Sexual dimorphism in pollen foraging and sensory traits in *Heliconius* butterflies

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Abstract

Sexual dimorphism in foraging behaviour is widespread in insects and may arise from differences in nutritional demands, sensory systems, or cognition. In *Heliconius*, pollen-feeding is an evolutionary innovation among butterflies that supports extended lifespans and sustained reproduction. However, how foraging behaviour varies between the sexes and how it relates to sexually dimorphic traits remains poorly understood. We investigated sex-specific foraging strategies in wild *Heliconius himera*, a highland specialist from southern Ecuador, using field surveys and DNA metabarcoding. Females carried more pollen than males, consistent with higher nutritional demands. Yet both sexes used similar plant richness and composition, indicating that differences reflect foraging effort rather than shifts in plant choice. Gut samples revealed greater pollen diversity and a more consistent community profile than proboscis samples, suggesting they better capture cumulative foraging history. To place these behavioural differences in a sensory context, we quantified sexually dimorphic sensory traits. Males had larger eyes and more ommatidia, whereas females had larger mushroom bodies, brain regions that support associative learning and memory. These patterns are consistent with sexual dimorphism reported across *Heliconius* and may contribute to the observed sex differences in

pollen foraging. Our findings highlight how sex-specific foraging differences may arise from effort on shared floral resources and co-occur with divergent sensory and neural investment, offering insights into the ecological basis of intraspecific variation in pollen use.

Introduction

Sexual dimorphism is widespread across animals and often involves divergence in morphology, physiology, and behaviour. Among insects, sex differences in foraging behaviour may arise from distinct dietary needs or sensory biases. Many species exhibit sex-specific specializations in visual and olfactory systems that support different ecological roles, such as mate location in males and resource acquisition in females (Smith et al., 2019). For instance, in the oil bee *Macropis fulvipes*, both sexes forage on the same flowers, but males rely more on visual cues and only collect pollen, whereas while females show a stronger preference for olfactory cues and collect both pollen and floral oils (Dötterl et al., 2011). Similarly, in the fly *Bibio rufiventris*, males and females differ in their responses to floral volatiles over the course of the day, reflecting sex-specific attraction to scent cues (Zhu et al., 2025). Despite such examples, the ecological and sensory drivers of sex-specific foraging behaviour remain poorly understood in most insect taxa.

Pollen-feeding is a widespread and ecologically important behaviour in insects, particularly among bees, flies, and beetles, where it plays a central role in adult nutrition, reproduction, and pollination (Romeis et al., 2005). These lineages have evolved diverse morphological and behavioural adaptations to collect and digest nutrient-rich pollen (Roulston & Cane, 2000; Ruedenauer et al., 2019). Unlike most butterflies that feed only on nectar, *Heliconius* uniquely feed on pollen, a strategy central to their nutrition, reproduction, and mutualism with the plants they pollinate (Gilbert, 1972; Young & Montgomery, 2020). Sex differences in pollen feeding have been documented in some *Heliconius* species, but how these differences align with sexually dimorphic sensory traits, and whether they involve shifts in pollen load versus composition is understudied.

Insects that feed on pollen gain access to amino acids and lipids otherwise scarce in nectar (Romeis et al., 2005). In *Heliconius*, pollen feeding has been linked to enlarged mushroom bodies that support sensory integration and learning, possibly facilitating this unique behaviour (Sivinski, 1989; Montgomery et al., 2016). Both sexes benefit from pollen-derived nutrients that extend lifespan and sustain reproduction (Dunlap-Pianka et al., 1977; Pinheiro de Castro et al., 2020). But the direct benefits differ: in females, amino acids and lipids directly support oogenesis and sustained egg production, and in older females, pollen-feeding helps maintain adult cyanogenic defences that contribute to Müllerian mimicry (O'Brien et al., 2003, 2004; Pinheiro de Castro et al., 2025). whereas in males direct benefits appear more limited, where it mainly supports spermatophore production and mating effort (Boggs et al., 1981; Boggs & Gilbert, 1979). These differences in direct benefits likely drives a female bias in pollen foraging.

Multiple studies have found evidence for sex differences in pollen feeding in *Heliconius*. Field observations of *H. charithonia*, a species within the *erato/sara/sapho* clade, have revealed sex-specific pollen use: females are more often recorded on *Hamelia patens* plants, while males favor *Lantana camara* (Mendoza-Cuenca & Macías-Ordóñez, 2005). Similarly, broader studies across *Heliconius* have documented larger pollen loads and longer foraging duration in females than males (Boggs et al., 1981; Cardoso, 2001; Gilbert, 1991). These patterns mirror broader trends in other Lepidoptera, where females actively seek out nectar with higher amino acid content than males (Alm et al., 1990; Erhardt & Rusterholz, 1998; Mevi-Schütz & Erhardt, 2005).

In *Heliconius*, differences in foraging may be influenced by sexually dimorphic visual systems. Unlike most butterflies, *Heliconius* possess two UV-sensitive opsins, and in the erato/sara/sapho clade one of these (UVRh1) is linked to the sex chromosome (W) and expressed only in females, enabling UV wavelength discrimination in females, which may enhance floral detection capabilities (Chakraborty et al., 2023; Finkbeiner & Briscoe, 2021). Its ecological relevance

remains unclear; for instance, UV sensitivity does not appear to affect oviposition in *H. erato* and *H. himera* (Borrero et al., 2023). Beyond opsin expression, other visual traits such as facet number, facet diameter, and corneal area shape acuity and sensitivity and can differ between sexes (Land, 1997; Wright et al., 2023). As such, integrated studies of foraging behaviours and sensory traits may help elucidate the basis of sex-specific pollen foraging.

