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Letter

Tempo and mode of winter diapause evolution in butterflies

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Abstract

Quantifying the tempo and mode via modern phylogenetic comparative methods can provide key insights into how selection and constraints shape trait evolution on a macroevolutionary time scale. Here, we elucidate the evolution of hibernation (winter) diapause, a complex and defining life-history trait that allows temporal escape from harsh winters in temperate regions for many insects, including our model system, butterflies. Butterflies can diapause in all major life stages, and the availability of global-scale phylogenies makes them an ideal model system for studying diapause evolution. First, using a thorough literature survey, we scored the developmental stage of hibernation diapause (egg, larva, pupa, adult) vs. absence of diapause. We find that larval diapause is most common, while pupal, egg, and adult diapause are relatively rare. Next, we determined that the loss of diapause occurred at a much higher rate and that gains primarily occurred from the non-diapause state. While ancestral state estimation at deeper nodes remained uncertain, we found consistent patterns for some families and strong evidence for extensive convergence in diapause evolution. Contrary to expectations, we find no support for increased gain of diapause during the Eocene–Oligocene glaciation (~35 million years ago). Overall, the evolution of diapause in butterflies has a complex history, has evolved convergently, and has likely predated the major glaciation event consistent with the deep history of diapause evolution in insects. This study advances our understanding of the evolution of a complex and important life-history trait and establishes a macroevolutionary foundation for future studies on the ultimate and proximate basis of diapause evolution.

Keywords: convergent evolution, Eocene–Oligocene glacial maximum, life-history trait, Mk models, phylogenetic comparative methods, seasonality

Lay Summary

Animals living in seasonal environments, with harsh winters and mild summers in temperate regions and wet–dry seasons in the tropics, are confronted with alternating cycles of favorable and unfavorable seasons. While some migrate, many animals, especially insects, undergo hibernation diapause—a deep resting stage—during the unfavorable season to survive harsh winters. Hibernation diapause, thus, is a key evolutionary strategy for surviving in temperate environments. Butterflies, which can diapause in all life stages—egg, larva, pupa, and adult—serve as a model system for understanding diapause evolution. Through a comprehensive literature survey, we find that larval diapause is most common in temperate regions. Next, by employing statistical methods that allow inferring evolutionary patterns across millions of years, we show that hibernation diapause has evolved independently multiple times, and its loss occurs at a much higher rate than its gain. We also find that the major glacial cycle that led to the global cooling of the Earth around 35 million years ago did not influence diapause evolution. Our study sheds light on diapause evolution across ca. 100 million years, laying the foundations for future studies on ecological drivers and genetic mechanisms behind species-specific diapause strategies.

Introduction

Few habitats on Earth are stable across the year. From temperate seasonality with harsh winters and mild summers to tropical seasonality with alternating dry and wet periods, organisms across taxonomic and trophic levels are faced with seasonal variation in both abiotic and biotic environmental conditions (Williams et al., 2017). While some organisms have the capacity to *spatially avoid* seasonal stress and seek out better resources elsewhere (e.g., migratory birds, caribou herds, and large marine mammals) (Fudickar et al., 2021; Satterfield et al., 2020), many smaller

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organisms such as insects instead *temporally avoid* seasonal stress by entering a resting stage (Denlinger, 1986; Masaki, 1980; Tauber et al., 1986). A particularly deep resting stage is diapause, a preprogrammed form of dormancy that is usually cued well in advance of environmental deterioration (Wilsterman et al., 2021).

The capacity to diapause is a key innovation that allows populations/species to colonize and persist in harsh seasonal environments. For example, killifishes inhabiting seasonal marshes in tropical habitats have convergently evolved embryonic diapause (Furness et al., 2015). Similarly, the timing of the evolution of reproductive diapause coincides with habitat transitions into seasonal environments in African *Bicyclus* butterflies (Halali et al., 2020). Apart from such sporadic examples, investigations of macroevolutionary dynamics of diapause evolution in animals, especially in insects, are scant. In a recent commentary, Denlinger, (2023) even acknowledged that "*we still lack ambitious phylogenetic analyses that enable us to draw conclusions about the deep history of diapause*." In this study, by capitalizing on the rich information on the natural history of butterflies, we provide insights into the tempo and mode of winter (hibernation) diapause evolution in butterflies.

Butterflies diapause in all major life stages (egg, larva, pupa, and adult) (Scott, 1981) and are found in a variety of environments, including many strongly seasonal biomes. Butterflies evolved from nocturnal moth ancestors ~101 million years ago (MYA) (Allio et al., 2020; Wahlberg et al., 2013) and have since radiated to comprise around 18,800 extant species spread across all continents barring Antarctica (Van Nieukerken et al., 2011). Butterflies are perhaps the best-studied insects on Earth, and the availability of large-scale phylogenies (Chazot et al., 2021; Kawahara et al., 2023) is opening up avenues for macroevolutionary analyses using global diversity. Hibernation diapause is a key adaptation and likely a prerequisite to survive in harsh temperate environments. We have a good understanding of the underlying environmental, physiological, and genetic mechanisms regulating hibernation diapause in representatives from some butterfly families (Nylin, 2013 and references therein). As such, butterflies lend themselves to the study and meaningful interpretation of diapause macroevolution.

The present study tackles several general questions on the macroevolution of hibernation diapause. A large literature on insect diapause physiology (Denlinger, 2022) suggests that diapause is not merely a cessation of development and suppression of metabolism but a highly complex alternative developmental pathway (Koštál, 2006). This pathway involves numerous physiological mechanisms, including biological clocks, developmental and cell-cycle regulators, abiotic stress resistance mechanisms, energetic remodeling processes, and reactive oxygen species protection, among many others (Koštál et al., 2017). These mechanisms are, in some cases, found exclusively in the diapause stage (de Kort, 1996). As such, it is crucial to understand whether such a complex physiological adaptation has evolved independently multiple times or, in general, how labile or conserved the evolution of diapause is. Furthermore, the deep evolutionary history of butterflies, tracing back to ~100 million years, allows for investigating the dynamics of diapause macroevolution in the context of dramatic climate change events in the past. Specifically, the Eocene–Oligocene glacial event (~35 MYA) had a major impact on Earth's climate, leading to global cooling (Condamine et al., 2013; Zachos et al., 2001). As the capacity to diapause is a key adaptation that allows survival in temperate environments, we specifically ask if the rate of gain of diapause would be higher during this period.

