

Rewiring of two conserved signalling hubs into one axis underlies convergent female-specific wing regression in insects

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Sexual dimorphism represents a striking dimension of morphological diversity both within and across taxa. Female-specific wing regression (FSWR) is common in insects, but the mechanisms underlying this regression remain largely unknown. Here we show that FSWR is widespread among Pterygota insects, including cockroaches, where it contributes to ecological adaptation. We reveal that FSWR regulation involves sex-specific isoforms of DSX, a master regulator in the insect sex differentiation pathway, which oppositely regulate the expression of *E93*, the insect metamorphosis-controlling ‘adult factor’, during wing development. Mechanistically, DSX^F represses, while DSX^M activates, *E93* expression through binding to DSX-binding motifs in FSWR lineages. This differential binding remodels chromatin accessibility and establishes a male-specific three-dimensional topology, facilitating the formation of an enhancer–promoter loop. Consequently, wing morphogenesis gene expression and wing blade cell type specification are promoted in males but suppressed in females. Taken together, our findings provide mechanistic insights into how the rewiring of two conserved signalling hubs into one axis can create regulatory degrees of freedom, thereby generating novel phenotypic variation able to fuel the exploitation of new ecological niches.

Sexually dimorphic traits represent a striking and widespread dimension of morphological diversity¹. While numerous studies in evolutionary developmental biology (evo-devo) have focused on the acquisition or exaggeration of sex-biased traits^{2–5}, far less attention has been paid to the sex-specific regression or loss of traits^{6–11}, despite its ubiquity in nature. A particularly enigmatic example is female-specific wing regression (FSWR), a phenomenon widespread across insect orders^{12–14}. FSWR is thought to be shaped by both sexual and natural selection, promoting mobility and dispersal ability in males, while favouring

resource allocation towards fecundity, cryptic lifestyles and burrowing efficiency in females^{13,15}. Insect FSWR thus serves as a powerful model for examining how selective pressures drive sex-biased modifications of a deeply conserved wing formation process, leading to sex-biased, alternative and adaptive phenotypic outcomes. Nevertheless, the precise genetic, molecular and cellular mechanisms underlying FSWR remain largely unknown.

Sexual dimorphism typically emerges from a sex-specific or sex-biased genetic switch that modulates key developmental

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processes, often through phylogenetically conserved gene regulatory networks^{16,17}. A classic example involves the insect sex differentiation pathway, in which two master regulators, Transformer (TRA) (a splicing factor) and Doublesex (DSX) (a transcription factor and the orthologue of mammalian *Doublesex* and *Mab-3* related transcription factor), serve as key modules coordinating sex-biased development^{18–22}. TRA generates sex-specific alternative splicing isoforms of *Dsx*, *Dsx^M* and *Dsx^F*, and their coding proteins share DNA binding motifs but differ in regulatory regions. *DSX^M* and *DSX^F* can regulate distinct gene repertoires in males and females, exert opposing effects on the same target genes, or both, thereby acting as a complex genetic switch to instruct male- or female-specific developmental trajectories^{18,23,24}. Concurrently, a growing body of research highlights the critical roles of *cis*-regulatory element (CRE) evolution in reconfiguring the expression of key developmental genes, enabling novel phenotypic variation without disrupting core gene functions. This includes the origin and diversification of complex traits (for example, amphibian limbs, bird wings and butterfly wings^{25–36}) as well as their secondary loss or regression (for example, limbs in snakes, legless lizards and cetaceans; wings in penguins; and tails in hominids^{37–42}). Beyond the evolution of novel CREs per se, evolutionary changes in their context-dependent chromatin accessibility are increasingly recognized as an important contributor to phenotypic diversity^{43,44}. Recent efforts have therefore shifted towards understanding the precise spatial organization of genomic elements and the roles of three-dimensional (3D) chromatin topology in orchestrating the precise regulation of gene expression and changes therein^{37,45–47}. However, these advances remain to be systematically integrated with the mechanisms of sex differentiation to achieve a better understanding of the development and evolution of sexually dimorphic traits.

Here we show that DSX has evolved the ability to act as a switch regulating the expression of *E93* through remodelling chromatin accessibility and 3D topology and thus promotes the specification of wing blade cell subtypes. Moreover, we show that the *DSX–E93* regulatory axis has been similarly rewired in other taxa exhibiting partial FSWR but not in non-FSWR insects. These findings establish a framework for how ancient and deeply conserved developmental regulators, linked through the evolution of CRE accessibility and chromatin topology, can facilitate the emergence of novel phenotypic variation, thereby driving the remarkable diversification of sexually dimorphic traits in insects.

Results

FSWR is an adaptive innovation in cockroaches with multiple independent origins

To investigate the evolution of insect FSWR, we first performed a comparative survey of the subclass Pterygota^{14,48}. Our analysis revealed that FSWR is particularly common in the Polyneoptera, occurring at frequencies ranging from 30% to 60% in three orders: Phasmatodea (stick insects), Embioptera (web-spinners) and Blattodea (cockroaches and termites; Fig. 1a and Supplementary Table 1). We constructed a phylogenetic tree of Blattodea using genomic and/or transcriptomic reference

data from 21 species to determine the distribution of FSWR across lineages⁴⁹. Within this order, all three cockroach superfamilies, including Blaberoidea, Corydioidea and Blattoidea, were found to exhibit FSWR with variable degrees of regression. Nevertheless, FSWR is absent in the families Lamproblattidae, Cryptocercidae and Tryonicidae, as well as in termites. Integrative analyses of maximum-likelihood- and parsimony-based ancestral state reconstruction, stochastic mapping (~470 changes), Pagel's λ (7.6393×10^{-5} , $P = 1$) and Blomberg's K (0.2757, $P = 0.668$) revealed a highly labile evolutionary history of FSWR in Blattodea with multiple independent origins across lineages, supporting the convergent evolution of FSWR^{50–52} (Fig. 1b, Supplementary Table 1 and Extended Data Fig. 1a). Taken together, the widespread convergent occurrence of FSWR in cockroaches offers a promising framework for investigating the mechanisms underlying sex-specific phenotypic regression in insects.

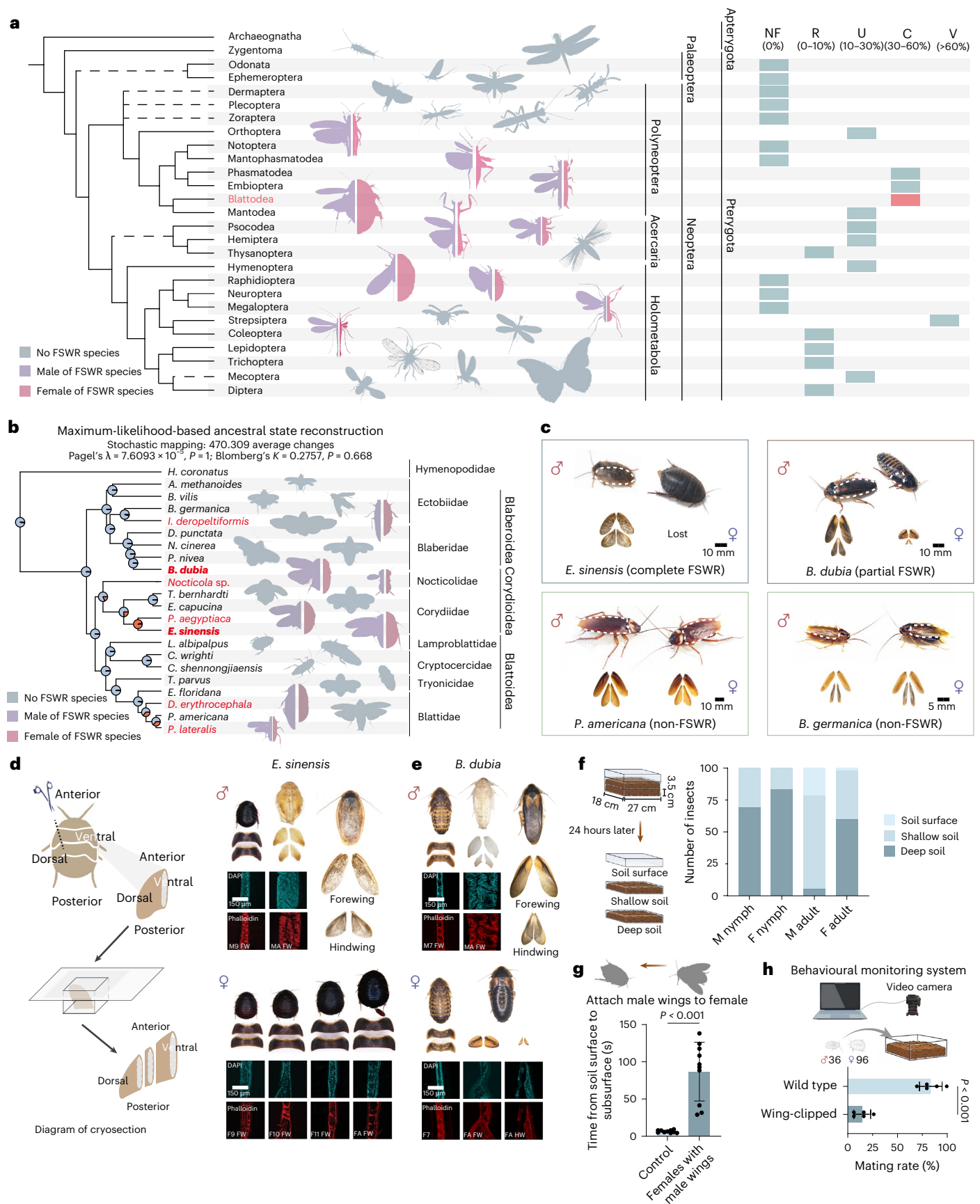
Next, we selected four cockroach species for evo-devo studies on the basis of their distinct wing morphologies and their phylogenetic placement: *Eupolyphaga sinensis* (Corydioidea, complete FSWR), the Dubia cockroach *Blaptica dubia* (Blaberoidea, partial FSWR), the American cockroach *Periplaneta americana* (Blattoidea, non-FSWR) and the German cockroach *Blattella germanica* (Blaberoidea, non-FSWR) (Fig. 1b,c). Phylogenetic analysis reveals that *E. sinensis* (Corydioidea) and *P. americana* (Blattoidea) belong to two sister superfamilies, whereas *B. dubia* and *B. germanica* belong to the more distantly related superfamily Blaberoidea, consistent with the convergent evolution of FSWR in both lineages. Previous work has shown that in both *P. americana* and *B. germanica*, wing pads undergo pronounced tissue folding during nymph-to-adult metamorphosis, followed by rapid wing expansion upon adult emergence^{53–55}. We first compared wing/wing pad morphology between sexes in *E. sinensis*. Males and females in this species undergo 9 (M9) and 11 (F11) nymphal moults, respectively, and the relative wing pad area is greater in males than in females across the 6–9 nymphal instars (N6–N9). In males, wings emerge and expand following adult emergence, whereas adult females retain wing pads that closely resemble those of the F11 nymphal stage (Fig. 1d and Extended Data Fig. 1b,d). Wing pad tissue folding and wing expansion occur exclusively in males (Fig. 1d and Extended Data Fig. 1f). We also compared wing/wing pad morphology between sexes in *B. dubia* (partial FSWR), where both sexes undergo seven nymphal moults. Compared with males, females exhibit partially reduced wing pad tissue folding, wing expansion and wing size (Fig. 1e and Extended Data Fig. 1c,e). These findings illustrate that the varying degrees of FSWR in cockroaches are underpinned by diverse developmental and morphogenetic processes.