Heliconius himera is a highland specialist inhabiting seasonally dry forests of southern Ecuador (~1500 m asl). Compared with its lowland relatives, it occupies more open habitats with higher light intensity, stronger temperature fluctuations, and distinct floral communities, conditions that likely shape both resource availability and foraging strategies (Descimon & Mast, 1984; Jiggins et al., 1996; Dell'Aglio, Mena, et al., 2022). Reflecting adaptation to this environment, *H. himera* differs from lowland *H. erato* in brain morphology and sensory processing, investing more in antennal lobes and less in visual neuropils (Montgomery & Merrill, 2017). When visual and olfactory cues conflict, *H. himera* relies more heavily on olfactory input than *H. erato* (Dell'Aglio, McMillan, et al., 2022), a pattern also seen in other Lepidoptera with altered neural investment (Stöckl et al., 2016; Borrero et al., 2024). As an incipient species within the *erato/sara/sapho* clade, it likely shares the sexually dimorphic opsin expression and vision reported in related taxa (McCulloch et al., 2017; Chakraborty et al., 2023). This combination of distinct sensory weighting and potential sexually dimorphic vision makes *H. himera* a compelling model for exploring how environmental adaptation and sex-specific sensory traits shape foraging behaviour.

Despite its ecological importance, pollen use in *Heliconius* remains incompletely understood. Traditional methods such as field observations and palynological keys have revealed diverse diets dominated by *Psychotria*, *Psiguria*, and *Lantana* (Boggs et al., 1981; Cardoso, 2001; Estrada & Jiggins, 2002; Mendoza-Cuenca & Macías-Ordóñez, 2005). However, these methods are slow, expertise-dependent, and limited in taxonomic resolution. DNA metabarcoding provides a powerful alternative: targeting regions such as ITS2, enables high-throughput, species-level identification of mixed pollen samples (Bell et al., 2016; Keller et al., 2015). Already widely applied in bee pollination studies (Martins et al., 2023; Piko et al., 2021; Wilson et al., 2021), this method can open new perspectives on *Heliconius* foraging ecology and sex-specific pollen use.

In this study, we integrate field surveys, DNA metabarcoding, and morphological analyses to investigate sex differences in foraging behaviour in *H. himera*. Specifically, we tested the hypothesis that sexes differ in the amount and diversity of pollen collected, reflecting distinct nutritional demands, foraging strategies, or sensory capabilities. We further quantified eye and brain morphology to place behavioural differences in a sensory context. This study provides one of the first molecular characterizations of pollen resource use in *Heliconius* and advances our understanding of how sexual dimorphism in sensory traits shapes intraspecific variation in foraging, with implications for pollinator ecology more broadly.

Materials & Methods:

Study sites:

Field surveys were conducted at five sites in Loja Province, Ecuador, around the city of Vilcabamba (4°15′S, 79°13′W), at elevations of 1500–1700 m (Fig. 1A–B). All sites are characterized by thorn scrub dry forest dominated by Acacia macracantha and xerophytic vegetation (Jiggins et al., 1996). Rumi Wilco is a 40-ha private reserve containing ~700 plant species (Jorgensen & Leon-Yanez, 1999), while Cerro Mandango is a nearby mountain site dominated by Acacia. The other sites (Quebrada Puliche, Agua de Hierro, and San Pedro) occur

***Figure 1**: **Sampling region and pollen foraging behavior in H. himera.** (**A**) Study area in southern Ecuador. (**B**) Overview map showing the location of butterfly survey sites in Loja Province. Points represent individual collection records, with colour indicating sex (green = female, orange = male). (**C**) Satellite map of survey sites, including Rumi Wilco, Cerro Mandango, Quebrada Puliche, and San Pedro. (**D**) Close-up photograph of a H. himera with a visibly pollen-loaded proboscis.*

along small streams with similar vegetation, adjacent to coffee plantations and pastures.

Butterfly sampling:

From April to September 2023, we sampled *H. himera* along trails at all five sites, with most effort concentrated at Rumi Wilco and Cerro Mandango, which were visited at least five days per week. Daily sampling took place between 08:00 and 17:00, coinciding with peak activity of *H. himera* (Davison et al., 1999), spanning the wet-to-dry season transition.

Each butterfly was captured, marked on the forewing, and released after recording GPS location, time, sex, body length, pollen load and observer. Pollen load was visually classified as none, small, or large depending on the proportion of the proboscis covered (Fig. 1D). Recaptured individuals were noted for movement analyses. In total, 877 butterflies were recorded (371 females, 506 males).

To test for sex differences in microhabitat, we measured temperature and humidity at capture sites using HOBO Temp/RH U23-002 data loggers that recorded continuously for at least 24 h post-capture. Environmental data were obtained for 49 individuals (24 females, 25 males) over 2–3 sampling days per month throughout the study period.

Pollen collection and ITS2 sequencing:

To identify pollen sources, we collected 71 *H. himera* (25 females, 46 males) with hand nets between July and August 2023. Specimens were placed in entomological envelopes; proboscises were dissected and stored in PCR tubes, while bodies (containing guts) were preserved in DMSO/EDTA/NaCl solution. Samples were kept at −15 °C until DNA extraction and sequencing in February 2024. All proboscises and a subset of guts (13 females, 26 males) were analysed using ITS2 DNA metabarcoding following the protocol of Sickel *et al.*, (2015), with positive and negative controls included.

Sequence reads were processed with a custom bioinformatics pipeline (https://github.com/chiras/metabarcoding_pipeline), generating amplicon sequence variants (ASVs) and assigning taxonomy against curated ITS2 plant databases (Keller et al., 2020; Quaresma et al., 2024). Remaining unclassified reads were further annotated with the SINTAX algorithm (Edgar, 2016). Data were analysed in R with the *phyloseq* package (McMurdie & Holmes, 2012). Analyses were restricted to Streptophyta, and taxa were retained if they reached ≥1% relative read abundance in at least one sample.

