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Brain size scaling through development in the whitelined sphinx moth (*Hyles lineata*) shows mass and cell number comparable to flies, bees, and wasps

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ABSTRACT

Factors regulating larval growth and determinants of adult body size are described for several holometabolous insects, but less is known about brain size scaling through development. Here we use the isotropic fractionation ("brain soup") method to estimate the number of brain cells and cell density for the whitelined sphinx moth (Lepidoptera: *Hyles lineata*) from the first instar through the adult stage. We measure mass and brain cell number and find that, during the larval stages, body mass shows an exponential relationship with head width, while the total number of brain cells increases asymptotically. Larval brain cell number increases by a factor of ten from nearly 8000 in the first instar to over 80,000 in the fifth instar. Brain cell number increases by another factor of 10 during metamorphosis, with the adult brain containing more than 900,000 cells. This is similar to increases during development in the vinegar fly (*Drosophila melanogaster*) and the black soldier fly (*Hermetia illucens*). The adult brain falls slightly below the brain-to-body allometry for wasps and bees but is comparable in the number of cells per unit brain mass, indicating a general conservation of brain cell density across these divergent lineages.

1. Introduction

Holometabolous insects (Insecta: Endopterygota) have complex life cycles, experiencing two life stages that are radically different in form and function. This modularity allows natural selection to act on phenotypes of each life stage somewhat independently (Moran, 1994), and has likely contributed to the species diversity observed in holometabolous lineages (Yang, 2001). Organisms with complex life cycles often show some degree of decoupling between growth and reproduction and in holometabolous insects mature size is determined by the final juvenile stage, with adult female body size correlated with fecundity for many clades (Honěk, 1993). Thus, during the larval stage much of the insect's physiology and behavior serve to maximize growth, while in the adult stage they serve purposes of dispersal, mating, and egg-laying. In conjunction with this, the larval brain contains cells differentiated for larval behaviors and those in stages of quiescence for differentiation and growth into the adult brain (Hartenstein et al., 2008; Andrade et al., 2019; Farnworth et al., 2022). Given the focus on growth in the larval stage, it is not surprising that larval foraging behavior varies widely across Holometabola. For example, the precocial larvae of moths, predaceous beetles, and sawflies often actively seek food sources (e.g., Hileman et al., 1994; Flowers and Costa, 2003; Singer et al., 2004), whereas the altricial larvae of many aculeate wasps and bees, along with all ants, are provisioned by their mother or sisters (Field, 2005; Hunt, 2007; Field et al., 2020; da Silva, 2021); the similarly altricial parasitoid wasps and fly species complete development in the food source where they hatch (Shaw, 2006; Mills, 2009; Labandeira and Li, 2021; Sýkora et al., 2022). While much is known about regulation of larval body size and physiological cascades that determine the timing of metamorphosis in holometabolous insects, relatively less is known about brain development or the neuroethology of larval behaviors outside of model organisms.

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The most well-characterized insect nervous system is that of the vinegar fly (Diptera: *Drosophila melanogaster*), for which detailed information on brain morphology (Court et al., 2023), development (Hartenstein et al., 2008), gene expression (Brunet Avalos et al., 2019), and even a synaptic-level connectome of the first instar larval brain (Winding et al., 2023) are available. While providing an impressive amount of detail, this is likely a representative example of the structure and development of the nervous system of a holometabolous insect, rather than the canonical one. For example, while the general patterns of embryonic brain development are highly conserved across insects (Urbach and Technau, 2003), there are differences in larval cell number and phenotypes that likely reflect selection on behavior in the brain. In Hymenoptera (sawflies, wasps, bees, and ants), behavioral shifts to parasitism and nest-building are associated with increasing mushroom body complexity (Oya et al., 2017), as measured by size and number of cell types (Kuwabara et al., 2023).

The whitelined sphinx (Lepidoptera: Sphingidae: *Hyles lineata*) is a large-bodied, fast-flying moth that nectars from and serves as important pollinator for a wide variety of plant species (Raguso et al., 2003; Alarcón et al., 2008; Smith et al., 2022). Most sphinx moths are nocturnal or show crepuscular activity patterns, but several genera in the subfamily Macroglossinae show diurnal foraging behavior. *H. lineata* can be observed foraging during the day, but is active at night and shows distinct, crepuscular activity peaks in lab assays (Broadhead et al., 2017). In controlled conditions *H. lineata* caterpillars develop through five larval instars before pupating to become adults, but it is likely that variation in food quality or quantity and genetic background influence instar number, as has been documented in another sphinx moth, the tobacco hornworm, *Manduca sexta* (Esperk et al., 2007; Grunert et al., 2015). Casey (1976) notes that *H. lineata* shows greater intraspecific variation in adult body size than other sphinx moths, but the variables contributing to this are not documented. Caterpillars display a highly variable cuticular coloration polyphenism, making them particularly useful in studies of phenotypic plasticity and gene–environment interactions (Francois and Davidowitz, 2020).

