Adaptive evolution of sperm proteins depends on sperm competition in a pair of Lepidoptera

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Abstract

Sperm are among the most variable cells in nature. Both within and between species, sperm display more diversity in form than one would expect of an ostensibly single-function cell. Morphologically, some of this variation within species has been demonstrated to be non-adaptive, but for many species that consistently produce multiple sperm morphs, the significance of variable sperm remains unknown. Here, we investigate the molecular evolution of dimorphic sperm in Lepidoptera, the butterflies and moths; males of this order produce both fertilizing eupyrene sperm and a secondary apyrene type that lacks DNA. Based on population genetic analyses in two species, the monandrous Carolina sphinx moth and the highly polyandrous monarch butterfly, we see evidence for increased selection 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, indicative of strong purifying selection. Additionally, rates of non-synonymous divergence are comparable between sperm genes and the rest of the genome, suggesting that many alleles reach fixation owing to positive selection as well. Investigation of the distribution of fitness effects of new non-synonymous mutations in monarch sperm confirms stronger selection on sperm proteins in monarchs, with very few neutral variants and a preponderance of deleterious and beneficial variants. These results mirror findings on sperm evolution in other taxa; increased sperm competition decreases within-population morphological variation. Our results suggest that sperm competition can be a powerful selective force at the sequence level as well.

Introduction

Sperm cells display remarkable diversity throughout the animal kingdom (Pitnick et al. 2009), from small and plentiful to gigantic (Pizzari 2006) or super-structure-forming (Higginson et al. 2012). Indeed, this variation exists at every level, from fixed differences between species to variability within individual males (Buckland-Nicks 1998; Swallow and Wilkinson 2002; Tavares-Bastos et al. 2002; Marks et al. 2008; Sasakawa 2009). In many independently evolved cases, males consistently produce two different sperm types in a phenomenon known as sperm dimorphism. Intriguingly, in all cases examined, only one of the two is capable of fertilization (Wilms 1986; Eckelbarger et al. 1989; Bressac et al. 1991; Carcupino et al. 1999; Sasakawa 2009). The evolutionary causes and consequences of variation in sperm morphology both within and between morphs are immediately intriguing. As gametes, these cells are the final step in the long chain of events leading to reproductive success. Why should such important components of fitness be so variable? Different fields of research have pursued largely independent explanations.

Morphological and behavioral reproductive biologists note that much of this diversity, at least within sperm morphs, can be attributed to deleterious variation, e.g. genetic defects (Chenoweth 2005) or age-related decline in sperm quality (Preston et al. 2015). This deleterious variation has also been shown to be inversely correlated with rates of sperm competition between species (Kleven et al. 2008), *i.e.* taxa that experience more sperm competition tend to have less morphologically variable sperm at both population and individual levels. From this perspective, sperm vary *in spite of* constraint imposed by their reproductive importance, with postcopulatory selection through sperm competition and cryptic female choice weeding out the suboptimal sperm variants in species with high rates of polyandry (Birkhead 1998; Immler et al. 2008). By extension of this paradigm, non-fertilizing sperm are often posited as specialized agents of sperm competition, acting as final combatants in male-male competition (Buckland-Nicks 1998; Swallow and Wilkinson 2002; Buckland-Nicks et al. 2010), though conclusive evidence to demonstrate as much is less abundant.

Separately, molecular biologists and geneticists have been mostly concerned with understanding the diversity of sperm (and other reproductive) proteins between species. One common observation is that reproductive proteins diverge rapidly between species. These proteins often appear as outliers compared to the rest of the genome and, in many cases, adaptive evolution is implicated as the cause of this elevated divergence (Civetta and Singh 1995; Willie J. Swanson and Vacquier 2002; Dorus et al. 2004). Typical reasons proposed include roles in sexual antagonism or speciation through post-copulatory, pre-zygotic isolation (Willie J Swanson and Vacquier 2002; Martin and Hosken 2004). These arguments posit that sperm and other reproductive proteins vary precisely *because of* their importance to fitness. However, experimental evidence to demonstrate the role of adaptation here is far less common (but see Swanson and Vacquier 1998). Moreover, high rates of non-adaptive variation within species could also generate elevated divergence in evolutionary time when coupled with relaxed selection (Willie J Swanson and Vacquier 2002; Willie J. Swanson and Vacquier 2002; Gershoni and Pietrokovski 2014). Only recently have theoreticians attempted to unify these patterns of molecular evolution with the pressures exerted by sperm competition (Dapper and Wade 2016).

As explained by Nearly Neutral Theory (Ohta 1992), the efficacy of selection on a set of proteins depends on the effective population size on which selection can act. Genes encoding reproductive proteins are typically expressed in only half of a given population (males or females), thus cutting in half the size of the population under selection (Whitlock and Wade 1995; Barker et al. 2005). Furthermore, proteins involved in postcopulatory events like sperm competition may only experience selection in the

presence of a competitor's proteins, which substantially decreases the opportunity for selection if females seldom mate more than once in a breeding season. This logic predicts that reproductive proteins can diverge more quickly than the rest of the genome due to relaxed selection and that adaptive evolution, particularly in sperm proteins, should only be a significant force in species with high rates of polyandry (Dapper and Wade 2016). Few molecular evolutionary studies have sought to disentangle the signal of adaptive evolution from the more easily observed patterns of divergence, and no studies have addressed the function of dimorphic sperm from this paradigm. Here, we present the first molecular evolutionary investigation of dimorphic sperm, integrating perspectives from both morphological reproductive biology and theoretical population genetics to better understand some of the most perplexing dimorphic sperm observed in the animal kingdom.

Sperm Dimorphism in Lepidoptera

Males of almost all species in the order Lepidoptera (butterflies and moths) produce two distinct sperm types: one fertilizing sperm type (**eupyrene**) and a second type (**apyrene**) which lacks a nucleus and nuclear DNA (Meves 1902). The function of apyrene sperm is poorly understood, but it is incapable of fertilizing eggs. Nevertheless, its production is hormonally regulated and occurs in a developmentally predictable way, implying a novel gain of function rather than loss of fidelity in spermatogenesis (Friedlander 1997), and evidence from organismal studies suggests that it plays some functional role(s) in reproduction (Takemura et al. 2006). Males can apparently control the ratio of the two sperm types in their ejaculate and typically transfer to females 10-20 times as much apyrene sperm as eupyrene sperm (Oberhauser 1988), leading some to suggest that apyrene sperm play a role in sperm competition (Silberglied et al. 1984; Swallow and Wilkinson 2002). Here, we assess patterns of both polymorphism and divergence among sperm proteins using two newly published proteomic datasets from both eupyrene and apyrene sperm of two species: the monarch butterfly, *Danaus plexippus* (Whittington et al. 2017), and the Carolina sphinx moth, *Manduca sexta* (Whittington et al. 2015).