Here, we use taxa across the globe to ask fundamental questions on diapause macroevolution in an iconic group of animals—butterflies. We focus on the hibernation (winter) diapause in temperate taxa because of the scattered nature of data on diapause in tropical species. We provide first-hand insights into the tempo and mode of this complex key trait and establish a base for future comparative and functional genomics research.

Methods

Collection of the diapause data

The choice of genus/species used in the study was guided by the global genus-level phylogeny of butterflies (Chazot et al., 2019), which was used for comparative analyses. We adhered to the definition of diapause; it is a state of arrested development in a species-specific developmental stage (egg, larva, pupa, or adult), which is entered *before* the onset of adverse environmental conditions (Koštál, 2006). Induction of diapause relies on the use of seasonal cues, for example, photoperiod in temperate environments where decreasing day length indicates that the favorable or adverse season is approaching (Tauber et al., 1986). Diapause typically occurs in a single species-specific developmental stage (i.e., egg, larva, pupa, or adult). In contrast, other "resting" phenotypes associated with surviving adverse conditions, such as quiescence, are direct responses to such conditions and can often occur in several developmental stages in a given species (Denlinger, 1986, 2022; Masaki, 1980).

For tropical taxa, it is often challenging to distinguish between diapause and other resting phenotypes because reliable data is sparse, and diapause strategies are often variable within and among populations (Denlinger, 1986; Halali et al., 2020, 2021; Tauber & Tauber, 1981). Thus, we restricted the scope of the analysis to hibernation or winter diapause in taxa from temperate and temperate-like areas (e.g., high altitude). In such areas, winter survival generally demands a diapause because of the cold and lack of food for larvae and adults. Phenology and distribution of the genus/species were checked using the literature, online databases, and observations from the Global Biodiversity Information Facility to make sure the taxa had representatives in temperate or temperate-like areas and that adults of these species are not active in the winter months. We only included genera for which we could find reliable information on the hibernating stage in the literature and online databases from at least one such species.

Since no part of Africa can be considered truly temperate or is at very high altitude, no genus restricted to this continent was classified as having hibernation diapause. Furthermore, several taxa from high altitudes and/or high latitudes in South America and Asia were excluded from the analysis even though they probably are capable of hibernation diapause since the overwintering stage is unknown. Mainly tropical or subtropical genera with one or a few species extending to the parts of temperate areas closest to the equator (Southern United States and Europe, parts of Asia) where seasonality is less severe, and overwintering may be possible without diapause, were not classified as having hibernation diapause.

Hibernation diapause was classified as egg, larval, pupal, or adult diapause. A few genera/species in our database can diapause in both the larval and pupal stages (e.g., *Pararge aegeria*), and these were classified as having larval diapause (Supplementary Table S1). This allowed us to reduce the number of rare states and parameter estimates when performing comparative analyses. Furthermore, species that overwinter in eggs as fully formed (i.e., pharate) larvae (e.g., *Parnassius* species) were classified as having larval diapause, with the rationale that this phenotype is distinct from overwintering as an undeveloped egg (in a diapause induced by the mother, e.g., *Bombyx mori*) and frequently variable in that some individuals may emerge from the egg before winter and some not (Denlinger, 2022; Tauber et al., 1986). We acknowledge that some taxa reported as having egg diapause may, in fact, overwinter as fully formed larvae in the egg, although this detail has not been reported, meaning that the "egg diapause" state may be somewhat inflated in the data. In some rare cases where hibernation diapause in a genus occurs in different stages in different species, the most common stage was chosen for classification at the genus level. The developmental stage of diapause state in butterflies is, however, largely conserved at the genus level, as exemplified by the well-studied European species (see Supplementary Table S1).

Genera were classified as lacking a true hibernation diapause if none of its species met the criteria given above and coded as "no hibernation diapause" (henceforth no diapause). Note that this classification includes both tropical taxa (often active all year) and taxa at higher latitudes or altitudes with more flexible non-diapause resting phenotypes, such as dormancy seen in several developmental stages.

Also, note that recently, two butterfly trait databases have been published, one for European butterflies (Middleton-Welling et al., 2020) and one global (Shirey et al., 2022), that contain information on diapause states. However, after a brief exploration, we found mismatches (and missing data) for several taxa between databases (see Supplementary Table S2). We thus chose to gather the information ourselves from the primary literature.

Phylogenetic comparative analysis

All analyses were carried out in R ver 4.3.2 (R Core Team, 2023). Details of the R packages used for specific tasks are given in the description of each step of the analysis. Base R and R packages *tidyverse* ver. 2.0.0 (Wickham et al., 2019), *ggplot2* ver. 3.5.0 (Wickham, 2016) and *plotrix* ver 3.8.4 (Lemon, 2006) were used for general data processing and plotting figures, and *ape* ver 5.7.1 (Paradis & Schliep, 2019) for handling phylogenies and related data.

Trait classification and phylogenetic tree

The original diapause data comprised five diapausing states: egg, larva, pupa, adult, and no diapause. From these, two additional classifications were derived: (1) three-state classification—juvenile (egg, larval, and pupal combined together), adult and no diapause; (2) binary-state classification—diapause (egg, larval, pupal, and adult combined together) and no diapause. These classifications comprising five-, three-, and two states provide different levels of resolution into the dynamics of diapause evolution, decrease the frequency of rare states, and reduce model complexity (see below).

The genus-level phylogeny of butterflies from Chazot et al. (2019) comprising 994 tips was used for comparative analyses. The diapause data could be collected for 952 taxa (948 unique genera, covering ~52% of butterfly genera) with 42 taxa excluded and pruned from the phylogeny, as reliable information on diapause could not be obtained. All excluded taxa were distributed across the phylogeny and comprised of both short and long branches (Supplementary Figure S1).

Fitting Markov models for modeling the evolution of diapause

Time-continuous Markov (Mk) models (Harmon, 2019; Lewis, 2001; Pagel, 1994) are at the heart of modeling the evolution of discrete traits. Three models—the equal rates (ER), symmetric (SYM), and all rates different (ARD)—were fitted to five-, three-, and binary-state diapause classifications. For two states, the SYM model is equivalent to the ER model; hence, only ER and ARD models were fitted for binary-state diapause classification. The models were fitted using maximum likelihood (function *fitMK*) in the R package *phytools* ver 2.0.9 (Revell, 2012, 2024), and the best fitting model was chosen based on the Akaike Information Criteria score. Furthermore, each model was fitted with two different root priors—"flat" where all states have equal probabilities occurring at the root (Schluter et al., 1997), and "fitzjohn" which treats root probability as a nuisance parameter (FitzJohn et al., 2009). In total, we fitted 16 Mk models (Supplementary Table S3), and transition rates obtained from the best-fitting model were used to draw inferences on the evolutionary tempo.

