

RESEARCH

Open Access



Butterfly brains change in morphology and in gene splicing patterns after brief pheromone exposure

Emilie Dion^{1,2*}, Yi Peng Toh^{1,3}, Dantong Zhu¹ and Antónia Monteiro^{1*}

Abstract

How insect brains differ between the sexes and respond to sex-specific pheromones is still not well understood. Here we briefly exposed female *Bicyclus anynana* butterflies to wild type and modified male sex pheromone blends, previously shown to modify females' sexual preferences, and examined how their brains were modified at the morphological and molecular levels 3 days later. First, we 3D-reconstructed male and female brains of this species and explored changes in the size of the 67 glomeruli present in the olfactory lobe. Then we showed that one glomerulus changed in volume after a blend exposure, potentially implicating it in sex pheromone perception. Finally, we found that a few genes were differentially expressed but many more were differentially spliced between male and female naïve brains, and between naïve and pheromone blend-exposed brains. These code for primarily calcium-binding channel proteins and RNA-binding proteins, respectively. A learned preference for changed levels in a single pheromone component was linked to different protein isoforms involved in synaptic transmission. Our work shows that naïve male and female brains differ primarily in gene splicing patterns and that a brief, 3-min, exposure to pheromones produces slight changes in brain volume and large changes in the splicing of genes involved in neural development, which correlate with changes in sexual preferences in females.

Keywords Plasticity, Olfactory learning, Immunostaining, Brain, Transcriptomics, Sex pheromone, Butterfly, Glomeruli

Background

Odour learning in insects can impact sex pheromone preferences and the evolution of populations and species. For instance, individuals of several species can learn to prefer new odours via exposure to those blends [1, 2]. If these odours are novel sex pheromone blends,

populations that evolve a preference for these blends can potentially undergo rapid reproductive isolation and speciation [3–5]. It is, thus, interesting to investigate how pheromone odour learning might take place at the mechanistic level.

Odour learning is a plastic behavioural response that affects all levels of the olfactory circuitry. It can be measured and examined using different physiological and molecular tools. For instance, mechanisms of chemosensory learning have been explored with electrophysiology experiments at the periphery and in the central nervous system, via the measurement of neuronal activities (e.g. [6–11]). In addition, these mechanisms have been explored at the molecular level in the insect peripheral sensory structures, such as antennae and legs, via monitoring of molecular changes after an odour exposure

*Correspondence:

Emilie Dion
emilie.dion@u.nus.edu
Antónia Monteiro
antoniamonteiro@nus.edu.sg

¹ Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, Singapore 117543, Singapore

² Fulbright University Vietnam, Tan My Ward, Ho Chi Minh City, Vietnam

³ Division of Evolutionary Biology, Faculty of Biology, Ludwig-Maximilians-Universität München, Munich, Germany



© The Author(s) 2026. **Open Access** This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

(e.g. [12, 13]). However, the molecular actors of olfactory learning in the insect's central nervous system remain underexplored.

The antennal lobes (ALs) and the mushroom bodies (MBs) are the main brain structures involved in olfactory learning [14, 15]. The AL is the primary olfactory-processing centre where olfactory sensory neurons (OSNs) from the antennae connect with projection neurons (PNs) to create globular neuropils called glomeruli. From these glomeruli, PNs connect to the MB, which are critical in olfactory learning and memory in many species [16–19]. Odours are represented in distinct glomeruli working as a functional unit, with OSNs expressing the same receptor type converging to one or two glomeruli [4, 20]. Generally, the size of a single glomerulus is assumed to reflect the number of sensory axons terminating in this structure and the size and shape of the AL, comprising all glomeruli, can be affected by odour exposure, sex, and other factors [2, 21–27]. Because of these properties, these neuropiles are good candidates to explore for plasticity in odour sensitivity at the molecular and morphological levels in males and females.

In most insects, olfactory behaviours, neuronal circuitry, and learning abilities are sexually dimorphic and reflect sex-specific adaptations to chemical environments. The olfactory systems of females and males are finely tuned towards their different ecological roles and reproductive strategies, often related to the female location of host odours for oviposition and foraging and the male detection of female sex pheromones [26]. Structural and mechanistical differences include antennal morphology (e.g. male moths' elaborate feathery antennae [28]), morphology and size of the glomeruli (e.g. the large macroglomerular complex of male moths [26]), tuning of the sensory neurons and expression of sensory-related genes (e.g. biased expression of ORs (olfactory receptors) and OBPs (olfactory binding proteins) in *Drosophila* [29] or honeybees [30]). Females and males also differ in their capabilities for olfactory memory and learning. These differences are often reflected in the sexual dimorphism of the MB size caused by changes in Kenyon cell numbers (e.g. in orchid bees [31]; in mosquitoes [32]). While sexual dimorphism in brain anatomy and structures is well documented, the molecular differences in the olfactory system between males and female insects require more examination.

To explore molecular and morphological mechanisms of olfactory preference development we used *Bicyclus anynana* butterflies as our model species. Lepidopterans use olfaction and olfactory learning in foraging and sexual selection processes (e.g. [33–37]), and olfactory learning has previously been demonstrated in *B. anynana*. Caterpillars can learn novel food odour

preferences [34, 37], and adult females of the species alter their male sex pheromone (MSP) preferences after a short exposure to new blends [35]. For example, naïve females are normally attracted to the wildtype (Wt) MSP blend, but they can learn to prefer novel blends after a short exposure to them. The Wt MSP comprises 3 components (named MSP1, 2, and 3; [38, 39]). Females exposed, upon emergence, to males having lower amounts of MSP1 and absence of MSP2 (called New Blend 1, NB1) lose this preference and accept mating with both NB1 and Wt males indiscriminately. Females exposed to blends with increased amounts of MSP2 (called NB2), later prefer these males over Wt males [35] (Fig. 1A). Although no sex-specific sensory genes were previously identified being differentially expressed in the heads of naïve butterflies [40], three OBPs and one OR were recently found upregulated in female brains compared to males immediately after both sexes were visually trained, which is unexpected [41]. Molecular details of how direct odour-learning becomes encoded and retained in the brain, however, are unknown.

Here we explore molecular and morphological correlates of pheromone olfactory learning in naïve females and in females of the same age after exposure to various pheromone blends. We also examine how male and female naïve brains differ from each other at the morphological and molecular levels. We hypothesised that brief pheromone blend exposures, as described in Dion et al. [35] (Fig. 1A), would alter the volumes of specific glomeruli of the AL responding to specific MSP components, and alter gene expression and/or splicing in the female brain a few days after the exposure event. We repeated the odour exposure experiments of Dion et al. [35] (Fig. 1A), dissected the brains of the butterflies on the second day (48 h after the exposure), instead of submitting them to mate choice assays, and then either imaged the brains or subjected them to RNA sequencing. We compared (1) naïve male and female brains to identify sex-specific differences in individuals kept completely isolated from any environmental cue for 2 days; (2) naïve and Wt-exposed female brains to discover processes triggered by environmental stimuli and specifically by Wt MSP exposure; (3) Wt-blend versus new blend (NB)-exposed female brains; as well as (4) NB1 versus NB2-exposed female brains, to identify mechanisms associated with detailed perceptions of specific MSP component change. We hypothesised that genes specifically related to changes in MSP2 would be up- or downregulated in treatments with higher amounts of these components relative to other treatments (e.g. higher in NB2 versus Wt and NB1; higher in Wt versus NB1).

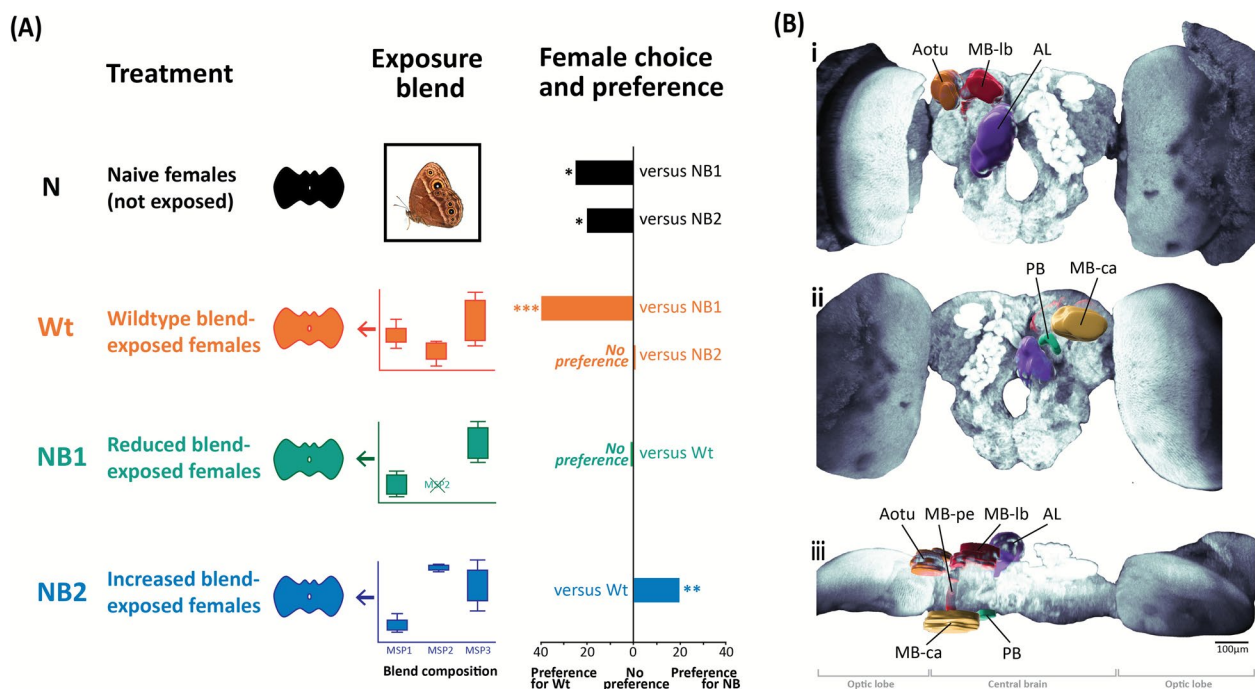


Fig. 1 Experimental procedures and brain structure identification. **A** Naive females and those exposed to different pheromone blends (Treatment, left) were previously shown to have different preferences for the blends in a mate choice assay. The blend composition shows amounts of the three male sex pheromones (MSP1, 2 and 3) used for exposure (Exposure blend, middle). Naive females were isolated and not exposed until mate choice on day 2. Female preferences (right) are shown as significant increases in choice percentage from random (50%) for the Wt or new blend (NB1 and NB2) perfumed male. Asterisks illustrate significant preferences at p value < 0.05 *, $p < 0.001$ ***, and $p < 1E-4$ **** (from [35]). **B** 3D reconstruction of the midbrain structures shown on the animal right side from an (i) anterior, (ii) posterior, and (iii) top (upper side is anterior, lower side is posterior) views. Aotu = anterior optic tubercle; AL = antennal lobe; PB = protocerebral bridge; MB-lb = mushroom body lobe; MB-ca = mushroom body calyx; MB-pe = mushroom body pedunculus

Results

B. anynana brain morphology is similar to that of other lepidopterans

To better visualise the brain's finer structures, and to perform 3D volumetric reconstructions, we stained brains with an anti-synapsin antibody. The overall layout and morphology of the brain of *B. anynana* is similar to that of other Lepidoptera (e.g. [42–46]) (Fig. 1B). The large optic lobes surround the central complex, and two pairs of ALs and MBs are present on the front and the back of the central complex, respectively. *B. anynana* ALs are

located at the anterior part of the brain tissue (~10 µm from the anterior of the whole brain) and span about 88 µm in depth. They are distinguishable by their spherical shape on either side of the oesophageal foramen, and contain “berry-like” subunits called glomeruli, arranged around the central fibrous neuropil (CFN) (Figs. 1B & 2A).

We identified an average of 67 and 68 glomeruli making up the AL in naïve females and males, respectively (Fig. 2A & B; Additional File 1, Fig. 1; Additional. File 2). This number varied from 64 to 69 across individuals, but the glomeruli count did not vary significantly across

(See figure on next page.)