In contrast to the surface-inhabiting species *P. americana* and *B. germanica*, *E. sinensis* and *B. dubia* are well-known cryptic species that commonly feed on leaf litter⁵⁶. To evaluate the ecological importance of FSWR in *E. sinensis*, we examined its distribution across soil depths and assessed individual mobility between soil layers in relation to relative wing size. We found that wingless nymphs of both sexes and wingless adult females predominantly occupied deep soil layers, whereas winged

Fig. 1 | FSWR is both widespread and ecologically important in cockroaches.

a, Phylogenetic distribution of FSWR within Insecta. Controversial or uncertain nodes in the evolutionary tree are indicated by dashed lines. The orders are classified according to the prevalence of FSWR at the familial level: not found (NF) (0%), rare (R) (0–10%), uncommon (U) (10–30%), common (C) (30–60%) and very common (V) (>60%). The order Blattodea is denoted in red. **b**, Maximum-likelihood-based ancestral state reconstruction of the FSWR phenotype in Blattodea, with results from stochastic mapping analysis, Pagel's λ and Blomberg's K value indicated. Species with FSWR are highlighted in red, with *E. sinensis* and *B. dubia* shown in bold. The pie charts at the nodes represent the posterior probabilities of the ancestral states. **c**, Wing phenotypes in *E. sinensis*, *B. dubia*, *P. americana* and *B. germanica* in both sexes. **d**, Imaging of wings/wing pads in N9–adult males and females of *E. sinensis*, along with

DAPI and phalloidin staining of nuclei and the F-actin cytoskeleton in wing pads. **e**, Imaging of wings/wing pads in N7–adult males and females of *B. dubia*, along with DAPI and phalloidin staining of nuclei and the F-actin cytoskeleton in wing pads. **f**, Vertical niche distribution of *E. sinensis* during nymphal and adult stages. **g**, Soil burrowing time for females with male wings attached and control wild-type females in *E. sinensis*. **h**, Mating rate statistics for wild-type males compared to wing-clipped males in *E. sinensis*. All differences were analysed using two-tailed Student's *t*-tests. The data are presented as mean $\pm$ s.d. All staining experiments were performed with three biological replicates, while all behavioural experiments were conducted with at least ten biological replicates. M, male; F, female; FW, forewing; HW, hindwing; A, adult. Schematic diagrams in **h** created with biogdp.com (ref. 156).



adult males were largely restricted to the soil surface and the shallowest soil layer (Fig. 1f). We observed that the time required for adult males to migrate from the soil surface to deeper layers was substantially longer than that for adult females (Extended Data Fig. 1g, ~1.9-fold). When male wings were experimentally attached to F9 nymphs, the time required for these artificially winged females to migrate from the surface to deeper layers increased dramatically (Fig. 1g, ~13.7-fold). Moreover, we observed that males frequently flapped their wings during courtship (Supplementary Video 1), and experimental wing clipping in males significantly reduced mating success ($P < 0.001$) (Fig. 1h and Supplementary Video 2). These findings support the hypothesis that FSWR in *E. sinensis* represents an adaptive phenotypic innovation, facilitating efficient burrowing in females while enhancing courtship performance in males.

The somatic sex differentiation pathway determines FSWR in insects

The origin and evolution of sexual dimorphism are influenced by key genes in the conserved somatic sex differentiation pathway, beginning with TRA inducing the expression of sex-specific alternative splicing isoforms of *Dsx*^{16–24}. To characterize these sex differentiation factors and assess their roles in the development and evolution of FSWR in cockroaches, we constructed chromosome-level genome assemblies for *E. sinensis* and *B. dubia* (previously unsequenced) and improved assemblies for *P. americana*⁵⁷ and *B. germanica*⁵⁸ (Extended Data Fig. 2a–i and Supplementary Table 3). We identified five *EsTra*, two *EsDsx*, four *BdTra* and two *BdDsx* transcript isoforms (Extended Data Fig. 2j–k). The female-specific transcripts of *EsTra^f*, *EsDsx^f*, *BdTra^f* and *BdDsx^f* as well as the male-specific *EsDsx^m* were validated by reverse transcription PCR (RT-PCR) (Extended Data Fig. 2l,m). The injection of *dsTra^{CF}* (mixed double-stranded RNAs (dsRNAs) targeting the common region and the female-specific region of *Tra*) downregulated *Dsx^f* in both *E. sinensis* and *B. dubia* and concurrently upregulated *Dsx^m* in *E. sinensis* (Extended Data Fig. 2n,o), consistent with the established model whereby TRA is required for female-specific *Dsx* splicing, and the loss of *Tra* function leads to the production of the default male-type *Dsx* isoform^{16,18,20}. Using published *Tra* and *Dsx* isoform data from *P. americana* and *B. germanica*^{19–21} as well, we further investigated whether and how *Tra* and *Dsx* regulate FSWR across cockroaches upon RNA interference (RNAi) knockdown. In *E. sinensis* and *B. dubia*, *Dsx* knockdown in nymphs markedly suppressed adult wing size and wing morphogenesis in males (Fig. 2a,b, left). In both species, *Tra^{CF}* knockdown induced winged females, whereas *Dsx^f* knockdown moderately increased wing pad expansion in females (Fig. 2a,b, right, and Extended Data Fig. 2p,q). In contrast, in *P. americana* and *B. germanica*, neither *Tra* nor *Dsx* knockdown affected wing morphology in either sex, except for mild deformities observed for female wings upon *Tra* knockdown (Fig. 2c,d). These results indicate that the somatic sex differentiation pathway has been co-opted in the regulation of FSWR.

DSX-mediated *E93* expression instructs sexual wing dimorphism in *E. sinensis*

To identify DSX target genes that determine FSWR, we focused on *E. sinensis* and used a stepwise screening approach. First, a comprehensive RNA-seq screen revealed 258 differentially expressed genes (DEGs) upregulated in the wing pads of M9 individuals compared with both F9 and F11 individuals. Second, transcriptomic analysis revealed that 27 of these 258 DEGs were downregulated following *EsDsx^m* RNAi in male wing pads. Third, an assay for transposase-accessible chromatin using sequencing (ATAC-seq) in M9 wing pads demonstrated that 8 of these 27 DEGs exhibited detectable chromatin accessibility in their promoter regions (Fig. 3a and Extended Data Fig. 3a–d). Fourth, three of these eight candidate genes harboured predicted DSX-binding motifs in their promoter regions, as identified using the online JASPAR database⁵⁹ (Fig. 3b). While RNAi targeting *Esin_12894* or *Esin_04848* did not affect

sexual wing dimorphism, *EsE93^{RNAi}* resulted in markedly malformed and incomplete developed wings in males, or induced a supernumerary male nymphal stage lacking wings, yet produced no phenotypic defects in females (Fig. 3c and Extended Data Fig. 3e,f). *E93* is generally recognized as the ‘adult factor’, a canonical 20-hydroxyecdysone (20E)-responsive transcription factor gene that triggers organ and/or tissue morphogenesis (that is, wing morphogenesis) during insect metamorphosis^{60–65}. This result raised the possibility that *E93* may interact with DSX, thereby promoting wing morphogenesis in males but not in females, ultimately controlling sexual wing dimorphism in *E. sinensis*.

