**Title:** Novel genetic control of migratory diapause in Australian Monarch Butterflies

**Authors:** William Hemstrom1,2, Micah Freedman3, Myron Zalucki4, Michael Miller1

**Author Affiliations:**

1. Department of Animal Science, University of California, Davis
2. Department of Biological Sciences, Purdue University
3. Department of Botany, University of British Columbia
4. School of Biological Sciences, The University of Queensland

# Abstract

Monarch Butterflies (Danaus plexippus) are a charismatic and culturally important North American butterfly species that are famous for their dramatic and unique migratory life history. While non-migratory populations of the species are widespread and not of particular conservation concern, migratory populations in North America have recently seen dramatic declines, prompting concern that the migratory life history of the species may vanish over the coming years as selective factors begin to favor residency over migration. However, one relatively new non-native monarch population in Australia rapidly re-acquired a migratory life history following hundreds of generations of residency and successive bottlenecks as the species stepping-stoned across the Pacific during the late 1800’s and early 1900’s. The genetic process by which this happened is not currently known. We raised and sequenced individuals from Queensland, Australia and found a novel association between reproductive diapause, a key migratory trait, and a genetic locus that has not previously been identified to be involved in migration in North America. This may imply that the species might be able to rapidly re-evolve migration in other places as well and thus provides some hope that the feared loss of migration in North American monarchs may not be permanent.

# Introduction

Since they rely on multiple distinct ecosystems and consistent seasonal timings, migratory species are acutely at risk from global anthropogenic environmental change (Both et al., 2010; Chowdhury et al., 2021).The loss of breeding, wintering, or any transitional habitats may all cause declines in migrants (Robbins et al., 1989), as may shifts in resource phenology due to seasonal changes which can cause resource availability and species life-histories to fall out of synchrony (T. Jones & Cresswell, 2010). It is therefore not surprising that widespread declines have been observed in many migratory taxa (Limburg & Waldman, 2009; Robbins et al., 1989; Runge et al., 2014).

Population-level adaptability in migratory life-history should therefore be expected to increase long-term population viability by conferring resistance to phenological and environmental changes (Pulido & Berthold, 2010). Migratory life-history adaptation can essentially occur along three distinct axes: 1) *temporal*, or adaptation that changes the phenology of migration; 2) *spatial*, or adaptation that alters migratory pathways and orientation; and 3) *residency*, or adaptation that shifts populations between migratory and resident life histories. Inter-population variation and adaptation in each of these pathways is well known from nature: temporal changes or variation in migration are well documented in many taxa including as birds (Chu et al., 2022; Gordo, 2007; Mondain-Monval et al., 2021; Pinszke & Remisiewicz, 2023; Prytula et al., 2022; Rubolini et al., 2007), fish (Kovach et al., 2012), mammals (Shuert et al., 2022), and butterflies (Culbertson et al., 2022; Fitchett et al., 2022); as is short-stopping, extension of migratory pathways, or complete re-direction of migration (Ambrosini et al., 2011; Dufour et al., 2021, 2022; Jia et al., 2021; La Sorte & Thompson III, 2007; Rushing et al., 2020; Sonnleitner et al., 2022; Visser et al., 2009); and shifts towards residency or partial migration have been observed and/or predicted in birds (Bonnet-Lebrun et al., 2020; Falchi et al., 2023; Haran et al., 2021; Møller et al., 2014; Plummer et al., 2015; Pulido & Berthold, 2010), butterflies (Howard et al., 2010), mammals (Fleming et al., 2003; J. D. Jones et al., 2014; Van Der Ree et al., 2006), fish (Adams et al., 2022), and other taxa.

Adaptation via a transition to residency is of both particular interest and concern for the conservation of migratory species. While switching to a resident life history can allow species to avoid the increasing risks of migration and the intrinsically large energetic costs of migration in general (McWilliams et al., 2004; Rankin & Burchsted, 1992; Wikelski et al., 2003), there is reasonable concern that the “extinction” of migration may not be easily reversible in some species. This is concerning since migratory life-history variation is expected to increase population adaptability (Pulido, 2007), and thus the permanent loss of migration could potentially compromise long-term population persistence. Beyond this, migratory species often provide critical ecosystem services (López-Hoffman et al., 2017; D. J. Semmens et al., 2011) and have cultural and/or ecological significance (Burger et al., 2015; Reynolds & Clay, 2011; Schuetz & Johnston, 2019; D. J. Semmens et al., 2018) that may not survive the transition to permanent residency.

The loss of migratory behavior is a major concern for monarch butterflies (*Danaus plexippus*) (Brower & Malcolm, 1991; Satterfield et al., 2018). Specifically, North American migratory monarch populations have experienced severe declines over the last several decades, sparking a fear that those populations may go extinct in the near future (B. X. Semmens et al., 2016). However, many individual butterflies ostensibly from the North American migratory population are now year-round residents in some areas in the southern United States (Howard et al., 2010), where they can survive due to the presence year-round of introduced tropical milkweed (*Asclepias curassavica*) (Batalden & Oberhauser, 2015), and there are wide-spread, non-migratory monarch populations that are not of any immediate conservation concern (Walker et al., n.d.). While the species as a whole may therefore not be at risk, their migratory behavior almost certainly is. In addition to the damage to the long-term population persistence that could result from the loss of migration in North American populations (as discussed above), the loss of the publicly beloved annual monarch migration and concomitant overwintering would be culturally costly (Gustafsson et al., 2015).

Interestingly, however, there is a population of monarchs in Australia in which migratory behavior has apparently re-emerged after it was previously lost. This population, which was established sometime before 1871 (Zalucki & Clarke, 2004), is the product of a series of sequential introductions that crossed the Pacific Ocean from North America beginning sometime in the early 1840s after the introduction of their host plants enabled individuals to survive after being blown from the mainland (W. B. Hemstrom et al., 2022; Pierce et al., 2014; Figure 1, see Zalucki & Clarke, 2004). Monarchs are not known to be migratory on any Pacific islands, and no migratory behavior was observed in Australia until the 1930s, when non-reproductive overwintering aggregations were observed in northern New South Wales (NSW, see James & James, 2019). These and other individuals were later shown to migrate hundreds of kilometers each year to their wintering grounds from more southerly, inland locations in NSW (James, 1983; James & James, 2019). After over 90 years (and hundreds of generations) without any apparent migration, the behavior re-emerged in these populations. Understanding how this happened could provide crucial context around the “extinction” of migration in monarchs and other species.

To better understand how migration re-emerged in Australian monarchs, we studied continuously breeding monarch populations in Queensland. While these populations do not experience substantial seasonal fluctuations in resource availability, and thus do not migrate (James & James, 2019), recent work has shown that reproductive diapause can be induced in some, *but not all* individuals from these populations if they are exposed to a reduction in photoperiod during development (Freedman et al., 2018), a trait that these non-migratory populations do not display in the wild. In brief, reproductive diapause, where individuals delay their reproductive maturation, is a critical and tightly linked phenotype for migratory monarchs because it allows them to invest more heavily in lipid storage and increase their average longevity (Herman & Tatar, 2001). Like other migratory behaviors, diapause is initiated by changes in day length, temperature, and other seasonal fluctuations (Goehring & Oberhauser, 2002; Iiams et al., 2019) and is thought to be directly controlled by juvenile hormone (JH) concentration during development (Green & Kronforst, 2019; Herman & Tatar, 2001).