Fig. 2 Glomeruli number and volumes in the different treatments. **A** Characterized and measured individual *B. anynana* glomeruli in the antennal lobes. Glomeruli are colour-coded based on their spatial position within the AL, (i) anterior to posterior, (ii) posterior to anterior, (iii) sliced image of an AL through the X–Y plane with the central fibrous neuropil labelled. A = anterior, L = lateral, V = ventral, D = dorsal, AM = Antero-Medial, AL = Antero-Lateral, AC = Antero-Central, AD = Antero-Dorsal, PL = Postero-Lateral, PV = Postero-Ventral, PM = Postero-Medial, PC = Postero-Central, PD = Postero-Dorsal, CFN = Central Fibrous Neuropil (glomeruli positions and identification are detailed in Additional File 1, Additional Fig. 1 & Result 1; Additional File 2, Table 1). **B** Each treatment AL had similar number of glomeruli. Large dots are averages; error bars are 95% confidence intervals and small dots are each data point ($n = 7$ in each treatment). **C** Glomerulus PD2 is significantly bigger in Wt-exposed compared to NB1-exposed females. PD2 sizes are equivalent in other comparisons. Large dots are averages, error bars are 95% confidence intervals, small dots are each data point ($n = 7$ in each treatment) and ** means $0.001 < \text{adjusted } p < 0.01$ (WtvsNB1 Adj $p = 0.008$)

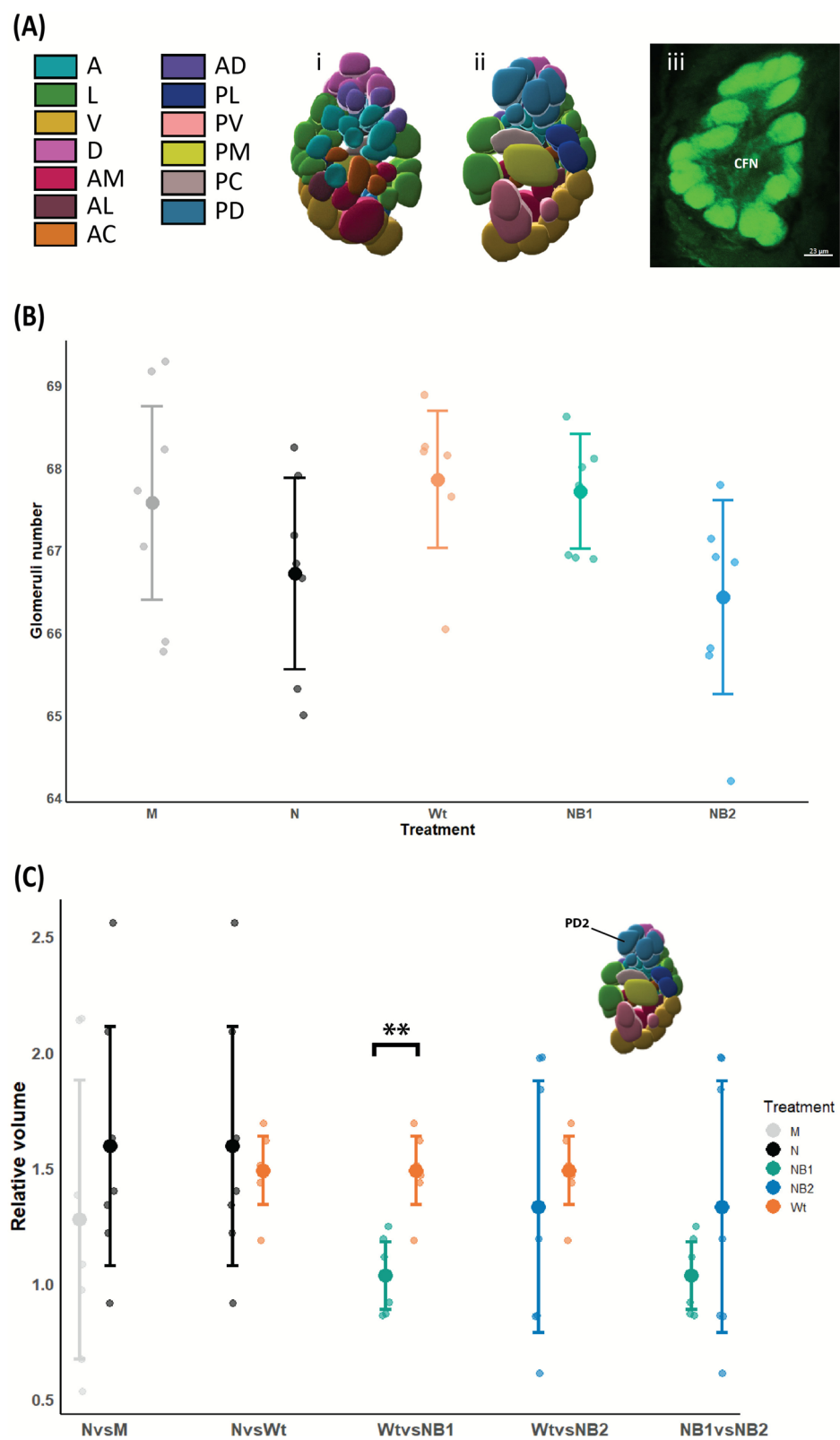


Fig. 2 (See legend on previous page.)

treatments and sexes (Additional File 1; Fig. 2). The glomeruli were categorised according to the region of the AL they are located in (A, anterior; P, posterior; D, dorsal; V, ventral; L, lateral; M, medial; C, central) and assigned a unique number to differentiate them from neighbouring glomeruli in the same region (Fig. 2A; Additional File 2, Table 1; Additional File 1, Additional Fig. 1 & Result 1). Fifty five glomeruli were successfully identified across all individuals ($n=35$) while 13 glomeruli (A2, A4, A10, AC3, AD2, AD4, AM3, D4, D6, D7, L6, L13, V10) could not be identified in at least one individual across our samples, but were still present in more than half of the individuals sampled (Additional File 2, Table 3). Glomerulus PD8 was present in all male individuals sampled, absent in all naive females, and found in 24% of exposed female brains only.

Glomeruli were of the same size in naïve males and females

To test for sex-specific differences in the AL, we compared the sizes of glomeruli of naive males and naive females. Both sexes had similar total AL volume (♀: $\bar{x}=1.14 \times 10^6 \mu\text{m}^3$, ♂: $\bar{x}=1.25 \times 10^6 \mu\text{m}^3$; $t_{10}=0.556$, $P=0.59$) and similar total volume of all glomeruli (♀: $\bar{x}=7.76 \times 10^6 \mu\text{m}^3$, ♂: $\bar{x}=9.23 \times 10^6 \mu\text{m}^3$; $t_{11}=0.432$, $P=0.674$) (Fig. 2B, Additional File 2, Table 2). All glomeruli for both females and males were similar in volume (all adjusted p values >0.05 ; Additional File 2, Table 4 and 5).

Exposure to Wt blend did not significantly affect the size of glomeruli

We tested whether exposure to the Wt male pheromone blend affected the growth of brain neuropiles in females, and compared the volumes of all glomeruli between naive females and Wt-exposed females after immunostaining. We found that no glomeruli changed significantly after exposure (all adjusted p values >0.05 ; Additional File 2, Table 5).

One of the new pheromone blends affected the size of a specific glomeruli in female brains

Next, we tested whether exposure to new pheromone blends altered the size of specific female glomeruli. We compared the volume of glomeruli of naive and exposed females after immunostaining and found that females exposed to NB1 blend (reduced blend) had a significantly smaller PD2 glomerulus compared to those exposed to Wt blend. This glomerulus was also smaller in NB1-exposed relative to NB2-exposed individuals, but this difference was not significant (Fig. 2C; Additional File 2, Table 5).

The number of OR genes, expressed at low levels in the brain, approximates the number of glomeruli

Because the number of glomeruli closely approximates the number of ORs normally expressed in cells of the peripheral nervous system, such as the antennal tips [47], we examined the number of transcripts for chemical receptors and olfactory-related proteins that are expressed in the brain using our transcriptome sequences. We counted 55 olfactory receptors genes (similar to [40]), the OR co-receptor ORCO, and three joint olfactory and gustatory receptors (OGR). We also found 41 gustatory receptors (GR), 14 olfactory binding proteins (OBP) including one pheromone-specific (PBP), 41 ionotropic receptors (IR), six sensory neuron membrane proteins (SNMP), one chemosensory protein (CSP), and 20 members of the ejaculatory bulb protein 3 (EBP3) family. We also identified ten pickpocket (*ppk*) proteins and five transient receptor potential channels (*trp*) (Additional File 3, Table 5). Most of these proteins (about 70%) are expressed at very low levels in the brains, with less than ten cumulative read counts over all libraries. It is interesting to note, however, that we identified 55 ORs and that only 55 glomeruli were consistently identified across all individuals, suggesting a close association or specificity between ORs and glomeruli number.

Sex pheromone exposure affected gene splicing more than gene expression

We generated RNAseq libraries from the butterfly brains to evaluate the impact of sex and pheromone blend exposure on gene expression and alternative splicing. Specifically, we assessed changes in total gene expression levels, types of alternative splicing events (e.g. exon skipping, intron retention), and variation in splicing patterns. We identified differentially expressed genes (DEGs), which showed significant differences in expression levels between treatments (when the log2 fold change in expression between conditions is statistically significant at adjusted $p < 0.05$). We also identified differentially spliced genes (DSGs), which exhibited significant changes in the relative inclusion or exclusion of specific splice junctions between treatments (at $\text{FDR} < 0.05$). A gene is DS when the percent spliced in index (ΔPSI , the proportion of transcripts from a gene that include a specific exon or splice junction) differs significantly between conditions. The PCA done on a random sample of 500 gene counts and the hierarchical clustering analysis showed low levels of clustering according to treatment or sex, with a small variance across samples (cumulative 38%), suggesting that brain exposure to pheromones and butterfly sex do not correlate with large shifts in gene expression (Additional File 1, Additional Fig. 2A). Three outliers belonging

to naive males, NB1-exposed and NB2-exposed females deviated significantly from the cluster on the PCA and were removed from the dataset before further differential expression analysis. The unsupervised clustering of normalised gene expression data for all samples showed only partial grouping suggesting that overall gene expression patterns are not strongly shaped by sex or treatment. Several gene clusters showed consistent expression across subsets of samples, suggesting shared transcriptional responses across sexes and treatments. We also built heatmaps based on gene expression at adjusted p value < 0.1 because we found less than 30 DEGs at adjusted $p < 0.05$ in each comparison (see results below). A limited number of DEGs were identified at adjusted $p < 0.1$ (139 in total), but the heatmaps effectively separate the sample groups, showing that the genes still capture the transcriptional difference between the treatments (Additional File 1, Additional Fig. 2B).

Clustering based on normalised PSI values showed stronger structure than based on gene counts. In naive individuals, partial grouping by sex was observed, consistent with sex-biased splicing. In naive versus Wt-exposed females, treatment-specific clustering emerged, though not complete. Samples from all exposed female treatments remained largely intermixed suggesting that exposure effects on splicing remain low across individuals (Additional File 1, Additional Fig. 3). In all comparisons, the DEGs were a different set of genes than the DSGs. No gene was both DE and DS (Fig. 3A–E).

There were large differences in the number of DEG and DSGs. A small number of 18 to 29 genes were identified as DEGs ($\text{Padj} < 0.05$) between treatments. The highest number of DEGs was found in the naive versus Wt-exposed comparisons (Fig. 4A; Additional File 3, Table 2; Additional File 4). No enrichment was found in any of the DEG clusters based on gene ontology. In contrast, an average of 55,470 alternative splicing events were detected in a total of 1937 genes across libraries, including an average of 857 DS variants across all comparisons ($\text{FDR} < 0.05$, IJC and SJC > 20), with most differences also being found between the naive versus Wt-exposed comparison (1048 significant splice variants, Fig. 4A). Overall, a higher proportion of retained intron sites ($\sim 31\%$ on average), followed by spliced exon sites (average $\sim 26\%$), were detected relative to the other types of splicing events (Fig. 4B; Additional File 3, Table 3).

Naive male and female brains had similar gene expression but different splicing patterns

To examine sex-specific gene expression and splicing in *B. anynana* brains, we compared data from naive male and naive female brains. We found a small number of nine downregulated and nine upregulated genes in female

brains compared to males, but a much larger number, and distinct set of 394 DSG each showing at least one splicing event that occurs in significantly different proportions between the sexes (Fig. 3A). Among the DEGs, 15 were uncharacterized or non-annotated proteins. The remaining identified DEGs were mostly involved in membrane building and structural constitution of the cuticle, or cellular signalling and homeostasis (Additional File 4, Table 2). The GO analysis revealed that the DSGs were primarily involved in calcium binding and organ morphogenesis, mostly at the cell periphery and cell projections (Additional File 1, Additional Fig. 2D; Additional File 3, Table 4). The potassium voltage-gated channel *Shaker* and the zinc finger protein DZIP1 are examples of such genes with high significant absolute ΔPSI (0.43 and 0.35 respectively; Fig. 3A). Interestingly, genes from the sex-determination pathway, such as *doublesex*, *transformer*, *sex-lethal*, and *fruitless* did not show sex-biased expression of alternative transcripts in the naive butterfly brains.

