


RESEARCH ARTICLE

Open Access



# The genome and sex-dependent responses to temperature in the common yellow butterfly, *Eurema hecabe*

Ivy H. T. Lee<sup>1†</sup>, Wenyan Nong<sup>1†</sup>, Wai Lok So<sup>1†</sup>, Chris K. H. Cheung<sup>1</sup>, Yichun Xie<sup>1</sup>, Toby Baril<sup>2</sup>, Ho Yin Yip<sup>1</sup>, Thomas Swale<sup>3</sup>, Simon K. F. Chan<sup>4</sup>, Yingying Wei<sup>5</sup>, Nathan Lo<sup>6</sup>, Alexander Hayward<sup>2</sup>, Ting Fung Chan<sup>7</sup>, Hon-ming Lam<sup>8</sup> and Jerome H. L. Hui<sup>1\*</sup> 

## Abstract

**Background** Lepidoptera (butterflies and moths) is one of the most geographically widespread insect orders in the world, and its species play important and diverse ecological and applied roles. Climate change is one of the biggest challenges to biodiversity this century, and lepidopterans are vulnerable to climate change. Temperature-dependent gene expression differences are of relevance under the ongoing climate crisis. However, little is known about how climate affects gene expression in lepidopterans and the ecological consequences of this, particularly with respect to genes with biased expression in one of the sexes. The common yellow butterfly, *Eurema hecabe* (Family Pieridae), is one of the most geographically widespread lepidopterans that can be found in Asia, Africa, and Australia. Nevertheless, what temperature-dependent effects there may be and whether the effects differ between the sexes remain largely unexplored.

**Results** Here, we generated high-quality genomic resources for *E. hecabe* along with transcriptomes from eight developmental stages. Male and female butterflies were subjected to varying temperatures to assess sex-specific gene expression responses through mRNA and microRNA transcriptomics. We find that there are more temperature-dependent sex-biased genes in females than males, including genes that are involved in a range of biologically important functions, highlighting potential ecological impacts of increased temperatures. Further, by considering available butterfly data on sex-biased gene expression in a comparative genomic framework, we find that the pattern of sex-biased gene expression identified in *E. hecabe* is highly species-specific, rather than conserved across butterfly species, suggesting that sex-biased gene expression responses to climate change are complex in butterflies.

**Conclusions** Our study lays the foundation for further understanding of differential responses to environmental stress in a widespread lepidopteran model and demonstrates the potential complexity of sex-specific responses of lepidopterans to climate change.

**Keywords** Butterfly, Sex-biased, Temperature, Transcriptome, Genome, Sesquiterpenoid, MicroRNA cluster

<sup>†</sup>Ivy H. T. Lee, Wenyan Nong, and Wai Lok So contributed equally to this work.

\*Correspondence:

Jerome H. L. Hui

jeromehui@cuhk.edu.hk

Full list of author information is available at the end of the article

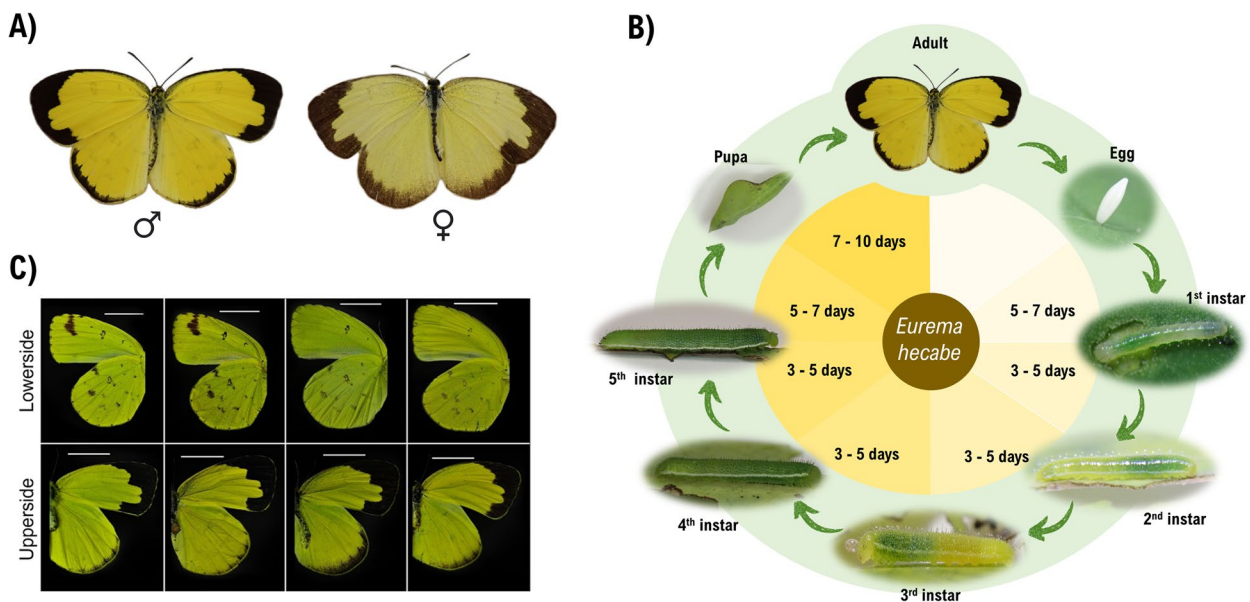


**Background**

Lepidoptera is a large insect order that contains more than 150,000 described species of butterflies and moths and accounts for about 10% of all the described living species in the world [1]. Aside from their great biodiversity, lepidopterans also play important roles in the environment and agriculture either as pollinators, herbivores, or prey [2, 3]. Climate change, including shifting patterns of temperature and precipitation, is one of the biggest challenges to biodiversity this century. Insects such as lepidopterans may be especially vulnerable to climate change, as their body temperature can be highly correlated with the surrounding temperature [4]. Indeed, over recent years, several populations of butterflies around the globe have been affected by the changing climate and extreme temperature events [5, 6]. In general, higher temperatures result in decreases of reproductive success, changes to behaviour, and sometimes lethality in investigated species [7–11].

Pieridae, the whites and sulphurs family of butterflies, contains about 1100 described species worldwide [12, 13]. The pierid species, the common grass yellow butterfly, *Eurema hecabe*, is an abundant and particularly widely distributed species, found in Asia, Africa, and Australia (Fig. 1A, B) [14]. Given the abundance and geographical distribution of *E. hecabe*, this species offers a useful model for understanding the effects of climate change on a common lepidopteran species across three continents.

Increasing studies have demonstrated within species divergence in traits other than morphology, such as in physiological and molecular traits. For example, environmental responses of populations from different geographical locations can differ, while the sexes of the same species can also exhibit a wide range of differences in biological processes [15]. It has been hypothesized that between sex differences in animal physiology can result in different degrees of responses to environmental change [16]. In Pieridae and Nymphalidae butterflies, protandry (males appearing earlier than females) has been reported to maximize mating opportunities of males [17–20]. Meanwhile, males and females have been shown to exhibit different immune and reproductive responses, resulting in different adaptation abilities [21–24]. However, when it comes to understanding the effects of climate change (e.g. temperature increases) on animals, most contemporary studies still treat species as having a unified response. There are also relatively few studies examining how temperature may elicit different responses between lepidopteran sexes. One study which did consider this, focusing on *Bicyclus anynana* (Nymphalidae), demonstrated significant sexual dimorphism in both eye morphology and opsin gene expressions under different temperatures [25]. Thus, while the question of sex-specific responses to climate change in lepidopterans remains largely unexplored, there is evidence that this may have relevance to important traits of evolutionary and ecological importance.



**Fig. 1** Common yellow *Eurema hecabe*. **A** Photographs of male (left) and female (right) *E. hecabe*. **B** Life cycle of *E. hecabe* cultured in the laboratory. **C** Wing morphologies of B-type *E. hecabe* collected in different seasons

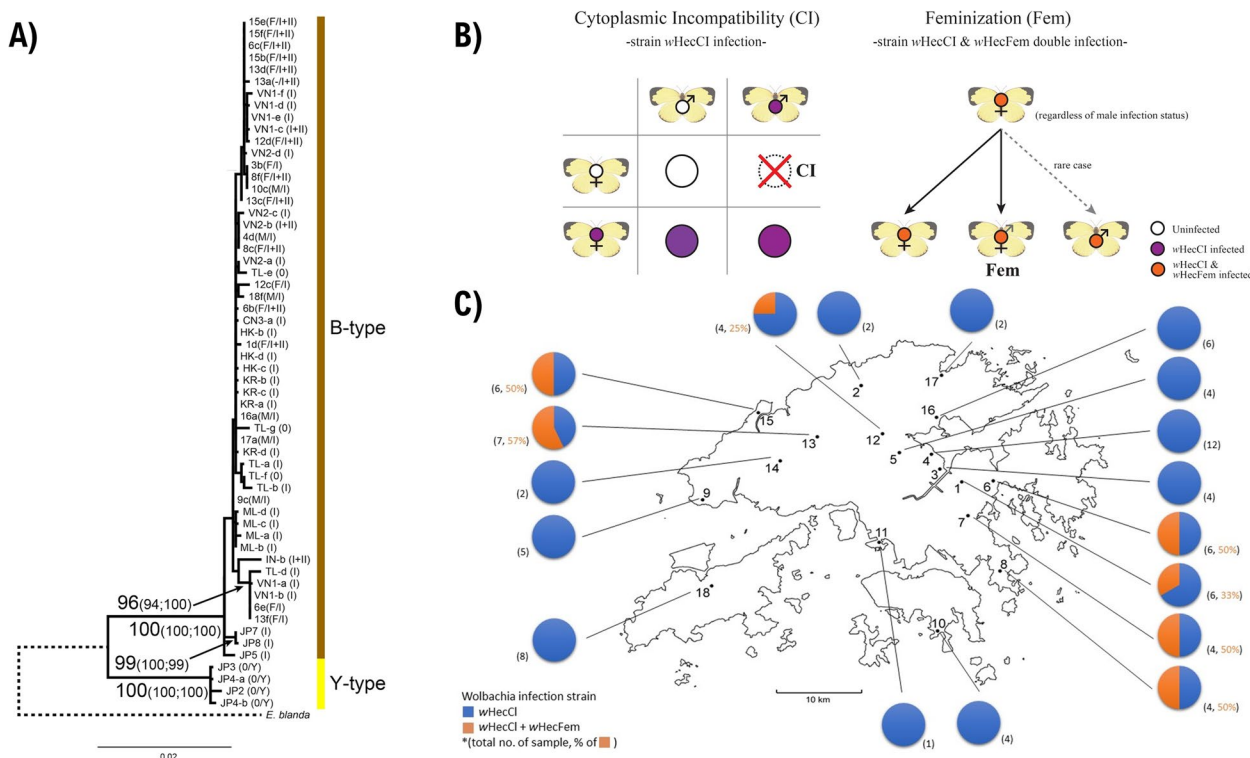
Here, we collected and sequenced the genome of *E. hecabe* and its associated *Wolbachia* strains collected in Hong Kong. In addition, a high-quality genome assembly is presented, together with accompanying transcriptomes from all available developmental stages (egg, first to fifth instars, pupa and adult). Adult butterflies were also cultured at different temperatures, and mRNA and micro-RNA transcriptomes were obtained from the two sexes, to analyse the genetic effects of variation in temperature upon *E. hecabe*.

