# Molecular evolution of non-fertilizing sperm in Lepidoptera suggests minimal direct involvement in sperm competition

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**Abstract**

Sperm are among the most variable cells in nature. Some of this variation results from non-adaptive errors in spermatogenesis, but many species consistently produce multiple sperm morphs, the adaptive significance of which remains unknown. Here, we investigate the evolution of dimorphic sperm in Lepidoptera, the butterflies and moths. Males of this order produce both fertilizing sperm and a secondary, non-fertilizing type that lacks DNA. Previous organismal studies suggested a role for non-fertilizing sperm in sperm competition, but this hypothesis has never been evaluated from a molecular framework. We combined published datasets with new sequencing in two species, the monandrous Carolina sphinx moth and the highly polyandrous monarch butterfly. Based on population genetic analyses, we see evidence for increased adaptive evolution in fertilizing sperm, but only in the polyandrous species. This signal comes primarily from a decrease in non-synonymous polymorphism in sperm proteins compared to the rest of the genome, suggesting stronger purifying selection, consistent with selection via sperm competition. Non-fertilizing sperm proteins, in contrast, do not show an effect of mating system and do not appear to evolve differently from the background genome in either species, arguing against the involvement of non-fertilizing sperm in direct sperm competition. Based on our results and previous work, we suggest that non-fertilizing sperm may be used to delay female remating in these insects and decrease the risk of sperm competition rather than directly affect its outcome.

**Introduction**

54 Sperm cells display remarkable diversity throughout the animal kingdom (Pitnick, Hosken, & Birkhead,
55 2009), from small and plentiful to gigantic and few (Pizzari, 2006) to super-structure-forming (Higginson,
56 Miller, Segraves, & Pitnick, 2012). This variation exists at every level, from fixed differences between
57 species to variability within individual males (John Buckland-Nicks, 1998; Marks, Biermann, Eanes, &
58 Kryvi, 2008; Sasakawa, 2009; Swallow & Wilkinson, 2002; Tavares-Bastos, Teixeira, Colli, & Báo, 2002). In
59 many independently evolved cases, males consistently produce two different sperm types, a
60 phenomenon known as sperm dimorphism. In all cases examined, only one of the two sperm morphs is
61 capable of fertilization (Bressac et al., 1991; Carcupino, Baldacci, Fausto, Scapigliati, & Mazzini, 1999;
62 Eckelbarger, Young, & Cameron, 1989; Sasakawa, 2009; Wilms, 1986). The evolutionary causes and
63 consequences of variation in sperm morphology, both within and between morphs, are immediately
64 intriguing. As gametes, these cells are the final step in the long chain of events leading to reproductive
65 success or failure. Why should such important components of fitness be so variable?

66 Much of this morphological diversity within morphs can be attributed to deleterious variation, *e.g.*
67 genetic defects (Chenoweth, 2005) or age-related decline in sperm quality (Preston, Saint Jalme, Hingrat,
68 Lacroix, & Sorci, 2015). This deleterious variation has been shown to be inversely correlated with rates
69 of sperm competition between species; taxa that experience more sperm competition tend to have less
70 morphologically variable sperm at both population and individual levels (Kleven, Laskemoen, Fossøy,
71 Robertson, & Lifjeld, 2008). In other words, sperm often vary *in spite of* constraint imposed by their
72 reproductive importance. In species with high rates of polyandry, postcopulatory selection through
73 sperm competition and cryptic female choice weeds out the suboptimal sperm variants, at least for
74 fertilizing sperm (Birkhead, 1998; Immler, Calhim, & Birkhead, 2008).

75 Production of multiple sperm morphs, conversely, is often posited to be adaptive in some way. The very
76 fact that sperm dimorphism has repeatedly evolved suggests that it has some fitness benefit. Most
77 commonly, non-fertilizing sperm in dimorphic systems are proposed to be specialized agents of male-
78 male competition, acting as final combatants in the struggle for reproductive success (J Buckland-Nicks,
79 Bryson, Hart, & Partridge, 2010; John Buckland-Nicks, 1998; Swallow & Wilkinson, 2002). Indeed, some
80 have suggested that sperm dimorphism allows specialization in the non-fertilizing sperm for a
81 competitor-inhibiting function, sometimes called "kamikaze sperm" (Baker & Bellis, 1989). Although this
82 hypothesis has fallen out of favor, it was proposed and mainly evaluated in the context of mammalian
83 sperm (A. Harcourt, 1991; A. H. Harcourt, 1989; Moore, Martin, & Birkhead, 1999), where non-fertilizing
84 sperm are not usually differentiated from fertilizing sperm in a sophisticated way.

85 One of the most extreme cases of sperm dimorphism occurs in butterflies and moths (Lepidoptera). In
86 nearly all species of this order, males produce both fertilizing (**eupyrene**) sperm and a second type
87 (**apyrene**) that lacks a nucleus and nuclear DNA (Meves, 1902). The function of apyrene sperm is poorly
88 understood, but because it lacks DNA, it is clearly incapable of fertilizing eggs. Nevertheless, it does not
89 appear to be the result of errors in spermatogenesis; apyrene sperm production is hormonally regulated
90 and occurs in a developmentally predictable way, implying a novel gain of function in these insects
91 (Friedlander, 1997). Organismal studies have demonstrated that males can control the ratio of the two
92 sperm types in their ejaculate and typically transfer to females 10 to 20 times as much apyrene sperm as
93 eupyrene sperm, depending in part on the female's past mating history (Oberhauser, 1988). These
94 observations have led some to suggest that apyrene sperm play a specialized role in sperm competition
95 (Silberglied, Shepherd, & Dickinson, 1984), yet there remain several other competing hypotheses for
96 apyrene sperm function that have not been resolved through organismal observations and experiments
97 (Swallow & Wilkinson, 2002).

98 Recently, characterizations of the proteins found in lepidopteran sperm has opened a new avenue to
99 assess their evolution and function (Whittington et al., 2017; Whittington, Zhao, Borziak, Walters, &
100 Dorus, 2015). Proteomic studies have revealed distinct protein profiles for these two cell types
101 (Whittington, Karr, Mongue, Walters, & Dorus, in press). In both morphs, these proteins are retained
102 through maturation, and, in the case of apyrene sperm, the discarding of the nucleus. Because distinct
103 cellular functions are ultimately the product of their expressed protein complement, the class of
104 proteins uniquely found in apyrene sperm make logical targets for understanding the function of these
105 cells from a molecular perspective.

106 At the molecular level, sperm and other reproductive proteins are often observed to evolve rapidly
107 (Civetta & Singh, 1995; Dorus, Evans, Wyckoff, Sun, & Lahn, 2004; Willie J. Swanson & Vacquier, 2002).
108 For certain reproductive proteins, like sperm-egg interaction pairs, there is compelling evidence that
109 adaptive co-evolution drives this accelerated change (Herberg, Gert, Schleiffer, & Pauli, 2018; W J
110 Swanson & Vacquier, 1998). Yet there are also many instances of reproductive proteins that diverge
111 quickly because of relaxed purifying selection owing to expression in a single sex instead of the whole
112 population (Barker, Demuth, & Wade, 2005; Wade, Priest, & Cruickshank, 2008). Many other factors,
113 including number of protein-protein interactions or importance of reproductive role, can also act to
114 shape the intensity of positive or purifying selection on reproductive proteins (Schumacher, Rosenkranz,
115 & Herlyn, 2014; Schumacher, Zischler, & Herlyn, 2017). Recent theoretical work has formalized the

prediction that strong purifying selection on sperm proteins should depend on high rates of polyandry to generate sperm competition (Dapper & Wade, 2016). Thus, with the appropriate datasets, the degree of each sperm morph's role in sperm competition can be assessed via molecular tests of evolution.

