Allometry and shared ancestry, rather than ecology, shapes the evolution of 3D eye size in temperate butterflies

Sridhar Halali[1*], Stephen A. Hall[2], Lars B. Pettersson[1], Romain Carrié[3,4], Paul Caplat[5], Emily Baird[6,7], Niklas Wahlberg[1,7]

[1] Biodiversity & Evolution Unit, Dept. of Biology, Lund University, Sweden
[2] Division of Solid Mechanics, Lund University, Lund, Sweden
[3] Centre for Environmental and Climate Science, Lund University, Lund, Sweden
[4] Dynafor, National Research Institute for Agriculture, Food and Environment, INRAE, Toulouse University, Toulouse, France
[5] School of Biological Sciences, Queen's University Belfast, Belfast, UK
[6] Department of Zoology, Stockholm University, Stockholm, Sweden
[7] Contributed equally
[*] Corresponding author (sridharhalali@gmail.com)

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ABSTRACT

Sensory traits shape animal lifestyles due to the central role they play in retrieving and processing environmental information. However, being some of the most energetically expensive tissues to build and maintain, ecological demands often modulate investment in these organs. Evidence that ecology shapes the evolution of sensory traits is plenty, but is heavily biased towards vertebrates and has only recently begun to emerge in invertebrates. Here, we elucidate the macroevolution of a key sensory organ – eye size – using temperate butterflies as models. Using micro-CT X-ray imaging of pinned museum specimens, we quantified the eye size of 444 individuals comprising 59 species. Further, using 12 years of long-term monitoring data to quantify species habitat preferences, we tested the hypothesis that forest-associated species, likely experiencing dimmer light conditions, should have larger eyes than those from open habitats. Our comparative analyses revealed tight allometric scaling between eye and wing size, and phylogeny alone explained 74% of eye size variation, with low heterogeneity in the evolutionary rates. Further, we found that habitat structure and a behavioural trait, male mate location strategy, had no association with eye size. Overall, allometry and shared ancestry, not ecology, shape the macroevolution of eye size in temperate butterflies, and demonstrate how non-invasive microCT imaging can be used on pinned museum specimens for studying phenotypic evolution.

INTRODUCTION

By retrieving and processing environmental information, sensory organs are essential for guiding animal behaviour playing an important role in shaping animal lifestyles. However, sensory organs are among the most energetically expensive tissues to build and maintain (Niven & Laughlin, 2008). As such, the degree to which an animal invests in them is expected to be under strong selection and modulated according to specific ecological demands (Laughlin et al., 1998; Niven & Laughlin, 2008). There is plenty of evidence that ecological factors drive the evolution of sensory traits, at both micro- and macroevolutionary scales and across animals, in line with adaptive explanations. For example, nocturnal Lepidoptera have larger antennae than diurnal species (Lin et al., 2025), many fishes living in caves have completely lost their eyes (Krishnan & Rohner, 2017), and birds living in forests have larger eyes than those from open habitats (Ausprey et al., 2021). However, our knowledge of the macroevolution of sensory traits (e.g. eye size) is heavily based on vertebrates, with only a few recent studies emerging on invertebrates (e.g. Chong et al., 2024; Keesey et al., 2019; Scales & Butler, 2016; Wainwright et al., 2023).

Several factors can complicate studying sensory traits, especially on a macroevolutionary scale: (1) sensory traits generally show tight allometric scaling with body size (e.g. Ausprey, 2021; Chong et al., 2024) and such scaling could act as a constraint; (2) sensory traits (e.g. eye size) tend to have a strong phylogenetic signal (e.g. Ausprey, 2021), suggesting that shared ancestry could shape the evolutionary trajectory; (3) several studies do not find evidence for ecological factors shaping investment in sensory traits across animals (e.g. Chen et al., 2021). Thus, disentangling the relative roles of allometry, shared ancestry, and ecological factors, is crucial to fully understand the evolution of sensory traits across species, a goal that can be achieved using a macroevolutionary framework.

The eye is a primary external sensory organ that is paramount for shaping most basic activities in visually guided animals. In both invertebrates and vertebrates, the light environment acts as a major selective force in determining eye size, anatomy, optics, and underlying molecular machinery (Caves et al., 2017; Sondhi et al., 2021; Warrant & Johnsen, 2013). For example, diurnal and nocturnal insects tend to show anatomically distinct apposition and superposition eyes, respectively, adapted for markedly different light environments (Land & Fernald, 1992; Land & Nilsson, 2012). While the light environment during the day and night is dramatically different, relatively subtle differences in the amount of light between habitats can affect eye size and optics in both invertebrates and vertebrates (e.g. Ausprey et al., 2021; Scales & Butler, 2016; Wainwright et al., 2023, 2024). Although the anatomy of the insect compound eye is fundamentally different from camera-type eyes of vertebrates (see Land & Nilsson, 2012), the general principle of how selection operates on the eye surface area or the cornea is similar – the larger the eye surface area, the more light will be captured increasing sensitivity of the eye (Rutowski et al., 2009). However, our understanding of, if, and how, light habitat drives the evolution of insect eye size on a large macroevolution scale remains unclear.

Here, we elucidate the macroevolution of eye size using temperate butterflies as a model system. By generating 3D images of pinned museum specimens using micro-computed X-ray tomography (micro-CT), we quantified the 3D eye surface area of 444 individuals comprising 59 European butterflies across major families spanning the evolutionary history of ~100 million years. Using micro-CT is a major advance compared to previous studies on the evolution of sensory traits in insects, as: (1) few studies have used a macroevolutionary framework and many of those are restricted to a small taxonomic group (e.g. Keesey et al., 2019); and (2) previous studies have mainly extrapolated eye surface area from linear measurements (e.g. Rutowski, 2000) and corneal spreads (Wainwright et al., 2023; Wright et al., 2024), which involves destructive specimen preperations. Next, using phylogenetic comparative methods, we investigate the allometry and tempo of eye size evolution. We further use 12 years of butterfly monitoring data from Sweden to quantify species habitat preference and test a hypothesis that forest-linked species, that would experience dimmer light conditions, should have relatively larger eyes (Ausprey et al., 2021; Wainwright et al., 2023). We also test if a behavioural trait (male mate locating strategy) affects eye size (Rutowski, 2000). Overall, we provide first-hand insights into the macroevolutionary dynamics of eye size using temperate butterflies as models and demonstrate how high-throughput non-invasive X-ray micro-CT technology can be used on pinned museum specimens for studying phenotypic evolution.