## Results and discussion

### B-type *E. hecabe* in Hong Kong with high *Wolbachia* infection rate

Previous studies have identified two sibling species, the B- and Y-types, in *E. hecabe* based on the mitochondrial markers: cytochrome *c* oxidase subunit I (COI), cytochrome *c* oxidase subunit III, and NADH dehydrogenase subunit 5 (e.g. [26]). The B-type is generally widespread in tropical and subtropical areas, while the Y-type is usually restricted to temperate areas in the northern Japan, with the exception of a Y-type individual collected in Hong Kong in 1995 [26]. Variations among different aspects of the biology of these sister species have been

observed. For example, morphological variation including polymorphic forms of wing patterns have been found during different seasons and in different geographical locations in Japan [27, 28], and the colour of the forewing fringe on the B-type is brown while it is yellow on the Y-type [29]. Furthermore, behavioural and ecological variation, such as host plant utilization [30], larval feeding response [30], and mate choice [31] has also been reported between the two genetic subtypes. Due to the above distinctions, Y-type *E. hecabe* is now reclassified as the separate species *E. mandarina*, while the B-type *E. hecabe* is regarded as a monophyletic and homogenous lineage [26]. Among the 89 COI sequences (1326 bp) we collected, 40 polymorphic sites were identified with 24 being parsimony-informative. The wing morphology of individuals collected from different seasons were also compared; however, no identifiable differences were observed (Fig. 1C). Using COI sequences together with sequences deposited in GenBank, all collected butterflies were identified as B-type *E. hecabe*, supported by high bootstrap values or probabilities in all phylogenetic analyses (94% in maximum-likelihood, 95% in neighbour-joining, and 100% in Bayesian inference, Fig. 2A).



**Fig. 2** Population genetics of *E. hecabe* and their *Wolbachia* infection status in Hong Kong. **A** Phylogenetic analysis of concatenated COI, COIII, and ND5 gene in this study with sequences from other countries (sequences from Narita et al., 2007a), showing all samples collected in this study were found to be B-type. **B** Schematic diagram showing the reproductive phenotypes of *E. hecabe* infected with different *Wolbachia* strains. For details, please refer to the main text. **C** The proportion of wHecCI infection and wHecCI + wHecFem double infection are shown in pie charts

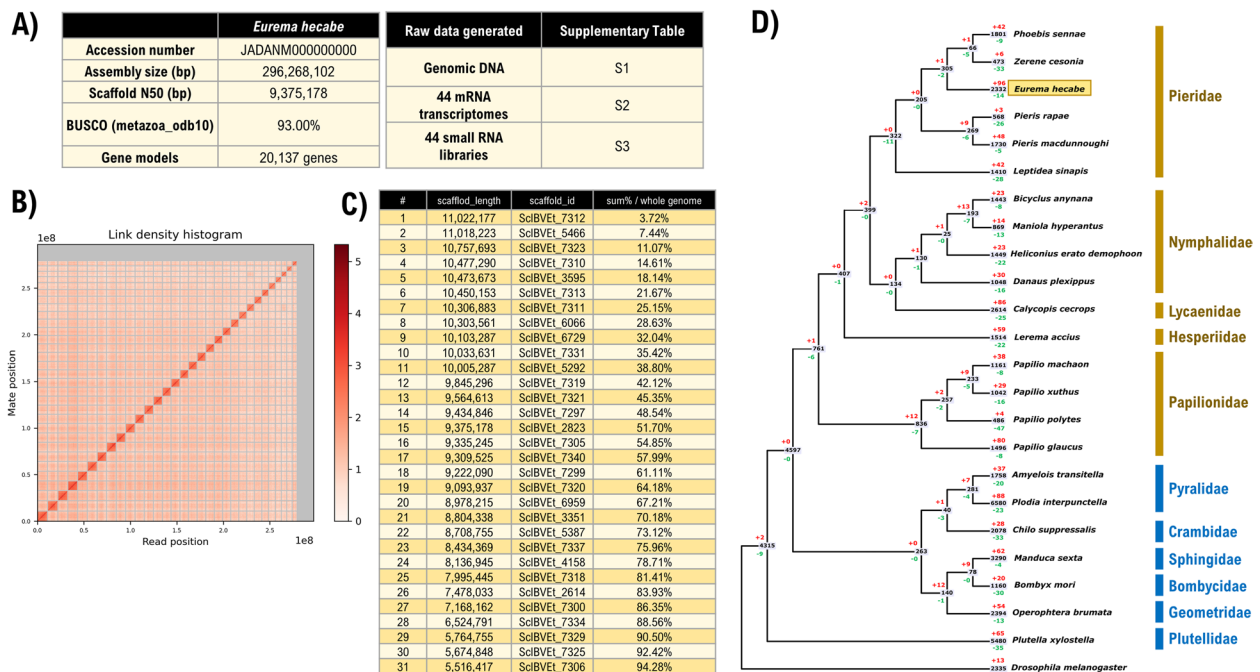
The endosymbiont *Wolbachia* represents one of the most abundant bacteria present in butterflies [32]. There are at least four major subgroups/strains of *Wolbachia* (A–D), with strains A and B thought to have diverged ~60 million years ago and be confined to arthropods [33]. Two *Wolbachia* B subgroup strains, *wHecCI* (strain I) and *wHecFem* (strain II), have been found to infect *Eurema* butterflies, with profound influences on host sex determination well documented in *Eurema mandarina* [32, 34, 35]. Infection with *wHecCI* occurs both in isolation and together with *wHecFem*, while infection with *wHecFem* appears to only occur together with *wHecCI* [36]. Infection with *wHecCI* induces cytoplasmic incompatibility (CI) when an infected male mates with an uninfected female [36, 37] (Fig. 2B). Meanwhile, in *wHecFem* infected lineages, the W chromosome has been lost [34, 35], and associated loss of the female-determining W chromosome is functionally compensated by *wHecFem* [34, 38]. Additionally, a meiotic drive mechanism appears to prevent inheritance of the maternal Z chromosome due to non-random segregation of the sex chromosomes [34, 35]. Thus, in *wHecFem* infected individuals, the production of ZZ progeny is inhibited resulting in only 0Z eggs being produced (with a paternally inherited Z), and the resultant offspring are feminized by *wHecFem* to become functional females, effectively leading to all female progeny [34, 35]. Among 89 analysed samples, all

were found to be infected by *Wolbachia* strain *wHecCI*, while 18 (20.2%) were co-infected with *Wolbachia* strain *wHecFem* (Fig. 2C).

**High-quality *E. hecabe* genome, macrosynteny, and gene expansion**

We generated a high-quality chromosome-level genome assembly of *E. hecabe* ( $2n=62$ ), for which 94.28% of the genomic sequences are contained on 31 pseudomolecules (~296.3 Mb) (Fig. 3A, B), which is compatible with the estimated genome size of 314.7 Mb by k-mer 21 (Additional file 1: Figure S1 & 2). The BUSCO (Benchmarking Universal Single-Copy Orthologs) score for complete genes is 93%, and the scaffold N50 and L50 of the genome are 15 Mb and 9.375 Mb, respectively (Fig. 1C).

The *E. hecabe* genome contains ~20% repetitive sequences, with 999 distinct transposable element (TE) classifications identified by our annotation (Additional file 1: Figure S3, Additional file 2). The dominant TE type is rolling circle elements (8.7%), with the next most abundant TE type being unclassified elements (4.6%), followed by LINES (4.5%). There are few LTR elements (0.8%) and very few SINES (0.001%) present in the genome (Additional file 2). LINES are the most diverse TE type in the *E. hecabe* genome, with 385 distinct LINE families identified by our analysis. A repeat landscape analysis suggests a gradual increase in TE activity over time, followed by a slight recent



**Fig. 3** Genomic analyses of *E. hecabe* genome. **A** Tables of genome statistics and resources generated in this study. **B** Link density histogram of the *E. hecabe* genome. **C** Information of the 31 pseudochromosomes. **D** Gene family expansion and contraction in insects. Red and green colours indicate the number of significantly ( $p < 0.05$ ) expanded (+) or contracted (-) gene families at each node, respectively



decline in activity (Additional file 1: Figure S3). Consistent with their abundance in the *E. hecabe* genome, rolling circle elements and LINES are the most recently active TEs, but there appears to have been a considerable very recent increase in DNA and LTR element activity (Additional file 1: Figure S3).

Using the transcriptomic data, we predicted a total of 20,137 gene models, including 18,197 protein coding genes and 1940 tRNA genes in this genome, which is in the range similar to other published Pieridae genomes (Fig. 3C, D). The pseudochromosomes of *E. hecabe* displayed significant one-to-one relationships against the genomic scaffolds in other butterflies (Additional file 1: Figure S2). Lineage blocks were also visible when compared with the fruit fly *D. melanogaster*, suggesting that the genome architecture of these lineages had not undergone active chromosomal rearrangements from their last common ancestor.

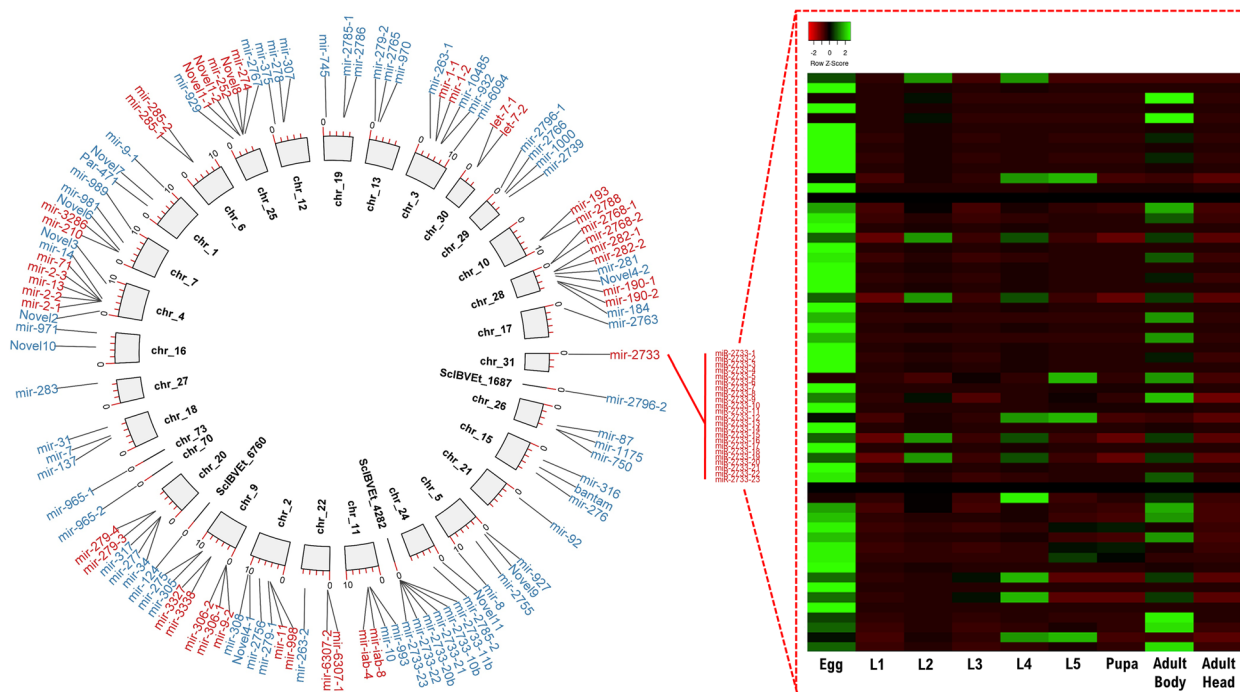
**Conserved and novel microRNAs in *E. hecabe***