To verify this hypothesis, we first extracted the developmental expression profiles of *EsTra^f*, *EsDsx^f*, *EsDsx^m* and *EsE93* from RNA-seq data. In males, *EsE93* exhibited a similar but slightly delayed expression pattern compared with that of *EsDsx^m* (Fig. 3d, top). In females, *EsE93* expression remained very low, whereas the expression patterns of *EsTra^f* and *EsDsx^f* were closely correlated (Fig. 3d, bottom). We then examined whether *EsE93* expression may be affected by TRA and DSX. As measured by quantitative RT-PCR (qRT-PCR), *EsDsx^m* RNAi in males decreased *EsE93* expression, whereas *EsTra^{CF}* and *EsDsx^f* RNAi in females increased it (Fig. 3e). To confirm their genetic relationship, *EsTra^{CF}* and *EsE93* were knocked down simultaneously. As expected, cotreatment with *dsEsE93* completely abolished *dsEsTra^{CF}*-induced wing morphogenesis in females (Fig. 3f).

We next investigated the molecular mechanisms by which DSX-mediated *E93* expression may control sex-biased wing morphogenesis in *E. sinensis*. We used multiscale resampling and hierarchical clustering to analyse male-biased genes from RNA-seq data across two control and two RNAi treatment groups: M9dsMock, M9dsEsDsx^m, M9dsEsE93 and F9dsMock. Notably, M9dsEsE93 revealed a substantial shift in the transcriptional profile from M9dsMock to F9dsMock, whereas M9dsEsDsx^m induced a moderate shift in the same direction (Fig. 3g). The same results were obtained using 3D principal component analysis (PCA) (Extended Data Fig. 3g). Moreover, genes downregulated in both the *dsEsDsx^m* and *dsEsE93* groups involved four prominent signalling pathways known to play key roles in wing morphogenesis in diverse insects: Wingless (Wnt/Wg), Janus kinase-signal transducer and activator of transcription (JAK-STAT), Notch and Hedgehog^{54,66} (Fig. 3h). Consistent with this, knocking down representative genes (*Wg*, *hopscotch* (*hop*), *Notch* (*N*) and *Hedgehog* (*Hh*)) from each of the four pathways led to malformed and smaller wings in males (Fig. 3i). Taken together, these findings document DSX as the genetic switch controlling male-biased *E93* expression, in which DSX^m-activated and DSX^f-inhibited *E93* expression instruct sexual wing dimorphism in *E. sinensis* by differentially regulating wing morphogenesis genes in males and females.

Rewiring the DSX–*E93* axis is a shared feature of FSWR across insect clades

To determine whether and how the DSX–*E93* axis controls FSWR across insect clades, we expanded our study to other species with varying degrees of FSWR, including *B. dubia* and the tropical house cricket, *Gryllobates sigillatus* (both with partial FSWR), as well as *B. germanica* and *P. americana* (non-FSWR). In *B. dubia*, *BdDsx^C* knockdown reduced *BdE93* expression in male wing pads, while *BdTra^{CF}* and *BdDsx^f* knockdown increased it (Fig. 4a). *BdE93* RNAi caused nymph–adult intermediates with deformed wings or supernumerary nymphs lacking wings in both sexes, with more severe defects in males, consistent with its higher expression in males (Fig. 4b and Extended Data Fig. 4a). Similarly, in *G. sigillatus*, knocking down the common regions of *GsTra* and *GsDsx* in females, and *GsDsx* in males, oppositely affected male-biased *E93* expression and sexual wing dimorphism (Extended Data Fig. 4b–d)⁶⁷. These results indicate that the role of the DSX–*E93* axis in controlling sexual wing dimorphism in *B. dubia* and *G. sigillatus* is functionally similar to, though weaker than, that observed in *E. sinensis*.

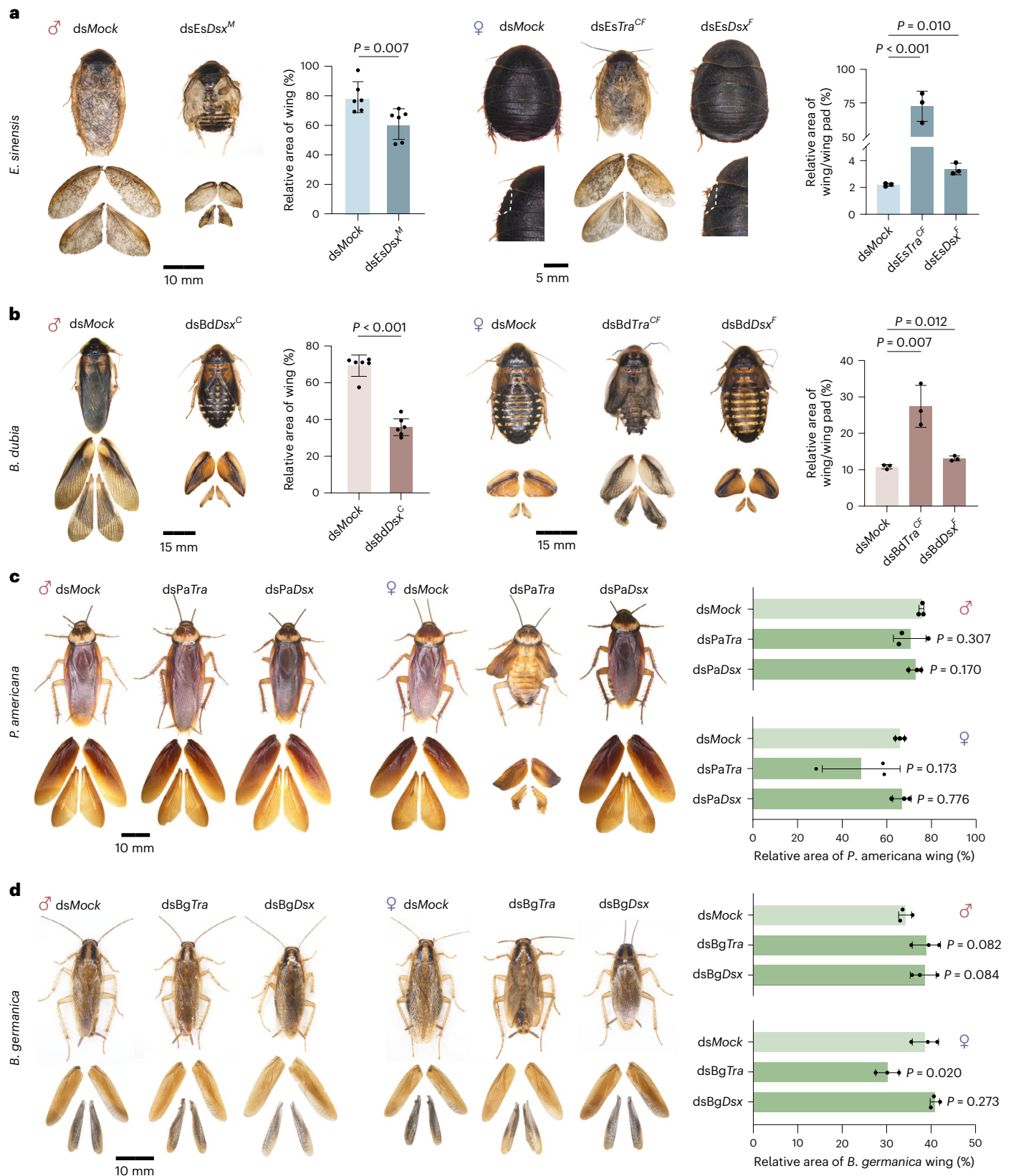


Fig. 2 | The sex differentiation pathway determines FSWR in cockroaches. **a–d**, *Tra* and *Dsx* functional validation and relative wing/wing pad area quantification in *E. sinensis* (**a**), *B. dubia* (**b**), *P. americana* (**c**) and *B. germanica* (**d**).

Wing/wing pad area was measured from individual forewings. All differences were analysed using two-tailed Student's *t*-tests. The data are presented as mean $\pm$ s.d. All experiments were performed with at least three biological replicates.