Diapause control is also an phenotype of interest because it highlights a theoretical dichotomy in the types of genes that are associated with migratory life histories: migratory genes can either be directly causal, proximate migratory genes that are required prior for migration to occur (such as genes that control diapause, directional motion, and navigation), or facultative migratory genes that increase fitness in already migratory populations but are not strictly required for migratory behavior to occur (such as genes that change wing or limb shapes, respiration, lipid storage, and muscle strength). For a newly migratory population, like monarchs in Queensland, we would expect to the strongest genetic associations with migration to occur in proximate migratory genes that cause traits like diapause rather than facultative genes which may take longer to increase in frequency following the evolution of migratory behavior.

The variance in diapause induction in Queensland monarchs allows for a direct search for genetic associations with this trait and thus for an examination of the genetic underpinnings of the re-evolution of migration in Australian monarchs. Here, we used reduced representation sequencing to search for genes associated with migratory diapause in Queensland monarch butterflies. We found one strongly associated locus co-located with an E3 ubiquitin ligase gene. This gene has been previously identified as associated with intermolt transitions in silk moths (Bombyx mori), which is controlled by the same major hormone (juvenile hormone, hereafter JH) as reproductive diapause. This gene had not previously been identified as associated with migration in North American monarch butterflies, and we did not find that any previously identified migratory genes were associated with diapause in our study.

# Materials and Methods

## Sample Collection, Incubation, and Phenotyping

We collected 22 female monarch butterflies from Pinjarra Hills, Queensland, Australia (27°32′26.7″S, 152°54′22.7″E) between the 5th and 9th of July 2018. Individuals in all life stages were present at the field site during our sample collections, consistent with continuous, year-round breeding. We enclosed each of these 22 females individually on either *Asclepias curassavica* or *Gomphocarpus sp.* host plants found on-location and subsequently successfully collected eggs produced by 21 of them. While these eggs represent 21 total maternal families, each may consist of multiple groups of half-siblings since females may mate multiple times and lay clutches with mixed parentage. We did not remove *Ophryocystis elektroscirrha* (a protozoan parasite) spores, which were uniformly present on our eggs (consistent with previous reports from this location, see Freedman et al., 2018).

We then incubated all eggs using the same incubators and following the “decreasing photoperiod” experimental scheme of Freedman et al. (2018) in order to induce reproductive diapause and ensure that our data could be later pooled with that produced in the earlier study. Briefly, larvae were reared at a constant temperature of 28°C under a photoperiod regime that declined from 14:10 L:D to 12:12 L:D over the course of 30 days (Δ4 mins/day). We released all male butterflies following emergence, since phenotyping diapause in male monarchs can be challenging due to the difficulty in extracting and weighing the ejaculatory duct, seminal vesicle, and accessory glands, which are likely the best indicator of male reproductive development (Goehring & Oberhauser, 2002). This left us with a total of 165 females from 17 maternal families.

We assessed reproductive diapause by determining the degree of oocyte development in each of our adult female butterflies. We observed that individuals fell into two clearly defined bins: 97 out of 165 had fully developed, chorionated eggs (visible ridges along the exterior of the egg), while 68 females did not. Of these latter individuals, a few (11) had no yolk in their eggs whatsoever, but most had some degree of yolking. Since vitellogenesis generally only begins to a substantial degree after eclosion in monarchs (Pan & Wyatt, 1976), we called any individuals with more than a small amount of yolking in their eggs as reproductively mature (N = 38 and 126 for reproductively immature and mature individuals, respectively, images of egg development in each individual are available from the authors upon request). Wings and bodies from all individuals were preserved in dry coin envelops or 95% ethanol, respectively. We note that while we refer to the postponement of reproductive development under otherwise suitable conditions as diapause, other authors refer to this process in monarchs as oligopause (James, 1982) or quiescence, since diapause implies an extended refractory period.

We supplemented these samples with preserved, dry butterfly wings from each of the “decreasing photoperiod” female individuals previously scored for reproductive development by Freedman et al. (2018), constituting an additional 40 total female butterflies from 11 maternal families, of which 12 and 28 were scored by the authors as reproductively immature and mature, respectively. Together, these two datasets contained 204 monarch butterflies, of which 50 and 154 were reproductively immature and mature, respectively (see Table 1).

## Sequencing and Genotyping

We removed and extracted DNA from one leg from each of our samples collected in 2018 or from a wing base from each of the samples collected by Freedman et al (2018) using the magnetic bead protocol of Ali et al (2016). We quantified the resulting DNA on a BioTek Instruments FLx800 Fluorescence Reader using Thermo Fisher Scientific Quant-iT PicoGreen dsDNA Reagent, then prepared Restriction Associated Digest (RAD) libraries using the Pst1 restriction enzyme according to Ali et al (2016). We sequenced these 150 bp paired-end sequencing libraries using an Illumina Hi-Seq 4000.

We aligned the resulting raw sequence data to the v4 monarch butterfly genome assembly (Gu et al., 2019) using the mem algorithm of the Burrows-Wheeler Aligner (Li & Durbin, 2009). We then filtered out PCR duplicates, improperly paired, or poorly mapped reads using SAMtools (Li et al., 2009). We then called gentoypes using the ANGSD software package with the following parameters: -doMajorMinor 1 (determine major and minor alleles using a genotype likelihood approach), -doMaf 2 (determine minor allele frequencies), -SNP\_pval 1e-8 (keep only loci with a SNP p-value ), -doGeno 4 (call genotypes), -doPost 2 (calculate genotype posterior probabilities using a uniform prior), -postCutoff 0.95 (keep only loci where the highest genotype posterior probability ), -minQ 20 (keep only loci with a sequencing quality ), -minMapQ 20 (keep only loci with a mapping quality ), -minInd 130 (keep only loci sequenced in at least 130 individuals), and -minMaf 0.05 (keep only loci with a minor allele frequency ).

## Statistical Analysis

In order to determine if any genomic regions were significantly associated with reproductive diapause, we used the snpR R package to filter out loci significantly out of Hardy-Weinburg Equilibrium () in either year in order to help remove any paralogous loci and interpolated missing data by conducting binomial draws at missing genotypes using the minor allele frequencies at those loci (Hemstrom & Jones, 2023). To assess the basic level of genetic diversity amongst our samples, we then calculated the average number of pairwise differences (), observed heterozygosity (), and according to Weir and Cockerham (1984) for all of our samples and for each year independently using snpR (Hemstrom & Jones, 2023). We also calculated according to Weir and Cockerham (1984) between sample years and between both sample year and reproductive phenotype jointly (Hemstrom & Jones, 2023). We bootstrapped a significance level for by bootstrapping individuals among populations with replacement 100 times. To determine if there was any substantial unexpected genetic structuring (other than family groups) in our samples, we conducted a Principal Component Analysis using snpR (Hemstrom & Jones, 2023). Since some individuals escaped their rearing dishes (but not the incubator) during rearing, we also confirmed the parentage of our samples using the Colony2 program, assuming polygamous, random mating (O. R. Jones & Wang, 2010).

In order to determine the degree to which diapause status was biased between families, we constructed a Fisher’s exact test for independence using 2000 simulations in R version 4.2.2 (R Core Team, 2022). We then conducted a Genome-Wide Association Study (GWAS) in order to determine if any loci were significantly associated with reproductive status. Since our samples were composed of many groups of full and half-siblings and came from two genetically different years, we used the GMMAT R package to conduct a population and family structure-corrected GWAS (Chen et al., 2016; Chen, 2020). We visualized the results by constructing a Manhattan plot using snpR (Hemstrom & Jones, 2023). We identified the genes underlying any candidate regions by hand using the MonarchBase genome browser online tool (Zhan & Reppert, 2013).

To determine if diapause is part of a generalized migratory syndrome in Australia we also briefly re-analyzed the connection between reproductive status and wing morphology in our 2016 samples (Freedman et al., 2018). To determine if wing shape or size was correlated with reproductive status, we fit a pair of linear mixed effect models with either with wing shape or size as response variables, the number of yolked oocytes as a fixed effect, and maternal family as a random effect using the R package nlme (Pinheiro et al., 2007).