Wing reflectance spectrometry and age estimation:

To estimate the relative age of wild individuals, we adapted a spectrometric method developed by (Dell'Aglio et al., 2017), who used wing colour metrics to infer butterfly age. We collected full-spectrum reflectance data (300–750 nm) from red and yellow wing bands of insectary-reared individuals of known age across seven *Heliconius* species (n = 320), including 108 reared *H. himera*. Spectra were measured using an Ocean Optics Flame spectrometer with a PX-2 xenon light source and WS-1 white standard. Reflectance was analysed in R with the pavo package (Maia et al., 2019), and the area under the reflectance curve (AUC) was calculated for each colour patch.

Age was predicted using linear models with log-transformed AUC values as predictors. A combined model including both red and yellow AUCs provided the best fit (adjusted $R^2 = 0.13$, $P < 0.001$) and was used to estimate the age of wild *H. himera*. Predicted values were backtransformed to days, and each individual was assigned a relative age score. This continuous relative age metric provided a standardized measure across individuals and was used to test how pollen foraging behaviour varies with age in the wild population.

Eye cuticle morphology:

To test for sexual dimorphism in eye cuticle morphology, we analysed wild-caught and insectary-reared H. himera following established protocols (Wright et al., 2024; Wright & Merrill, 2025b, 2025a). Eyes were dissected, cleaned, mounted on slides, and imaged at 25× magnification. Corneal surface area and facet number were measured using image thresholding and the Analyze Particles function in ImageJ/Fiji (Schindelin et al., 2012). Inter-ocular distance and hind tibia length were also measured to account for body size. Given the strong bilateral symmetry of these traits (Wright et al., 2024), analyses were performed on the left eye unless damaged, in which case the right eye was used.

Brain immunohistochemistry and Imaging:

We examined sexual dimorphism in brain morphology in 43 *H. himera* (21 females, 22 males), combining previously analysed wild individuals from Vilcabamba (Ecuador) and common-garden reared butterflies from Cambridge (UK) (Montgomery & Merrill, 2017) with newly processed specimens reared in Tena (Ecuador; 8 females, 9 males). Brains were labelled using anti-synapsin immunofluorescence (Montgomery et al., 2016; Ott, 2008) imaged with confocal microscopy, and neuropils segmented in Amira. We measured volumes of key optic and central brain regions previously shown to be ecologically and evolutionarily relevant in *Heliconius* (Montgomery et al., 2021; Montgomery & Merrill, 2017), Including the medulla, lamina, lobula, lobula plate, accessory medulla, ventral lobula, antennal lobe, anterior optic tubercle, and mushroom body calyx and lobes plus pedunculus. The rest-of-central-brain (rCB) volume was used as an allometric control. Paired structures were quantified in one hemisphere and doubled, and volumetric data were z-axis corrected using a scaling factor of 1.52 (Montgomery & Ott, 2015) and log10-transformed before analysis.

Statistical analysis:

Pollen presence was modelled with binomial GLMMs including sex and time of day as fixed effects and random intercepts for individual and date. Pollen load categories were analysed with cumulative link models with sex and time of day as fixed effects. Activity patterns were analysed with multinomial regression, modelling sex as a function of hour of day. Body size was tested with linear mixed models including sex as a fixed effect and observer as a random intercept. Movement distances of recaptured individuals were calculated with Vincenty ellipsoid methods and analysed with log-transformed linear models including sex and body length as fixed effects. Microhabitat associations (Temperature (°C) and relative humidity (%)) with sex were examined with binomial GLMMs including temperature and relative humidity as continuous predictors and date as a random intercept.

To test effects of sex, body part (proboscis vs. gut), and predicted age on pollen composition, we used PERMANOVA on Bray–Curtis dissimilarities. Pollen richness (observed taxa) and diversity (Shannon index) were modelled with GLMMs, including date as a random effect. Age was modelled as a continuous predictor; results were compared with likelihood ratio tests and visualized with estimated marginal means.

Sexual dimorphism in eye and brain morphology was tested using linear models and standardized major axis (SMA) regression. For eyes, log10-transformed corneal area and facet number were modelled with sex, $\log_{10}$-transformed hind tibia length, and interocular width as fixed effects. For neuropils, $\log_{10}$- transformed volumes were modelled with sex, rearing condition (wild vs. reared, with reared individuals from Tena or Cambridge), and log10 rest-of-central-brain volume (rCB) as fixed effects. Model residuals were checked for normality, and fixed effects were evaluated with Type II ANOVA. Likelihood ratio tests assessed the contribution of predictors, and post hoc contrasts were performed with *emmeans* using Bonferroni correction. SMA regression (smatr package (Warton et al., 2012)) was used to test sex-specific differences in slopes, intercepts (elevation shifts), and major axis shifts. Mushroom body calyx and lobes plus pedunculus were analysed separately, consistent with previous *Heliconius* studies (Montgomery et al., 2016; Montgomery & Merrill, 2017).

Results:

Sex Differences in Pollen Presence and Load

Our field collections resulted in records from 371 females and 506 males, which we used to test whether the probability of carrying pollen differed between the sexes. Overall, females were more likely to both carry pollen and tend to carry greater quantities compared to males. Specifically, inclusion of sex significantly improved model fit in our binomial GLMM ($\chi^2 = 16.59$, $P < 0.001$). Fitting a cumulative link model analysing pollen load categories, “no pollen”, “little pollen” and “lot pollen” (Figure 2A), also revealed that females were significantly more likely to carry both small (estimate = 0.09, z = 4.31, P < 0.001) or large pollen loads (estimate = 0.06, z = 4.00, P = 0.0001) than males.