While monarchs are typically studied to understand migration, insect-plant interactions, and disease ecology (de Roode et al. 2007; de Roode et al. 2008; Merlin et al. 2013), and Carolina sphinx moths are studied for physiology, immunology, and pest management (Kanost et al. 2004; Soberón et al. 2007; Sears et al. 2012), the pair also present an interesting contrast in mating ecology. North American monarchs spend time at incredibly high density in overwintering colonies in Mexico (Urquhart 1976). Owing to these unique population dynamics, female remating rates are among the highest observed in Lepidoptera. Females mate up to 14 times in the wild (Hill Jr. et al. 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.

We investigate the evolutionary genetics of these insects' sperm proteomes with two goals: to examine patterns of selection on sperm proteins between two species with differing mating systems and, if possible, to detect signatures of differing selection between sperm morphs within species. We also tested the general predictions for relaxed selection in sex-limited proteins using RNA-seq datasets for these two species from previously published data for *Manduca sexta* (Cao and Jiang 2017) and newly generated data for the monarch butterfly (summarized in Table S2). To do complete population genetic analyses, we have also generated the first published set of whole-genome resequencing data for *Manduca sexta* from a wild population.

Results

Differences Between Sperm Proteins and the Genome Background

First, we considered the sperm proteome as a whole and compared genes found in sperm to those in the genome background (here defined as all protein coding genes not present in the sperm proteome). Due to the lower gene count of the Z sex chromosome compared to the autosomes and the complications of mixed-sex sampling creating differing allele counts between autosomes and sex chromosomes, estimates from Z-linked genes were not investigated in these or subsequent analyses. In both species this subset contained >90% of the sperm proteome (Mongue and Walters 2017). Using permutation tests, we found no difference in the proportion of adaptive substitutions (α) between the sperm proteome and the rest of the genome in *Manduca sexta* ($p = 0.40892$, Figure 1A, left); for monarchs, however, the sperm proteome showed a significantly greater proportion of adaptive substitutions than the rest of the genome ($p = 0.00006$, Figure 1A, right).

To better understand the source of these results, we investigated the individual components of α: non-synonymous polymorphism (Pn), synonymous polymorphism (Ps), non-synonymous divergence (Dn), and synonymous divergence (Ds). We compared the scaled estimates of each (*e.g.* non-synonymous polymorphisms per non-synonymous site) to the genome background within each species using a Wilcoxon-Mann-Whitney test (Figure 1B). We found no differences between sperm and the genome background for any class of variants in *M. sexta* (Pn: $W = 3014100$, $p = 0.5964$; Ps: $W = 2879300$, $p = 0.1830$; Dn: $W = 3068300$, $p = 0.2009$; Ds: $W = 2895700$, $p = 0.2686$). The signal for elevated α in monarchs came primarily from Pn, which was greatly depressed in sperm ($W = 3062400$; $p = 3.224 * 10^{-}$

[11]) while other classes were comparable between sperm and the genome background (Ps: W = 2684200, p = 0.2720; Dn: W = 2506400, p = 0.1300; Ds: W = 2544400, p = 0.3437).

Next, we leveraged orthology, as established by Whittington *et al.* (2017), to further examine the apparent difference in selection occurring in monarch sperm proteins. A substantial portion of the monarch sperm proteome (~42%, 216 genes, Figure 2A) shares an ortholog in the sperm proteome of *M. sexta*; reciprocally, there are 236 genes in the *Manduca* sperm proteome that share an ortholog in the monarch sperm proteome (due to a few one-to-many orthologs). These proteins, hereafter referred to as *sperm homologs*, offer the opportunity to directly assess the selective pressures experienced by the same genes with conserved function but found in species with different levels of postcopulatory selection. We tested for differences in adaptive evolution between sperm homologs (containing an ortholog in the other species' sperm proteome) and proteins unique to one sperm proteome (orthology outside of sperm or no detectable orthology).

In *Manduca*, genes of these two classes did not differ in the proportion of adaptive substitutions with permutation testing (p = 0.6174, Figure 2B). In monarchs, we detected an increased proportion of adaptive substitution in the sperm homologs (p = 0.0372, Figure 2B). In fact, comparing between species, these sperm homologs had much higher α values in monarchs than in Carolina sphinx moths (p = 0.00008), while genes with unique expression in either species did not show differences between species (p = 0.5922).

Site-frequency-based methods

Based on different patterns of selection between sperm proteins and the genome background in monarch butterflies, we investigated the distribution of fitness effects (DFE) of new non-synonymous mutations in these genes. Using the same samples as above, we generated site frequency spectra to estimate the DFE and α for both the Carolina sphinx and monarch genome background, whole sperm proteome, and sperm homolog subset using a more complex likelihood model in the program polyDFE (Tataru et al. 2017).

The distribution of fitness effects of new non-synonymous mutations suggests stronger selection on sperm genes in monarchs but not Carolina sphinx moths. The DFE is largely unchanged from the genome background to whole sperm proteome to sperm homologs in the Carolina sphinx moth (Figure 3, left panels). For monarchs, the sperm proteome shows a dearth of weakly deleterious and effectively neutral variants, with a concurrent increase in strongly deleterious and beneficial variants. This pattern

178 is even more exaggerated in the sperm homologs, where almost no neutral variants are detectable
179 (Figure 3, right).

180 With polyDFE, we see a slightly higher α for the sphinx moth sperm proteome compared to the genome
181 background, but this pattern is not localized in the sperm homologs. Moreover, we see a much larger
182 difference in the way selection acts on sperm protein variants compared to the rest of the genome only
183 in monarch butterflies. Here, upwards of 90% of substitutions are inferred to be a result of adaptive
184 evolution in both the whole sperm proteome and the shared sperm orthologs (Supplemental Figure 1).
185 We note that estimates of α are influenced by the ways in which demography are (or are not) accounted
186 for, so the values obtained with this more complex likelihood method differ from our estimates based
187 solely on count-data. Likely, the point-estimates here are closer to the true proportions of adaptive
188 substitutions for these species, but we are more interested in relative patterns between classes of genes
189 than accurately estimating the true, absolute value of α per se.

190 *Molecular evolution in dimorphic sperm*

191 The two sperm proteomic datasets consisted of three classes of sperm proteins: unique to eupyrene
192 sperm, unique to apyrene sperm, or found in both cell types (henceforth "shared"). We assessed
193 differences in selective pressures between the sperm morphs with another series of permutation tests,
194 both comparing parts of the sperm proteome to the genome background and comparing parts of the
195 proteome to each other.

196 As expected based on the whole-proteome results, neither eupyrene-specific ($p = 0.55912$), shared ($p =$
197 0.4647), nor apyrene-specific proteins ($p = 0.96496$) differed from the genome background in the
198 Carolina sphinx (Figure 4). In monarchs, both eupyrene-specific proteins ($p =0.00018$) and shared
199 proteins ($p = 0.01038$) showed elevated α, but apyrene-specific proteins did not evolve differently from
200 the rest of the genome ($p = 0.55934$).

201 In *Manduca sexta*, α did not vary between apyrene-specific and eupyrene-specific proteins ($p = 0.7271$),
202 between apyrene-specific and shared ($p = 0.7176$) or eupyrene-specific and shared proteins ($p = 0.9979$).
203 Similarly, neither apyrene nor eupyrene sperm differed significantly from the shared set in monarchs (p
204 $= 0.6332$ & $p = 0.6234$, respectively). There was, however, a trend for increased α in eupyrene-specific
205 proteins compared to apyrene-specific proteins ($p = 0.0986$). In these analyses, we did not investigate
206 the role of orthology due to a loss of statistical power that would result from further subdividing our
207 datasets.