Exposure to Wt males induced the upregulation of several sensory-related genes in female brains

To test whether females exposed to Wt male sex pheromones changed their gene expression and splicing patterns relative to naive females of the same age, we performed DEGs and DSGs analyses. In Wt-exposed females, 13 genes were downregulated, and 16 genes upregulated, compared to naives. The upregulated set contained sensory-related genes such as both copies of the ejaculatory bulb-specific protein 3 (EBP3) (also known as chemosensory protein (CSP)), insect odourant-binding protein A10 [48, 49], or lipoid-binding proteins [50], the olfactory neuron axon outgrowth protein alaserpin (SPI, serine protease inhibitor), and the eye-specific gene *calphotin* (*Cpn*), all between 2.5 and 4 times upregulated in exposed females (Fig. 3B; Additional File 4, Table 3). Exposure to the Wt blend induced 474 significant DS events (at $p < 0.05$), with an overrepresentation of genes involved in RNA binding, microtubule binding, and mitochondrial inner membrane functions (Additional File 1, Additional Fig. 3H; Additional File 3, Table 4). An example of a DSG of a binding protein, with some of the largest values of inclusion level differences between the two treatments, include the ubiquitin-protein ligase Hakai, affecting RNA splicing and methylation in fruit flies (A5SS, $\Delta\text{PSI} = -0.78$, $\text{fdr} = 1.6749\text{e} - 10$; Fig. 2E).

Exposure to new blends affected the expression of cellular growth and developmental genes, and the splicing pattern of RNA-binding genes

To identify genes affected by the sex pheromone components, we identified those commonly up- and downregulated in the Wt versus NB comparisons, and between NB1 and NB2. Twenty-seven genes were DE in the Wt

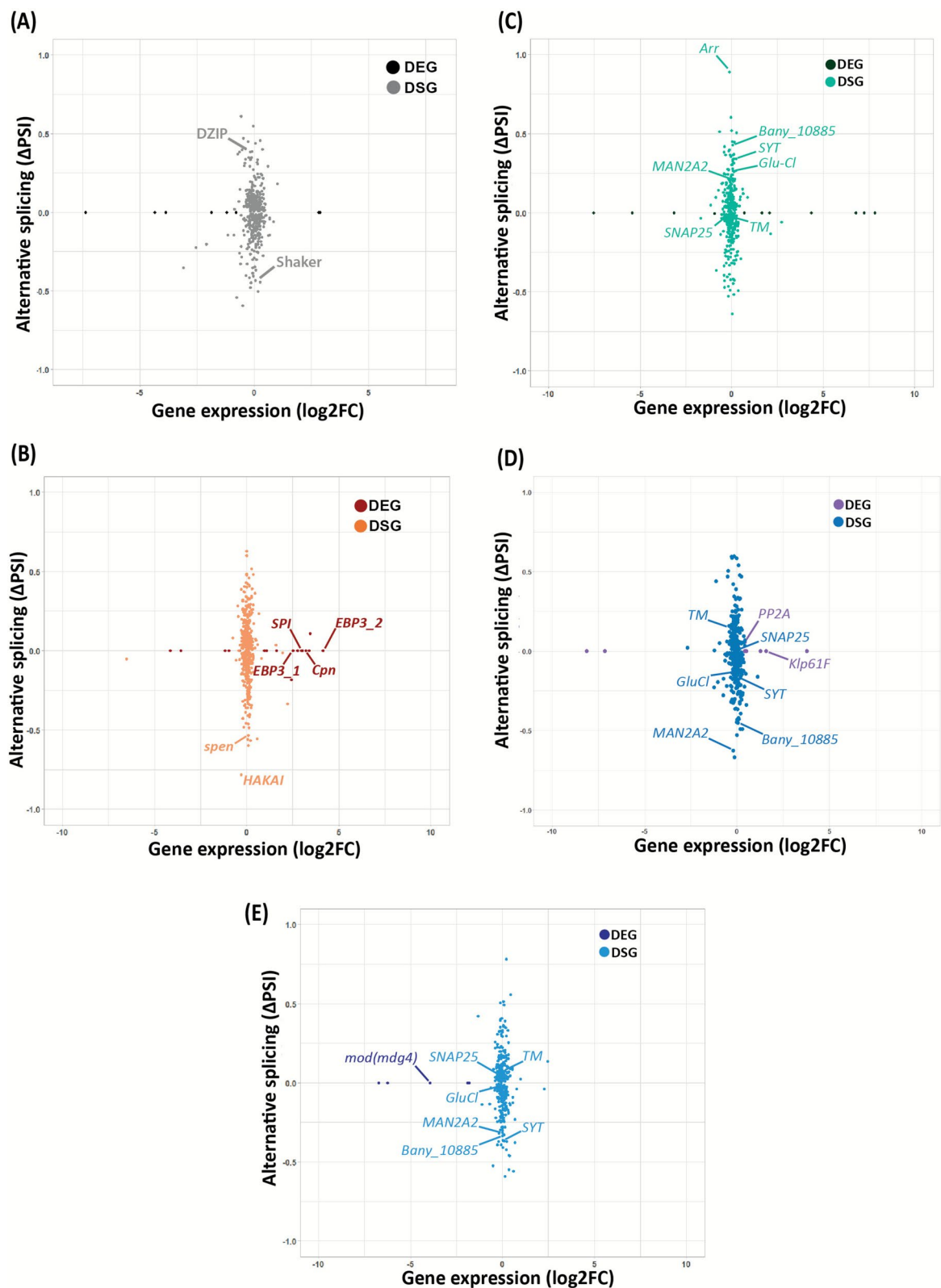


Fig. 3 Sex and exposure to new pheromone blends affected gene expression and splicing patterns in butterflies' brains. **A–E** show the gene expression differences (log2FC) of DEGs (padj < 0.05), and inclusion level differences (ΔPSI) of DSGs (for FDR < 0.05) in the M versus N; N versus Wt; Wt versus NB1, Wt versus NB2, and NB1 versus NB2 comparisons. Labeled are examples of genes of interest, involved in olfaction, neurogenesis, metabolism, and development. Five libraries per treatment were sequenced

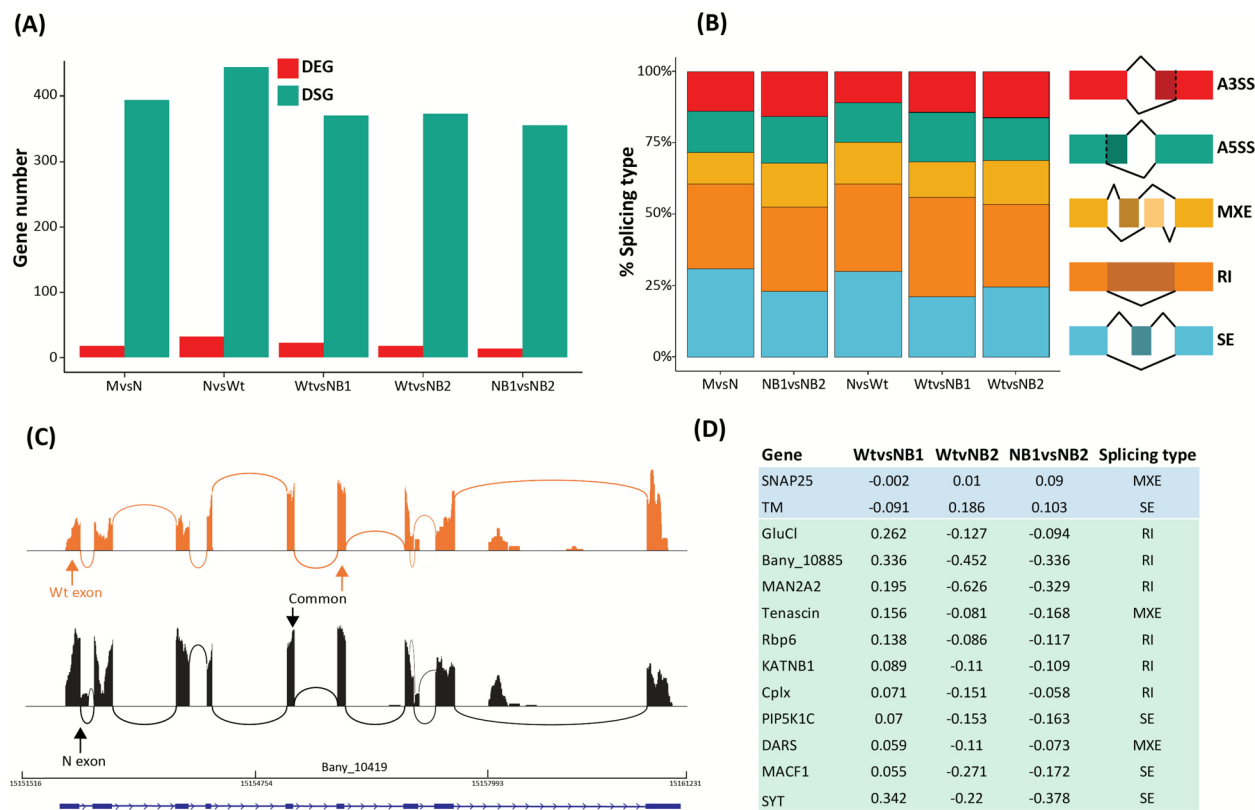


Fig. 4 There are more significant changes in splicing events than changes in gene expression between treatments. **A** Number of DEG and DSG identified in the different comparisons. **B** Proportions of significant splicing types found in the different comparisons (at FDR < 0.05, IJC and SJC > 20). **C** Sashimi plots showing HAKAI A5SS isoforms in a Wt and Naïve sample. **D** PSI of genes associated with an increase and a decrease in exposed MSP2 levels (in blue and green, respectively). Five libraries per treatment were sequenced

vs NB1 comparison (30% were not annotated or characterised (NAC)), twenty-one were DE in the Wt vs NB2 comparison (37% NAC), and nineteen genes were DE in the NB1 vs NB2 comparison (67% NAC) (Fig. 3C, D & E; Additional File 4, Tables 4, 5 & 6). The annotated DEGs were mostly involved in DNA biosynthetic processes and catalytic activities. For instance, the cell growth control and development protein phosphatase *PP2A* and the cell proliferation kinesin-like protein *Klp61F* were both upregulated in NB2-exposed brains compared to Wt (Fig. 3D), while a chromatin remodelling *mod(mdg4)-like* gene was downregulated in NB2 compared to NB1 exposed brains (Fig. 3E). We found no gene that was both upregulated in NB2 (versus Wt, and versus NB1) and downregulated in NB1 (versus Wt). Similarly, no gene was downregulated in NB2 (versus Wt and NB1) and upregulated in NB1 (versus Wt).

Most of the differences observed across treatments were in differential gene splicing. DSGs involved in mitochondrial inner membrane and RNA binding were over-represented in the Wt versus NB1 and in NB1 versus NB2 comparisons. For example, the gene with the highest

value of inclusion level difference (PSI of 89%) in NB1 (compared to Wt) is the regulator of GPCR (G Protein Coupled receptor) signal transduction arrestin (*Arr*), also having different splice variants in the other treatment groups (Fig. 3B). Other genes with high inclusion found DS after exposure to new blends function in neurogenesis (e.g. the neurite growth and transcription factor Zinc-finger protein *DZIP* [51]) (Additional File 4).

To identify splicing events specifically affected by MSP2 levels, we compared the three exposed treatments with each other. We selected splice variants that have a significantly higher Δ PSI (greater exon inclusion) in NB2 (where MSP2 levels were increased) compared to Wt and NB1, and in Wt compared to NB1 (where MSP2 is absent). We found two genes annotated as synaptosomal-Associated Protein 25 (SNAP25) and tropomyosin (TM). The percent splice indexes for these genes are lower than 10% in most comparisons (Fig. 4D; Additional File 3, Table 6). Similarly, we selected splice variants that have a significantly lower Δ PSI (reduced exon inclusion) in Wt and NB1 (where MSP2 levels were decreased) compared to NB2, and in NB1 (where MSP2 is absent) compared

to Wt. We found 11 genes, among which, synaptotagmin (SYT), alpha-mannosidase (MAN2A2), a glutamate-gated chloride channel (GluCl), and an uncharacterized ion transporter (Bany_10885) have the highest Δ PSI (differences in inclusion levels) (above 20%) (Figs. 3C,D, E & 4D; Additional File 3, Table 6). To get an additional list of candidate genes showing interesting trends in splice variant proportions in response to exposure to different MSP2 pheromone levels, and evaluate whether splicing changes were consistent across treatments, we identified DSGs in at least two of the three comparisons. Significant correlations of Δ PSI values across treatments for these genes (WtvsNB2 versus WtvsNB1: $R=0.44$, $p=0.03$; NB1vsNB2 versus WtvsNB1: $R=0.84$, $p=1.5e-7$; NB1vsNB2 versus WtvsNB2: $R=0.85$, $p=5.3e-8$; Additional File 1 Additional Fig. 3E, F, G) suggest that splicing changes co-vary in exposed females with MSP2 changes. The identified genes (listed in Additional File 3, Table 7) are mainly involved in intracellular transport, gene regulation, protein processing, and ion transport across key cellular compartments.

Discussion

In previous work, we showed that a brief, 3-min exposure of naive female butterflies to male sex pheromone blends, which differ in the quantity of at least one or two known pheromone components, led to changes in female preferences for the male blends, 2 days after the exposure treatment. Here we show that these brief pheromone exposures affected the size of at least one antennal lobe glomeruli, altered the expression level of a handful of genes, and affected the splicing of hundreds of genes in the brains of females. We also show that antennal lobe size is not sexually dimorphic and that male and female brains differ little in gene expression but markedly in splicing patterns.