In this study, we report the first molecular evolutionary analyses of dimorphic sperm. We assessed patterns of both polymorphism and divergence among sperm proteins from both eupyrene and apyrene sperm using proteomic datasets of two species: the monarch butterfly, *Danaus plexippus*, and the Carolina sphinx moth, *Manduca sexta* (Whittington et al., in press). North American monarchs spend time at incredibly high density in overwintering colonies in Mexico and California (Urquhart, 1976) and, owing to these unique population dynamics, have some of the highest female remating rates observed in Lepidoptera. Female monarchs mate an average of 2.6 times (and up to 14 times) in overwintering colonies in the wild (Hill Jr., Wenner, & Wells, 1976; Smith, 1984), creating ample opportunity for sperm competition. In contrast, Carolina sphinx moths are typically monandrous (Snow et al., 1974), making sperm competition rarely relevant as a selective force. Taking advantage of this contrast, we investigate the differences in patterns of selection between the two sperm morphs in each species to assess the role of apyrene sperm in sperm competition. If apyrene sperm are involved in sperm competition, their proteins should show evidence of stronger purifying selection in the monarch butterfly. To complete these analyses, we have generated the first published set of whole-genome resequencing data for *Manduca sexta* from a wild population. To test the general predictions for relaxed selection in sex-limited proteins, we used RNA-seq gene expression datasets from previously published data for Carolina sphinx moths (Cao & Jiang, 2017) and newly generated data for the monarch butterfly.

## Materials and Methods

### *Sources of data*

We used gene sets from the published genomes of each species (Kanost et al., 2016; Zhan & Reppert, 2013) with sperm genes identified from their respective proteomes (Whittington, Karr, Mongue, Walters, & Dorus, in press) . We inferred selection from patterns of polymorphism and divergence from congeners using whole genome Illumina resequencing data for both species: a previously published dataset for North American monarch butterflies (Zhan et al., 2014) and a new dataset of North Carolinian sphinx moths. Focal moths were collected with a mercury vapor light trap in July of 2017 in Rocky Mount, North Carolina (see supplemental table S1 for sequencing summary statistics and accessions). Divergences were called by comparison to the queen butterfly (*Danaus gilippus*, previously

146 published in Zhan et al. (2014)) for monarchs, and the five-spotted hawkmoth *(Manduca*
147 *quinquemaculata*, sequenced for this project) for the Carolina sphinx moth.

148 In both focal species, we used twelve wild-caught individuals for sampling of polymorphism. In the case
149 of Carolina sphinx moths, these were twelve males caught over the course of three nights. The sex-
150 biased sampling reflects a sex bias in dispersal and collection at the light trap. In the case of monarchs,
151 samples were selected based on depth of sequencing coverage in the published dataset and included 8
152 females and 4 males from the panmictic North American migratory population. This mixed-sex sampling
153 added the complication of unequal sampling between the autosomes (n = 24) and Z sex chromosome (n
154 = 16). Despite the male-biased gene accumulation on the Z chromosome, the vast majority of sperm
155 genes (92% in the Carolina sphinx, 90% in the monarch) are autosomal in both species (Mongue &
156 Walters, 2017). Due to the sampling complication and limited inference to be gained from Z-linked
157 genes, we focused on the autosomal genes in both species in subsequent analyses.

158 *SNP-based methods*

159 We aligned sequenced reads with bowtie2 (Langmead & Salzberg, 2012) for conspecifics to their
160 reference genome or with stampy (Lunter & Goodson, 2011) with an increased allowance for
161 substitution for heterospecific alignments. Alignments were taken through GATK's best practices
162 pipeline (McKenna et al., 2010), including hard filtering, to yield a set of high quality variants both within
163 and between species. Effect-class of each variable site (synonymous, non-synonymous, intergenic, etc.)
164 was determined using custom databases for the two species created with SnpEff (Cingolani et al., 2012).
165 Annotated SNPs were curated to remove false divergences (ancestral polymorphism) and then
166 differences in adaptive evolution were calculated using an estimator of the neutrality index to calculate
167 α, the proportion of substitutions driven by adaptive evolution (Stoletzki & Eyre-Walker, 2011). This
168 form of α corrects the inherent bias in a ratio of ratios while also allowing summation across multiple
169 genes to reduce noise associated with small numbers in count data. For any set of *i* genes with non-zero
170 counts of synonymous (s) polymorphism (P) and divergence (D):

$$\alpha = 1 - \frac{\sum(Dsi * Pni)/(Psi + Dsi)}{\sum(Dni * Psi)/(Psi + Dsi)}$$

171 This statistic was calculated with custom scripts in R (R Core Team, 2017).

172 *Assessment of adaptive evolution and statistical significance*

173 In each analysis, we calculated α for a biologically meaningful set of genes, *e.g*. the sperm proteome and
174 the background genome, and generated a test statistic from the absolute difference of the two point-
175 estimates. To determine significance, we combined the two sets and randomly assigned genes into two
176 new sets of sizes equal to the originals. The difference of these two datasets was determined and the
177 process was repeated for 50,000 permutations to build a distribution of differences between the point
178 estimates of two gene sets of these relative sizes. The p-value was taken as the proportion of times a
179 greater absolute difference was observed between the two random data sets than between the original
180 sets.

181 We used this permutation approach to make within-species comparisons of α for several different
182 groupings of genes. We first examined differences between the whole sperm proteome and background
183 genome (i.e. all autosomal non-sperm proteins). Next, we considered differences between sperm
184 homologs and sperm proteins unique to one species to assess how selection acted on the same genes in
185 different species. We identified sperm homologs as predicted orthologs that are present in the sperm of
186 both species, with orthology predicted via the proteinOrtho pipeline, as previously reported in Mongue
187 & Walters (2017). Unique sperm proteins may or may not have an ortholog in the other species but are
188 present in the sperm of only one species. Finally, we compared among proteins grouped by their
189 presence in apyrene versus eupyrene sperm. To do so, we classified sperm proteins into three subsets:
190 specific to eupyrene sperm, specific to apyrene sperm, or shared in both types. Pairwise comparisons
191 were made between each subset. For these analyses, we did not consider orthology status owing to the
192 reduction in power that would accompany multiple layers of subdivision of the dataset. For the whole
193 proteome and morph subset comparisons, we further assessed the relative contributions of
194 synonymous and non-synonymous polymorphism and divergence to the α calculation, using a Wilcoxon-
195 Mann-Whitney test to assess significant differences.

196 *Site-frequency-based methods*

197 We also investigated molecular evolution by leveraging site-frequency-spectrum-based approaches as
198 complimentary evidence. Owing to the redundancy in results, we have included these analyses in the
199 supplement rather the main text. In brief, we used the population genetics software suite ANGSD
200 (Korneliussen, Albrechtsen, & Nielsen, 2014) to generate site frequency spectra at putatively neutral
201 (four-fold degenerate) and selected (zero-fold-degenerate) sites in the genome. We unfolded site
202 frequency spectra and analyzed these spectra with the software polyDFE (Tataru, Mollion, Glémin, &
203 Bataillon, 2017) to examine rates of adaptive evolution in the whole sperm proteomes and background

204 genomes with a more complex likelihood model that corrects for effects of demography and potential
205 misattribution of ancestral state.