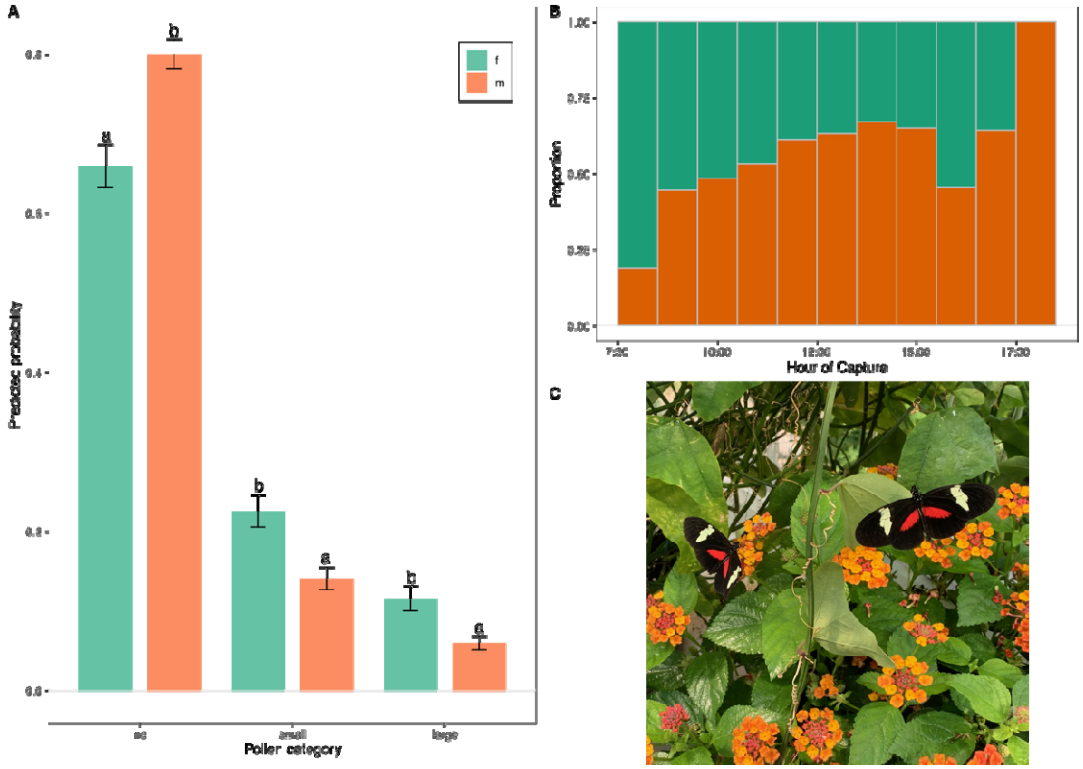

Figure 2: Pollen loads and temporal activity patterns in H. himera. (A) *Estimated marginal means of predicted probabilities for each pollen load category by sex, derived from a cumulative link mixed model. Letters denote significant pairwise differences between sexes within each category (α = 0.05).* ***(B)*** *Proportional distribution of captured males (orange) and females (green) across the day, binned by hour of capture* ***(C)*** *H. himera actively foraging on Lantana flowers.*

Pollen richness and diversity differ by body part but not by sex

To investigate the diversity of pollen resources used by *H. himera* and assess potential differences between sexes, sample types, and individual age, we performed DNA metabarcoding on pollen collected from 25 females and 46 males proboscis samples, as well as gut samples from a subset of these individuals (13 females and 26 males). Metabarcoding yielded 1,309,788 high-quality, taxonomically classified reads (mean = 11,907 per sample), detecting 54 families, 122 genera, and 163 species. All samples passed quality filtering and were retained for downstream analysis. The most common taxa included *Lantana camara* (Verbenaceae), *Stellaria media* (Caryophyllaceae), and several Asteraceae species: *Chromolaena odorata*, *Kaunia rufescens*, and *Fleischmannia pycnocephala* (Fig. 3A; Fig S3-S4).

Non-metric multidimensional scaling (NMDS) ordination revealed substantial overlap in pollen composition, with no significant differences between sexes (PERMANOVA: $R^2 = 0.0096$, $F = 1.05$, $P = 0.378$). In contrast, pollen composition differed significantly between body parts ($R^2 = 0.0325$, $F = 3.63$, $P = 0.001$; Fig. 3B), suggesting some differentiation in the pollen sources detected externally versus internally, possibly reflecting variation in sampling windows or digestion. We found no evidence that predicted age explained variation in pollen composition ($R^2 = 0.014$, $F = 1.03$, $P = 0.415$; Fig. S2).

Generalized linear and linear mixed models confirmed that observed plant species richness and Shannon diversity differed significantly by body part, with gut samples containing greater richness ($\chi^2 = 29.80$, df = 1, $P < 0.001$) and diversity ($\chi^2 = 15.56$, df = 1, $P < 0.001$) than proboscis samples (Fig. 3C). In contrast, sex had no effect on either metric (richness: $\chi^2 = 1.00$, $P = 0.32$; Shannon: $\chi^2 = 0.002$, $P = 0.97$). Predicted age did not influence richness or diversity when modelled as a continuous variable (richness: $\chi^2 = 0.19$, $P = 0.66$; Shannon: $\chi^2 = 1.78$, $P = 0.18$).