Patterns of Adaptive Evolution in Sex-Specific Tissues

Using RNA-seq data for a number of tissues in both monarchs and *Manduca sexta*, we calculated the tissue specificity metric, SPM (Kryuchkova-Mostacci and Robinson-Rechavi 2017), of every gene in the genome assembly with a sliding cutoff. Owing to the non-independence of estimates at each point, *e.g.* all genes that pass a strict threshold of SPM > 0.9 are represented in every estimate at lower thresholds, these data were not significance-tested. Nevertheless, the results from both species' sperm proteomes hold at all SPM thresholds. In monarchs, sperm genes show consistently higher α than the whole genome (Figure 5A). For the Carolina sphinx moth, sperm genes do not diverge from the whole genome, except at very high specificity thresholds, where sperm genes show lower α (Figure 5B). Finally, we note a general trend for increasing rates of adaptive evolution in more tissue-specific genes in monarchs but not in the Carolina sphinx moth. This result mirrors the inferred DFEs; there is little evidence for adaptive evolution, as indicated by positively selected variants, in the Carolina sphinx moth genome background.

Demographic estimates

Finally, using site frequency from 4-fold degenerate sites in the two species' genomes, we estimated population size history (Figure S2). Both have effective population sizes near 2,000,000, as expected of herbivorous invertebrates with high dispersal potential, numerous host plants, and a large range over North America. We also recover a population size increase in monarch butterflies in the recent past, which has been previously reported with genomic data (Zhan et al. 2014). We note that our inferred timing of this event differs from that of the previous authors, who used mutation rate estimates from *Drosophila melanogaster*. Such parameter differences affect the estimated time of events, but not the trajectories.

Discussion

We found elevated adaptive evolution in the sperm proteome of monarch butterflies compared to the genome background, but not in *Manduca sexta* (Figure 1). In particular, this difference is greatest for sperm genes with a sperm homolog in *M. sexta* (Figure 2B), suggesting that the same genes experience stronger selection in the polyandrous species. Intriguingly, the source of this signal was not increased divergence, but a reduction in non-synonymous polymorphism in sperm proteins (Figure 1B, top left). This pattern suggests an increased role of purifying selection on sperm protein variants in monarchs. These results echo the work of morphological reproductive biologists, who have shown that bird species

with higher rates of sperm competition show less intraspecific and intra-male variation in sperm length (Immler et al. 2008; Kleven et al. 2008). On the sequence level, similarly increased purifying selection has been observed in genes expressed in pollen, the main male-male competitors in flowering plants (Arunkumar et al. 2013). Our results are, to our knowledge, the first indication that this pattern of intensifying selection and decreasing intraspecific variation extends to the molecular level in sperm specifically.

That stronger purifying selection results in increased adaptation may seem counterintuitive, but greater selective scrutiny of within-population variation necessarily changes the patterns of fixation of alleles. Stronger positive selection obviously increases the proportion of adaptive divergences, but stronger purifying selection has a similar, albeit indirect effect. With greater purifying selection, fewer weakly deleterious alleles can fix by drift, meaning that the alleles that do reach fixation and contribute to divergence are more likely to be positively selected. Thus, theoretically, reproductive proteins experiencing stronger selection should not have elevated rates of divergence (Dapper and Wade 2016).

This reasoning runs contrary to the conventional wisdom of reproductive protein evolution, but as stated above, while observations of elevated divergence of reproductive proteins are common (Civetta and Singh 1995; Willie J. Swanson and Vacquier 2002; Dorus et al. 2004; Martin and Hosken 2004), evidence directly linking this pattern to adaptive processes is much more limited. In one recent exception, a role for sperm competition in adaptive evolution was suggested for wrasses, but this study assessed genes with male-biased expression rather than sperm genes directly and did not account for demography in modeling evolution (Dean et al. 2017). Furthermore, interpretation of results in spawning fish is complicated by the increased importance of abiotic selective pressures on sperm associated with external fertilization, as observed by Liao *et al.* (2018). We directly assessed the evolution of sperm proteins in two internally fertilizing species, avoiding such confounding environmental variables.

Targets of selection

Without better functional annotation of genes in either genome, it is difficult to identify the exact roles of the proteins under stronger selection in monarchs, but the signal from sperm homologs suggests that these genes have had conserved sperm function since the divergence of the two species some 100 million years ago (Heikkila et al. 2012). Such genes relate to core traits in sperm, according to recent gene ontology analyses (Whittington et al., in submission). In a similar vein, both proteins shared in the

two sperm types and those unique to fertilizing eupyrene sperm show an elevated α compared to the genome background (Figure 4). Shared sperm proteins are enriched for structural proteins that give rise to the sperm tail and thus impact motility (Whittington et al., in submission), while those expressed only in eupyrene sperm doubtless include important mediators of fertilization. On the morphological scale, variation in universal sperm traits like swimming ability, longevity, and overall viability are known to affect sperm competition outcomes (Burness et al. 2004; Kim et al. 2017) and have been shown to have a polygenic basis in other taxa (Hering et al. 2014). For traits like longevity and motility there is a threshold below which fertilization becomes significantly impaired, but in the absence of competitor alleles, there is likely a larger range of effectively-neutral trait-values, allowing for more variation to be maintained in the population. In the presence of competitor alleles, however, marginal differences in fertilization success come under selection, leading to the removal of deleterious variants.

Indeed, while the exact mechanisms of fertilization in Lepidoptera remain unclear, eggs are known to possess multiple micropyle openings for sperm (Kumar et al. 2007) and eupyrene sperm possess structures resembling an acrosome while their apyrene counterparts do not (Wolf 1992). This somewhat rare combination of male and female gamete structures is also found in sturgeon, in which the multiple micropyles give several sperm potential access to the egg nucleus and there is competition among sperm to initiate karyogamy via the acrosome reaction (Psenicka et al. 2010). If a similar dynamic exists in Lepidoptera, acrosomal proteins in eupyrene sperm would be likely targets for selection in polyandrous systems.

Whatever the mechanics of fertilization are, paternity outcomes in polyandrous species are routinely observed to be strongly bimodal (Simmons and Siva-Jothy 1998; Wedell and Cook 1998), including in monarch butterflies (Mongue et al. 2015). In other words, one of the two males in a double mating typically fathers most, if not all, of the observed offspring produced by the female, but there is little consistency in whether it is the first or second male. With these dynamics, fitness differences between winning and losing sperm phenotypes are large and selection can reliably remove less successful genotypes.