206 *Investigation of sex-limited and tissue-specific expression*

207 Next, we used RNA-seq data to assess whether or not differences in tissue specificity of expression
208 impacted our results from the sperm proteomes in these taxa. For *Manduca sexta*, there already existed
209 a wealth of tissue-specific data at multiple developmental timepoints (Cao & Jiang, 2017). Because we
210 were primarily interested in sperm involvement, we focused on data from adult males, specifically RNA
211 from the testes, head, thorax, and gut. Expression (measured as fragments per kilobase of transcript per
212 million mapped reads, FPKM) was averaged across biological replicates where available in this species.
213 Monarchs had no comparable published data, so we generated separate RNA-seq data sets from the
214 head, thorax, gut, testes, and accessory gland of three adult males (summarized in Table S2 with
215 accessions).

216 We quantified tissue-specificity of expression using the *specificity metric* (SPM) statistic, a ratio ranging
217 from 0 to 1 indicating the proportion of gene expression occurring in a given focal tissue (Kryuchkova-
218 Mostacci & Robinson-Rechavi, 2017). For instance, a gene with SPM = 0.8 for the testes shows 80% of its
219 total expression across all sampled tissues in the testes. This same gene would have a much lower SPM
220 value in head, thorax, or other tissues. We observed a bimodal distribution of tissue specificities, which
221 allowed us to bin genes into one of two classes: those that displayed low levels of specificity (SPM < 0.5)
222 and those that displayed high levels (SPM > 0.5). After separating genes by specificity, we calculated α
223 for three classes of genes in these two specificity bins.

224 We had two goals with these analyses: (1) to determine if patterns of adaptive evolution between
225 classes remained the same at both low- and high-specificities and (2) if α increased within a class of
226 genes at higher specificity compared to low. First, we considered background genome genes (i.e. non-
227 sperm genes) ranked by maximum specificity observed in the head, thorax, or gut for each of these
228 genes. Next, we considered only genes identified in the sperm proteome and ranked them by SPM in the
229 testes. Finally, for putatively male-limited non-sperm genes, we excluded sperm proteome genes and
230 considered again those ranked by specificity in the testes (or testes and accessory glands for monarchs).
231 As with our other α calculations, we used non-parametric bootstrapping to generate 95% confidence
232 intervals. For cases in which confidence intervals overlapped, we assessed significance with permutation
233 testing. These analyses were completed with custom R scripts.

234 *Demographic estimates*

235 Finally, to contextualize the previous analyses and take full advantage of our newly-generated data, we
236 characterized present and historical population sizes of our study species from genomic data. Using
237 folded four-fold degenerate site frequency spectra, we estimated neutral coalescence patterns with
238 Stairway Plot (Liu & Fu, 2015). For estimated generation time, we used four generations per year for
239 monarchs and three for the Carolina sphinx moth. For mutation rate, we chose the estimate $2.9*10^{-9}$
240 from the butterfly *Heliconius melpomene*, the closest relative with a spontaneous mutation rate
241 estimate (Keightley et al., 2015).

242 **Results**

243 *Differences Between Sperm Proteins and the Background Genome*

244 First, we considered the sperm proteome as a whole (*i.e.* all apyrene, shared, and eupyrene proteins)
245 and compared adaptive evolution of genes found in sperm to those in the background genome, defined
246 as all autosomal protein coding genes not present in the sperm proteome. Z-linked genes were excluded
247 from the analysis. We counted and classified synonymous and non-synonymous single nucleotide
248 polymorphisms within species and divergences to a congener (*Danaus gilippus* for the monarch, and
249 *Manduca quinquemaculata* for the Carolina sphinx). These quantities were used to generate an estimate
250 of the proportion of adaptive substitutions (α) per gene-class for both the sperm proteome and the
251 background genome. We found no difference in α between the sperm proteome and the rest of the
252 genome in the Carolina sphinx ($p = 0.40892$ by permutation testing, Figure 1A, left); for monarchs,
253 however, the sperm proteome showed a significantly greater proportion of adaptive substitutions than
254 the rest of the genome ($p = 0.00006$, Figure 1A, right). Note that in the strict sense, negative α values are
255 not biologically meaningful and likely point to an abundance of weakly deleterious variants within
256 populations or complex demographic histories (Eyre-Walker & Keightley, 2009); nevertheless, these
257 confounding variables should not differentially affect genes within species, so our observed differences
258 point to true differences in selection in gene sets.

259 To better understand the relative roles of polymorphism and divergence in sperm and background
260 genes, we investigated the individual components of α: counts of non-synonymous polymorphism (Pn),
261 synonymous polymorphism (Ps), non-synonymous divergence (Dn), and synonymous divergence (Ds).
262 We compared the scaled estimates of each (*e.g.* non-synonymous polymorphisms per non-synonymous
263 site) to the background genome within each species using a Wilcoxon-Mann-Whitney test (Figure 1B).

264 We found no differences between sperm and the background for any class of variants in *M. sexta* (Pn: W
265 = 3014100, p = 0.5964; Ps: W = 2879300, p = 0.1830; Dn: W = 3068300, p = 0.2009; Ds: W = 2895700, p =
266 0.2686). The signal for elevated α in monarch sperm primarily reflects non-synonymous polymorphism,
267 which was greatly depressed (W = 3062400; p = $3.224 * 10^{-11}$), as would be expected under strong
268 purifying selection, while other classes were comparable between sperm and the background genome
269 (Ps: W = 2684200, p = 0.2720; Dn: W = 2506400, p = 0.1300; Ds: W = 2544400, p = 0.3437).

270 Next, we leveraged orthology, as established by Whittington *et al.* (2017), to test for differences in
271 mating system while controlling for the effects of sperm proteome content. Substantial numbers of
272 orthologous proteins are found in the sperm proteomes of both species, which we hereafter referred to
273 as *sperm homologs*. Sperm homologs offer the opportunity to directly assess the selective pressures
274 experienced by the same genes with putatively conserved function but found in species with different
275 levels of postcopulatory selection. Nearly half of the monarch sperm proteome (~42%, 216 genes, Figure
276 2A) shares an ortholog in the sperm proteome of *M. sexta*; reciprocally, there are 236 genes (37%) in the
277 Carolina sphinx sperm proteome that share an ortholog in the monarch sperm proteome; these
278 numbers are not equal due to lineage-specific duplications among sperm homologs creating a few cases
279 of one-to-many orthology. We tested for differences in adaptive evolution between sperm homologs
280 and sperm proteins unique to one species (orthology outside of sperm or no detectable orthology). In
281 Carolina sphinx moths, genes of these two classes did not differ in the proportion of adaptive
282 substitutions with permutation testing (p = 0.6174, Figure 2B). In monarchs, we detected an increased
283 proportion of adaptive substitution in the sperm homologs compared to unique proteins (p = 0.0372,
284 Figure 2B). Comparing between species, sperm homologs had much higher α values in monarchs than in
285 Carolina sphinx moths (p = 0.00008), while genes with unique expression in either species did not show
286 differences between species (p = 0.5922). Thus, the same sperm proteins appear to be evolving more
287 adaptively in the polyandrous species.

288 *Site-frequency based methods*

289 We also took a likelihood approach to modeling adaptive evolution using site frequency spectra
290 generated from the same samples we used for SNP-counting. These results are detailed in the
291 supplement. In short though, we found a shift in the predicted distribution of fitness effects of new
292 mutations in monarch sperm proteins compared to the background consistent with stronger purifying
293 selection (Figure S1) and drastically higher α in sperm genes in monarchs alone (Figure S2).