MicroRNAs are 21–23 nucleotides of noncoding RNAs that regulate gene expression post-transcriptionally and have been suggested to be a potential contributor to the evolution of arthropod developmental processes and morphologies (e.g. [39–42]). Previous sequencing of small RNA from lepidopterans identified a burst of microRNA innovation in these lineages [43]. Here, we

sequenced the small RNA transcriptomes from 8 developmental stages, including egg, first to fifth instars, pupa, and adult of *E. hecabe* (Fig. 1D). Among all microRNA precursor candidates identified in *E. hecabe*, a total of 130 microRNAs were identified (Fig. 4). Similar to a previous study [43], we also identify novel or lineage-specific microRNAs, finding 12 such sequences in *E. hecabe*. Further, a total of 118 conserved microRNAs, including 43 lepidopteran lineage-specific microRNAs, which were previously thought to be species-specific, were identified (Fig. 4). An interesting phenomenon is that a cluster of tandemly duplicated miR-2733 on chromosome 31 was significantly expanded in this genome similar to other lepidopterans such as the moths *B. mori* and *Heortia vitessoides* [44, 45], and members of this miR-2733 cluster are relatively highly expressed in the egg and adult bodies, but not in the pupae stages (Fig. 4).

**Sex-specific responses upon temperature change**

Sexual dimorphism refers to the phenomenon of females and males of the same species exhibiting a wide range of differences in various biological processes. The availability and feasibility of transcriptomic profiling made new genome-wide discovery of underlying mechanisms possible. One such discovery is sex-biased gene expression, where morphological differences between sexes of the same species are caused by differential expression of



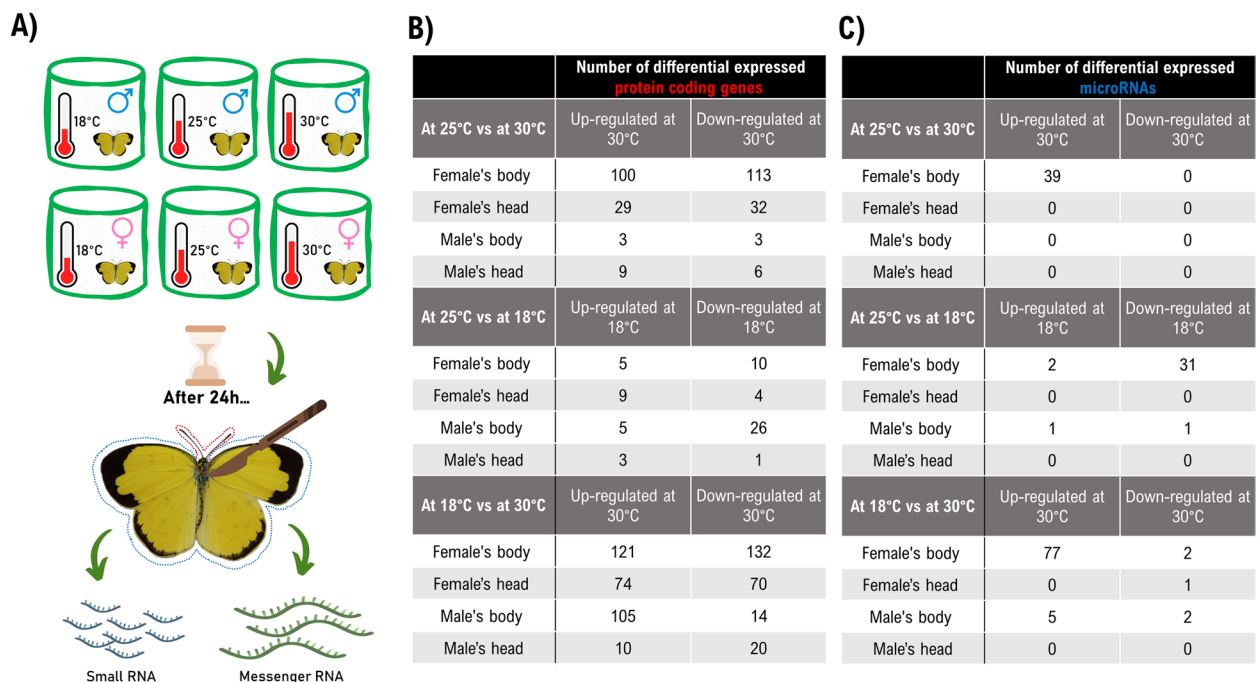
**Fig. 4** Conserved and novel microRNA loci annotated in the *E. hecabe* genome. Blue colour indicated non-clustered microRNAs while red colour indicated the clustered microRNAs

genes that are present in both sexes [46]. In lepidopterans, *BmOr19* has been shown to have a female-biased expression in the antennae of *Bombyx* for seeking host plants, while female-biased expression of *ultraviolet opsin gene 1 (UVRh1)* in *Heliconius* might also link to searching of oviposition sites [47–49]. Deep sequencing of microRNAs in different animals is now also showing microRNAs exhibiting sex-biased expression in different groups of animals. In insects, sex-biased microRNA expression has been revealed in different developmental stages, for instance, in the flies *Bactrocera dorsalis* and *D. melanogaster* [50–54], the beetle *Tribolium castaneum* [55], the termite *Reticulitermes speratus* [56], the plant hopper *Sogatella furcifera* [57], the wasp *Ceratosolen solmsi* [58], the bee *Apis mellifera* [59], and the mosquito *Anopheles gambiae* [60]. Studies have shown that environmental cues can also relate to sex-biased protein coding gene expression. For example, 11 microRNAs showed sex-biased expression patterns in the red flour beetle *Tribolium castaneum* during stresses including temperature, starvation, and bacterial infection [55]. Nevertheless, sex-biased genes and microRNAs responding to environmental cues such as temperature changes in lepidopterans remain poorly known.

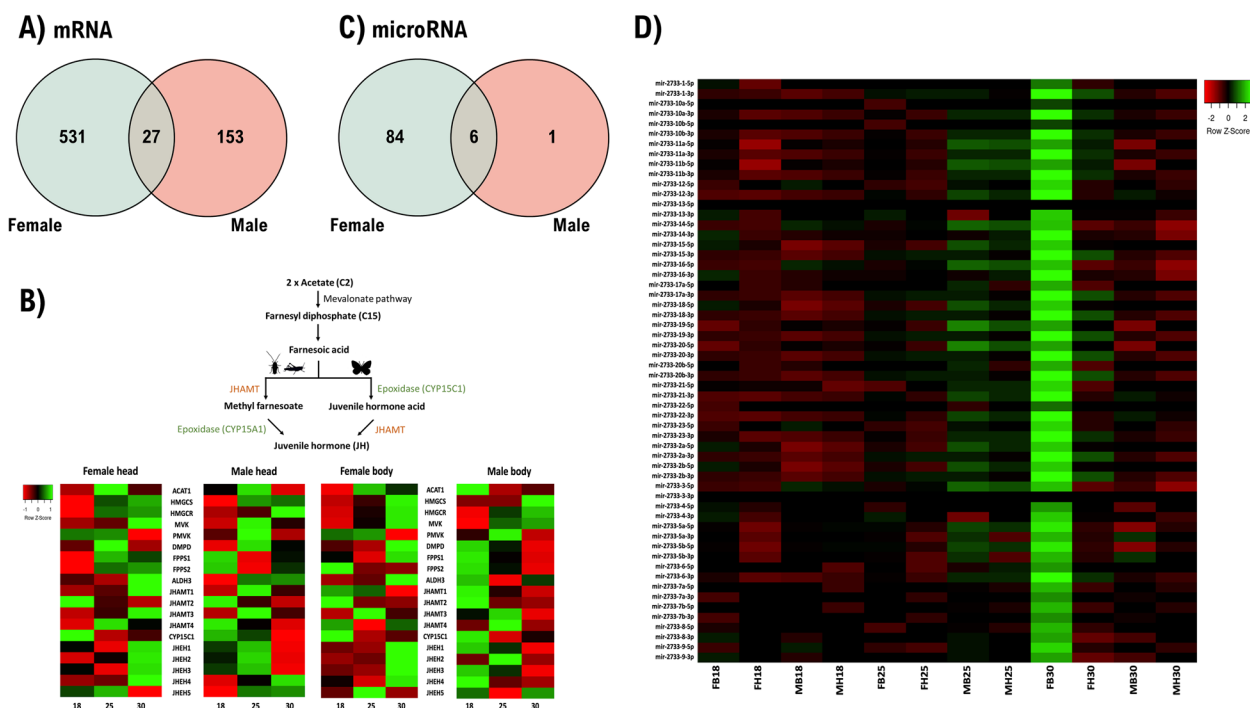
Here, we treated male and female adult *E. hecabe* at three different temperatures and revealed mRNA and

sRNA transcriptomic responses in their heads and bodies (Fig. 5A; Additional file 1: Figure S4). The temperature ranges were chosen based on those that this species will experience in its natural environment. As shown in Fig. 5B, the protein coding gene responses in heads and bodies between males and females are largely similar, except between 25 °C and 30 °C, where there are much more differentially expressed genes in different tissues of females than males. Comparison of all the differentially expressed genes between males and females found that there is only a subset of genes in common (Fig. 6A; Additional file 1: Figure S5 & S6; Additional file 3; Additional file 4), suggesting that the genetic responses of *E. hecabe* to temperature changes are largely sex-specific. Gene pathway enrichment analyses suggested that different gene ontology and pathways are enriched in different contexts between sexes (Additional file 5; Additional file 1: Figure S7 – S9).