In contrast, wing-monomorphic *P. americana* and *B. germanica* showed comparable *E93* levels between sexes during the last nymphal instar (Extended Data Fig. 4e,f). We further investigated whether *DSX*

and *E93* also formed a *DSX–E93* axis in the context of wing metamorphosis in these two non-FSWR cockroach taxa. Our results revealed that neither *Tra* nor *Dsx* knockdown affected *E93* expression in either

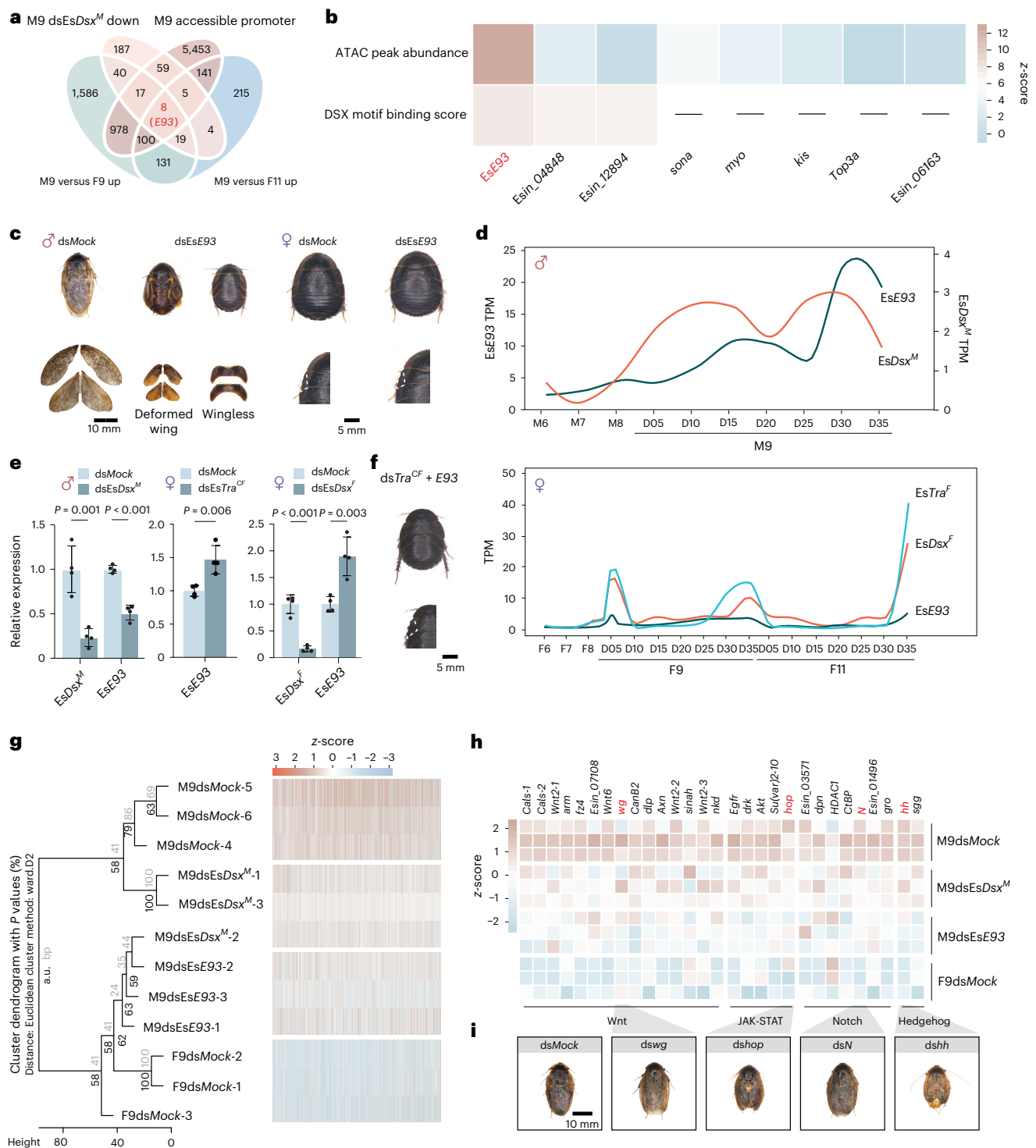


Fig. 3 | DSX-mediated E93 expression controls sexual wing dimorphism in *E. sinensis*. **a**, Integrated analysis of RNA-seq and ATAC-seq data from wing pads for the identification of genes with accessible promoters that are differentially expressed under several conditions in *E. sinensis*. **b**, Heat map showing ATAC-seq peak abundance and DSX motif binding scores for the genes overlapping in **a**. **c**, Functional assessment of *EsE93* in wings of both sexes. Six micrograms of dsRNA was injected into final-instar nymphs on day 20. **d**, Expression patterns of *EsTra^F*, *EsDsxF*, *EsDsxF* and *EsE93* across developmental stages in males (top) and females (bottom) according to transcriptome data. TPM, transcripts per million. **e**, Relative expression of *EsDsxF*, *EsDsxF* and *EsE93* on day 5 post RNAi-mediated knockdown (*EsDsxF* in males, *EsTra^{CF}/EsDsxF* in females) via dual injections of 6 µg of dsRNA (days 1 and 3). **f**, Validation of the wing phenotype

mediated by co-knockdown of *Tra^{CF}* and *E93* in males. **g**, Hierarchical clustering of multiscale resampling and heat maps of male-biased genes (N9) from Extended Data Fig. 3b under different conditions. **h**, Heat map of genes involved in signalling pathways related to wing morphogenesis selected from the genes in **g**. **i**, Functional validation of representative genes from the four signalling pathways in **h**. Six micrograms of dsRNA was injected into males during the final nymphal instar. The injections were replicated on days 1, 10, 12, 23 and 28 for a total of five injections per male. All differences were analysed using two-tailed Student's *t*-tests. The data are presented as mean ± s.d. All qRT-PCR analyses were performed using pooled forewing and hindwing pads (including primordia and pterothecae) with four biological replicates. Functional validation experiments were performed with three biological replicates.

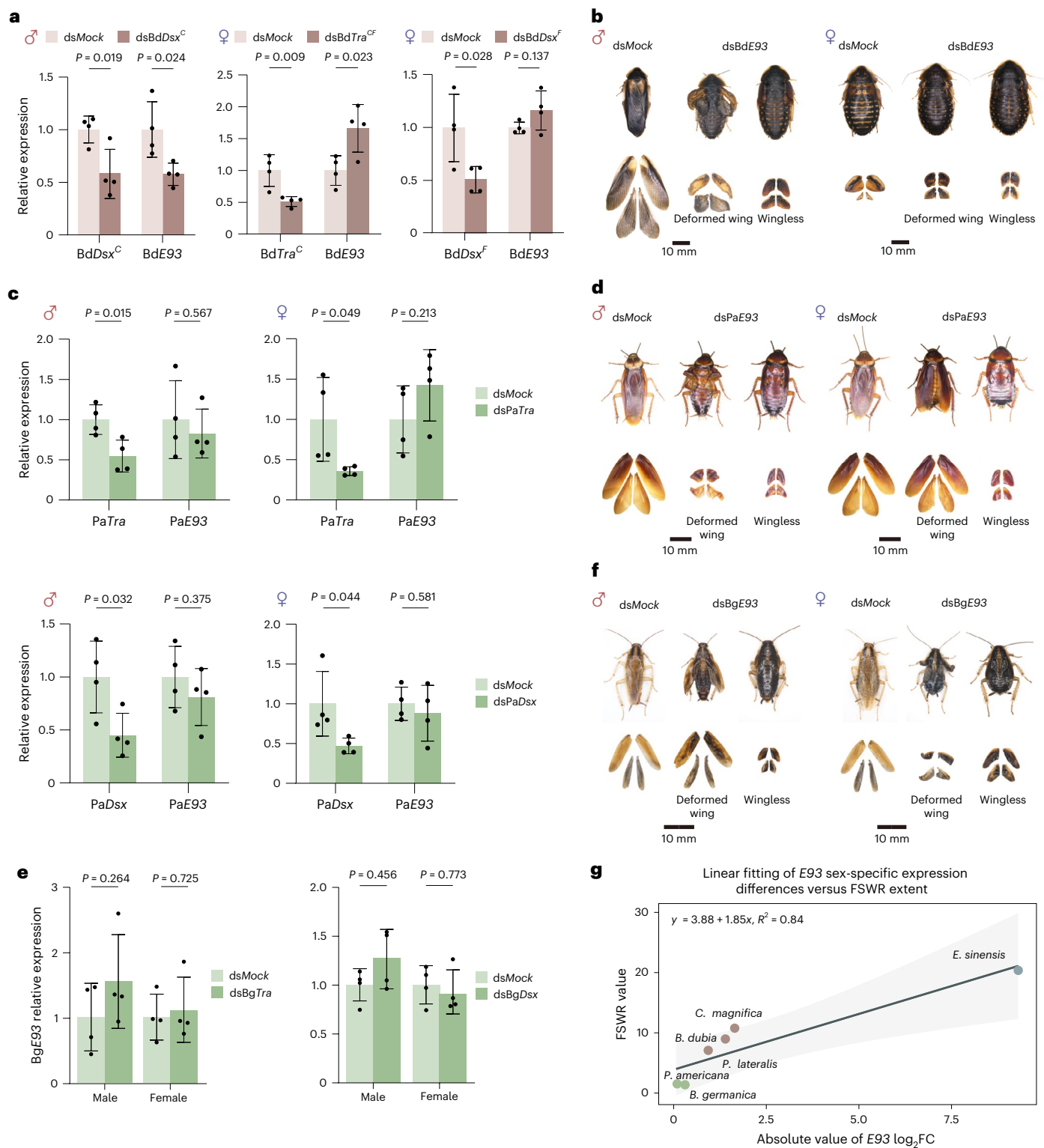


Fig. 4 | Rewiring the DSX–E93 axis underlies convergent FSWR in insects.

a, Substantial downregulation of *BdE93* in the wing pads of *B. dubia* in both sexes. Expression levels were assessed in N7 on day 5 post-*BdTra^{CF}*, *BdDsx^F* or *BdDsx^C* dsRNA injection (6 μ g of dsRNA on days 1 and 3). **b**, Wing/wing pad phenotypes in N7 following *BdE93* knockdown (6 μ g of dsRNA injected on day 20) in *B. dubia*. **c**, Expression levels of *PaE93* in *P. americana* N14 wing pads (both sexes, day 25) following *PaTra* (top) and *PaDsx* (bottom) dsRNA injection (3 μ g of dsRNA on day 23). **d**, Wing/wing pad phenotypes under *PaE93* knockdown treatment in the final instar (3 μ g of dsRNA on days 1 and 23) in *P. americana*. **e**, Expression levels of *BgE93* in *B. germanica* N6 wing pads (both sexes, day 9) following *BgTra* (left)

and *BgDsx* (right) dsRNA injection (3 μ g of dsRNA on day 6). **f**, Wing/wing pad phenotypes under *BgE93* dsRNA injection in the final instar (1 μ g of dsRNA on day 6) in *B. germanica*. **g**, Linear regression of *E93* expression fold changes between sexes (males/females) across six cockroach species against the degree of FSWR. The shaded area represents the 95% confidence interval. All differences were analysed using two-tailed Student's *t*-tests. The data are presented as mean $\pm$ s.d. All qRT-PCR analyses were performed using pooled forewing and hindwing pads (including primordia and pterothecae) with four biological replicates. Functional validation experiments were performed with five biological replicates.

sex of *P. americana* or *B. germanica*, although *E93* knockdown itself caused severe adult emergence defects or supernumerary nymphal phenotypes in all cases (Fig. 4c–f and Extended Data Fig. 4e,f). These findings suggest that the DSX–*E93* axis identified in FSWR cockroaches is absent in non-FSWR species.