Naive male and female brains have similar morphologies upon emergence

The number of glomeruli, total AL volume, and the volume of all glomeruli were similar in the two sexes, which is consistent with previous studies in the Monarch *Dan-*aus plexippus** and the Neotropical butterfly *Godyris zavaleta*. These studies have identified 65–70 and 67 glomeruli on average respectively in the two species, with no sex-specific differences in total AL volume [44, 52]. Naive *B. anynana* males had, however, one glomerulus that was absent in most females, which is possibly responding to still unknown olfactory cues used primarily by males of the species. This glomerulus is likely not involved in sex pheromone perception [38, 53], because it is mostly absent in females. The lack of sexually dimorphic glomeruli volume suggests that pheromone detection in

the species may not be restricted to one sex, and be useful to males for social aggregation or competition for a mate. Functional differences between the sexes may also occur at the level of receptor expression in the peripheral organs, in neural tuning, sensitivity or central processing, rather than in glomerular volume. Sex-specific differences in olfactory coding remain an open question and need to be further explored in both central and peripheral structures.

Naïve brains were sexual dimorphic primarily in gene splicing

Naive brains exhibited few sex-specific differences in gene expression, with the few DEGs identified being involved in cuticle development. A previous study found many more sex-biased genes using whole butterflies' heads at emergence [40], suggesting that early sex differences are affected by peripheral tissues and/or decline as the brains matures. Sex differences in gene expression in butterflies [40, 54] and *D. melanogaster* brains were also previously shown to be low, with flies' male heads being closer to female heads than to other male tissues [55, 56]. In insects, sex-biased gene expression was found in reproductive tissues (e.g. in *Drosophila* [56, 57]); while in heads, sex-biased gene expression occurs primarily in non-nervous tissue [58, 59]. Cuticle genes being primarily DE in the male and female brains of *B. anynana* support these findings.

Alternative splicing, however, appears to be an important mechanism governing sex differences in the brains of young butterflies. In both invertebrate and vertebrate species, such as fruit flies, fishes, bats and humans, the sexes show large differences in gene splicing [56, 60–65], and brains/heads usually show some of the highest transcriptional diversity (along with gonads) across all tissues. Generally, sex-specific spliced genes are thought to derive from the sex differentiation pathway, crucial to neurological development. In insects, these genes include the sex-specific isoforms of *doublesex* (*dsx*) and *fruitless* (*fru*) [66, 67], which are known to impact sexual behaviours and sex-specific morphologies across species [68–71]. In our study, it was surprising that none of the well-known sex differentiation pathway genes were DS (or DE) between naive male and female brains, unlike hundreds of other genes. It is possible that sex-specific expression or variants of these genes might be affecting brains at earlier stages of development, as it is the case for *dsx* in our butterfly species [41] and *fru* in *Drosophila* larval nervous system development [72]. Alternatively, the recent discovery that *B. anynana* uses a unique primary sex determination signal [73], distinct from that used in *Drosophila* or *Bombyx mori*, could have led to yet

unexplored diversification of the sex-determination pathway in this species.

Gene ontology analysis for the set of DSGs indicated that male and female brains differed in signalling processes involved in neurogenesis and happening mostly at axons or dendrites. Calcium ion binding proteins, which function in signal transduction and regulate many aspects of the cell's functioning, homeostasis, neuron excitation, and synaptic transmission were overrepresented in the set of DSGs [74, 75]. DSGs involved in brain morphogenesis and located at the cell projection and periphery were also overrepresented, but these functions were not reflected by sex-specific size changes in the antennal lobe. Cellular or connection growth might still be happening in other parts of the brain at this stage. An interesting candidate DSG with a high PSI was *Shaker* (*Sh*), a potassium voltage-gated channel known to be expressed as multiple splice variants that encode proteins with distinct structural features in the *Drosophila* brain [76–78]. The response of cation channel splice variants to the same ligand can be very different, e.g. it can lead to opposite responses to an insecticide [79], but the functions of *Sh* variants are unknown. *Drosophila* brain *Sh* expression affects male sex discrimination [80], visual motion detection, and photoreceptor sensitivity [81, 82], so the different variants expressed in male and female *B. anynana* brains should be examined in future in connection to similar behavioural essays.

Glomeruli volume did not correlate with female exposure to specific MSP blends

Odour-exposure experiments associated with a sucrose reward in honey bees lead to an increase in the odourant-specific glomeruli volume, associated with a stronger response of the glomeruli to the odour and increased appetitive behaviour in individuals [7, 83]. In our experiment, however, we found little support for the role of altered glomeruli volume in mediating female preference change for a new sex pheromone blend. We found one glomerulus (PD2) decreasing in size when females were exposed to a pheromone blend lacking MSP2; however, naïve females did not show similar size reductions despite also lacking MSP2 exposure. In general, changes in specific MSP did not lead to consistent and specific glomeruli size changes, and thus this experiment did not help us identify the glomeruli tuned to the specific MSP components. It is possible that MSP quantity and ratio changes between Wt, NB1, and NB2 males might have been too minor to impact glomeruli volume, our sample size may have been too small for enough statistical power (only 7 brains were measured per treatment), or our sampling time, 2 days after the exposure, might have been too soon to detect potential size differences.

Despite the lack of differences in glomeruli size, we clearly detected molecular changes in the brain after exposure to new blends, some connected to brain growth and development. The DEGs and DSGs identified were involved in gene expression regulation (RNA binding, transcription factors) and neuronal tissue development and maturation. At this stage, changes might be taking place in the number of synaptic connections, or in other olfactory neuropiles like the mushroom bodies (2, [84]. For instance, DEG *Klp61F* impacts axons and dendrite microtubule growth [85, 86], and DEG *mod(mdg4)* affects synaptic plasticity in the *Drosophila* brain [87]. DSG *arrestin* regulates the signalling of G coupled receptors, impacting the development of the olfactory transduction machinery. An arrestin-related protein called mKast is also expressed in pupal and adult honeybee Kenyon cells in the MB, key to olfactory learning and memory. The protein is thought to affect cell fate, but its exact function is still unknown [88–92]. Arrestin is also upregulated in the eyes of *B. anynana* males trained to prefer visual cues from female wings [41], suggesting that the gene responds to learning regardless of the type of sensory cue involved. Here, the short 3-min exposure to the male blends could have created cellular growth or differentiation of new neurons in the females' sensory and memory structures 2 days later, but the precise mechanism behind this plasticity needs to be determined.

Expression differences in an odorant binding protein may be associated with MSP3 learning

The OBP homologue EBP3, an odourant-binding protein (OBP), could be involved in the butterfly olfactory perception and learning process of MSP3. Two EBP3 copies were upregulated in the brains of females exposed to Wt blends relative to naïve individuals. Because these two *EBP3* genes were not differentially expressed in the other comparisons, these OBPs could be binding to MSP3 specifically, as this pheromone is present in similar amounts in Wt, NB1 and NB2 males (Fig. 1A). EBP3 carries semiochemicals with different proposed roles and is known to bind to several pheromone components from fruit flies and moths (e.g. [93–96]), and to react to plant volatiles in pest flies [97] and psyllids, where its upregulation is correlated with increased preference for phytochemicals [98]. A previous study [41] showed that the two *EBP3* were upregulated in the brains of *B. anynana* females exposed to a male with modified wing ornaments compared to exposed males, supporting the gene role in female sensory perception. Similar changes in the level of these proteins also happen in other insect species: in *D. melanogaster*, a mushroom body-expressed *EBP3* was recently associated with long term memory formation [99], two *EBP3s* are overexpressed in host odour-exposed

mosquitoes [100], and other OBPs were previously shown to be differentially regulated in the brains of bees after olfactory learning [101]. Here, the two genes are relevant candidates for MSP perception and blend preference memory formation in exposed females. Functional analyses for these genes are needed in *B. anynana*, with perhaps a focus on mushroom bodies, to elucidate their role in odour memory formation.

A few additional differences in gene expression between naive and Wt-exposed females could have affected olfactory perception and growth of sensory structures. For example, two Wt-female upregulated genes, *calphotin* and *alaserpin* (SERA or antichymotrypsin-2, a serine protease inhibitor) indicate the growth of visual and/or olfactory organs. *Calphotin* functions in rhabdomere development [102–104] and *alaserpin* functions in antennal neuron extension [105]. The new sensory experience of these Wt pheromone-exposed females may have facilitated the expansion of associated sensory organs needed by females to navigate their new environment.

There is little support for changes in gene expression correlating with changes in female preference for new sex pheromone blends. This is because no gene commonly changed in expression in the three training blend comparisons that differed in MSP2 levels. MSP quantity and ratio changes between the three treatments might be too minor to impact gene expression, or it may impact gene expression at different time points. Most importantly, small gene expression changes in the mushroom bodies, the main centres for learning in insects [23, 106, 107], might not have been detected in our whole-brain transcriptomics analysis and may require a single-cell sequencing approach.

Gene splicing is different between pheromone exposure treatments

In response to early exposure to new sex pheromone blends, a much larger number of genes were differentially spliced, relative to differentially expressed, and genes were exclusively differentially spliced or up- and down-regulated in the different treatments. Similar results, of non-overlapping gene sets that are either DS or DE, have been described in aphid polyphenic morphs or in butterfly wings from different seasonal forms, including *B. anynana* [108–110].

We found that DSGs with RNA and microtubule binding functions were overrepresented in the Naive vs Wt, and NB1 vs NB2 comparisons. This suggests a role for the splice variants in the formation of neuronal networks that involve substantial RNA processing and cytoskeletal modifications. *Drosophila* and mammalian nervous systems are also known to use alternative splicing at high

frequency for mechanisms like cell differentiation and morphogenesis [62, 111–113].

We noted a higher proportion of IR events, where unspliced introns remain in mature mRNA, relative to other types of splicing events. IRs have been shown to affect RNA binding proteins and contribute to the plasticity of the transcriptome and regulation of gene expression during cell development, cell differentiation, and in response to cellular stress [114, 115]. In mammals, IR affects the synaptic plasticity of neuronal cells and can be triggered by a short stimulation, allowing a fast neuronal plastic response to a stimulus [116–118]. The precise role of the IR variants and how they affect pheromone perception and learning in butterflies remains to be investigated.

A learned preference for new blends was linked to variants of proteins involved in synaptic transmission. Levels of expression of these proteins affect learning and memory in other species. For instance, synaptic glutamate-gated chloride channels regulate fast inhibitory synaptic transmission and have six known variants (across insects) with different spatial and developmental expression patterns, and response strength to the substrates (neurotransmitter and insecticides) [119–121]. These channel proteins are necessary for plant volatile olfactory memory retention in honeybees [122, 123]. Synaptotagmin and SNAP25 interact to promote neurotransmission and synaptic growth in *Drosophila* [124, 125]. SYT are calcium sensors with multiple variants [126], where the syt-1 variant affects long-term synapse potentiation and promotes learning in mice [127]. SYT is also upregulated in the eyes of *B. anynana* trained with visual cues [41], suggesting its involvement in neuronal learning processes across different types of sensory modalities. SNAP25 is part of the attachment protein receptor complex responsible for exocytosis at synapses in insects [128–130]. Bee olfactory learning reduces SNAP25 expression levels [131], and the protein is involved in memory consolidation in rats [132] and humans [133–135]. However, the roles of specific variants of these proteins in butterflies remain to be tested.

Conclusions

We showed extensive sexual dimorphism in gene splicing in male and female *B. anynana* butterfly brains and showed that a short exposure of a female butterfly brain to various male pheromone blends alters the size of a glomeruli in the antennal lobe and changes additional splicing patterns in the brain. Specific changes in one pheromone component affected the splicing of genes involved in synaptic transmission. Functional experiments will need to be performed in some of the genes identified in this study to probe how and which of these

changes mediate pheromone odour learning that ultimately impact female mate choice.

Methods

Insect rearing and odour treatments

Insect production and odour treatments were the same as in [35]. Individuals were reared at 27 °C, 80% humidity and 12:12 h light:dark photoperiod. Caterpillars and butterflies were fed unlimited *Zea mays* corn leaves and mash banana, respectively. Sexes were separated at the pupal stage, with each pupa placed individually in a plastic box and, upon emergence, in plastic cages. Males used for exposure were kept in groups in age-specific cages. After blend manipulation, males were allowed to rest for 30 min, and then manually exposed to the females. Males were all aged 4 to 6 days old and all animals were virgins. Females within 3 h of emergence were exposed to males for 3 min, and then isolated in cups for 2 days (~48 h). This protocol was used previously to identify changes in female behaviour. The treatments (male sex pheromone blends used for female exposure) and female responses were as follows (Fig. 1A) [35]:

- Naive females (N) and naïve males (M) were kept isolated in individual boxes until dissection on day 2. They were not fed or used for behavioural experiments. Naive females have significant preferences for the Wt blend when tested against NB1 or NB2 in choice assays.
- Wt-exposed females (Wt) were manually exposed upon emergence to males carrying the Wt blend, then they were placed in individual containers and dissected 2 days later. These females have a strong preference for the Wt males when tested against NB1 males, but they randomly choose between Wt and NB2 males.
- NB1- and NB2-exposed females (NB1 and NB2) were manually exposed upon emergence to males carrying the respective reduced and increased blends, placed in individual containers, and dissected 2 days later. NB1-exposed females choose randomly between the two males. NB2-exposed females significantly prefer NB2 males.

