Tempo and mode of diapause evolution in butterflies

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ABSTRACT

Quantifying the tempo and mode via modern phylogenetic comparative methods can provide crucial insights into the role of selection and constraints in trait evolution. Here we elucidate the evolution of diapause, a complex and defining life-history trait that allows temporal escape from unfavorable conditions in many insects, including our model system, butterflies. Using a thorough literature survey, we first scored the developmental stage of diapause (egg, larva, pupa, adult) vs. absence of diapause. We find that larval diapause is most common in temperate lineages while pupal, egg, and adult diapause are relatively rare. Next, we determined that the loss of diapause occurred at a much higher rate than the gain, and its gain 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 the convergent evolution of diapause across butterflies. We found no support for the hypothesis that the rate of the gain of diapause should be higher during the Eocene-Oligocene glacial maximum event (~35 MYA). Overall, the evolution of diapause in butterflies has a complex history, has evolved convergently, and has likely evolved much earlier than the Eocene-Oligocene glaciation event consistent with the deep history of diapause evolution in insects. These findings fill a deep gap in much-needed studies for future comparative research.

KEYWORDS

Convergent evolution, Eocene-Oligocene glacial maximum, Life-history trait, Mk models, Phylogenetic comparative methods, Seasonality

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). Seasonality, thus, imposes contrasting selection pressures for reproduction and survival across seasons (Tauber et al., 1986; Varpe, 2017). While some organisms have the capacity to *spatially avoid* seasonal stress and seek out better resources elsewhere (e.g. migratory birds, caribou herds, large marine mammals) (Fudickar et al., 2021; Satterfield et al., 2020), many smaller 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 pre-programmed form of dormancy that usually is cued well in advance of environmental deterioration (Wilsterman et al., 2021).

The capacity to diapause is most likely a key innovation allowing populations/species to colonize and persist in harsh seasonal environments. For example, killifishes inhabiting extremely seasonal marshes in South America and Africa have convergently evolved embryonic diapause (Furness et al., 2015). Similarly, the timing of the evolution of reproductive diapause coincides with when the African *Bicyclus* butterflies started colonizing savannah grasslands from ancestral forest habitats (Halali et al., 2020). Capacity to diapause in mosquitoes has also been thought be a key adaptation allowing their diversification in temperate environments (Diniz et al., 2017). Apart from these sporadic examples (which also had a relatively low number of taxa), investigations of macroevolutionary dynamics of diapause evolution in animals, especially in insects, have not received much attention. 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 diapause evolution using a large number of taxa in a macroevolutionary framework.

Butterflies diapause in all major life-stages (egg, larva, pupa, and adult) (Scott, 1981) and are found in a variety of environments across earth, including many biomes that are characterized by strong seasonality. Butterflies evolved from nocturnal moth ancestors around 101 million years ago (MYA) (Allio et al., 2020; Wahlberg et al., 2013) and have since radiated to comprise around 18800 extant species spread across all continents barring Antarctica (Van Nieukerken et al., 2011), and are perhaps one of the best studied insect groups on Earth. Moreover, large scale phylogenies are becoming increasingly available (Chazot et al., 2021; Kawahara et al., 2023) opening up new avenues for carrying out large-scale macroevolutionary analyses of key traits that may have influenced the diversification of groups. Diapause is such a key adaptation, and likely was pre-requisite to survive in harsh temperate environments. We have a comparatively good understanding of the underlying environmental, physiological and genetic mechanisms regulating 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 a number of general questions surrounding diapause and its evolution. As evidenced by a large literature on diapause physiology, summarized for insects by Denlinger (2022), diapause is not merely a cessation of development and suppression of metabolism, but a highly complex alternative developmental pathway (Košťál, 2006). This pathway involves a large number of physiological mechanisms including biological clocks, developmental and cell-cycle regulators, abiotic stress resistance mechanisms, energetic remodeling processes, immune defense-related adjustments, lipid bilayer modifications, reactive oxygen species protection, among many others (Košťál 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 rather, is derived from an ancestral deep proto—diapause state. This further leads to the question of why butterfly species (or insects in general) diapause at different developmental stages or why diapause in certain developmental stages is more common than diapause in other (Denlinger, 2023; Hayes, 1982). This might suggest some life-stage is inherently more favourable for diapause than other. In this respect, instances of intra-tribe variation in diapause strategy (e.g. in Nymphalini) (Friberg et al., 2023; Gotthard 2004) are particularly interesting, since they raise the general question of evolutionary lability of diapause, and how commonly diapause strategy shifts from one life-stage to another. One might consider the most common diapause life-stage to represent an optimal solution, and that convergent evolutionary processes lead towards diapause in this life-stage, but it is entirely possible that different constraints, be these intrinsic (e.g. body size, host preference, reproductive strategy) or extrinsic (e.g. temperature, humidity, among-year seasonal variability), favor diapause in different life-stages (Hayes, 1982).

Finally, the deep evolutionary history of butterflies tracing back to ~100 million years allows investigating dynamics of diapause evolution in the context of dramatic climate change events Earth has experienced 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). Given that the capacity to undergo diapause is one of the key adaptations that allows survival in freezing environments in temperate regions, we specifically ask if the rate of gain of diapause would be higher during this period. Given the deep evolutionary origin of diapause in insects (Denlinger, 2022; Tougeron 2019), de novo evolution of diapause in butterflies is unlikely but rather may have evolved from pre-existing ‘proto’ diapause/dormancy like state from their tropical ancestors.

The present study uses a large dataset including taxa across the globe to ask fundamental questions about diapause - a complex and important adaptive life-history trait - in an iconic group of animals - butterflies – in a macroevolutionary framework.

METHODOLOGY

Collection of the diapause data

Data on diapause was collected from the literature and online databases, supplemented by phenological data from GBIF (https://www.gbif.org/). The choice of genus/species used in the study was guided by the global genus-level phylogeny of butterflies which was used in our comparative analyses (Chazot et al., 2019, see below). We adhered to the definition of diapause that 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 (Košťál, 2006). Induction or breaking of diapause relies on the use of seasonal cues, for example, photoperiod in the temperate environments where increasing or decreasing daylength indicates that the favorable or adverse season is approaching, respectively (Tauber et al., 1986). 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).

Only comparatively few butterfly species have been studied experimentally in order to investigate diapause strategy and among these, the majority of studies are focused on species in temperate regions. As such the diapausing life-stage (i.e. egg, larva, pupa, adult or a combination thereof) in most cases had to be inferred from the seasonality of the habitat, the seasonal occurrence (phenology) of the butterflies and observations of diapause stages by naturalists. Such inference can be problematic for tropical and subtropical habitats, where the seasonality is variable according to latitude and altitude, phenology often little studied, and diapause can occur only in some populations (Denlinger, 1986; Halali et al., 2020, 2021; Tauber & Tauber, 1981). We therefore decided to restrict the scope of this investigation to hibernation (winter) diapause in temperate or very high-altitude areas, where winter survival is typically not possible without diapause. 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.

Following these criteria, a genus was classified as having hibernation diapause if it contains any species inhabiting temperate or very high-altitude areas and where literature and/or GBIF records show that adults are not active in winter. However, we included only taxa where the overwintering life-stage is known for at least one species. This had the consequence that several taxa from high-altitudes and/or high latitudes in South America and Asia had to be excluded from the analysis, even though they probably are capable of hibernation diapause. Some uncertainty also remains for species in the parts of temperate areas closest to the equator (South USA and Europe, parts of Asia) where seasonality is less severe, and overwintering may be possible without diapause. In addition, many species are present only as occasional strays to temperate areas. Here, a judgement call had to be made, so that a genus was not classified as having hibernation diapause if its distribution is mainly tropical and/or subtropical but one or a few species extends to temperate areas, unless there is strong evidence for true hibernation in a species-specific developmental stage, with active adults absent in winter.

Hibernation diapause was classified as egg, larval, pupal or adult diapause. A few species in our database can diapause in both the larval and pupal stage (e.g. *Pararge aegeria*) and these species were classified as having larval diapause. This allowed us to reduce the number of rare states and state space when performing comparative analyses (see below). 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). In some rare cases where hibernation diapause in a genus occurs in different stages in different species, the most common stage was chosen. Genera were classified as lacking a true hibernation diapause if none of its species met the criteria given above, and coded as "none/flexible", in 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 in one of several developmental stages.

Also, note that recently two butterfly trait databases have been published, one for European butterflies (Middelton-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 1 for some examples). We thus chose not to gather the information ourselves from the primary literature.

Phylogenetic comparative analysis

Software usage

Unless specified, all analyses were carried in R ver 4.2.3 (R Core Team 2023) using following R packages. For reading .xlsx files- *openxlsx* ver. 4.2.5.2 (Schauberger & Walker, 2023); data handling- base R functions and *tidyverse* ver. 2.0.0 (Wickham et al., 2019); generating figures (without phylogenies) - *ggplot2* ver. 3.4.4 (Wickham, 2016); for phylogenetic comparative analyses including preprocessing steps, plotting figures with phylogenies and related tasks: *ape* ver. 5.7.1 (Paradis & Schliep, 2019), *geiger* ver 2.0.11 (Harmon et al., 2008; Pennell et al., 2014) and *phytools* ver 2.0.9 (Revell, 2012, 2024).

Trait classification

The original diapause data comprised of five diapausing states: egg, larva, pupa, adult, and 'none/flexible' state (called "flexible" henceforth). From these classifications, two additional classifications were derived: (1) 'three-state classification' which includes juvenile diapause (species diapausing as egg, larva and pupa combined together), adult diapause and flexible state; (2) 'two-state classification' which includes diapause (species diapausing as egg, larva, pupa and adult combined together) and flexible state. These different classifications comprising of five-, three- and binary states, offer different advantages, for example, by providing different resolution into the dynamics of diapause evolution. Combining different states in a biologically meaningful way also decreases the frequency of infrequent states and helps in reducing the model complexity, mainly, number of parameters that needs to be estimated.

Phylogenetic tree for comparative analysis

The genus level phylogeny of butterflies from Chazot et al. (2019) comprising 994 tips (including outgroups) was used for comparative analyses. The diapause data could be collected for 952 taxa (948 unique genera) with 42 taxa excluded and pruned from the phylogeny as reliable information on diapause could not be obtained.

Fitting Markov (Mk) models for modelling the evolution of diapause

Time-continuous Markov (Mk) models are at the heart of modelling the evolution of discrete traits for estimating transition rates between states (Harmon, 2019; Lewis, 2001). In the most basic Mk model with binary states (1 & 2), the transition rate between the states is equal ($q_{12} = q_{21}$). This model is hence referred as the 'equal rates' model. However, one could increase the complexity by allowing q_{12} and q_{21} to have different transition rates. This is the 'all rates different' model. Thus, for a character with binary states, the equal rates and all rates different will have one and two parameters that will need to be estimated, respectively. For a character with more than three states, an additional model (symmetric model) could be fitted where forward and backward transitions between adjacent states are allowed to have same rates.

All three models – the equal rates (ER), symmetric (SYM) and all rates different (ARD) – were fitted to five, three and binary diapause classifications. Note that the symmetric model is same as the equal rates model for a binary state, hence, only ER and ARD could be fitted for binary diapause classification (see Table 1). The models were fitted using maximum likelihood (*fitMK* function) in the R package *phytools* (Revell, 2012, 2024) and the best fitting model was chosen based on the Akaike Information Criteria (AIC) score. Furthermore, within a model (i.e. ER, SYM and ARD), two different root priors – 'flat root prior' where all states have equal probabilities occurring at the root (Schluter et al., 1997) and 'fitzjohn root prior' which treats root probability as a nuisance parameter (FitzJohn et al., 2009) – were fitted. It is important to note that model comparisons (using AIC score in our case) cannot be carried out across diapause classifications and root priors. In total, we fitted 16 Mk models (see Table 1). Transition rates obtained from the best fitting model were used to draw inferences on the tempo of diapause evolution in butterflies.

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) in *phytools.* Ancestral states are estimated by employing Felsenstein's pruning algorithm which computes the likelihood of each state at the node given the phylogeny, tip states and the model of evolution (Felsenstein, 1973; Harmon, 2019). The model of evolution in this case is the best fitting Mk model which was ARD in all cases (see above, Table 1). The difference between both approaches is that maximum likelihood estimates the ancestral state only at the nodes, while stochastic mapping generates a range of character histories or 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 reconstruction of ancestral states using both maximum likelihood (function *ancr*) and stochastic mapping (function *make.simmap*) for both flat and fitzjohn root prior was carried out using the best fitting model. 1000 stochastic maps were generated by running 1000 simulations and these maps were summarized to calculate the probability of each state to be at the node, for counting the number of transitions (function *countSimmap*) and further downstream analyses (calculating rate through time, see below). It should be noted that the stochastic mapping we carry out here is not fully hierarchical Bayesian, as such, we do not sample the transition rate matrix from the posterior. Rather, the transition matrix estimated using the maximum likelihood is supplied to the algorithm. Ancestral reconstructions were carried out using the *phytools* R package.

