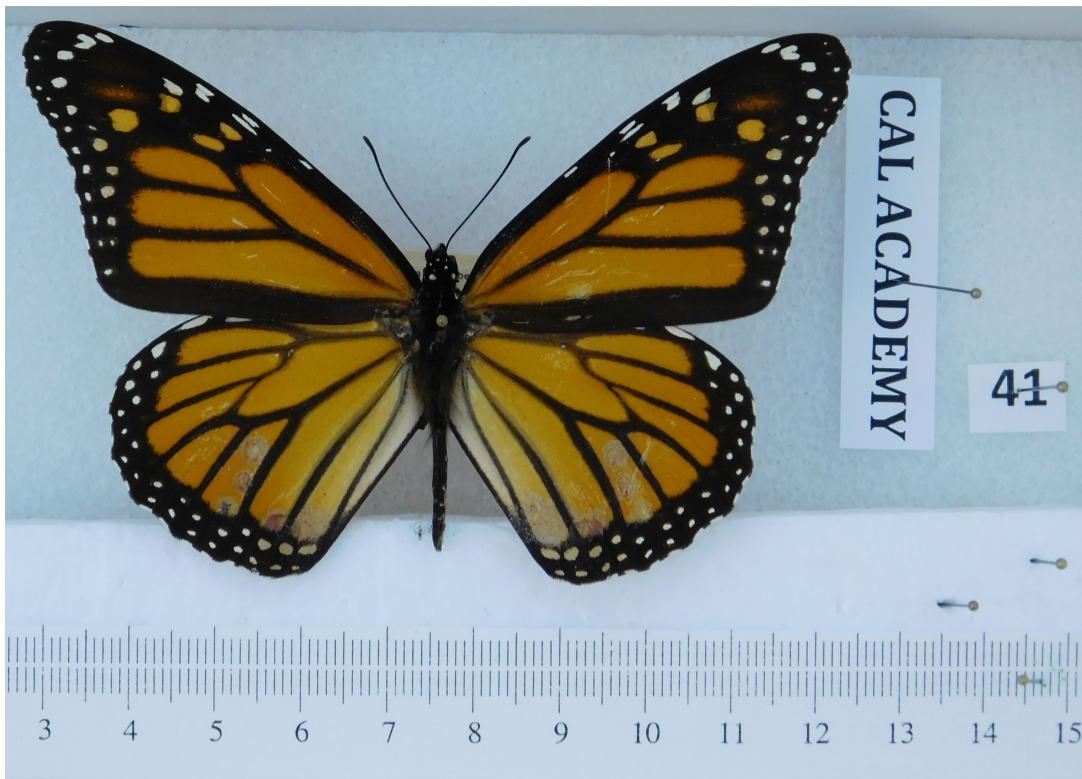
<b>(a). Response variable: Forewing area</b>			
Predictor	DF	$\chi^2$	p
<b>Milkweed species</b>	3	<b>17.76</b>	<b>&lt;0.001***</b>
Sex	1	2.95	0.086
Monarch population	3	5.10	0.164

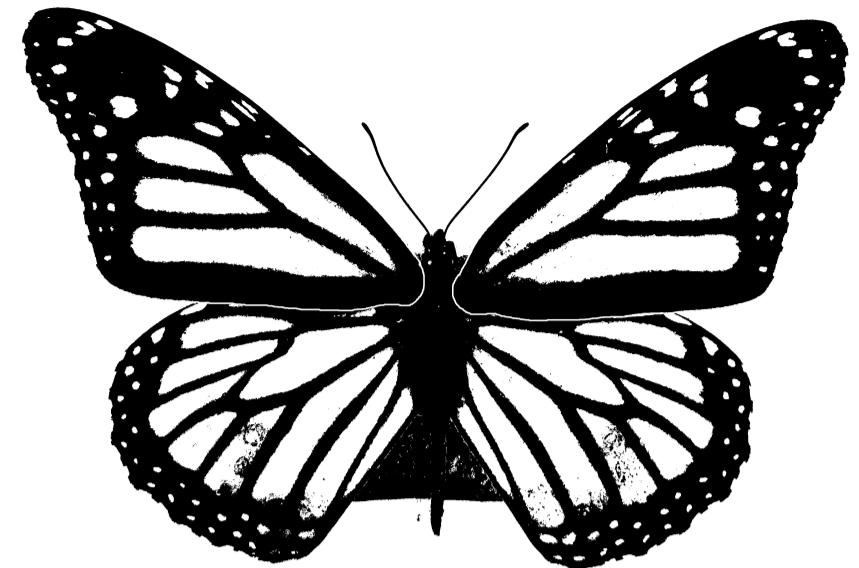
<b>(b). Response variable: Forewing elongation</b>			
Predictor	DF	$\chi^2$	p
Milkweed species	3	5.23	0.155
<b>Sex</b>	<b>1</b>	<b>5.25</b>	<b>0.022*</b>
Monarch population	3	2.24	0.524