Being the earliest diverging lineage of its genus (Hundsdoerfer et al., 2009), *H. lineata* is also particularly interesting from the perspective of trait evolution. For example, the caterpillars are broad generalists, foraging on plant species from at least 20 families (Robinson et al., 2023a), while within the genus, monophagy of toxic host plants has likely evolved multiple times (Hundsdoerfer et al., 2009) and coincides with shifts in caterpillar coloration (Robinson et al., 2023b). Overall, given their powerful flight, large size, and role as pollinators and herbivores, sphinx moths are interesting both ecologically (e.g., Balbuena et al., 2022) and evolutionarily (e.g., Bischoff et al., 2015; Kay et al., 2019), serving as important sources of study for neuroecology and neuroethology (Reisenman and Riffell, 2015; Stöckl et al., 2016; Bisch-Knaden et al., 2018; Stöckl and Kelber, 2019). Additionally, given the precocial nature of larval Lepidoptera, moths are an apt system to study how the nervous system allocates resources to two distinct, behaviorally charismatic life stages, and add to our understanding of the ways environmental variables affect brain growth and differentiation.

Estimates of larval brain size at different instars are available for *D. melanogaster* (Power, 1952; Mu et al., 2021; Jiao et al., 2022; Winding et al., 2023), the black soldier fly (Diptera: *Hermetia illucens*, Barrett et al., 2022), the red flour beetle (Coleoptera: *Tribolium castaneum*, Farnworth et al., 2022), but brain size scaling is not well-characterized in any butterfly or moth species, where, unlike these others, the larval stage often wanders widely and makes foraging decisions (Heinrich, 1979; Singer and Stireman, 2001; Bernays et al., 2004). While mass and volume provide important insights about how brains scale in size, these methods cannot distinguish changes in cell number from neuropil volume, which may have important implications for understanding how natural selection drives changes in brain size. Here, we use the isotropic fractionator (IF) to estimate developmental brain size scaling by mass and cell number in *H. lineata*. We compare our findings with estimates from developmental and adult studies of brain mass and cell density in other highly visual insects. These are the first estimates for brain cell number in a moth and serve as an important addition to comparative work on developmental and interspecific brain size scaling in holometabolous insects.

2. Materials and methods

2.1. Insect specimens

Specimens for this study were obtained from the lab colony of *H. lineata* maintained by the Davidowitz lab at the University of Arizona, except a single adult female collected at a porch light in Oro Valley, AZ (Supplemental Table S1, Specimen ID 22080701). The lab colony originated from eggs and larvae collected in the Colorado Springs area (Eldorado County, CO) in 2009. To maintain diversity, freshly collected individuals from the source population are introduced into the lab colony every 4–5 years and lab populations are kept at a minimum 250 adults per generation. Larvae are reared in environmental chambers at 27 °C with 40–50% humidity and a 16-h photoperiod, and provided ad libitum access to an artificial wheat germ-based diet (Davidowitz et al., 2003). Adults were maintained in a flight cage with ad libitum access to a sponge saturated with a 20% sucrose solution and host plants (*Oenothera caespitosa*) for oviposition.

2.2. Sample preparation

Individual larvae or adults were immobilized on ice, then for second (L2) through fourth (L4) instar larva and adults, total body mass was measured with an analytical balance (Mettler Toledo AT261, Marshall Scientific, Hampton, NH) and head width was measured with digital calipers (VXB Ball Bearings Company, Anaheim, CA). First instar (L1) larvae were too small to collect morphometric data in this way. Heads were severed from bodies and placed in 4% paraformaldehyde in phosphate buffered saline (PBS; Sigma–Aldrich cat. #P4417) for 24–48 h on a shaker at room temperature. Brains were dissected out on SYLGARD® coated petri dishes (Sigma–Aldrich, St. Louis, MO, USA). Brains damaged during dissection were discarded. Larval brains were separated from the subesophageal ganglion (SOG). Retinas were removed from adult brains and the brains were then separated into optic lobes and central brain using microscissors. Excess buffer was first wicked from brains using a small piece of paper towel and, to prevent desiccation during weighing, brains were transferred to a small droplet of 80 % glycerol in a 1.5 mL centrifuge tube cap tared on the analytical balance. L1, L2, and third (L3) instar brains did not have enough mass to register on the analytical balance and therefore their mass was approximated using volume (see below) and the measured mass per unit volume of L4 brains. Brains were stored in 0.5 % cacodylate buffer solution at 4 °C until homogenization.

2.3. Isotropic fractionation

We followed the IF and counting protocols described in detail in Godfrey et al. (2021) and adapted from Herculano-Houzel and Lent (2005). Brains were homogenized with a 0.5 mL tissue grinder (Cole–Parmer, Vernon hills, IL, U.S.A.) in a small volume of (100–200 μL) dissociation solution (40 mM sodium citrate in 1%

Triton-PBS). Following homogenization, the solution was diluted with PBS and nuclei were labeled with 10–50 μL of 1:1000 SYTOX™ Green Nucleic Acid Stain (5 mM in PBS; Invitrogen Cat. S7020). The final concentration of SYTOX™ in homogenized samples ranged from 1:15,000 to 1:30,000 (Supplemental Table S1). Following homogenization, 10 μL of IF brain solution was loaded into a hemocytometer (Neubauer Improved Bright-Line Cell Counting Chamber; Jiangsu Co. Ltd, China) and counted. Cells were counted under epifluorescence using a 40× objective on an Axioplan upright microscope (Zeiss, Göttingen, Germany).