While standard Mk models assume homogenous evolutionary rates across time/clades, possible transition rate heterogeneity through time was investigated using the *fitmultiMk* function with flat root prior (see Revell et al., 2021) in *phytools*. This method requires defining time regimes where transition rates are expected to change *apriori*. According to our hypothesis, two regimes were defined—before Eocene–Oligocene glacial maximum (EOGM) (~35MYA) and from 35 million years to the present time—where dynamics in diapause evolution are expected to be different. The model estimates transition rates between states across these regimes, and the fit of these models was compared to the standard Mk models (Supplementary Table S4).

Ancestral state estimation of diapause using standard Mk models

Marginal ancestral state reconstructions were carried out using both maximum likelihood (Schluter et al., 1997) and Bayesian stochastic mapping (Bollback, 2006; Huelsenbeck et al., 2003) using the best-fitting model in *phytools*. Maximum likelihood estimates the ancestral state only at the nodes, while stochastic mapping generates many stochastic maps and allows changes to occur on the branches (Revell, 2013). These stochastic maps can then be summarized to get an estimate of ancestral states at each node (Revell, 2013; Revell & Harmon, 2022). Marginal ancestral estimation were carried out using maximum likelihood (function *ancr*) and stochastic mapping (function *make.simmap*) for both flat and fitzjohn root prior. For stochastic mapping, 1,000 stochastic maps were simulated and then summarized to calculate the probability of each state to be at the node, for counting the number of transitions (function *countSimmap*) and calculating rate through time (see below).

Ancestral state estimation using hidden rate Mk and gamma-distributed rate heterogeneity model

Hidden rate Markov models (HRM) allow for relaxing the assumption of a homogenous rate of evolution across the phylogeny as assumed by standard Mk models (Beaulieu et al., 2013). At its core, HRM allows incorporating the effect of "unobserved" factors (hence hidden) or rate classes that may influence the rate of trait evolution in different parts of the phylogeny (Beaulieu et al., 2013; Boyko & Beaulieu, 2021).

HRMs were fitted by using two rate classes (R1 & R2) and ARD model that allows each transition to have a different rate. Two hidden rate models (each for flat and fitzjohn root prior) were fitted that differed in the transitions between both rate categories (diapause states) and rate classes: (1) transitions between all rate categories and rate classes (R1 & R2) were allowed to occur at different rates (i.e., full HRM); and (2) an "umbral model" (see Revell

& Harmon, 2022) where each state has one hidden state and changes are only permitted between a particular observed state and its hidden state. For all HRMs, dual transitions (e.g., transition between (1, R1) → (2, R2)) are not permitted. The fit of all HRMs was compared with the standard Mk ARD models (Supplementary Table S5). The HRMs were fitted using the *corHMM* function in the *corHMM* package ver. 2.8 (Beaulieu et al., 2022) and marginal ancestral estimation were carried out using maximum likelihood.

Similar to HRM, the recent gamma-distributed rate heterogeneity model (Revell & Harmon, 2024; based on Yang, 1994) allows for relaxing the assumption of homogenous rates across clades. This model allows the transition rates to vary branch-wise according to the discretized gamma distribution (Revell & Harmon, 2024). The model was fitted using the *fitgammaMk* function (with fitzjohn root prior) in *phytools* with eight discrete rates for the ER, SYM, and ARD models (Supplementary Table S6). The ancestral estimation was carried out for the best-fitting model using maximum likelihood. Estimates from this model were used to qualitatively compare patterns with those from standard Mk and HRM models.

Summarizing uncertainty in ancestral estimation across models and root priors

The sum of squared Euclidean distances between the six different ancestral estimations (i.e., maximum likelihood, stochastic mapping, and HRM, each fitted with flat and fitzjohn root prior) for each node was calculated for summarizing how (in)congruent the ancestral estimates were across reconstructions. Here, the probability of each state to be at the node was treated as different features for each reconstruction, resulting in distance calculation across six data points with either five, three, or two dimensions corresponding to five, three, or binary diapause classification. The sum of squared distances was extracted from the return value of the *kmeans* R function.

Calculating the rate of state transitions through time

The rate of each state's transition through evolutionary time was calculated to test if certain transitions were more common during certain periods in the evolutionary history. Specifically, we expected that the gain of diapause from no diapause state would be higher during or around the Eocene–Oligocene glacial event that occurred ~35 MYA. The tree was first divided into 30 equal bins (the total edge length of the phylogenetic tree is 107.60 million years), resulting in time blocks of 3.58 million years each. Next, the rate through time was calculated as the ratio of the mean number of changes in each time block and the total edge length of the tree after accounting for the number of lineages in that time block. Rate through time was calculated using 1,000 stochastic maps for all three diapause classifications and root priors. Finally, the same analysis was repeated by using 20,000 stochastic maps (200 posterior trees with 100 stochastic maps/tree) to test the influence of variation in divergence time estimates (Supplementary Figure S12) on rate through time (details in Supplementary section). R codes from Hughes et al., (2021, available here: https://github.com/jakeberv/mammal_arboreality) were used/modified for this analysis. We want to emphasize here that interpretation of such rates should be made with caution, especially in the deeper parts of the tree, as the amount of information available (e.g., number of branching events) tends to be very low.

Results

Diversity of diapause strategies across butterflies

In the five-state classification, out of 952 total taxa (948 unique genera), the majority belonged to the no diapause state ($n = 790$), followed by the larval ($n = 113$), pupal ($n = 39$), egg ($n = 5$), and adult ($n = 5$) diapausing state (Figure 1A and Supplementary Figure S2). In temperate regions, larval diapause is most common, followed by pupal diapause, with egg and adult diapause rather uncommon. This finding corroborates with the species-level data on diapause states for European butterflies (Middleton-Welling et al., 2020, see Supplementary Figure S3). Overall, the "diapause" state (includes egg, larva, pupa, and adult winter diapause) comprised 17% of taxa in our dataset.