Figure 1

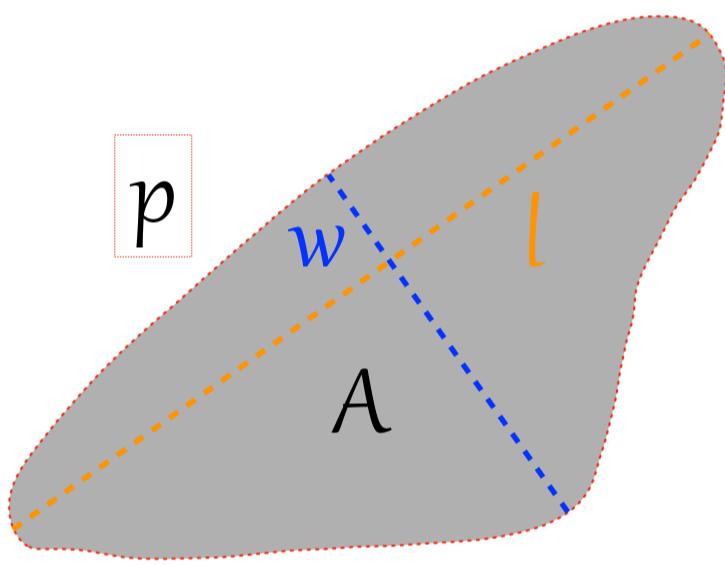
(a)



(b)



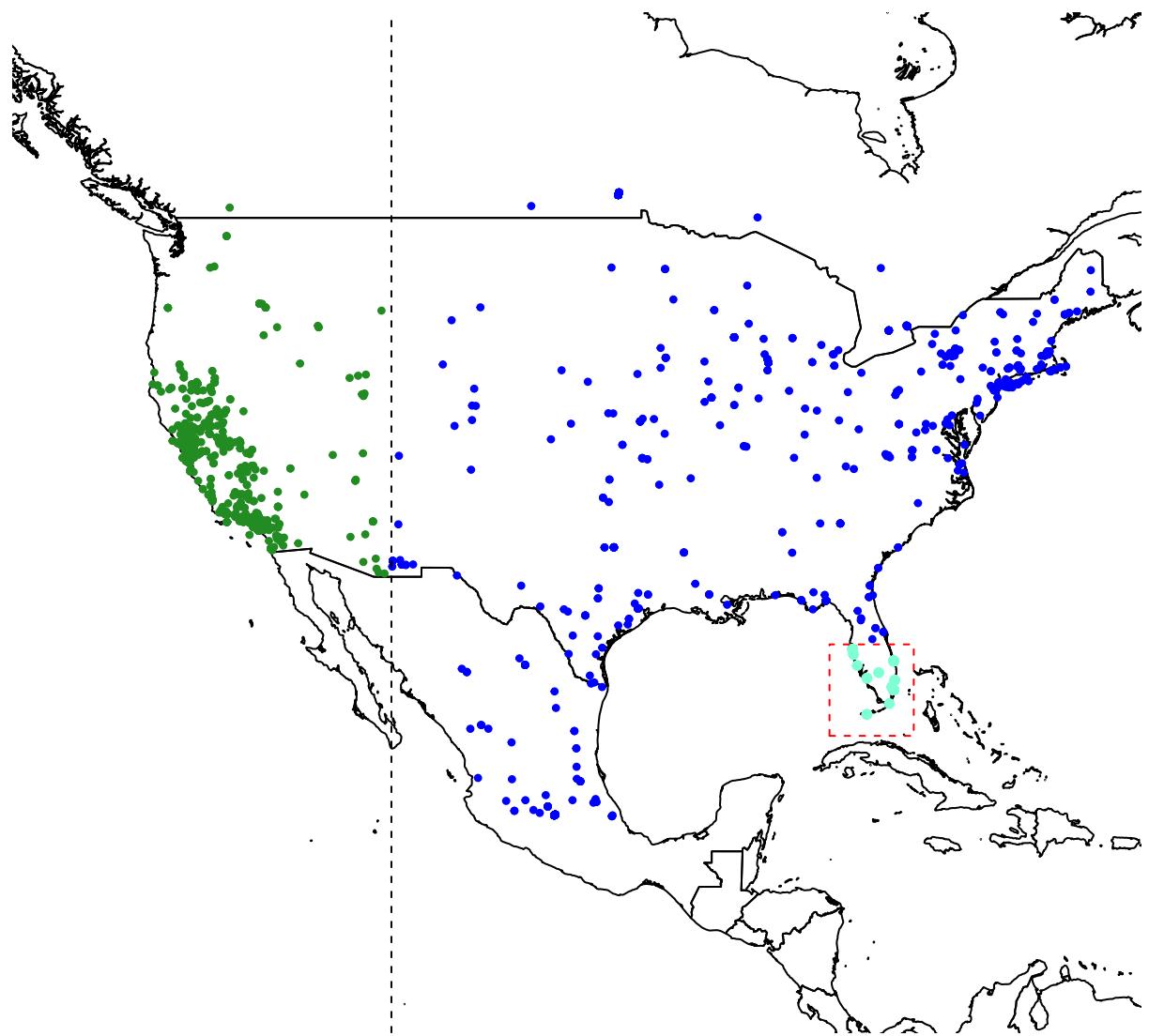
(c)