MATERIALS & METHODS

X-ray microCT scanning of museum specimens

We used pinned butterfly specimens from the Biological Museum at Lund University for micro-CT scanning without any pre-processing of the samples (i.e. no staining with heavy metals that is usually done for fresh samples and leaving specimens on their pins). The micro-CT scanning was carried out using an RX Solutions EasyTom150 (Chavanod, France) at the 4D Imaging Lab at Lund University (see Supplementary Information for more details on imaging). We aimed to scan four males and four females of each species, but this was not always possible (see Table S1 for sample size). After excluding bad quality scans, we included 444 scans comprising 59 European butterflies for measuring eye surface area.

Measuring the eye surface area and wing traits

The 3D reconstructions, comprised of the 16-bit greyscale image stacks, were used for measuring the surface area of one of the eyes (Fig.1, Fig.S1). Knowing whether the right or left eye was measured is difficult as the order of image stacks can flip, thus, affecting the symmetry. To check for any potential bias this may have caused, we measured the surface area (henceforth eye size) of both eyes on a subset of 20 randomly selected scans and found them to be highly similar (R^2=0.99). We analysed all 3D scans in the open-source software 3D Slicer (Fedorov et al., 2012; Kikinis et al., 2013), https://www.slicer.org/) using additional functionalities from the *SlicerMorph* plugin (Rolfe et al., 2021). Additionally, we took 2D photographs of the same individuals and, using a custom macro in the Fiji software (Schindelin et al., 2015), measured forewing length and proxy forewing area (Fig. S2). Both wing traits were highly correlated in our dataset (R^2=0.98, Fig. S3). We note that studies on butterfly eye size use different body size proxies including hindleg femur/tibia length (Rutowski, 2000; Wright et al., 2024), inter-ocular distance (Wainwright et al., 2023), and thorax size (Moradinour et al., 2021). We use forewing length as this trait is often used as a body size proxy in butterflies (Swanson et al., 2016) and can be easily measured, especially when working with museum specimens. Details on processing 3D and 2D images are provided in the Supplementary Information.

Repeatability of trait measurements

Since we manually measured both eye size (e.g. by manually placing a curve which demarcates eye boundary) and wing traits (by manually placing the landmarks) and by two individuals (see Supplementary Information), we measured repeatability for both traits as R^2 values from the regressions. For eye size, we re-measured a subset of 20 randomly selected 3D images for: (1) intra-individual repeatability – original eye size vs. measurements carried out after 2-3 weeks; (2) inter-individual repeatability; (3) across software repeatability by measuring eye size in another popular 3D image analyses software Amira (Thermo Fisher Scientific, USA). We also quantified the repeatability of wing trait measurements by measuring them twice. The measurements were highly repeatable for both eye size and wing traits (R^2=0.99, Fig. S4).

General software usage

Unless specified, we carried out all analyses in R ver 4.5.0 (R Core Team, 2025) and details of R packages used for specific analyses are provided when describing the analyses. Base R and *tidyverse* ver. 2.0.0 (Wickham et al., 2019) were used for general handling, *ggplot2* ver. 3.5.2 (Wickham, 2016) and *ggridges* ver 0.5.6 (Wilke, 2024) for plotting non-phylogenetic figures, *ape* ver. 5.8.1 (Paradis & Schliep, 2019) for handling phylogenies and related data, and *ggtree* ver 3.16.0 (Yu et al., 2017) for visualizing phenotypic data on phylogenies, and *viridis* ver 0.6.5 (Garnier, 2024) for colour palette. Figure panels were made using Inkscape (https://inkscape.org/).

Evolutionary allometry and the contribution of phylogeny in explaining eye size evolution

We used the published phylogeny of the European butterflies (Wiemers et al., 2020) for all comparative analyses and pruned it to match the number of species (n=59) in the study. Unless specified, all trait data was log10 transformed before analysis. We carried out evolutionary allometry between eye size and forewing length by fitting several phylogenetic regressions with a phylogenetic covariance matrix as a random factor. First, we fitted a global regression (eye size ~ forewing length) on the entire dataset (444 individuals of 59 species) to explore general allometric scaling. Next, we included 'sex' as an additive effect (eye size ~ forewing length + sex) to test for sexual size dimorphism in the eye size. Visual inspection of the allometry indicated that the butterfly families occupy a unique morphospace (Fig. 2). We thus fitted two additional models including only 'family' and both 'family' and 'sex' as additive effects. The Hesperiidae family has superposition eyes (Horridge et al., 1972), unlike most diurnal butterflies which have apposition eyes. As the sensitivity and resolution constraints on superposition eyes are different from apposition eyes (for details, see Land & Nilsson, 2012), this family acts as an 'anatomical' outlier. We therefore fitted a global regression model by removing Hesperiidae to test its effect on the estimates. Phylogenetic regressions were fitted using the *pGLMM* function in the *phyr* ver 1.1.0 R package (Ives et al., 2020). Lastly, we quantified the contribution of shared ancestry by computing residual variation (R^2) between the global phylogenetic to a non-phylogenetic (*glm* function) regression using the rr^2 ver 1.1.1 package (Ives & Li, 2018).

Extracting eye size residuals

To account for allometric scaling between eye size and forewing length, we obtained relative eye size as residuals by fitting phylogenetic generalized least squares (PGLS) regression on species averaged eye size and forewing length using the *gls* function from the *nlme ver* 3.1.168 package (Pinheiro et al., 2025). Initially, we fitted separate PGLS for males and females, however, their eye size residuals followed the same pattern across species (Fig S5). Thus, unless specified, we carried out further comparative analyses on pooled species-averaged traits. PGLS was supplied with the Brownian Motion, Ornstein-Uhlenbeck and Pagel's λ correlation structures, along with a non-phylogenetic model (Pagel's λ=0). Models with the Brownian Motion and Pagel's lambda correlation structure had a similar fit (Table S2) and the likelihood ratio test was non-significant (P=0.23). Thus, we used residuals from the Brownian Motion model for downstream analyses. However, where possible, we included forewing length as a covariate to control for allometric scaling.