Ancestral state estimation using hidden rate Mk models

Using hidden rate Markov models offer an added advantage compared to the standard Mk models by relaxing the assumption of homogenous rate of evolution across the phylogeny (Beaulieu et al., 2013). At its core, this model 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). The observed state could be any discrete trait, which is diapause states in our case. The rationale for using hidden rate models was in general to test for congruency in the evolutionary rates with that of the standard Mk model and especially if incongruencies across root priors (which was observed for standard Mk models, see Results) still persisted.

Hidden rate models were fitted by using two rate classes (say R1 & R2) and all rates different model which allows each transition to have a different rate. Three different hidden rate models (each for flat and fitzjohn root prior) were fitted that different in the transitions between both rate categories (diapause states) and rate classes. The models are as follows: (1) transitions between all rate categories and rate classes (R1 & R2) were allowed to occur at different rates (full hidden rates model); (2) transitions between all rate categories were allowed to occur different rates but transition between rate classes had same a rate; (3) an 'umbral model' (see Revell & Harmon, 2002) where each state has one hidden state and changes are only permitted between a particular observed state and its hidden state. For all hidden rates models, dual transitions (e.g. transition between (1, R1) → (2, R2)) are not permitted.

The fit of all hidden rate models was compared with the standard Mk ARD models. The hidden rates models (function *corHMM*) were fitted in the *corHMM* package (Boyko et al. 2021, Beaulieu et al. 2022). Note that the fitzjohn root prior is called 'maddfitz' root prior in *corHMM.*

The marginal reconstructions were carried out using maximum likelihood. We note that extensive set of models can be fitted in the hidden rates framework, for example, by fitting different Mk models (ER/SYM/ARD) or using different number of rate classes and so on (see Boyko et al. 2021). However, our main goal was to compare ancestral reconstructions using standard Mk and 'simple' hidden rates models and see if the (in)congruency still persists.

Summarizing uncertainty in ancestral estimation across models and root priors

Sum of squared Euclidean distances between the six different ancestral estimations (i.e. maximum likelihood, stochastic mapping and hidden rates model each fitted with flat and fitzhon 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. Sum of squared distances were extracted from the return value of the *kmeans* R function.

Calculating rate of state transitions through time

The rate of each state transition through time (in millions of years) 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 (or gain of each developmental diapause state) from flexible state would be higher during or around the Eocene-Oligocene glacial event that occurred ~35 million years ago. We first divided the tree 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 total edge length of the tree after accounting for the number of lineages in that time block (see Hughes et al. (2021) for similar analyses; the original R code is available here - https://github.com/jakeberv/mammal_arboreality - was modified for our analyses). Rate through time was calculated using 1000 stochastic maps obtained from ancestral state reconstructions for all three diapause classifications and root priors.

RESULTS

Diversity of diapausing strategies across butterflies

In the five-state classification, out of 952 total taxa (948 unique genera), the majority belonged to the flexible state (n=788) followed by the larval (n=112), pupal (n=41), egg (n=6) and adult (n=5) diapausing state (Figure 1a & S1). In temperate regions, larval diapause is most common, followed by pupal diapause, with egg and adult diapause rather uncommon, at least when analysed at the genus level. This finding corroborates with the species-level data on diapause states for European butterflies (Middleton-Welling et al. 2019, see Supplementary Figure S8). Overall, the 'diapause' state (which includes egg, larva, pupa and adult diapause) across the five-, three- and binary-state classification, comprised 17% of the genera in our dataset.

Next, exploring relative proportions of genera comprising of different diapausing states across butterfly families indicated some patterns. For example, taxa diapausing as pupa comprised 52% in Papilionidae but only 16% in Pieridae, 12% in Lycaenidae and <2% in remaining families (Figure 1b). Similarly, ~8% of Lycaenidae and 1.4% of Pieridae taxa diapaused in the egg stage, with egg diapause being completely absent in the other families (Figure 1b). Only the larval diapausing state was represented across all families (excluding Hedylidae which comprised only two *Macrosoma* species in our tree). The flexible 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). This reflects the general fact that the majority of butterfly lineages are restricted to tropical regions (Chazot et al., 2021), and thus were classified into the flexible state (see Methods). See Supplementary Figure S2 for the relative proportions of each diapausing states for three- and binary state diapause classification.

Number and rate of transitions between states

Fitting three Mk models – equal rates (ER), symmetric (SYM) and all rates different (ARD) – for all diapause classifications (five-, three- and binary-state) using maximum likelihood, the ARD model always had the best fit, irrespective of the root prior used (Table 1). Moreover, the number and rate of transitions among almost all states remained similar across root priors (Figure 2 & 3).

For the five-state classification, the most common transition was the loss of larval diapause to flexible state (Figure 2a). A few transitions also included gain of larval diapause from the flexible state, gain and loss of pupal diapause from the flexible state and gain of larval diapause from pupal diapause (Figure 2a). Apart from these few transitions, most other transitions were estimated to be zero (Figure 2a). Overall, the gain of diapause mainly occurred from the flexible state whereas transitions among diapausing states (e.g. larva <-> egg, adult <-> pupa and so on) were low or entirely absent. Similarly, for the three-state classification, the gain and loss of juvenile diapause from flexible state comprised the most common transitions, while transitions leading towards or from adult diapause was estimated to be zero in most instances (Figure 2b). For two-state classification, loss of diapause was more common than gain of diapause, similar to the five-state classification (Figure 2c).

As for transition rates, the rate of loss of diapause was always higher than the gain of diapause across all three diapause classifications (all arrows pointing towards flexible strategy in Figure 3). Interestingly, a close look at the five-state classification, in fact, indicated that the transition rate for the gain of larval diapause from pupal diapause was much higher than the gain from flexible 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).

Comparing different hidden rates and standard Mk models, the 'umbral' hidden rates model had a better fit than the standard Mk ARD model for three and binary diapause classification (Table 2). For the five-state classification, AIC values were very close between the umbral hidden rates and standard Mk model (we used umbral hidden rate model for ancestral estimation for consistency, see below). Furthermore, 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 across all classifications (Supplementary Figure S3).

Table 1: List of different Markov (Mk) models (ER = equal rates, SYM = symmetric rates, ARD = all rates different) and their fits across diapause classification and root priors. Models were fitted using the *fitMk* function in *phytools.* Rows highlighted in bold indicates the best fitting models based on the AIC score. Note that the models cannot be compared across diapause classifications and root priors. For binary state, ER and SYM models are the same hence only the ER model was fitted.

State classification	Mk model	Root prior	Log Likelihood	No. of parameters	AIC
Five	ER	flat	-540.84	1	1083.68
Five	SYM	flat	-476.32	10	972.64
Five	**ARD**	**flat**	**-435.48**	**20**	**910.96**
Five	ER	fitzjohn	-539.24	1	1080.48
Five	SYM	fitzjohn	-474.73	10	969.45
Five	**ARD**	**fitzjohn**	**-434.22**	**20**	**908.44**
Three	ER	flat	-445.78	1	893.56
Three	SYM	flat	-387.99	3	781.98
Three	**ARD**	**flat**	**-371.82**	**6**	**755.65**
Three	ER	fitzjohn	-444.69	1	891.39
Three	SYM	fitzjohn	-386.92	3	779.83
Three	**ARD**	**fitzjohn**	**-371.81**	**6**	**755.62**
Binary	ER	flat	-370.64	1	743.28
Binary	**ARD**	**flat**	**-359.54**	**2**	**723.08**
Binary	ER	fitzjohn	-369.97	1	741.94
Binary	**ARD**	**fitzjohn**	**-359.54**	**2**	**723.08**

Table 2: Fit of the standard Mk and several hidden rate models (HRM) across diapause classification and root priors. Models were fitted using the *corHMM* function in the *corHMM* package. Note that the *fitzjohn* root prior is same as the *maddfitz* in the *corHMM* package. Rows highlighted in bold indicates the best fitting models based on the AIC score. Note that the models cannot be compared across diapause classifications and root priors. Also note the slight differences in the log likelihood and AIC values for standard Mk (all rates different models) models between *corHMM* and *phytools*. See Methods section for the details on the fitted hidden rates model.

Diapause classification	Model	Root prior	log Likelihood	AIC
Five	**Standard Mk**	**flat**	**-433.64**	**907.28**
Five	HRM full	flat	-424.36	932.72
Five	HRM equal rate class	flat	-424.52	931.03
Five	HRM umbral	flat	-436.02	908.04
Five	**Standard Mk**	**fitzjohn**	**-433.64**	**907.28**
Five	HRM full	fitzjohn	-424.36	932.72
Five	HRM equal rate class	fitzjohn	-424.52	931.03
Five	HRM umbral	fitzjohn	-436.06	908.12
Three	Standard Mk	flat	-371.81	755.62
Three	HRM full	flat	-357.20	742.40
Three	HRM equal rate class	flat	-358.99	743.97
Three	**HRM umbral**	**flat**	**-357.80**	**735.61**
Three	Standard Mk	fitzjohn	-371.81	755.62
Three	HRM full	fitzjohn	-359.13	746.27
Three	HRM equal rate class	fitzjohn	-358.99	743.97
Three	**HRM umbral**	**fitzjohn**	**-357.29**	**734.57**
Binary	Standard Mk	flat	-359.54	723.08
Binary	HRM full	flat	-345.76	703.52
Binary	HRM equal rate class	flat	-347.06	704.12
Binary	**HRM umbral**	**flat**	**-344.99**	**701.98**
Binary	Standard Mk	fitzjohn	-359.54	723.08
Binary	HRM full	fitzjohn	-345.76	703.52
Binary	HRM equal rate class	fitzjohn	-347.06	704.12
Binary	**HRM umbral**	**fitzjohn**	**-344.99**	**701.98**

Ancestral state estimation

Ancestral state estimates were generally congruent among diapause classifications across methods (maximum likelihood, stochastic mapping and hidden rates model) and across root priors for standard MK models. The effect of root prior on ancestral estimates was more apparent for five-state compared to the three- and binary-state classification at deeper nodes (Figure 4, Supplementary Figure S4 & S5). Moreover, there were large differences in the ancestral estimates, especially at the deeper nodes, when compared between the standard Mk and hidden rate models (Figure 4, Figure S4-S6).

For example, in the five-state classification using the standard Mk model, both maximum likelihood and stochastic mapping with fitzjohn root prior estimated high probability for pupal diapause at the base of the Pieridae, while the flat root prior estimated a ~60% probability for the flexible state (Figure 4, Figure S5). The hidden rates model (using the umbral model) on the other hand estimated adult diapause at the root of Pieridae with high probability (Figure 4). Note that the standard Mk model had a slightly better fit compared to the umbral hidden rate model for five-state diapause classification (Table 2, delta AIC = 0.76 for flat prior and 0.84 for fitzjohn prior). Similarly, a major conflict occurred at the root of Nymphalidae - the hidden rates model estimated adult diapause at the root but was largely incongruent in maximum likelihood and stochastic mapping (Figure 4, Figure S5).

More incongruencies occurred when comparing reconstructions across diapause classifications and methods. For example, in the five-state classification using the standard Mk model, there was a high probability for larval diapause to be at the base of Hesperiidae but the flexible state was estimated to be ancestral in the three- and binary-state classification (Figure 4, Figure S4 & S5). This means that the evolution of larval diapause was estimated to be more ancestral in the five-state classification but more recent in the three- and binary-state classification. Similarly, the ancestral state for Nymphalidae was ambiguous in the five-state classification but was estimated to be the flexible state with high probability in the three- and binary-state classification using standard Mk models (Figure 4, Figure S4 & S5).

Overall, the ancestral state estimates were ambiguous when comparing across methods and classifications, especially the deeper nodes (Figure S6). But these estimates appeared to be more stable in more recent nodes, especially 50 million years onwards. Despite uncertainties, some patterns remained consistent across all methods and classifications. Pupal diapause estimated to be ancestral for Papilionidae with high probability and there was consistent evidence for repeated evolution of diapause (or diapause at specific developmental stages) in butterflies (Figure 4, Figure S4 & S5).

Rate through time

Rate through time was calculated to test if gain in diapause would be more frequent during Eocene-Oligocene glacial maximum event which occurred around 35 million years ago. There was no clear evidence for this trend, however, for the three and two state classification, there was a slight peak in the rate of gain of diapause appeared to peak at around or slightly before

40 million years ago (Figure 5, Figure S7). This period predates the Eocene-Oligocene glacial maximum event, but it is important to consider that some uncertainty could exist in the dating resolution. The rate through time remained broadly similar across root priors for the three- and binary-state classification but affected for five-state classification (Figure 5, Figure S7).