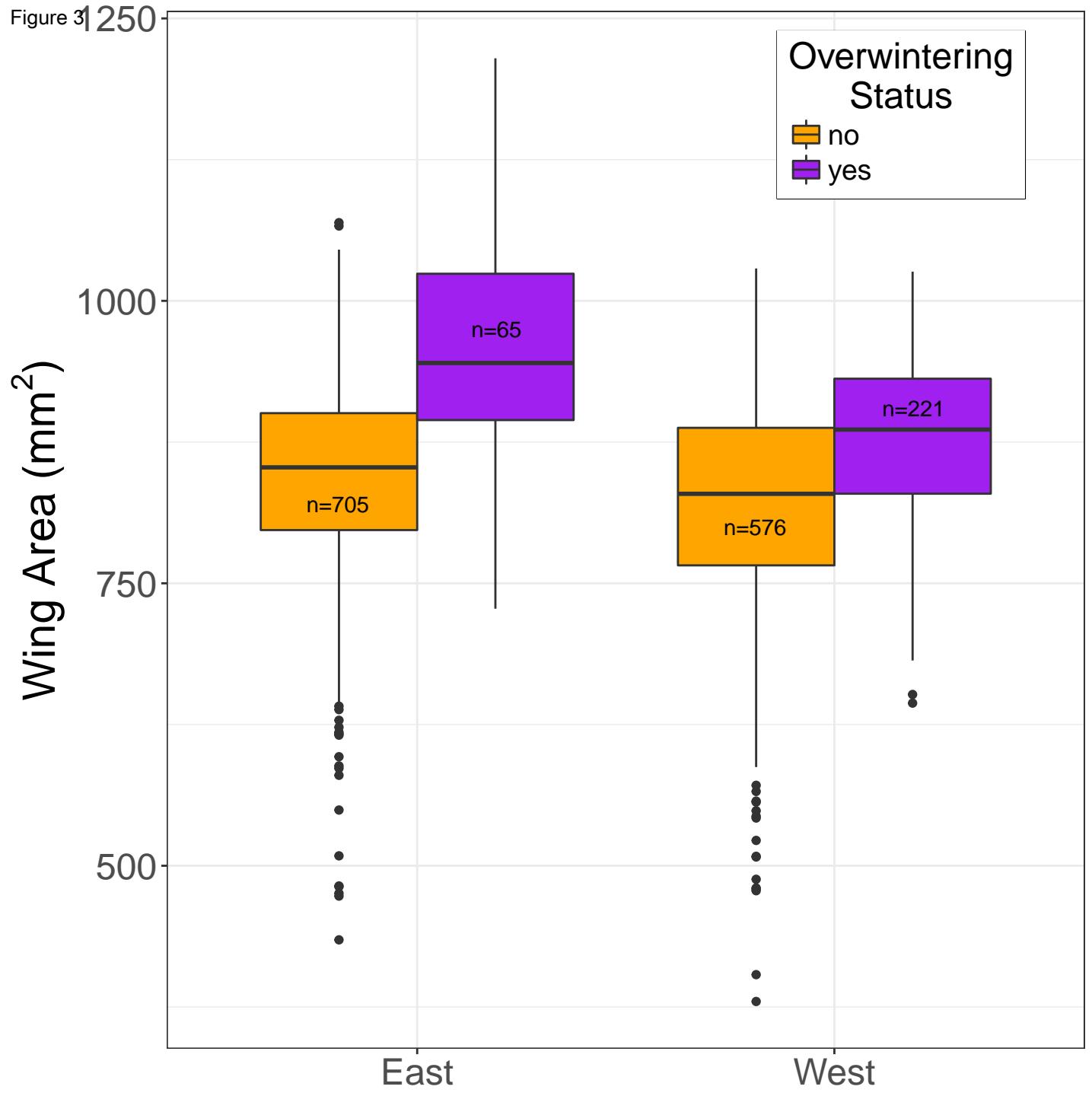


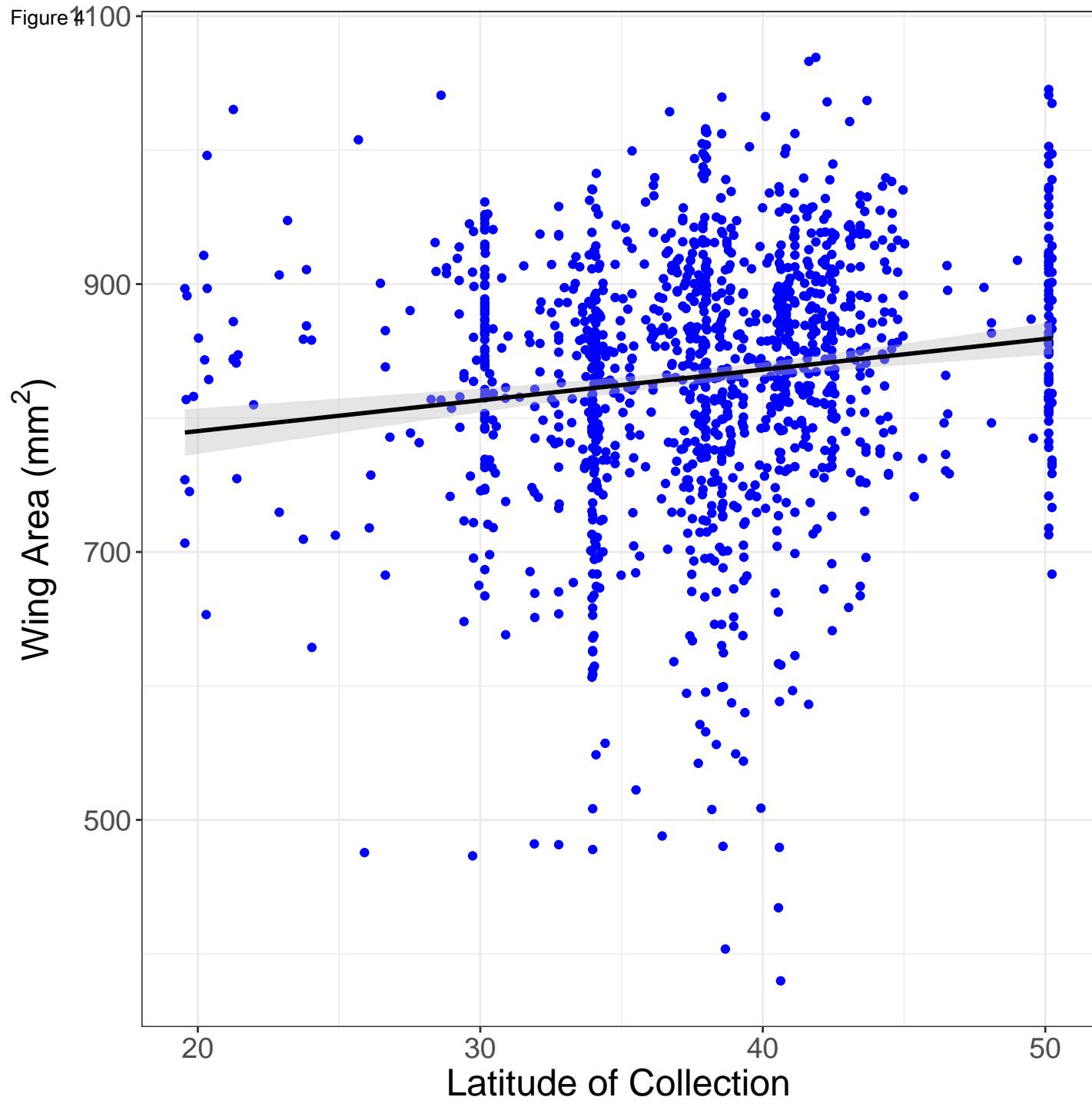