We used shape, brightness, and size to consistently identify nuclei, only counting an object as a nucleus if it had a clear boundary that comprised more than half of a circular or oval shape (i.e., not fragmented into less than half of a circle or oval) but acknowledge there is subjectivity to counting nuclei in both cell chambers and FIJI images (see Supplemental Fig. S1 for examples of nucleus morphology). To increase consistency, during training two observers counted the same brain until counts were similar and estimates of total brain cells for each life stage were obtained by two observers. Rarely, homogenized samples would contain clumps of >35 nuclei and, when these were encountered repeatedly in the first four sampling grids, samples were re-homogenized and counting proceeded. In such cases, only counts recorded after re-homogenization were used in estimates of total brain cells. If re-homogenization did not resolve the presence of these clusters, the sample was discarded and data were not included in further analyses. We observed this clumping primarily with adult brain tissue homogenization.

2.4. Whole-mount cell counting

Given that L1 were brains were too small to homogenize individually and mass could not be measured from L1, L2, or L3 using the analytical balance, we employed confocal microscopy to gather data for these life stages. To estimate the nuclei numbers in first (L1) and second (L2) instar brains and volume of L1, L2, and L3 brains, we used a confocal microscope to image entire intact brains mounted on slides. Whole-mount counts from L2 brains were compared with those from IF to ensure estimates between the two methods were comparable. To approximate the mass of L1, L2, and L3 brains, we imaged and measured volume from two fourth instar (L4) brains for which we had measured mass (Supplemental Table S2). Unfortunately, the quality of the L3 brain and of the images were poor and we could not confidentially measure the volume of the L3 brain. Therefore, while we have IF estimates for this life stage, we are missing brain mass. Brains used for whole-mount quantification were submerged in 1:1000 SYTOX™ on a glass slide for 30 min. This solution was replaced with increasing concentrations of glycerol in PBS (40% for 1 h, 60% for 3–5 h, and 80% overnight). The glycerol was then replaced with Mowiol® (Sigma–Aldrich Cat. 81,381) mounting media. Brains were imaged at 1–3 μm optical sections using a 20× or a 40× objectives on a Zeiss LSM880 inverted confocal microscope. Using the polygon tool in FIJI, the cell rind and whole-brain volumes were traced in each section to estimate total rind and brain volume, respectively. Using the multi-point tool in FIJI (Schindelin et al., 2012), nuclei were counted in two or three adjacent sections at once to prevent over-counting nuclei that spanned multiple sections. For one of the L2 brains two observers counted different couplets of adjacent sections to ensure consistency. This allowed us to estimate the average number of nuclei per section in two ways, averaged over two or three sections at a time. These methods produced similar estimates of whole-brain nuclei number from a sample, but the three-section method was generally more conservative and chosen for comparative analyses (Supplemental Table S2, Estimate 2).

2.5. Comparisons with published data

Black soldier fly (referred to here by its genus, *Hermetia,* to prevent confusion with *H. lineata*) brain size and cell number estimates were obtained from Barrett et al. (2022). *Hermetia* brains had been fixed in an alcoholic fixative, which dehydrates the tissue, therefore to perform cell density comparisons with *H. lineata*, we scaled the mass of these brains using the previously reported equation for comparison with brain tissue fixed with an aqueous fixative ($y = 1.3295x + 0.0261$, Godfrey et al., 2021). Data on body mass, brain mass, and brain cell number for bees, ants, and wasps (Hymenoptera) were obtained from Godfrey et al. (2021). Brain cell estimates for *H. lineata* were plotted on top of the existing phylogenetic least squares regressions to illustrate similarities or differences.

2.6. Statistics

Statistical analyses and data visualization were performed in R (version 4.1.2 (2021-11-01) – "Bird Hippie") using the Rstudio interface (Ghost Orchid" Release, 077589bc, 2021-09-20). Non-linear least squares were performed using the *nls* function in R (Bates and Chambers, 1992) to describe the relationship between larval head width and either body mass or brain cell number. Analysis of variance was used to compare mean brain cell number across life stages. This was achieved by first building a linear model using the *lm* function from the R Stats package (R Core Team and contributors) and then running the *Anova* function from the 'car' (Companion to Applied Regression; Fox and Weisberg, 2019) package on this linear model. Pairwise comparisons of life stage-specific brain cell numbers were performed using the *lsmeans* function with Tukey's HSD to adjust the p-value for multiple comparisons. Citations for all R packages used for data anlaysis and visualization are reported in the Supplementary materials reference section. A comparison of mean cell densities between brain regions of *H. lineata* and *Hermetia* was performed using an unpaired t-test (MedCalc Software Ltd, 2023) with p-values adjusted for multiple comparisons using the *p.adjust* function in R with a Bonferroni correction.