Immunohistochemistry

Brains were dissected, fixed, and stained following prior protocols [136, 137] with minor modifications. Briefly, the head was submerged in HEPES-buffered saline (HBS; 150 mM NaCl; 5 mM KCl; 5 mM CaCl₂; 25 mM sucrose; 10 mM HEPES; pH 7.4) and fixed for 22–24 h under agitation at room temperature in zinc-formaldehyde solution (ZnFa; 0.25% [18.4 mM] ZnCl₂; 0.788% [135 mM]

NaCl; 1.2% [35 mM] sucrose; 4% formaldehyde). The brains were then dissected in HBS and washed 3 times in HBS before further staining. Samples were then submerged in 80% methanol/20% dimethyl sulfoxide (DMSO) for 2 h under agitation and subsequently stored in 100% methanol in –20 °C till further processing. To prepare these samples for staining, they were brought to room temperature and rehydrated in decreasing methanol concentrations (90%, 70%, 50%, 30%, 0% in 0.1 M Tris buffer, pH 7.4, 10 min each). Subsequently, they were pre-incubated for 1.5 h at room temperature in a mixture of 5% bovine serum albumin (BSA; Sigma Aldrich, St. Louis, Missouri, USA) and 0.1 M phosphate-buffered saline (PBS; pH 7.4 containing 1% DMSO and 0.005% NaN₃) (PBSd). They were then stained with synapsin antibodies (anti-SYNORF1 obtained from the Developmental Studies Hybridoma Bank DSHB, product 3C11; <https://dshb.biology.uiowa.edu/>) at a 1:30 dilution in PBSd-BSA for 3.5 days at 4 °C and washed thereafter with PBSd (3×1.5 h). Alexa Fluor 488 (AF 488) conjugated goat anti-mouse IgG antibody (ThermoFisher Scientific) was added in a 1:100 PBSd-BSA ratio for 2.5 days at 4 °C. The brains were then dehydrated in increasing glycerol concentrations (4% for 2 h, 15%, 50%, and 80% for 1 h each) in 0.1 M Tris buffer with DMSO to 1%. After which they were submerged into 100% ethanol in a drop of 80% glycerol. The ethanol was agitated for 20 min and refreshed twice (30 min each) without agitation. Clearing of the brain tissues was done by dripping methyl salicylate into brains immersed in 100% ethanol and waiting for the brains to sink before refreshing the methyl salicylate once (30 min incubations).

Confocal imaging

The cleared brains were mounted in methyl salicylate onto glass slides with a depression of 0.6–0.8 mm (Marienfeld Superior, Germany) and the coverslip sealed with clear nail polish. All imaging was done using the Olympus FV3000 confocal laser-scanning microscope with either the 4× or 10× dry objective lens and an aperture of 120 µm. A 488-nm laser line was used to excite the dye (AF 488). For each whole brain sample, we visually identified the different brain structures (Fig. 1B) and took a stack of images from either the left or right Antennal Lobe (AL) selected at random using the ×10 air objective with the following image settings: a mechanical z-step of 2 µm and an x–y resolution of 1024×1024 pixels. An additional zoom factor of ×4.21 was applied for optimal z sampling. Finally, a 1.52× correction factor was applied to the voxel size in the z-dimension to correct for the artefactual shortening caused by the ×10 air objective [44].

Imaging for the whole brain required stacking two images stitched side by side taken with an overlap of 10%. We used the $\times 4$ objective with a mechanical z -step of 2 μm and an x - y resolution of 512×512 pixels. Stacking and stitching were done in FIJI [138].

Glomerular identification and volumetric reconstructions

We used naïve individuals to first identify each glomerulus based on their location, size and shape within each AL sample by comparing the confocal stacks directly at different depths throughout the brain. Several easily distinguishable glomeruli were used as landmarks paving the way for identification of the other glomeruli in the region (Fig. 2A; Additional File 2, Table 1; Additional file 1, Additional Fig. 1, Additional Result 1). We followed the nomenclature used in several other studies including Solari, Corda [139] and Stocker, Lienhard [140], for naming of the glomerulus in *B. anynana*.

After identification, each glomerulus was reconstructed in Imaris 9.9.0 (Oxford Instruments Group) based on the stack of confocal images taken. We manually delineated individual glomerulus using the Imaris *Add New Surface* module by outlining with the *Draw* function the boundaries of each. We performed the outline on every alternate image slice within one stack for each respective glomerulus. Then a surface rendering of the glomeruli was done with the *Create Surface* function to obtain a 3D model, allowing us to visualise the spatial position of each glomerulus within the antennal lobe. The colours of the glomeruli were assigned using the *Colour* tab and glomeruli belonging to the same region were coded in the same colour (Fig. 2A; Additional File 1, Additional Fig. 1; Additional File 2, Table 1). We subsequently obtained the volume of each glomerulus using the *Statistic* tab. We also obtained, in a similar fashion, the volume of the whole AL (consisting of the volume of all glomeruli subunits and the central fibrous neuropil) (Fig. 2A; Additional File 2, Table 2). A reference key detailing the process of glomerulus identification based on the relative positions to the landmark glomeruli is available in Additional File 1, Additional Fig. 1, Additional Result 1 and Additional File 2, Table 1.

Glomeruli number and volume statistical analysis

Glomeruli counts were compared between naïve males and naïve females using a T -test, between naïve females and Wt-exposed females using a Wilcoxon-Mann-Whitney test, and we used a Kruskal-Wallis test followed by a pairwise Wilcoxon test with Holm correction to assess differences in counts across exposure treatments. Normality and homoscedasticity were tested a priori with Shapiro and Levene tests.

Volume comparisons were performed with a two-tailed Student's t test unless otherwise stated, followed by a Benjamini-Hochberg FDR correction. To account for the individual variation in body size of each sample, we normalised (a) the absolute volume of the whole AL to the volume of the midbrain for comparisons of total AL volume and (b) the volume of each glomerulus to total glomerular volume (total AL volume excluding the CFN; Fig. 2A) for comparisons between naïve males and females. We also normalised the absolute volume of each glomerulus to the volume of the whole AL for comparisons between females exposed to different MSP blends. If the relative volumes were found to deviate from normality through Shapiro Wilk's test, non-parametric Mann-Whitney U test was used instead for comparison. Sample size was seven brains in each treatment group. All statistical analyses were done using R Statistical Software v4.0.1 [141] and Rmisc (Hope, 2022), coin (Hothorn et al., 2008), car (Fox & Weisberg, 2019), and rcompanion (Mangiafico & Salvatore, 2023) packages.

RNA extraction, sequencing, and assembly building

Two days after exposure and isolation, butterflies were flash frozen in liquid nitrogen, their brains dissected following Toh, Dion [136] and stored in RNALater (Invitrogen, ThermoFischer Scientific, no. 7020). Five brains were pooled per replicate, and five replicates were made for each of the five treatments. RNA extraction was performed using phenol and chloroform. The samples and RNA extractions were randomised to minimise batch effects. The 25 samples were sent to NovogeneAIT Genomics, Singapore, for library preparation using the NEBNext® Ultra™ Directional RNA Library Prep Kit and RNA sequencing was done with 150-bp paired end reads using the Illumina NovaSeq6000 platform.

The initial quality control of the sequencing files was performed with FastQC v0.11.9 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc>), followed by adaptor trimming and cleaning of low-quality raw reads with Trimmomatic v0.39 [142]. The bbsplit script from the bbmap toolbox [143] was used to filter-out the bacterial sequences from the dataset by aligning the reads to a concatenation of bacterial genomes downloaded from the National Center for Biotechnology Information (NCBI) in June 2018. We identified and removed ribosomal RNA sequences by aligning the reads to the eukaryotic rRNA database available in sortmeRNA [144]. We obtained the gene and transcript count matrices by aligning the clean reads to the *B. anynana* genome V2 [145] in Hisat2 [146, 147]. Resulting bam files for each library were aligned to the.gff3 *B. anynana* genome with stringtie and the abundance tables were compiled with the python prepDE.py script [146]. On average, 86.49% of clean reads mapped

to the genome (Additional File 3, Table 1). For local blast purposes, we used stringtie to build a brain assembly by converting the libraries' bam to .gtf files that were merged into the assembly file and converted to a fasta file with the gffread utility [146, 148, 149].

Gene expression analysis

All analysis were done in R v4.1.3 [141] implemented in RStudio v2022.07.01 [150]. To capture overall variance structure in the dataset and detect outliers, unbiased principal component analysis (PCA) plots were built on normalised gene counts using ggplot2 [151] using a random sample of 500 genes. Three libraries (one M, one NB1 and one NB2) were excluded from the DE analysis as they were considered outliers based on the PCA (Additional File1, Additional Fig. 2A). We built the heatmaps based on normalised counts using a random set of 500 genes following the DESeq2 package protocol [152]. We identified differentially expressed genes (DEGs) from the gene count matrix using the DESeq2 package [152]. By incorporating the SmartSVA algorithm [153], we did not detect any surrogate variable, indicating no significant hidden batch effects in the dataset. A PCA and the heatmaps for each treatment group comparisons were also generated using DEGs with adjusted p values < 0.1 to provide sufficient features for pattern visualisation, because we found less than 30 DEG at adjusted p value < 0.05 overall (see Results).

Expression changes in naïve individuals and after Wt-exposure compared to naïves were analysed with a Wald test. Changes across exposure treatments (Wt versus NB1 versus NB2) were tested both by direct treatment pair comparisons, and by applying a likelihood ratio test followed by pairwise comparisons using the 'contrast' command. We compared naïve males and naïve females, naïve females and Wt-exposed females, Wt-exposed females and NB1-exposed females, Wt-exposed females and NB2-exposed female, and finally NB1- and NB2-exposed females. All genes with less than 10 reads in total over all libraries and with adjusted p values above 0.05 were filtered out.

Alternative splicing analysis

rMATS v4.1.2 [154–156] was used to assess the alternative splicing events in each treatment, and provide the relative abundance of splicing isoforms by quantifying Percent Spliced In (Δ PSI) for specific splicing events. Five types of alternative splicing events were considered: skipped exon (SE), alternative 5' splice site (A5SS), alternative 3' splice site (A3SS), mutually exclusive exon (MXE), and retained intron (RI). We compared the treatments by assessing the differences in inclusion level (Δ PSI). rMATS provides adjusted p values after using a

Bayesian hierarchical model that accounts for biological variability across replicates, followed by multiple gene comparison correction within comparisons [150–152]. These corrections were done within each analysis and not across the entire experiment. Using R [141], the package reshape [157], and adapted methods from Tian and Monteiro [110], we selected the differentially spliced genes (DSG) with at least one differentially spliced site between treatments, a false discovery rate (adjusted p value) below 0.05 and a total junction count (for inclusion and skipping junctions) above 20. We excluded low abundance splicing events likely to be splicing mistakes ($|\text{PSI}| < 50$) [110]. If a DSG had multiple spliced sites, we used the splicing event with the maximum absolute value of inclusion level difference between the treatments to represent the differential splicing level of the gene. We built the heatmaps based on total splicing events and spliced genes without treatment comparison using normalised PSI values obtained from the rMATS 'statoff' function, and ran the additional filtering, processing, and figure building steps in R. Heatmaps were built with the Pheatmap and Rcolorbrewer packages (Neuwirth, 2022; Kolde, 2025). Percent splicing type, gene number, and Δ PSI correlations plots were built using packages ggplot2 [151], ggpubr [158], and wesanderson [159]. To visualize the gene junctions, we indexed the bam files using samtools [160], aligned the sequences with the genome (V2), and built the sashimi plots in the Integrative Genomics Viewer (IGV v2.17.3; [161]).

Functional annotation

Gene descriptions were retrieved from the *Bicyclus anynana* genome annotation [145] and completed by locally blasting the assembly genes against the insecta (taxid: 50,557) NCBI non-redundant (nr) protein database (built using the BLAST+ command line tool suite [162]) in diamond v2.0.8.146 [163, 164]. The alignment was imported in Blast2go v6.0.3 to get the GO annotations [165]. Protein function prediction was done in parallel using InterProScan in Blast2go [166] and the eggNOG-mapper online tool (eggnog-mapper.embl.de [167, 168]). GO enrichment analyses were done with the R package clusterprofiler [169].

Abbreviations

A3SS	Alternative 3' splice site
A5SS	Alternative 5' splice site
AL	Antennal lobe
CFN	Central fibrous neuropile
CSP	Chemosensory protein
DE	Differentially expressed
DEG	Differentially expressed gene
DSG	Differentially spliced gene
EBP	Ejaculatory bulb protein
FDR	False discovery rate
GO	Gene ontology

GR	Gustatory receptor
IR	Ionotropic receptor
MB	Mushroom bodies
MSP	Male sex pheromone
MXE	Mutually exclusive exon
NAC	Not annotated or characterized
NB	New blend
OBP	Olfactory binding protein
OGR	Olfactory and gustatory receptor
OR	Odourant receptors
OSN	Olfactory sensory neuron
PB	Protocerebral bridge
PBP	Pheromone binding protein
PCA	Principal component analysis
PSI	Percentage spliced index
PN	Projection neurons
RI	Retained intron
SE	Skipped exon
SNMP	Sensory neuron membrane protein
Wt	Wild type

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12915-026-02514-w>.