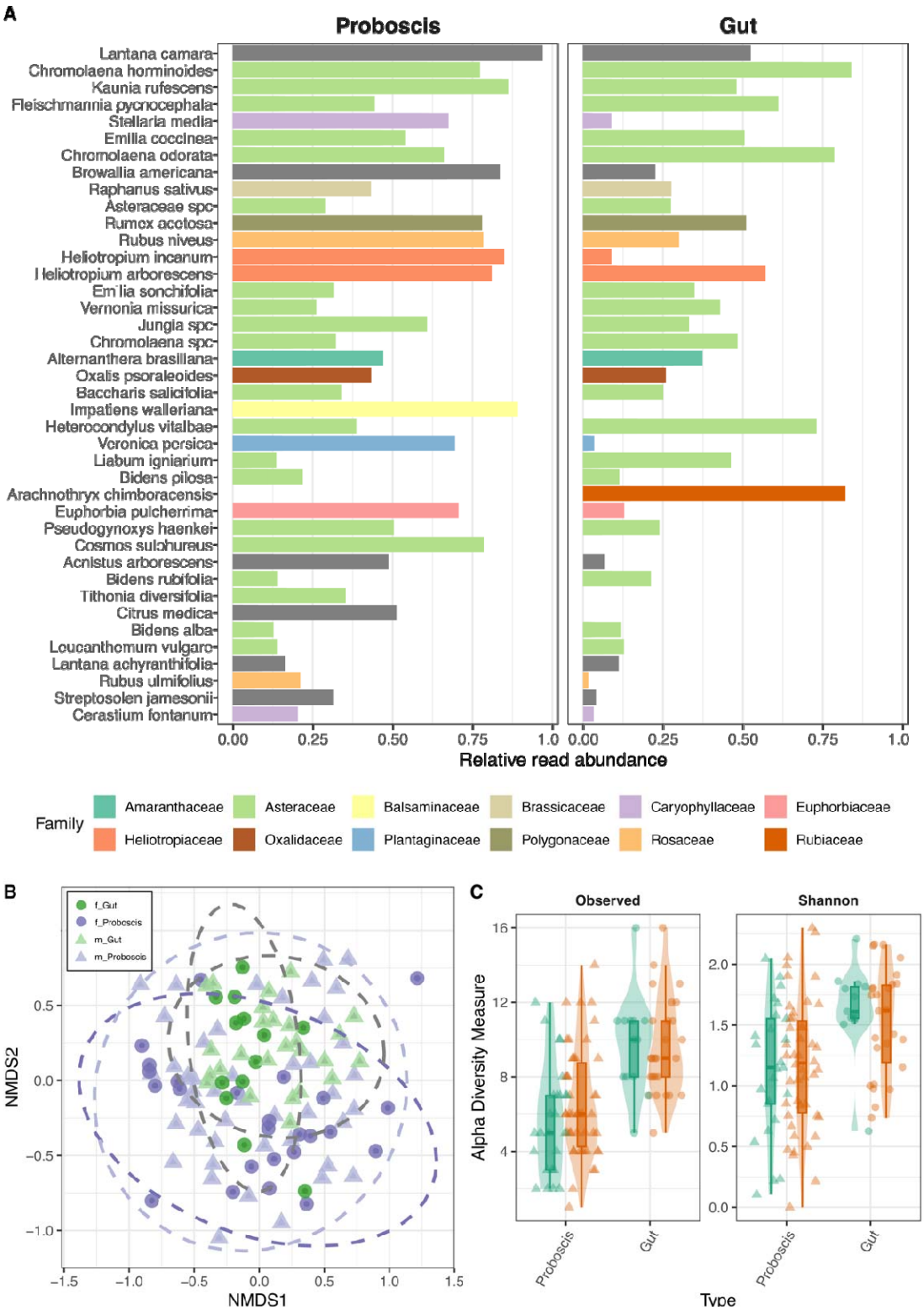

Figure 3: Diversity and composition of pollen collected from different sexes and tissues. *(**A**) Relative read abundance of the most common plant taxa identified in proboscis and gut samples. Bars represent the mean relative abundance across individuals, with colors indicating plant families. (**B**) NDMS plot across samples, showing clustering by body part (proboscis = purple, gut = green) and sex (female = circles, male = triangles). 95% confidence ellipses are shown for each group. (**C**) Observed richness and Shannon diversity of pollen samples collected from proboscis and gut tissues of H. himera, separated by sex.*

Daily activity, movement, habitat use and age

Given the sex differences in pollen presence and load size, we next tested for variation in body size, daily activity, movement, and microhabitat use (Fig. S1). Females averaged 24.4 mm in length (SD = 1.66, IQR = 23–26), while males averaged 25.3 mm (SD = 1.55, IQR = 24–26). Linear models confirmed males were larger ($\chi^2 = 129.73$, $P < 0.001$; Fig. S1)

Daily activity also differed (LR = 19.11, $P < 0.001$), with females captured earlier in the day (Fig. 2B). To test whether time of day independently influenced pollen presence, we included capture time as a fixed effect in the binomial GLMM. After accounting for sex ($\chi^2 = 16.59$, $P < 0.001$), capture time did not improve the model ($\chi^2 = 0.46$, $P = 0.50$).

Movement was analysed from 109 recaptured females and 162 males. Recaptured distances varied widely but were comparable: females moved a mean of 87.2 m (median = 24.8, SD = 176.0) and males 66.9 m (median = 36.2, SD = 95.1). A linear model of log-transformed distances showed no effect of sex on movement distance ($\chi^2 = 0.63$, $P = 0.61$; Fig. S1).

Both sexes were recaptured under similar microclimatic conditions. Females were recaptured at a mean temperature of 25.8 °C (median = 25.9, SD = 3.5) and 50.9% relative humidity (median = 48.0, SD = 16.8), while males were recaptured at 26.4 °C (median = 26.2, SD = 3.4) and 49.5% relative humidity (median = 48.3, SD = 15.0). These distributions overlapped strongly, with interquartile ranges of 23.3–27.9 °C for females and 24.1–29.3 °C for males. Binomial GLMMs confirmed that neither temperature ($\chi^2 = 1.05$, df = 1, $P = 0.31$) nor humidity ($\chi^2 = 0.11$, $P = 0.75$) significantly affected the probability of capturing either sex (Fig. S1).