Phylogenetic signal and fitting homogenous-rate evolutionary models

Eye size residuals were used to calculate phylogenetic signal (as Pagel's λ) and for fitting models of continuous trait evolution. We quantified Pagel's λ using the *phylo.sig* function in the *phytools* ver 2.4.4 package (Revell, 2012, 2024). Pagel's λ ranges from 0 to 1, where $\lambda = 0$ indicates no effect of shared ancestry on trait evolution, and $\lambda = 1$ indicates a strong phylogenetic signal, consistent with a Brownian Motion model of evolution. We further fitted several homogenous-rate evolutionary models (Brownian Motion, Ornstein-Uhlenbeck, Early Burst, Brownian Motion with a trend and a non-phylogenetic white noise model) using *fitContinuous* function in the *geiger* ver 2.0.11 package (Harmon et al., 2008; Pennell et al., 2014). Model fits were assessed using the AICc score and AIC weights. We tested for the effect of phylogenetic uncertainty on phylogenetic signal using the *sensiPhy ver* 0.8.5 package (Paterno et al., 2018) and fit of continuous trait evolution models by replicating analyses on 500 posterior trees.

Fitting a heterogenous-rate evolutionary model

Variation in the rate of trait evolution through time or across lineages is pervasive (Chira & Thomas, 2016). To account for rate heterogeneity in evolutionary rates across clades, we used the Bayesian variable-rates regression model (Venditti et al., 2011). The model first computes the expected trait values based on the homogenous-rate Brownian Motion model (with global background rate) and compares them to the observed trait values. If the observed trait values do not conform to the background rate, the model will then either stretch or compress the branches or clades such that trait evolution conforms to the Brownian Motion model. The rate scalar (r) determines the magnitude at which the branches/clades are stretched or compressed and can indicate branches/clades evolving at exceptional rates: $r>1$ or $0 \leq r < 1$ indicates the evolutionary rate is higher or lower than the background rate, respectively, while $r=1$ indicates the rate is same as the background Brownian rate (Baker et al., 2016).

The variable-rates model uses the Markov Chain Monte Carlo (MCMC) approach with reversible-jump and is implemented in the software *BayesTraits* ver 4.1.3 (Meade & Pagel, 2024). Species-averaged trait data was supplied in the form of regression (mean eye size ~ mean forewing length) and the model then returns a posterior distribution of regression coefficients, branch-specific rate scalars (r), background Brownian rate and Pagel's λ value. We ran three independent MCMC chains for 110 000 000 iterations with a burn-in of 10 000 000 and sampling every 10 000 iterations thereafter. Following previous studies (Baker & Venditti, 2019; Furness et al., 2022), the gamma prior (α=1.1, β rescaled to give a median of 1) was provided for the scalar parameter, uniform prior ranging between -100 to 100 for regression coefficients, and uniform prior ranging between 0 to 1 for Pagel's λ. MCMC diagnostics were assessed visually by inspecting trace plots and calculating effective sample size (>1000 for all estimated parameters) and Gelman-Rubin metric (value of 1-1.2 is expected for converged chains) using the *coda* ver 0.19.4.1 package (Plummer et al., 2006). Convergence diagnostics are reported in Supplementary Information (see Table S3, Fig. S6).

Quantifying species habitat

We used species habitat preference (affinity towards closed or open habitats) as a proxy for the light environment – species in more forested habitats will experience relatively dimmer light conditions than in open habitats. For quantifying species habitat preference, we carried out spatial analyses using the aggregated long-term monitoring data (from 2010-2021) from the Swedish Butterfly Monitoring Scheme (SEBMS, Pettersson, 2025) and the Copernicus tree cover density raster (10x10m^2 grid size) for Sweden (workflow shown in Fig. 5). SEBMS conducts systematic butterfly surveys every year on fixed transects all over Sweden (Figure 5B). Such systematic survey data should have lower observation bias which is typically observed in citizen science databases. Each transect is divided into several segments depending on the transect length (Fig. 5C) and is generally composed of a similar habitat. Since our goal was to quantify broad species habitat preference, we used tree cover density as a proxy of habitat structure for each segment on the transect and assigned this value to all species recorded on the segment. Thus, we obtained the distribution of habitat preference for each species, and we used the median value of this distribution (Fig. 5D, 5E) for further analyses. In total, we quantified habitat across 1427 unique transect segments on which 19 889 individuals of 57 species were recorded.

We used the tree cover density raster and extracted the values for all grids where the raster intersected with butterfly monitoring transects using the *exactextractr* ver 0.10.0 package (Baston, 2023). We set the buffer of 10m on each side of each transect and then calculated the weighted mean of canopy cover for each segment of the transect by weighting with the coverage fraction. Coverage fraction is the degree of overlap between the buffered transects, with a value of 1 indicating 100% overlap and 0 indicating no overlap. We excluded points with <0.002 coverage fraction.

Testing the effect of habitat and male mate location behaviour on eye size

We tested the association between eye size and habitat preference across species by fitting PGLS (same steps as described above) with species-averaged eye size as a response, forewing length as a covariate, and median tree cover density as a predictor. We fitted an additional model to test how absolute eye size (i.e. by removing forewing length) and canopy cover are associated. Both covariate and predictor were standardized (mean=0, SD=1) before fitting the models. Additionally, using the aggregated data across years (since we do not investigate the putative temporal changes in the species assemblages and traits), we calculated community-weighted means for species-averaged eye size, eye size residuals and species-averaged forewing length. Community-weighted means were calculated by averaging the trait values of recorded species at each segment of the transect weighted by their observed average abundance across years. These weighted means were regressed against the median canopy cover of the segment separately using linear models.

Finally, we tested if eye size is influenced by perching versus patrolling male mate location behaviour (Rutowski, 1991, 2000). In patrolling species, males actively patrol the habitat or territory to spot the females, while in perching species, males sit and wait for the females. According to Rutowski (2000), perching species are expected to have larger eyes, hence a larger visual field, allowing to detect rival males, females and predators from large distances. While Rutowski (2000) did not find a significant difference, we again tested this hypothesis by fitting PGLS with both absolute and relative eye size as response variables. We gathered data on male mate location behaviour for 36 species (Fig. S7) from Rutowski (1991) and Wickman (1992).

RESULTS

Tight allometric scaling and family-specific morphospace

Global phylogenetic regression using *pGLMM* on the entire data set revealed tight scaling between 3D eye surface area (henceforth eye size, Fig. 1) and forewing length (estimate = 0.52, P<0.001; Fig. 2A). Excluding the family Hesperiidae from the global regression resulted in a slightly steeper slope (estimate = 0.64, P<0.001, Fig. 2A). Including 'sex' as an additive effect with forewing length as a covariate further suggested that males generally have larger eyes than females relative to wing length (estimate = 0.11, p<0.001, Fig. 2B). When 'sex' was included as an interactive effect, females had slightly steeper slope than males (Table S4, Fig. S8).