# Results

## Background

Although the ancestral range of monarch butterflies is believed to be in North and Central America, they have fairly recently expanded into South America and the Caribbean, and very recently to several locations throughout the Atlantic and Pacific (Haeger et al., 2015; Opler, 1999; Zhan et al., 2014). In North America, the species is well known for its unique, multi-generational migratory life history wherein individuals dispersing northward in the spring do so over three to four generations and then return to their wintering grounds in a single step (Cockrell et al., 1993; Malcolm et al., 1993).

In Southern Florida, the Caribbean, Central America, South America, and throughout most of their introduced range monarchs are non-migratory (Altizer & Davis, 2010; Dockx, 2007; North, 2004). Residency in these populations appears to be a derived trait which has arisen multiple times after the species’ split from *Danaus eripus*, their closest extant relative (Zhan et al., 2014). The Pacific expansion of monarchs is relatively recent, with historical records indicating that they established first in Hawaii in the 1840s, likely as a result of individuals blown in during storm events that were able to survive on recently introduced milkweeds (Pierce et al., 2014; Zalucki & Clarke, 2004). Genetic evidence is consistent with an introduction to Hawaii during this time period (Hemstrom et al., 2022). As described above, they then spread across the Pacific and reached Australia in approximately 1871 (Zalucki & Clarke, 2004), and were recorded migrating again in Australia sometime in the 1930s (see Figure 1, James & James, 2019).

## Sequencing results

We incubated female monarch butterflies from a total of 17 clutches of eggs produced by females collected from a continuously-breeding population from southern Queensland, Australia under a decreasing photoperiod following the methods of Freedman et al. (2018). The resulting 165 females were phenotyped for oocyte development as a proxy for diapause status. Butterflies were phenotyped as reproductively mature (n = 126 not in diapause and thus with a non-migratory phenotype) or as reproductively immature (n = 38 in diapause and thus with a migratory phenotype). We sequenced these individuals along with an additional 40 butterflies raised under very similar conditions by Freedman et al. (2018), of which 28 and 12 were scored in that study as reproductively mature and immature, respectively.

We sequenced a total of 417,846,352 reads across all individuals, 98.8% of which mapped to the monarch reference genome and 244,443,414 of which were maintained after filtering (1,158,500 per sample, on average), encompassing 6,578,062 total sequenced bases. From this, we called 179,735 individual SNPs following filtering.

## Diversity and Structuring

Genetic diversity levels were extremely similar between our two years ( and and and for 2016 and 2018, respectively). Each year had a slight heterozygote excess ( = -0.011 and -0.008 for 2016 and 2018, respectively), although that excess was lower if all samples were considered jointly ( = -0.003). Pairwise between the two years was small but significant (0.019, 0.01). We found no significant genetic divergence between the two sample years on either the PCA or the NGSadmix results, with clustering instead driven by family, although we did find that three of our 2018 maternal families were substantially different from all other samples on our first two PC axes (Figure 2).

## Genetic Basis of Diapause

Diapause status was strongly biased by maternal family (Fisher’s Exact Test, , see Figure 3a). We identified one small region of chromosome 11 (between positions ~5,589,000 to ~5,620,000) containing many loci associated with diapause status well beyond the background level of association, including three SNPs from two different RAD-tags that had a -value (Figure 3b). This region overlaps the gene DPOGS208560 (LOC116768596), an E3 ubiquitin ligase, and was still strongly associated with diapause if only the 2018 butterflies alone were examined. We also identified one locus with a -value on chromosome 16, but there were no other significantly associated loci nearby.

We found that wing elongation was significantly negatively correlated with the number of yolked oocytes in Freedman et al.’s (2018) earlier study ( = 0.034) after accounting for maternal family. Wing size was also negatively correlated with the number of yolked oocytes, but not significantly so after accounting for maternal family ( = 0.26).

# Discussion

## Re-emergence of Diapause Control in Australian Monarch Butterflies

We found that a region on Chromosome 11 containing the gene DPOGS208560 was strongly associated with diapause initiation in Monarch Butterflies in Queensland, Australia. This gene is an E3 ubiquitin ligase, a class of proteins broadly involved with proteosomal degradation of diverse substrates, including the circadian clock protein CRY2 (Xing et al., 2013; Yoo et al., 2013), which is known to play a role in monarch photoperiodism including migratory responses to declining photoperiod (Iiams et al., 2019). Interestingly, a predicted homolog of this gene in silk moths has been previously found to be differentially expressed during larval molt-intermolt transitions (Hu et al., 2016), which are generally controlled by fluctuations in JH and ecdysone (Jindra et al., 2013). JH is known to be a major driver of reproductive development and diapause in monarchs (Green & Kronforst, 2019; Herman & Tatar, 2001). Taken together, it is not surprising that DPOGS208560 may participate in diapause control in monarchs.

What is surprising, however, is that this gene has not been previously identified as a candidate migration gene in monarchs. Specifically, DPOGS208560 was not among the 536 genes located in regions of the genome found to be significantly associated with contrasts between migration and residency in North and South American, Pacific, and European populations by Zhan et al. (2014), nor does it overlap with the genes suggested to be involved in photoperiodic responses by Iiams et al. (2019) or the genes associated with diapause termination in western North American monarchs (Green & Kronforst, 2019). There are at least two distinct explanations for this difference: 1) the DPOGS208560 variant we observed is either present in North America but was not previously recognized as associated with migration or 2) is the result of a new mutation which occurred in Australian monarchs. The former case would imply that this allele was either maintained as monarchs passed through repeated population bottlenecks during their Pacific expansion or was secondarily re-introduced by immigrant individuals from North America (which would have likely been anthropogenic in origin).

Since we observed that *only* the DPOGS208560 gene was associated with diapause in Australian monarchs, it seems unlikely that a secondary re-introduction of migratory monarch butterflies from North America could explain the re-emergence of migratory behavior in Australia, since there would be no reason for other known migratory associated genes to have not also made the jump back across the Pacific. Unfortunately, the SNP loci that we located are not perfectly associated with diapause since only 56 % of individuals phenotyped as not in diapause carried the associated allele (compared to 14 % of individuals phenotyped as reproductively mature) and are likely to only be in linkage with any causal variation rather than the causal variants themselves. Our work cannot therefore currently delineate between the other two options (maintenance of variance at DPOGS208560 during the Pacific crossing or a new mutation). If the direct, causal loci were to be identified, it would then be possible to determine which gene versions are present in North America or across the Pacific and thus determine the origin of the DPOGS208560 diapause variant in Australia.

Note that it is unlikely that DPOGS208560 alone controls diapause induction under declining day-length in Australian monarchs. While our study features many *individuals*, it features only 32 maternal families and thus far fewer independent samples. Our power is therefore limited, and it is entirely likely that we failed to detect many causal loci for diapause onset. This is consistent with the fact that our top associated SNP that overlaps DPOGS208560 was not perfectly associated with diapause, although that may also be due to imperfect linkage with the actual (and possibly un-sequenced) causal variants. Additional studies with larger sample sizes are therefore still needed to better understand the mechanisms underlying diapause induction in Australian monarch butterflies.