Evidence of this can be seen in the estimated distribution of fitness effects of new mutations in monarch sperm proteins. Compared to the genome background, we see a decrease in the proportion of effectively neutral and weakly deleterious mutations ($-10 \leq s \leq 0$) and an increase in both strongly deleterious ($s \leq -10$) and beneficial ($s > 0$) mutations (Figure 3, right). The increase in apparently beneficial mutations follows the logic above. In the absence of competition, not only are mildly

299 suboptimal variants effectively neutral, but novel beneficial variants have no point of comparison for
300 fertilization efficiency. Thus, more efficient sperm variants should have no selective advantage in
301 monandrous species unless they markedly increase fitness in a single mating. This reasoning is
302 supported by the estimated distribution of fitness effect for the complimentary gene sets in the Carolina
303 sphinx moth; in this species, we see little variation in the DFE between the genome background and the
304 sperm proteome (Figure 3, left).

305 *Sperm dimorphism*

306 We failed to detect a strong difference between eupyrene (fertilizing) and apyrene (non-fertilizing)
307 sperm in either species (Figure 4). Moreover, apyrene sperm did not show a distinct pattern of evolution
308 compared to the genome background. If these sperm were truly involved in interfering with competitors'
309 sperm, as some have suggested (Silberglied et al. 1984; Swallow and Wilkinson 2002; Solensky and
310 Oberhauser 2009), we would expect evidence for stronger selection in apyrene sperm of monarch
311 butterflies; if anything, however, there was a trend for more adaptive evolution in eupyrene-specific and
312 shared-sperm proteins (Figure 4, bottom), likely due to fertilization dynamics coupled with sperm
313 competition as discussed above. Moreover, there appears to be proportionally less detectable orthology
314 in apyrene-specific than eupyrene-specific proteins in both species (Whittington et al., in submission),
315 suggesting that the apyrene sperm proteome is less conserved and potentially less selectively
316 constrained over evolutionary time.

317 These results suggest a few possibilities for apyrene sperm. Firstly, it is possible that the function(s) of
318 apyrene sperm is governed by a small subset of apyrene-specific proteins. Because our methods
319 aggregate signal for selection across multiple genes or sites to counteract high variance in variant counts
320 within genes (Stoletzki and Eyre-Walker 2011), the importance of these few genes could be lost in the
321 heterogeneous selection on different proteins. Still, the observation that few or no apyrene specific
322 genes show mating-system-dependent evolution argues against an *active* role for apyrene sperm in
323 sperm competition, but apyrene sperm may still have adaptive significance without specialized
324 molecular function.

325 The filler hypothesis also relates to sperm competition, but posits that apyrene sperm are employed
326 proactively, to fill the female's sperm storage organ and delay remating, thus decreasing the risk of
327 sperm competition rather than impacting its outcome (Swallow and Wilkinson 2002). Indeed, both in
328 monarchs and another butterfly (*Pieris napi*), female time to remating increases with the number of

apyrene sperm received from males (Oberhauser 1988; Cook and Wedell 1999). Such observations are somewhat confounded by the size of the spermatophore nuptial gift that males provide during mating, but apyrene sperm themselves have been proposed as a form of nutritional nuptial gift, so these two hypotheses are not mutually exclusive (He et al. 1995; Lamunyon 2000). Under both the nutrient and filler hypothesis, the actual protein content of apyrene sperm should be less important than its physical presence and abundance, so factors affecting the rate of apyrene sperm production would be more likely targets for selection in polyandrous species than the proteins sequences themselves.

Finally, we consider the most intriguing hypothesis for apyrene sperm function: capacitation of fertilization. This hypothesis is based on the observation that apyrene sperm appear to be necessary for successful fertilization in *Bombyx mori* (Takemura et al. 2006); the mechanism here is unclear, but could conceivably involve one or a few genes that interact with the female reproductive tract to make conditions more favorable for eupyrene sperm. In such a case, these proteins would behave more akin to the broader class of reproductive proteins with sex-limited expression and evolve independently of rates of polyandry in a species. Moreover, the experiment demonstrating the capacitation role in *Bombyx* used apyrene sperm from a different male to restore fertilization success to the experimental male (Sahara and Takemura 2003; Takemura et al. 2006), so whatever the method of capacitation is, it lacks the allorecognition to be involved in offensive sperm competition.

Evolution of genes with sex-limited expression

One corollary to the model of reproductive protein evolution proposed by Dapper and Wade is the prediction that genes with sex-limited expression can diverge more quickly under relaxed selection due to the smaller effective population size of males or females compared to the population as a whole (2016). As discussed above, we recovered their predictions for variable selection on sperm proteins but did not observe a strong pattern of difference in the evolution of genes with testes-specific expression, our proxy for sex-limited expression (Figure 5). This pattern was not formally tested, but we did not see relaxed selection for the apyrene-specific set of genes either, which, as discussed above, may behave more like male-limited genes than sperm-competition genes.

Nearly Neutral Theory may provide an explanation for these discrepancies. As mentioned above, larger populations have more efficient selection and a smaller range of slightly deleterious mutations that behave neutrally (Ohta 1992). Formally, mutations with a selective effect less than $1/N_e$ are expected to behave neutrally. For instance, one commonly cited estimate for human population size is Ne ≈ 10,000

over our species' evolutionary history (Zhao et al. 2000). Based on this, mutations with selective effects less than 0.0001 should behave neutrally for alleles expressed in both sexes, while those with effects of 0.0002 are effectively neutral for alleles only expressed in one sex. And indeed, there is evidence that genes expressed only in men have a higher mutational load than those expressed in both sexes (Gershoni and Pietrokovski 2014). Chimpanzees, another species with a similar effective population size (Won and Hey 2005), also show increased non-synonymous divergence in reproductive proteins (Wong 2010). Moreover, male reproductive protein evolution appears to depend more on effective population sizes than intensity of sperm competition in the great apes in general (Good et al. 2013).

Consider, however, that the effective population sizes of insects are orders of magnitude higher than those of most mammals. Using neutral site frequency spectra, we estimate effective populations near 2,000,000 for both North American monarchs and Carolina sphinx moths (Figure S2). Consequently, selection is much more effective in these populations; mutations with effects above $5*10^{-7}$ should be subject to selection in both sexes and those with effects on the order of $1*10^{-6}$ under selection if expression is sex-limited. In other words, even selection on alleles with sex-limited expression in these insects should be 100 times stronger than selection on the entire human population. There could indeed be a two-fold difference in selection, but the absolute magnitude of the difference would be miniscule, and the effects of mating system would be more apparent.

Conclusions

Established literature on sperm morphology has long recognized the effects of sperm competition on variation in sperm traits (Immler et al. 2008; Kleven et al. 2008). Whether this pattern extends to the nucleotide level has been unclear because research on molecular evolution in sperm proteins has focused less on non-adaptive variation and more on explaining positive selection. Our investigation of the sperm proteome in two Lepidoptera sits at the intersection of these fields and provides important insights for the influence of post-copulatory selection in molecular evolution of reproductive proteins. In a polyandrous species, sperm genes experience a strikingly different selective environment than the rest of the genome. When considering genes with a sperm homolog in both species, we see weaker selection in the monandrous species, consistent with selection through sperm competition. The results on genetic variation are visible with both straightforward count-based and sophisticated model-based population genetic analyses. The evolution of dimorphic sperm, however, does not show such strong differences, but patterns of increased selection in fertilizing-sperm-specific proteins are consistent with sperm competition as a driver of selection.