Additional file 1. Additional figures and results.

Additional file 2. Identification and volumes of glomeruli.

Additional file 3. RNAseq summary and data description.

Additional file 4. List of DEG and DSG in all comparisons.

Acknowledgements

We thank Shen Tian, Jocelyn Wee, and Suriya Murugesan for help with the RNAseq data analysis. We also thank Greenology for providing the corn plants for feeding the caterpillars. We acknowledge support from the Ministry of Education, Singapore (awards MOE2018-T2-1-092 and MOE-T2EP30223-0007), and the National Research Foundation, Singapore, Competitive Research Program (award NRF-CRP25-2020-0001).

Authors' contributions

Conceptualization: ED, AM; Investigation: ED, YPT, DZ; Funding acquisition: ED, AM; Project administration: AM; Supervision: ED, AM; Writing – original draft: ED, YPT; Writing – review & editing: ED, YPT, AM. All authors read and approved the final manuscript.

Funding

Ministry of Education, Singapore (awards MOE2018-T2-1-092 and MOE-T2EP30223-0007).

Data availability

All data generated or analysed during this study are included in this published article, its additional files and in publicly available repositories. All Illumina reads of RNA-seq are available under NCBI BioProject PRJNA1308762 (<http://www.ncbi.nlm.nih.gov/bioproject/PRJNA1308762>). RNAseq data analysis scripts are available at both https://github.com/Deguydion/RNAseq_ButterflyBrain_Dionetal/tree/main and <https://doi.org/10.6084/m9.figshare.30856208> [170, 171]. Antennal lobe volume datasets and analysis scripts re available at <https://doi.org/10.6084/m9.figshare.27873258.v2> [172].

Declarations

Ethics approval and consent to participate

No ethical approval was needed to work with *B. anynana* wild type laboratory strains.

Not applicable.

Consent for publication

All authors consent for this manuscript to be published.

Competing interests

The authors declare no competing interests.

Received: 8 January 2025 Accepted: 12 January 2026

Published online: 21 January 2026

References

- Adam E, Hansson BS, Knaden M. Fast Learners: One Trial Olfactory Learning in Insects. *Front Ecol Evolution*. 2022;10.
- Fabian B, Sachse S. Experience-dependent plasticity in the olfactory system of *Drosophila melanogaster* and other insects. *Front Cell Neurosci*. 2023. <https://doi.org/10.3389/fncel.2023.1130091>.
- Dion E, Monteiro A, Nieberding CM. The Role of Learning on Insect and Spider Sexual Behaviors, Sexual Trait Evolution, and Speciation. *Front Ecol Evol*. 2019;6(225).
- Zhao Z, McBride CS. Evolution of olfactory circuits in insects. *J Comp Physiol A*. 2020;206(3):353–67.
- Anton S, Rössler W. Plasticity and modulation of olfactory circuits in insects. *Cell Tissue Res*. 2021;383(1):149–64.
- Anderson P, Hansson BS, Nilsson U, Han Q, Sjöholm M, Skals N, et al. Increased behavioral and neuronal sensitivity to sex pheromone after brief odor experience in a moth. *Chem Senses*. 2007;32(5):483–91.
- Arenas A, Giurfa M, Farina WM, Sandoz JC. Early olfactory experience modifies neural activity in the antennal lobe of a social insect at the adult stage. *Eur J Neurosci*. 2009;30(8):1498–508.
- Guerrieri F, Gemenio C, Monsempes C, Anton S, Jacquin-Joly E, Lucas P, et al. Experience-dependent modulation of antennal sensitivity and input to antennal lobes in male moths (*Spodoptera littoralis*) pre-exposed to sex pheromone. *J Exp Biol*. 2012;215(13):2334–41.
- López S, Guerrero A, Bleda MJ, Quero C. Short-term peripheral sensitization by brief exposure to pheromone components in *Spodoptera littoralis*. *J Comp Physiol A*. 2017. <https://doi.org/10.1007/s00359-017-1205-5>.
- Seeholzer LF, Seppo M, Stern DL, Ruta V. Evolution of a central neural circuit underlies *Drosophila* mate preferences. *Nature*. 2018;559(7715):564–9.
- Baracchi D, Cabirol A, Devaud JM, Haase A, d'Ettorre P, Giurfa M. Pheromone components affect motivation and induce persistent modulation of associative learning and memory in honey bees. *Commun Biol*. 2020;3(1):447.
- Claudianos C, Lim J, Young M, Yan S, Cristino AS, Newcomb RD, et al. Odor memories regulate olfactory receptor expression in the sensory periphery. *Eur J Neurosci*. 2014;39(10):1642–54.
- Wan X, Qian K, Du Y. Synthetic pheromones and plant volatiles alter the expression of chemosensory genes in *Spodoptera exigua*. *Sci Rep*. 2015;5:17320.
- Stopfer M. Central processing in the mushroom bodies. *Curr Opin Insect Sci*. 2014;6:99–103.
- Amin H, Lin AC. Neuronal mechanisms underlying innate and learned olfactory processing in *Drosophila*. *Curr Opin Insect Sci*. 2019;36:9–17.
- Vogt K, Schnaitmann C, Dylla KV, Knapek S, Aso Y, Rubin GM, et al. Shared mushroom body circuits underlie visual and olfactory memories in *Drosophila*. *Elife*. 2014;3:e02395.
- Farris SM, Van Dyke JW. Evolution and function of the insect mushroom bodies: contributions from comparative and model systems studies. *Curr Opin Insect Sci*. 2015;12:19–25.
- Groh C, Rössler W. Analysis of synaptic microcircuits in the mushroom bodies of the honeybee. *Insects*. 2020;11(1):43.
- Couto A, Young FJ, Atzeni D, Marty S, Melo-Flórez L, Hebberecht L, et al. Rapid expansion and visual specialisation of learning and memory centres in the brains of *Heliconiini* butterflies. *Nat Commun*. 2023;14(1):4024.
- Thomas H. Neuronal architecture and functional organization of olfactory glomeruli. In: Thomas H, editor. *Neurophysiology*. Rijeka: IntechOpen; 2022. p. Ch. 4.

21. Devaud JM, Acebes A, Ferrus A. Odor exposure causes central adaptation and morphological changes in selected olfactory glomeruli in *Drosophila*. *J Neurosci*. 2001;21(16):6274–82.
22. Devaud J-M, Acebes A, Ramaswami M, Ferrus A. Structural and functional changes in the olfactory pathway of adult *Drosophila* take place at a critical age. *J Neurobiol*. 2003;56(1):13–23.
23. Giurfa M. Cognition with few neurons: higher-order learning in insects. *Trends Neurosci*. 2013;36(5):285–94.
24. Grabe V, Baschwitz A, Dweck HKM, Lavista-Llanos S, Hansson BS, Sachse S. Elucidating the neuronal architecture of olfactory glomeruli in the *Drosophila* antennal lobe. *Cell Rep*. 2016;16(12):3401–13.
25. Anton S, Chabaud M-A, Schmidt-Büsser D, Gadenne B, Iqbal J, Juchaux M, et al. Brief sensory experience differentially affects the volume of olfactory brain centres in a moth. *Cell Tissue Res*. 2016;364(1):59–65.
26. Williams AT, Verhulst EC, Haverkamp A. A unique sense of smell: development and evolution of a sexually dimorphic antennal lobe – a review. *Entomol Exp Appl*. 2022;170(4):303–18.
27. Eriksson M, Nylin S, Carlsson MA. Insect brain plasticity: effects of olfactory input on neuropil size. *R Soc Open Sci*. 2019;6(8):190875.
28. Johnson TL, Elgar MA, Symonds MRE. Movement and olfactory signals: Sexually dimorphic antennae and female flightlessness in moths. *Front Ecol Evolution*. 2022;Volume 10 – 2022.
29. Ahn S-J, Oh H-W, Corcoran J, Kim J-A, Park K-C, Park CG, et al. Sex-biased gene expression in antennae of *Drosophila suzukii*. *Arch Insect Biochem Physiol*. 2020;104(1):e21660.
30. Du H, Su W, Huang J, Ding G. Sex-biased expression of olfaction-related genes in the antennae of *Apis cerana* (Hymenoptera: Apidae). *Genes*. 2022;13(10):1771.
31. Yamhure-Ramírez D, Wainwright PC, Ramírez SR. Sexual dimorphism and morphological integration in the orchid bee brain. *Sci Rep*. 2025;15(1):8915.
32. Li J, Merchant A, Zhou S, Wang T, Zhou X, Zhou C. Neuroanatomical basis of sexual dimorphism in the mosquito brain. *iScience*. 2022. <https://doi.org/10.1016/j.isci.2022.105255>.
33. Couto A, Wainwright JB, Morris BJ, Montgomery SH. Linking ecological specialisation to adaptations in butterfly brains and sensory systems. *Curr Opin Insect Sci*. 2020;42:55–60.
34. Gowri V, Dion E, Viswanath A, Piel FM, Monteiro A. Transgenerational inheritance of learned preferences for novel host plant odors in *Bicyclus anynana* butterflies. *Evolution*. 2019;73(12):2401–14.
35. Dion E, Pui LX, Weber K, Monteiro A. Early-exposure to new sex pheromone blends alters mate preference in female butterflies and in their offspring. *Nat Commun*. 2020;11(1):53.
36. González-Rojas MF, Darragh K, Robles J, Linares M, Schulz S, McMillan WO, et al. Chemical signals act as the main reproductive barrier between sister and mimetic *Heliconius* butterflies. *Proceedings Biological sciences*. 1926;2020(287):20200587.
37. Gowri V, Monteiro A. Acquired preferences for a novel food odor do not become stronger or stable after multiple generations of odor feeding in *Bicyclus anynana* butterfly larvae. *Ann N Y Acad Sci*. 2024;1531(1):84–94.
38. Nieberding CM, de Vos H, Schneider MV, Lassance J-M, Estramil N, Andersson J, et al. The male sex pheromone of the butterfly *Bicyclus anynana*: towards an evolutionary analysis. *PLoS One*. 2008;3(7):e2751.
39. Dion E, Monteiro A, Yew JY. Phenotypic plasticity in sex pheromone production in *Bicyclus anynana* butterflies. *Sci Rep*. 2016;6:39002.
40. Ernst DA, Westerman EL. Stage- and sex-specific transcriptome analyses reveal distinctive sensory gene expression patterns in a butterfly. *BMC Genomics*. 2021;22(1):584.
41. Ernst DA, Agcaoili GA, Merrill AN, Westerman EL. A learning experience elicits sex-dependent neurogenomic responses in *Bicyclus anynana* butterflies. *Mol Ecol*. 2023;32(12):3220–38.
42. El Jundi B, Huetteroth W, Kurylas AE, Schachtner J. Anisometric brain dimorphism revisited: implementation of a volumetric 3D standard brain in *Manduca sexta*. *J Comp Neurol*. 2009;517(2):210–25.
43. Huetteroth W, Schachtner J. Standard three-dimensional glomeruli of the *Manduca sexta* antennal lobe: a tool to study both developmental and adult neuronal plasticity. *Cell Tissue Res*. 2005;319(3):513–24.
44. Heinze S, Reppert SM. Anatomical basis of sun compass navigation I: the general layout of the monarch butterfly brain. *J Comp Neurol*. 2012;520(8):1599–628.
45. Montgomery SH, Merrill RM, Ott SR. Brain composition in *Heliconius* butterflies, posteclosion growth and experience-dependent neuropil plasticity. *J Comp Neurol*. 2016;524(9):1747–69.
46. Sehadová H, Podlahová Š, Reppert SM, Sauman I. 3d reconstruction of larval and adult brain neuropils of two giant silk moth species: *Hyalophora cecropia* and *Antheraea pernyi*. *J Insect Physiol*. 2023;149:104546.
47. Carlsson MA, Schapers A, Nassel DR, Janz N. Organization of the olfactory system of nymphalidae butterflies. *Chem Senses*. 2013;38(4):355–67.
48. Pikielny CW, Hasan G, Rouyer F, Rosbash M. Members of a family of *Drosophila* putative odorant-binding proteins are expressed in different subsets of olfactory hairs. *Neuron*. 1994;12(1):35–49.
49. Angeli S, Ceron F, Scaloni A, Monti M, Monteforti G, Minnocci A, et al. Purification, structural characterization, cloning and immunocytochemical localization of chemoreception proteins from *Schistocerca gregaria*. *Eur J Biochem*. 1999;262(3):745–54.
50. Liu G, Sun B, Fan W, Yue S, He Q, Picimbon J-F. Renaming ‘Chemosensory’ Proteins (CSPs): ‘Lipoid-Binding Proteins’ — Molecular Nomenclature, Structure, Expression, Evolution, and Intracellular Functions. Preprints: Preprints; 2024.
51. Bu S, Lv Y, Liu Y, Qiao S, Wang H. Zinc finger proteins in neuro-related diseases progression. *Front Neurosci*. 2021. <https://doi.org/10.3389/fnins.2021.760567>.
52. Montgomery SH, Ott SR. Brain composition in *Godyris zavaleta*, a diurnal butterfly, reflects an increased reliance on olfactory information. *J Comp Neurol*. 2015;523(6):869–91.
53. Costanzo K, Monteiro A. The use of chemical and visual cues in female choice in the butterfly *Bicyclus anynana*. *Proc R Soc Lond [Biol]*. 2007;274(1611):845–51.
54. Catalán A, Macías-Muñoz A, Briscoe AD. Evolution of sex-biased gene expression and dosage compensation in the eye and brain of *Heliconius* butterflies. *Mol Biol Evol*. 2018;35(9):2120–34.
55. Catalán A, Hutter S, Parsch J. Population and sex differences in *Drosophila melanogaster* brain gene expression. *BMC Genomics*. 2012;13(1):654.
56. Gibilisco L, Zhou Q, Mahajan S, Bachtrog D. Alternative splicing within and between *Drosophila* species, sexes, tissues, and developmental stages. *PLoS Genet*. 2016;12(12):e1006464.
57. Parisi M, Nuttall R, Naiman D, Bouffard G, Malley J, Andrews J, et al. Paucity of genes on the *Drosophila* X chromosome showing male-biased expression. *Science*. 2003;299(5607):697–700.
58. Goldman TD, Arbeitman MN. Genomic and functional studies of *Drosophila* sex hierarchy regulated gene expression in adult head and nervous system tissues. *PLoS Genet*. 2007;3(11):e216.
59. Chang PL, Dunham JP, Nuzhdin SV, Arbeitman MN. Somatic sex-specific transcriptome differences in *Drosophila* revealed by whole transcriptome sequencing. *BMC Genomics*. 2011;12:364.
60. Wang ET, Sandberg R, Luo S, Khrebtkova I, Zhang L, Mayr C, et al. Alternative isoform regulation in human tissue transcriptomes. *Nature*. 2008;456(7221):470–6.
61. Trabzuni D, Ramasamy A, Imran S, Walker R, Smith C, Weale ME, et al. Widespread sex differences in gene expression and splicing in the adult human brain. *Nat Commun*. 2013;4(1):2771.
62. Mohr C, Hartmann B. Alternative splicing in *Drosophila* neuronal development. *J Neurogenet*. 2014;28(3–4):199–215.
63. Karlebach G, Veiga DFT, Mays AD, Chatzipsantziou C, Barja PP, Chatzou M, et al. The impact of biological sex on alternative splicing. *bioRxiv*. 2020:490904.
64. Naftaly AS, Pau S, White MA. Long-read RNA sequencing reveals widespread sex-specific alternative splicing in threespine stickleback fish. *Genome Res*. 2021;31(8):1486–97.
65. Chen W, Zhou W, Li Q, Mao X. Sex differences in gene expression and alternative splicing in the Chinese horseshoe bat. *PeerJ*. 2023;11:e15231.
66. Prakash A, Monteiro A. Molecular mechanisms of secondary sexual trait development in insects. *Curr Opin Insect Sci*. 2016;17:40–8.
67. Hopkins BR, Kopp A. Evolution of sexual development and sexual dimorphism in insects. *Curr Opin Genet Dev*. 2021;69:129–39.
68. Salvemini M, Polito C, Saccone G. Fruitless alternative splicing and sex behaviour in insects: an ancient and unforgettable love story? *J Genet*. 2010;89(3):287–99.