DISCUSSION

Diapause is a complex and defining life-history adaptation in a large number of organisms 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). In insects, diapause exists across all major orders (Denlinger 2022) and in the present study we described diapause states across 952 butterfly species from seven major families and elucidate the tempo and mode of diapause evolution across the last ca. 100 million years.

Our diapause classification at the genus level suggested that larval diapause is the most widespread strategy in temperate lineages of butterflies, followed by pupal diapause. Egg and adult diapause are overall rare. A brief exploration suggests that this macroecological pattern corroborates with species-level data on diapause in European butterflies using a recent dataset (Middleton-Welling et al. (2020), Supplementary Figure S8). It is currently unclear why butterfly species (or even insects in general) diapause at different developmental stages or why diapause in certain developmental stages is common (Denlinger, 2023). We suspect the evolution of different diapausing strategies is likely driven by several interacting factors such as latitude, season length, temperature, hostplant (e.g. short or long-lived), voltinism and so on. For example, the stage at which the Lepidoptera species diapause has a strong effect on their voltinism with those diapausing in non-egg stage generally tend to be bi- or multivoltine (Hayes, 1982; Teder, 2020). Using diapause and other ecological data on 182 species of North American butterflies, Hayes (1982) found that non-diapausing species are confined to warmer habitats, egg diapausing species are found in mostly colder habitats while larval and pupal diapausing species are widely distributed, and also that pupal diapausing species tend to use woody plants as hosts. Future research using large species-level data and causal modelling (as the cause-and-effect relationship between the factors mentioned above is not always clear) would allow disentangling of the role of environmental and ecological factors driving diapause evolution in butterflies.

Our macroevolutionary analyses indicate that egg, larval, pupal or adult diapause can evolve independently and do not require any particular developmental stage to act as a precursor (see below). This hints at the possibility that overcoming inherent mechanistic constraints to evolve diapause might be relatively easy on both micro- (Batz et al., 2020) as well as macroevolutionary scales, for example, by remodeling the ancestral gene regulatory networks (Singh et al., 2021). But the extent of constraint might also be group-specific, for example, some insect groups can diapause at different developmental stages (like butterflies) but others can diapause only in a particular developmental stage (Denlinger, 2023). Whether such constraints exist or not remains an open question, however. One interesting general question that arises from the current analysis is whether families/genera with species undertaking annual migrations (e.g. Pieridae, Nymphalidae, Papilionidae) show a different mode of diapause evolution than those without, as one might expect migratory behavior to pose highly different selection pressures on the phenotype than diapause does, for example, high aerobic scope vs metabolic suppression (Sattersfield et al., 2020).

Modelling diapause evolution across classifications (five-, three- and binary-state), evolutionary models (standard and hidden rate Mk models) and root priors (flat and fitzjohn) provided consistent support for the loss of diapause occurring at a higher rate than its gain. Specifically, loss of diapause across all developmental stages (i.e. egg, larva, pupa and adult) independently led to the 'flexible' strategy which was assigned to many tropical species. The rate was highest for the transition from adult diapause to the flexible strategy which is slightly perplexing given that the adult diapause an extremely infrequent state (Figure 3). A higher rate in the loss of diapause likely arises due to the fact the diapause has a deep origin in butterflies (see below for more discussion) and many lineages with flexible strategy are nested within these clades. Moreover, this pattern could arise when diapausing temperate lineages disperse into the tropics, which happened frequently during the Miocene (23-5 MYA) or even before that (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. For example, experiments using populations of the butterfly *Pararge aegeria* collected from different latitudes in Europe have shown that populations from lower latitudes (= less harsh winters), lack the capacity to enter diapause (Nylin et al., 1995). Thus, reduced sensitivity or loss of diapause could readily occur if selection for maintaining this complex life-history trait is relaxed.

We found a striking pattern of convergence in diapause or the developmental stage at which taxa enter the diapause 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 most common among temperate lineages has evolved convergently multiple times in each and across families. Egg, pupal and adult diapause have also evolved independently multiple times in distantly related lineages or even families. For example, egg diapause has evolved independently in Lycaenidae and Pieridae (Figure 4). As discussed above, why species in temperate regions diapause at different developmental stages or why larval diapause is widespread is an open question. However, the number of transitions and transition rates (Figure 2 & 3) indicates that transitions between the developmental stages are extremely rare, and the gain of diapause almost always seems to occur from the flexible state assigned to tropical species. This suggests that the capacity to diapause at different stages has evolved independently and does not require diapause in a particular stage to evolve first or act as a precursor. Perhaps, these are different solutions (diapausing at different stages) to the same problem (surviving a harsh season) but targeted future studies are needed to investigate the ultimate factors shaping this phenomenon. Independent gain of diapause from the flexible state (assigned to tropical species) also lends some support to the 'tropical origin of diapause' hypothesis and that insects would not have acquired the capacity to diapause de novo during the glaciation event (Tauber & Tauber, 1981; Tougeron, 2019). Thus, it is possible that the ability of many tropical species to undergo some form of dormancy (Denlinger, 1986) may act as a precursor for the evolution of hibernation diapause in temperate lineages. Future functional genomic studies will be able to provide much needed insight on the origin of diapause in insects or butterflies specifically.

Given that hibernal diapause in temperate environments is essential for surviving harsh winters, this life-history trait represents a key adaptation to survive in cold environments. Thus, on a macroevolutionary scale, we expected that the rate of the gain of diapause during the major glacial cycle – the Eocene-Oligocene glacial maximum (EOGM) which occurred ~35 MYA (Condamine et al., 2013; Zachos et al., 2001) – would be higher or at peak during or around this period. Such evidence, for example, was found in the Antarctic Notothenioid fishes where the timing of the evolution of anti-freeze glycoproteins roughly matches the EOGM event (Near et al., 2012) or in grasses (Pooidae) where gene-families involved in cold-tolerance showed expansion during this period (Sandve & Fjellheim, 2010). Hawkins & DeVries (2009) proposed such a hypothesis for butterflies that cold-related adaptations or lineages should become more apparent during or after the EOGM event, but this hypothesis remains untested. The age of butterflies in our tree dates back to ~108 million years, thus, if there is such a pattern, we would be able to discover it. Firstly, our ancestral state reconstructions across different diapause classifications and evolutionary models suggested that the evolution of diapause predates the EOGM event. For example, the evolution of pupal diapause was reconstructed with high probability at the base of the family Papilionidae which is ~68 MYA (Figure 4, Figure S4 & S5). There were also instances in the family Nymphalidae where the larval diapause was reconstructed with high probability at ~45 MYA. Furthermore, calculating the rate of transition through time did not reveal any obvious trend in the increased rate of gain of diapause during or around the EOGM event (Figure 5, Figure S7). Perhaps the lack of such a pattern is not entirely surprising as diapause has deep origins in insects and the physiological and genetic machinery required for entering diapause is likely already available. In other words, the evolution of diapause is not a de novo innovation in 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, checking the recently published database on European species (Middleton-Welling et al., 2020) suggests that most often the developmental stage at which the species enters diapause remains conserved at the genus level. Future studies using species-level sampling, for example, by focusing on a specific family, will provide coherent insights into the diapause evolution. However, these studies will still face the problem, especially if using species globally, as we still have meagre knowledge on diapause in tropical species (Denlinger, 1986). This brings us to the second caveat where we assigned most of the taxa from the tropics, unless for those high altitudes where clear information on diapause was available, to the flexible state. We acknowledge here that we have likely underestimated the cases of true diapause due to the extreme lack of information in tropical species except few sporadic studies (Braby, 1995; Halali et al., 2020, 2021). Third, there was strong bias in the tip frequencies in our dataset, that is, some states were either too frequent, or rarely represented, in the data. For example, the flexible state was dominant (but this is also because the diversity of butterflies is high in the tropics) and in temperate lineages, egg and adult states were too infrequent. This could affect the estimate of the number or rate transitions. We tried minimizing this bias by having different classifications (five-, three- and binary-state) derived by merging states in a biologically meaningful way which also considerably also decreases the model complexity. However, bias in the tip frequency is not a consequence of genus-level sampling but is a real pattern and this bias would still persist even if species-level data is used (see Figure S8 showing that most European species diapause in the larval stage).

Estimating ancestral states across millions of years is an exciting endeavor but general doubts on its accuracy, especially at deeper nodes and in the absence of fossil evidence, is a recurring theme in macroevolution (Cunningham, 1999; Cunningham et al., 1998; Omland, 1999). For example, striking discrepancies in the evolution of parity modes (ovo- vs viviparity) at the root of squamates between different methods particularly exemplifies these issues (see King & Lee, 2015; Pyron & Burbrink, 2014). Furthermore, assuming homogenous rate of evolution (which is assumed by standard Mk models) can affect ancestral reconstructions (King & Lee, 2015). We found that although in general there was high congruency in ancestral estimation between both homogenous and heterogenous (hidden rates) rate Mk models, there were often major conflicts at the deep nodes (Supplementary Figure S6) and when comparing estimates between root priors. We, thus, avoided drawing any conclusions, for example, on the likely ancestral state for all butterflies or even for the families. We also want to highlight one such perplexing pattern here. As discussed above, in some cases, especially for the Family Papilionidae, pupal diapause was consistently estimated at the base of this family going back to ~68 MYA. Paleoclimatic studies have suggested that the climate during early Eocene was warmer than today and hosted tropical forests in much of the Northern Hemisphere (Condamine et al., 2012; Morley, 2007). Thus, such deep origin of pupal diapause in our ancestral estimation is a bit puzzling. Perhaps including more taxa (representing both short and long branches) or including outgroups (however, the backbone of Lepidoptera still remains unresolved, see Rota et al., 2022) may help identifying ancestral diapause state of butterflies and more accurate estimates for each family.

After accounting for the caveats and deriving conservative conclusions, we still identified some major patterns in the evolution of diapause in butterflies. Mainly, that diapause has deep roots in butterflies, diapause at different developmental stages shows striking convergence and that the loss of diapause occurs at a much higher rate than its gain. Our study, thus, provides the macroevolutionary framework for future comparative and functional (genomic) studies.

CONCLUSIONS

The capacity to diapause is a key life-history adaptation for survival in harsh environments, especially in harsh winters in temperate regions. Here, using a large dataset on butterflies, we elucidate the tempo and mode of diapause evolution in an explicitly macroevolutionary framework. Broadly, using the genus-level data, we show that larval diapause is most common among temperate lineages. Furthermore, we show that loss of diapause occurs at a much faster rate than the gain and all diapause stages show extensive convergence across butterflies. Ancestral reconstructions at the base of most families remained uncertain, however, there was consistent support for a very deep evolutionary origin of diapause. Our analysis suggests diapause is a highly evolvable trait on a macroevolutionary scale and that mechanistic constraints to diapause in a particular life-stage appear easily overcome through evolution. Finally, we find no direct evidence for increased evolutionary rates in the gain of diapause during the Eocene-Oligoene glacial maximum around 35 MYA. We, thus, establish a macroevolutionary base for future studies on diapause evolution, especially for investigating the ultimate and proximate basis of diapause at different developmental stages. We also emphasize that studies on tropical species are desperately needed for elucidating the evolution of diapause in temperate lineages and using species-level data to advance our understanding of the evolution of this key life-history trait.

AUTHOR CONTRIBUTION

All authors contributed to the study design. Sören Nylin collected the diapause data, Sridhar Halali carried out the phylogenetic analyses with inputs from everyone, Etka Yapar wrote/modified R functions for summarizing uncertainty in ancestral estimation and for calculating rate through time, Sridhar Halali and Philipp Lehmann wrote the first draft of the manuscript and everyone contributed with refining the draft.