Allometry further indicated that families occupy a unique morphospace (Fig. 2D). For example, species in the family Lycaenidae are generally smaller (hence smaller forewing length) and have smaller eyes. While species in the family Nymphalidae show large variation in forewing length, and thus eye size is more spread out in the morphospace (Fig. 2D, 2E). The exception was the family Hesperiidae, which has a much larger relative eye size (Fig. 2A). This pattern was also mimicked in the phenogram of eye size residuals, which showed that Hesperiidae and a clade of fritillary butterflies had the largest eyes (Fig. 3). Next, fitting two models by including family and both family and sex as additive effects, respectively, suggested that relative eye size (i.e. intercepts) of Pieridae and Lycaenidae were significantly smaller than Hesperiidae, but only in the latter model (Fig. 2C, Fig. S8). Regression estimates from all all fitted models are reported in Table S4.

Finally, calculating the residual variation by comparing the phylogenetic and non-phylogenetic regression (eye size~forewing length) suggested that phylogeny accounted for 74% of the variation in eye size evolution.

Eye size residuals have a strong phylogenetic signal and follow the Brownian Motion model

The phylogenetic signal (Pagels λ) in eye size residuals was high (λ=0.98, Fig. 3, see Fig. S9 for likelihood profile) and remained similarly high across 500 posterior trees (Fig. S9). Further, the Brownian Motion model had a better fit among other models and was the case across 500 posterior trees (Table S5, Fig. S10). However, we interpret this result with caution as model convergence was low for Ornstein-Uhlenbeck, BM with trend and Early Burst models (Table S5).

Rate heterogeneity in eye size evolution is low and is family-specific

Fitting the Bayesian variable-rates model showed that almost all median rate scalars (*r*) were 1, suggesting that the residual variation in the Brownian rate and background Brownian rate were similar, except for the Hesperiidae family with median *r*>10 (Fig. 4). This is apparent in the consensus tree obtained from the variable-rates model where the entire Hesperiidae clade is stretched out, showing a higher evolutionary rate (Fig. 4). The consensus trees looked similar across different runs (Fig. S11). Notably, the clade of fritillary butterflies (especially from genera *Fabriciana* and *Speyeria*) also have relatively large eyes (Fig. 2A) and the mean r value across three runs was 1.24.

Habitat preference has a weak negative and male mate strategy does not effect on eye size

PGLS indicated habitat preference did not influence either relative (estimate= -0.01, P = 0.08, Fig. 5F, Table S6, S7) or absolute (estimate = -0.001, P = 0.90, Fig. S12, Table S6, S7) eye size. However, regressions between community weighted means of relative (estimate = -0.0003, P=0.004) or absolute eye size (estimate = -0.0003, P <0.001) and canopy cover showed a very weak negative association, with large confidence intervals around estimates (Fig. S13, Table S8). The association between community-weighted forewing length and canopy cover was non-significant (Fig. S13). Male mate location behaviour strategy had no effect on both relative and absolute eye size (Fig. S14, Table S9, S10).

DISCUSSION

Sensory tissues are some of the energetically most expensive to build and maintain, and their investment is often strongly modulated by ecological factors. Here, we elucidate macroevolutionary dynamics and drivers of eye size evolution in temperate butterflies.

Our evolutionary allometry indicates a tight scaling between eye size and forewing length (a body size proxy) and that butterfly families occupy a unique position in the morphospace. This pattern arises because the forewing length, or body size in general, tends to be family-specific in butterflies. For example, Papilionidae are often large, most Pieridae are medium-sized and species in the Hesperiidae and Lycaenidae are generally much smaller than species from other families, while Nymphalidae show a high degree of variation in forewing lengths across species (Fig. 1C). Although our study has limited sampling, the family-specific pattern of body size holds across European butterflies (Fig. S15, data from Middleton-Welling et al. (2020)). The family Hesperiidae stands out in the morphospace as they have much larger eyes despite small forewing lengths compared to the same sized butterflies from other families (Fig. 1). This is explained by their superposition eyes with optics designed to optimize light capture in dark environments (e.g. nocturnal moths typically have superposition eyes) despite being diurnal, while other diurnal butterflies mainly have apposition eyes (Horridge et al., 1972). For a given size, superposition eyes are more light sensitive than apposition eyes (Land & Nilsson, 2012). By having superposition eyes that are relatively larger than butterflies from other families with similar body sizes, Hesperiidae would have a far greater light sensitivity and may facilitate their typical fast flight allowing them to have fast motion vision. Thus, in a sense, Hesperiidae are 'anatomical' outliers within the diurnal lineage of butterflies. Removal of Hesperiidae from allometry, as expected, increased the slope by a small magnitude (Fig. 1A).

The allometry also indicated that males generally have larger eyes than females relative to wing length, which is often the case in butterflies and insects (Rutowski, 2000; Stanger-Hall et al., 2018). It is hypothesized that larger eyes (or greater visual sensitivity) in male butterflies might aid in spotting rival males, mates or predators (Bergman et al., 2021; Rutowski, 2000). We posit another mutually non-exclusive hypothesis that females are faced with a larger energetic trade-off between investment in sensory and reproductive organs than males, leading to smaller sensory tissues such as eye size.

Eye size residuals had a strong phylogenetic signal and phylogeny explained ~74% variation in the eye size evolution. This pattern corroborates with the findings of other studies. For example, along with tight allometric scaling between eye size and body size, Ausprey (2021) and Ausprey (2024) found a high phylogenetic signal (Pagel's $\lambda \geq 0.9$) for relative eye size and that phylogeny explained >60% variation in eye size in birds. Such a strong phylogenetic signal in the eye size residuals likely also explains the better fit for the Brownian Motion model of evolution. Furthermore, the Bayesian variable-rates method to model rate heterogeneity, broadly, failed to detect clades or branches with exceptional rates, except for Hesperiidae and a clade of Fritillary butterflies which had relatively higher rates (Fig. 4). Such a strong phylogenetic signal, conformity to the Brownian Motion model and low heterogeneity in evolutionary rates is striking, especially when sensory organs are expected to evolve under selective regimes such as light environment (Ausprey, 2021; Ausprey et al., 2021; Wainwright et al., 2023).