We also examined two additional cockroach species with partial FSWR, the emerald cockroach, *Corydidarum magnifica*, and the Turkestan cockroach, *Periplaneta lateralis*, and found that *E93* expression in wing pads was also substantially higher in males than in females (Extended Data Fig. 4g). Linear regression analysis revealed a strong positive correlation ($y = 3.88 + 1.85x$, $R^2 = 0.84$, $n = 6$) between sex-biased *E93* expression divergence and the extent of FSWR (Fig. 4g), further supporting the notion that DSX-mediated modulation of *E93* expression independently evolved in association with varying degrees of FSWR in cockroaches. Our findings thus suggest that rewiring the DSX–*E93* axis underlies the convergent evolution of FSWR across diverse insect lineages.

DSX alters *E93* chromatin accessibility and 3D topology in FSWR taxa

We next aimed to understand how DSX sex-specifically regulates *E93* expression in sexually wing-dimorphic *E. sinensis* and *B. dubia* but not in wing-monomorphic *P. americana* from molecular and cellular sides. We first confirmed the precise transcription start site (TSS) of *E93* in all species using 5' rapid amplification of cDNA ends (RACE) PCR (Extended Data Fig. 5a–d). In *E. sinensis*, a chromatin accessibility peak around the *EsE93* promoter region was detected in the wing pads of both sexes via ATAC-seq, along with a putative male-specific enhancer peak located approximately 16 kb upstream of the promoter (Fig. 5a, tracks 1–6). Using the online JASPAR database, we predicted two DSX-binding motifs within the promoter peak and one within the putative enhancer peak. To confirm these predictions, we performed an electrophoretic mobility shift assay (EMSA) to detect protein–DNA binding between *EsDSX* and the predicted DNA motifs. The EMSA results revealed binding bands between *EsDSX*^M and 5-FAM-labelled probes containing binding motifs in both the promoter and enhancer regions; moreover, binding bands were competitively eliminated by cold probes, and band intensity decreased substantially when core binding motifs in the probe were mutated (Fig. 5b), which confirmed the specificity of the DSX-binding motifs. Likewise, we observed specific binding bands between *EsDSX*^F and the 5-FAM-labelled probe containing binding motifs in promoter regions (Extended Data Fig. 5e). Taken together, these results show that both *EsDSX*^M and *EsDSX*^F bind to the same DSX-binding motifs. Furthermore, the above data indicate that *EsDSX*^M induces *EsE93* expression through binding to DSX-binding motifs in the promoter and enhancer regions in males, whereas *EsDSX*^F represses *EsE93* expression through binding to the corresponding motif in the promoter region of females, solidifying the role of *EsDSX* as a genetic switch in the sex-specific regulation of *EsE93* expression. To further investigate whether TRA/DSX can remodel sex-specific chromatin accessibility in wing pads, we performed loss-of-function experiments followed by ATAC-seq analyses. On the basis of differential peak analysis using log₂ fold change (log₂FC) and false discovery rate (FDR) metrics, RNAi-mediated knockdown of *EsDsx*^M in males resulted in significantly reduced chromatin accessibility at both the *E93* promoter (log₂FC = −0.4967, FDR = 0.0002) and enhancer (log₂FC = −0.5295, FDR = 0.0055) regions. Conversely, knockdown of *EsTra*^{CF} in females led to markedly increased accessibility at the enhancer region (log₂FC = 2.8582, FDR = 8.62×10^{-18}), thereby resulting in a male-like chromatin accessibility at the *E93* locus (Fig. 5a, tracks 7–14). These findings suggest that *EsDSX*^M may function akin to a pioneer transcription factor in facilitating male-specific chromatin accessibility in *E. sinensis*.

Recent 3D genomic studies show that genome organization, particularly enhancer–promoter (E–P) loops within topologically associated domains, orchestrates precise regulation of gene expression controlled by transcription factors^{37,45–47}. The accessibility of

DSX-binding sites relative to the *E93* promoter and enhancer as revealed by our ATAC-seq data suggests the possibility that the corresponding promoter and enhancer regions may form such an E–P loop, thereby facilitating *EsDSX*^M-upregulated *EsE93* expression in males. To test this hypothesis, we performed circular chromosome conformation capture sequencing (4C-seq) in wing pads and found that spatial adjacency between the promoter and enhancer regions was 4.1-fold greater in males than in females (Fig. 5a, tracks 15–16). *EsDSX*^M thus appears to alter 3D chromatin topology, thereby establishing a male-biased *EsE93* E–P loop, which in turn promotes male-biased *E93* expression. These results show that DSX-binding motifs in the *E93* enhancer and promoter regions function as crucial CREs in cockroach development. By virtue of binding to either *DSX*^M or *DSX*^F, *E93* CREs alter chromatin accessibility and 3D topology in *E. sinensis* in a sex-specific manner, enabling the formation of an *E93* E–P loop exclusively in males and thereby promoting sexually dimorphic *E93* expression in wing pads.

We then replicated the same approach in *B. dubia* and detected accessible chromatin peaks at the *BdE93* promoter region in male (M7) and female (F7) wing pads using ATAC-seq (Fig. 5c, tracks 1–4, and Extended Data Fig. 5f,h). Moreover, a male-biased enhancer peak was revealed approximately 17 kb upstream of the promoter, whereas JASPAR supported the prediction of conserved DSX-binding motifs in both the promoter (one site) and enhancer (two sites) regions. As in *E. sinensis*, higher levels of chromatin accessibility in both the promoter and enhancer regions were detected in males than in females. Furthermore, *BdDsx*^C RNAi in males showed a slight reduction in chromatin accessibility at both the promoter (log₂FC = −0.0152, FDR = 0.7426) and enhancer (log₂FC = −0.0186, FDR = 0.7060) regions, whereas *BdTra*^{CF} RNAi in females led to significantly increased accessibility at both the promoter (log₂FC = 1.1493, FDR = 5.41×10^{-15}) and enhancer (log₂FC = 1.5233, FDR = 5.68×10^{-22}) regions (Fig. 5c, tracks 5–12). These results indicate that the *Tra*–*Dsx* axis similarly regulates sex-specific chromatin accessibility in this species. Consistent with these findings, 4C-seq data revealed that spatial proximity between the promoter and enhancer was 2.4-fold greater in males than in females (Fig. 5c, tracks 13–14), indicating a highly similar mechanism using *BdDSX*^C-induced *E93* expression via an E–P loop in *B. dubia*.

In the wing-monomorphic *P. americana*, in contrast, we observed chromatin accessibility peaks in the *PaE93* promoter region in both male (M10) and female (F10) wing pads, but failed to identify a male-specific enhancer peak (Fig. 5d and Extended Data Fig. 5g,i). We also found no DSX-binding motif within the *PaE93* promoter accessibility peak and no evidence of an E–P loop, consistent with the finding that *PaE93* expression is not regulated by *PaDSX*. Although we predicted putative DSX-binding sites outside the *PaE93* promoter accessibility peak, these sites remain inaccessible during the relevant developmental time period (Figs. 4c and 5d). These results demonstrate that DSX and *E93* engage in sex-specific interactions through DSX-binding sites whose accessibility appears to be mediated by DSX itself, driving the formation of a male-biased E–P loop to promote sexual wing dimorphism. This regulatory mechanism is present only in taxa with complete or partial FSWR such as *E. sinensis* and *B. dubia*, but absent in wing-monomorphic cockroaches such as *P. americana*.