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REFERENCES

Allio, R., Scornavacca, C., Nabholz, B., Clamens, A.-L., Sperling, F. A., & Condamine, F. L. (2020). Whole Genome Shotgun Phylogenomics Resolves the Pattern and Timing of Swallowtail Butterfly Evolution. *Systematic Biology*, *69*(1), 38–60. https://doi.org/10.1093/sysbio/syz030

Batz, Z. A., Clemento, A. J., Fritzenwanker, J., Ring, T. J., Garza, J. C., & Armbruster, P. A. (2020). Rapid adaptive evolution of the diapause program during range expansion of an invasive mosquito. *Evolution*, *74*(7), 1451–1465. https://doi.org/10.1111/evo.14029

Beaulieu, J. M., O’Meara, B. C., & Donoghue, M. J. (2013). Identifying Hidden Rate Changes in the Evolution of a Binary Morphological Character: The Evolution of Plant Habit in Campanulid Angiosperms. *Systematic Biology*, *62*(5), 725–737. https://doi.org/10.1093/sysbio/syt034

Bollback, J. P. (2006). SIMMAP: Stochastic character mapping of discrete traits on phylogenies. *BMC Bioinformatics*, *7*(1), 88. https://doi.org/10.1186/1471-2105-7-88

Boyko, J. D., & Beaulieu, J. M. (2021). Generalized hidden Markov models for phylogenetic comparative datasets. *Methods in Ecology and Evolution*, *12*(3), 468–478. https://doi.org/10.1111/2041-210X.13534

Braby, M. F. (1995). Reproductive seasonality in tropical satyrine butterflies: Strategies for the dry season. *Ecological Entomology*, *20*(1), 5–17. https://doi.org/10.1111/j.1365-2311.1995.tb00423.x

Chazot, N., Condamine, F. L., Dudas, G., Peña, C., Kodandaramaiah, U., Matos-Maraví, P., Aduse-Poku, K., Elias, M., Warren, A. D., Lohman, D. J., Penz, C. M., DeVries, P., Fric, Z. F., Nylin, S., Müller, C., Kawahara, A. Y., Silva-Brandão, K. L., Lamas, G., Kleckova, I., … Wahlberg, N. (2021). Conserved ancestral tropical niche but different continental histories explain the latitudinal diversity gradient in brush-footed butterflies. *Nature Communications*, *12*(1), 5717. https://doi.org/10.1038/s41467-021-25906-8

Chazot, N., Wahlberg, N., Freitas, A. V. L., Mitter, C., Labandeira, C., Sohn, J.-C., Sahoo, R. K., Seraphim, N., de Jong, R., & Heikkilä, M. (2019). Priors and Posteriors in Bayesian Timing of Divergence Analyses: The Age of Butterflies Revisited. *Systematic Biology*, *68*(5), 797–813. https://doi.org/10.1093/sysbio/syz002

Condamine, F. L., Rolland, J., & Morlon, H. (2013). Macroevolutionary perspectives to environmental change. *Ecology Letters*, *16*, 72–85. https://doi.org/10.1111/ele.12062

Condamine, F. L., Sperling, F. A. H., Wahlberg, N., Rasplus, J.-Y., & Kergoat, G. J. (2012). What causes latitudinal gradients in species diversity? Evolutionary processes and ecological constraints on swallowtail biodiversity. *Ecology Letters*, *15*(3), 267–277. https://doi.org/10.1111/j.1461-0248.2011.01737.x

Cunningham, C. W. (1999). Some Limitations of Ancestral Character-State Reconstruction When Testing Evolutionary Hypotheses. *Systematic Biology*, *48*(3), 665–674. https://doi.org/10.1080/106351599260238

Cunningham, C. W., Omland, K. E., & Oakley, T. H. (1998). Reconstructing ancestral character states: A critical reappraisal. *Trends in Ecology & Evolution*, *13*(9), 361–366. https://doi.org/10.1016/S0169-5347(98)01382-2

de Kort, C. a. d. (1996). Cosmic influences on the expression of a specific gene in the Colorado potato beetle: The diapause protein 1 gene. *Archives of Insect Biochemistry and Physiology*, *32*(3–4), 567–573. https://doi.org/10.1002/(SICI)1520-6327(1996)32:3/4<567::AID-ARCH28>3.0.CO;2-9

Denlinger, D. L. (1986). Dormancy in Tropical Insects. *Annual Review of Entomology*, *31*(1), 239–264. https://doi.org/10.1146/annurev.en.31.010186.001323

Denlinger, D. L. (2022). *Insect Diapause* (1st ed.). Cambridge University Press. https://doi.org/10.1017/9781108609364

Denlinger, D. L. (2023). Insect diapause: From a rich history to an exciting future. *Journal of Experimental Biology*, *226*(4), jeb245329. https://doi.org/10.1242/jeb.245329

Diniz, D. F. A., De Albuquerque, C. M. R., Oliva, L. O., De Melo-Santos, M. A. V., & Ayres, C. F. J. (2017). Diapause and quiescence: Dormancy mechanisms that contribute to the geographical expansion of mosquitoes and their evolutionary success. *Parasites & Vectors*, *10*(1), 310. https://doi.org/10.1186/s13071-017-2235-0

Felsenstein, J. (1973). Maximum likelihood and minimum-steps methods for estimating evolutionary trees from data on discrete characters. *Systematic Zoology*. *22*(3), 240-249.

FitzJohn, R. G., Maddison, W. P., & Otto, S. P. (2009). Estimating Trait-Dependent Speciation and Extinction Rates from Incompletely Resolved Phylogenies. *Systematic Biology*, *58*(6), 595–611. https://doi.org/10.1093/sysbio/syp067

Friberg, M., Lehmann, P., & Wiklund, C. (2023). Autumn mass change and winter mass loss differ between migratory and nonmigratory butterflies. *Animal Behaviour*, *204*, 67–75. https://doi.org/10.1016/j.anbehav.2023.08.005

Fudickar, A. M., Jahn, A. E., & Ketterson, E. D. (2021). Animal Migration: An Overview of One of Nature's Great Spectacles. *Annual Review of Ecology, Evolution, and Systematics*, *52*(1), 479–497. https://doi.org/10.1146/annurev-ecolsys-012021-031035

Furness, A. I., Reznick, D. N., Springer, M. S., & Meredith, R. W. (2015). Convergent evolution of alternative developmental trajectories associated with diapause in African and South American killifish. *Proceedings of the Royal Society B: Biological Sciences*, *282*(1802), 20142189. https://doi.org/10.1098/rspb.2014.2189

Gotthard, K. (2004). Growth Strategies and Optimal Body Size in Temperate Pararginii Butterflies. *Integrative and Comparative Biology*, *44*(6), 471–479. https://doi.org/10.1093/icb/44.6.471

Halali, S., Brakefield, P. M., Collins, S. C., & Brattström, O. (2020). To mate, or not to mate: The evolution of reproductive diapause facilitates insect radiation into African savannahs in the Late Miocene. *Journal of Animal Ecology*, *89*(5), 1230–1241. https://doi.org/10.1111/1365-2656.13178

Halali, S., Halali, D., Barlow, H. S., Molleman, F., Kodandaramaiah, U., Brakefield, P. M., & Brattström, O. (2021). Predictability of temporal variation in climate and the evolution of seasonal polyphenism in tropical butterfly communities. *Journal of Evolutionary Biology*, *34*(9), 1362–1375. https://doi.org/10.1111/jeb.13895

Harmon, L. J. (2019). *Phylogenetic Comparative Methods*. *EcoEvoRxiv*. 234.

Harmon, L. J., Weir, J. T., Brock, C. D., Glor, R. E., & Challenger, W. (2008). GEIGER: investigating evolutionary radiations. *Bioinformatics*, *24*, 129–131. https://doi.org/10.1093/bioinformatics/btm538

Hawkins, B. A., & DeVries, P. J. (2009). Tropical niche conservatism and the species richness gradient of North American butterflies. *Journal of Biogeography*, *36*(9), 1698–1711. https://doi.org/10.1111/j.1365-2699.2009.02119.x

Hayes, J. L. (1982). A Study of the Relationships of Diapause Phenomena and Other Life History Characters in Temperate Butterflies. *The American Naturalist*, *120*(2), 160–170. https://doi.org/10.1086/283979

Huelsenbeck, J. P., Nielsen, R., & Bollback, J. P. (2003). Stochastic Mapping of Morphological Characters. *Systematic Biology*, *52*(2), 131–158. https://doi.org/10.1080/10635150390192780

Hughes, J. J., Berv, J. S., Chester, S. G. B., Sargis, E. J., & Field, D. J. (2021). Ecological selectivity and the evolution of mammalian substrate preference across the K–Pg boundary. *Ecology and Evolution*, *11*(21), 14540–14554. https://doi.org/10.1002/ece3.8114

Kawahara, A. Y., Storer, C., Carvalho, A. P. S., Plotkin, D. M., Condamine, F. L., Braga, M. P., Ellis, E. A., St Laurent, R. A., Li, X., Barve, V., Cai, L., Earl, C., Frandsen, P. B., Owens, H. L., Valencia-Montoya, W. A., Aduse-Poku, K., Toussaint, E. F. A., Dexter, K. M., Doleck, T., … Lohman, D. J. (2023). A global phylogeny of butterflies reveals their evolutionary history, ancestral hosts and biogeographic origins. *Nature Ecology & Evolution*, *7*(6), 903–913. https://doi.org/10.1038/s41559-023-02041-9

King, B., & Lee, M. S. Y. (2015). Ancestral State Reconstruction, Rate Heterogeneity, and the Evolution of Reptile Viviparity. *Systematic Biology*, *64*(3), 532–544. https://doi.org/10.1093/sysbio/syv005

Koštál, V. (2006). Eco-physiological phases of insect diapause. *Journal of Insect Physiology*, *52*(2), 113–127. https://doi.org/10.1016/j.jinsphys.2005.09.008

Koštál, V., Štětina, T., Poupardin, R., Korbelová, J., & Bruce, A. W. (2017). Conceptual framework of the eco-physiological phases of insect diapause development justified by transcriptomic profiling. *Proceedings of the National Academy of Sciences*, *114*(32), 8532–8537. https://doi.org/10.1073/pnas.1707281114

Lewis, P. O. (2001). A Likelihood Approach to Estimating Phylogeny from Discrete Morphological Character Data. *Systematic Biology*, *50*(6), 913–925. https://doi.org/10.1080/106351501753462876

Masaki, S. (1980). Summer Diapause. *Annual Review of Entomology*, *25*(1), 1–25. https://doi.org/10.1146/annurev.en.25.010180.000245

Middleton-Welling, J., Dapporto, L., García-Barros, E., Wiemers, M., Nowicki, P., Plazio, E., Bonelli, S., Zaccagno, M., Šašić, M., Liparova, J., Schweiger, O., Harpke, A., Musche, M., Settele, J., Schmucki, R., & Shreeve, T. (2020). A new comprehensive trait database of European and Maghreb butterflies, Papilionoidea. *Scientific Data*, *7*(1), 351. https://doi.org/10.1038/s41597-020-00697-7

Morley, R. J. (2007). Cretaceous and Tertiary climate change and the past distribution of megathermal rainforests. In: *Tropical Rainforest Responses to Climatic Changes* (ed. Bush, M.B. & Flenley, J.). Praxis Publishing, Chichester, pp. 1–31.

Near, T. J., Dornburg, A., Kuhn, K. L., Eastman, J. T., Pennington, J. N., Patarnello, T., Zane, L., Fernández, D. A., & Jones, C. D. (2012). Ancient climate change, antifreeze, and the evolutionary diversification of Antarctic fishes. *Proceedings of the National Academy of Sciences*, *109*(9), 3434–3439. https://doi.org/10.1073/pnas.1115169109

Nylin, S. (2013). Induction of diapause and seasonal morphs in butterflies and other insects: Knowns, unknowns and the challenge of integration. *Physiological Entomology*, *38*(2), 96–104. https://doi.org/10.1111/phen.12014

Nylin, S., Wickman, P.-O., & Wiklund, C. (1995). Life-cycle regulation and life history plasticity in the speckled wood butterfly: Are reaction norms predictable? *Biological Journal of the Linnean Society*, *55*(2), 143–157. https://doi.org/10.1111/j.1095-8312.1995.tb01056.x

Omland, K. E. (1999). The Assumptions and Challenges of Ancestral State Reconstructions. *Systematic Biology*, *48*(3), 604–611. https://doi.org/10.1080/106351599260175

Paradis, E., & Schliep, K. (2019). ape 5.0: An environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics*, *35*, 526–528. https://doi.org/10.1093/bioinformatics/bty633

Pennell, M. W., Eastman, J. M., Slater, G. J., Brown, J. W., Uyeda, J. C., Fitzjohn, R. G., Alfaro, M. E., & Harmon, L. J. (2014). geiger v2.0: An expanded suite of methods for fitting macroevolutionary models to phylogenetic trees. *Bioinformatics*, *30*, 2216–2218. https://doi.org/10.1093/bioinformatics/btu181

Pyron, R. A., & Burbrink, F. T. (2014). Early origin of viviparity and multiple reversions to oviparity in squamate reptiles. *Ecology Letters*, *17*(1), 13–21. https://doi.org/10.1111/ele.12168

R Core Team (2023). R: A Language and Environment for Statistical Computing_. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>

Revell, L. J. (2012). phytools: An R package for phylogenetic comparative biology (and other things). *Methods in Ecology and Evolution*, *3*(2), 217–223. https://doi.org/10.1111/j.2041-210X.2011.00169.x

Revell, L. J. (2013). Two new graphical methods for mapping trait evolution on phylogenies. *Methods in Ecology and Evolution*, *4*(8), 754–759. https://doi.org/10.1111/2041-210X.12066

Revell, L. J. (2024). phytools 2.0: An updated R ecosystem for phylogenetic comparative methods (and other things). *PeerJ*, *12*, e16505. https://doi.org/10.7717/peerj.16505

Revell, L. J., & Harmon, L. J. (2022). *Phylogenetic comparative methods in R*. Princeton University Press.