## Proximate vs Facultative Migratory Alleles

Regardless, it is interesting that DPOGS208560 had not been previously noted as associated with migration in monarch butterflies. In their 2014 study, Zhan et al. generally focused their discussion of migratory genetics on Collagen IV -1, one of three genes they found that were strong outliers for association with migration. This gene is involved in muscle functioning in insects (Schnorrer et al., 2010), and Zhan et al. hypothesized that divergence at this gene was driven by selection for increased wing strength and muscle efficiency to facilitate long-distance migration. While they mentioned that the remaining significantly associated genes were enriched for the “morphogenesis, neurogenesis, and extracellular matrix/basement membrane” functional terms, they were not otherwise discussed (DPOGS208560 does not have any of these functional terms). It is possible that DPOGS208560 also contributes to diapause and migration in butterflies outside of Australia, but that the quantitative effect of other genes (a product of the effect sizes of the migratory alleles and their frequencies) dwarfs that of DPOGS208560, thus concealing the relative impact of the latter gene. It is worth noting, however, Zhan et al. (2014) also noted that the FBXO45 gene is located adjacent to Collagen IV -1 and was thus also associated with migration. Although Zhan et al. (2014) did not discuss the potential role of FBXO45 in migration and focused instead on Collagen IV -1, F-box proteins are part of SCF complexes that, along with E3 ubiquitin ligases such as DPOGS208560, are likely involved in processing circadian clock proteins (Grima et al., 2002; Xing et al., 2013).

As mentioned above, we did not identify loci near the Collagen IV -1 gene as associated with diapause, despite the fact that we did sequence several nearby loci. This is surprising, since although monarchs in Australia (like other non-migratory populations), have substantially smaller and shorter wings than their migratory counterparts (Freedman et al., 2020), we also found from re-analyzing data from Freedman et al.’s (2018) study that wing shape is significantly correlated with reproductive development in Australian monarchs. This implies that wing morphology is still a part of the correlated migratory syndrome in Australian monarchs, and so we might expect to see some potential causal genetic elements for wing morphology to also be associated with diapause.

However, it does make some sense that Collagen IV -1 would not be a key gene in populations that are *newly* migratory. We would argue that migratory genes can be divided into two basic categories: facultative migratory genes and those that are the proximate drivers of migratory behavior. Collagen IV -1 is, for example, a “facultative” migratory gene in that selection acting on it would serve to make already migratory individuals more fit, but would not itself *cause* or *enable* migratory behavior. DPOGS208560, on the other hand, is likely a proximate migratory gene, since individuals that do not delay their reproductive investment have much shorter life-spans and often cannot complete their full migration (Herman & Tatar, 2001). Genes that control orientation, navigation, and directed flight would also fall into this category to varying extents. Any population that is newly migratory must have some proximate causal gene migratory variants or they would not display migratory behavior in the first place, but may only acquire facultative migratory gene variants over time as selection favors migratory individuals that are better able to complete their migration. That we found DPOGS208560 but not Collagen IV -1 to be associated with migration in Australia supports this hypothesis, and, in general, a complex interpretation of the genetic control of migration in monarchs (Green, 2021; Merlin & Liedvogel, 2019).

## Persistence of Migration in Monarchs and Beyond

Our work suggests that even if migration is lost in monarchs, it may be temporary. Despite the loss of allelic variance during the successive bottlenecks, monarch butterflies either maintained the genetic variation needed to migrate or secondarily re-acquired it via mutation. This gives us some hope that the contemporary loss of migration that we have observed in many migrants may be reversible over relatively short evolutionary timescales. At the very least, we can be assured that for monarchs in particular, migrants in Australia constitute a reservoir of migratory alleles that could potentially be tapped for North America if needed.

While monarchs may be able to quickly recover from the loss of migratory behavior should conditions improve, it is unlikely that this is the case for most other migratory species of conservation concern, particularly for vertebrates. For example, if we assume the latter hypothesis (migratory variance in Australian monarchs is the result of a new mutation), it is clear that monarchs have had ample evolutionary opportunity to re-acquire migratory mutations: monarchs have a generation time of approximately seven generations per year if continuously breeding, which means that monarchs in Australia went through roughly ~ 350 generations in the 50 or so years since they were first reported on the continent, and although they likely experienced a strong bottleneck initially, they were probably at a relatively large effective size for most of that time. Since the rate at which new mutations appear in a population is proportional to both effective populations size and generation time, monarchs have had a large opportunity space for the generation of new migratory alleles.

Alternatively, it is safe to assume that if migratory variance in Australian monarchs was maintained from North America this would only have been possible because selection was not actively acting against that variance. This is actually quite likely: monarchs in the Pacific are not exposed to substantial annual changes in day length or average temperature, and thus do not receive their main migratory cues. Environmentally triggered migratory variation, therefore, is probably selectively nearly neutral in the Pacific. In contrast, individually fixed migratory variance, such as wing morphology, was likely selected against across the Pacific, thus driving the observed, repeated decreases in wing size and length observed in newly non-migratory monarch populations (Freedman et al., 2020). Environmentally triggered migratory variation is therefore more likely type of migratory variation to be maintained in non-migratory populations.

In contrast to monarchs, however, a species with slower generation times and smaller effective population sizes would be unlikely re-evolve lost phenotypic variance on anything approaching a similar time frame, nor would they as easily hold on to phenotypic variance in the long run under neutral conditions. Nonetheless, our findings are still optimistic for the future of migration in monarch butterflies or in other species where selection on migratory alleles is merely relaxed and not becoming increasingly negative.

# Conclusion

This study suggests that reproductive diapause in Australian monarch butterflies is influenced by a novel genetic mechanism via the DPOGS208560 gene, and that the previously identified migratory genetic elements were not associated with diapause in this populations. Research to determine if the migratory variant of this gene is the result of ancestral variation maintained from North America or a new mutation is needed. Regardless, our work is consistent with the hypothesis that proximate genetic mechanisms for migration are more likely to quickly re-emerge during a transition to a migratory life history than facultative mechanisms.

# Acknowledgements and Funding Sources

We would like to thank Dr. Shawan Chowdhury for his assistance with Monarch feeding and maintenance during the rearing process and Dr. Sean O’Rourke for assistance during library preparation and sequencing. Travel and sequencing funding was provided by the Jastro-Shields Graduate Research Award and the UC Davis Department of Animal Science. MF was funded by NSF Postdoctoral Fellowship Award 2010658.

# References

Adams, C. E., Chavarie, L., Rodger, J. R., Honkanen, H. M., Thambithurai, D., & Newton, M. P. (2022). An opinion piece: The evolutionary and ecological consequences of changing selection pressures on marine migration in Atlantic salmon. *Journal of Fish Biology*, *100*(4), 860–867. <https://doi.org/10.1111/jfb.15024>

Ali, O. A., O’Rourke, S. M., Amish, S. J., Meek, M. H., Luikart, G., Jeffres, C., & Miller, M. R. (2016). Rad capture (Rapture): Flexible and efficient sequence-based genotyping. *Genetics*, *202*(2), 389–400. <https://doi.org/10.1534/genetics.115.183665>

Altizer, S., & Davis, A. K. (2010). Populations of monarch butterflies with different migratory behaviors show divergence in wing morphology. *Evolution*, *64*(4), 1018–1028. <https://doi.org/10.1111/j.1558-5646.2009.00946.x>

Ambrosini, R., Rubolini, D., AP, M., Bani, L., Clark, J., Karcza, Z., Vangeluwe, D., C, du F., Spina, F., & Saino, N. (2011). Climate change and the long-term northward shift in the African wintering range of the barn swallow Hirundo rustica. *Climate Research*, *49*(2), 131–141. <https://www.int-res.com/abstracts/cr/v49/n2/p131-141/>

Batalden, R. V., & Oberhauser, K. S. (2015). Potential changes in eastern North American monarch migration in response to an introduced milkweed, Asclepias curassavica. *Monarchs in a Changing World: Biology and Conservation of an Iconic Butterfly*, 215–224.