3. Results

3.1. The isotropic fractionation method

As observed for other insects, we found the IF method suitable for estimating brain cell number in *H. lineata*. The coefficient of variation (CV) for a particular sample gives an indication of how well the samples have been homogenized (Herculano-Houzel and Lent, 2005). For larval brains, we observed a mean CV of 0.232 ($s = 0.025$), which decreased for our adult estimates to 0.146 ($s = 0.032$) for the central brain and 0.156 ($s = 0.057$) for the optic lobes (Supplemental Table S1). Overall, these CVs are larger than the <0.10 reported for vertebrates (Herculano-Houzel et al., 2011), but comparable to the CV = 0.203 reported for adult ant, bee, and wasp brains (Godfrey et al., 2021) and the CV = 0.2479 for singly counted adult *D. melanogaster* brains (Raji and Potter, 2021). As a second assessment of homogenization, we plotted brain cell estimates by subsample (counting grid) to test if there was a correlation between total brain cell estimate and the order in which the subsample was taken from the homogenate. We detected a negative correlation for the smallest brains (L2, $r^2 = -0.604$, $p < 0.001$; Fig. S2), which were also diluted the least for counting (Supplemental Table S1). This effect is driven in part by only four estimates from grids 12–16 for one L2 brain. In this instance the observer included nuclear fragments (less than half a circular

boundary) in the counts, and therefore the remaining homogenate was re-counted with only four grids. However, a correlation is still detected if we remove this brain ($r^2 = -0.494$, $p = 0.014$), suggesting adequate homogenization is difficult with such a small amount of tissue. Notably, the CV L2 brains is 0.197 ($s = 0.12$), indicating variation in intra-sample estimates is similar to other life stages. We did not detect a significant correlation at any of the other larval stages (L3, $p = 0.95$; L4: $p = 0.67$; L5, $p = 0.07$).

3.2. Changes in cell number through larval development

H. lineata caterpillars experience exponential growth in body size between the second and fifth instar (Figs. 1A and 2A). As with other holometabolous insects, the larvae hatch with a fully developed larval brain (Fig. 1B) and brain cell number increases asymptotically during larval development (Fig. 2B). Over the course of metamorphosis body size decreases slightly (Fig. 2C, Table S3), as is commonly observed in holometabolous insects (Odell, 1998; Kraus et al., 2014; Ormerod et al., 2017; Hanna et al., 2023) and may be in part due to water loss. Brain mass increases nearly 30-fold at this time, from 0.0867 mg ($s = 0.028$) in the fifth instar to 2.502 mg ($s = 0.471$; Fig. 2C) in the adult, while brain cell number increases over 10-fold, from 81,806 ($s = 6163$) to 914,816 ($s = 56{,}334$; Fig. 2D).

We did not detect a significant increase in cell number during the first molt, but with 8002 ($s = 544$) cells in the first instar and 15,054 ($s = 2818$) in the second, it is likely that sample sizes and the variation in L2 estimates affected our ability to detect differences using an analysis of variance that included all stages. Second instar estimates varied from 12,800 to 18,433 (Supplemental Table S1) and the two whole-mount brains were estimated to have 15,734 and 11,953 cells, respectively (Supplemental Table S2). We also detected significant differences across observer IF based estimates at this life stage (Supplemental Fig. S3). As noted with our homogenization analysis, it seems that the small size of these brains, potentially combined with nuclei morphology or active cell replication at this stage, affected estimates. In studies of smaller brains, authors have pooled samples to reduce variation in estimation (Raji and Potter, 2021), but we did not have the sample material to do that here.