Habitat structure influencing light environment is one of the major drivers of eye size evolution in diurnal lineages. For example, butterflies and birds living in forested or close canopy habitats (=dim light) tend to have larger eyes than those living in open habitats (=bright light) (Ausprey, 2021; Ausprey et al., 2021; Wainwright et al., 2023; Wright et al., 2024). The test of this hypothesis in insects on a large macroevolutionary scale is, however, lacking. Here, we tested for the association between the light habitat preference of a species (quantified as tree cover density) and both absolute and relative eye size using both phylogenetically controlled regressions and community-weighted means. Intriguingly, we found a weak negative association between relative eye size and habitat preference in males but not females, and no association between absolute eye size and habitat. In fact, species in the clade of Fritillary butterflies which have the largest relative eye size (along with Hesperiidae), included in our study prefer open habitats (Fig.5). The lack of relationship between eye size and light habitat or other factors that affect light environment (e.g. water depth) has also been found, for example, in butterflies (Seymoure et al., 2015), bumblebees (Bartholomée et al., 2023; Tichit et al., 2024), fishes (Yoder et al., 2022) and frogs (Chen et al., 2021; Huang et al., 2019). Finally, in line with the findings of Rutowski (2000), we did not find any association between eye size with perching versus patrolling male mate location behaviour.

Taken together, our results show that the evolution of eye size is largely explained by allometry and shared ancestry. How, then, are butterflies able to occupy such a large range of different light habitats? We posit that selection is operating on the other components of the compound eye morphology (see below), and not eye size *per se*.

In the apposition compound eye type (which is the eye type of all butterflies tested here except for the Hesperiidae) each facet is one light-capturing optical unit (Land, 1997; Land & Nilsson, 2012). The number of facets and their distribution across the eye, facet diameter, the angle between the facets (i.e. interommatidial angle), and the eye curvature, determine the sensitivity and resolution. For example, increasing the facet diameter *(D)* which increases the interommatidial angle ($\Delta\Phi$), allows greater capture of the light, increasing eye sensitivity (Land, 1997; Rutowski, 2000). In contrast, increasing the number of facets results in a decrease of the interommatidial angle increasing resolution. The eye parameter (calculated as: $D\times\Delta\Phi$) reflects the trade-off between eye sensitivity and resolution. For example in bumblebees, species associated with dim light environments have higher eye parameters (i.e. larger D and $\Delta\Phi$) than those associated with bright light environments, but there was no correlation with eye size (Bartholomée et al., 2025, 2023; Tichit et al., 2024). This relationship gets even further complicated when eye curvature enters the equation. For example, flatter eyes can have fewer larger facets increasing both sensitivity and resolution, but the same facets on a curved eye would maintain sensitivity at the expense of lower resolution due to an increase in the inter-ommatidial angle (Rutowski, 2000). To deal with the sensitivity-resolution trade-off, species often show regional differences in density of facets and facet diameter across an eye (Rutowski et al., 2009). Thus, in principle, even if the eye size remains similar across species irrespective of the habitat (as seen in our data), selection can operate and optimise different aspects of eye morphology. In the context of our study, even if a forest and open habitat species may have similar eye size, it is possible that eyes may differ in facet number, facet diameter or eye curvature, which ultimately determine how a butterfly or an insect would see its surroundings.

The discussion above highlights the important caveat of our study which is not being able to quantify details of eye morphology (e.g. facet number, size, interommatidial angle), limiting it from fully elucidating how ecology shapes visual adaptation. Quantifying detailed eye morphology requires high-resolution scans (usually <3µm) on fresh tissues (stained with heavy metal) for which the internal anatomy, such as the orientation of cones, is intact. The resolution of our micro-CT scans (voxel sizes of 6-8µm) does not allow us to resolve individual facets or cones and internal anatomical structures are collapsed in dried museum specimens. However, an approach like ours can be used to identify interesting clades for more in-depth studies. Additionally, testing how habitat shapes the evolution of visual adaptation in tropical lineages (Wainwright et al., 2024) would be interesting, as habitats such as rainforests and savannahs or other grasslands differ dramatically in light environments. Lastly, we note that our taxon sampling is not exhaustive, although representative of forested and open habitat species from diverse phylogenetic backgrounds (see Fig. 5E). Increasing the number of species and incorporating global diversity in future studies would be valuable for understanding the dynamics of eye size evolution.

In Summary, we have leveraged high-throughput X-ray micro-CT imaging of museum specimens and long-term biomonitoring data to quantify 3D eye size and species habitat of European butterflies, respectively. Using a range of phylogenetic comparative methods, we show that allometry and shared ancestry, and not ecology, shape the evolution of eye size. Future studies on the evolution of eye morphology and anatomy on a macroevolutionary scale will provide holistic insights into how ecology shapes visual adaptations in invertebrates.

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Data availability statement: All the data and scripts are available on Github (https://github.com/sridhar-halali/eye_size_macroevolution). 3D models of eye surface or cornea will be made available upon acceptance of the manuscript.

Author contributions: EB, PC and NW conceived the original idea and SH refined these ideas further; SH performed all the imaging and measurements (with the help of assistants) using the setup and inputs from SAH; LBP provided the long-term field monitoring data on Swedish butterflies and the associated spatial data; SH carried out the data analyses with inputs from RC on spatial analyses; SH wrote the first draft with subsequent inputs from all authors; EB, PC and NW obtained the funding.

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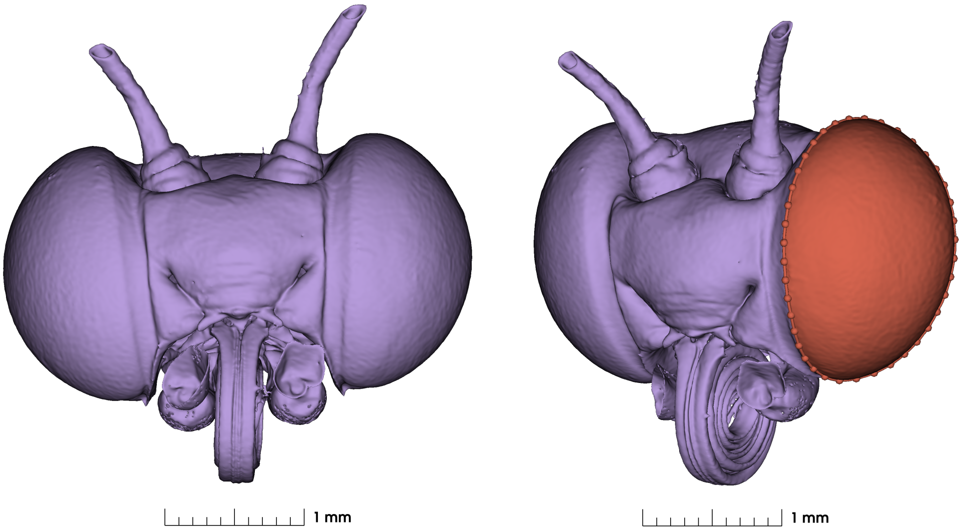

Figure 1: 3D reconstructed head of a butterfly (*Issoria lathonia*, left) and the segmented corneal surface (highlighted in red, right) representing the 3D eye surface area.