Sexual dimorphism in eye and brain morphology

We tested for sexual dimorphism in eye and brain morphology (Fig. 4). Males had larger eyes than females ($F_{1,39} = 26.25$, $P < 0.001$). SMATR analysis showed no sex difference in slope between corneal area and tibia length but a significant elevation shift (Wald $\chi^2 = 68.82$, P <

0.001), with males consistently having larger eyes for a given tibia length (Fig. 4A). Facet number was also higher in males ($F_{1,40}$ = 6.07, P = 0.018). Using corneal area as the allometric control, SMATR again detected no sex difference in slope or elevation but a significant major axis shift (Wald χ^2 = 21.23, P < 0.001), showing that males had larger eyes and thus more facets (Fig. 4D).

We also found sex differences in brain morphology, particularly in higher-order integrative regions (Fig. 4B–F). Linear models revealed that females had significantly larger mushroom body calyces (MBCA; χ^2_1 = 36.0, P < 0.001), after accounting for overall brain size and rearing condition (χ^2_2 = 2.57, P =0.28). SMATR analysis confirmed a significant elevation shift in the scaling relationship between MBCA volume and overall brain size (Wald χ^2_1 = 34.25, P < 0.001), indicating that females consistently had larger MBCA for a given brain size (Fig. 4C). A similar pattern, with larger female investment. was observed for the mushroom body lobes and pedunculus (Fig. 4F). Linear models showed significant effects of sex (χ^2_1 = 29.5, P < 0.001), after accounting for overall brain size and rearing condition (χ^2_2 = 49.8, P < 0.0001). SMATR analysis detected a significant elevation shift in the scaling relationship (Wald χ^2_1 = 8.17, P =0.01).

By contrast, optic lobes showed no sex differences (χ^2_1 = 0.91, P = 0.34; Wald χ^2_1 = 1.15, P = 0.28; Fig. 4B), nor did analyses of individual visual neuropils (Supplementary Table 1). Antennal lobes also showed no significant differences (χ^2_1 = 3.6, P = 0.06; Wald χ^2_1 = 0.23, P = 0.63; Fig. 4E). Full model results are provided in Supplementary Table 1.

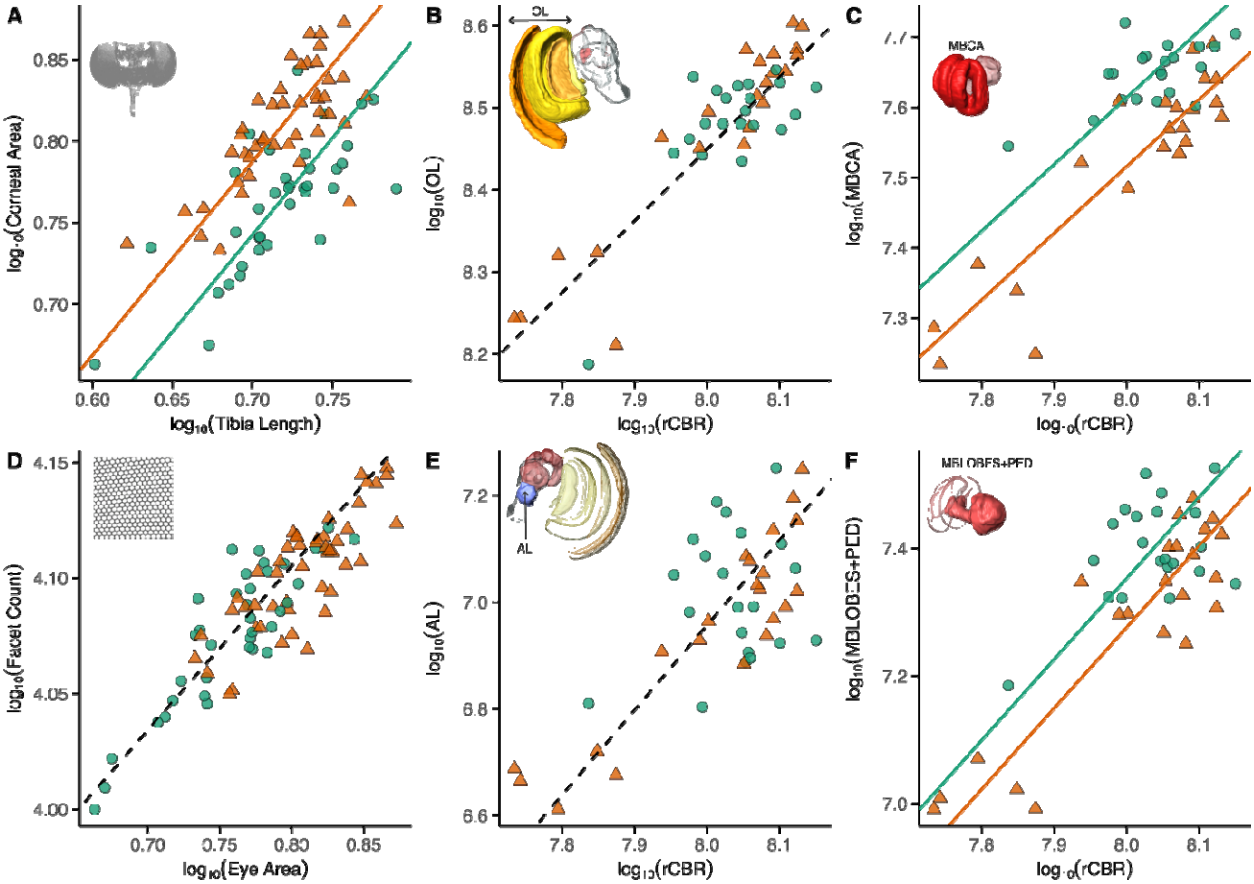

Figure 4: ***Sexual dimorphism in eye and brain morphology.*** *Males = orange; females = green. All traits are log_{10}-transformed. (**A**) Corneal area shows a significant elevation shift, with males having larger eyes. (**B, E**) Optic lobe (OL) and antennal lobe (AL) volumes scale with rest-of-central-brain volume (rCB) but show no sex differences. (**C, F**) Mushroom body calyx (MBCA) and mushroom body lobes and pedunculus (MBLOBES+PED) exhibit a significant elevation shift, with females having larger volumes than males. (**D**) Facet count shows a significant major axis shift, with males having more facets. Lines indicate sex-specific SMATR regressions (solid) or common slopes (dashed). Insets show the corresponding anatomical structures: eye (gray), OL (yellow/orange), MB (red), AL (blue).*