Our findings alone do not confirm the function of apyrene sperm in Lepidoptera, but they do contextualize established hypotheses. We suggest that apyrene sperm are unlikely to be active agents of sperm competition, but rather, may play a passive role in reducing the risk of competition. The method by which apyrene sperm capacitate fertilization in some species remains unclear based solely on genomic approaches and will likely require functional experiments to completely understand.

Materials and Methods

Sources of data

We used gene sets from the published genomes of each species (Zhan et al. 2011; Kanost et al. 2016) with sperm genes identified from their respective proteomes (Whittington et al. 2015; Whittington et al. 2017). 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). Divergences were called by comparison to the queen butterfly (*Danaus gilippus*, previously published) for monarchs, and the five-spotted hawkmoth *(Manduca quinquemaculata*, sequenced for this project) for the Carolina sphinx moth.

In both focal species, we used twelve wild-caught individuals for sampling of polymorphism. In the case of Carolina sphinx moths, these were twelve males caught over the course of three nights. The sex-biased sampling reflects a sex bias in dispersal and collection at the light trap. In the case of monarchs, samples were selected based on depth of sequencing coverage in the published dataset and included 8 females and 4 males from the panmictic North American migratory population. These samples added the complication of unequal sampling between the autosomes (n = 24) and Z sex chromosome (n = 16). Despite the Z's enrichment in the sperm proteome however, the vast majority of sperm genes (92% in the Carolina sphinx, 90% in the monarch) are autosomally linked in both species (Mongue and Walters 2017). Due to the lowered power for statistical testing and limited inference to be gained from Z-linked genes, we focused on the autosomal genes in both species in subsequent analyses.

SNP-based methods

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

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

This statistic was calculated with custom R scripts in R version 3.3.3 (R Core Team 2017).

Assessment of adaptive evolution and statistical significance

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

These analyses were applied within-species at several levels: differences between the sperm proteome and genome background, differences between sperm homologs and unique proteins in the proteome, and, finally, differences between the two sperm morphs. The whole proteome comparison is relatively straightforward. For the sperm homolog to novel proteins comparison, we considered orthology in the same manner as we did in a previous investigation of genomic architecture in these two species (Mongue and Walters 2017). Sperm proteins were divided into one of two classes based on orthology and expression: either sperm homolog or unique if the ortholog was not found in the sperm proteome

or there was no detectable ortholog. In these analyses, the sperm proteome was taken as a whole, agnostic of sperm dimorphism. To examine differences in dimorphic sperm, we finally considered the gene product's location in the proteome, either unique to eupyrene sperm, unique to apyrene sperm, or shared in both types. For these analyses, we did not consider orthology status owing to the reduction in power that would accompany multiple layers of subdivision of the dataset.

Site-frequency-based methods

We also investigated molecular evolution by leveraging site-frequency-spectrum-based approaches as complimentary evidence. We used the population genetics software suite ANGSD (Korneliussen et al. 2014) to generate site frequency spectra at putatively neutral (four-fold degenerate) and selected (zero-fold-degenerate) sites in the genome. We unfolded site frequency spectra using parsimonious inference of ancestral state of alleles. These unfolded spectra were fed into the sfs-based tool polyDFE (Tataru et al. 2017) to examine rates of adaptive evolution with a more complex likelihood model that corrects for effects of demography and misattribution of ancestral state. We compared sites from the genome backgrounds to sites from the sperm proteomes to see if estimates of α or the distribution of fitness effects of new mutations differed between these two gene sets in each species. Divergence counts were omitted here to simplify the likelihood computation for these large datasets and remove any error for misattributed divergence. To test the robustness of results, input data were bootstrapped 100 times to obtain confidence intervals for parameter estimates. Processing of model inputs and outputs was accomplished with custom R scripts.

Investigation of sex-limited and tissue-specific expression

Next, we investigated the robustness of results from the sperm proteome analyses using RNA-seq data in these taxa. For *Manduca sexta*, there exists a wealth of tissue-specific data at multiple developmental timepoints (Cao and Jiang 2017). Because we were primarily interested in sperm involvement, we focused on sequencing from adult males, specifically RNA from the testes, head, thorax, and gut. Expression (as measured by FPKM) was averaged across biological replicates where available in this species. Monarchs had no comparable published data, so we sequenced the head, thorax, gut, testes, and accessory gland of three adult males.

In order to localize the signal for specific expression, we calculated tissue-specificity as SPM (specificity metric), a ratio ranging from 0 to 1 on the proportion of gene expression limited to a focal tissue (Kryuchkova-Mostacci and Robinson-Rechavi 2017). For instance, a gene with an SPM value of 0.8 for

the testes shows 80% of its total expression in the sampled tissues in the testes. Rather than strictly defining a single threshold for tissue-specific expression compared to general expression, we implemented a sliding cut-off. For a series of SPM thresholds ranging from 0 to 1 in increments of 0.5, all genes of a given class that showed specificity higher than the threshold were included in a calculation of α. This methodology created substantial non-independence between point estimates and precluded significance testing, but still allowed us to investigate whether results from the sperm proteome were sensitive to filtering of the included genes.

We first compared the effect of SPM threshold value on all genes in the genome that had non-zero expression in sampled tissues. These genes were evaluated based on the maximum SPM across all tissues. For sperm proteins, we considered only genes identified in the sperm proteome and ranked them by SPM in the testes. Finally, for male-limited non-sperm genes, we excluded sperm proteome genes and considered again those ranked by specificity in the testes (or testes and accessory glands for monarchs). These analyses were completed with custom R scripts as well.

Demographic estimates

Finally, to contextualize the previous analyses, we characterized present and historical population size from genomic data. Using 4-fold degenerate frequency spectra, we estimated neutral coalescence patterns with the program stairway plot (Liu and Fu 2015). For estimated generation time, we used the widely cited four generations per year for monarchs and three for the Carolina sphinx moth. Finally, for mutation rate, we chose the estimate $2.9*10^{-9}$ from the butterfly *Heliconius melpomene*, the closest relative with a spontaneous mutation rate estimate (Keightley et al. 2015).

Acknowledgments

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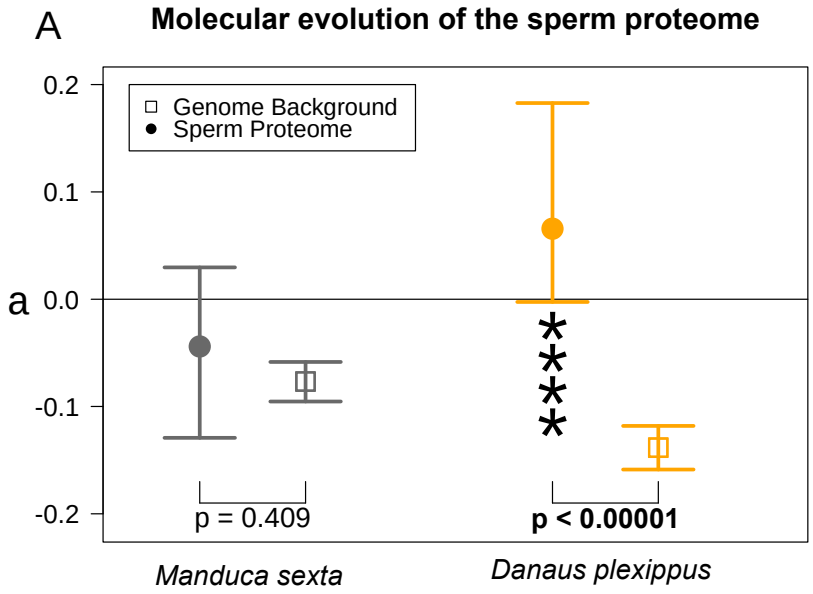