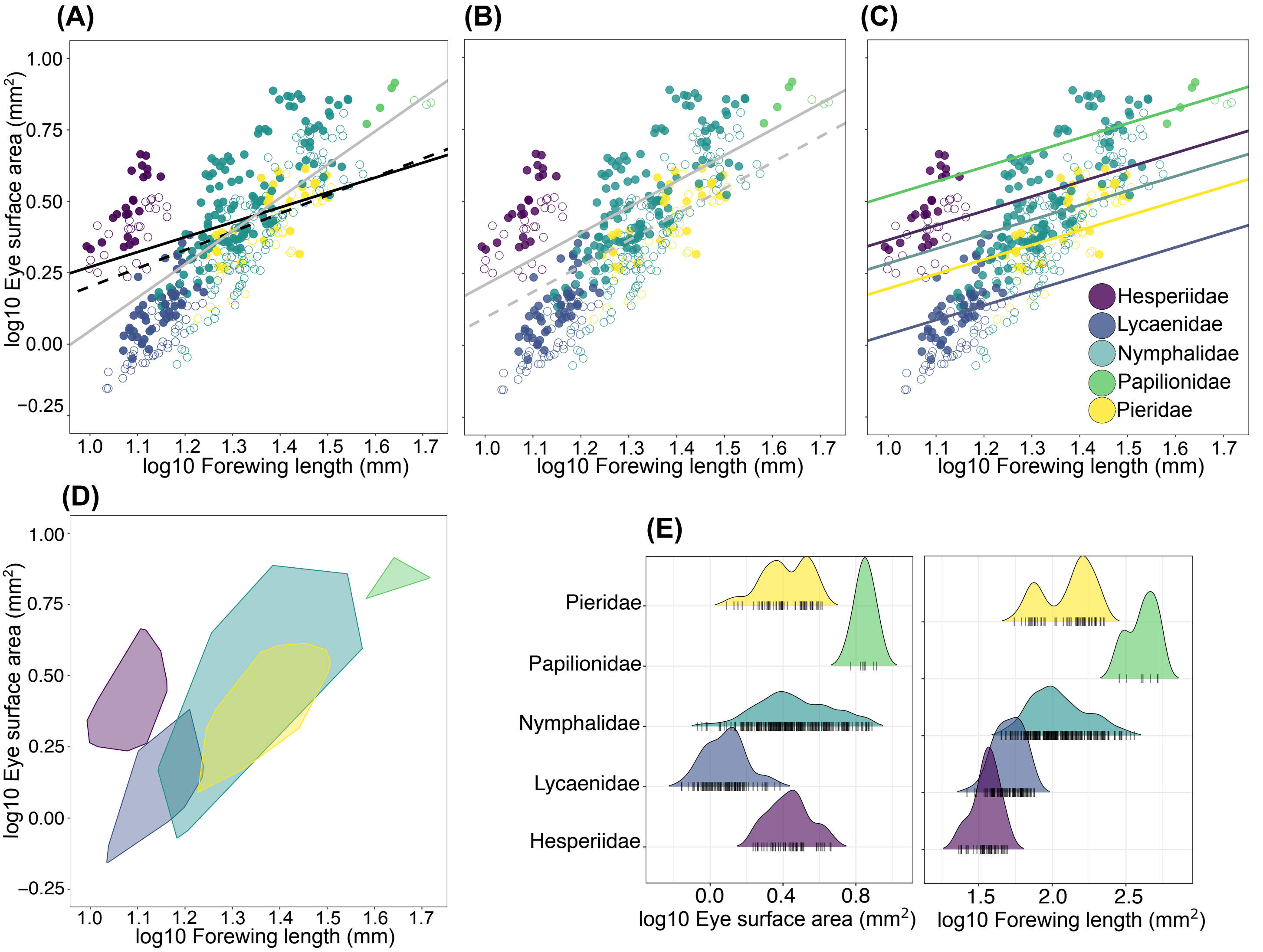

Figure 2: Allometry between 3D eye surface area and forewing length for all individuals (444 individuals, 59 species). In (A), the grey regression line represents the estimates from the ordinary least squares regression, while the solid and dashed regression lines represent estimates from PGLS with and without Hesperiidae, respectively. In (B), solid and dashed lines are for male and female, respectively, and in (C), each line represents family-specific regression. In (A-D), points and polygons are colored according to the family, and filled and open circles (in A-C) represent males and females, respectively. Figure (D) shows morphospace, and (E) shows the combined distribution of the eye surface area and forewing length for males and females for all individuals.