Discussion

Systematic field sampling combined with DNA metabarcoding revealed high floral diversity and clear sex differences in pollen foraging in wild *H. himera*. Females carried larger pollen loads than males, consistent with sex-specific nutritional demands; yet both sexes used similar plant richness and composition, indicating shared floral resources and differences in effort rather than choice. Notably, gut samples contained higher pollen diversity and more consistent community profiles than proboscis samples, suggesting they better capture cumulative foraging history. We

further confirmed sexual dimorphism in eye morphology and mushroom body volume, consistent with patterns reported across Heliconius (Couto et al., 2023; Wright et al., 2024). While we did not directly test functional links, these sensory traits may contribute to sex differences in foraging effort. Overall, our study provides one of the first molecular characterizations of pollen resource use in Heliconius, showing that sex-specific foraging reflects effort on shared floral resources and may be shaped by divergent sensory investment.

The floral resources used by *H. himera* differed markedly from those of lowland *Heliconius*. Metabarcoding revealed a diverse diet of 163 plant species spanning, genera and 54 families. While *Lantana camara* (*Verbenaceae*) was the most frequent resource, *Asteraceae* were also well represented, particularly *Chromolaena* and *Kaunia*, which produce amino acid–rich nectar and are widely visited by butterflies and other pollinators (Layek et al., 2022; Rathnayake & Wijetunga, 2016; Wist & Davis, 2008). By contrast, *Gurania* and *Psiguria* (*Cucurbitaceae*), central to the foraging ecology of lowland *Heliconius (Estrada & Jiggins, 2002; Jiggins, 2017)*, are entirely absent in the range and diet of *H. himera*. Instead, *H. himera* relied heavily on *Lantana*, highlighting its role as a core floral resource in montane dry forests. Larval resource use also reflects this environment: *H. himera* oviposits on *Passiflora* species of the *Decaloba* subgenus, which tolerate dry conditions and occur in open habitats (Ulmer & MacDougal, 2004). Thus, both larval and adult foraging are shaped by the montane dry-forest environment: larvae exploit drought-tolerant host plants, while adults rely on a broad array of floral resources. Together, these patterns suggest that *erato* clade species, including *H. himera,* have retained a generalist strategy across life stages, facilitating colonisation of high-altitude habitats.

In our study, both sexes of *H. himera* experienced similar environmental conditions and movement patterns, indicating shared microhabitat use and strong spatial overlap in access to floral resources. In patchy, resource-limited montane habitats, such overlap may favour a generalist foraging strategy over sex-specific specialization. Accordingly, sex-specific nutritional

demands appear to be met through differences in foraging effort rather than consumption of distinct floral species. In other *Heliconius*, microhabitat differences often underlie floral preferences rather than intrinsic species identity (Cardoso, 2001; Estrada & Jiggins, 2002). For example, *H. melpomene* and *H. cydno* rely on *Lantana* and *Psychotria*, respectively, due to habitat use rather than innate preference (Jiggins, 2017). Likewise, long-term studies of *Argynnis idalia* show that sex differences in floral visitation diminish when males and females forage during overlapping phenological windows (Chmielewski et al., 2023). By contrast, *H. charithonia* exhibits clear sex-specific floral preferences (Mendoza-Cuenca & Macías-Ordóñez, 2005). The absence of strong floral partitioning in *H. himera* therefore suggests that sex-specific strategies are expressed mainly through pollen collection effort, as an adaptive response to ecological constraints of montane environments. While *H. himera* belongs to the erato/sara/sapho clade, where sexual dimorphism in UV opsin expression is common, its opsin profile remains unknown (Chakraborty et al., 2023; McCulloch et al., 2016, 2017). A reduction or absence of such dimorphism could further contribute to convergence in floral use between sexes.

Our comparison of gut and proboscis samples confirmed that tissue type strongly affects pollen detection and must be considered in dietary inference. Gut samples showed higher richness and diversity, likely reflecting a longer integration window and cumulative foraging history or more passive uptake from nectar. Proboscis samples were often dominated by *Lantana*, suggesting its importance as a core pollen source actively processed through prolonged proboscis manipulation and digestion (Eberhard et al., 2007; Gilbert, 1972; Harpel et al., 2015). Meanwhile, floral taxa visited primarily for nectar may not persist externally, indicating that gut samples capture longer-term foraging, whereas proboscis samples reflect more recent plant interactions.

Beyond these tissue-specific insights, our results highlight the power of DNA metabarcoding for ecological research in butterflies. This approach enabled species-level identification of complex pollen mixtures from small tissue samples, detecting a broad taxonomic spectrum not easily resolved by classical palynology (Bell et al., 2016). The successful use of DMSO-preserved samples further demonstrates its potential for archived collections, opening opportunities to compare historical and spatial variation in pollen use, shifts in foraging behaviour, and interspecific differences across the *Heliconius* clade. For example, seasonal or local changes in pollen availability may influence investment in cyanogenic glycosides or egg-laying effort (Jiggins, 2017; Young & Montgomery, 2020). Leveraging DNA metabarcoding across temporal and environmental gradients can thus deepen our understanding of the ecological and evolutionary dynamics structuring pollinator–plant interactions in tropical ecosystems.