$$\text{aspect ratio} = \frac{l}{w}$$

$$\text{roundness} = \frac{4\pi A}{p^2}$$

Figure 2







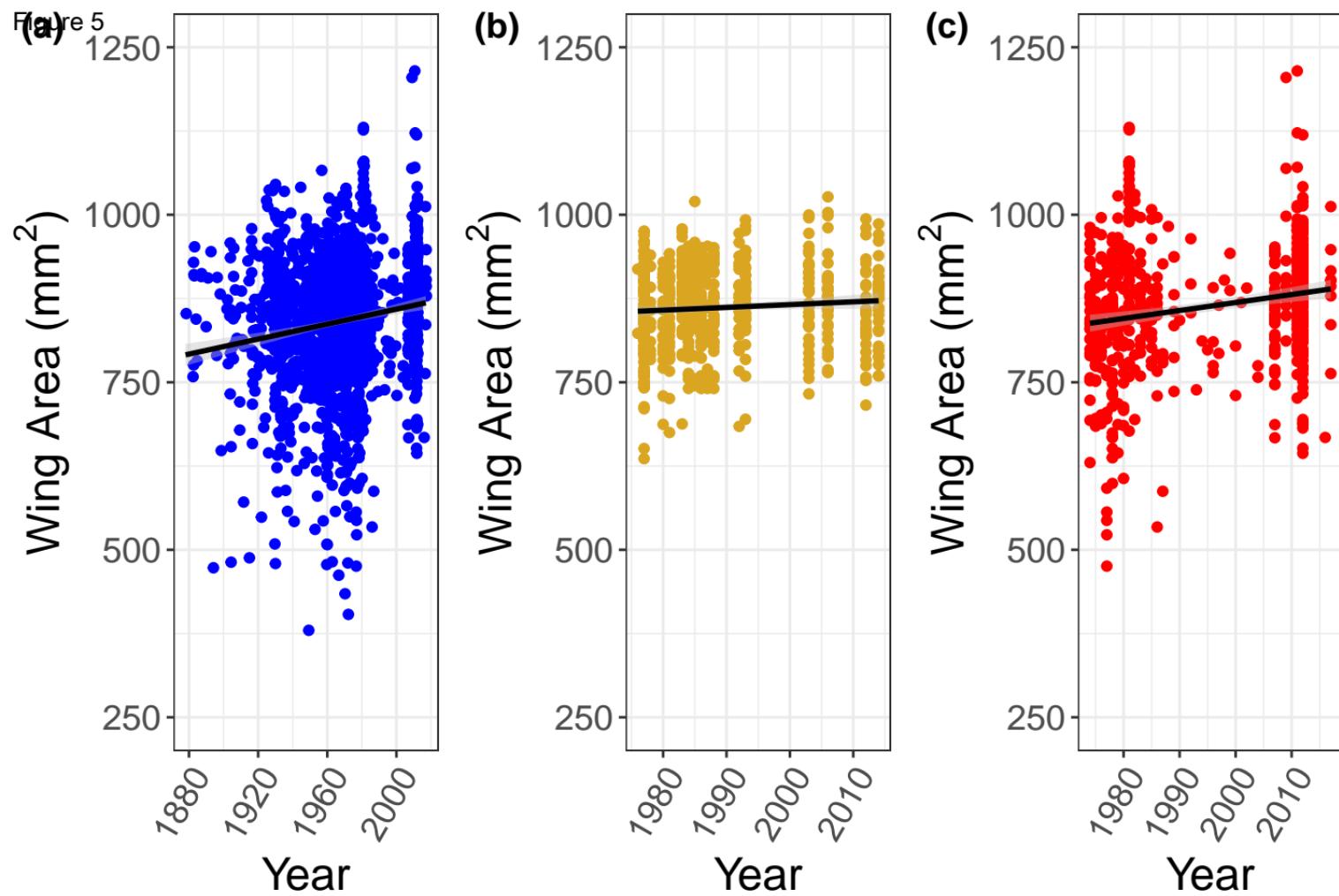


Figure 6