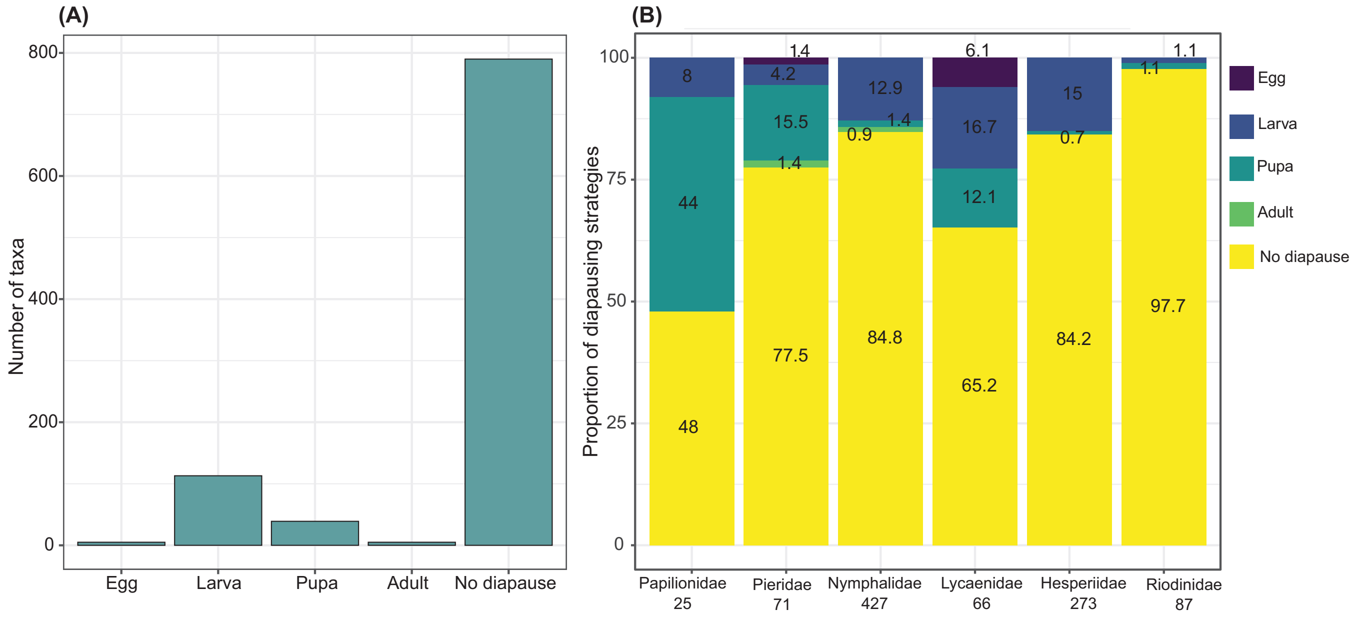

Figure 1. Number of butterfly taxa (i.e., 952 total taxa comprising 948 unique genera, A) and relative proportion of each diapause state (B) for five-state classification. In figure (B), the numbers below the family names on the x-axis indicate the number of taxa in each family. Note that the family Hedylidae is not shown, as it was represented by only two taxa in our tree.

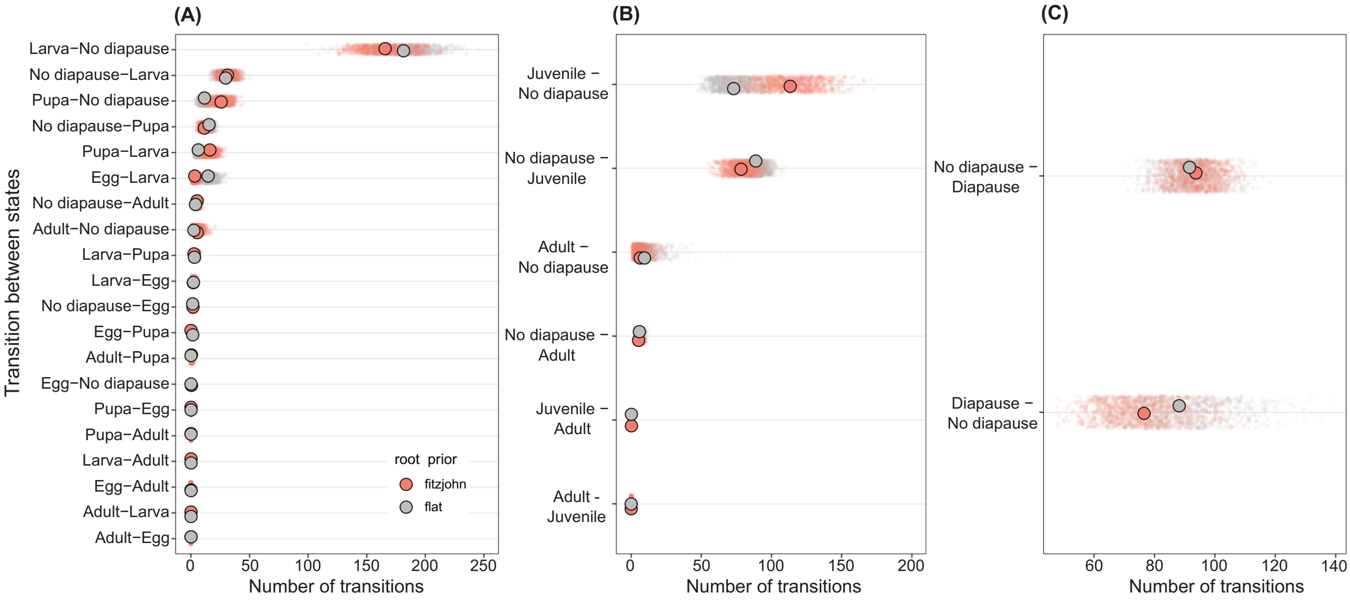

Figure 2. Number of transitions between states for five (A), three (B), and binary (C) diapause classification for fitzjohn and flat root prior. Points in the background indicate the number of transitions across 1,000 stochastic maps, and the larger points indicate the mean number of transitions for a particular transition. Note that the points have been slightly jittered to avoid complete overlap between data points.

Next, relative proportions of genera comprising different diapausing states across families showed a family-specific pattern. For example, taxa diapausing as pupa comprised 44% in Papilionidae but only 16% in Pieridae, 12% in Lycaenidae, and <2% in the remaining families (Figure 1B). Similarly, ~6% of Lycaenidae and 1.4% of Pieridae taxa were egg diapausers but this state was absent in the other families. Only the larval diapausing state was represented across all families, excluding Hedylidae, which comprised only two *Macrosoma* species in our tree. The no diapause state was predominant across all families, even accounting for up to 98% in families such as Riodinidae that are primarily restricted to tropical regions (Figure 1B), reflecting the pattern that the majority of butterfly lineages are tropical (Chazot et al., 2021), and thus were classified into the no diapause state (see Methods). Relative proportions of each diapausing state for three- and binary-state classification are shown in Supplementary Figure S4.