DSX-mediated *E93* expression controls wing blade cell subtype specification

To systematically characterize the regulatory pattern of the DSX–*E93* axis in wing development and evolution at the cellular level, we performed single-nucleus RNA sequencing (snRNA-seq) on wing pad samples from all three above-mentioned cockroach species in parallel^{68,69}. Following sample processing and integration methods comparison, 49 transcriptionally distinct cell clusters were identified via the sorted LIGER strategy from eight samples across three species (Extended Data Fig. 6a,b). On this basis, six main functional cell types—wing blade, sensory organ precursor, myoblast, trachea,

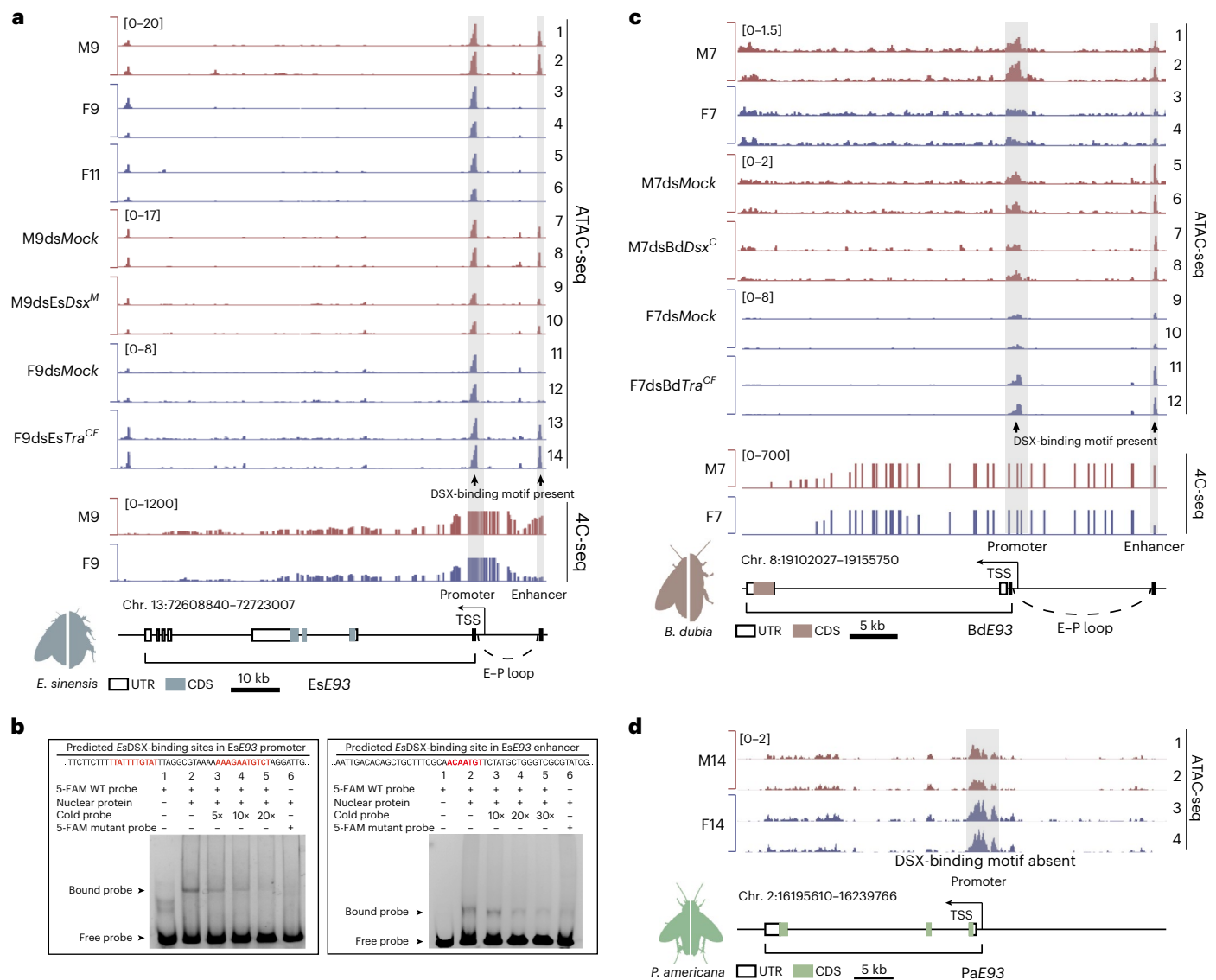


Fig. 5 | Sex-specific DSX isoforms remodel and bind to E93 DSX-binding motifs. **a**, Peak abundance of ATAC-seq and 4C-seq data from wing pads around EsE93 loci in *E. sinensis*. Potential promoter and enhancer regions are highlighted in grey. UTR, untranslated region; CDS, coding sequence. **b**, EMSA confirmation of EsDSX^M binding at predicted motifs within accessible chromatin regions of the

EsE93 promoter and enhancer. Representative results from three independent experiments are shown. WT, wild type. **c**, Peak abundance of ATAC-seq and 4C-seq data from wing pads around BdE93 loci in *B. dubia*. Potential promoter and enhancer regions are highlighted in grey. **d**, Peak abundance of ATAC-seq data from wing pads around PaE93 loci in *P. americana*. Potential promoter regions are highlighted in grey.

cuticle-secreting epidermis and fat body—were identified using the conserved *Drosophila melanogaster* core markers^{70–73} (Fig. 6a,b and Extended Data Fig. 6c).

To identify the key cell (sub)types contributing to sexual wing dimorphism, we focused on the genes downstream of DSX-mediated E93 (that is, genes in the Wnt/Wg, JAK-STAT, Notch and Hedgehog signalling pathways), which were shown to be involved in regulating wing morphogenesis in *E. sinensis* (Fig. 3g,h). Hypergeometric tests revealed that these genes were specifically enriched in two wing blade cell subtypes, cluster 7 and cluster 18 (Fig. 6c and Extended Data Fig. 6d). Gene Ontology enrichment analysis suggested that these two wing blade cell subtypes were distinctly involved in wing morphogenesis, and cluster 18 was also associated with cell division (Fig. 6d). In *E. sinensis*, EsdsDs^{xM} and EsdsE93M revealed substantial shifts in transcriptional profiles in clusters 7 and 18 when compared with EsdsMockM and EsdsMockF with respect to both wing blade cell marker genes (Fig. 6e, left) and wing morphogenesis genes (Extended Data Fig. 6e, top). Consistently, cell proportion analysis revealed that clusters 7 and

18 were more abundant in males than in females, and this prevalence was reduced by EsdsDs^{xM} and EsdsE93M (Fig. 6f, left). These results indicate that DSX-mediated E93 expression promotes the specification of two wing blade cell subtypes in males by inducing the expression of wing morphogenesis genes in *E. sinensis*.

In *B. dubia* (partial FSWR), we again observed male-biased expression of wing blade cell marker genes and wing morphogenesis genes in cluster 18. Surprisingly, cluster 7 exhibited higher gene expression levels and cell proportions in females than in males (Fig. 6e,f, middle, and Extended Data Fig. 6f, middle). In *P. americana* (non-FSWR), neither differential expression nor cell proportion changes were detected in cluster 18, while cluster 7 displayed female-biased patterns similar to those in *B. dubia* (Fig. 6e,f, right, and Extended Data Fig. 6f, bottom). We speculate that elevated gene expression in wing blade cluster 7 may support the incomplete degree of wing regression typical for *B. dubia* females. More generally, these results show that the DSX–E93 axis exerts its wing morphogenetic effects via the sex-specific alteration of wing cell type specification to varying degrees. More generally,