Higher pollen loads in females likely reflect reproductive and physiological demands. This pattern aligns with earlier observations in other *Heliconius* species, where females forage more actively and carry larger pollen loads than males (Boggs et al., 1981; Cardoso, 2001; Mendoza-Cuenca & Macías-Ordóñez, 2005). Female *Heliconius* require a steady intake of amino acids and lipids for oogenesis and to maintain cyanogenic glycosides that serve as chemical defenses for adults and offspring (Dunlap-Pianka et al., 1977; Gilbert, 1972; O'Brien et al., 2003; Pinheiro de Castro et al., 2025). These demands cannot be met through nectar alone, making pollen essential for replenishing protein reserves. Males also benefit from amino acids for spermatophore production and maintenance (Boggs et al., 1981; Boggs & Gilbert, 1979), but carried lower pollen loads, possibly reflecting weaker nutritional demands, greater time investment in mate search, or greater efficiency in resource extraction. Female-biased foraging extends beyond *Heliconius*: across Lepidoptera, females preferentially use nutrient-rich resources supplying amino acids, lipids, and sterols required for egg production (Alm et al., 1990; Chmielewski et al., 2023; Erhardt & Rusterholz, 1998; O'Brien et al., 2004; Mevi-Schütz &

Erhardt, 2005). This sex-specific investment pattern is consistent with optimal foraging theory, where allocation tracks net fitness benefits (Houston, 1995). More broadly, sex-based differences in resource use are an important source of intraspecific variation that may influence pollination networks and community dynamics (Kishi & Kakutani, 2020; Smith et al., 2019, 2021).

We used full-spectrum wing reflectance to estimate the relative age of wild butterflies, adapting a model trained on known-age individuals. Although wing wear and colouration are widely used as age proxies in Lepidoptera (Dell'Aglio et al., 2017; Kemp, 2006), they are influenced by environmental exposure and behaviour, so reflectance-based estimates should be interpreted cautiously. In our dataset, predicted age did not explain variation in pollen richness or diversity. This contrasts with earlier studies reporting age-related peaks in pollen load (Boggs et al., 1981) and suggests that age effects in *Heliconius* may be weak or context dependent.

Our analyses revealed pronounced sexual dimorphism in the visual system, with males possessing significantly larger eyes and more facets than females. This mirrors patterns across insects, where males often exhibit enlarged eyes or acute zones that improve spatial resolution and sensitivity to moving stimuli, aiding in mate detection and tracking (Somanathan et al., 2017; Wright et al., 2024; Ziemba & Rutowski, 2000). In butterflies, such traits may help males navigate visually complex habitats, track mates more efficiently, or evade predators while exposed during mate search. By contrast, female *H. himera* may rely more on chromatic cues during foraging, especially in species where dimorphic UV opsin expression supports female-specific flower detection (Chakraborty et al., 2023; Finkbeiner & Briscoe, 2021). Alternatively, females may accumulate larger pollen loads through greater persistence at flowers; recent behavioural work shows that *Heliconius* females often revisit flowers repeatedly, leading to increased pollen accumulation (Dell'Aglio et al., 2024). Both mechanisms are plausible, and direct behavioural data from *H. himera* will be needed to determine their relative importance.

Among insects, neural investment reflects ecological demands such as foraging, navigation, and reproduction (Smid & Vet, 2016). In *Heliconius*, mushroom bodies, centres for sensory integration, learning, and memory, are markedly enlarged compared to other Lepidoptera, a pattern linked to the evolution of pollen feeding and long-term spatial memory (Sivinski, 1989; Montgomery et al., 2016; Young & Montgomery, 2020; Couto et al., 2023). In our study, *H. himera* females had significantly larger mushroom body volumes than males, consistent with the *Heliconius*-wide pattern identified by Couto et al. (2023) but absent in outgroup *Heliconiini*. One possibility is that female-biased mushroom bodies reflect greater foraging demands. However, their functional basis remains uncertain: laboratory assays of learning and memory in *Heliconius* have not detected sex effects (Hodge et al., 2025; Toure et al., 2020; Young et al., 2024). Alternatively, larger mushroom bodies may arise as a by-product of higher reproductive investment. Females typically carry larger pollen loads and engage in prolonged or repeated flower visits (Dell'Aglio et al., 2024), and the nutrients obtained may enable greater allocation to neural tissue. At present, these explanations remain speculative, and there is no direct evidence that larger mushroom bodies enhance cognitive or foraging performance in females.

Together, our findings show that female *H. himera* carry larger pollen loads than males, yet both sexes exploit a similar diversity and composition of floral resources, indicating that sex-specific differences arise from foraging effort rather than plant choice. In patchy montane environments, such a generalist strategy may facilitate colonisation of high-altitude habitats. Gut samples proved particularly informative, capturing a broader and more integrated record of pollen use than proboscis samples and underscoring the importance of tissue choice in dietary inference. We also documented sexual dimorphism in eye and brain morphology, consistent with patterns across *Heliconius*, although the functional significance of these traits remains unresolved. Finally, our use of DNA metabarcoding highlights the value of molecular tools for reconstructing

butterfly diets and linking sex-specific foraging to broader questions of intraspecific variation and pollination ecology.

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Conflict of interest

The authors have no conflict of interest to declare.

Author Contributions

J.B.: conceptualization, data curation, formal analysis, investigation, visualization, writing—original draft, writing—review and editing; D.F.R.S.: conceptualization, investigation, writing—review and editing; D.S.W.: methodology, writing—review and editing; S.H.M.: conceptualization, supervision, writing—review and editing; A. K.: resources, formal analysis, writing—review and editing.; R.M.M.: conceptualization, resources, supervision, writing—original

draft, writing—review and editing.

Data Availability Statement

All data and analysis are available from the Dryad Digital Repository.

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