Number and rate of transitions between states

The ARD model was the best fitting model for all diapause classifications across models (Supplementary Tables S3, S4, and S6). Moreover, the transition number and rates among states remained similar across root priors (Figures 2 and 3).

For the five-state classification, the loss of larval to no diapause state was the most common transition (Figure 2A). A few transitions also included gain of larval diapause from no diapause, pupal, and egg diapause, as well as gain and loss of pupal diapause from no diapause (Figure 2A). Most other transitions were estimated to be zero. Overall, most of the gains of diapause predominantly occurred from the no diapause state. Whereas transitions among diapausing states, except a few from pupal → larval diapause and egg → larval diapause, were estimated to be zero. For the three-state classification, transitions between juvenile and no diapause were most common (Figure 2B). For the binary-state classification, the gain of diapause was estimated to be slightly higher than its loss from the no diapause state (Figure 2C).

As for transition rates, the rate of loss of diapause to the no diapause state was always higher than the gain of diapause across all three diapause classifications (Figure 3). Interestingly, a close look at the five-state classification indicated that the transition rate for the gain of larval diapause from egg and pupal diapause was much higher than the gain from no diapause state (Figure 3A). All transitions from juvenile diapausing stages (i.e., larval, pupal, and egg) to adult diapause were estimated to be zero (Figure 3A). There was also a large effect of root prior on egg → no diapause transition rate for the five-state classification. Comparing the standard Mk and two-time regime model (*fitmultiMk*), the standard Mk model had a better fit (Supplementary Table S4).

Finally, the "umbral" hidden rates model (HRM) and the gamma rate ARD model generally had a better fit compared to the standard MK ARD model (Supplementary Tables S5–S7). However, AIC values were very close between the umbral and the full HRM model for the three- and binary-state classification. Also, the fit of the umbral HRM model was better than the gamma rate model (Supplementary Table S7). The overall pattern of transition rates corroborated with the standard Mk models—loss of diapause occurred at a higher rate compared to the gain of diapause, which is especially apparent in the three- and binary-state classifications (Supplementary Figures S5 and S6).

Ancestral state estimation

Uncertainty in the ancestral state estimation was generally high at the deeper nodes across diapause classifications, methods (maximum likelihood, stochastic mapping, and HRM) and root priors. The effect of root prior on ancestral estimates was more apparent for the five-state classification (Figure 4; Supplementary Figures S7 and S8). Comparing umbral HRM and the gamma rate model, ancestral estimation across states remained qualitatively similar, but ambiguity in estimations seemed to be much lower for the latter model (Figure 4; Supplementary Figures S6 and S8). Despite uncertainties, there was strong evidence for convergent evolution of diapause (or diapause at specific developmental stages) across families.

For example, in the five-state classification, stochastic mapping with fitzjohn root prior estimated a high probability for

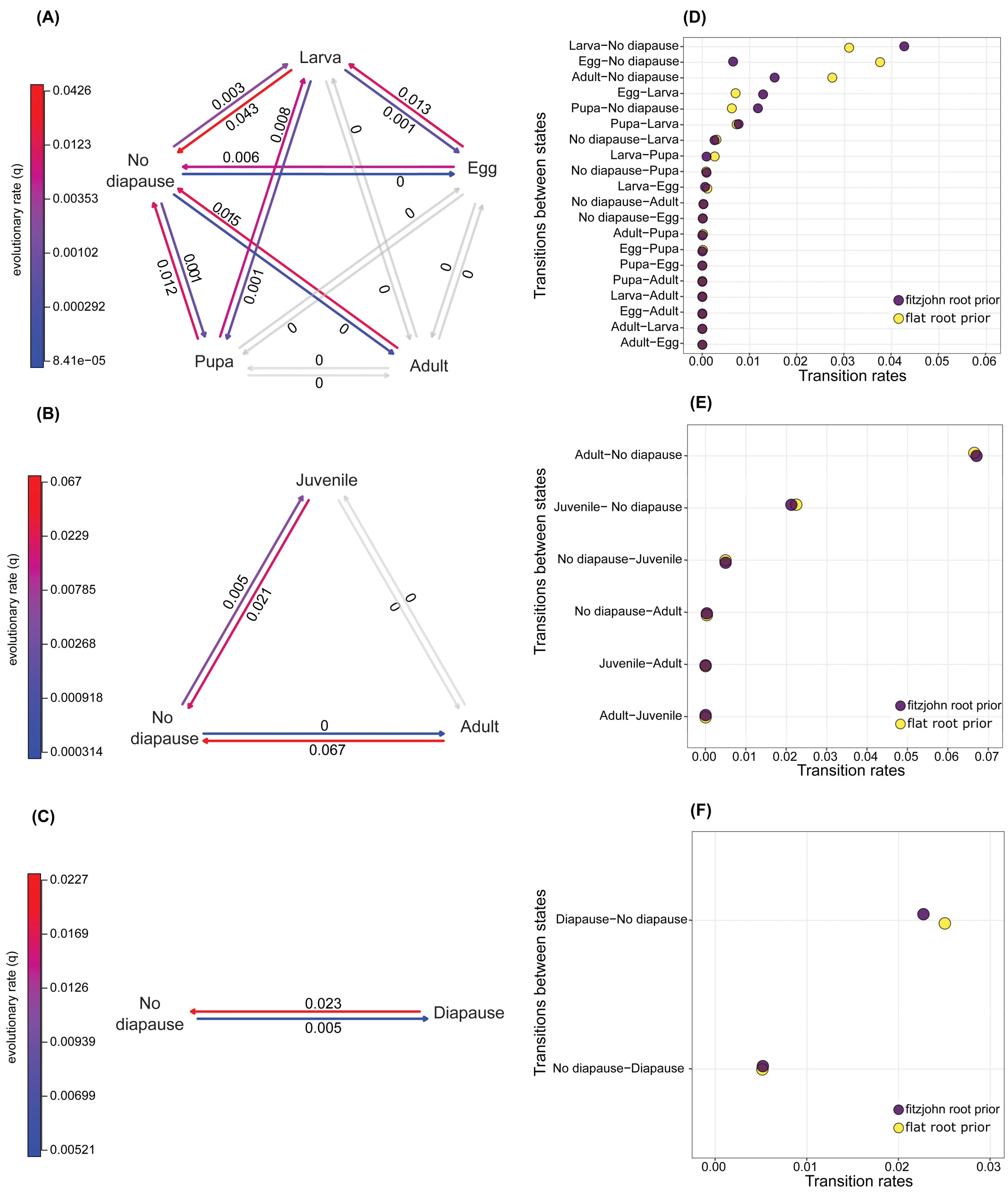

Figure 3. Transition rates across five (A), three (B), and binary (C) state classification using fitzjohn root prior (left panel). Transition rates for both fitzjohn and flat root prior for comparison between two root priors (D–F).