69. Wexler J, Delaney EK, Belles X, Schall C, Wada-Katsumata A, Amicucci MJ, et al. Hemimetabolous insects elucidate the origin of sexual development via alternative splicing. *Elife*. 2019;8:e47490.
70. Prakash A, Monteiro A. Doublesex mediates the development of sex-specific pheromone organs in *Bicyclus* butterflies via multiple mechanisms. *Mol Biol Evol*. 2020;37(6):1694–707.
71. Takahashi M, Okude G, Futahashi R, Takahashi Y, Kawata M. The effect of the doublesex gene in body colour masculinization of the damselfly *Ischnura senegalensis*. *Biol Lett*. 2021;17(6):20200761.
72. Neville MC, Nojima T, Ashley E, Parker DJ, Walker J, Southall T, et al. Male-specific fruitless isoforms target neurodevelopmental genes to specify a sexually dimorphic nervous system. *Curr Biol*. 2014;24(3):229–41.
73. Van't Hof AE, Whiteford S, Yung CJ, Yoshida A, Zrzavá M, de Jong MA, et al. Zygosity-based sex determination in a butterfly drives hypervariability of *Masculinizer*. *Sci Adv*. 2024;10(18):eadj6979.
74. Luan S, Wang C. Calcium signaling mechanisms across kingdoms. *Annu Rev Cell Dev Biol*. 2021;37(Volume 37, 2021):311–40.
75. Saghian R, Wang L-Y. Voltage-gated calcium channels (VGCCs) and synaptic transmission. In: Zamponi GW, Weiss N, editors. *Voltage-Gated Calcium Channels*. Cham: Springer International Publishing; 2022. p. 359–83.
76. Kamb A, Tseng-Crank J, Tanouye MA. Multiple products of the *Drosophila* Shaker gene may contribute to potassium channel diversity. *Neuron*. 1988;1(5):421–30.
77. Pongs O, Kecskemethy N, Müller R, Krah-Jentgens I, Baumann A, Kiltz HH, et al. Shaker encodes a family of putative potassium channel proteins in the nervous system of *Drosophila*. *EMBO J*. 1988;7(4):1087–96.
78. Schwarz TL, Tempel BL, Papazian DM, Jan YN, Jan LY. Multiple potassium-channel components are produced by alternative splicing at the Shaker locus in *Drosophila*. *Nature*. 1988;331(6152):137–42.
79. Tan J, Liu Z, Nomura Y, Goldin AL, Dong K. Alternative splicing of an insect sodium channel gene generates pharmacologically distinct sodium channels. *J Neurosci*. 2002;22(13):5300–9.
80. Houot B, Fraichard S, Greenspan RJ, Ferveur JF. Genes involved in sex pheromone discrimination in *Drosophila melanogaster* and their background-dependent effect. *PLoS One*. 2012;7(1):e30799.
81. Peretz A, Abitbol I, Sobko A, Wu CF, Attali B. A Ca²⁺/calmodulin-dependent protein kinase modulates *Drosophila* photoreceptor K⁺ currents: a role in shaping the photoreceptor potential. *J Neurosci*. 1998;18(22):9153–62.
82. Gür B, Sporar K, Lopez-Behling A, Silies M. Distinct expression of potassium channels regulates visual response properties of lamina neurons in *Drosophila melanogaster*. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol*. 2020;206(2):273–87.
83. Arenas A, Giurfa M, Sandoz JC, Hourcade B, Devaud JM, Farina WM. Early olfactory experience induces structural changes in the primary olfactory center of an insect brain. *Eur J Neurosci*. 2012;35(5):682–90.
84. Alcalde Anton A, Young FJ, Melo-Flórez L, Couto A, Cross S, McMillan WO, et al. Adult neurogenesis does not explain the extensive post-eclosion growth of *Heliconius* mushroom bodies. *R Soc Open Sci*. 2023;10(10):230755.
85. Heck MM, Pereira A, Pesavento P, Yannoni Y, Spradling AC, Goldstein LS. The kinesin-like protein KLP61F is essential for mitosis in *Drosophila*. *J Cell Biol*. 1993;123(3):665–79.
86. Feng C, Cleary JM, Kothe GO, Stone MC, Weiner AT, Hertzler JJ, et al. Trim9 and Klp61F promote polymerization of new dendritic microtubules along parallel microtubules. *J Cell Sci*. 2021;134(11).
87. Lin YH, Maaroufi HO, Kucerova L, Rouhova L, Filip T, Zurovec M. Adenosine receptor and its downstream targets, mod(mdg4) and hsp70, work as a signaling pathway modulating cytotoxic damage in *Drosophila*. *Front Cell Dev Biol*. 2021;9:651367.
88. Raming K, Freitag J, Krieger J, Breer H. Arrestin-subtypes in insect antennae. *Cell Signal*. 1993;5(1):69–80.
89. Alvarez CE. On the origins of arrestin and rhodopsin. *BMC Evol Biol*. 2008;8:222.
90. Kaneko K, Suenami S, Kubo T. Gene expression profiles and neural activities of Kenyon cell subtypes in the honeybee brain: identification of novel 'middle-type' Kenyon cells. *Zool Lett*. 2016;2(1):14.
91. Yamane A, Kohno H, Ikeda T, Kaneko K, Ugajin A, Fujita T, et al. Gene expression and immunohistochemical analyses of mkast suggest its late pupal and adult-specific functions in the honeybee brain. *PLoS One*. 2017;12(5):e0176809.
92. Kohno H, Kubo T. Mkast is dispensable for normal development and sexual maturation of the male European honeybee. *Sci Rep*. 2018;8(1):11877.
93. Bohbot J, Sobrio F, Lucas P, Nagnan-Le Meillour P. Functional characterization of a new class of odorant-binding proteins in the moth *Mamestra brassicae*. *Biochem Biophys Res Commun*. 1998;253(2):489–94.
94. Kitabayashi AN, Arai T, Kubo T, Natori S. Molecular cloning of cDNA for p10, a novel protein that increases in the regenerating legs of *Periplaneta americana* (American cockroach). *Insect Biochem Mol Biol*. 1998;28(10):785–90.
95. Nagnan-Le Meillour P, Cain AH, Jacquin-Joly E, François MC, Ramachandran S, Maida R, et al. Chemosensory proteins from the proboscis of *Mamestra brassicae*. *Chem Senses*. 2000;25(5):541–53.
96. Jacquin-Joly E, Vogt RG, François M-C, Nagnan-Le Meillour P. Functional and expression pattern analysis of chemosensory proteins expressed in antennae and pheromonal gland of *Mamestra brassicae*. *Chem Senses*. 2001;26(7):833–44.
97. Zhu J, Wang F, Zhang Y, Yang Y, Hua D. Odorant-binding protein 10 from *Bradysia odoriphaga* (Diptera: Sciaridae) binds volatile host plant compounds. *J Insect Sci*. 2023. <https://doi.org/10.1093/jisesa/iead004>.
98. Lin Y, Huang J, Akutse K, Hou Y. Phytopathogens increase the preference of insect vectors to volatiles emitted by healthy host plants. *J Agric Food Chem*. 2022;70(16):5262–9.
99. Widmer YF, Bilcan A, Bruggmann R, Sprecher SG. Regulators of long-term memory revealed by mushroom body-specific gene expression profiling in *Drosophila melanogaster*. *Genetics*. 2018;209(4):1167–81.
100. Mappin F, Bellantuono AJ, Ebrahimi B, DeGennaro M. Odor-evoked transcriptomics of *Aedes aegypti* mosquitoes. *PLoS One*. 2023;18(10):e0293018.
101. Wang ZL, Wang H, Qin QH, Zeng ZJ. Gene expression analysis following olfactory learning in *Apis mellifera*. *Mol Biol Rep*. 2013;40(2):1631–9.
102. Charlton-Perkins M, Cook TA. Chapter five - Building a fly eye: Terminal differentiation events of the retina, corneal lens, and pigmented epithelia. In: Cagan RL, Reh TA, editors. *Current Topics in Developmental Biology*. 93: Academic Press; 2010.129–173.
103. Martin JH, Benzer S, Rudnicka M, Miller CA. Calphotin: a *Drosophila* photoreceptor cell calcium-binding protein. *Proc Natl Acad Sci U S A*. 1993;90(4):1531–5.
104. Allen ML, Rhoades JH, Sparks ME, Grodowitz MJ. Differential gene expression in red imported fire ant (*Solenopsis invicta*) (Hymenoptera: Formicidae) larval and pupal stages. *Insects*. 2018;9(4):185.
105. Hanneman EH, Kanost MR. Differential alarperin expression during development of the antennae in the tobacco hawkmoth, *Manduca sexta*. *Arch Insect Biochem Physiol*. 1992;19(1):39–52.
106. Zars T. Behavioral functions of the insect mushroom bodies. *Curr Opin Neurobiol*. 2000;10(6):790–5.
107. Baltruschat L, Prisco L, Ranft P, Lauritzen JS, Fiala A, Bock DD, et al. Circuit reorganization in the *Drosophila* mushroom body calyx accompanies memory consolidation. *Cell Rep*. 2021;34(11):108871.
108. Kalsotra A, Cooper TA. Functional consequences of developmentally regulated alternative splicing. *Nat Rev Genet*. 2011;12(10):715–29.
109. Grantham ME, Brisson JA. Extensive differential Splicing underlies phenotypically plastic Aphid morphs. *Mol Biol Evol*. 2018;35(8):1934–46.
110. Tian S, Monteiro A. A transcriptomic atlas underlying developmental plasticity of seasonal forms of *Bicyclus anynana* butterflies. *Mol Biol Evol*. 2022. <https://doi.org/10.1093/molbev/msac126>.
111. Norris A, Calarco J. Emerging roles of alternative pre-mRNA splicing regulation in neuronal development and function. *Front Neurosci*. 2012. <https://doi.org/10.3389/fnins.2012.00122>.
112. Zheng S, Black DL. Alternative pre-mRNA splicing in neurons: growing up and extending its reach. *Trends Genet*. 2013;29(8):442–8.
113. Su C-H, D D, Tarn W-Y. Alternative splicing in neurogenesis and brain development. *Front Mol Biosci*. 2018. <https://doi.org/10.3389/fmolb.2018.00012>.
114. Grabski DF, Broseus L, Kumari B, Rekosh D, Hammarskjöld ML, Ritchie W. Intron retention and its impact on gene expression and protein diversity: a review and a practical guide. *Wiley Interdiscip Rev RNA*. 2021;12(1):e1631.