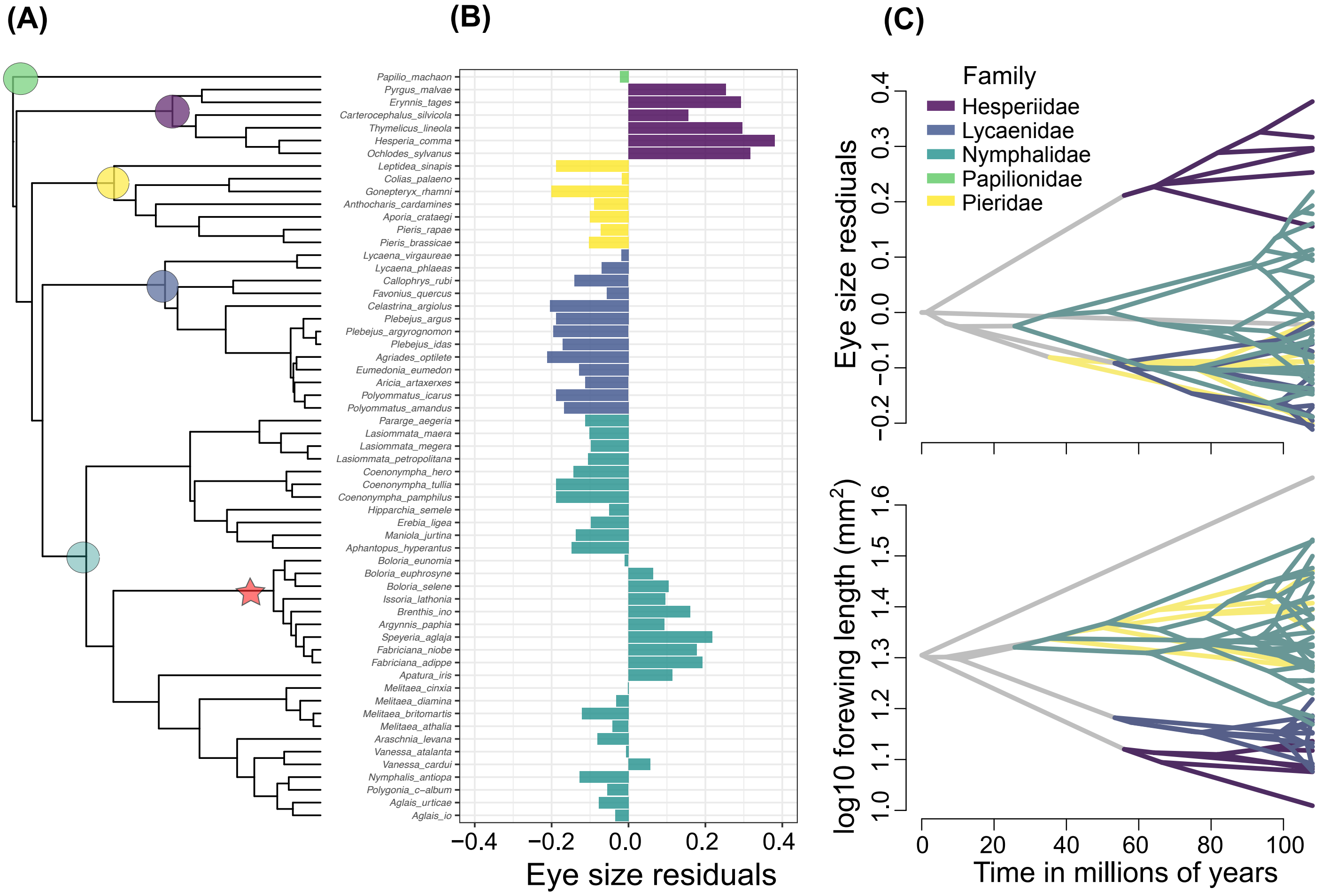

Figure 3: The phylogeny (A) of 59 temperate butterflies included in the study. The families are highlighted using solid circles at the nodes, and the clade of fritillary butterflies is highlighted by a star. In B, eye size residuals were obtained from phylogenetic regression (log10 eye size~log10 forewing length) using species-averaged trait values. In C, eye size residuals and forewing length are projected onto a phylogeny according to the Brownian Motion model of evolution. In all panels, colors represent butterfly families.