Bonnet-Lebrun, A.-S., Manica, A., & Rodrigues, A. S. L. (2020). Effects of urbanization on bird migration. *Biological Conservation*, *244*, 108423. <https://doi.org/10.1016/j.biocon.2020.108423>

Both, C., Van Turnhout, C. A. M., Bijlsma, R. G., Siepel, H., Van Strien, A. J., & Foppen, R. P. B. (2010). Avian population consequences of climate change are most severe for long-distance migrants in seasonal habitats. *Proceedings of the Royal Society B: Biological Sciences*, *277*(1685), 1259–1266. <https://doi.org/10.1098/rspb.2009.1525>

Brower, L. P., & Malcolm, S. B. (1991). Animal Migrations: Endangered Phenomena. *American Zoologist*, *31*(1), 265–276. <https://doi.org/10.1093/icb/31.1.265>

Burger, J., Gochfeld, M., Niles, L., Powers, C., Brown, K., Clarke, J., Dey, A., & Kosson, D. (2015). Complexity of bioindicator selection for ecological, human, and cultural health: Chinook salmon and red knot as case studies. *Environmental Monitoring and Assessment*, *187*(3), 102. <https://doi.org/10.1007/s10661-014-4233-4>

Chen, H. (2020). *GMMAT: Generalized linear Mixed Model Association Tests Version 1.2. 0*.

Chen, H., Wang, C., Conomos, M. P., Stilp, A. M., Li, Z., Sofer, T., Szpiro, A. A., Chen, W., Brehm, J. M., Celedón, J. C., Redline, S., Papanicolaou, G. J., Thornton, T. A., Laurie, C. C., Rice, K., & Lin, X. (2016). Control for Population Structure and Relatedness for Binary Traits in Genetic Association Studies via Logistic Mixed Models. *The American Journal of Human Genetics*, *98*(4), 653–666. <https://doi.org/10.1016/J.AJHG.2016.02.012>

Chowdhury, S., Zalucki, M. P., Amano, T., Woodworth, B. K., Venegas-Li, R., & Fuller, R. A. (2021). Seasonal spatial dynamics of butterfly migration. *Ecology Letters*, *24*(9), 1814–1823. <https://doi.org/10.1111/ele.13787>

Chu, J. J., Gillis, D. P., & Riskin, S. H. (2022). Community science reveals links between migration arrival timing advance, migration distance and wing shape. *Journal of Animal Ecology*, *91*(8), 1651–1665. <https://doi.org/10.1111/1365-2656.13755>

Cockrell, B. J., Malcolm, S. B., & Brower, L. P. (1993). Time, temperature, and latitudinal constraints on the annual recolonization of eastern North America by the monarch butterfly. *Biology and Conservation of the Monarch Butterfly*, *38*, 233–251.

Culbertson, K. A., Garland, M. S., Walton, R. K., Zemaitis, L., & Pocius, V. M. (2022). Long-term monitoring indicates shifting fall migration timing in monarch butterflies (Danaus plexippus). *Global Change Biology*, *28*(3), 727–738. <https://doi.org/10.1111/gcb.15957>

Dockx, C. (2007). Directional and stabilizing selection on wing size and shape in migrant and resident monarch butterflies, Danaus plexippus (L.), In Cuba. *Biological Journal of the Linnean Society*, *92*(4), 605–616.

Dufour, P., Åkesson, S., Hellström, M., Hewson, C., Lagerveld, S., Mitchell, L., Chernetsov, N., Schmaljohann, H., & Crochet, P.-A. (2022). The Yellow-browed Warbler (Phylloscopus inornatus) as a model to understand vagrancy and its potential for the evolution of new migration routes. *Movement Ecology*, *10*(1), 59. <https://doi.org/10.1186/s40462-022-00345-2>

Dufour, P., Franceschi, C. de, Doniol-Valcroze, P., Jiguet, F., Guéguen, M., Renaud, J., Lavergne, S., & Crochet, P.-A. (2021). A new westward migration route in an Asian passerine bird. *Current Biology*, *31*(24), 5590–5596.e4. <https://doi.org/10.1016/j.cub.2021.09.086>

Falchi, V., Cerritelli, G., Barbon, A., Catoni, C., Cutroneo, A., dell’Omo, G., Dragonetti, M., Giovacchini, P., Meschini, A., Panzarin, L., Picciau, A., & Giunchi, D. (2023). Inter and intra-population variability of the migratory behaviour of a short-distance partial migrant, the Eurasian Stone-curlew Burhinus oedicnemus (Charadriiformes, Burhinidae). *Journal of Ornithology*, *164*(1), 85–100. <https://doi.org/10.1007/s10336-022-02020-0>

Fitchett, J. M., Pandazis, A., & Pillay, S. (2022). Advance in the timing of the annual migration of the brown-veined white butterfly through Johannesburg, South Africa, over the period 1914–2020. *International Journal of Biometeorology*, *66*(11), 2251–2258. <https://doi.org/10.1007/s00484-022-02353-8>

Fleming, T. H., Eby, P., Kunz, T. H., & Fenton, M. B. (2003). Ecology of bat migration. *Bat Ecology*, *156*, 164–165.

Freedman, M. G., Dingle, H., Strauss, S. Y., & Ramírez, S. R. (2020). Two centuries of monarch butterfly collections reveal contrasting effects of range expansion and migration loss on wing traits. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(46), 28887–28893. <https://doi.org/10.1073/pnas.2001283117>

Freedman, M. G., Dingle, H., Tabuloc, C. A., Chiu, J. C., Yang, L. H., & Zalucki, M. P. (2018). Non-migratory monarch butterflies, Danaus plexippus (L.), Retain developmental plasticity and a navigational mechanism associated with migration. *Biological Journal of the Linnean Society*, *123*(2), 265–278. <https://doi.org/10.1093/biolinnean/blx148>

Goehring, L., & Oberhauser, K. S. (2002). Effects of photoperiod, temperature, and host plant age on induction of reproductive diapause and development time in Danaus plexippus. *Ecological Entomology*, *27*(6), 674–685. https://doi.org/<https://doi.org/10.1046/j.1365-2311.2002.00454.x>

Gordo, O. (2007). Why are bird migration dates shifting? A review of weather and climate effects on avian migratory phenology. *Climate Research*, *35*(1-2), 37–58. <https://www.int-res.com/abstracts/cr/v35/n1-2/p37-58/>

Green, D. A. (2021). Monarch Butterfly Migration as an Integrative Model of Complex Trait Evolution. *The American Naturalist*, *198*(1), 142–157. <https://doi.org/10.1086/714526>

Green II, D. A., & Kronforst, M. R. (2019). Monarch butterflies use an environmentally sensitive, internal timer to control overwintering dynamics. *Molecular Ecology*, *0*(ja). <https://doi.org/10.1111/mec.15178>

Grima, B., Lamouroux, A., Chélot, E., Papin, C., Limbourg-Bouchon, B., & Rouyer, F. (2002). The F-box protein Slimb controls the levels of clock proteins Period and Timeless. *Nature*, *420*(6912), 178–182. <https://doi.org/10.1038/nature01122>

Gu, L., Reilly, P. F., Lewis, J. J., Reed, R. D., Andolfatto, P., & Walters, J. R. (2019). Dichotomy of Dosage Compensation along the Neo Z Chromosome of the Monarch Butterfly. *Current Biology*, *29*(23), 4071–4077.e3. https://doi.org/<https://doi.org/10.1016/j.cub.2019.09.056>

Gustafsson, K. M., Agrawal, A. A., Lewenstein, B. V., & Wolf, S. A. (2015). The Monarch Butterfly through Time and Space: The Social Construction of an Icon. *BioScience*, *65*(6), 612–622. <https://doi.org/10.1093/biosci/biv045>

Haeger, J., Jordano, D., & Zalucki, M. (2015). Monarchs across the Atlantic Ocean: What’s happening on the other shore. *Monarchs in a Changing World: Biology and Conservation of an Iconic Butterfly. Cornell University Press, Ithaca, NY*, 247–256.