To understand the above observation, we further investigated the expression of sesquiterpenoid pathway genes, as sesquiterpenoids such as juvenile hormone regulate development, physiology, metamorphosis, and reproduction in insects [61–63]. In the *E. hecabe* genome, we were able to identify all genes in the mevalonate and juvenile hormone biosynthesis pathways (Fig. 6B; Additional file 6). It is worth noting that there were two copies



**Fig. 5** Transcriptomes of heads and bodies of male and female adults of *E. hecabe* treated at different temperatures. **A** Schematic diagram showing the experimental design. **B** Table summarizing the number of differentially expressed protein-coding genes in different tissues of different sexes at different temperatures. **C** A table summarizing the number of differentially expressed microRNAs in different tissues of different sexes at different temperatures



**Fig. 6** Sex-specific responses of different tissues under different temperatures of *E. hecabe*. **A** A Venn diagram showing the total number of differentially expressed protein-coding genes identified in both sexes under different temperatures. **B** Schematic diagram showing the canonical biosynthetic pathway of sesquiterpenoid hormones in insects (upper), and the heatmaps showing expression of sesquiterpenoid biosynthetic pathway genes in different tissues at different temperatures. **C** A Venn diagram showing the total number of differentially expressed microRNAs identified in both sexes under different temperatures. **D** Heatmap showing expression of members in the miR-2733 cluster (F, female; M, male; B, body; H, head; 18, at 18 °C; 25, at 25 °C; 30, at 30 °C)

of farnesyl diphosphate synthase (FPPS) identified in *E. hecabe*. Multiple copies of FPPS are commonly reported in other butterflies and moths, including *Bombyx mori*, *Choristoneura fumiferana*, *Danaus plexippus*, *Helicoverpa armigera*, *Mythimna unipuncta*, *Papilio glaucus*, *Papilio xuthus*, and *Pieris rapae* [64–68]. In addition, no CYP15A1 could be identified in *E. hecabe*, similar to other lepidopterans [63]. In the heads of females and males *E. hecabe* treated at different temperatures, we found that most sesquiterpenoid biosynthetic pathway genes have similar differential responses, with the exception of a few genes, such as mevalonate kinase (MVK), which was upregulated in the female head at 25 °C but downregulated in male head at 30 °C (Fig. 6B). This result suggests the hormonal system responds differently in the different sexes of lepidopterans upon temperature changes.

Considering microRNAs, similar to the responses of protein coding genes, we observed sex-biased responses in both the heads and bodies of the females and males of *E. hecabe*, and there are many more differentially expressed microRNAs in different tissues of females than males, similar to the picture for protein genes (Figs. 5C and 6C; Additional file 1: Figure S10; Additional file 7).

As mentioned above, a lepidopteran miR-2733 cluster is revealed in the *E. hecabe* genome, and members of insect microRNA cluster have been hypothesized to reinforce and modify the selection force of cluster regulation and gene regulatory network of existing microRNAs [42]. We found that members of miR-2733 were upregulated in temperature 30 °C only in the body of female *E. hecabe*, but not in other treatments (Fig. 6D). Although the way in which this microRNA cluster contributes to the adaptation of *E. hecabe* to its environment remains to be functionally tested, we have demonstrated the microRNAs and cluster of microRNAs respond to environmental cues differently between sexes, rather than as a largely unified or similar response.

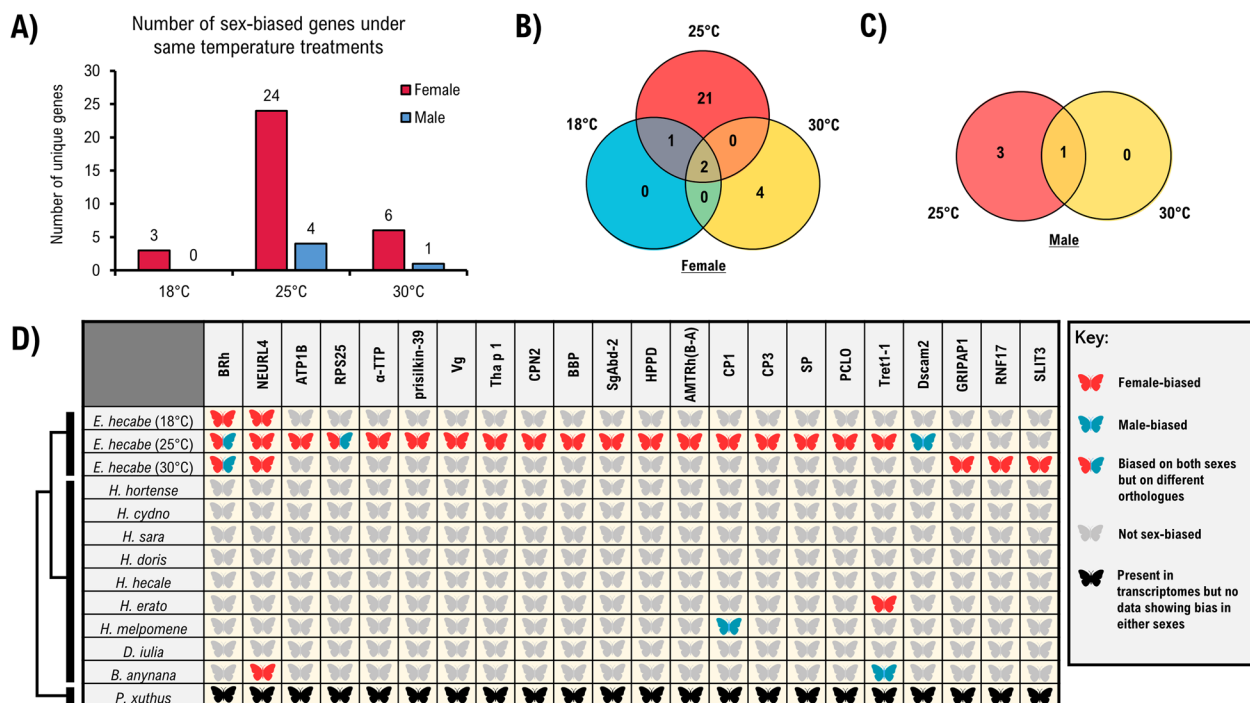
In the analysis of neuropeptides expression profiles between the head and body transcriptomes, we found that there is a clear discrepancy (Additional file 8). Meanwhile, most of the neuropeptides were expressed in the early developmental stages (i.e. egg and 1st instar), and some were specifically expressed higher in the adult stage, including adipokinetic hormone (AKH), crustacean hyperglycemic hormone (CHH), diapause hormone/PBAN/pyrokinin, insulin-like peptide, ion-transport peptide, neuroparsin, partner of bursicon

(pburs), prothoracicotrophic hormone (PTTH), short neuropeptide F (sNPF), SIFamide, and Trissin 2 (Additional file 1: Figure S11). These neuropeptides have been found to be important regulators in lepidopterans, for instance, insulin controls the seasonal plasticity, development, and diapause in some species [69–71], while other hormones such as ecdosone have been shown to drive seasonality plasticity in some species of butterflies [72, 73]. Furthermore, we discovered several sex-biased neuropeptides, including CHH, IMFamide, ion-transport peptides, and pburs (red boxes highlighted in Additional file 1: Figure S11). How these different neuropeptides function in butterflies remains to be explored.

Given there is data available on sex-biased gene expression for a range of butterfly models [49, 74–77], we then compared sexual dimorphic gene expression across lineages (Additional file 9). In our analysis of *E. hecabe* head transcriptomes, more differential expression of sex-biased genes was found when butterflies were reared at 25 °C compared to 18 °C and 35 °C, with the number of

sex-biased genes being 24 and 4 genes in females and males, respectively (Fig. 7A). Only 2 genes (*blue-sensitive rhodopsin*, *BRh* and *neuralized-like protein 4*, *NEURL4*) were found to be consistently expressed in a sex-biased manner in females at different temperatures (Fig. 7B), while only 1 gene was found in the males (another form of *BRh*) (Fig. 7C). Surprisingly, we identified a list of genes with female-biased expression in *H. hecabe*, but not in other available butterflies, while *NEURL4* also displayed a similar pattern in *Bicyclus anynana* (Fig. 7D). In addition, we observed a pattern of opposite sex-specific expression of *facilitated trehalose transporter Tret1-like*, *Tret-1* among one set of species, while this gene is female-biased in *E. hecabe* and *Heliconius erato*, it is male-biased in *B. anynana*. While efforts have been made here to understand sex-biased gene expression in butterflies, our current understanding of this topic is still in its infancy.

This study established a high-quality genome assembly and transcriptomic resources for a butterfly species *E. hecabe* that can be commonly found in three continents.



**Fig. 7** Sex-specific responses of *E. hecabe* head tissue compared to other different published butterfly transcriptomics. **A** Bar plot showing the number of sex-biased genes under same temperature treatments in both sexes of *E. hecabe*. **B** Venn diagram illustrating the female-biased genes under different temperature (18 °C, 25 °C, and 30 °C) treatments. **C** Venn diagram illustrating the male-biased genes under different temperature (25 °C and 30 °C) treatments. **D** Comparative analysis showing the sex-biased genes in *E. hecabe* and other related published sex-biased transcriptomic studies. The expression data for *Bicyclus anynana* is extracted from Ernst and Westerman (2021). BRh, blue (B-) sensitive rhodopsin; NEURL 4, neuralized-like protein 4; ATP1B, sodium/potassium-transporting ATPase subunit beta-2; RPS25, 40S ribosomal protein S25; α-TTP, alpha-tocopherol transfer protein; Vg, vitellogenin-like; Tha p 1, allergen Tha p 1-like; CPN2, carboxypeptidase N subunit 2-like; BBP, bilin-binding protein-like; SgAbd-2, endocuticle structural glycoprotein SgAbd-2-like; HPPD, 4-hydroxyphenylpyruvate dioxygenase-like; AMTRh(B-A), ammonium transporter Rh type B-A; CP1, cuticle protein 1; CP3, cuticle protein 3-like; SP, serine protease snake-like; PCLO, protein piccolo; Tret1-1, facilitated trehalose transporter Tret1-like; Dscam2, Down syndrome cell adhesion molecule-like protein; GRIPAP1, GRIP1-associated protein; RNF17, RING finger protein 17; SLIT3, slit homolog 3 protein-like



Differential genetic responses between different sexes upon environmental parameter changes were tested and revealed. It is envisioned that this established model can be easily adopted and used to understand other aspects of lepidopteran biology and responses to climate change.

## Conclusions

We provide high-quality genomic resources for one of the most common widely distributed butterfly species, *Eurema hecabe*, the common grass yellow, which can be found across three continents (Asia, Africa, Australia). For the first time, we provide a chromosomal-level genome assembly for this species, as well as 87 new mRNA and small RNA transcriptomes. In addition, we demonstrate considerable feasibility to work on this species as a model for a range of pertinent questions in evolution and ecology. We revealed that different tissues of different sexes of this butterfly species respond differently across temperatures, including differences in the expression of key hormonal genes as well as noncoding microRNAs. The foundation set up in this study will be useful for future molecular ecology studies on the following aspects, including but not limited to population genomics to reveal their migratory patterns, subpopulation and speciation event, climate change effects on them, and compare to other well established butterfly species found on the same and other continents.