115. Wong JLL, Schmitz U. Intron retention: importance, challenges, and opportunities. *Trends Genet.* 2022;38(8):789–92.
116. Buckley PT, Lee MT, Sul JY, Miyashiro KY, Bell TJ, Fisher SA, et al. Cytoplasmic intron sequence-retaining transcripts can be dendritically targeted via ID element retrotransposons. *Neuron.* 2011;69(5):877–84.
117. Mauger O, Lemoine F, Scheiffele P. Targeted intron retention and excision for rapid gene regulation in response to neuronal activity. *Neuron.* 2016;92(6):1266–78.
118. Petrić Howe M, Crerar H, Neeves J, Harley J, Tyzack GE, Klein P, et al. Physiological intron retaining transcripts in the cytoplasm abound during human motor neurogenesis. *Genome Res.* 2022;32(10):1808–25.
119. Furutani S, Ihara M, Nishino Y, Akamatsu M, Jones AK, Sattelle DB, et al. Exon 3 Splicing and Mutagenesis Identify Residues Influencing Cell Surface Density of Heterologously Expressed Silkworm (Bombyx mori) Glutamate-Gated Chloride Channels. *Mol Pharmacol.* 2014;86(6):686–95.
120. Kita T, Ozoe F, Ozoe Y. Expression pattern and function of alternative splice variants of glutamate-gated chloride channel in the housefly *Musca domestica*. *Insect Biochem Mol Biol.* 2014;45:1–10.
121. Wu S-F, Mu X-C, Dong Y-X, Wang L-X, Wei Q, Gao C-F. Expression pattern and pharmacological characterisation of two novel alternative splice variants of the glutamate-gated chloride channel in the small brown planthopper *Laodelphax striatellus*. *Pest Manag Sci.* 2017;73(3):590–7.
122. El Hassani AK, Schuster S, Dyck Y, Demares F, Lebouille G, Armen-gaud C. Identification, localization and function of glutamate-gated chloride channel receptors in the honeybee brain. *Eur J Neurosci.* 2012;36(4):2409–20.
123. Demares F, Drouard F, Massou I, Crattelet C, Lœuillet A, Bettiol C, et al. Differential involvement of glutamate-gated chloride channel splice variants in the olfactory memory processes of the honeybee *Apis mellifera*. *Pharmacol Biochem Behav.* 2014;124:137–44.
124. Schiavo G, Stenbeck G, Rothman JE, Söllner TH. Binding of the synaptic vesicle v-SNARE, synaptotagmin, to the plasma membrane t-SNARE, SNAP-25, can explain docked vesicles at neurotoxin-treated synapses. *Proc Natl Acad Sci U S A.* 1997;94(3):997–1001.
125. Kikuma K, Li X, Kim D, Sutter D, Dickman DK. Extended synaptotagmin localizes to presynaptic ER and promotes neurotransmission and synaptic growth in *Drosophila*. *Genetics.* 2017;207(3):993–1006.
126. Dean C, Dunning FM, Liu H, Bomba-Warczak E, Martens H, Bharat V, et al. Axonal and dendritic synaptotagmin isoforms revealed by a pHluorin-syt functional screen. *Mol Biol Cell.* 2012;23(9):1715–27.
127. Wu X, Hu S, Kang X, Wang C. Synaptotagmins: beyond presynaptic neurotransmitter release. *Neuroscientist.* 2020;26(1):9–15.
128. Johard HA, Risinger C, Nässel DR, Larhammar D. The highly conserved synapse protein SNAP-25 displays sequence variability in the cockroach *Leucophaea maderae*. *Comp Biochem Physiol B Biochem Mol Biol.* 1999;122(1):63–8.
129. Vilinsky I, Stewart BA, Drummond J, Robinson I, Deitcher DL. A *Drosophila* SNAP-25 null mutant reveals context-dependent redundancy with SNAP-24 in neurotransmission. *Genetics.* 2002;162(1):259–71.
130. Nagy G, Milosevic I, Fasshauer D, Müller EM, de Groot BL, Lang T, et al. Alternative splicing of SNAP-25 regulates secretion through nonconservative substitutions in the SNARE domain. *Mol Biol Cell.* 2005;16(12):5675–85.
131. Zhang L-Z, Yan W-Y, Wang Z-L, Guo Y-H, Yi Y, Zhang S-W, et al. Differential protein expression analysis following olfactory learning in *Apis cerana*. *J Comp Physiol A.* 2015;201(11):1053–61.
132. Ren Z, Yu J, Wu Z, Si W, Li X, Liu Y, et al. MicroRNA-210-5p contributes to cognitive impairment in early vascular dementia rat model through targeting Snap25. *Front Mol Neurosci.* 2018. <https://doi.org/10.3389/fnmol.2018.00388>.
133. Hou Q, Gao X, Zhang X, Kong L, Wang X, Bian W, et al. SNAP-25 in hippocampal CA1 region is involved in memory consolidation. *Eur J Neurosci.* 2004;20(6):1593–603.
134. Spellmann I, Müller N, Musil R, Zill P, Douhet A, Dehning S, et al. Associations of SNAP-25 polymorphisms with cognitive dysfunctions in Caucasian patients with schizophrenia during a brief trial of treatment with atypical antipsychotics. *Eur Arch Psychiatry Clin Neurosci.* 2008;258(6):335–44.
135. Golimbet VE, Alfimova MV, Gritsenko IK, Lezheiko TV, Lavrushina OM, Abramova LI, et al. Association between a synaptosomal protein (SNAP-25) gene polymorphism and verbal memory and attention in patients with endogenous psychoses and mentally healthy subjects. *Neurosci Behav Physiol.* 2010;40(4):461–5.
136. Toh YP, Dion E, Monteiro A. Dissections of larval, pupal and adult butterfly brains for immunostaining and molecular analysis. *Methods Protoc.* 2021;4(3):53.
137. Ott SR. Confocal microscopy in large insect brains: zinc-formaldehyde fixation improves synapsin immunostaining and preservation of morphology in whole-mounts. *J Neurosci Methods.* 2008;172(2):220–30.
138. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods.* 2012;9(7):676–82.
139. Solari P, Corda V, Sollai G, Kreissl S, Galizia CG, Crnjar R. Morphological characterization of the antennal lobes in the Mediterranean fruit fly *Ceratitis capitata*. *J Comp Physiol A.* 2016;202(2):131–46.
140. Stocker RF, Lienhard MC, Borst A, Fischbach KF. Neuronal architecture of the antennal lobe in *Drosophila melanogaster*. *Cell Tissue Res.* 1990;262(1):9–34.
141. R Development Core Team. R: A language and environment for statistical computing Vienna, Austria: ISBN 3–900051–07–0; 2020 [Available from: <http://www.R-project.org>].
142. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics.* 2014;30(15):2114–20.
143. Bushnell B. BBMap: A Fast, Accurate, Splice-Aware Aligner. United-States2014 [Available from: <https://www.osti.gov/biblio/1241166>].
144. Kopylova E, Noé L, Touzet H. SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. *Bioinformatics.* 2012;28(24):3211–7.
145. Murugesan SN, Connahs H, Matsuoka Y, Das Gupta M, Tiong GJL, Huq M, et al. Butterfly eyespots evolved via cooption of an ancestral gene-regulatory network that also patterns antennae, legs, and wings. *Proc Natl Acad Sci.* 2022;119(8):e2108661119.
146. Pertea M, Kim D, Pertea GM, Leek JT, Salzberg SL. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. *Nat Protoc.* 2016;11(9):1650–67.
147. Kim D, Paggi JM, Park C, Bennett C, Salzberg SL. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat Biotechnol.* 2019;37(8):907–15.
148. Pertea G, Pertea M. GFF utilities: GffRead and GffCompare. *F1000Res.* 2020. <https://doi.org/10.12688/f1000research.23297.1>.
149. Pertea M, Pertea GM, Antonescu CM, Chang T-C, Mendell JT, Salzberg SL. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nat Biotechnol.* 2015;33(3):290–5.
150. Posit Team. RStudio: Integrated Development Environment for R. <http://www.posit.co/> Boston, MA. 2023 [
151. Wickham H. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York; 2016.
152. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 2014;15(12):550.
153. Chen J, Behnam E, Huang J, Moffatt MF, Schaid DJ, Liang L, et al. Fast and robust adjustment of cell mixtures in epigenome-wide association studies with SmartSVA. *BMC Genomics.* 2017;18(1):413.
154. Park JW, Tokheim C, Shen S, Xing Y. Identifying differential alternative splicing events from RNA sequencing data using RNASeq-MATS. In: Shomron N, editor. Deep sequencing data analysis. Totowa, NJ: Humana Press; 2013. p. 171–9.
155. Shen S, Park JW, Huang J, Dittmar KA, Lu Z-x, Zhou Q, et al. MATS: a Bayesian framework for flexible detection of differential alternative splicing from RNA-Seq data. *Nucleic Acids Res.* 2012;40(8):e61–e.
156. Shen S, Park JW, Lu Z-x, Lin L, Henry MD, Wu YN, et al. rMATS: Robust and flexible detection of differential alternative splicing from replicate RNA-Seq data. *Proc Natl Acad Sci USA.* 2014;111(51):E5593–601.
157. Wickham H. Reshaping data with the reshape package. *J Stat Softw.* 2007;21(12):1–20.
158. Kassambara A. ggpubr: 'ggplot2' Based Publication Ready Plots. R package version 0.6.1. 2025:170–2. <https://rpkgs.datanovia.com/ggpubr/>.
159. Ram K, Wickham H. wesanderson: A Wes Anderson Palette Generator (<https://CRAN.R-project.org/package=wesanderson>) 2023 [

160. Danecek P, Bonfield JK, Liddle J, Marshall J, Ohan V, Pollard MO, et al. Twelve years of SAMtools and BCFtools. *Gigascience*. 2021. <https://doi.org/10.1093/gigascience/giab008>.
161. Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, et al. Integrative genomics viewer. *Nat Biotechnol*. 2011;29(1):24–6.
162. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, et al. BLAST+: architecture and applications. *BMC Bioinformatics*. 2009;10(1):421.
163. Buchfink B, Reuter K, Drost H-G. Sensitive protein alignments at tree-of-life scale using DIAMOND. *Nat Methods*. 2021;18(4):366–8.
164. Buchfink B, Xie C, Huson DH. Fast and sensitive protein alignment using DIAMOND. *Nat Methods*. 2015;12(1):59–60.
165. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene Ontology: tool for the unification of biology. *Nat Genet*. 2000;25(1):25–9.
166. Jones P, Binns D, Chang HY, Fraser M, Li W, McAnulla C, et al. InterPro-Scan 5: genome-scale protein function classification. *Bioinformatics*. 2014;30(9):1236–40.
167. Huerta-Cepas J, Szklarczyk D, Heller D, Hernández-Plaza A, Forslund SK, Cook H, et al. eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. *Nucleic Acids Res*. 2019;47(D1):D309–d14.
168. Cantalapiedra CP, Hernández-Plaza A, Letunic I, Bork P, Huerta-Cepas J. eggNOG-mapper v2: functional annotation, orthology assignments, and domain prediction at the metagenomic scale. *Mol Biol Evol*. 2021;38(12):5825–9.
169. Yu G, Wang LG, Han Y, He QY. ClusterProfiler: an R package for comparing biological themes among gene clusters. *Omics*. 2012;16(5):284–7.
170. Dion E. Scripts used in Dion et al. Butterfly brain transcriptomics. *BMC Biol*. figshare; 2025. <https://doi.org/10.6084/m9.figshare.30856208>.
171. Dion E. RNAseq analysis scripts of exposed butterfly brains from Dion et al. *BMC Biol*. GitHub; 2025. https://github.com/Deguydion/RNAseq_ButterflyBrain_Dionetal/tree/main.
172. Toh YP, Dion E. Dataset of AL volumes_Dionetal. *BMC Biol*. figshare; 2025. <https://doi.org/10.6084/m9.figshare.27873258.v2>.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.