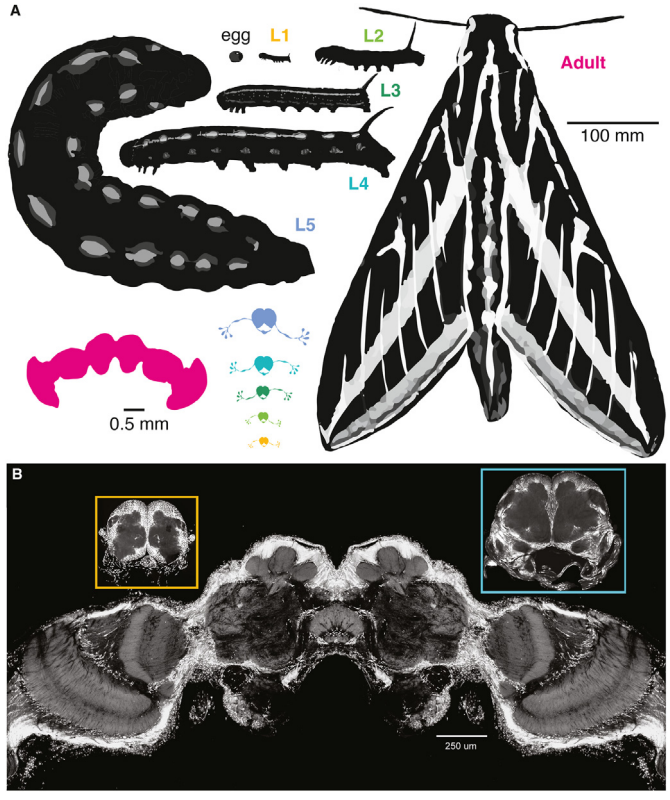

Fig. 1. Size scaling through development in *Hyles lineata*. (A) Greyscale silhouettes showing relative size scaling for five larval instars and adult of *H. lineata*, with relative brain sizes through development (lower left). (B) Section through first instar (L1, yellow outlined inset, left), second instar (L2, teal outlined inset, right), and adult (retina included) brains; nuclei labeled with SYTOX. Adult brain image presented as a mirrored image of the left side of the brain.

3.3. Adult brain size and region-specific cell numbers

In adults we measured brain mass and cell number in two brain regions separately, the optic lobes and the central brain. We did not detect sexual dimorphism in adult brain mass (Mann–Whitney U test: $W = 15$, $p = 0.2857$) nor in total brain cell number (Mann–Whitney U test: $W = 9$, $p = 0.9048$), but note that male cell estimates, particularly for the optic lobes, are more variable than female estimates (Fig. 3A). The optic lobes comprise nearly two thirds of adult brain mass (~1.5 mg, 61.19%) and brain cells (~590,000, 64.45%), meaning cell density is comparable in the optic lobes and central brain (Fig. 3B; Supplemental Table S4). This is in contrast with the *He. illucens*, in which the optic lobes comprise the majority brain mass and show higher cell density than the central brain (Barrett et al., 2022, Fig. 3B). Thus, while *H. lineata* has higher cell density in the central brain than the black soldier fly (unpaired t-test vs. females: $t = 4.801$, $df = 19$, $p_{adj} = 0.0001$, vs. males: $t = 3.326$, $df = 18$, $p_{adj} = 0.0028$), total brain cell density is lower than that of male black soldier flies, specifically (unpaired t-test, $t = -5.945$, $df = 18$, $p_{adj} = 0.0006$), an effect driven by male optic lobe cell density (unpaired t-test, $t = -5.286$, $df = 18$, $p_{adj} = 0.0006$; Fig. 2B; Table S5).

Since these are the first estimates of brain cell number from the IF method for a moth, we also compared our findings with existing brain cell number estimates for Hymenoptera (Godfrey et al., 2021). When we superimpose our *H. lineata* data onto these estimates, we find this moth has fewer brain cells than bees and wasps of a similar body size (Fig. 4A), but comparable brain cell density (Fig. 4B). While estimates for Hymenoptera are only available for adults, we included larval estimates for *H. lineata* in these plots, which illustrates that fifth instar larva have brains comparable in size and cell number to ants (Formicidae).

4. Discussion

Bilaterian brains likely share a common origin (Bailly et al., 2013; Strausfeld and Hirth, 2013; Wolff and Strausfeld, 2016) and, despite astounding differences in size and architecture, all begin as a small number of ectoderm germ layer cells and progress through generally analogous patterns of proliferation and differentiation (Arendt and Nübler-Jung, 1999; Brand and Livesey, 2011; Chen and Konstantinides, 2022). Final brain size is determined largely by a developmental relationship with body size, and deviations from general allometric brain-body mass slopes are hypothesized to be indicative of selection on cognitive function (Jerison, 1973; Striedter, 2005). However, selection on body size is complex and clade-specific shifts in brain-body allometry appear to be common, at least in vertebrates (Smaers et al., 2021). Comparisons of regional differences in brain size (e.g., Stöckl et al., 2016; Godfrey and Gronenberg, 2020; Barrett et al., 2021) or changes in the number of cells (e.g., Surchev et al., 2007; Azevedo et al., 2009; Herculano-Houzel et al., 2011) across clades can add acuity to evolutionary and functional analyses. And, while insect brain size is generally