## Methods

### Field collections and detection of *Wolbachia* strain *wHecCI* and *wHecFem*

Eighty-nine individuals including 79 adults and 10 larvae of *E. hecabe* were collected from different locations in Hong Kong from 2014 to 2015 (Additional file 10). Sampling was undertaken at geographical sites where *E. hecabe* is common. An adult of *E. blanda* was collected at Wong Nai Tun (22.415, 114.022) and used as the outgroup in phylogenetic analyses. The sex of the adult individuals was determined by the sexually dimorphic wing patterns and genital morphology at the end of abdomen, while the presence/absence of testes was used to determine sex in the last instar larvae. All specimens were labelled with collection locality, GPS coordinates, date, and stored at  $-20^{\circ}\text{C}$  in the laboratory before further processing. Details of the collection sites are listed in Additional file 10. Genomic DNA was extracted from the abdominal/thoracic muscle of adults or gut-removed abdominal tissue from larvae using PureLink Genomic DNA Mini Kit (Invitrogen). The mitochondrial COI gene, encoding cytochrome *c* oxidase subunit I, was amplified using primers 5'-ATTCAACCAATCATAAAGATAT-3' and 5'-ATCAGAATAACGTCGAGGTAT-3' following a previous study [78]. The infection status of

*Wolbachia* was first screened by performing PCR with 16S rRNA marker using primers (5'-TTGTAGCCTGCTATGGTATAACT-3') and (5'-GAATAGGTATGATTTTCATGT-3') [79], and specific strains were further identified using primers *wsp81F*, 5'-TGGTCCAATAAGTGA TGAAGAAAC-3' and *WHecFem1*, 5'-ACTAACGTCGTTTTTGTTTAG-3' for strain *wHecCI*; and primers *WHecFem2*, 5'-TTACTCACAATTGGCTAAAGAT-3' and *wsp691R*, 5'-AAAAATTAAACGCTACTCCA-3' for strain *wHecFem* [80]. PCR was performed as follows: 95 °C for 3 min, followed by 35 cycles of 95 °C for 1 min, 58 °C for 1.5 min, and 72 °C for 1.5 min, and a final extension at 72 °C for 7 min. PCR products were subjected to gel electrophoresis.

### Butterfly culture

Adult *E. hecabe* were collected from different locations in Hong Kong during 2019 to 2021, including The Chinese University of Hong Kong at Ma Liu Shui (22.421, 114.208), Tai Po Kau (22.422, 114.178), Shing Mun Country Park (22.385, 114.143), Sai Kung (22.419, 114.349), Hok Tau (22.489, 114.182), and Luk Keng (22.520, 114.214). Animals were kept in a net cage (width: 60 cm, length: 50 cm, height: 90 cm) at room temperature ( $\sim 23^{\circ}\text{C}$ ), with a humidity 40% and 14:10 h of light–dark cycle; 10% honey solution was prepared from commercially available “Bee easy wild flower honey” (Langnese, Germany) and provided to the animals. Fresh leaves of *Senna surattensis* were collected from The Chinese University of Hong Kong and were provided to the adult animals in cage for oviposition. Photo documentation of animals was carried out with a digital camera (Olympus Tough TG-6).

### Genome sequencing

Sample for genome sequencing originates from a single *E. hecabe* individual within the established laboratory culture (Fig. 1A, B), with genomic DNA (gDNA) extracted using the PureLink Genomic DNA Mini Kit (Invitrogen) following the manufacturer's protocol. Extracted gDNA was subjected to quality control using a Nanodrop spectrophotometer (Thermo Scientific) and gel electrophoresis. Qualifying samples were sent to Novogene and Dovetail Genomics for library preparation and sequencing. The resulting library was sequenced on Illumina HiSeq X platform to produce  $2\times 150$  paired-end sequences. The length-weighted mean molecule length is 10,226.69 bases, and the raw data can be found at NCBI's Small Read Archive (SRR12799420) (Additional file 10).

### Chicago library preparation and sequencing

A Chicago library was prepared from another *E. hecabe* individual, which was collected from the

established laboratory culture as described previously [81]. Briefly, ~500 ng of HMW gDNA (mean fragment length = 85 kbp) was reconstituted into chromatin in vitro and fixed with formaldehyde. Fixed chromatin was digested with DpnII, the 5' overhangs filled in with biotinylated nucleotides, and free blunt ends were ligated. After ligation, crosslinks were reversed, and the DNA purified from protein. Purified DNA was treated to remove biotin that was not internal to ligated fragments. The DNA was then sheared to ~350 bp mean fragment size and sequencing libraries were generated using NEBNext Ultra enzymes and Illumina-compatible adapters. Biotin-containing fragments were isolated using streptavidin beads before PCR enrichment of each library. The libraries were sequenced on an Illumina HiSeq X to produce 46,027,927 2×150 bp paired end reads (1–100 kb pairs) (Additional file 10).

#### Dovetail HiC library preparation and sequencing

An additional Dovetail HiC library was prepared with the same individual used in Chicago library preparation. Briefly, for each library, chromatin was fixed in place with formaldehyde in the nucleus and then extracted fixed chromatin was digested with the restriction enzyme DpnII, the 5' overhangs were filled in with biotinylated nucleotides, and free blunt ends were ligated. After ligation, crosslinks were reversed, and the DNA purified from protein. Purified DNA was treated to remove biotin that was not internal to ligated fragments. The DNA was sheared to ~350 bp mean fragment size and sequencing libraries were generated using NEBNext sUltra enzymes and Illumina-compatible adapters. Biotin-containing fragments were isolated using streptavidin beads before PCR enrichment of each library. The libraries were sequenced on an Illumina HiSeq X sequencer to produce 46,890,786 2×150 bp paired end reads (10–10,000 kb pairs) (Additional file 10).

#### Transcriptome sequencing

Total RNA from different developmental stages and adult body tissues (head and body) were isolated using a combination method of cetyltrimethylammonium bromide (CTAB) pre-treatment [82] and mirVana™ miRNA Isolation Kit (Ambion) following the manufacturer's instruction (details can be found in Additional file 10). Different developmental stages RNA (one individual for each stage) was obtained for sequencing in order to better annotate the gene models of this newly established genome. Total RNA from different tissues were extracted from different sexes of butterflies at different temperature, in order to test whether different tissues of males and females respond similarly/differently at different temperatures. The extracted total RNA was subjected to quality control

using a Nanodrop spectrophotometer (Thermo Scientific), gel electrophoresis, and an Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit). Qualifying samples underwent library construction and sequencing at Novogene; polyA-selected RNA-Sequencing libraries were prepared using TruSeq RNA Sample Prep Kit v2. Insert sizes and library concentrations of final libraries were determined using an Agilent 2100 bioanalyzer instrument (Agilent DNA 1000 Reagents) and real-time quantitative PCR (TaqMan Probe), respectively. Details of the sequencing data can be found in Additional file 10.

#### Genome assembly

Chromium WGS reads were used to make a de novo assembly using Supernova (v 2.1.1) with default parameters (raw coverage = 54.18×) (Additional file 1: Figure S11). The Supernova output pseudohaplotype assembly, shotgun reads, Chicago library reads, and Dovetail HiC library reads were used as input data for HiRise, a software pipeline designed specifically for scaffolding genome assemblies using proximity ligation data [83]. An iterative analysis was conducted as follows. First, Shotgun and Chicago library sequences were aligned to the draft input assembly using a modified SNAP read mapper (<http://snap.cs.berkeley.edu>). The separations of Chicago read pairs mapped within draft scaffolds were analysed by HiRise to produce a likelihood model for genomic distance between read pairs, and the model was used to identify and break putative misjoins, to score prospective joins, and make joins. In order to identify a misjoin, the likelihood model was used to compute the log likelihood change gained by joining both sides of each position of each contig in the initial assembly. A score would then be calculated. If this score fell below the threshold values over a maximal internal segment of an input contig, the segment was thus defined as a “low support” segment and a break was introduced [83]. After aligning and scaffolding Chicago data, Dovetail HiC library sequences were aligned and scaffolded following the same method. After scaffolding, shotgun sequences were used to close gaps between contigs.

#### Gene model and repetitive elements prediction

The gene model was predicted as described in the previously published Hong Kong oyster (*Magallana hongkongensis*) genome paper [84]. Briefly, the gene models were trained and predicted by funannotate (v1.7.4, <https://github.com/nextgenusfs/funannotate>) [85] with parameters “-repeats2evm -protein\_evidence uniprot\_sprot.fasta -genemark\_mode ET -busco\_seed\_species fly -optimize\_augustus -busco\_db arthropoda -organism other -max\_intronlen 350,000”, the gene models from several prediction sources

including GeneMark, high-quality Augustus predictions (HiQ), pasa, Augustus, GlimmerHM, and snap were passed to Evidence Modeler to generate the gene model annotation files, PASA was then used to update the EVM consensus predictions, UTR annotations and models for alternatively spliced isoforms were added. The protein-coding gene models were then interrogated using blastp with the nr and swissprot databases in diamond (v0.9.24) with the parameters “-more-sensitive -evalue 1e-3”, and then mapped by HISAT2 (version 2.1.0) with transcriptome reads. Gene matrix count tables were then generated by stringtie (version 2.1.1) and used for further gene expression analyses. Gene models with no homology to any known protein in the GenBank nr database and no mRNA support were removed from the final version. Repetitive elements were predicted as previously described [45].

#### Identification of orthologous gene families and macrosynteny analysis

Orthologues and orthogroups in the proteomes of 22 other lepidopteran species (*Amyelois transitella*, *Bicyclus anynana*, *Bombyx mori*, *Calycopsis cecrops*, *Chilo suppressalis*, *Danaus plexippus*, *Heliconius erato demophoon*, *Leptidea sinapis*, *Lerema accius*, *Manduca sexta*, *Maniola hyperantus*, *Operophtera brumata*, *Papilio glaucus*, *Papilio machaon*, *Papilio polytes*, *Papilio xuthus*, *Phoebis sennae*, *Pieris macdunnoughi*, *Pieris rapae*, *Plodia interpunctella*, *Plutella xylostella* and *Zerene cesonia*) and *Drosophila melanogaster* were retrieved from NCBI and Lepbase [86], and inferred using OrthoFinder v. 2.5.2 [87] with default values and ‘-M msa’ activated. To symbolize the gene families, the longest protein of each gene was taken as the representative in OrthoFinder analysis. Gene gain and loss rates were computed from gene families using CAFE5 [88]. Single-copy orthologues were anchored by mutual best Diamond blastp hits (–evalue 0.001) between each species pairs. Oxford synteny plots were generated following previously described method [89] using R package ‘ggplot2’ [90].

#### Functional terms enrichment analysis

The functional term annotations were performed using eggNOG [91]. Orthogroups were assigned with Gene Ontology (GO), EuKaryotic Orthologous Groups (KOG), Kyoto Encyclopedia of Genes and Genomes (KEGG), and KEGG Orthology (KO) terms by inheriting the terms from genes found within the groups. Functional enrichment was tested using function ‘compareCluster()’ in R package ‘clusterProfiler’ v.4.2.2 [92] under the environment of R 4.1.0 [93]. Significantly enriched terms were determined with pvalueCutoff=0.05, pAdjustMethod=‘BH’, and qvalueCutoff=0.2. The genome of internal nodes (ancestral populations) was inferred

according to the gene count results of CAFE5. Data was visualized using R packages ‘ggplot2’ [90], ‘ggtree’ [94] and ‘pathview’ [95].

#### Specific gene family annotation and gene tree building

Gene family sequences were first retrieved from the butterfly genome with the use of tBLASTn, in reference to the sequences from species including *D. melanogaster* and *Bombyx mori* via data from the National Center for Biotechnology Information (NCBI) [96]. The retrieved genes were then compared to sequences found in the NCBI nr database with the use of BLASTx to examine their identities. For neuropeptides, amino acid sequences identified in *B. mori* [97], *Spodoptera frugiperda* [98], *Atrijuglans hetaohei* [99], *Cydia pomonella* [100], *Chilo suppressalis* [101], and *Phaouda fammans* [102] were used as queries in TBLASTN searches of the *E. hebeca* genome and transcriptomes. Signal sequences were predicted using the SignalP 3.0 Server [103] (<http://www.cbs.dtu.dk/services/SignalP>). Potential peptide processing sites within the prepropeptides were identified using guidelines outlined in previous study [104]. For the phylogenetic analyses of gene families, DNA sequences were first translated into amino acid sequences and were aligned to the sequences of the members of the respective gene family. Gapped sites were removed from the alignments with the use of MEGA7.0. Phylogenetic trees were constructed with MEGA7.0. Maximum likelihood gene tree was constructed using IQ-TREE [105] with ‘-T AUTO -B 1000 -bnni -alrt 1000’. Gene trees were visualized using R package ‘ggtree’ [94].