294 *Patterns of Adaptive Evolution in Sex-Specific Tissues*

295 Next, we used RNA-seq data to examine the effect of tissue-specificity on selection in these insects.
296 With these data, we calculated the tissue specificity metric, SPM (Kryuchkova-Mostacci & Robinson-
297 Rechavi, 2017), which ranges from ubiquitous expression (near 0) to single-tissue specific (1). Although
298 the sperm proteomes of both of our species were enriched for gene products specifically expressed in
299 testes, they also contained broadly expressed gene products (Figure 3B). To assess the effect of these
300 broadly expressed genes on our inference of selection, we recalculated the α statistic for two bins of
301 genes (Figure 3C): those with broad expression (SPM < 0.5) and those with high tissue-specificity (SPM >
302 0.5).

303 In Carolina sphinx moths, there were no significant changes in α between low- and high-specificity genes
304 in any part of the genome (background genes: $p = 0.3868$, sperm proteome genes: $p = 0.3248$, male-
305 limited genes: $p = 0.5579$; Figure 3C, left), nor did any of the gene classes differ from each other within a
306 specificity bin. In monarchs, however, both sperm proteome genes ($p = 0.0242$) and testes genes ($p =$
307 $0.0137$) showed higher α in the high-specificity group than the low-specificity group, though somatically
308 expressed genes did not ($p = 0.6831$). Additionally, we found that sperm genes showed much greater α
309 than the background genome or other genes expressed in the testes at both low- and high-specificities
310 (Figure 3C, right). This result indicates that our initial results (considering the whole sperm proteome)
311 are not dependent on the underlying specificity of sperm genes.

312 *Molecular evolution in dimorphic sperm*

313 Having verified the patterns of evolution in the whole sperm proteomes with several approaches, we
314 turned to our primary question, assessing apyrene sperm function through analysis of molecular
315 evolution. We considered the different subsets of the sperm proteomes based on the two sperm types.
316 The two datasets consisted of three classes of sperm proteins: unique to eupyrene sperm, unique to
317 apyrene sperm, or found in both cell types (henceforth "shared", Figure 4A). We assessed differences in
318 selective pressures between the sperm morphs with another series of permutation tests, both
319 comparing parts of the sperm proteome to the background genome and comparing parts of the
320 proteome to each other.

321 As expected based on the whole-proteome results from Carolina sphinx moth, neither eupyrene-specific
322 ($p = 0.55912$), shared ($p = 0.4647$), nor apyrene-specific proteins ($p = 0.96496$) differed from the
323 background genome (Figure 4B). α did not vary between apyrene-specific and eupyrene-specific

324 proteins (p = 0.7271), between apyrene-specific and shared (p = 0.7176) or eupyrene-specific and
325 shared proteins (p = 0.9979). In monarchs, both eupyrene-specific proteins (p =0.00018) and shared
326 proteins (p = 0.01038) showed elevated α, but apyrene-specific proteins did not evolve differently from
327 the background genome (p = 0.55934). Neither apyrene nor eupyrene sperm differed significantly from
328 the shared set in monarchs (p = 0.6332 & p = 0.6234, respectively), but there was a trend towards
329 significantly increased α in eupyrene-specific proteins compared to apyrene-specific proteins (p =
330 0.0986).

331 As with the whole sperm proteome, we investigated which classes of variants contributed to our
332 observed differences in α (Figure 4C). Consistent with the results above, none of the variant classes
333 significantly differed from the genome background in the sphinx moth eupyrene-specific proteins (Pn: W
334 = 995190, p = 0.0857; Ps: W = 943550, p = 0.6782; Dn: W = 966630, p = 0.3183; Ds: W = 963410, p =
335 0.3596). Shared proteins also showed the same level of variation as the background across all variants
336 (Pn: W = 1470300, p = 0.3277; Ps: W = 1444400, p = 0.1369; Dn: W = 1540700, p = 0.6883; Ds: W =
337 1437100, p = 0.1030). And finally, apyrene-specific proteins were not significantly different either (Pn: W
338 = 548570, p = 0.4974; Ps: W = 491410, p = 0.2149; Dn: W = 560910, p = 0.2741; Ds: W = 495180, p =
339 0.2653). In summary, there was no evidence for stronger selection on either sperm morph in Carolina
340 sphinx moths.

341 For monarchs, we found that the elevated α in the eupyrene-specific and shared subsets was driven
342 primarily by a decrease in non-synonymous polymorphism compared to the background genome (W =
343 1291700, p = 0.0003 for eupyrene; W = 1486100, p = $1.167*10^{-8}$ for shared). Apyrene-specific proteins
344 did not show a reduction in non-synonymous polymorphism (W = 284620, p = 0.1684). Synonymous
345 polymorphism did not significantly differ from the background in any subset of the sperm proteome
346 (eupyrene: W = 1164200, p = 0.4492; shared: W = 1249900, p = 0.5570, apyrene: W = 270160, p =
347 0.4927). Nor did synonymous divergence (eupyrene: W = 1056000, p = 0.0928; shared: W = 1209000, p =
348 0.7665, apyrene: W = 279420, p = 0.2594). Intriguingly, non-synonymous divergence was elevated
349 compared to the background in eupyrene-specific proteins (W = 1021800, p = 0.0151), but not the
350 shared (W = 121800, p = 0.9185) or apyrene-specific portions of the proteome (W = 266580, p = 0.6042).
351 This suggests periodic sweeps of positively selected variants in fertilizing sperm proteins.

352 We did not examine orthology within dimorphic sperm owing to small gene counts giving reduced
353 statistical power. Nor could we could examine tissue specificity here because apyrene and eupyrene
354 sperm are produced at different developmental timepoints and we did not have suitable expression

355 data in both species. Nonetheless, the consistency of results in the whole proteome datasets gives us no
356 reason to expect that within-proteome results would be idiosyncratic to our methodology.

357 *Demographic estimates*

358 Finally, to contextualize our results with population dynamics, we estimated population size history
359 using site frequency from 4-fold degenerate sites in the two species' genomes (Figure S3). Both have
360 effective population sizes near 2,000,000, as expected of herbivorous invertebrates with high dispersal
361 potential, numerous host plants, and a large range over North America. We also recovered a population
362 size increase in monarch butterflies in the recent past, which has been previously reported with genomic
363 data (Zhan et al., 2014). We note that our inferred timing of this event differs from that of the previous
364 authors, who used mutation rate estimates from *Drosophila melanogaster*. Such input parameter
365 differences affect the estimated time of events, but not the trajectories.

366 **Discussion**

367 We investigated the molecular evolution of eupyrene (fertilizing) and apyrene (non-fertilizing) sperm,
368 the ubiquitous lepidopteran cell-type of unknown functional significance. These sperm have long been
369 posited to interfere with competitors' sperm, in part because their quantity varies with levels of male-
370 male competition (Silberglied et al., 1984; Solensky & Oberhauser, 2009; Swallow & Wilkinson, 2002). In
371 contrast to these organismal observations, the results of our molecular analyses cast doubt on this
372 hypothesis. If apyrene sperm played an active role in sperm competition, we would expect evidence for
373 stronger selection in apyrene sperm compared to the background genome in monarchs. We found a
374 signal for elevated adaptive evolution (α) in the sperm proteome compared to the background genome
375 in these polyandrous butterflies, but this signal did *not* include apyrene-sperm-specific proteins. Instead,
376 genes encoding apyrene sperm proteins evolve similarly to the background genome in both monarchs
377 and Carolina sphinx moths. This result is unlikely to have arisen from a lack of power in our
378 methodologies, as eupyrene-specific and shared sperm proteins showed patterns in line with
379 expectations for a role of sperm competition in molecular evolution in monarchs.