pupal diapause at the base of the Pieridae but was ambiguous when flat root prior was used for both stochastic mapping and maximum likelihood estimation (Figure 4; Supplementary Figure S7). Ancestral estimation using the umbral HRM model estimated pupal diapause at the root of Pieridae (Figure 4). Similarly, a major conflict occurred at the root of Nymphalidae—ancestral estimated remained ambiguous using maximum likelihood and stochastic mapping but was estimated to be pupal

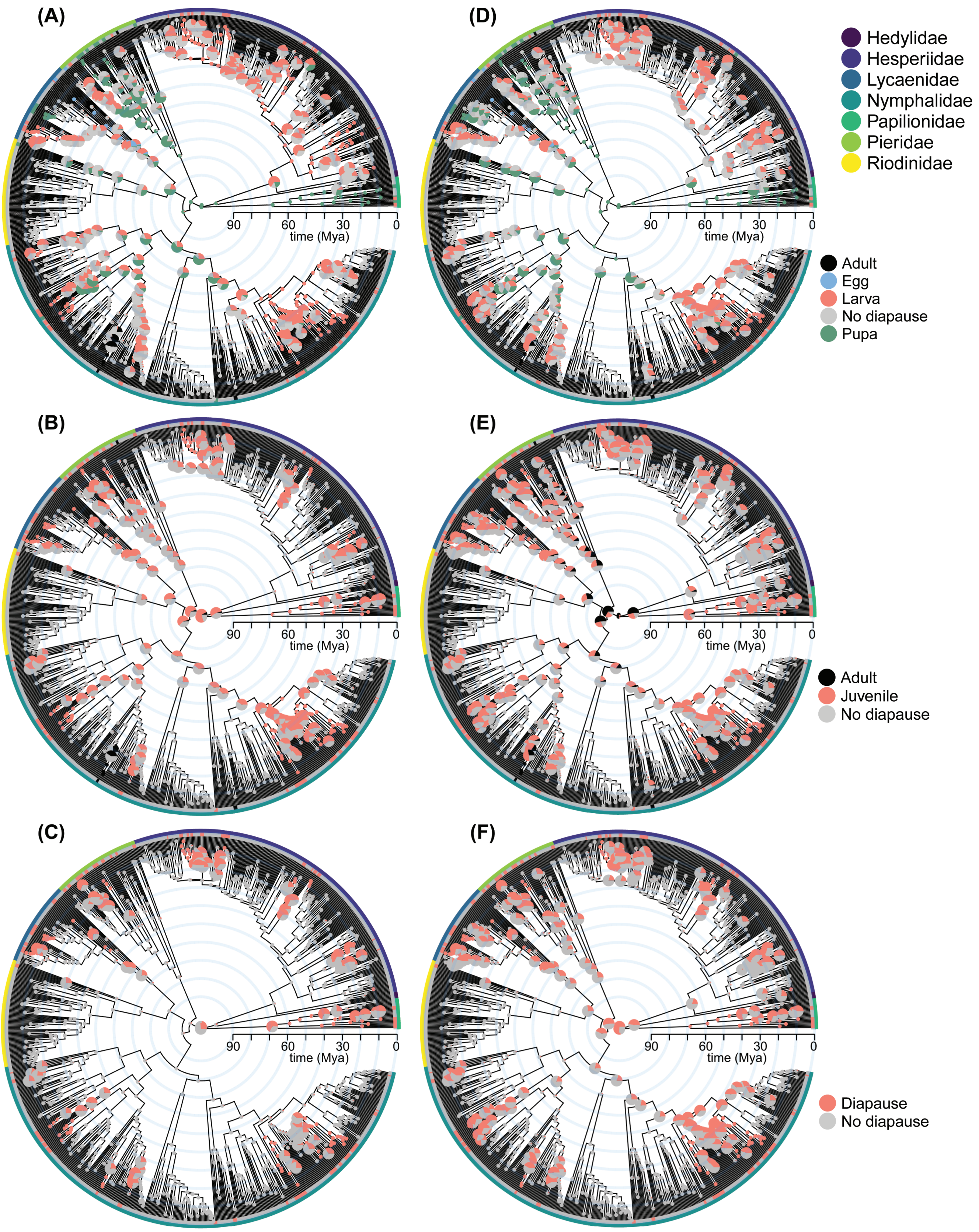

Figure 4. Marginal ancestral state estimation using stochastic mapping (left panel, A–C) and hidden rates model (HRM) (right panel, D–F) for all diapause classifications using fitzjohn root prior. Ancestral estimations using maximum likelihood for the fitzjohn root prior and all three methods (maximum likelihood, stochastic mapping, HRM) for flat prior are provided in Supplementary Figures S7 and S8. The inner concentric ring at the rim of the phylogeny indicates the tip state for each taxon, and the outer ring indicates the family (see the top left figure for family demarcation). Note that pies having > 0.80 probability for a state to occur at the nodes are reduced in size.