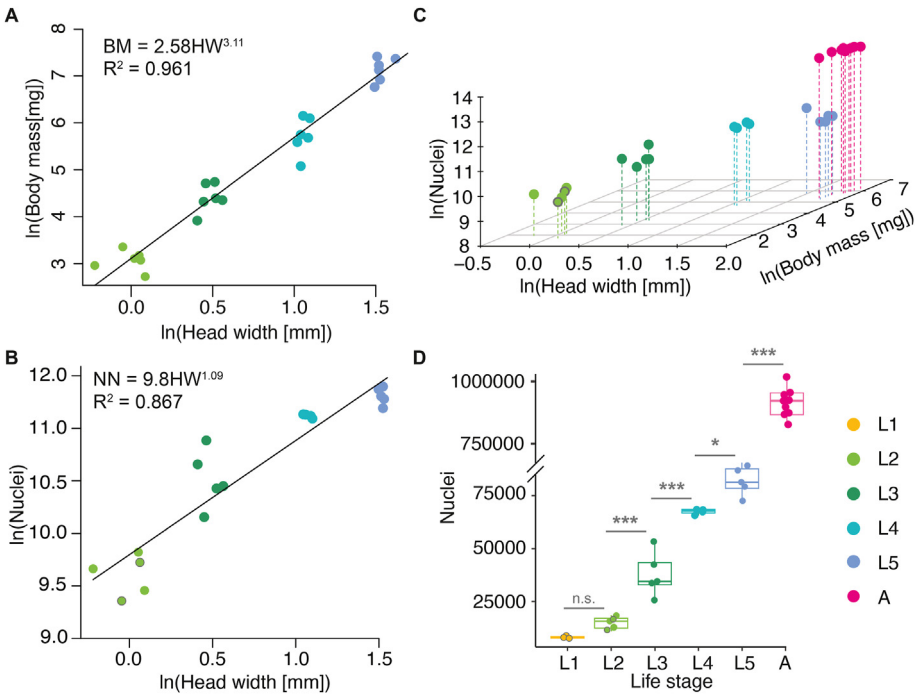

Fig. 2. Brain scaling through development in *Hyles lineata*. Exponential regression for (A) Body mass and (B) total brain nuclei over the course of larval development. (C) Log-transformed values of body mass, head width, and nuclei number across development for life stages where all three variables were measured. (D) Changes in brain nuclei number over development for all life stages. Circles with gray borders indicate nuclei were estimated from whole-mount brain images. BM = body mass, HW = head width, NN = nuclei number, L1 = 1st instar, L2 = 2nd instar, L3 = 3rd instar, L4 = 4th instar, L5 = 5th instar, A = Adult. * = $p < 0.05$, *** = $p < 0.001$.

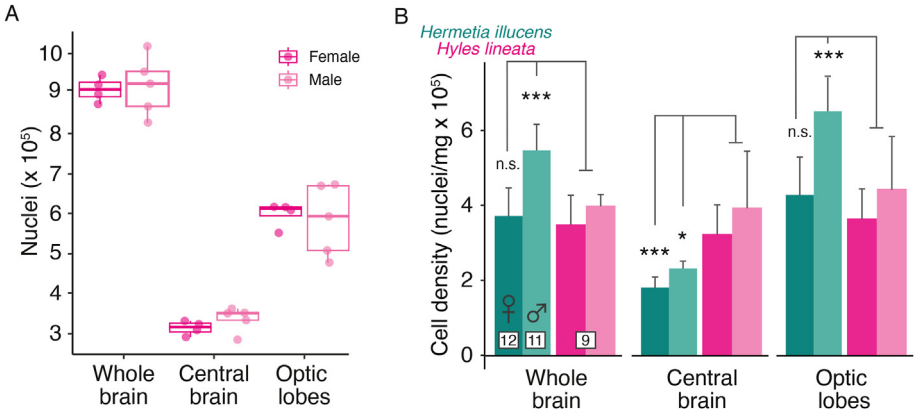

Fig. 3. *Hyles lineata* adult brain size by total cell number and density. (A) Total brain and region-specific cell numbers in males and females. (B) Comparisons of brain cell density between *H. lineata* and the black soldier fly (*Hermetia illucens*). Numbers at the base of the leftmost bars indicate sample sizes, with *H. lineata* sexes pooled. Data for *H. illucens* from 2 of Barrett et al. (2022). Summary statistics for (A) listed in Supplemental Table S3, summary and test statistics for (B) listed in Supplemental Tables S4 and S5, respectively. * = $p < 0.05$, *** = $p < 0.001$.

measured by mass or volume (e.g., Wehner et al., 2007; Seid et al., 2011; Godfrey and Gronenberg, 2020), data on total brain cell number are now available for an increasing number of invertebrates (e.g., Barrett et al., 2021; Godfrey et al., 2021; Jiao et al., 2022). Here we provide the first estimates of brain cell scaling through development and in the adult optic lobes and central brain for a moth, the whitelined sphinx (*H. lineata*). A crepuscular and nocturnally active pollinator with fast and hovering flight capabilities, its behavioral ecology is somewhat unique from the Hymenoptera and Diptera for which brain cell estimates currently exist.