380 *Selection consistent with sperm competition, but only in fertilizing sperm*

381 The source of the apparently elevated α in the monarch sperm proteome came mainly from a dearth of
382 non-synonymous polymorphisms in sperm proteins compared to the background genome, indicating the
383 action of purifying selection to remove many variants before fixation in monarchs. Strong purifying

384 selection has been similarly observed in genes expressed in pollen, the main male-male competitors in
385 flowering plants (Arunkumar, Josephs, Williamson, & Wright, 2013). A similar pattern can also be
386 observed in passerine birds, in which species with higher rates of sperm competition show less
387 intraspecific and intra-male variation in sperm length compared to sperm of less polyandrous species
388 (Immler et al., 2008; Kleven et al., 2008).

389 Moreover, the elevated α in sperm homologs in monarchs suggests that genes that have had conserved
390 sperm function since the divergence of the two species some 100 million years ago (Heikkila, Kaila,
391 Mutanen, Pena, & Wahlberg, 2012) are under stronger purifying selection in the polyandrous species.
392 According to recent gene ontology analyses, such genes are enriched for core traits in sperm, such as
393 mitochondrial function, respiration, and flagellar structure. Similarly, proteins shared between the two
394 sperm types and those unique to eupyrene sperm show an elevated α compared to the background
395 genome in monarchs. Sperm proteins shared between morphs are enriched for structural proteins that
396 give rise to the sperm tail and thus impact motility (Whittington et al., in press), while those expressed
397 only in eupyrene sperm doubtless include important mediators of fertilization. At the cellular level,
398 variation in sperm traits like swimming ability, longevity, and overall viability affects sperm competition
399 outcomes (Burness, Casselman, Schulte-Hostedde, Moyes, & Montgomerie, 2004; Kim et al., 2017) and
400 has a polygenic basis in other taxa (Hering, Olenski, & Kaminski, 2014). For traits like longevity and
401 motility there is a threshold below which fertilization becomes significantly impaired, but in the absence
402 of competitor alleles, there is a larger range of effectively-neutral trait-values, allowing for more
403 variation to be maintained in the population. In the presence of competitor alleles, however, marginal
404 differences in fertilization success come under selection, leading to the removal of deleterious variants
405 through sperm competition.

406 Stronger selection from competition may include even the event of fertilization itself. Lepidopteran eggs
407 are known to possess multiple micropyle openings for sperm (Kumar, Kariappa, Babu, & Dandin, 2007)
408 and eupyrene sperm possess structures resembling an acrosome (while their apyrene counterparts do
409 not) (Friedlander, 1997). This rare combination of male and female gamete structures is also found in
410 sturgeon, in which the multiple micropyles give several sperm potential access to the egg nucleus and
411 there is competition among sperm to initiate karyogamy via the acrosome reaction (Psenicka, Rodina, &
412 Linhart, 2010). Consistent with micropyle-mediated competition, it has been shown that more
413 polyandrous species of Lepidoptera tend to have more micropyles on their egg surfaces than
414 monandrous species (Iossa, Gage, & Eady, 2016). If this truly does extend the opportunity for male-male

415 competition and cryptic choice, then acrosomal proteins in eupyrene sperm would be likely targets for
416 selection in polyandrous systems.

417 Whatever the mechanics of fertilization are, paternity outcomes in polyandrous species are often
418 bimodally distributed (Simmons & Siva-Jothy, 1998; Wedell & Cook, 1998), including in monarch
419 butterflies (Mongue, Ahmed, Tsai, & De Roode, 2015). For females that mate twice, one of the two
420 males typically fathers most, if not all, of the observed offspring produced by the female, but there is
421 little consistency in whether it is the first or second male. With these dynamics, fitness differences
422 between winning and losing sperm phenotypes are large and selection can reliably remove less
423 successful genotypes.

424 Evidence of this can be seen in the estimated distribution of fitness effects of new mutations in monarch
425 sperm proteins. Compared to the background genome, we see a decrease in the proportion of
426 effectively neutral and weakly deleterious mutations and an increase in both strongly deleterious and
427 beneficial mutations. In the absence of competition, not only are mildly suboptimal variants effectively
428 neutral, but novel, more efficient competitors should have no selective advantage in monandrous
429 species unless they also markedly increase fitness in a single mating. This reasoning is supported by the
430 estimated distribution of fitness effect for the complimentary gene sets in the Carolina sphinx moth; in
431 this species, we see little variation in the DFE between the background genome and the sperm
432 proteome. Moreover, there is no decrease (and indeed) an increase in non-synonymous divergence of
433 eupyrene sperm proteins in monarchs compared to the rest of the genome. This pattern suggests that in
434 addition to strong purifying selection there must be periodic sweeps of beneficial alleles. Without a
435 broader, phylogenetically controlled study, these results between a single pair of species are not
436 conclusive, but they fit well with the prediction that sperm protein evolution depends on the rates of
437 polyandry in a species (Dapper & Wade, 2016).

438 *Evolution of tissue-specific and male-limited genes*

439 Other studies have demonstrated that tissue specificity of expression can strongly influence the
440 molecular evolution of reproductive proteins (Schumacher & Herlyn, 2018), in some cases more than
441 mating system (Carnahan-Craig & Jensen-Seaman, 2014). Because our proteomic data did not contain
442 information on tissue specificity on their own, we examined this dynamic with RNA-seq data. We found
443 increased adaptive evolution in monarch sperm genes with higher specificity compared to low-
444 specificity sperm genes. Furthermore, while not significantly different from background genome, α for

445 non-sperm genes expressed in the testes increased with greater specificity in monarchs, suggesting that
446 they too may be subject to stronger sexual selection in this polyandrous species. Neither of these
447 patterns held for Carolina sphinx moths, which showed no differences based on tissue specificity. This
448 consistency further suggests the difference in mating system as an explanation for differences in
449 intensity of selection.

450 Finally, we did not observe relaxed constraint in reproductive proteins predicted due to the smaller
451 effective population size of males or females compared to the population as a whole, as predicted by
452 theory (Dapper & Wade, 2016; Wade et al., 2008). Specifically, we did not observe a difference in the
453 adaptive evolution of genes with testes-specific expression, our proxy for sex-limited expression,
454 compared to the background genome. To explain this discrepancy between theory and observation, we
455 turn to Nearly Neutral Theory. Large populations have more efficient selection than small populations
456 and a smaller range of slightly deleterious mutations that behave neutrally (Ohta, 1992). Mutations with
457 a selective effect less than $1/N_e$ are expected to behave neutrally. For instance, one commonly cited
458 estimate for human population size is Ne ≈ 10,000 over evolutionary history (Zhao et al., 2000). Based
459 on this, mutations with selective effects less than 0.0001 should behave neutrally for alleles expressed in
460 both sexes, while those with effects of 0.0002 are effectively neutral for alleles only expressed in one
461 sex. And indeed, there is evidence that genes expressed only in men have a higher mutational load than
462 those expressed in both sexes (Gershoni & Pietrokovski, 2014). Chimpanzees, another species with a
463 similar effective population size (Won & Hey, 2005), also show increased non-synonymous divergence in
464 reproductive proteins (Wong, 2010). Broadly, male reproductive protein evolution appears to depend
465 more on effective population sizes than intensity of sperm competition in the great apes in general
466 (Good et al., 2013), as one would expect for species with relatively small effective population sizes.