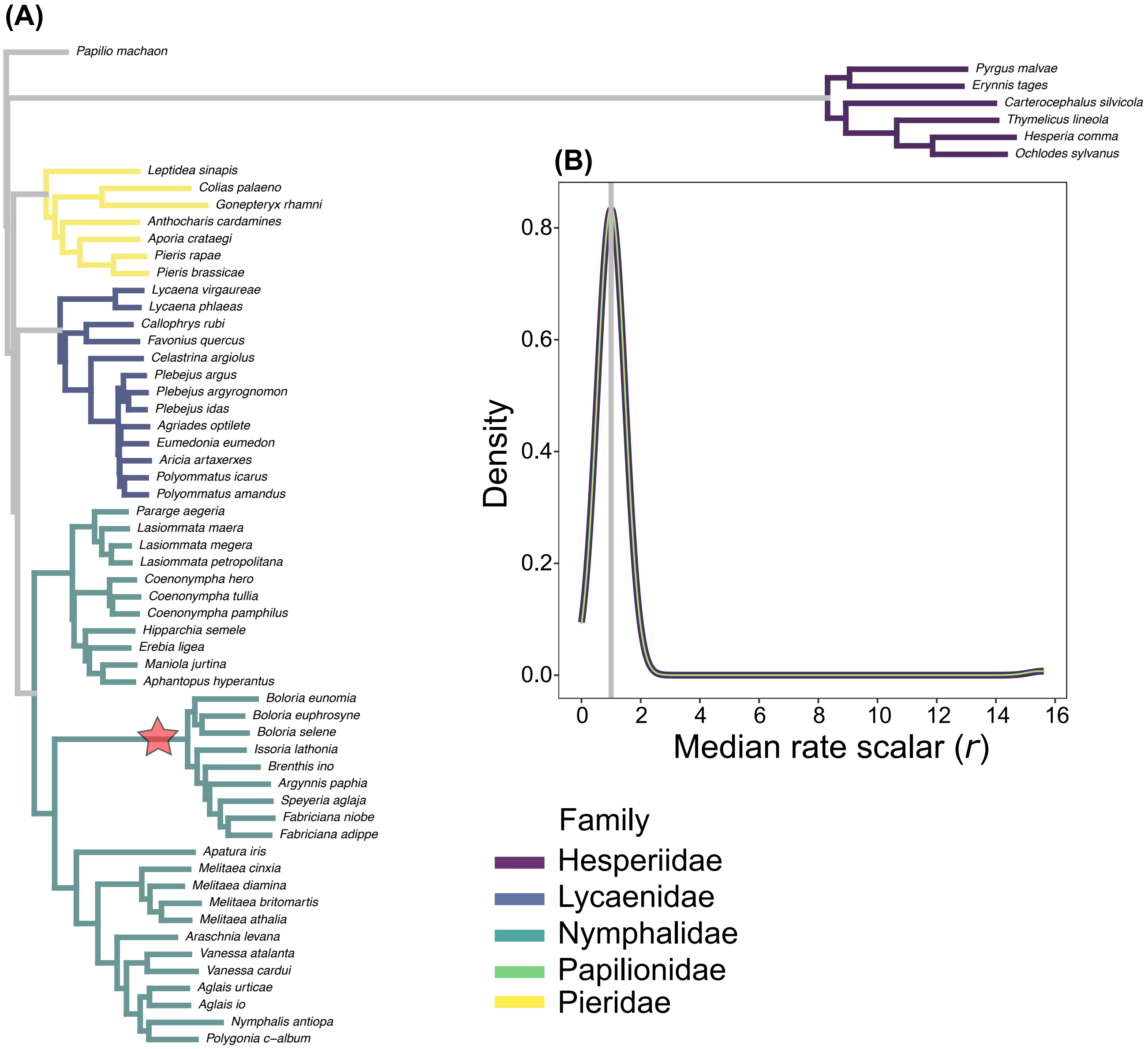

Figure 4: Consensus tree (A) obtained from the Bayesian variable-rate model used for quantifying rate heterogeneity in the eye size evolution. The branches/clades are stretched in proportion to the rate scalar (r), with $r>1$ and $r<1$ indicating higher and lower rates, respectively, while $r=1$ indicates rates equal to the homogenous Brownian Motion rate. The tree shows that the Hesperiidae clade has the highest rate and hence has been stretched the most. The clade of fritillary butterflies (indicated by a star) has been stretched only slightly, with r slightly exceeding 1. Colors of clades correspond to butterfly families. Figure B shows the posterior distribution of median rate scalar (r) for three independent runs (note that the lines are overlapping), with majority of rate scalars equal to 1, suggesting overall rate heterogeneity is low.

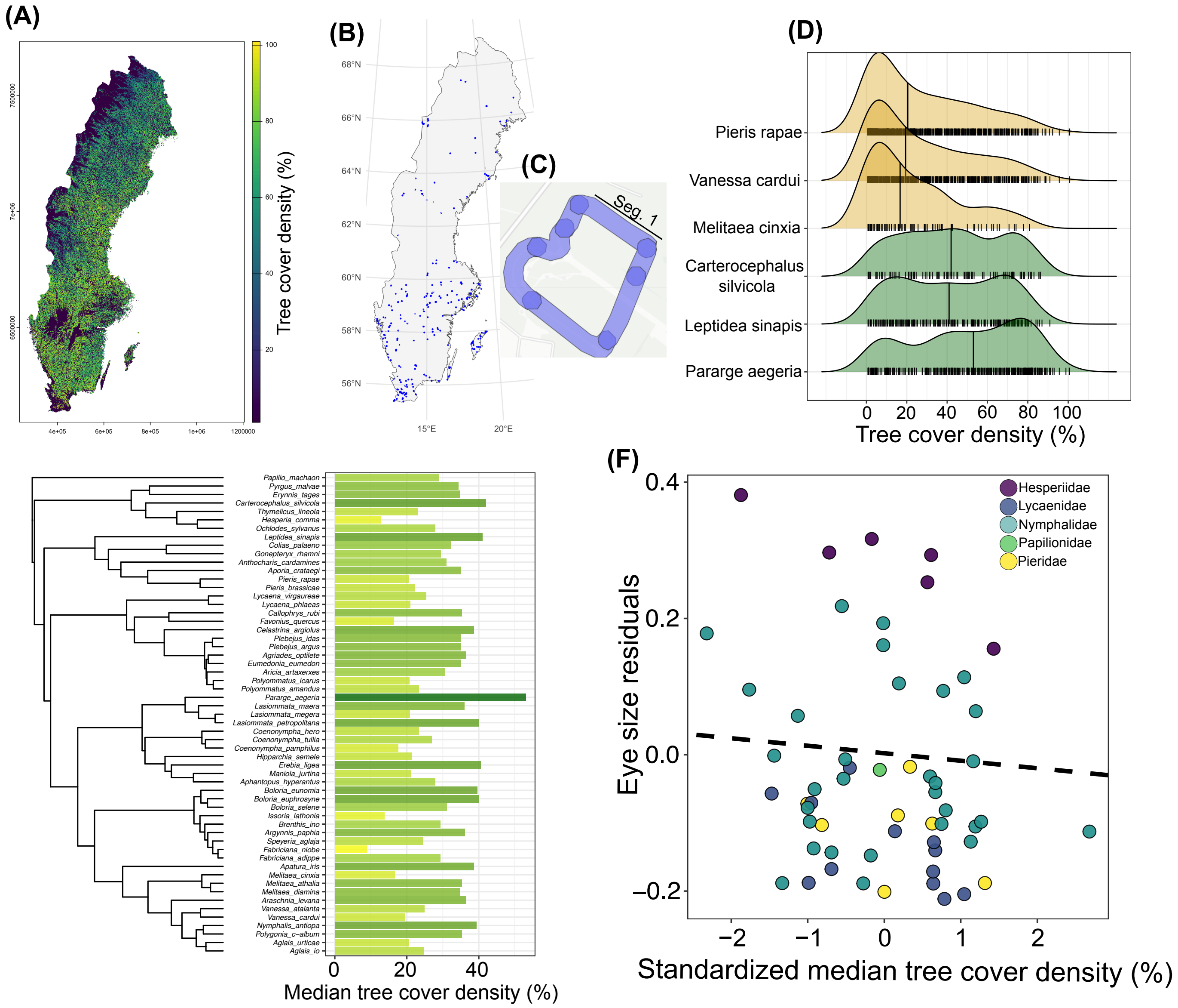

Figure 5: Workflow of the spatial analyses to quantify species habitat preference and test the association between eye size and habitat. Copernicus tree cover density (in %) raster (A) and the transects (B) used for monitoring butterflies by the Swedish Butterfly Biomonitoring Scheme across Sweden. Each transect is composed of different segments (C, intersections represent the start and end of the transect), depending on the length of the transect. Aggregated butterfly presence data (across 12 years, from 2010-2021) for each segment were used, and the habitat was quantified at the segment level such that all butterflies recorded at a particular segment would be assigned the same value of habitat metric (i.e. % tree cover density). But note that not all segments have the sampling effort of 12 years. From this, a distribution (D) for each species was obtained, and the median tree cover density value (black vertical line) was used as a predictor for phylogenetic regression. Median tree cover density for 57 species is shown in (E). The relationship between eye size residuals and median tree cover density (F) was tested using PGLS (estimate= -0.01, P=0.09). Colors in scatter plots (F) represent butterfly families. Note that PGLS were also fitted including forewing length as a covariate to control for allometric scaling as well as for the absolute eye size (see Methods).