Rota, J., Twort, V., Chiocchio, A., Peña, C., Wheat, C. W., Kaila, L., & Wahlberg, N. (2022). The unresolved phylogenomic tree of butterflies and moths (Lepidoptera): Assessing the potential causes and consequences. *Systematic Entomology*, *47*(4), 531–550. https://doi.org/10.1111/syen.12545

Sandve, S. R., & Fjellheim, S. (2010). Did gene family expansions during the Eocene–Oligocene boundary climate cooling play a role in Pooideae adaptation to cool climates? *Molecular Ecology*, *19*(10), 2075–2088. https://doi.org/10.1111/j.1365-294X.2010.04629.x

Satterfield, D. A., Sillett, T. S., Chapman, J. W., Altizer, S., & Marra, P. P. (2020). Seasonal insect migrations: Massive, influential, and overlooked. *Frontiers in Ecology and the Environment*, *18*(6), 335–344. https://doi.org/10.1002/fee.2217

Schauberger, P., & Walker, A. (2023). *openxlsx: Read, Write and Edit xlsx Files*. https://CRAN.R-project.org/package=openxlsx

Schluter, D., Price, T., Mooers, A. Ø., & Ludwig, D. (1997). Likelihood of Ancestor States in Adaptive Radiation. *Evolution*, *51*(6), 1699–1711. https://doi.org/10.2307/2410994

Scott, J. A. (1981). Hibernal diapause of North American Papilionoidea and Hesperioidea. *The Journal of Research on the Lepidoptera*, *18*(3), 171–200. https://doi.org/10.5962/p.266881

Singh, P. P., Reeves, G. A., Contrepois, K., Ellenberger, M., Hu, C.-K., Snyder, M. P., & Brunet, A. (2021). *Evolution of diapause in the African turquoise killifish by remodeling ancient gene regulatory landscape* (p. 2021.10.25.465616). bioRxiv. https://doi.org/10.1101/2021.10.25.465616

Tauber, C. A., & Tauber, M. J. (1981). Insect Seasonal Cycles: Genetics and Evolution. *Annual Review of Ecology and Systematics*, *12*(1), 281–308. https://doi.org/10.1146/annurev.es.12.110181.001433

Tauber, M., Tauber, C., & Masaki, S. (1986). *Seasonal adaptations of insects*. Oxford University Press, USA

Teder, T. (2020). Phenological responses to climate warming in temperate moths and butterflies: Species traits predict future changes in voltinism. *Oikos*, *129*(7), 1051–1060. https://doi.org/10.1111/oik.07119

Tougeron, K. (2019). Diapause research in insects: Historical review and recent work perspectives. *Entomologia Experimentalis et Applicata*, *167*(1), 27–36. https://doi.org/10.1111/eea.12753

Van Nieukerken, E. J., Kaila, L., Kitching, I. J., Kristensen, N. P., Lees, D. C., Minet, J., Mitter, C., Mutanen, M., Regier, J. C., Simonsen, T. J., Wahlberg, N., Yen, S.-H., Zahiri, R., Adamski, D., Baixeras, J., Bartsch, D., Bengtsson, B. Å., Brown, J. W., Bucheli, S. R., … Zwick, A. (2011). Order Lepidoptera Linnaeus, 1758. In: Zhang, Z.-Q. (Ed.) Animal biodiversity: An outline of higher-level classification and survey of taxonomic richness. *Zootaxa*, *3148*(1). https://doi.org/10.11646/zootaxa.3148.1.41

Varpe, Ø. (2017). Life History Adaptations to Seasonality. *Integrative and Comparative Biology*, *57*(5), 943–960. https://doi.org/10.1093/icb/icx123

Wahlberg, N., Wheat, C. W., & Peña, C. (2013). Timing and Patterns in the Taxonomic Diversification of Lepidoptera (Butterflies and Moths). *PLoS ONE*, *8*(11), e80875. https://doi.org/10.1371/journal.pone.0080875

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York. https://ggplot2.tidyverse.org

Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L. D., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J., Kuhn, M., Pedersen, T. L., Miller, E., Bache, S. M., Müller, K., Ooms, J., Robinson, D., Seidel, D. P., Spinu, V., … Yutani, H. (2019). Welcome to the tidyverse. *Journal of Open Source Software*, *4*(43), 1686. https://doi.org/10.21105/joss.01686

Williams, C. M., Ragland, G. J., Betini, G., Buckley, L. B., Cheviron, Z. A., Donohue, K., Hereford, J., Humphries, M. M., Lisovski, S., Marshall, K. E., Schmidt, P. S., Sheldon, K. S., Varpe, Ø., & Visser, M. E. (2017). Understanding Evolutionary Impacts of Seasonality: An Introduction to the Symposium. *Integrative and Comparative Biology*, *57*(5), 921–933. https://doi.org/10.1093/icb/icx122

Wilsterman, K., Ballinger, M. A., & Williams, C. M. (2021). A unifying, eco-physiological framework for animal dormancy. *Functional Ecology*, *35*(1), 11–31. https://doi.org/10.1111/1365-2435.13718

Zachos, J., Pagani, M., Sloan, L., Thomas, E., & Billups, K. (2001). Trends, Rhythms, and Aberrations in Global Climate 65 Ma to Present. *Science*, *292*(5517), 686–693. https://doi.org/10.1126/science.1059412

Figures

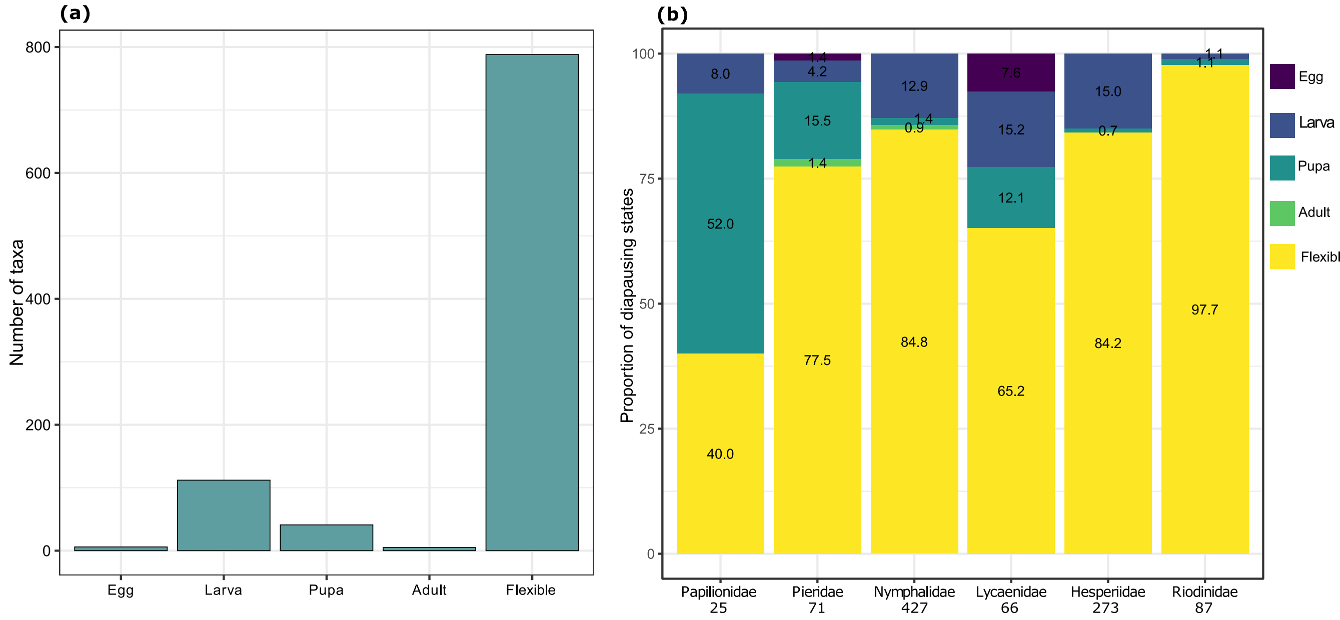

Figure 1: Number of butterfly taxa (A) and relative proportion of each diapause state (B) for five state classification. In Figure (B), the numbers below the family names on the x-axis indicates the number of taxa in each family. Note that the family Hedylidae is not shown as it was represented by only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

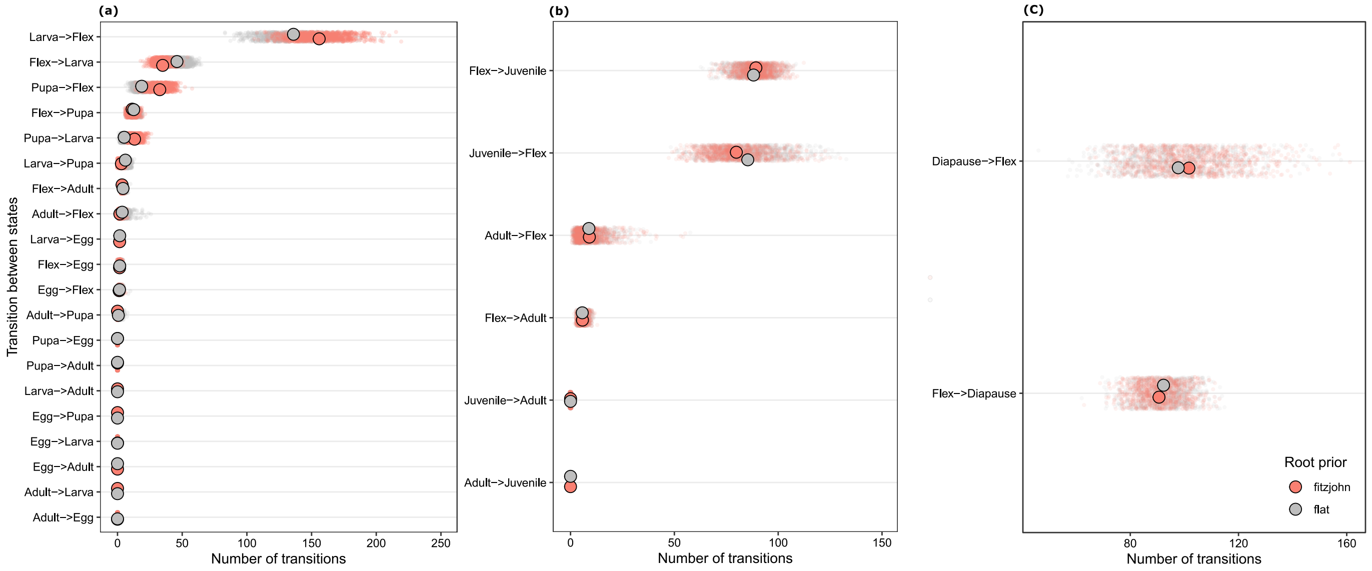

Figure 2: Number of transitions between states for five, three, and binary diapause classification for fitzjohn (pink) and flat (grey) root prior. Note that 'flexible' state is abbreviated as 'flex'. Points in the background indicate number of transitions across 1000 stochastic maps and the larger points indicate mean number of transitions for a particular transition. Note that the points have been slightly jittered to avoid complete overlap between data points.