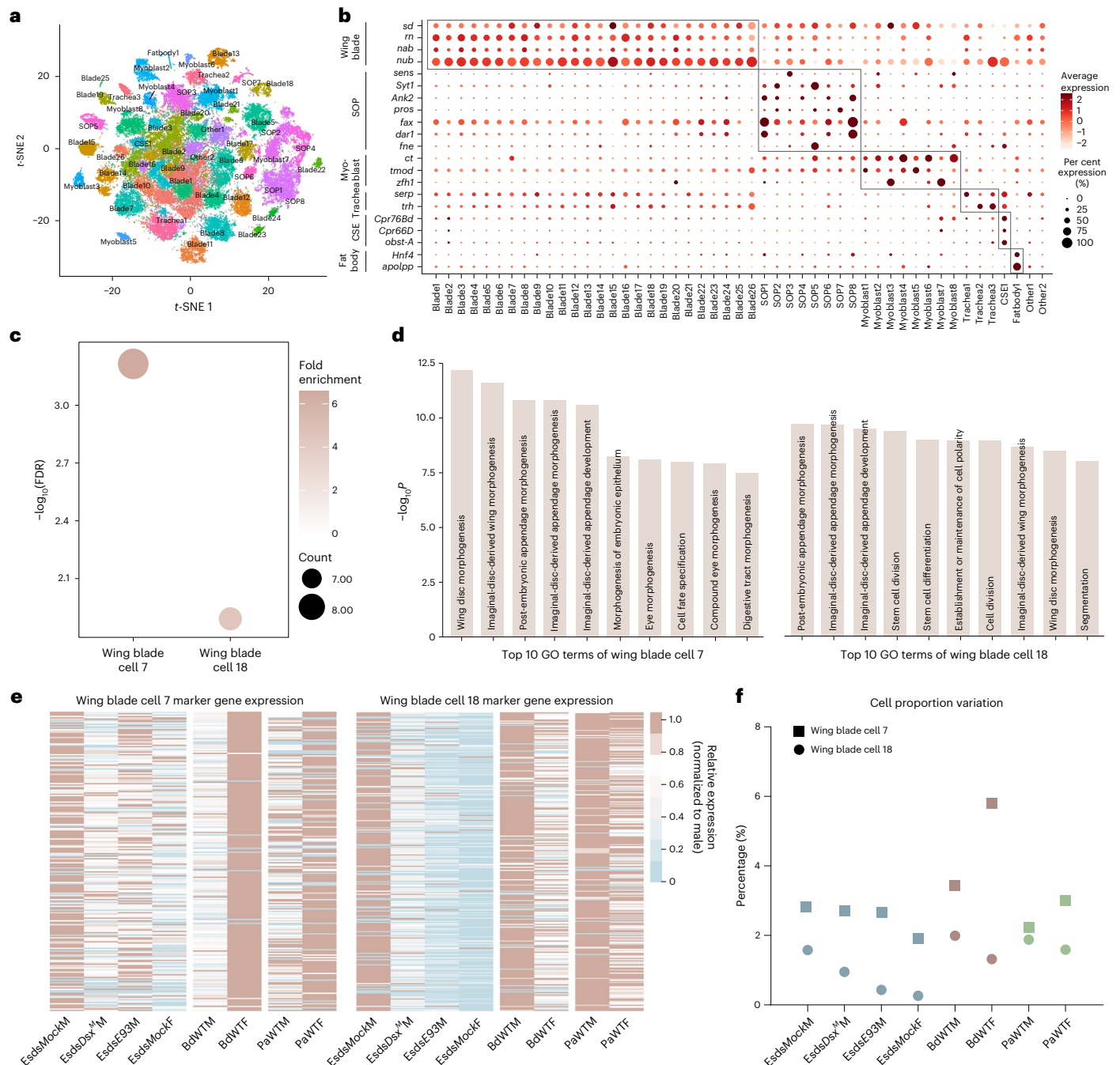


Fig. 6 | DSX-mediated *E93* expression controls wing blade cell specification in wing pads. **a, *t*-distributed stochastic neighbour embedding (*t*-SNE) clustering of integrated snRNA-seq data from *E. sinensis*, *B. dubia* and *P. americana*. SOP, sensory organ precursor; CSE, cuticle-secreting epidermis. **b**, Cluster-specific cell (sub)type annotation. **c**, Cell subclusters 7 and 18 with significant enrichment of DSX-*E93*-axis-regulated wing morphogenesis genes identified by**

hypergeometric testing. **d**, Biological process Gene Ontology (GO) enrichment of marker genes from the wing blade cell 7 and 18 subclusters; the statistical significance of the enrichment was calculated using the hypergeometric test. **e**, Expression profiles of marker genes in wing blade cell 7 and 18 subclusters across different samples or treatments. **f**, Dynamic shifts in the proportion of wing blade cell 7 and 18 subclusters across different samples or treatments.

this study documents that lineage-specific interactions between DSX and *E93* CREs and the resulting changes in chromatin 3D topology instruct the convergent evolution of FSWR in cockroaches. This adaptive and phylogenetically widespread phenotype is achieved through the control of sex-specific gene expression and, ultimately, wing cell type specification.

Discussion

Wings represent a fundamental innovation that propelled insect diversification and radiation, yet FSWR is quite common in insects^{14,48,74–77}.

Unlike other forms of sexual dimorphism, which often involve the sex-limited development of a specific trait such as cephalic horns in male scarab beetles^{3,6,7,23,78}, or sex-biased exaggeration of the male trait version such as eye stalks in stalk-eyed flies^{79,80}, cockroach FSWR entails sex-biased phenotypic regression: the failure to develop complete wings during the nymphal-to-adult transition in females, thereby generating a female phenotype with enhanced burrowing ability. Here we show that rewiring the DSX-*E93* axis underlies convergent FSWR in cockroaches (Extended Data Fig. 7). Below we discuss the most important implications of our results.

DSX has been implicated in the regulation of sexually dimorphic traits in diverse insects and as such represents an ancient and deeply conserved regulator of sex-specific development. Here we demonstrate that in FSWR insects, DSX functionally interacts with *E93*. More generally, on one side, these findings offer a compelling case study of the power of gene co-option in the genesis of morphological diversity, a major theme in the evo-devo field. On the other side, it adds a striking example of how the integration of two deeply conserved signalling hubs, DSX and *E93*, into one axis can create novel regulatory degrees of freedom that neither component could achieve alone. This mechanism thus mirrors a growing number of findings in other taxa^{19,23,24,81,82}, highlighting a concurrent evolutionary strategy for generating sexual dimorphism in insects.

Mechanistically, this work underscores the general roles of CREs, their context-specific accessibility and chromatin topology in the development and evolution of phenotypic traits^{31,32,37–39,43,44}. We identified putative DSX-binding sites outside the *E93* promoter accessibility peak even in the wing-monomorphic *P. americana*, suggesting that the widespread occurrence of FSWR in pterygote insects may be underlain not just by the evolutionary lability of CREs but perhaps even more so by evolutionary changes in their accessibility during key developmental transitions, themselves driven by diversification in DSX function. Specifically, our findings raise the possibility that DSX may function like a pioneer transcription factor to remodel chromatin accessibility and topology in FSWR taxa⁸³, or alternatively may regulate the sex-specific activation of such a factor. Additional 4C-seq following *Tra/Dsx* knock-down will help provide more direct evidence for the spatial proximity between the *E93* promoter and enhancer. Moreover, future work will need to determine whether DSX indeed acts as a pioneer factor or exerts its regulatory function via other mechanisms. Our results nonetheless illustrate the power of spatial genome organization to fine-tune gene expression during ontogeny broadly and metamorphosis in particular. More generally, by integrating sex differentiation with multiscale evo-devo principles, from CREs and their chromatin accessibility to 3D chromatin topology, gene repertoire alteration and cell type specification, we establish wing-dimorphic cockroaches as a new paradigm for studying complex phenotype formation in development and evolution.

Sex-specific regression or degeneration of wings and other secondary sexual traits has been documented in other taxa, yet its ubiquity remains understudied. For example, insect hormones are known to be involved in FSWR evolution in other insects. A striking complete FSWR is present in the hemipteran mealybugs⁸, involving JH-repressed *E93* expression in females through the MEKRE93 pathway⁵⁴. Likewise, complete FSWR is observed in the tussock moth *Orgyia recens*^{9,10} and the winter moth *Nyssiodes lefuarius*¹¹, which is affected by 20E-induced and JH-regulated programmed cell death of the female wing disc during the pupal stage. Likewise, thoracic horns in many horned beetle species in the genus *Onthophagus* are extraordinarily sexually dimorphic in adults^{6,7,84}, and this phenomenon is induced by programmed-cell-death-mediated resorption. Intriguingly, previous studies in *B. germanica* established *Broad-complex* as essential for nymphal wing pad development^{55,85}, but we found *Broad-complex* was not involved in FSWR. In summary, our work shows that by rewiring the DSX–*E93* axis, hemimetabolous cockroaches and crickets have arrived at a powerful and novel developmental means to fuel the evolution of diverse and complex degrees of sexual dimorphism.

Given that DSX^M activates and DSX^F inhibits *E93* expression, which results in convergent FSWR in insects, it is likely that sexual selection played a key role in the evolution of this particular type of sexual dimorphism. However, sexual selection alone appears insufficient to explain the frequent recurrence of FSWR within and across insect lineages, both within the cockroaches and beyond. For example, although the 20E receptor gene *EcR* was equally required for *E93* upregulation in both sexes of *E. sinensis* (Extended Data Fig. 3h,i), 20E signalling in the two subtypes 7 and 18 of wing blade cells was lower in *E. sinensis* than in *B.*

dubia and *P. americana* (Extended Data Fig. 6f). Similar to mealybugs, *E. sinensis* females underwent two additional moults and obtained larger body sizes than males (Fig. 1d), thus implicating that hormonal regulation also contributes to FSWR development and evolution^{8,62}. Our previous studies have shown that the sex differentiation pathway and JH signalling jointly control other sexually dimorphic traits, such as sex pheromone production and oviposition behaviour in female insects^{19,22}. We therefore propose that both sex differentiation and insect hormones broadly underpin *E93*-controlled FSWR by driving the evolution of CRE accessibility and chromatin topology across insect orders, which may promote a new way of thinking about the origins of sexual dimorphism and their adaptive potential under both sexual and natural selection.

Methods

Source organisms

Cockroaches and crickets used in this study underwent prolonged and stable cultivation in the laboratory. All cockroaches were kept at 29 ± 1 °C and a relative humidity of 45–70% under natural light conditions in plastic cages. They were provided with a diet consisting of corn flour, wheat bran and fish meal (7:2:1), and water was supplied ad libitum.

Statistics of FSWR

Insect phylogenetic relationships were based on published literature⁸⁶. In-depth literature research quantified FSWR frequency at the family level across Pterygota orders (Supplementary Table 1). FSWR occurrence was categorized by frequency: not found (0%), rare (0–10%), uncommon (10–30%), common (30–60%) and very common (>60%).

PacBio isosequencing and phylogenetic analysis

Pacific Biosystems (PacBio) isosequencing was employed on *E. sinensis* and *B. dubia*. RNA was extracted from multiple tissues (wing pad, leg, antenna, head, testis, ovary and ootheca) and from multiple stages (early nymph, intermediate nymph, later nymph and adult) using AG RNAex Pro reagent (Accurate Biology, cat. no. AG21101) in accordance with the manufacturer's instructions. An SMRTbell Template Prep Kit (PacBio, cat. no. 100-938-900) was used to prepare cDNA libraries, which were subsequently sequenced on a PacBio Sequel II system. Subsequent data processing was conducted using PacBio Isoseq3 v.3.4.0.