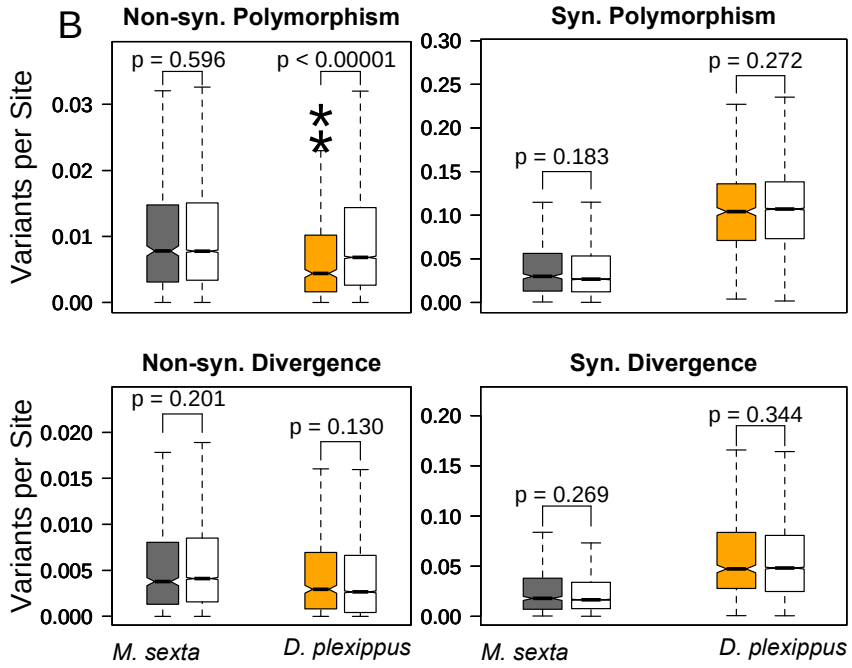
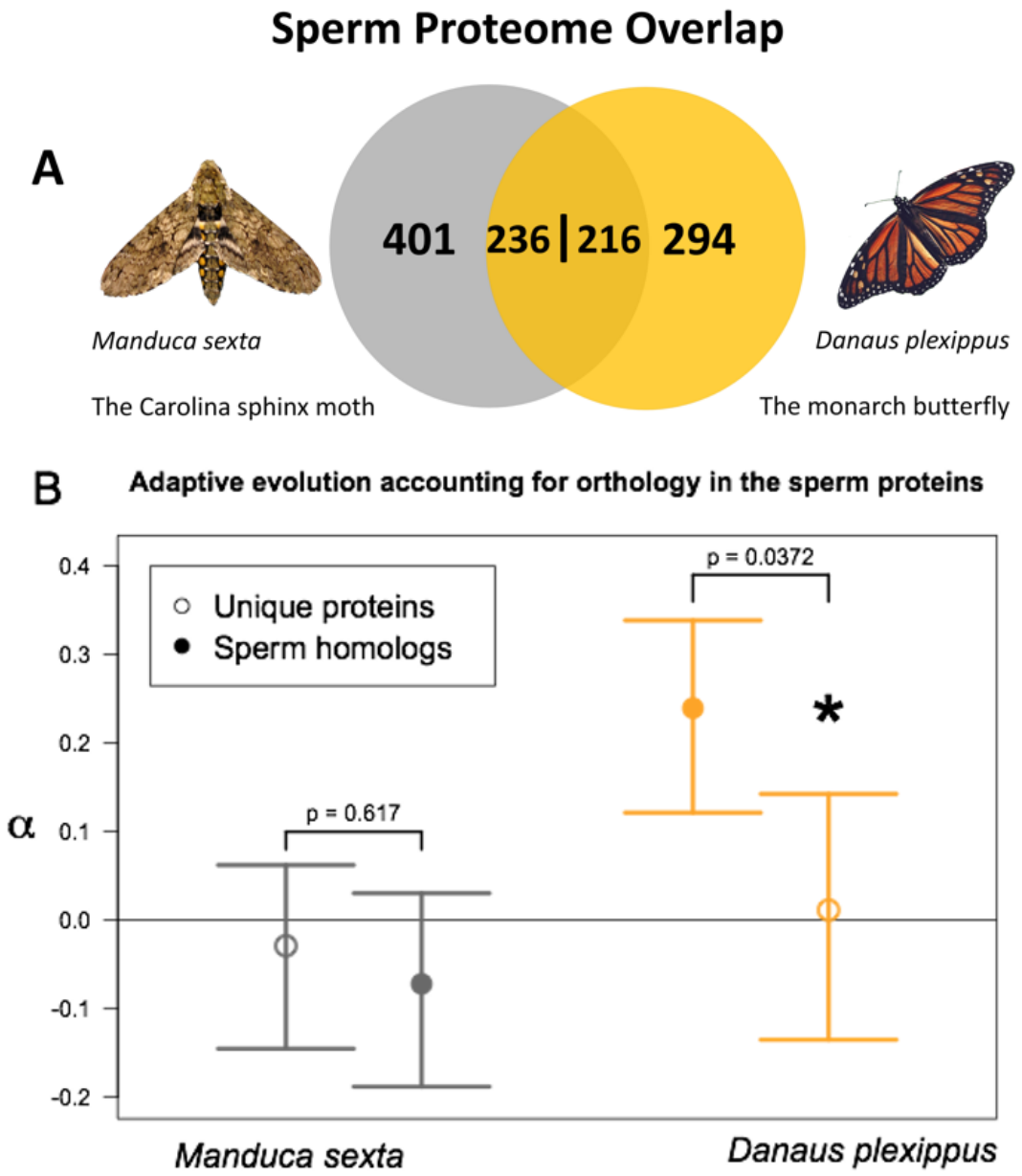


Figure 1. A. Genes in the sperm proteome of monarch butterflies (*D. plexippus*) show a significantly higher proportion of adaptive substitutions (α) than the rest of the genome (right); compare to the Carolina sphinx moth (*M. sexta*) in which there is no difference between the sperm proteome and the rest of the genome. 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 genome background (open boxes). There were no strong differences between sperm genes and the genome background 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 are derived from Wilcoxon-Mann-Whitney tests.