The brain of *H. lineata* increases over 100-fold in cell number during development from approximately 8000 in the first instar to over 914,000 in the adult. Naturally, the majority of these cells are added during metamorphosis, but *H. lineata* experiences growth in brain cell number at each larval molt. This fold change through development is intermediate to growth reported for fly development: *D. melanogaster* shows a nearly 45-fold increase in cell number through development from just over 3000 cells in the first instar (Winding et al., 2023) to 133,000 cells in the adult (Mu et al., 2021), whereas *Hermetia* increases over 140-fold from approximately 2000 cells in the first instar to over 330,000 cells in the adult (Barrett et al., 2022). In *H. lineata* brain cell numbers increase asymptotically during the larval stages; between the 3rd and 4th instar almost 30,000 cells are added, but between the 4th and 5th instar the brain grows by only approximately 15,000 cells. This pattern of growth may also exist for *Hermetia*, as there is a steeper

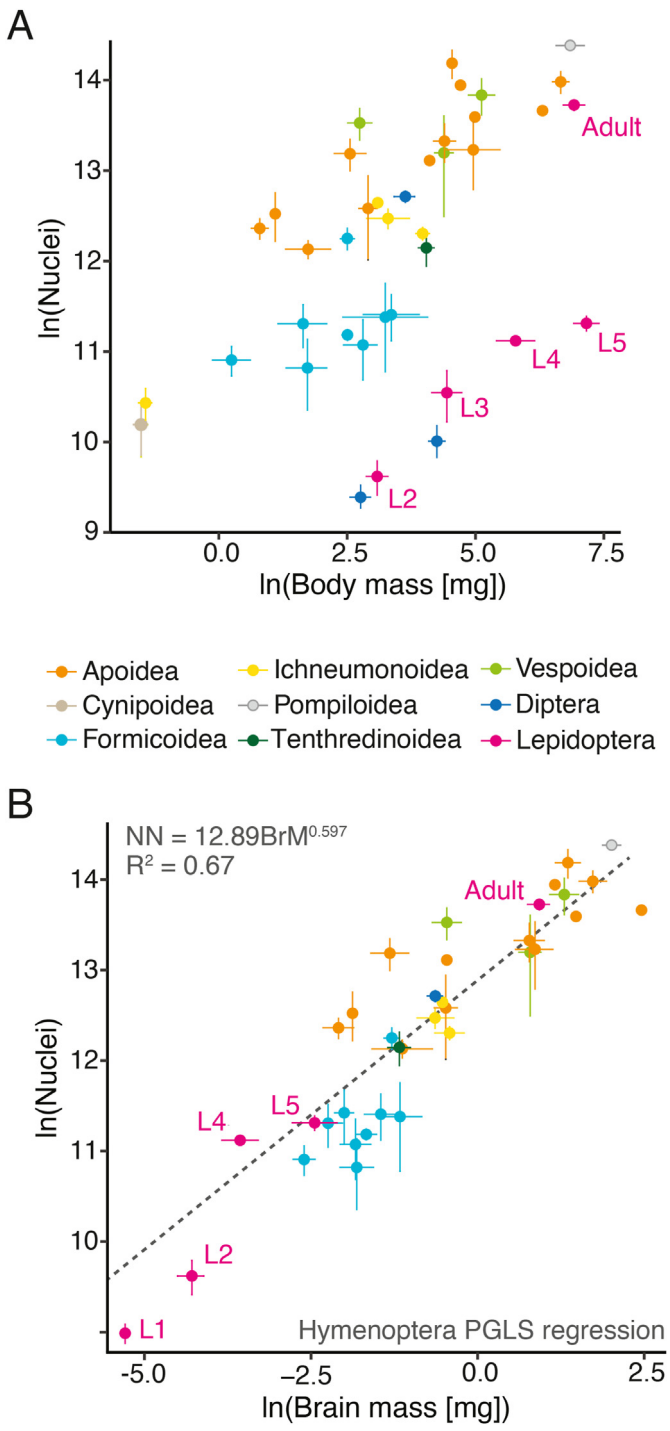

Fig. 4. Comparison of *Hyles lineata* brain cell number with other insects. The whitelined sphinx mean total brain cell number is (A) slightly lower than Hymenoptera of a similar body size, but (B) comparable to Hymenoptera brains of a similar mass. Diptera data points in (A) represent L4, L6, and adult values for *Hermetia illucens* from 1 of Barrett et al. (2022). Diptera data point in (B) from 2 of Barrett et al. (2022). Data for Hymenoptera and phylogenetic least squares regression statistics from Godfrey et al. (2021). Error bars represent standard deviation for each axis.

increase in cell number between the second and fourth instar than between the fourth and sixth (Barrett et al., 2021). Despite detailed information on brain development in *D. melanogaster*, second instar brain cell estimates are not available. However, the pattern of growth described in developmental studies suggests it might also grow asymptotically. In *D. melanogaster* the larval brain develops during embryogenesis and the majority of neuroblasts enter mitotic quiescence before hatching until the end of the first instar. At this time, different sets of neuroblasts are reactivated through the second instar until all are proliferating in the third instar (Hartenstein et al., 2008), with the precise timing of neuroblast re-entry into a proliferative state modulated by insulin signaling (Brogiolo et al., 2001) and nutrient availability (Chell and Brand, 2010). However, while many insects, including *Hermetia* and *H. lineata*, show developmental plasticity in larval instar number, often increasing instars under nutrient deficiency or environmental stress (Esperk et al., 2007), *D. melanogaster* develops invariably through three instars (Hutchinson et al., 1997). Therefore, the timing or pattern of brain cell proliferation may be different in insects with variable instar number and understanding how this trait influences larval brain development or adult brain size will require more detailed characterization of species that show such developmental plasticity.