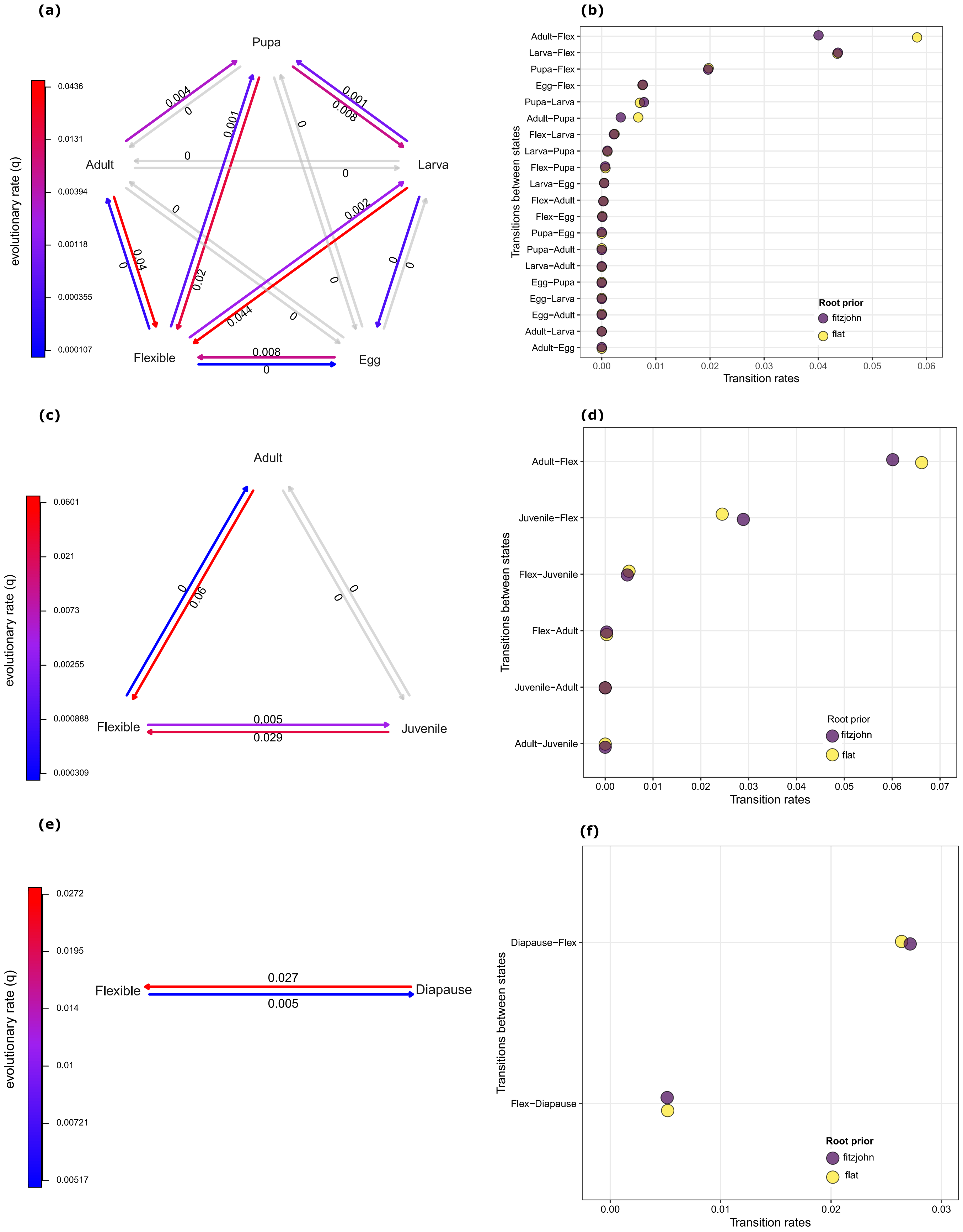

Figure 3: Transition rates across five, three and binary state classification using fitzjohn root prior (left panel). Transition rates for both fitzjohn (violet) and flat (yellow) root prior for comparison between two root priors. Note that ‘flexible’ state is abbreviated as ‘flex’ in the right figure panel.

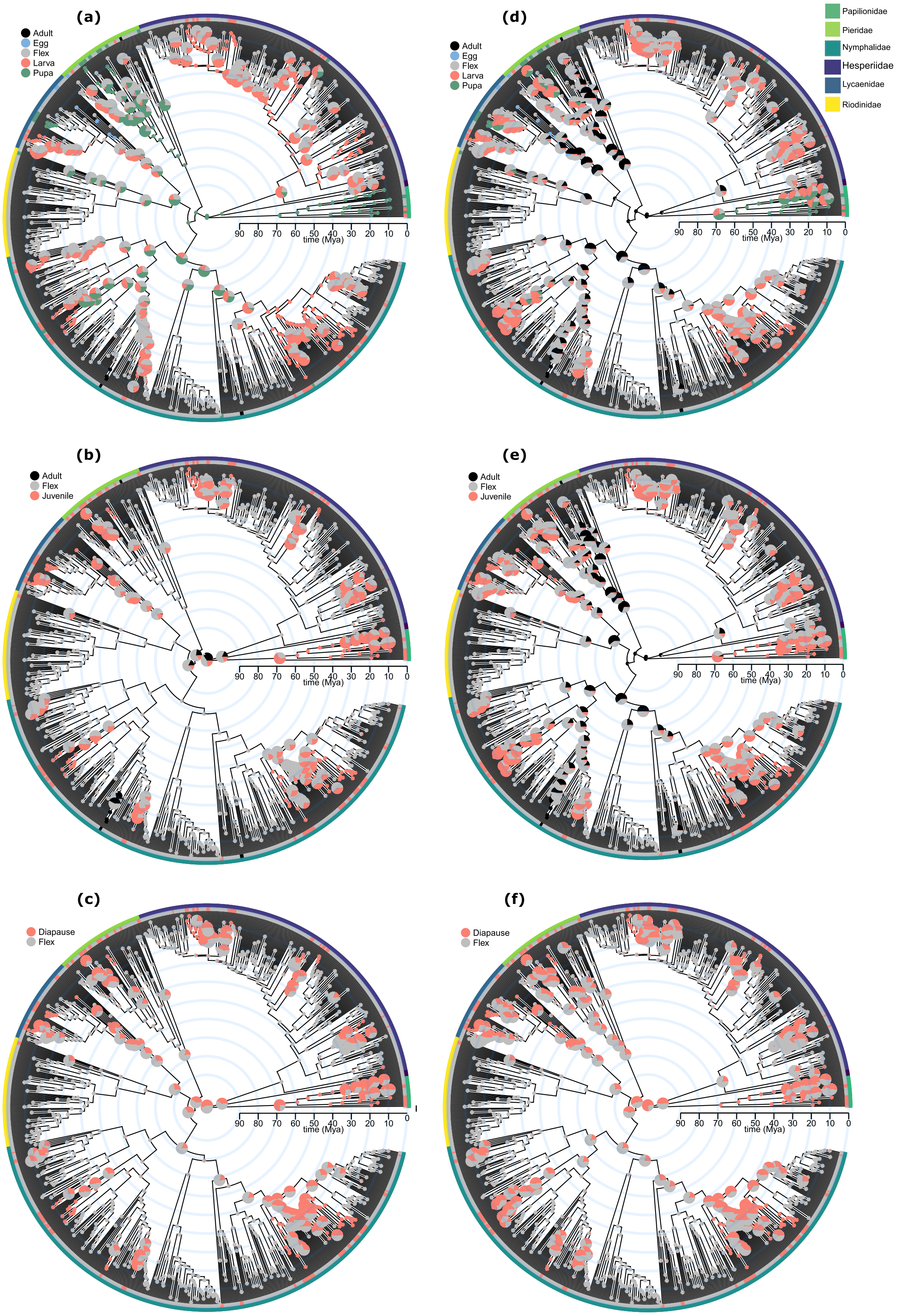

Figure 4: Marginal ancestral state estimation using stochastic mapping (left panel) and hidden rates model (right panel) for all diapause classifications using fitzjohn root prior. Ancestral estimations using maximum likelihood for the fitzjohn root prior and all three methods (maximum likelihood, stochastic mapping, hidden rates model) for flat prior are provided in supplementary files. The inner concentric ring at rim of phylogeny indicates tip state for each taxon and the outer ring indicates family (see top left figure for family demarcation). Note that pies having >0.85 probability for a state a occur at the nodes are reduced in size.

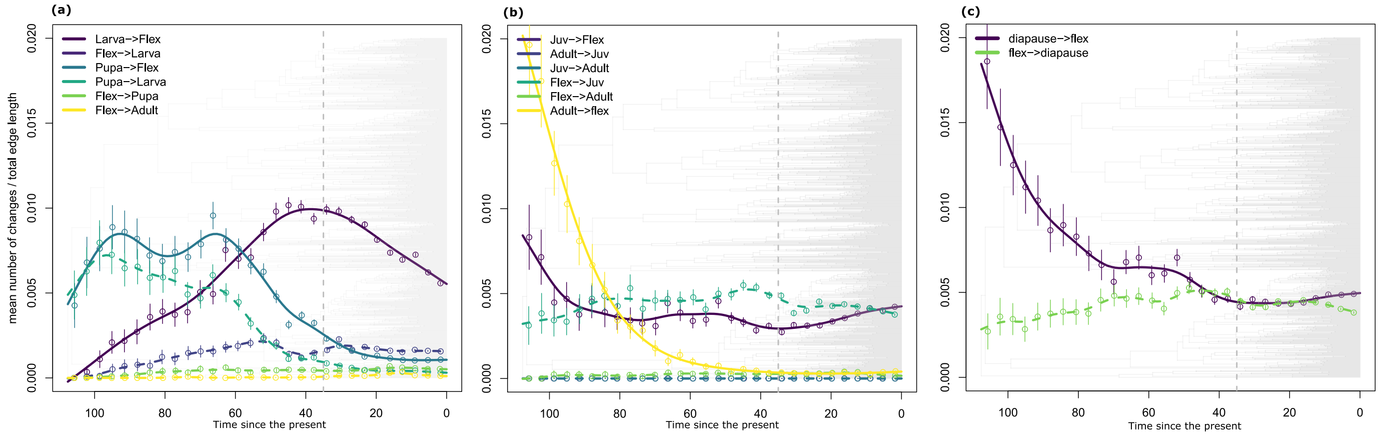

Figure 5: Rate through time plots for five, three and binary state classifications for fitzjohn root prior using stochastic maps. Each point (95% CI) represents average number of transitions in a 3.58-million-year time block (see Methods) and the smoothed line (spline) estimated from these points. Gain 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 five state classification, rates for only the most frequent transitions are shown as most transitions were estimated to be zero. Rate through time plots for stochastic maps using flat prior are provided in the supplementary file.

Supplementary Information

Table S1: Comparing diapause states across two recently published databases. Note that this is only a preliminary exploration. Abbreviations for diapause states as follows: A=adult, E=egg, L=larva, P=Pupa. Combination of multiple diapause states means that the species can diapause into different states according to both databases.

Taxon	*LepTraits (Shirey et al. 2022)*	*European trait database (Middleton-Welling et al. 2019)*	*Match*
Carcharodus_alceae	L	L	Yes
Carcharodus_floccifera	L	L	Yes
Carcharodus_lavatherae	L	L	Yes
Carterocephalus_palaemon	L	L	Yes
Carterocephalus_silvicola	L	L	Yes
Erynnis_tages	L	L	Yes
Heteropterus_morpheus	L	L	Yes
Pyrgus_andromedae	L	L	Yes
Pyrgus_carthami	L	L	Yes
Pyrgus_centaureae	L	L	Yes
Pyrgus_malvae	P	P	Yes
Pyrgus_malvoides	P	P	Yes
Pyrgus_serratulae	L	L	Yes
Pyrgus_sidae	L	L	Yes
Spialia_orbifer	L	L	Yes
Thymelicus_acteon	L	L	Yes
Thymelicus_lineola	LLEE	E	No
Thymelicus_sylvestris	L	L	Yes
Agriades_optilete	L	L	Yes
Celastrina_argiolus	P	P	Yes
Cupido_argiades	L	L	Yes
Kretania_pylaon	L	L	Yes
Lycaena_dispar	L	L	Yes
Lycaena_phlaeas	PLE	L	No
Phengaris_teleius	L	L	Yes
Plebejus_argus	E	E	Yes
Plebejus_idas	E	E	Yes
Polyommatus_amandus	L	L	Yes
Polyommatus_eros	E	L	No
Satyrium_pruni	E	E	Yes
Satyrium_w-album	E	E	Yes
Scolitantides_orion	PL	P	No
Thecla_betulae	E	E	Yes
Tongeia_fischeri	L	L	Yes
Aglais_io	LA	A	No
Aglais_urticae	LA	A	No

Apatura_ilia	L	L	Yes
Apatura_iris	L	L	Yes
Apatura_metis	L	L	Yes
Aphantopus_hyperantus	LE	L	No
Araschnia_levana	P	P	Yes
Argynnis_laodice	LE	L	No
Argynnis_pandora	L	L	Yes
Boloria_eunomia	L	L	Yes
Boloria_freija	L	L	Yes
Boloria_frigga	L	L	Yes
Boloria_improba	L	L	Yes
Boloria_napaea	L	L	Yes
Boloria_oscarus	L	L	Yes
Chazara_persephone	A	L	No
Coenonympha_amaryllis	L	L	Yes
Coenonympha_hero	L	L	Yes
Coenonympha_oedippus	L	L	Yes
Coenonympha_tullia	L	L	Yes
Erebia_cyclopius	L	L	Yes
Erebia_disa	L	L	Yes
Erebia_discoidalis	L	L	Yes
Erebia_medusa	L	L	Yes
Euphydryas_aurinia	L	L	Yes
Limenitis_camilla	L	L	Yes
Limenitis_populi	L	L	Yes
Lopinga_achine	L	L	Yes
Maniola_nurag	A	L	No
Melitaea_britomartis	L	L	Yes
Minois_dryas	L	L	Yes
Neptis_rivularis	L	L	Yes
Neptis_sappho	L	L	Yes
Nymphalis_antiopa	LA	A	No
Nymphalis_xanthomelas	LA	A	No
Oeneis_bore	L	L	Yes
Oeneis_jutta	L	L	Yes
Oeneis_polixenes	L	L	Yes
Polygonia_c-album	LA	A	No
Vanessa_atalanta	PPLAAA	A	No
Vanessa_cardui	LA	A	No
Vanessa_virginiensis	A	L	No
Archon_apollinus	P	P	Yes
Iphiclides_podalirius	P	P	Yes
Papilio_alexanor	P	P	Yes
Papilio_hospiton	P	P	Yes
Papilio_machaon	P	P	Yes
Papilio_saharae	P	P	Yes