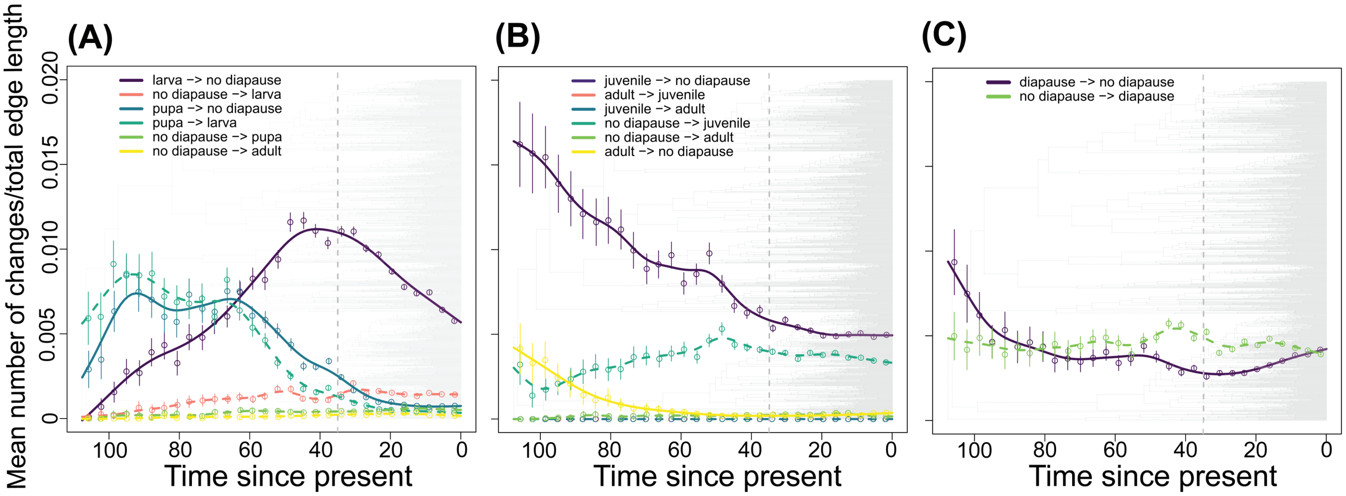

Figure 5. Rate through time plots for five (A), three (B), and binary (C) state classifications for fitzjohn root prior to using stochastic maps. Each point (95% CI) represents the average number of transitions in a 3.58-million-year time block (see *Methods* section) and the smoothed line (spline) estimated from these points. Gain in the diapause is represented with dashed lines. The vertical line represents the Eocene–Oligocene glacial maximum which occurred at ~35 MYA. Note that for the five-state classification, rates for only the most frequent transitions are shown as most transitions were estimated to be zero (see Figures 2 and 3). Rate through time plots for stochastic maps using flat prior is provided in Supplementary Figure S10.

diapause using umbral HRM (Figure 4; Supplementary Figures S7 and S8).

More incongruencies occurred when comparing reconstructions across diapause classifications and methods. For example, in the five-state classification using the standard Mk model, larval diapause had a high probability of being at the root of Hesperiidae but no diapause in the three- and binary-state classification (Figure 4; Supplementary Figures S7 and S8). Similarly, the ancestral state for Nymphalidae was ambiguous in the five-state classification but was estimated to be no diapause state in the three- and binary-state classifications using standard Mk models (Figure 4; Supplementary Figures S7 and S8).

Overall, the ancestral state estimates across methods were ambiguous at the deeper nodes, especially for the five-state classification. But these estimates generally appeared to be more stable in more recent nodes (~50 million years onwards), especially for the three- and binary-state classifications (Supplementary Figure S9). Despite uncertainties, some patterns remained consistent; for example, pupal diapause was consistently estimated to be ancestral to Papilionidae.

Rate through time

Rate through time was calculated to test if the gain in diapause would be more frequent during the EOGM event (~35 MYA). There was no clear evidence for this trend; however, for the three, and binary-state classifications, there was a slight peak in the rate of gain of diapause at around or slightly before 40 MYA (Figure 5; Supplementary Figure S10). This period predates the EOGM event. The rate through time remained broadly similar across root priors for the three- and binary-state classifications but not for the five-state classification (Figure 5; Supplementary Figure S10). Calculating rate through time across 200 posterior trees showed a similar trend to that using the single tree but with large variation in rates, especially at deeper time points (Supplementary Figure S12).

Discussion

Diapause is a complex and defining life-history adaptation that allows the temporal avoidance of seasonally harsh conditions and acts to synchronize life cycles with periods of seasonal abundance (Varpe, 2017; Wilsterman et al., 2021). Diapause exists across all major insect orders (Denlinger, 2022), and in the present study, we elucidate the evolutionary tempo and mode of this trait across the last ca. 100 million years.

Our diapause classification at the genus level suggested that larval diapause is the most widespread strategy in butterflies in temperate areas, followed by pupal diapause. Egg and adult diapause are overall rare. This macroecological pattern corroborates with species-level data on diapause in European butterflies (Middleton-Welling et al., 2020, Supplementary Figure S8). It is currently unclear why butterflies (or insects in general) diapause at different developmental stages or why diapause in certain developmental stages is common (Denlinger, 2023). Using data on 182 species of North American butterflies, (Hayes, 1982) found that nondiapausing species are confined to warmer habitats, egg diapausing species occur in mostly colder habitats, and both larval and pupal diapausing species are widely distributed. Also, pupal diapausing species tend to use woody larval hostplants. Future research utilizing extensive species-level data and causal modeling could clarify the role of ecology in driving diapause evolution in butterflies, as the cause-and-effect relationship between life history (e.g., voltinism, phenology) and ecological factors (e.g., temperature, hostplant preference) is often not clear.

Our macroevolutionary analyses indicated a striking pattern of convergence in diapause or diapause at a specific developmental stage across families. Moreover, families also differ in the proportion of taxa diapausing at a particular stage, suggesting family-specific evolutionary trajectories (Figure 1B). Larval diapause, which was predominant in temperate lineages, has evolved independently multiple times within and across families. Egg, pupal, and adult diapause have also evolved independently in distantly related lineages. For example, egg diapause has evolved independently in Lycaenidae and Pieridae (Figure 4). Furthermore, the gain of diapause mainly occurred from the no (winter) diapause state assigned to tropical species, and transitions among developmental stages were rare, except transitions from pupa and egg to larval diapause in the five-state classification (Figures 2 and 3). That is, the evolution of diapause does not require diapause in a particular stage to act as a precursor, and perhaps, diapausing at different developmental stages represents different solutions to the same problem (i.e., surviving a harsh winter season). In other words, it does not seem as if the physiological adaptations necessary for winter diapause can easily be carried over to another developmental stage once they have evolved. Extensive convergence in diapause evolution further hints at the possibility that overcoming inherent mechanistic constraints to evolve winter diapause might be relatively easy over the macroevolutionary timescale, perhaps evolving from dry season diapause or diapause-like states in warmer areas. However, the extent of constraint might also be group-specific, as some insect group species can diapause at different developmental stages (like butterflies), but others can diapause only in a particular developmental stage (Denlinger, 2023). Finally, one interesting question that arises is whether species undertaking annual migrations (e.g., in families Pieridae, Nymphalidae, Papilionidae) show a different mode of diapause evolution than those without, as one would expect migratory behavior to pose different selection pressures than diapause does, for example, high aerobic scope vs. metabolic suppression (Sattersfield et al., 2020).