467 In contrast to mammals, the effective population sizes of most insect species are orders of magnitude
468 higher. Using neutral site frequency spectra, we estimated effective populations near 2,000,000 for both
469 North American monarchs and Carolina sphinx moths. Selection is much more effective in these massive
470 populations; mutations with effects above $5*10^{-7}$ should be subject to selection in both sexes and those
471 above $1*10^{-6}$ should be subject to selection if expression is sex-limited. Thus, even selection on alleles
472 with sex-limited expression in these insects should be 100 times stronger than selection on the entire
473 human population. Even if there is a relative two-fold difference in selection, the absolute magnitude of
474 the difference should be miniscule, and the effects of mating system more apparent.

475 *Advancing understanding of apyrene sperm*

476 Previous morphological work found that eupyrene sperm traits (like sperm length) but not apyrene
477 sperm traits, varied with risk of sperm competition in other butterflies (Gage, 1994). Similarly, from a
478 molecular perspective, none of the patterns of increased purifying and positive selection that we
479 observed for monarch sperm proteins applied to the apyrene-specific proteins. That we also do not see
480 evidence for the action of sperm competition on apyrene-specific protein evolution is itself informative,
481 however. Research to-date has proposed four main hypotheses for apyrene sperm (Swallow &
482 Wilkinson, 2002): active sperm competition agents, passive competition agents, nutrient nuptial gifts, or
483 necessary facilitators of fertilization. Our molecular analyses argue against apyrene sperm as active
484 agents of sperm competition, but it is worth considering predictions for molecular evolution of apyrene
485 sperm under the other hypotheses.

486 Indeed, apyrene sperm may still have adaptive significance without specialized molecular function,
487 especially under the filler hypothesis. This proposed function also relates to sperm competition, but
488 posits that apyrene sperm are employed proactively, to fill the female's sperm storage organ and delay
489 remating, thus decreasing the risk of sperm competition, rather than impacting its outcome (Swallow &
490 Wilkinson, 2002). Both in monarchs and the butterfly *Pieris napi*, female time to remating increases with
491 the number of apyrene sperm received from males (Cook & Wedell, 1999; Oberhauser, 1988). Such
492 observations are somewhat confounded by the size of the spermatophore nuptial gift that males
493 provide during mating, but apyrene sperm themselves have been proposed as a form of nutritional
494 nuptial gift (He, Tanaka, & Miyata, 1995; Lamunyon, 2000). Under both the nutrient and filler
495 hypotheses, the actual sequence of apyrene sperm proteins should be less important than their physical
496 presence and abundance, so factors affecting the rate of apyrene sperm production would be more
497 likely targets for selection in polyandrous species than the proteins sequences themselves.

498 Finally, apyrene sperm appear to capacitate fertilization in *Bombyx mori* (Takemura, Sahara, Mochida, &
499 Ohnuma, 2006); the mechanism here is unclear and the phenomenon is untested in other taxa, but it
500 could conceivably involve proteins that modulate female reproductive physiology to make conditions
501 more favorable for eupyrene sperm or induce oviposition. In such a case, these proteins would behave
502 more akin to the broader class of reproductive proteins and evolve independently of rates of polyandry
503 in a species. If there is an evolutionarily conserved capacitation effector in our study taxa, it is possible
504 that this function is governed by a small subset of apyrene-specific proteins. Because our methods
505 aggregate signal for selection across multiple genes or sites to counteract high variance in variant counts

506 within genes (Stoletzki & Eyre-Walker, 2011), the importance of one or a few genes could be lost in the
507 heterogeneous selection on different proteins.

508 Conclusions

509 Variation in reproductive traits has long been studied at the morphological and molecular level,
510 generally. Yet sperm dimorphism, one of the most striking and enigmatic reproductive traits, has not
511 previously been assessed using population genetic analyses. Our investigation of the sperm proteome in
512 two Lepidoptera demonstrates a pattern of stronger purifying selection on fertilizing-sperm genes in a
513 species with higher rates of sperm competition. In this polyandrous species, these genes experience a
514 strikingly different selective environment than the rest of the genome, with strong purifying selection
515 reducing variation in sperm genes. In contrast, fertilizing-sperm genes in the monandrous species hold
516 as much deleterious variation as other parts of their genome. Our new molecular findings fit well with
517 established studies on sperm morphology which show that sperm competition results in decreased
518 variation in sperm traits.

519 The evolution of non-fertilizing sperm, however, does not show a strong influence of sperm competition.
520 This lack of pattern itself argues against apyrene sperm as active agents of sperm competition, one of
521 the long-held hypotheses for non-fertilizing sperm function. Instead, apyrene sperm may play a passive
522 role in reducing the risk of competition by delaying female remating. The method by which apyrene
523 sperm capacitate fertilization in some species remains unclear based solely on genomic approaches and
524 will likely require functional experiments to completely understand.

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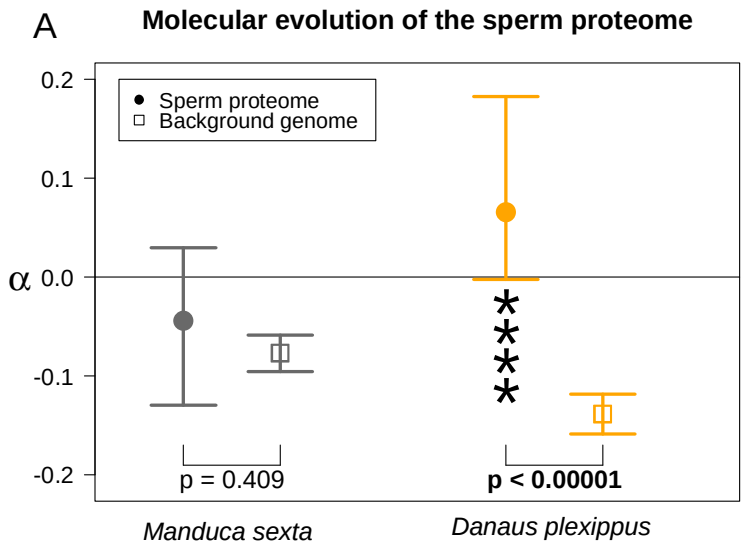
**Author Contributions:** AJM designed the experiments, collected samples, performed analyses, and wrote the manuscript. MEH collected samples and conducted analyses. LG provided data and performed analyses. CES planned and facilitated sample collection and edited the manuscript. JRW assisted in experiment design and manuscript editing.

**Data accessibility:** *Manduca sexta* whole genome resequencing data can be found on NCBI's Sequence Read Archive with the following accession: SRP144217. *Danaus plexippus* RNA sequencing data can be retrieved with accessions: SRR8580831 - SRR8580842. Analysis scripts can be found at https://github.com/WaltersLab/DimorphicSpermMolEvo.