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

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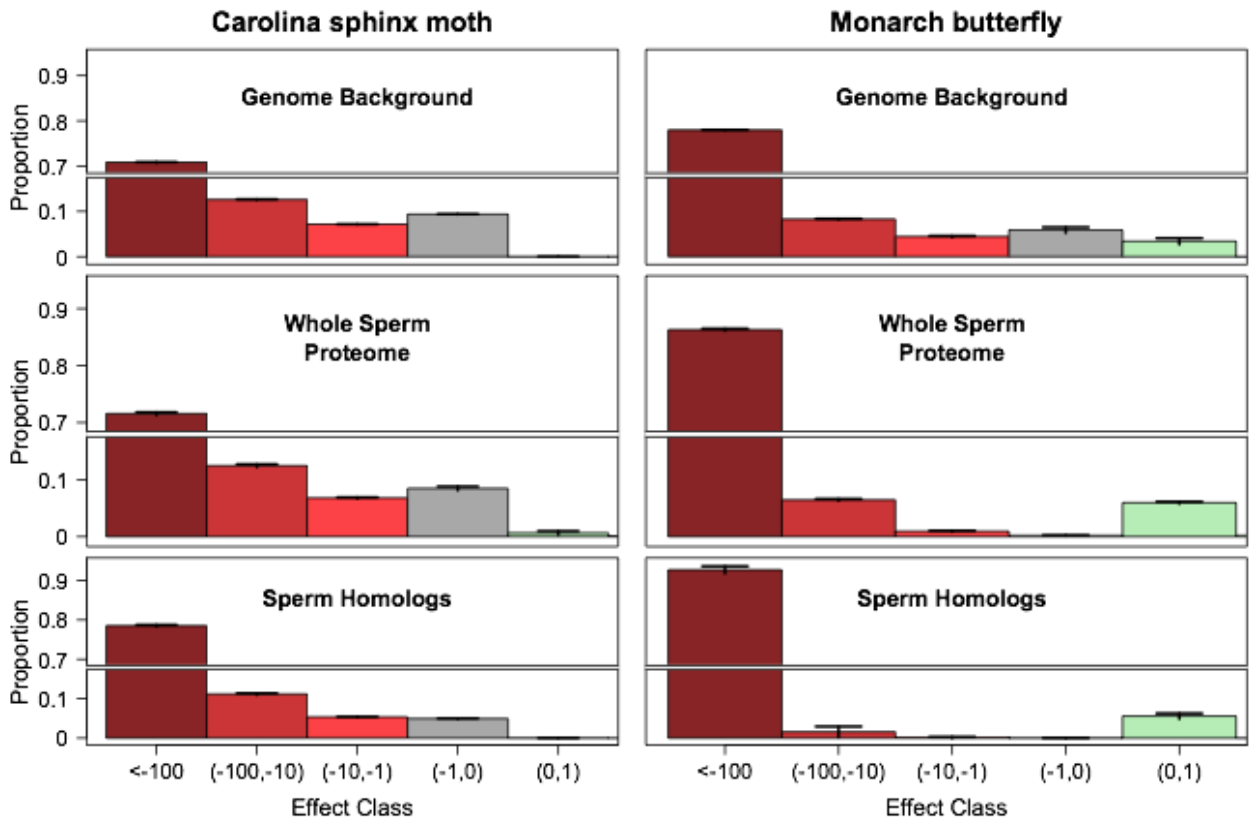
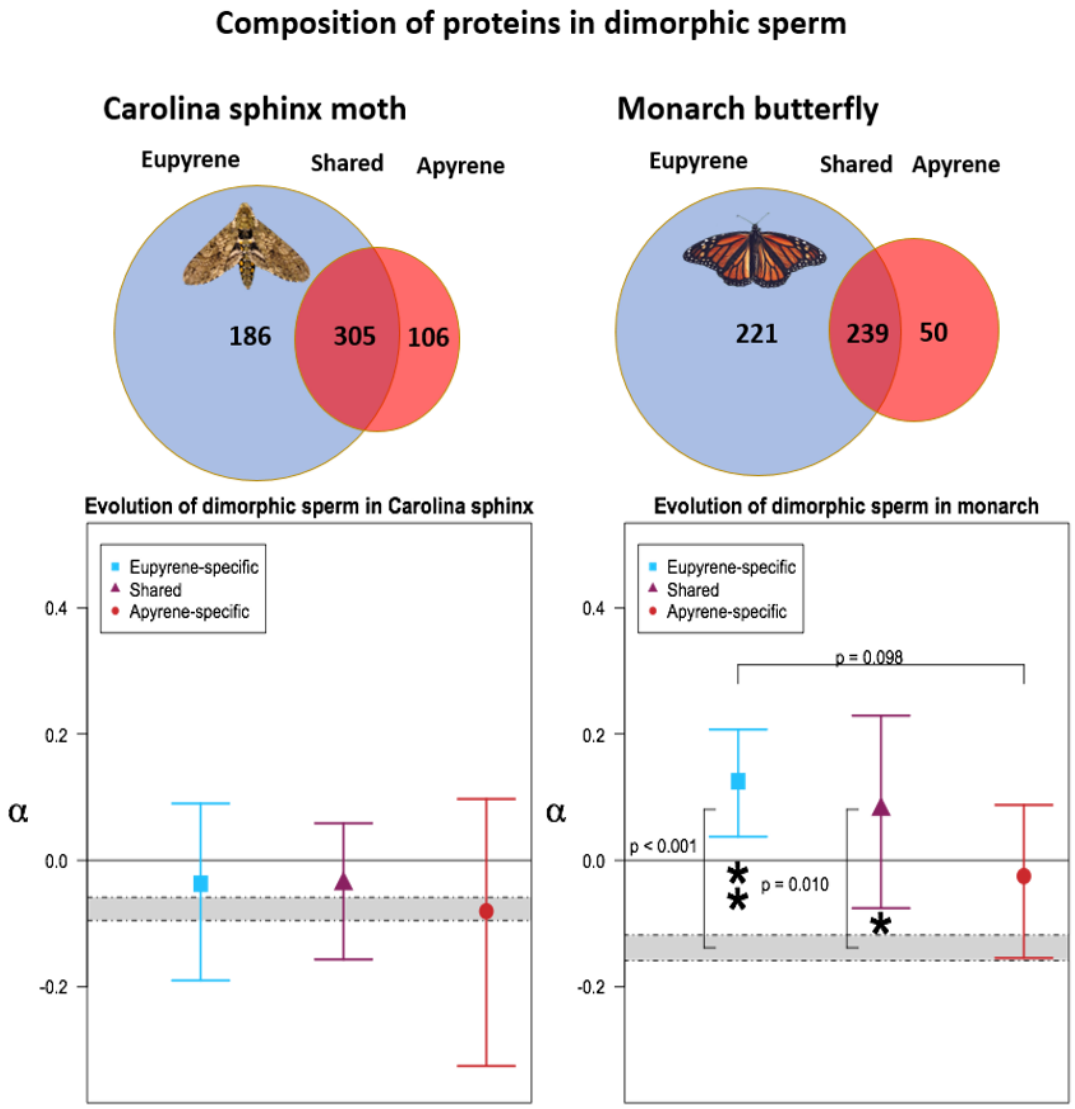


Figure 3. Predicted distribution of fitness effects of new non-synonymous mutations for the gene sets investigated in Figures 1 and 2. From top to bottom: autosomal genome background, autosomal sperm proteome, and the subset of sperm homologs found in both species. Bars represent the mean proportion for each selective class, with error bars representing twice the standard error of the mean from 100 bootstrap replicates of the input data. Note the gap in the y-axis due to the preponderance of strongly deleterious (s <-100) mutations. **Left.** The DFE shows little variation between the background and the sperm data sets, baring a slight increase in the proportion of strongly deleterious mutations. **Right.** In monarch butterflies, note the increasingly bimodal distribution of fitness effects that coincides with increased selection inferred from earlier analyses. In the sperm proteome (middle), there is a decrease in effectively neutral (gray, $-1 < s < 0$) and weakly deleterious (light red, $-100 < s < -1$) variants, with a concomitant increase in both strongly deleterious (dark red, $s < -100$) and beneficial (green, $0 < s < 1$) variants. In sperm homologs this effect is even more pronounced, with nearly all variants coming under selection.