Haran, R., Kiat, Y., & Izhaki, I. (2021). Patterns of partial migration of the Dead Sea Sparrow (Passer moabiticus) along the Great Rift Valley in Israel. *Journal of Arid Environments*, *192*, 104534. <https://doi.org/10.1016/j.jaridenv.2021.104534>

[dataset] Hemstrom, W., Freedman, M., Zalucki, M. P. & Miller, M. R.; 2023; NCBI Short Read Archive; PRJNA984345

Hemstrom, W. B., Freedman, M. G., Zalucki, M. P., Ramírez, S. R., & Miller, M. R. (2022). Population genetics of a recent range expansion and subsequent loss of migration in monarch butterflies. *Molecular Ecology*, *31*(17), 4544–4557. https://doi.org/<https://doi.org/10.1111/mec.16592>

Hemstrom, W., & Jones, M. (2023). snpR: User friendly population genomics for SNP data sets with categorical metadata. *Molecular Ecology Resources*, *23*(4), 962–973. <https://doi.org/10.1111/1755-0998.13721>

Herman, W. S., & Tatar, M. (2001). Juvenile hormone regulation of longevity in the migratory monarch butterfly. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *268*(1485), 2509–2514. <https://doi.org/10.1098/rspb.2001.1765>

Howard, E., Aschen, H., & Davis, A. K. (2010). Citizen Science Observations of Monarch Butterfly Overwintering in the Southern United States. *Psyche*, *2010*, 689301. <https://doi.org/10.1155/2010/689301>

Hu, W., Liu, C., Cheng, T., Li, W., Wang, N., & Xia, Q. (2016). Histomorphometric and transcriptomic features characterize silk glands’ development during the molt to intermolt transition process in silkworm. *Insect Biochemistry and Molecular Biology*, *76*, 95–108. https://doi.org/<https://doi.org/10.1016/j.ibmb.2016.07.002>

Iiams, S. E., Lugena, A. B., Zhang, Y., Hayden, A. N., & Merlin, C. (2019). Photoperiodic and clock regulation of the vitamin A pathway in the brain mediates seasonal responsiveness in the monarch butterfly. *Proceedings of the National Academy of Sciences*, *116*(50), 25214–25221. <https://doi.org/10.1073/pnas.1913915116>

James, D. G. (1982). Ovarian dormancy in danaus plexippus (l.) (Lepidoptera: Nymphalidae)—oligopause not diapause. *Australian Journal of Entomology*, *21*(1), 31–35. <https://doi.org/10.1111/j.1440-6055.1982.tb01761.x>

James, D. G. (1983). Induction of Reproductive Dormancy in Australian Monarch Butterflies Danaus plexippus (L.). *Australian Journal of Zoology*, *31*(4), 491–498. <https://doi.org/10.1071/ZO9830491>

James, D. G., & James, T. A. (2019). Migration and Overwintering in Australian Monarch Butterflies (Danaus plexippus (L.) (Lepidoptera: Nymphalidae): A Review with New Observations and Research Needs. *The Journal of the Lepidopterists’ Society*, *73*(3), 177–190. <https://doi.org/10.18473/lepi.73i3.a7>

Jia, Y., Liu, Y., Jiao, S., Guo, J., Lu, C., Zhou, Y., Wang, Y., Lei, G., Wen, L., & Mo, X. (2021). Shifting of the Migration Route of White-Naped Crane (Antigone vipio) Due to Wetland Loss in China. *Remote Sensing*, *13*(15). <https://doi.org/10.3390/rs13152984>

Jindra, M., Palli, S. R., & Riddiford, L. M. (2013). The Juvenile Hormone Signaling Pathway in Insect Development. *Annual Review of Entomology*, *58*(1), 181–204. <https://doi.org/10.1146/annurev-ento-120811-153700>

Jones, J. D., Kauffman, M. J., Monteith, K. L., Scurlock, B. M., Albeke, S. E., & Cross, P. C. (2014). Supplemental feeding alters migration of a temperate ungulate. *Ecological Applications*, *24*(7), 1769–1779. https://doi.org/<https://doi.org/10.1890/13-2092.1>

Jones, O. R., & Wang, J. (2010). COLONY: A program for parentage and sibship inference from multilocus genotype data. *Molecular Ecology Resources*, *10*(3), 551–555. <https://doi.org/10.1111/j.1755-0998.2009.02787.x>

Jones, T., & Cresswell, W. (2010). The phenology mismatch hypothesis: Are declines of migrant birds linked to uneven global climate change? *Journal of Animal Ecology*, *79*(1), 98–108. https://doi.org/<https://doi.org/10.1111/j.1365-2656.2009.01610.x>

Kovach, R. P., Gharrett, A. J., & Tallmon, D. A. (2012). Genetic change for earlier migration timing in a pink salmon population. *Proceedings of the Royal Society B: Biological Sciences*, *279*(1743), 3870–3878. <https://doi.org/10.1098/rspb.2012.1158>

La Sorte, F. A., & Thompson III, F. R. (2007). Poleward shifts in winter ranges of north american birds. *Ecology*, *88*(7), 1803–1812. https://doi.org/<https://doi.org/10.1890/06-1072.1>

Li, H., & Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics (Oxford, England)*, *25*(14), 1754–1760. <https://doi.org/10.1093/bioinformatics/btp324>

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., & Subgroup, 1000. G. P. D. P. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, *25*(16), 2078–2079. <https://doi.org/10.1093/bioinformatics/btp352>

Limburg, K. E., & Waldman, J. R. (2009). Dramatic Declines in North Atlantic Diadromous Fishes. *BioScience*, *59*(11), 955–965. <https://doi.org/10.1525/bio.2009.59.11.7>

López-Hoffman, L., Chester, C. C., Semmens, D. J., Thogmartin, W. E., Rodríguez-McGoffin, M. S., Merideth, R., & Diffendorfer, J. E. (2017). Ecosystem Services from Transborder Migratory Species: Implications for Conservation Governance. *Annual Review of Environment and Resources*, *42*(1), 509–539. <https://doi.org/10.1146/annurev-environ-110615-090119>

Malcolm, S., Cockrell, B., & Brower, L. (1993). Spring recolonization of eastern North America by the monarch butterfly: Successive brood or single sweep migration. *Biology and Conservation of the Monarch Butterfly*, *38*, 253–267.

McWilliams, S. R., Guglielmo, C., Pierce, B., & Klaassen, M. (2004). Flying, fasting, and feeding in birds during migration: A nutritional and physiological ecology perspective. *Journal of Avian Biology*, *35*(5), 377–393. <https://doi.org/10.1111/j.0908-8857.2004.03378.x>

Merlin, C., & Liedvogel, M. (2019). The genetics and epigenetics of animal migration and orientation: Birds, butterflies and beyond. *Journal of Experimental Biology*, *222*(Suppl\_1), jeb191890. <https://doi.org/10.1242/jeb.191890>

Møller, A. P., Jokimäki, J., Skorka, P., & Tryjanowski, P. (2014). Loss of migration and urbanization in birds: A case study of the blackbird (Turdus merula). *Oecologia*, *175*(3), 1019–1027. <https://doi.org/10.1007/s00442-014-2953-3>

Mondain-Monval, T. O., Amos, M., Chapman, J.-L., MacColl, A., & Sharp, S. P. (2021). Flyway-scale analysis reveals that the timing of migration in wading birds is becoming later. *Ecology and Evolution*, *11*(20), 14135–14145. <https://doi.org/10.1002/ece3.8130>

North, J. (2004). *The monarch butterfly: Biology & conservation*. Cornell university press.