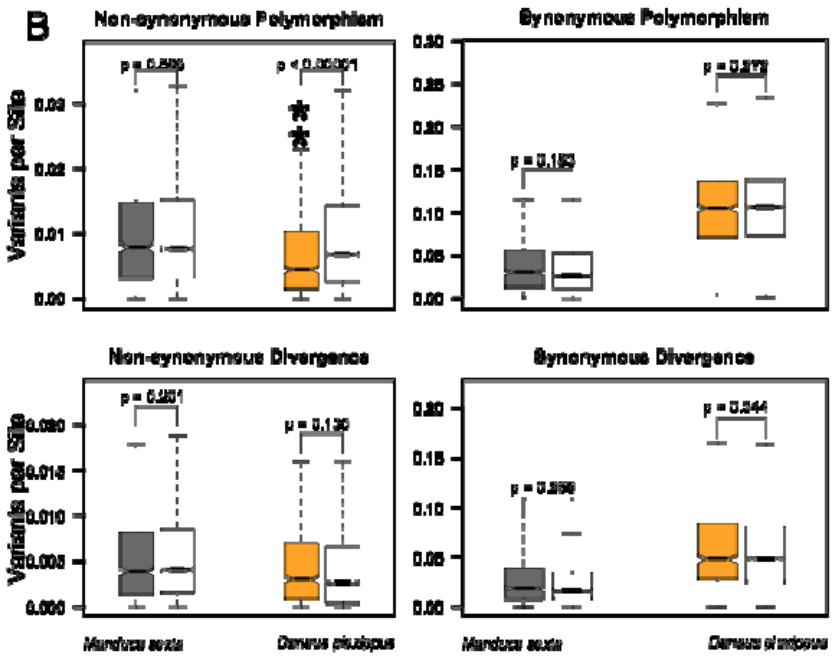



**Figure 1. A.** In the Carolina sphinx moth (*M. sexta*), there is no difference between the sperm proteome and the rest of the genome (left); conversely, genes in the sperm proteome of monarch butterflies (*D. plexippus*) show a significantly higher proportion of adaptive substitutions (α) than the rest of the genome (right). P-values come from permutation tests. Error bars represent 95% bootstrapped confidence intervals from the point estimates. **B.** Decomposing α into its components: Pn, Ps, Pn, and Ds and comparing the sperm proteome (filled boxes) to the background genome (open boxes). There were no strong differences between sperm genes and the background genome in Carolina sphinx moths. In monarch butterflies, the signal for increased adaptive substitution comes from a marginal increase in non-synonymous divergence (bottom left) combined with a great reduction in non-synonymous polymorphism in sperm genes compared to the rest of the genome (top left). P-values reflect Wilcoxon-Mann-Whitney tests, with * < 0.05, ** < 0.005, *** < 0.0005, etc.

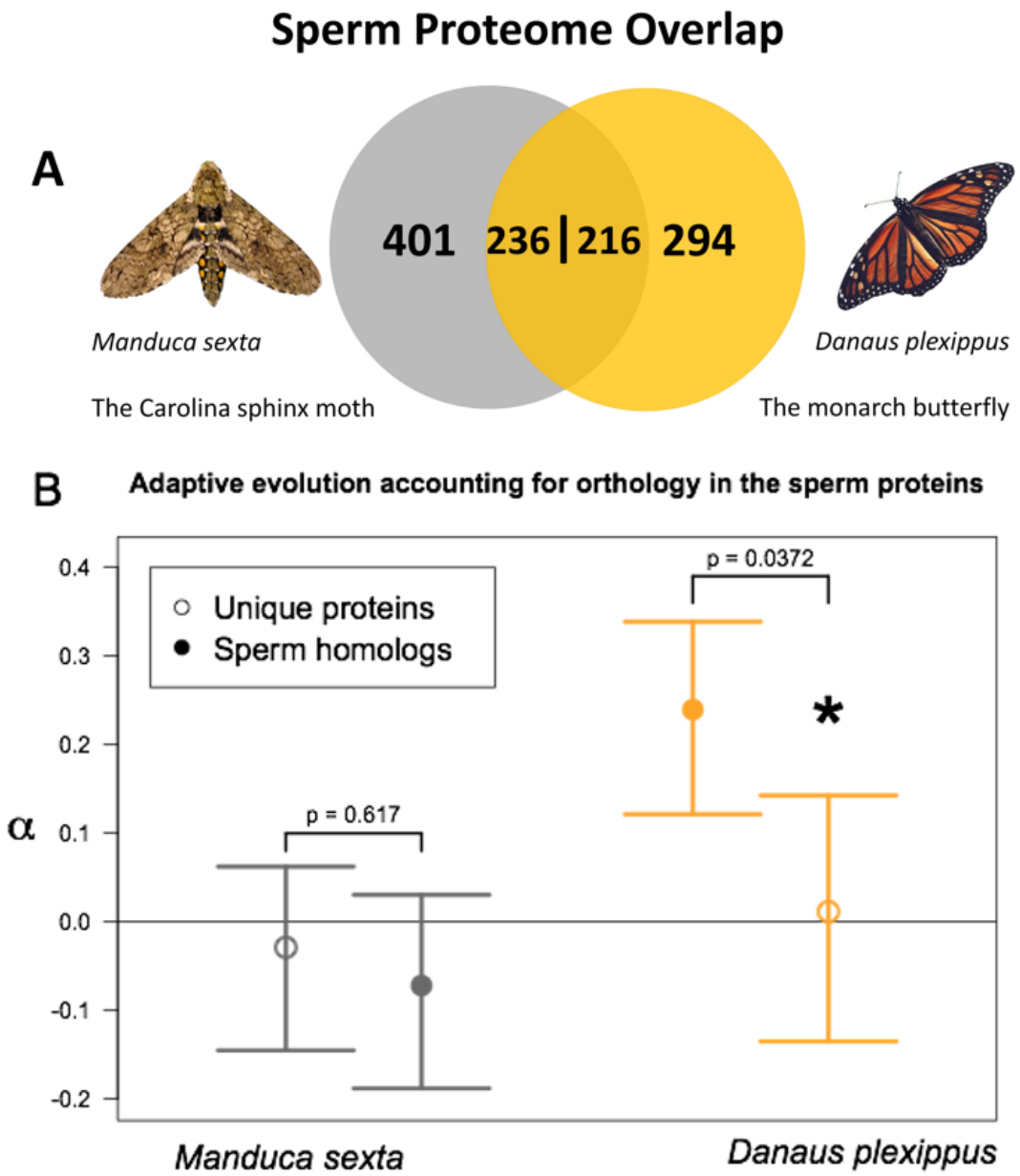



**Figure 2. A.** Composition of the portion of the sperm proteomes analyzed in this study. Numbers indicate counts of proteins unique to one species' sperm or with an ortholog in the other species' sperm (sperm homologs). Note that the overlap number varies between species due to the presence of a few one-to-many-orthologs. **B.** Sperm homologs show evidence for a greater proportion of adaptive substitutions (α) in monarch butterflies, but not in Carolina sphinx moths. P-values are based on permutation tests comparing the difference between two sets of genes randomly assigned from the sperm proteome in each species; error bars are 95% bootstrap confidence intervals.