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755 **Figure 4. Top.** Composition of the sperm proteome with respect to dimorphic sperm. A majority of
756 identified proteins were shared between the two cell types, followed by the set unique to eupyrene
757 sperm, and finally the smallest set was the proteins found only in apyrene sperm. **Bottom**. None of the
758 sets of sperm proteins evolved either differently from each other or distinctly from the genome
759 background (shaded regions represent 95% confidence intervals of the genome background) in the
760 Carolina sphinx (left). In the monarch however (right), the signal for elevated α was localized to the
761 eupyrene-specific and shared proteins. There was also a trend for increased α in eupyrene-specific
762 proteins as compared to apyrene-specific. Error bars represent 95% confidence intervals from
763 bootstrapping.

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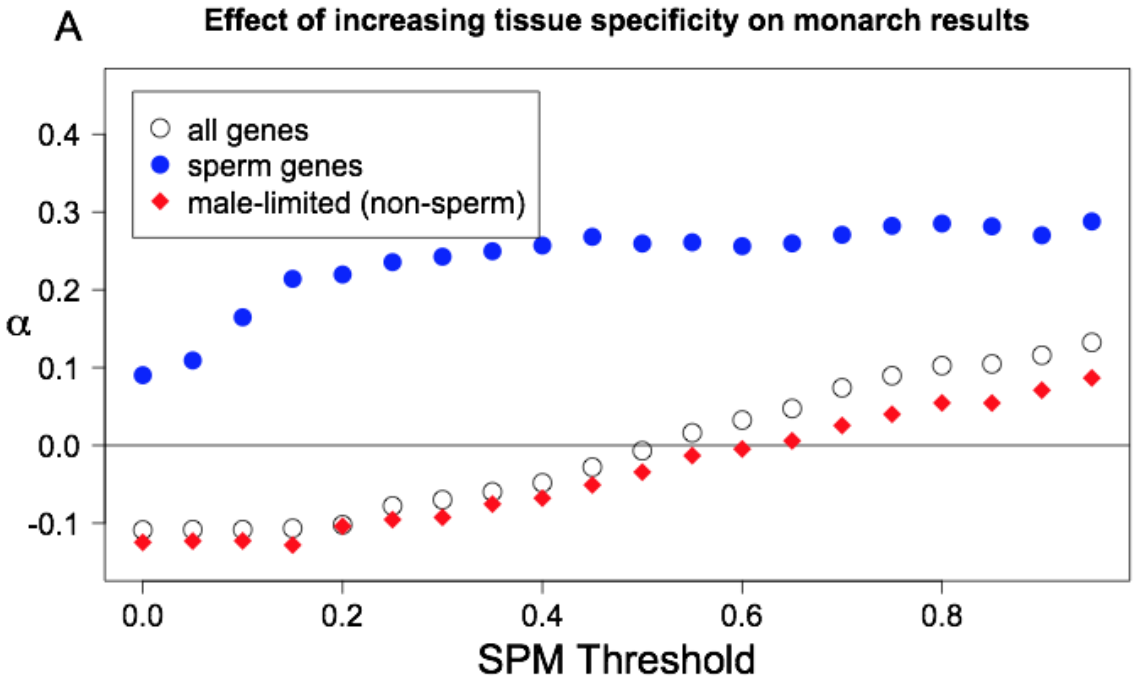
765



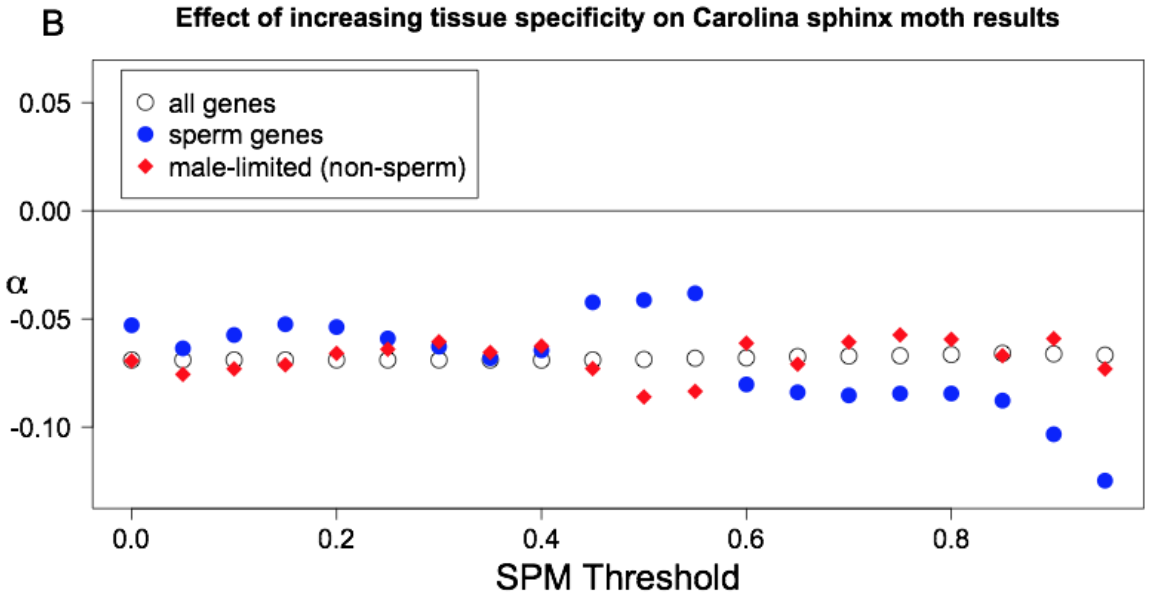


Figure 5. Estimated adaptive evolution as a function of specificity of gene expression, using a sliding cut-off for specificity (SPM). **A.** In monarchs, there is an overall increase in inferred α with increasing tissue specificity for all classes of genes. Adaptive evolution of the sperm proteome is higher than the genome as a whole at all cut-off thresholds. Interestingly, male-limited genes (as defined by high specificity of gonadal expression) appear to evolve similarly to somatic genes. **B.** Sperm proteins in the Carolina sphinx do not show differences from the genome for any but the strictest cut-off specificities, and even then, α slightly decreases compared to the rest of the genome. Genes with testes-limited expression again do not show differences from the genome background.