#### Sex-specific temperature challenge, mRNA and small RNA transcriptome analyses

Adult *E. hecabae* of both sexes (3 butterflies per condition) were exposed to different temperatures (18 °C, 25 °C, and 30 °C) respectively for 24 h continuously with a humidity at around 50% and a cycle of 14 h of light and 10 h of darkness. Total RNA of the treated individuals was extracted separately with the use of the mirVana miRNA Isolation Kit (Invitrogen) following the manufacturer’s instructions. Extracted RNA was subjected to quality control with gel electrophoresis and the Nanodrop spectrophotometer (Thermo Scientific). Samples that were qualified were sent out for transcriptome and sRNA library preparation and sequencing.

The transcriptome reads were separately aligned back to the genome guide trinity assembly generated in the previous gene model prediction step for downstream analyses of differential expression with the script of align\_and\_estimate\_abundance.pl (Trinity version 2.9.1). Gene matrix tables were used for different gene expression analyses by edgeR [106] with default parameters, at

least 2 of replicates must have cpm values > 1 cpm value ( $-\min\_reps\_min\_cpm$  2,1). Predicted miRNAs were manually examined to identify their identities. In order to identify the conserved miRNAs, the predicted miRNA hairpins from the butterflies were compared to miRNA precursor sequences found on miRbase with the use of BLASTn [107, 108]. Hairpin sequences that had no significant similarity to any miRNA sequences on miRbase were subjected to further manual checking on whether they fulfilled the criteria of miRNAs based on the information on MirGeneDB 2.0 [109]. Sequences that fulfilled the criteria were considered as novel miRNAs.

#### Abbreviations

AKH	Adipokinetic hormone
BRh	Blue-sensitive rhodopsin
BUSCO	Benchmarking universal single-copy orthologs
CHH	Crustacean hyperglycemic hormone
COI	Cytochrome oxidase I
FPPS	Farnesyl diphosphate synthase
LINE	Long interspersed nuclear element
LTR	Long terminal repeats
mRNA	Messenger RNA
MVK	Mevalonate kinase
NEURL4	Neuralized-like protein 4
pburs	Partner of bursicon
PTTH	Prothoracicotropic hormone
SINE	Short interspersed nuclear elements
sNPF	Short neuropeptide F
sRNA	Small RNA
TE	Transposable element
Tret-1	Facilitated trehalose transporter Tret1-like
UVRh1	Ultraviolet opsin gene 1

#### Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12915-023-01703-1>.

**Additional file 1: Figure S1.** GenomeScope v2.0 k-mer profile plot based on 21-mers in Illumina reads. The observed k-mer frequency distribution is depicted in blue, whereas the GenomeScope fitmodel is shown as a black line. The unique and putative error k-mer distributions are plotted in yellow and red, respectively. **Figure S2.** Oxford dot plots of orthologous genes between the different lepidopteran species. Orthologous genes are coloured according to their positions in the reference species at the horizontal axis. **Figure S3.** Transposable elements. **Figure S4.** Prcomp principal components and heatmaps of all samples. **Figure S5.** Differentially expressed gene clusters of different combinations of *E. hecabe* sexes and tissues under different heat-stress experiment. Genes differentially expressed between different temperatures (18°C, 25°C and 30°C) in different tissues (H: head; B: body) of both sexes (F: female; M: male) were identified from strand-specific RNA-Seq using EdgeR with three biological replicates (each replicate annotated with number 1, 2 and 3) per sample. **Figure S6.** Differentially expressed protein-coding genes under various temperature settings. Venn diagrams showing the numbers of common and sex-specific differential expressed protein-coding genes when comparing the expression at 18°C with 25°C, 25°C with 30°C and 18°C with 30°C. **Figure S7.** GO annotation of differentially expressed genes in female *Eurema hecabe* at 30°C when comparing to 18°C. **Figure S8.** GO annotation of differentially expressed genes in male *Eurema hecabe* at 30°C when comparing to 18°C. **Figure S9.** GO annotation of differentially expressed genes in female *Eurema hecabe* at 30°C when comparing to 25°C. **Figure S10.** Differentially expressed microRNAs at different temperatures. Venn diagrams showing the numbers of common and sex-specific differential

expressed miRNAs when comparing the expression at 18°C with 25°C and 18°C with 30°C. **Figure S11.** Neuropeptides in *E. hecabe*. (A) The neuropeptide genes identified in the *E. hecabe* genome and their expression in different life stages. (B) Expression of neuropeptide genes under different temperatures. F: female; M, male; H: head; B: body.

**Additional file 2.** Transposable elements information.

**Additional file 3.** Summary of differentially expressed protein coding genes and microRNAs.

**Additional file 4.** Expression data of mRNA.

**Additional file 5.** Gene ontology and pathway enrichment analyses data.

**Additional file 6.** Expression data of sesquiterpenoid pathway genes.

**Additional file 7.** Expression data of microRNAs.

**Additional file 8.** Expression data of neuropeptides

**Additional file 9.** Comparative sex-biased analysis.

**Additional file 10.** Sequencing data.

#### Acknowledgements

The authors would like to thank the editor and anonymous reviewers for constructive comments and suggestions.

#### Authors' contributions

IHTL: animal collection and culture, temperature experiments, transcriptomic analyses, microRNA annotation; WN: genome assembly, genome annotation, gene gain/loss analyses, transcriptomic analyses; WLS: transcriptomic analyses; CKHC: animal collection and culture, population genetic analyses, Wolbachia analyses; YX: gene gain/loss analyses, synteny plots; TB: genome annotation; HYY: animal collection and culture; TS: genome assembly; SKFC: conceived the study; YYW: conceived the study, funding acquisition; NL: conceived the study, funding acquisition; AH: conceived the study, funding acquisition; TFC: conceived the study, funding acquisition; HML: conceived the study, funding acquisition; JHLH: funding, conceived and supervised the study. All authors are involved in the manuscript writing. All authors read and approved the final manuscript.

#### Authors' information

##### Twitter

Yichun Xie = Yichun\_CH3CH2OH

Toby Baril = Toby\_Baril\_Bio

Alexander Hayward = zanderhayward

Jerome Hui = JeromeHui2

#### Funding

This study was supported by Hong Kong Research Grant Council Collaborative Research Fund CRF (C4015-20EF), General Research Fund GRF (14100420), Area of Excellence (AoE/M-403/16), CUHK Direct Grant (4053489, 4053547), CUHK Group Research Scheme (3110154), and CUHK Strategic Seed Funding for Collaborative Research Scheme (3133356). AH is supported by a Biotechnology and Biological Sciences Research Council (BBSRC) David Phillips Fellowship (BB/N020146/1). IHTL was supported by a postgraduate studentship provided by The Chinese University of Hong Kong.

#### Availability of data and materials

The raw reads generated in this study have been deposited to the NCBI database under the BioProject accession PRJNA664668 [110]. The final chromosome assembly was submitted to NCBI Assembly under accession number JADANM000000000 in NCBI [111]. The genome annotation files were deposited in the Figshare [112].

#### Declarations

##### Ethics approval and consent to participate

Not applicable.

##### Consent for publication

Not applicable.



### Competing interests

The authors declare that they have no competing interests.

### Author details

<sup>1</sup>School of Life Sciences, Simon F.S. Li Marine Science Laboratory, State Key Laboratory of Agrobiotechnology, Institute of Environment, Energy and Sustainability, The Chinese University of Hong Kong, Hong Kong, China. <sup>2</sup>University of Exeter, Exeter, UK. <sup>3</sup>Dovetail Genomics, Scotts Valley, USA. <sup>4</sup>Agriculture, Fisheries and Conservation Department, Hong Kong, China. <sup>5</sup>Department of Statistics, The Chinese University of Hong Kong, Hong Kong, China. <sup>6</sup>School of Life and Environmental Sciences, University of Sydney, Sydney, Australia. <sup>7</sup>School of Life Sciences, State Key Laboratory of Agrobiotechnology, The Chinese University of Hong Kong, Hong Kong, China. <sup>8</sup>School of Life Sciences, State Key Laboratory of Agrobiotechnology, Institute of Environment, Energy and Sustainability, The Chinese University of Hong Kong, Hong Kong, China.