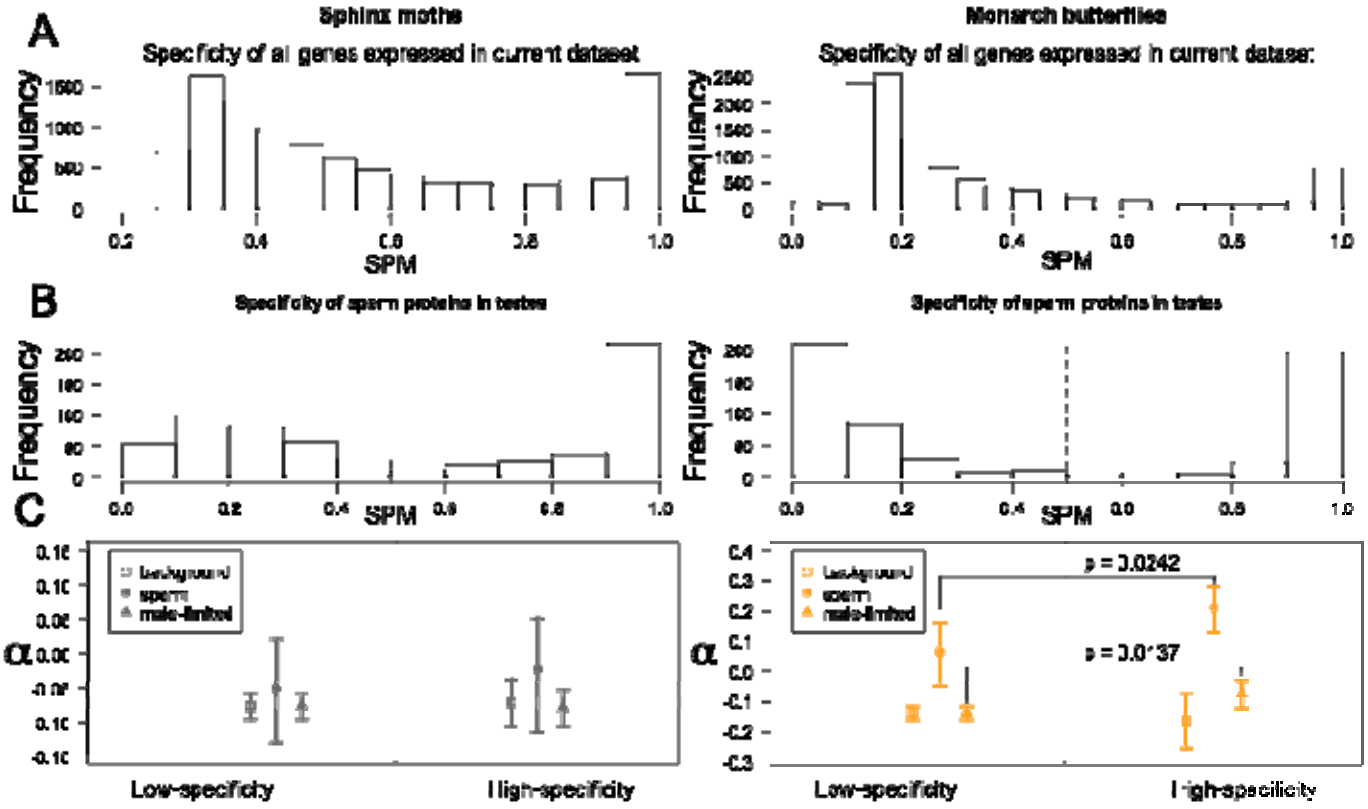



**Figure 3.** Investigating how tissue specificity of gene expression impacts adaptive evolution in both Carolina sphinx moths (**left column**) and monarchs (**right column**). **A.** Maximum specificity of all genes across all studied tissues using the RNA-seq data considered in these analyses. **B.** Observed distribution of specificity of sperm proteome genes expressed in the testes. Based on these distributions, we separated genes into one of two categories, low-specificity (SPM < 0.5) or high-specificity (SPM > 0.5), divided by the dashed line. **C.** Inferred proportion of adaptive substitutions (α) in background genes (squares), sperm proteome genes (circles), and male-limited genes (as defined by testes expression). Bars represent 95% confidence intervals from non-parametric bootstrapping. Non-overlapping confidence intervals imply significant differences generally, but we have also highlighted two significant differences that are less visibly apparent. Monarchs show evidence for increasing α with increasing tissue specificity in sperm and testes genes, but sphinx moths do not. Moreover, sperm proteome genes evolve more adaptively than background or testes-specific genes in both specificity groups for monarchs but not sphinx moths.

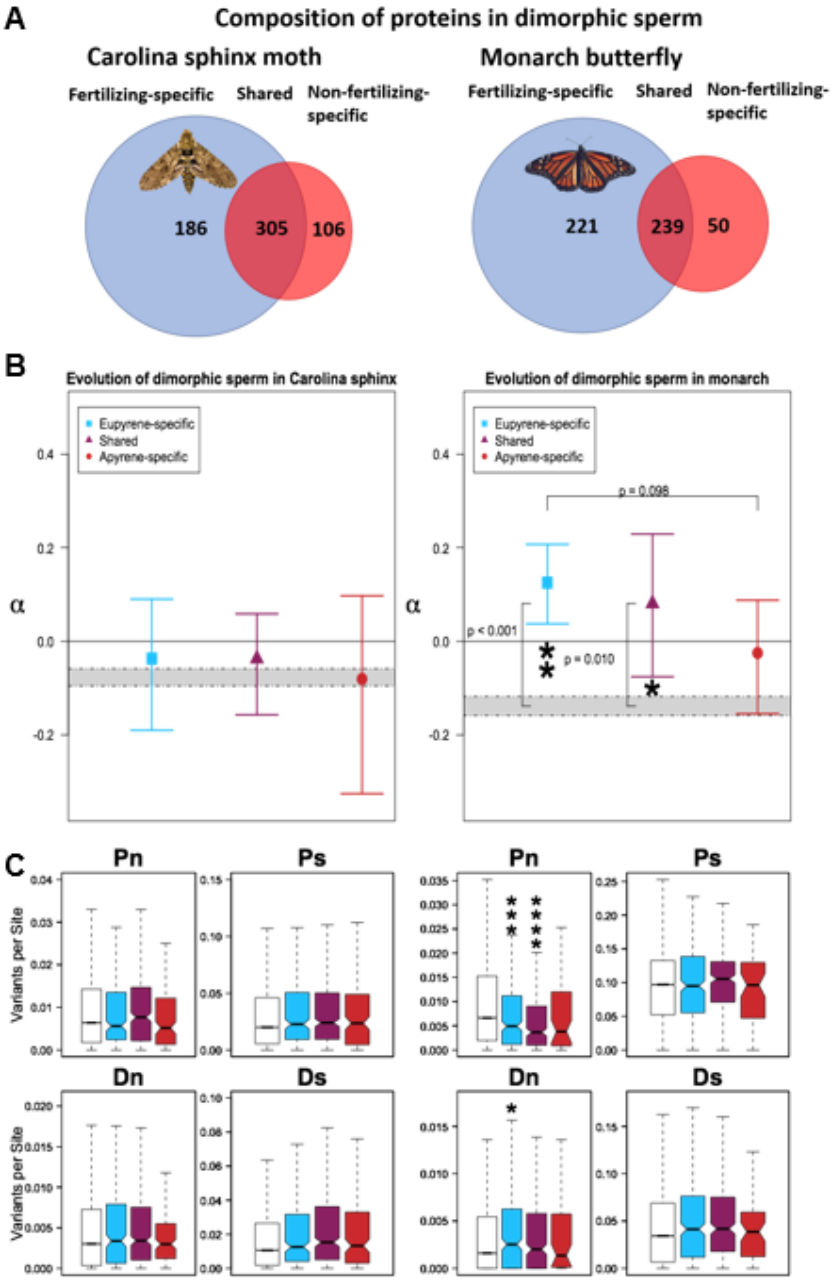



**Figure 4. A.** Composition of the sperm proteome with respect to dimorphic sperm. The majority of identified proteins were shared between the two cell types, followed by the set unique to eupyrene sperm, and finally the smallest set was the proteins found only in apyrene sperm. **B**. None of the sets of sperm proteins evolved either differently from each other or distinctly from the background genome (shaded regions represent 95% confidence intervals of the background) in the Carolina sphinx (left). In the monarch however (right), the signal for elevated α was localized to the eupyrene-specific and shared proteins. There was also a trend for increased α in eupyrene-specific proteins as compared to apyrene-specific. Error bars represent 95% confidence intervals from bootstrapping. **C**. Decomposing α into Pn, Ps, Dn, and Ds for dimorphic sperm. Plotting of variation follows the coloring and order in parts A and B; from left to right in each panel: background genome, eupyrene, shared, and apyrene sperm. Asterisks denote significant differences from the background genome based on a Wilcoxon-Mann-Whitney test, with * < 0.05, ** < 0.005, *** < 0.0005, etc.