Opler, P. A. (1999). *A field guide to western butterflies*. Houghton Mifflin Harcourt.

Pan, M. L., & Wyatt, G. R. (1976). Control of vitellogenin synthesis in the Monarch butterfly by juvenile hormone. *Developmental Biology*, *54*(1), 127–134. https://doi.org/<https://doi.org/10.1016/0012-1606(76)90291-8>

Pierce, A. A., Zalucki, M. P., Bangura, M., Udawatta, M., Kronforst, M. R., Altizer, S., Haeger, J. F., & Roode, J. C. de. (2014). Serial founder effects and genetic differentiation during worldwide range expansion of monarch butterflies. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1797), 1–9. <https://doi.org/10.1098/rspb.2014.2230>

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., & Team, R. C. (2007). Linear and nonlinear mixed effects models. *R Package Version*, *3*(57), 1–89.

Pinszke, A., & Remisiewicz, M. (2023). Long-term changes in autumn migration timing of Garden Warblers Sylvia borin at the southern Baltic coast in response to spring, summer and autumn temperatures. *The European Zoological Journal*, *90*(1), 283–295. <https://doi.org/10.1080/24750263.2023.2192239>

Plummer, K. E., Siriwardena, G. M., Conway, G. J., Risely, K., & Toms, M. P. (2015). Is supplementary feeding in gardens a driver of evolutionary change in a migratory bird species? *Global Change Biology*, *21*(12), 4353–4363. https://doi.org/<https://doi.org/10.1111/gcb.13070>

Prytula, E. D., McKellar, A. E., Schwitters, L., & Reudink, M. W. (2022). Rapid advancement of spring migration and en route adjustment of migration timing in response to weather during fall migration in Vaux’s Swifts (Chaetura vauxi). *Canadian Journal of Zoology*, *100*(1), 56–63. <https://doi.org/10.1139/cjz-2021-0089>

Pulido, F. (2007). The Genetics and Evolution of Avian Migration. *BioScience*, *57*(2), 165–174. <https://doi.org/10.1641/B570211>

Pulido, F., & Berthold, P. (2010). Current selection for lower migratory activity will drive the evolution of residency in a migratory bird population. *Proceedings of the National Academy of Sciences*, *107*(16), 7341–7346. <https://doi.org/10.1073/pnas.0910361107>

R Core Team. (2022). *R: A language and environment for statistical computing* [Manual]. <https://www.R-project.org/>

Rankin, M. A., & Burchsted, J. C. A. (1992). The Cost of Migration in Insects. *Annual Review of Entomology*, *37*(1), 533–559. <https://doi.org/10.1146/annurev.en.37.010192.002533>

Reynolds, H. L., & Clay, K. (2011). Migratory species and ecological processes. *Envtl. L.*, *41*, 371.

Robbins, C. S., Sauer, J. R., Greenberg, R. S., & Droege, S. (1989). Population declines in North American birds that migrate to the neotropics. *Proceedings of the National Academy of Sciences*, *86*(19), 7658–7662. <https://doi.org/10.1073/pnas.86.19.7658>

Rubolini, D., AP, M., Rainio, K., & Lehikoinen, E. (2007). Intraspecific consistency and geographic variability in temporal trends of spring migration phenology among European bird species. *Climate Research*, *35*(1-2), 135–146. <https://www.int-res.com/abstracts/cr/v35/n1-2/p135-146/>

Runge, C. A., Martin, T. G., Possingham, H. P., Willis, S. G., & Fuller, R. A. (2014). Conserving mobile species. *Frontiers in Ecology and the Environment*, *12*(7), 395–402. <https://doi.org/10.1890/130237>

Rushing, C. S., Royle, J. A., Ziolkowski, D. J., & Pardieck, K. L. (2020). Migratory behavior and winter geography drive differential range shifts of eastern birds in response to recent climate change. *Proceedings of the National Academy of Sciences*, *117*(23), 12897–12903. <https://doi.org/10.1073/pnas.2000299117>

Satterfield, D. A., Maerz, J. C., Hunter, M. D., Flockhart, D. T. T., Hobson, K. A., Norris, D. R., Streit, H., Roode, J. C. de, & Altizer, S. (2018). Migratory monarchs that encounter resident monarchs show life-history differences and higher rates of parasite infection. *Ecology Letters*, *21*(11), 1670–1680. <https://doi.org/10.1111/ele.13144>

Schnorrer, F., Schonbauer, C., Langer, C. C. H., Dietzl, G., Novatchkova, M., Schernhuber, K., Fellner, M., Azaryan, A., Radolf, M., Stark, A., Keleman, K., & Dickson, B. J. (2010). Systematic genetic analysis of muscle morphogenesis and function in Drosophila. *Nature*, *464*(7286), 287–291. <https://doi.org/10.1038/nature08799>

Schuetz, J. G., & Johnston, A. (2019). Characterizing the cultural niches of North American birds. *Proceedings of the National Academy of Sciences*, *116*(22), 10868–10873. <https://doi.org/10.1073/pnas.1820670116>

Semmens, B. X., Semmens, D. J., Thogmartin, W. E., Wiederholt, R., López-Hoffman, L., Diffendorfer, J. E., Pleasants, J. M., Oberhauser, K. S., & Taylor, O. R. (2016). Quasi-extinction risk and population targets for the Eastern, migratory population of monarch butterflies (Danaus plexippus). *Scientific Reports*, *6*(1), 23265. <https://doi.org/10.1038/srep23265>

Semmens, D. J., Diffendorfer, J. E., Bagstad, K. J., Wiederholt, R., Oberhauser, K., Ries, L., Semmens, B. X., Goldstein, J., Loomis, J., Thogmartin, W. E., Mattsson, B. J., & López-Hoffman, L. (2018). Quantifying ecosystem service flows at multiple scales across the range of a long-distance migratory species. *Global Flows of Ecosystem Services*, *31*, 255–264. <https://doi.org/10.1016/j.ecoser.2017.12.002>

Semmens, D. J., Diffendorfer, J. E., López-Hoffman, L., & Shapiro, C. D. (2011). Accounting for the ecosystem services of migratory species: Quantifying migration support and spatial subsidies. *Ecological Economics*, *70*(12), 2236–2242. <https://doi.org/10.1016/j.ecolecon.2011.07.002>

Shuert, C. R., Marcoux, M., Hussey, N. E., Heide-Jørgensen, M. P., Dietz, R., & Auger-Méthé, M. (2022). Decadal migration phenology of a long-lived Arctic icon keeps pace with climate change. *Proceedings of the National Academy of Sciences*, *119*(45), e2121092119. <https://doi.org/10.1073/pnas.2121092119>

Sonnleitner, J., LaZerte, S. E., McKellar, A. E., Flood, N. J., & Reudink, M. W. (2022). Rapid shifts in migration routes and breeding latitude in North American bluebirds. *Ecosphere*, *13*(12), e4316. <https://doi.org/10.1002/ecs2.4316>

Van Der Ree, R., McDonnell, M. J., Temby, I., Nelson, J., & Whittingham, E. (2006). The establishment and dynamics of a recently established urban camp of flying foxes (Pteropus poliocephalus) outside their geographic range. *Journal of Zoology*, *268*(2), 177–185. https://doi.org/<https://doi.org/10.1111/j.1469-7998.2005.00005.x>

Visser, M. E., Perdeck, A. C., Van Balen, J., & Both, C. (2009). Climate change leads to decreasing bird migration distances. *Global Change Biology*, *15*(8), 1859–1865. https://doi.org/<https://doi.org/10.1111/j.1365-2486.2009.01865.x>