Received: 16 December 2022 Accepted: 13 September 2023

Published online: 25 September 2023

### References

- Powell JA. Lepidoptera (moths, butterflies). In: Resh VH, Cardé RG, editors. Encyclopedia of insects. Burlington MA, USA: Academic Press; 2003. p. 631–63 1266pp.
- Stork NE. How many species of insects and other terrestrial arthropods are there on Earth? Annu Rev Entomol. 2018;63:31–45.
- Regier JC, Mitter C, Zwick A, Bazinet AL, Cummings MP, Kawahara AY, et al. A large-scale, higher-level, molecular phylogenetic study of the insect order lepidoptera (moths and butterflies). PLoS ONE. 2013;8:e58568.
- Stevenson RD. Body size and limits to the daily range of body temperature in *Terrestrial Ectotherms*. Am Nat. 1985;125:102–17.
- McDermott Long O, Warren R, Price J, Brereton TM, Botham MS, Franco AMA. Sensitivity of UK butterflies to local climatic extremes: which life stages are most at risk? J Anim Ecol. 2017;86:108–16.
- Zylstra ER, Ries L, Neupane N, Saunders SP, Ramírez MI, Rendón-Salinas E, et al. Changes in climate drive recent monarch butterfly dynamics. Nat Ecol Evol. 2021;5:1441–52.
- Overgaard J, Sørensen JG. Rapid thermal adaptation during field temperature variations in *Drosophila melanogaster*. Cryobiology. 2008;56:159–62.
- Régnière J, Powell J, Bentz B, Nealis V. Effects of temperature on development, survival and reproduction of insects: experimental design, data analysis and modeling. J Insect Physiol. 2012;58:634–47.
- Bodlah MA, Gu LL, Tan Y, Liu XD. Behavioural adaptation of the rice leaf folder *Cnaphalocrocis medinalis* to short-term heat stress. J Insect Physiol. 2017;100:28–34.
- Clarke A. Costs and consequences of evolutionary temperature adaptation. Trends Ecol Evol. 2003;18:573–81.
- Janowitz SA, Fischer K. Opposing effects of heat stress on male versus female reproductive success in *Bicyclus anynana* butterflies. J Therm Biol. 2011;36:283–7.
- Wahlberg N, Rota J, Braby MF, Pierce NE, Wheat CW. Revised systematics and higher classification of pierid butterflies (Lepidoptera: Pieridae) based on molecular data. Zool Scr. 2014;43:641–50.
- Espeland M, Breinholt J, Willmott KR, Warren AD, Vila R, Toussaint EFA, et al. A comprehensive and dated phylogenomic analysis of butterflies. Curr Biol. 2018;28:770–778.e5.
- Chan A, Cheung JKH, Sze P, Wong A, Wong E, Yau EYW. A review of the local restrictedness of Hong Kong butterflies. 2011.
- Oliva M, Muñoz-Aguirre M, Kim-Hellmuth S, Wucher V, Gewirtz ADH, Cotter DJ, Parsana P, Kasela S, Balliu B, Viñuela A, Castel SE, Mohammadi P, Aguet F, Zou Y, Khramtsova EA, Skol AD, Garrido-Martín D, Reverter F, Brown A, Evans P, Gamazon ER, Payne A, Bonazzola R, Barbeira AN, Hamel AR, Martínez-Pérez A, Soria JM, GTE Consortium, Pierce BL, Stephens M, Eskin E, Dermitzakis ET, Segrè AV, Im HK, Engelhardt BE, Ardlie KG, Montgomery SB, Battle AJ, Lappalainen T, Guigó R, Stranger BE. The impact of sex on gene expression across human tissues. Science. 2020;369(6509):eaba3066.
- Shine R. Ecological causes for the evolution of sexual dimorphism: a review of the evidence. Q Rev Biol. 1989;64:419–61.
- Forsberg J, Wiklund C. Protandry in the green-veined white butterfly, *Pieris napi* L. (Lepidoptera: Pieridae). Funct Ecol. 1988;2:81–8.
- Wiklund C, Wickman PO, Nylin S. A sex difference in the propensity to enter direct/diapause development: a result of selection for protandry. Evol. 1992;46:519–28.
- Zijlstra WG, Kesbeke F, Zwaan BJ, Brakefield PM. Protandry in the butterfly *Bicyclus anynana*. Evol Ecol Res. 2002;4:1229–40.
- Nève G, Singer MC. Protandry and postandry in two related butterflies: conflicting evidence about sex-specific trade-offs between adult size and emergence time. Evol Ecol. 2008;22:701–9.
- Stoehr AM. Inter- and intra-sexual variation in immune defence in the cabbage white butterfly, *Pieris rapae* L. (Lepidoptera: Pieridae). Ecol Entomol. 2007;32:188–93.
- Lindsey E, Altizer S. Sex differences in immune defenses and response to parasitism in monarch butterflies. Evol Ecol. 2009;23:607–20.
- Karl I, Stoks R, De Block M, Janowitz SA, Fischer K. Temperature extremes and butterfly fitness: conflicting evidence from life history and immune function. Glob Change Biol. 2011;17:676–87.
- Tigres N, Sass EM, Lewis SM. Sex-specific response to nutrient limitation and its effects on female mating success in a gift-giving butterfly. Evol Ecol. 2013;27:1145–58.
- Everett A, Tong X, Briscoe AD, Monteiro A. Phenotypic plasticity in opsin expression in a butterfly compound eye complements sex role reversal. BMC Evol Biol. 2012;12:1–12.
- Narita S, Nomura M, Kato Y, Yata O, Kageyama D. Molecular phylogeography of two sibling species of *Eurema* butterflies. Genetica. 2007;131:241–53.
- Kato Y. Seasonal polyphenism in a subtropical population of *Eurema hecabe* (Lepidoptera: Pieridae). Japanese J Entomol. 1992;60:304–17.
- Kato Y. Overlapping distribution of two groups of the butterfly *Eurema hecabe* differing in the expression of seasonal morphs on Okinawa-jima Island. Zool Sci. 2000;17:539–47.
- Kato Y. Fringe color, seasonal morph and host-plant use of the pierid butterfly *Eurema hecabe* (L.) (Lepidoptera, Pieridae) on Okinawa-jima Island. Trans Lepidopterol Soc Japan. 1999;50:111–21.
- Kato Y. Host-plant adaptation in two sympatric types of the butterfly *Eurema hecabe* (L.) (Lepidoptera: Pieridae). Entomol Sci. 2000;3:459–63.
- Kobayashi A, Hiroki M, Kato Y. Sexual isolation between two sympatric types of the butterfly *Eurema hecabe* (L.). J Insect Behav. 2001;14:353–62.
- Kageyama D, Narita S, Watanabe M. Insect sex determination manipulated by their endosymbionts: incidences, mechanisms and implications. Insects. 2012;2012(3):161–99.
- Werren JH, Baldo L, Clark ME. Wolbachia: master manipulators of invertebrate biology. Nat Rev Microbiol. 2008;6:741–51.
- Kageyama D, Ohno M, Sasaki T, Yoshido A, Konagaya T, Jouraku A, Kuwazaki S, Kanamori H, Katayose Y, Narita S, Miyata M, Riegler M, Sahara K. Feminizing Wolbachia endosymbiont disrupts maternal sex chromosome inheritance in a butterfly species. Evol Lett. 2017;1(5):232–44.
- Kern P, Cook JM, Kageyama D, Riegler M. Double trouble: combined action of meiotic drive and Wolbachia feminization in *Eurema* butterflies. Biol Lett. 2015;11(5):20150095.
- Kageyama D, Narita S, Noda H. Transfection of feminizing Wolbachia endosymbionts of the butterfly, *Eurema hecabe*, into the cell culture and various immature stages of the silkworm, *Bombyx mori*. Microb Ecol. 2008;56:733–41.
- Hiroki M, Tagami Y, Miura K, Kato Y. Multiple infection with Wolbachia inducing different reproductive manipulations in the butterfly *Eurema hecabe*. Proc R Soc London Ser B Biol Sci. 2004;271:1751–5.
- Narita S, Nomura M, Kageyama D. Naturally occurring single and double infection with Wolbachia strains in the butterfly *Eurema hecabe*: transmission efficiencies and population density dynamics of each Wolbachia strain. FEMS Microbiol Ecol. 2007;61:235–45.
- Bendena WG, Hui JHL, Chin-Sang I, Tobe SS. Neuropeptide and micro-RNA regulators of juvenile hormone production. Gen Comp Endocrinol. 2020;295:113507.
- Qu Z, Bendena WG, Nong W, Siggins KW, Noriega FG, Kai ZP, et al. MicroRNAs regulate the sesquiterpenoid hormonal