Parnassius_mnemosyne	LLEE	E	No
Zerynthia_polyxena	P	P	Yes
Zerynthia_rumina	P	P	Yes
Anthocharis_cardamines	P	P	Yes
Aporia_crataegi	PLL	L	No
Colias_tyche	PL	L	No
Colias_tyche	L	L	Yes
Euchloe_belemia	P	P	Yes
Gonepteryx_cleopatra	P	A	No
Gonepteryx_rhamni	A	A	Yes
Leptidea_morsei	PE	P	No
Leptidea_sinapis	P	P	Yes
Pieris_brassicae	P	P	Yes
Pieris_napi	P	P	Yes
Pieris_rapae	PPL	P	No
Pontia_daplidice	P	P	Yes
Pontia_glauconome	P	P	Yes
Hamearis_lucina	P	P	Yes

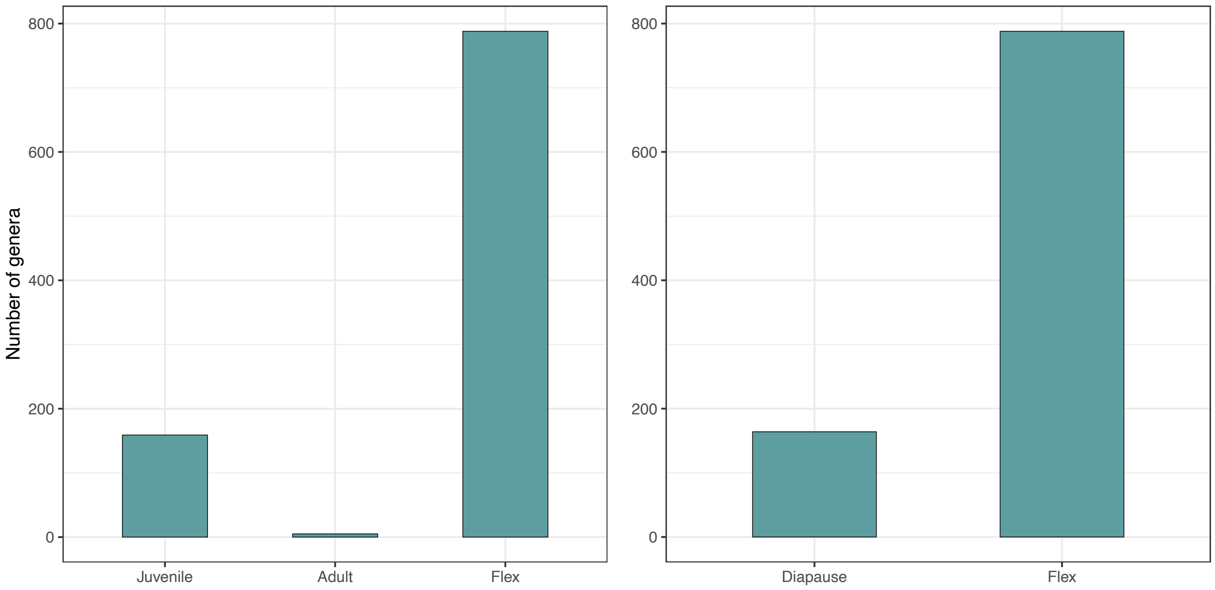

Figure S1: Number of butterfly taxa for three (left) and binary (right) diapause classifications.

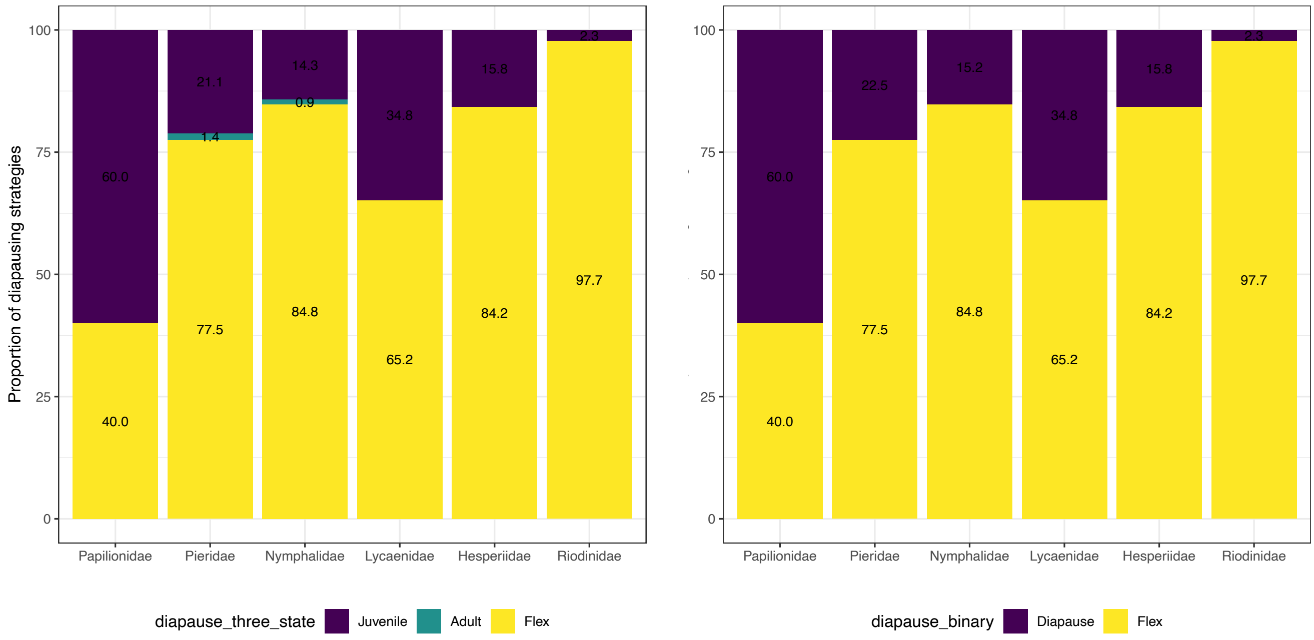

Figure S2: Relative proportion of each diapause state for three (left) and binary (right) diapause classifications. Note that the family Hedylidae is not shown as it was represented by only two taxa in our tree.

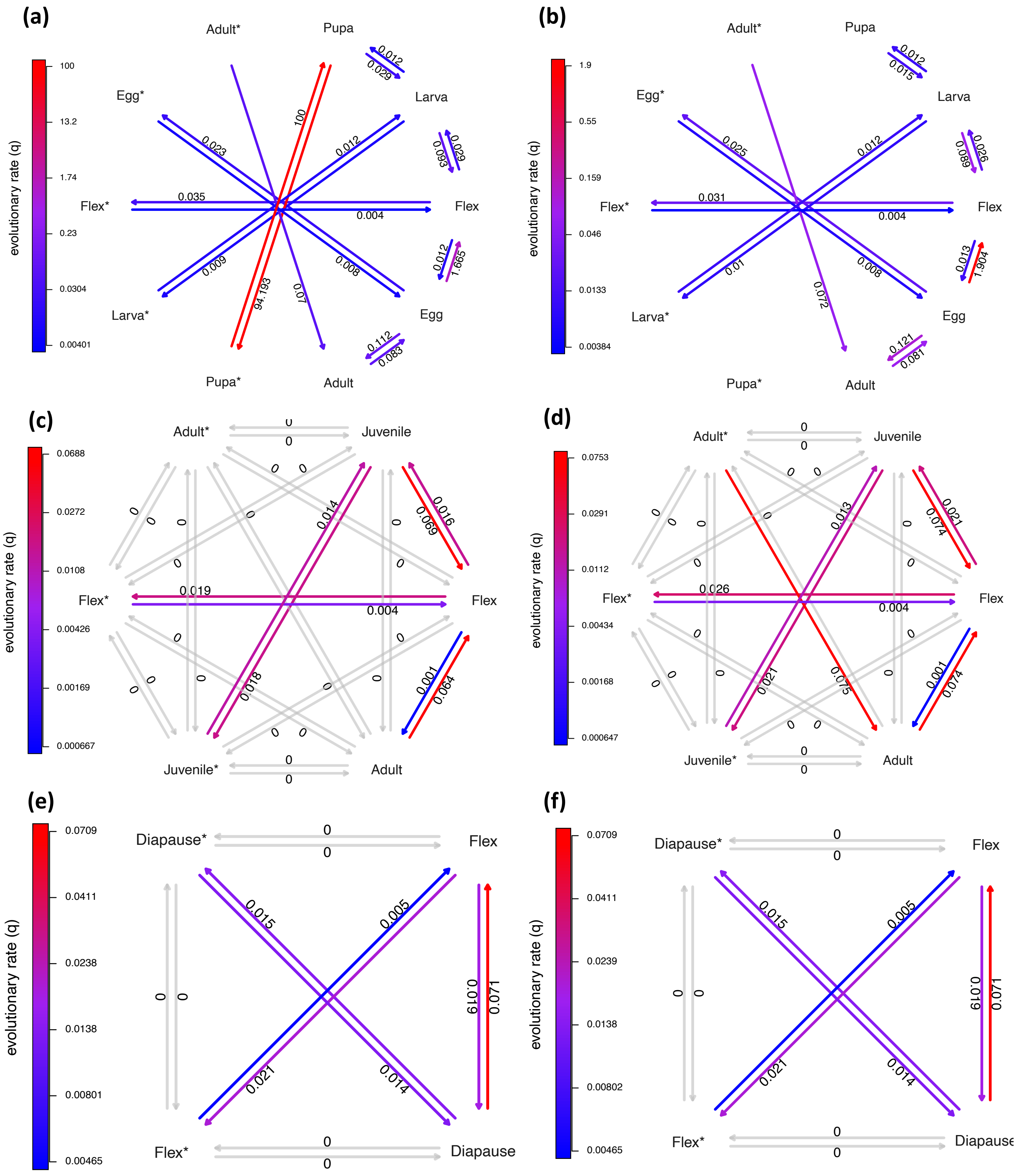

Figure S3: Transition rates between the states for five (a, b), three (c, d) and binary (e, f) diapause classification for flat (left panel) and fitzjohn (right panel) for the 'umbral' hidden rates model (see Methods). Note that transitions having zero rates are not shown for five state classification for clarity.

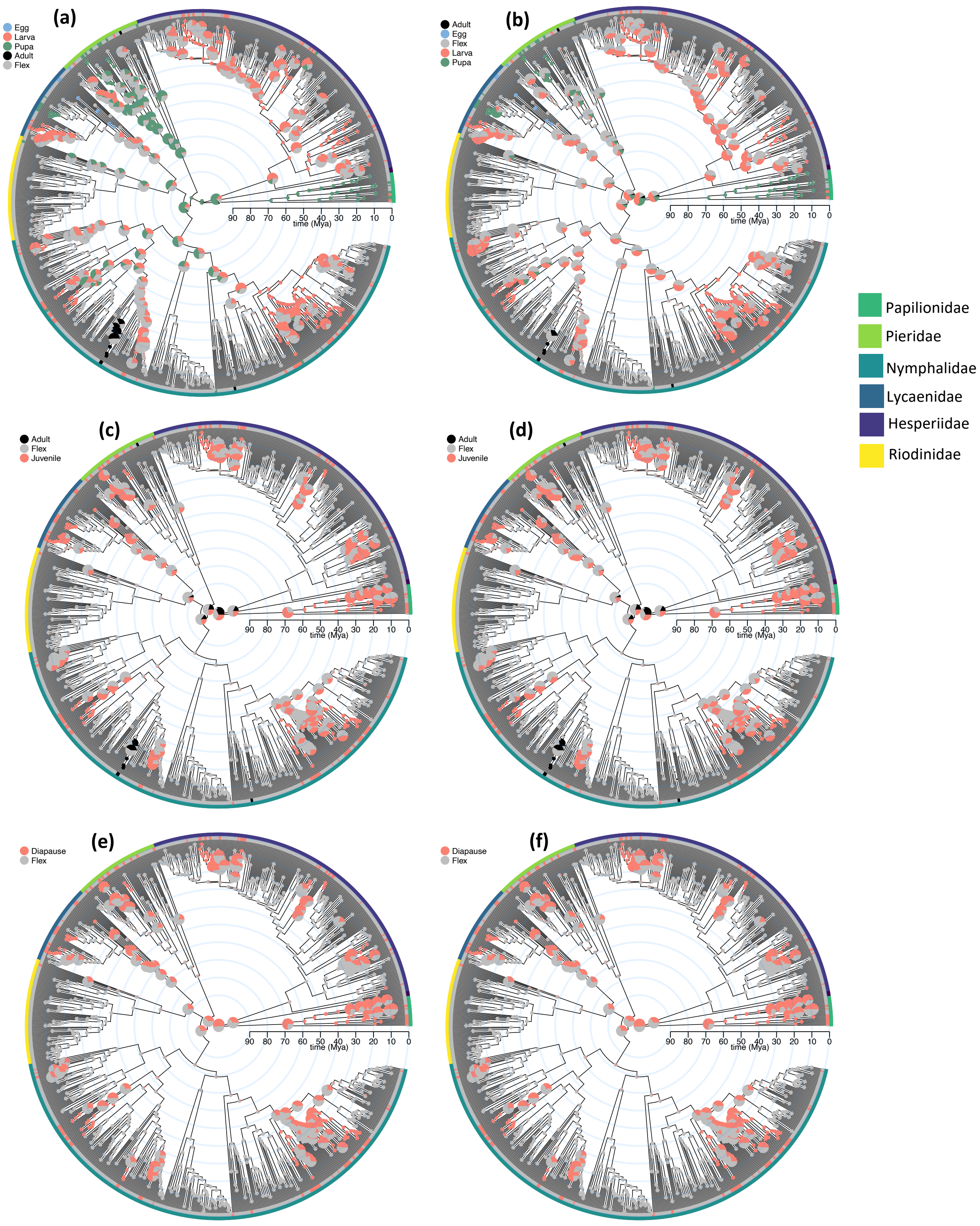

Figure S4: Marginal ancestral state estimation using maximum likelihood (left panel) and stochastic mapping (right panel) for five (a, b), three (c, d) and binary (e, f) diapause classifications using flat root prior. The inner concentric ring at rim of phylogeny indicates tip state for each taxon and the outer ring indicates family (see legend on the right). Note that pies having >0.85 probability for a state to occur at the nodes are reduced in size.