Walker, A., Oberhauser, K. S., Pelton, E. M., & Pleasants, J. M. (n.d.). Danaus plexippus (errata version published in 2022). *The IUCN Red List of Threatened Species*, *2022: e.T159971A219149911*. https://doi.org/<https://dx.doi.org/10.2305/IUCN.UK.2022-1.RLTS.T159971A219149911.en>

Weir, B. S., & Cockerham, C. C. (1984). Estimating F-Statistics for the Analysis of Population Structure. *Evolution*, *38*(6), 1358–1370. <https://doi.org/10.1111/j.1558-5646.1984.tb05657.x>

Wikelski, M., Tarlow, E. M., Raim, A., Diehl, R. H., Larkin, R. P., & Visser, G. H. (2003). Costs of migration in free-flying songbirds. *Nature*, *423*(6941), 704. <https://doi.org/10.1038/423704a>

Xing, W., Busino, L., Hinds, T. R., Marionni, S. T., Saifee, N. H., Bush, M. F., Pagano, M., & Zheng, N. (2013). SCFFBXL3 ubiquitin ligase targets cryptochromes at their cofactor pocket. *Nature*, *496*(7443), 64–68. <https://doi.org/10.1038/nature11964>

Yoo, S.-H., Mohawk, J. A., Siepka, S. M., Shan, Y., Huh, S. K., Hong, H.-K., Kornblum, I., Kumar, V., Koike, N., Xu, M., Nussbaum, J., Liu, X., Chen, Z., Chen, Z. J., Green, C. B., & Takahashi, J. S. (2013). Competing E3 Ubiquitin Ligases Govern Circadian Periodicity by Degradation of CRY in Nucleus and Cytoplasm. *Cell*, *152*(5), 1091–1105. <https://doi.org/10.1016/j.cell.2013.01.055>

Zalucki, M. P., & Clarke, A. R. (2004). Monarchs across the Pacific: The Columbus hypothesis revisited. *Biological Journal of the Linnean Society*, *82*(1), 111–121. <https://doi.org/10.1111/j.1095-8312.2004.00322.x>

Zhan, S., & Reppert, S. M. (2013). MonarchBase: The monarch butterfly genome database. *Nucleic Acids Research*, *41*(D1), D758–D763. <https://doi.org/10.1093/nar/gks1057>

Zhan, S., Zhang, W., Niitepõld, K., Hsu, J., Haeger, J. F., Zalucki, M. P., Altizer, S., Roode, J. C. de, Reppert, S. M., & Kronforst, M. R. (2014). The genetics of monarch butterfly migration and warning colouration. *Nature*, *514*, 317. <https://doi.org/10.1038/nature13812>

# Data Availability and Benefits-Sharing

Data Availability: The raw sequencing data produced for this study are available from the NCBI under accession number PRJNA984345. The filtered genotypes and the scripts used to produce both them and this paper are available at <https://github.com/hemstrow/aus_monarchs>. The exact script used to produce this paper from the genotypic data, including all non-map figures, is available at <https://github.com/hemstrow/aus_monarchs/blob/master/paper/paper_draft.Rmd>.

Benefit-Sharing: This paper represents the continuation of a collaboration between researchers in the United States and Australia. All major contributors are listed as co-authors. This paper also extends our knowledge of critical phenotypic variance in a newly-listed endangered species. All of the data produced in this paper are shared with the public at the repositories above.

# Author Contributions

WH and MM designed the study with input from MF and MZ. WH collected, raised, and phenotyped individuals. MZ provided, lab space, equipment, and expertise for each of those steps. WH analyzed the data and wrote the manuscript. MF, MZ, and MM provided feedback on the analysis and manuscript.

# Tables and Figures

Table 1: Counts of individual monarch butterfly reproductive status in each year.

| Year | # In Diapause | # Reproductive | # Total |
| --- | --- | --- | --- |
| 2016 | 12 | 28 | 40 |
| 2018 | 38 | 126 | 164 |
| Total | 50 | 154 | 204 |

Diagram

Description automatically generatedFigure 1: Approximate ancestral and introduced ranges with introduction dates for monarch butterflies in the Pacific, with sampling location noted in orange.

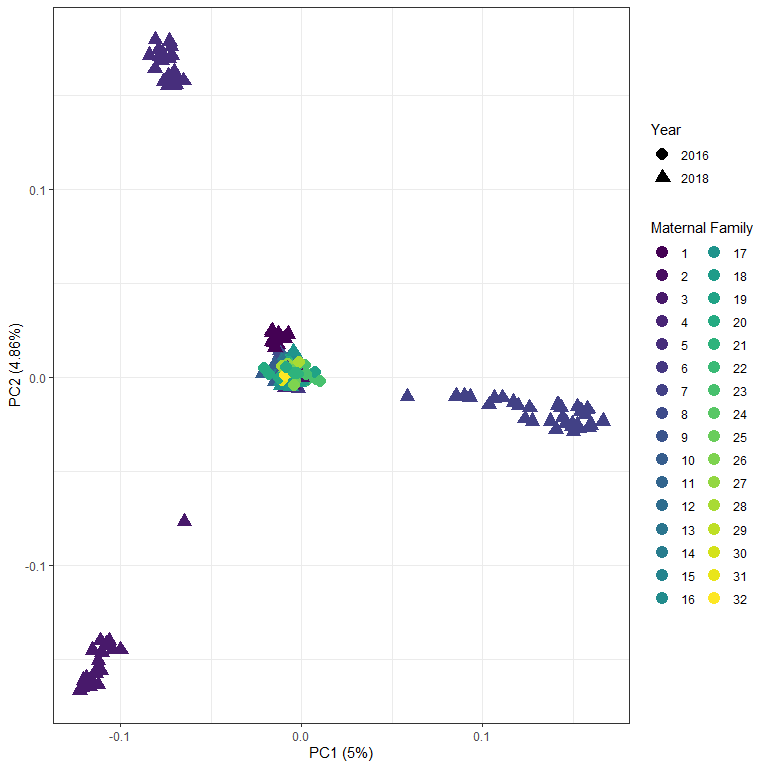


Figure 2: Principal Component Analysis of the monarchs from 2016 and 2018, colored by maternal family.

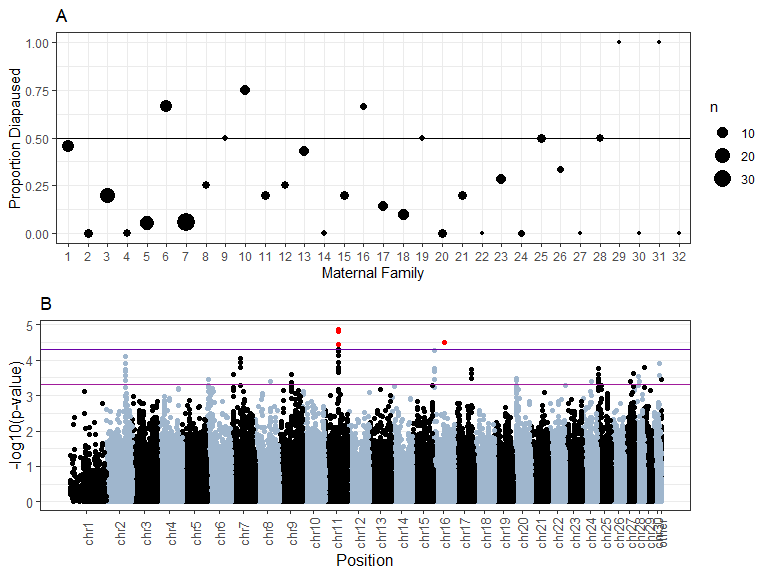


Figure 3: Percentage of individuals in reproductive diapause in each maternal family (A) and GMMAT p-values for association between each SNP and diapause status across all individuals (B). SNPs with a p-value lower that 5 x 10^-5 are highlighted in red.