- pathway in *Drosophila* and other arthropods. *Proc R Soc B Biol Sci.* 2017;284(1869):20171827.
41. Qu Z, Bendena WG, Tobe SS, Hui JHL. Juvenile hormone and sesquiterpenoids in arthropods: biosynthesis, signaling, and role of microRNA. *J Steroid Biochem Mol Biol.* 2018;184:69–76.
  42. Qu Z, Nong W, So WL, Barton-Owen T, Li Y, Leung TCN, et al. Millipede genomes reveal unique adaptations during myriapod evolution. *PLoS Biol.* 2020;18(9):e3000636.
  43. Quah S, Hui JHL, Holland PWH. A burst of miRNA innovation in the early evolution of butterflies and moths. *Mol Biol Evol.* 2015;32:1161–74.
  44. Yu X, Zhou Q, Cai Y, Luo Q, Lin H, Hu S, et al. A discovery of novel microRNAs in the silkworm (*Bombyx mori*) genome. *Genomics.* 2009;94:438–44.
  45. Law STS, Nong W, So WL, Baril T, Swale T, Chan CB, et al. Chromosomal-level reference genome of the moth *Heortia vitessoides* (Lepidoptera: Crambidae), a major pest of agarwood-producing trees. *Genomics.* 2022;114:110440.
  46. Grath S, Parsch J. Sex-biased gene expression. *Annu Rev Genet.* 2016;50:29–44.
  47. Wanner KW, Anderson AR, Trowell SC, Theilmann DA, Robertson HM, Newcomb RD. Female-biased expression of odourant receptor genes in the adult antennae of the silkworm, *Bombyx mori*. *Insect Mol Biol.* 2007;16:107–19.
  48. Bisch-Knaden S, Daimon T, Shimada T, Hansson BS, Sachse S. Anatomical and functional analysis of domestication effects on the olfactory system of the silkworm *Bombyx mori*. *Proc R Soc B Biol Sci.* 2014;281(1774):20132582.
  49. Catalán A, Macías-Munoz A, Briscoe AD. Evolution of sex-biased gene expression and dosage compensation in the eye and brain of *Heliconius* butterflies. *Mol Biol Evol.* 2018;35:2120–34.
  50. Zhang YE, Vibrationovski MD, Krinsky BH, Long M. Age-dependent chromosomal distribution of male-biased genes in *Drosophila*. *Genome Res.* 2010;20:1526–33.
  51. Marco A. Sex-biased expression of microRNAs in *Drosophila melanogaster*. *Open Biol.* 2014;4:140024.
  52. Yeh SD, Von Grothuss M, Gandasetiawan KA, Jayasekera S, Xia XQ, Chan C, et al. Functional divergence of the miRNA transcriptome at the onset of *Drosophila* metamorphosis. *Mol Biol Evol.* 2014;31:2557–72.
  53. Mohammed J, Flynt AS, Panzarino AM, Mosharraf Hossein Mondal M, DeCruz M, Siepel A, et al. Deep experimental profiling of microRNA diversity, deployment, and evolution across the *Drosophila* genus. *Genome Res.* 2018;28:52–65.
  54. Peng W, Yu S, Handler AM, Tu Z, Saccone G, Xi Z, et al. miRNA-1-3p is an early embryonic male sex-determining factor in the Oriental fruit fly *Bactrocera dorsalis*. *Nat Commun.* 2020;2020(11):1–11.
  55. Freitag D, Knorr E, Vogel H, Vilcinskis A. Gender- and stressor-specific microRNA expression in *Tribolium castaneum*. *Biol Lett.* 2012;8:860–3.
  56. Matsunami M, Nozawa M, Suzuki R, Toga K, Masuoka Y, Yamaguchi K, et al. Caste-specific microRNA expression in termites: insights into soldier differentiation. *Insect Mol Biol.* 2019;28:86–98.
  57. Chang ZX, Akinyemi IA, Guo DY, Wu Q. Characterization and comparative analysis of microRNAs in the rice pest *Sogatella furcifera*. *PLoS ONE.* 2018;13:e0204517.
  58. Wang YK, Li JP, Li TT, Liu R, Jia LY, Wei XQ, et al. Comparative profiling of microRNAs and their association with sexual dimorphism in the fig wasp *Ceratostolen solmsi*. *Gene.* 2017;633:54–60.
  59. Ashby R, Forêt S, Searle I, Maleszka R. MicroRNAs in honey bee caste determination. *Sci Reports.* 2016;6:1–15.
  60. Castellano L, Rizzi E, Krell J, Di Cristina M, Galizi R, Mori A, et al. The germline of the malaria mosquito produces abundant miRNAs, endo-siRNAs, piRNAs and 29-nt small RNAs. *BMC Genomics.* 2015;16:1–16.
  61. Hui JHL, Bendena WG, Tobe SS. Future perspectives for research on the biosynthesis of juvenile hormones and related sesquiterpenoids in arthropod endocrinology and ecotoxicology. in *Juvenile Hormone and Juvenoids: Modeling Biological Effects and Environmental*, ed. Devillers J. (New York, NY: CRC Press; ), 2013;15–30.
  62. Tsang SSK, Law STS, Li C, Qu Z, Bendena WG, Tobe SS, et al. Diversity of insect sesquiterpenoid regulation. *Front Genet.* 2020;11:1027.
  63. Qu Z, Kenny NJ, Lam HM, Chan TF, Chu KH, Bendena WG, et al. How did arthropod sesquiterpenoids and ecdysteroids arise? Comparison of hormonal pathway genes in noninsect arthropod genomes. *Genome Biol Evol.* 2015;7:1951–9.
  64. Cusson M, Béliveau C, Sen SE, Vandermoten S, Rutledge RG, Stewart D, et al. Characterization and tissue-specific expression of two lepidopteran farnesyl diphosphate synthase homologs: implications for the biosynthesis of ethyl-substituted juvenile hormones. *Proteins Struct Funct Genet.* 2006;65(3):742–58.
  65. Cheng D, Meng M, Peng J, Qian W, Kang L, Xia Q. Genome-wide comparison of genes involved in the biosynthesis, metabolism, and signaling of juvenile hormone between silkworm and other insects. *Genet Mol Biol.* 2014;37:444–59.
  66. Zhang W, Ma L, Xiao H, Liu C, Chen L, Wu S, Liang G. Identification and characterization of genes involving the early step of Juvenile Hormone pathway in *Helicoverpa armigera*. *Sci Rep.* 2017;7(1):16542.
  67. Picard MÈ, Cusson M, Sen SE, Shi R. Rational design of Lepidoptera-specific insecticidal inhibitors targeting farnesyl diphosphate synthase, a key enzyme of the juvenile hormone biosynthetic pathway. *J Pestic Sci.* 2021;46(1):7–15.
  68. Kinjoh T, Kaneko Y, Itoyama K, Mita K, Hiruma K, Shinoda T. Control of juvenile hormone biosynthesis in *Bombyx mori*: cloning of the enzymes in the mevalonate pathway and assessment of their developmental expression in the corpora allata. *Insect Biochem Mol Biol.* 2007;37(8):808–18.
  69. Nylin S. Induction of diapause and seasonal morphs in butterflies and other insects: knowns, unknowns and the challenge of integration. *Physiol Entomol.* 2013;38:96–104.
  70. Green DA, Kronforst MR. Monarch butterflies use an environmentally sensitive, internal timer to control overwintering dynamics. *Mol Ecol.* 2019;28:3642–55.
  71. Lobert GM. Kalamazoo College Diebold Symposium Presentation Collection. In: Does insulin regulate the pre migratory phenotype diapause in monarch butterflies? Kalamazoo, Mich: Kalamazoo College; 2019.
  72. Brakefield PM, Kesbeke F, Koch PB. The regulation of phenotypic plasticity of eyespots in the butterfly *Bicyclus anynana*. *Am Nat.* 1998;152(6):853–60.
  73. Monteiro A, Tong X, Bear A, Liew SF, Bhardwaj S, Wasik BR, Dinwiddie A, Bastianelli C, Cheong WF, Wenk MR, Cao H, Prudic KL. Differential expression of ecdysone receptor leads to variation in phenotypic plasticity across serial homologs. *PLoS Genet.* 2015;11(9):e1005529.
  74. Zhang W, Leon-Ricardo BX, van Schooten B, Van Belleghem SM, Counterman BA, McMillan WO, et al. Comparative transcriptomics provides insights into reticulate and adaptive evolution of a butterfly radiation. *Genome Biol Evol.* 2019;11:2963–75.
  75. Macías-Munoz A, Smith G, Monteiro A, Briscoe AD. Transcriptome-wide differential gene expression in *Bicyclus anynana* butterflies: female vision-related genes are more plastic. *Mol Biol Evol.* 2016;33(1):79–92.
  76. Ernst DA, Westerman EL. Stage- and sex-specific transcriptome analyses reveal distinctive sensory gene expression patterns in a butterfly. *BMC Genom.* 2021;22:584.
  77. Perry M, Kinoshita M, Saldi G, Huo L, Arikawa K, Desplan C. Expanded color vision in butterflies: molecular logic behind three way stochastic choices. *Nature.* 2016;535:280.
  78. Hebert PD, Penton EH, Burns JM, Janzen DH, Hallwachs W. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *PNAS.* 2004;101(41):14812–7.
  79. O'Neill SL, Giordano R, Colbert AM, Karr TL, Robertson HM. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *PNAS.* 1992;89(7):2699–702.
  80. Zhou W, Rousset F, O'Neill S. Phylogeny and PCR-based classification of *Wolbachia* strains using wsp gene sequences. *Proc R Soc London Ser B Biol Sci.* 1998;265:509–15.
  81. Lieberman-Aiden E, Van Berkum NL, Williams L, Imakaev M, Ragoczy T, Telling A, et al. Comprehensive mapping of long-range interactions reveals folding principles of the human genome. *Science.* 2009;326:289–93.
  82. Jordon-Thaden IE, Chanderbali AS, Gitzendanner MA, Soltis DE. Modified CTAB and TRIzol protocols improve RNA extraction from chemically complex Embryophyta. *Appl Plant Sci.* 2015;3:1400105.
  83. Putnam NH, Connell BO, Stites JC, Rice BJ, Hartley PD, Sugnet CW, et al. Chromosome-scale shotgun assembly using an in vitro method for long-range linkage. *Genome Res.* 2016;26:342–50.

84. Li Y, Nong W, Baril T, Yip HY, Swale T, Hayward A, et al. Reconstruction of ancient homeobox gene linkages inferred from a new high-quality assembly of the Hong Kong oyster (*Magallana hongkongensis*) genome. *BMC Genomics*. 2020;21:713.
85. Palmer J, Stajich J. Funannotate: eukaryotic genome annotation pipeline. 2018.
86. Challi RJ, Kumar S, Dasmahapatra KK, Jiggins CD, Blaxter M. Lepbase: the Lepidopteran genome database. *BioRxiv*. 2016;056994. <https://doi.org/10.1101/056994>.
87. Emms DM, Kelly S. OrthoFinder: phylogenetic orthology inference for comparative genomics. *Genome Biol*. 2019;20:238.
88. Mendes FK, Vanderpool D, Fulton B, Hahn MW. CAFE 5 models variation in evolutionary rates among gene families. *Bioinformatics*. 2021;36:5516–8.
89. Simakov O, Marlétaz F, Yue JX, O'Connell B, Jenkins J, Brandt A, et al. Deeply conserved syntenies resolves early events in vertebrate evolution. *Nat Ecol Evol*. 2020;4:820–30.
90. Wickham H, Chang W, Wickham MH. Package 'ggplot2'. Create elegant data visualisations using the grammar of graphics. Version. 2016;2(1):1–189.
91. Huerta-Cepas J, Szklarczyk D, Heller D, Hernández-Plaza A, Forslund SK, Cook H. eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. *Nucleic Acids Res*. 2019;47(D1):D309–14.
92. Wu T, Hu E, Xu S, Chen M, Guo P, Dai Z, et al. clusterProfiler 4.0: a universal enrichment tool for interpreting omics data. *Innov*. 2021;2:100141.
93. R Core Team. R: a language and environment for statistical computing. 2021.
94. Yu G, Smith DK, Zhu H, Guan Y, Lam TTY. ggtree: an r package for visualization and annotation of phylogenetic trees with their covariates and other associated data. *Methods Ecol Evol*. 2017;8:28–36.
95. Luo W, Brouwer C. Pathview: an R/Bioconductor package for pathway-based data integration and visualization. *Bioinformatics*. 2013;29:1830–1.
96. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol*. 1990;215:403–10.
97. Roller L, Yamanaka N, Watanabe K, Daubnerová I, Žitňan D, Kataoka H, et al. The unique evolution of neuropeptide genes in the silkworm *Bombyx mori*. *Insect Biochem Mol Biol*. 2008;38:1147–57.
98. Shi Y, Li J, Li L, Lin G, Bilal AM, Smaghe G, Liu TX. Genomics, transcriptomics, and peptidomics of *Spodoptera frugiperda* (Lepidoptera, Noctuidae) neuropeptides. *Arch Insect Biochem Physiol*. 2021;106:e21740.
99. Li F, Zhao X, Zhu S, Wang T, Li T, Woolfley T, Tang G. Identification and expression profiling of neuropeptides and neuropeptide receptor genes in *Atrijuglans hetaohei*. *Gene*. 2020;743:144605.
100. Garczynski SF, Hendrickson CA, Harper A, Unruh TR, Dhingra A, Ahn SJ, Choi MY. Neuropeptides and peptide hormones identified in codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae). *Arch Insect Biochem Physiol*. 2019;101:e21587.
101. Xu G, Gu GX, Teng ZW, Wu SF, Huang J, Song QS, et al. Identification and expression profiles of neuropeptides and their G protein-coupled receptors in the rice stem borer *Chilo suppressalis*. *Sci Rep*. 2016;6:1–15.
102. Wu HP, Wang XY, Hu J, Su RR, Lu W, Zheng XL. Identification of neuropeptides and neuropeptide receptor genes in *Phaуда flammans* (Walker). *Sci Rep*. 2022;12:1–13.
103. Bendtsen JD, Nielsen H, Von Heijne G, Brunak S. Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol*. 2004;340:783–95.
104. Veenstra JA. Review mono- and dibasic proteolytic cleavage sites in insect neuroendocrine peptide precursors. *Arch Insect Biochem Physiol*. 2000;43:49–63.
105. Nguyen LT, Schmidt HA, Von Haeseler A, Minh BQ. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol*. 2015;32:268–74.
106. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2010;26(1):139–40.
107. Kozomara A, Griffiths-Jones S. MiRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res*. 2014;42:14–22.
108. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol*. 1990;215(3):403–10.
109. Fromm B, Domanska D, Høye E, Ovchinnikov V, Kang W, Aparicio-Puerta E, et al. MirGeneDB 2.0: the metazoan microRNA complement. *Nucleic Acids Res*. 2020;48:132–41.
110. Lee IHT, Nong W, So WL So, Cheung CKH, Xie Y, Baril T, Yip HY, Swale T, Chan SKF, Wei Y, Lo N, Hayward A, Chan TF, Lam HM, Hui JHL. Sex-dependent responses to temperature in the common yellow butterfly *Eurema hecabe*. *BioProject* <https://identifiers.org/bioproject:PRJNA664668>.
111. Lee IHT, Nong W, So WL So, Cheung CKH, Xie Y, Baril T, Yip HY, Swale T, Chan SKF, Wei Y, Lo N, Hayward A, Chan TF, Lam HM, Hui JHL. Sex-dependent responses to temperature in the common yellow butterfly *Eurema hecabe*. *GenBank* <https://identifiers.org/ncbi/insdc:JADANM000000000>.
112. Lee IHT, Nong W, So WL So, Cheung CKH, Xie Y, Baril T, Yip HY, Swale T, Chan SKF, Wei Y, Lo N, Hayward A, Chan TF, Lam HM, Hui JHL. Sex-dependent responses to temperature in the common yellow butterfly *Eurema hecabe*. *figshare* <https://doi.org/10.6084/m9.figshare.19634646>.

## Publisher's Note

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

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more [biomedcentral.com/submissions](https://biomedcentral.com/submissions)