Modeling diapause evolution across classifications and evolutionary models provided consistent support for the loss of diapause occurring at a higher rate than its gain. Specifically, loss of diapause across all developmental stages (egg, larva, pupa, and adult) independently led to the no diapause strategy assigned to many tropical species. The transition from adult to no diapause state had the highest rate in the three-state classification, which is surprising as adult diapause is a very rare state (Figure 3). A higher rate of the loss of diapause likely arises as diapause has a deep origin in butterflies (more discussion below), and many lineages with no winter diapause are nested within these clades. Moreover, this pattern could arise when diapausing temperate lineages disperse into the tropics, which happened frequently before and during the Miocene (~23–5 MYA) (Chazot et al., 2021), where they may have lost the capacity to diapause. Microevolutionary studies suggest that populations can lose diapause, for example, if winters are not particularly harsh or the environmental cue(s) required for accurate initiation and termination of diapause are not reliable (Nylin et al., 1995). For example, populations of the butterfly *Pararge aegeria* at lower latitudes (= less harsh winters) in Europe lack the capacity to enter diapause. Thus, loss of diapause could readily occur if selection for maintaining this complex life-history trait is relaxed.

Hibernation diapause is a key adaptation to survive in cold temperate environments. We, thus, expected that the rate of diapause gain during the major glacial cycle—the EOGM (~35 MYA) (Condamine et al., 2013; Zachos et al., 2001)—would be higher during or around this period. Hawkins and DeVries (2009) proposed that cold-related adaptations or lineages should become more apparent during or after the EOGM event in butterflies, but this idea remains untested. The age of butterflies in our tree dates back to ~108 million years, allowing us to discover if such a pattern exists. Firstly, our ancestral estimations suggested that the evolution of diapause predates the EOGM event. For example, the origin of pupal diapause was estimated to be ~68 MYA in Papilionidae and larval diapause ~45 MYA in Nymphalidae (Figure 4). Moreover, the rate through time did not reveal any obvious trend in the increased rate of gain of diapause during or around the EOGM event (Figure 5; Supplementary Figures S10 and S11). Perhaps the lack of such a pattern is not entirely surprising as diapause (in a wider sense, including tropical areas) has deep origins in insects, and the physiological and genetic machinery required for entering diapause is likely already available (Ragland & Keep, 2017). In other words, the evolution of diapause is not a de novo innovation in butterflies. Alternatively, the speciation rates of temperate lineages that already had the capacity to diapause, and not the rate of gain of diapause, increased during this period. This idea could be tested in future studies. Diapause is widespread across insects (or arthropods), and it would be interesting to test whether past climatic events influenced the dynamics of diapause evolution in groups other than butterflies.

This study is not without caveats. First, we use the genus-level data, which likely may have affected the number and rate of transitions. However, data on European species (Middleton-Welling et al., 2020) suggests that the developmental stage of diapause remains conserved at the genus level (Supplementary Table S1). Future studies using species-level sampling will provide more coherent insights into diapause evolution; however, the meager knowledge of diapause in many species outside of Europe and North America will still be problematic. Second, including dry season diapause in tropical species would undoubtedly give interesting insights into diapause evolution, but it would currently be very challenging given the sporadic nature of data. Third, there is a strong bias in the frequency of diapause states; that is, some states were either too frequent or rarely represented. For example, egg and adult states were too infrequent in temperate areas. The presence of rare states has been observed to affect ancestral state estimation, which can then affect transition numbers and rates (see Schluter et al., 1997). We tried minimizing this bias by having different classifications derived by merging states in a biologically meaningful way; however, bias in state frequency is not a consequence of genus-level sampling but is a real pattern and would still persist even if species-level data is used (see Supplementary Figure S3). Fourth, incorporating variation in tree topology and divergence time estimates across all analyses would have been ideal. We have explored how variation in divergence time estimates could affect rate through time using posterior trees (Supplementary Figure S12). Large-scale butterfly phylogenies (e.g., Chazot et al., 2021; Kawahara et al., 2023) have converged to similar topology and divergence time estimates; hence, we believe lower-level uncertainties in topology would have a minimal effect on the broader patterns we infer in this study. Moreover, only maximum likelihood trees are available for these large butterfly phylogenies (close to 1,000 tips or more), as estimating uncertainty using Bayesian analyses is computationally demanding (see Chazot et al., 2021; Kawahara et al., 2023 for details).

Estimating ancestral states is an exciting endeavor, but general doubts about its accuracy, especially at deeper nodes and in the absence of fossil evidence, are a recurring theme in macroevolution (Cunningham, 1999; Cunningham et al., 1998; Omland, 1999). Furthermore, assuming a homogenous rate of evolution in standard Mk models can affect ancestral reconstructions (King & Lee, 2015). We found that although, in general, there was congruency in ancestral estimation between both homogenous and heterogenous (hidden rates) rate Mk models, there were often major conflicts at the deep nodes. We, thus, avoided drawing any conclusions, for example, on the likely ancestral state for all butterflies or even for the families. We want to highlight one such perplexing pattern here. As discussed above, pupal diapause was estimated to have a deep origin in the Family Papilionidae, going back to ~68 MYA. Paleoclimatic studies have suggested that the climate during the early Eocene was warmer than today and hosted tropical forests in much of the Northern Hemisphere (Condamine et al., 2012; Morley, 2007). Thus, such a deep origin of pupal diapause in our ancestral estimation is a bit puzzling. Perhaps including more taxa, species-level sampling, and outgroups (however, the backbone of Lepidoptera still remains unresolved; see Rota et al., 2022) may help identify the ancestral diapause state of each family and broadly of butterflies.

After accounting for the caveats and deriving conservative conclusions, we still identified major patterns in the evolution of diapause in butterflies. Mainly, diapause has deep roots in butterflies, diapause at different developmental stages shows striking convergence, and the loss of diapause occurs at a much higher rate than its gain. We emphasize that studies on tropical species are desperately needed to elucidate the evolution of hibernation diapause in temperate lineages. Our study establishes a solid macroevolutionary foundation for investigating the ultimate and proximate basis of diapause evolution in butterflies.

Supplementary material

Supplementary material is available online at *Evolution Letters*.

Data and code availability

Raw data and R scripts to reproduce the analyses can accessed through Dryad (https://doi.org/10.5061/dryad.mkkwh718t) and Github (https://github.com/sridhar-halali/butterfly-diapause-evolution/tree/19be561f6fff1c59bccfb2af69d27d88c4c4e16f).

Author contributions

All authors contributed to the study design. S.N. collected the diapause data, S.H. carried out the phylogenetic analyses with inputs from everyone, E.Y. wrote/modified R functions for summarizing uncertainty in ancestral estimation and for calculating rate through time, S.H. and P.L. wrote the first draft of the manuscript, and everyone contributed with refining the draft.

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