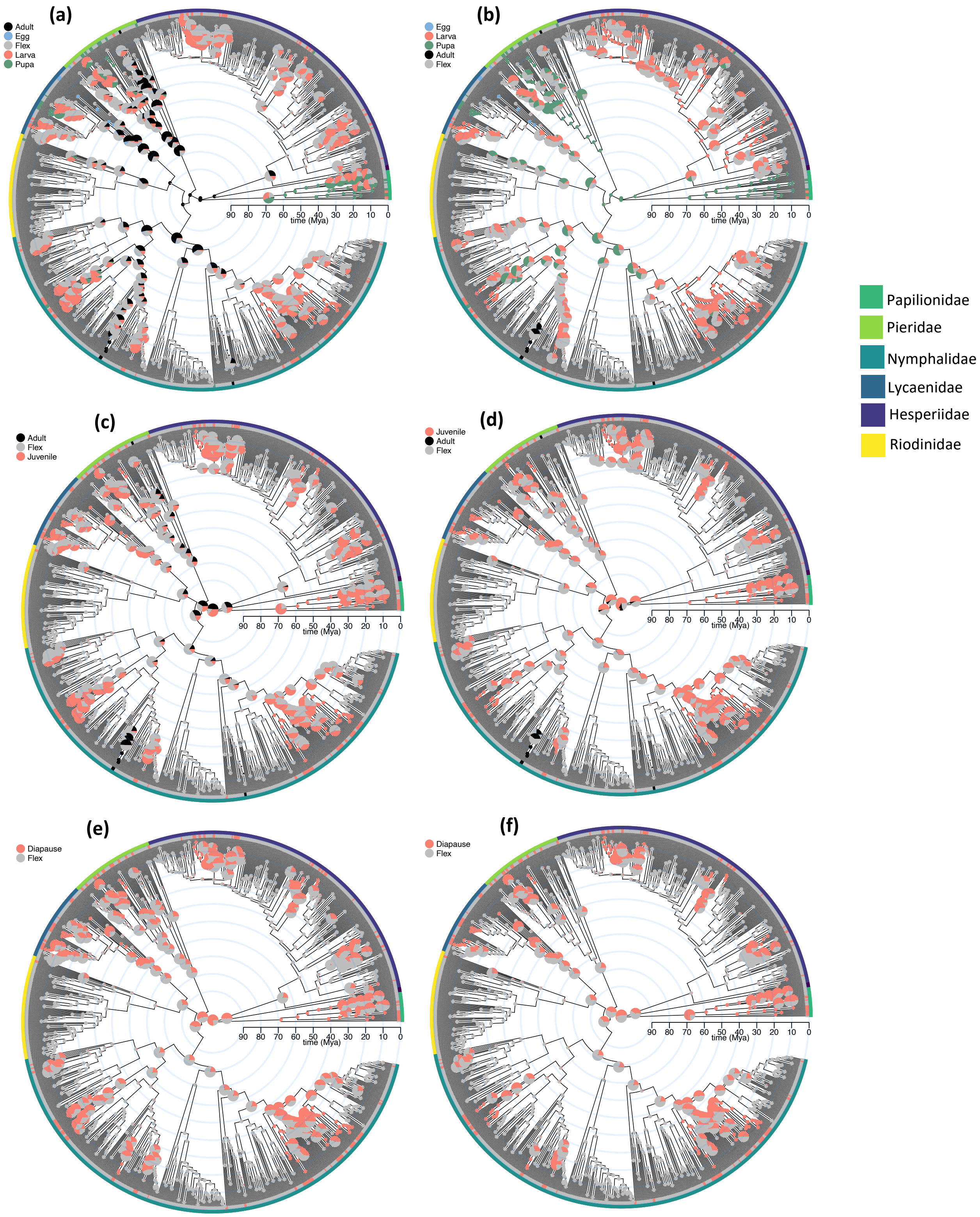

Figure S5: Marginal ancestral state estimation using hidden rates model for the flat root prior (left panel) and maximum likelihood using fitzjohn root prior (right panel) for five (a, b), three (c, d) and binary (e, f) diapause classifications. The inner concentric ring at rim of phylogeny indicates tip state for each taxon and the outer ring indicates family (see legend on the right). Note that pies having >0.85 probability for a state to occur at the nodes are reduced in size.

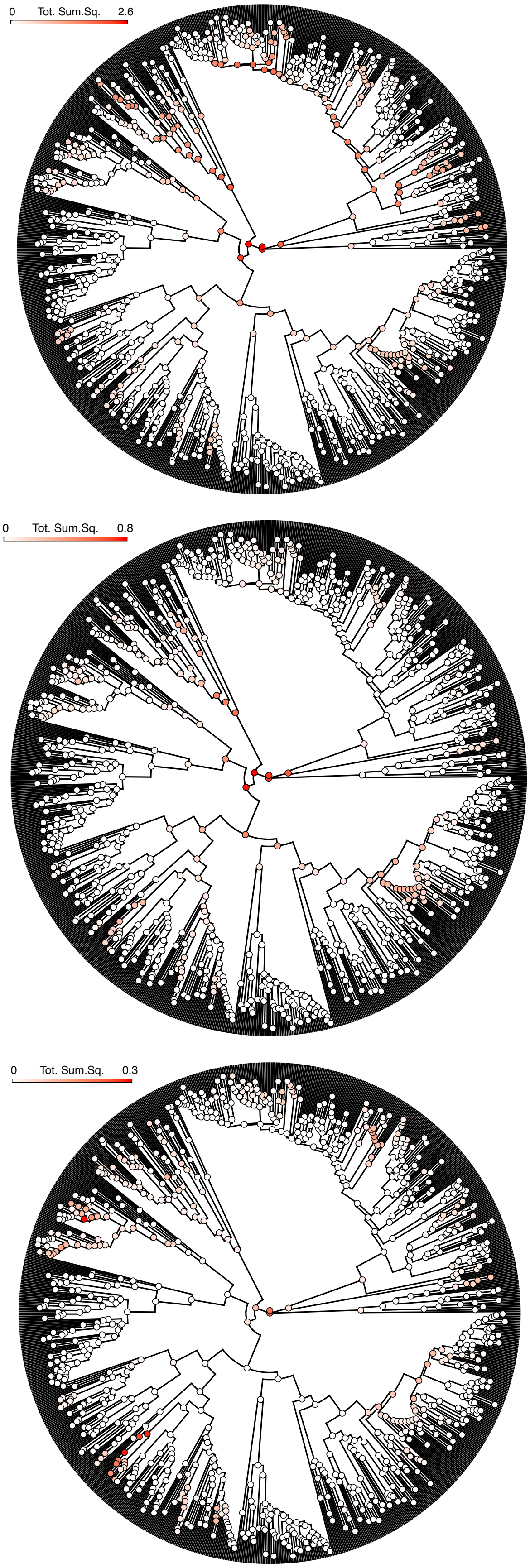

Figure S6: Depicting uncertainties (as total sum of squares) in the ancestral state estimation across six different reconstructions (maximum likelihood, stochastic mapping and hidden rates model each for flat and fitzjohn root prior) for five (top), three (middle) and binary (bottom) state diapause classification.

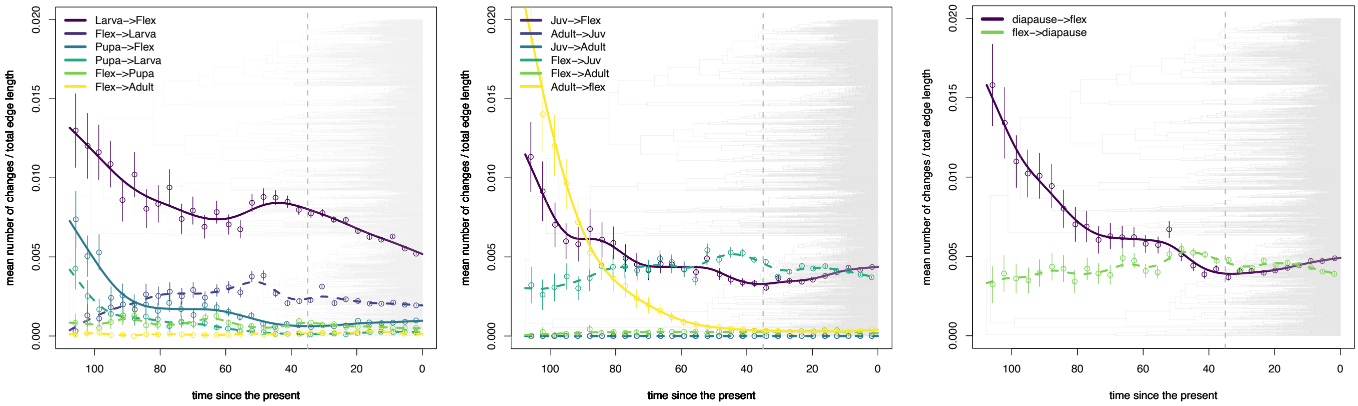

Figure S7: Rate through time plots for five, three and binary state classifications for flat root prior using stochastic maps. Each point (95% CI) represents average number of transitions in a 3.58-million-year time block (see Methods) and the smoothed line (spline) estimated from these points. Gain 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 five state classification, rates for only the most frequent transitions are shown as most transitions were estimated to be zero.

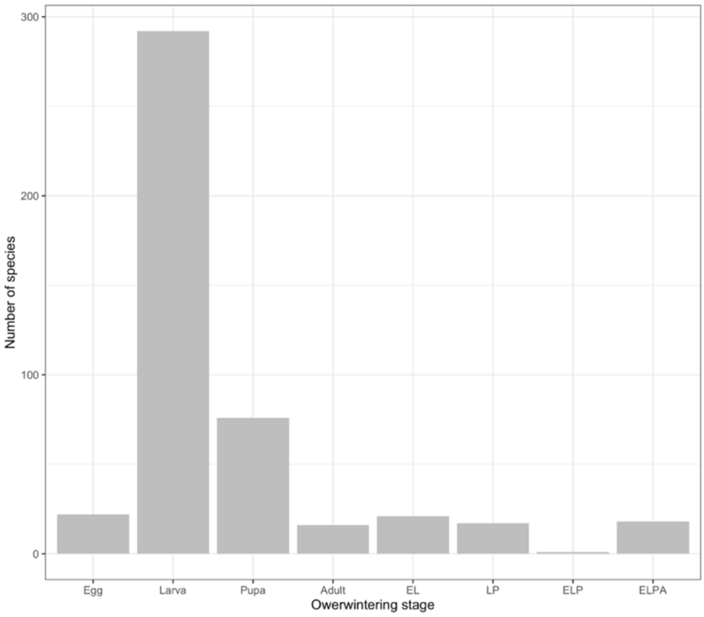

Figure S8: Frequency of diapause states in European butterflies based on the trait database of Middleton-Welling et al. (2020). The abbreviations are as follows: EL = egg, larva; LP= larva, pupa; ELP = egg, larva, pupa; ELPA= egg, larva, pupa, adult. The abbreviation states suggest that species can likely diapause in different developmental stages.