With over 900,000 cells, the whitelined sphinx moth brain is similar in cell number to the cicada killer (*Specius* sp., ~860,000) and the yellow paper wasp (*Polites flavus*, ~1 million cells). Estimates from Hymenoptera included portions of the retina, tissue that was completely removed prior to counting cells for *H. lineata*, so it is likely that *Hyles* brain cell numbers are close to the larger wasps from the Godfrey et al. (2021) dataset. The optic lobes comprise approximately 60% of *H. lineata*'s brain mass, more closely resembling the relative size of the optic lobes in the diurnal hummingbird hawkmoth (Sphingidae: *Macroglossum stellatarum*; ~57% total brain volume) than the nocturnal elephant hawk moth (Sphingidae: *Deilephila elpenor*, ~49% of total brain volume) (Stöckl et al., 2016). All three are in the hawk moth subfamily Macroglossinae, where diel shifts from nocturnal to diurnal patterns of activity have evolved at multiple times (Akiyama et al., 2022). *H. lineata* is not considered strictly diurnal, but exhibits a broad diel activity pattern (Broadhead et al., 2017) and can be observed nectaring during the day (personal observation). Approximately ~65 % *H. lineata* brain cells are in the optic lobes and it would be interesting to know the relative contribution of changes in cell number and size to observed differences across moths that vary in diel activity patterns.

We can make these comparisons with flies, for which brain region size and brain cell number estimates exist. In *D. melanogaster* the optic lobes comprise ~48% of brain volume (Özer and Carle, 2020), but contain between ~90,000 (Mu et al., 2021) and ~120,000 (Özel et al., 2021) cells to the central brain's ~43,000. *Hermetia* also have ~43,000 central brain cells and impressive ~145,000 cells in each optic lobe, which together comprise greater than 70% of brain mass (Barrett et al., 2022). Thus, in flies the optic lobes are more cellularly dense than the central brain, whereas in *H. lineata*, where the optic lobes comprise ~60% of the brain, mean cell density across regions is more homogenous, with comparable estimates for the optic lobes and central brain. In terms of overall size and cell number, the brain of *Hermetia* is a scaled down version of *H. lineata*, but because these flies have a greater majority of brain cells in the optic lobes, *H. lineata* has greater central brain cell density, suggesting a greater relative number of cells in circuits underlying olfactory or higher-order associative behaviors.

Adult *H. lineata* are active foragers, collecting nectar from a wide variety of plants and likely use multimodal sensory integration and learning for foraging and mating, as has been reported for other hawk moths (Raguso and Willis, 2002, 2005; Kelber et al., 2003; Balkenius and Kelber, 2006; KonDo et al., 2012). The behavioral ecology of *Hermetia* suggests they may use a smaller diversity of cues or less multimodal sensory integration than has been described for hawk moths. These flies are diurnal and require high-

intensity, natural lighting for males to recognize and intercept females (Tomberlin and Sheppard, 2001, 2002; Zhang et al., 2010); sexual dimorphism in optic lobe cell density may be an adaptation for this behavior (Barrett et al., 2022). Adults show innate preferences for color (Romano et al., 2020), and likely use olfactory cues to find oviposition sites (Scieuzo et al., 2021), but the extent of multimodal sensory integration or learning is not documented. Thus, while the behavioral ecology of these two species correlates with cell density in different brain regions, we advise caution in making direct comparisons with such coarse morphological data. It is likely that both species integrate sensory information and learn, and differences in cues used in feeding, mating, and oviposition, along with the large phylogenetic distance between these species, limits the utility of direct comparisons for insights into neuroethology or brain evolution. Instead, it will be more useful to compare shifts in brain cell number or density across a larger sample of taxa, or across specific brain regions of close relatives.

In this study we provide the first estimates of brain cell number during development and regions of the adult brain of a hawk moth (Lepidoptera: Sphingidae). We add to a rapidly increasing amount of data on comparative insect brain morphology (Heinze et al., 2021) and provide an important example of how brain cell number can contribute to understanding how Lepidoptera brains change in size over evolutionary time. More broadly, factors determining brain size have important implications for processes ranging from trait evolution (e.g., Evans et al., 2005; Zwarts et al., 2015; Jansen et al., 2020; Sayol et al., 2020) to disease (e.g., Coll-Tané et al., 2019; Jean et al., 2020) and measuring brain size and cell number allows us to begin to tease apart the relative contributions of these variables in brain size scaling both within species and across clades.

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CRediT authorship contribution statement

Isabel C. Aksamit: Validation, Resources, Methodology, Investigation, Data curation, Conceptualization. **Felipe Dorigão-Guimarães:** Validation, Methodology, Investigation. **Wulfila Gronenberg:** Visualization, Supervision, Resources, Methodology. **R. Keating Godfrey:** Visualization, Validation, Supervision, Project administration, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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