After these datasets were combined with three additional sequenced genomes and seven transcriptomic profiles of cockroaches (Supplementary Table 2)^{57,58,87,88}, a phylogenetic tree of cockroaches with *Hymenopus coronatus* as an outgroup was constructed. For species with available genomic resources, protein sets were filtered to retain the longest isoforms. For others, TransDecoder v.2.1.0 (ref. 89) was used to predict protein sets from RNA data obtained through the transcriptome. The predicted proteins were subsequently screened against the Pfam and Swiss-Prot databases using HMMER v.3.1b2 (ref. 90) and BLAST v.2.12.0 (ref. 91). To eliminate redundancy in the protein set, CD-HIT v.4.8.1 (ref. 92) was employed. Afterwards, orthology was automatically determined using the OrthoFinder⁹³ pipeline. OrthoFinder v.2.5.4 was used to identify orthologues. These were aligned with Muscle v.3.8.1551 (ref. 94) and trimmed with trimAl v.1.4 (ref. 95), and a tree was built with IQ-TREE v.2.1.4 (ref. 96) and visualized in iTOL (<https://itol.embl.de/>)⁹⁷. Ancestral state reconstruction used the R package phangorn v.2.12.1 (ref. 98), and stochastic mapping analysis and calculation of phylogenetic signal Pagel's λ and Blomberg's K were conducted using the R package phytools v.2.5 (ref. 99).

Imaging and quantitative analysis of wing/wing pad area

The imaging and quantitative assessments of relative wing/wing pad area were performed using a stereomicroscope in both sexes of *E. sinensis* (N6 to adult) and *B. dubia* (N4 to adult). Wing/wing pad area was measured from individual forewings. Relative wing/wing pad area was calculated as the ratio of wing/wing pad area to total body area.

Cryosectioning of wing pad tissues

To complement these observations, a comprehensive developmental dissection encompassed multiple nymphal instars of *E. sinensis* (N6 to adult) and *B. dubia* (N4 to adult). In addition, serial dissection was conducted at five-day intervals throughout the M9 instars in *E. sinensis*. Wing primordia from these stages were meticulously dissected and subjected to overnight fixation in 4% paraformaldehyde at 4 °C followed by overnight dehydration in a 50% sucrose solution. Subsequent embedding in OCT compound at −20 °C enabled cryosectioning of the tissues into 30-μm slices for slide preparation. These cryosections were stained with a 1:5,000 dilution of DAPI and a 1:500 dilution of phalloidin for 1 h to visualize nuclear and cytoskeletal components, respectively. Following five washes with PBS buffer, the slides were imaged under a confocal microscope with a ×10 objective lens.

Behavioural assay in *E. sinensis*

To quantify the vertical distribution of nymphs and adults of both sexes, plastic containers (27 × 18 × 3.5 cm³) were assembled vertically to form a three-tiered experimental apparatus. The lower two compartments were filled with soil to create a deep soil layer (3.5–7 cm) and a shallow soil layer (0–3.5 cm), while the uppermost compartment was left as a soil-free surface. Each group was replicated seven times and consisted of 100 individuals. To test the ecological role of wing reduction, wings from male adults were excised and glued onto F9 female nymph wing pads. Wingless females with adhesive only served as controls. Migration time from the surface to inner soil layers was quantified for both groups.

Mating experiments in adult *E. sinensis* were performed in standard 40 × 30 × 15 cm³ chambers. Control groups (wild-type adults, 36 males versus 96 females) were compared with the experimental groups (36 males subjected to tegminal ablation versus 96 wild-type females). Both groups maintained a 1:2.67 male-to-female ratio, and continuous 14-day video monitoring enabled the quantification of cumulative mating frequencies.

Genome studies of cockroaches

Genome sequencing and assembly. Genomic DNA was extracted from a single adult female of *E. sinensis*, *B. dubia*, *P. americana* and *B. germanica*. Genome size was estimated from Illumina data using Jellyfish v.2.2.10 (ref. 100) and GenomeScope v.1.0 (<http://qb.cshl.edu/genomescope/>)¹⁰¹. Subsequently, an additional 510 G continuous long-read data for *E. sinensis* and 100 G, 530 G and 277 G high-fidelity data for *B. dubia*, *P. americana* and *B. germanica* were obtained using the PacBio platform for de novo genome assembly. For *E. sinensis*, PacBio reads were assembled with Canu v.2.0 (ref. 102), haplotigs were purged with Purge_haplotigs v.1.1.3 (ref. 103) and the assembly was polished with the PacBio GenomicConsensus v.2.2.2 Arrow algorithm (using PacBio reads) and Pilon v.1.23 (using Illumina reads)¹⁰⁴. For *B. dubia*, *P. americana* and *B. germanica*, high-fidelity reads were assembled with Hifiasm v.0.20.0 (ref. 105), haplotigs were purged with purge_dups v.1.2.5 (ref. 106) and the assembly was polished with NextPolish v.1.4.1 (ref. 107).

Hi-C scaffolding. Hi-C data from a single female were used for scaffolding. For *E. sinensis*, reads were mapped with BWA v.0.7.17 (ref. 108) and processed with PreprocessSAMs.sh, and scaffolds were clustered/ordered with LACHESIS v.2e27abb (ref. 109). For *B. dubia*, *P. americana* and *B. germanica*, the Hi-C data were processed using Juicer v.1.6 (ref. 110) with BWA-MEM alignment followed by duplicate removal and iterative normalization. Scaffolds were clustered, ordered and oriented into chromosome-scale assemblies using 3D-DNA v.190716 (ref. 111). Misassemblies were manually corrected in Juicebox v.1.11 (ref. 112). Assembly completeness for all species was evaluated with BUSCO v.5.3.1 (insecta_odb10)¹¹³. The features of the genome assemblies and scaffolding are presented in Supplementary Table 3.

Genome annotation and functional annotation. Repetitive elements were identified with RepeatModeler v.2.0.1 (ref. 114) and Repbase v.20181026 (ref. 115), and genomes were soft-masked with RepeatMasker v.4.1.2 (<https://www.repeatmasker.org/>)¹¹⁶. For *E. sinensis*, 81 RNA-seq datasets (including replicates) from multiple developmental stages (five nymphal instars), tissues (wing pads, legs, antenna, heads, testis, ovaries and oothecae), stages (early nymphs, intermediate nymphs, later nymphs and adults) and pooled transcriptomes were aligned to the soft-masked genome using STAR v.2.7.10 (ref. 117). Additionally, the iso-seq data from the PacBio platform were aligned to the soft-masked genome using Minimap2 v.2.24 (ref. 118). For *B. dubia*, pooled RNA-seq data spanning multiple tissues (wing pads, legs, antennae, heads, testes, ovaries and oothecae) and developmental stages (early nymphs, intermediate nymphs, late nymphs and adults) were sequenced on the BGI platform and aligned to the soft-masked genome using STAR v.2.7.10 with the default parameters. Additionally, PacBio iso-seq reads were aligned to the genome with Minimap2 v.2.24. The resulting BAM files were subsequently sorted and indexed using SAMtools v.1.12 (ref. 119). BRAKER v.2.1.6 (ref. 120) was subsequently used to annotate protein-coding genes with untranslated region training and prediction. The gene set completeness was assessed using BUSCO v.5.3.1 (insecta_odb10), wherein the longest transcript of each gene served as the representative transcript for analysis. Functional annotation of the predicted genes was performed by referencing the publicly available databases NR, Swiss-Prot, TrEMBL, InterPro¹²¹ and EggNOG^{122,123}. Details regarding genome annotation can be found in Supplementary Table 3.

Transcript identification with isosequencing data and RT-PCR

The isoforms for *Tra* and *Dsx* transcripts were identified from isosequencing-generated data by tBLASTn v.2.12.0 (ref. 91). All specific splice junctions were verified with RT-PCR. Specifically, total RNA was extracted from the wing pads of *E. sinensis* on day 5 and *B. dubia* on day 9 using AG RNAex Pro reagent (Accurate Biology, cat. no. AG21101). cDNA synthesis was carried out with reverse transcriptase M-MLV (RNase H-) (Takara, cat. no. 2641Q). RT-PCR amplification was performed with PrimeSTAR Max DNA Polymerase (Takara, cat. no. R045B).

RNAi in vivo

For RNAi treatments, 200–500-bp fragments of the target genes were amplified via PCR from the cDNA. The T7-promoter-sequence-attached primers were subsequently used for PCR amplification of the dsRNA templates. dsRNA was synthesized using a T7 RiboMAX Express RNAi kit (Promega, cat. no. P1700) according to the manufacturer's instructions. A 92-bp noncoding sequence from the pSTBlue-1 vector (dsMock) was used as negative control dsRNA¹²⁴. dsRNA was quantified and adjusted to 3 μg μl^{−1}. The prepared dsRNA was injected into the abdomens of insects. The injection time points and dsRNA amounts are described in the figure legends. The dsRNA synthesis primers are listed in Supplementary Table 4.

qRT-PCR for gene expression detection

For our gene expression analysis, we pooled entire forewing and hindwing pads, including both the wing primordia and the surrounding pterothecae from at least two individuals for RNA extraction. qRT-PCR was performed using Hieff qPCR SYBR Green Master Mix (Yeasen, cat. no. 11202ES03) with three technical replicates. Relative gene expression levels were calculated using the 2^{−ΔΔCt} method. Primer sequences are listed in Supplementary Table 4.

Bulk RNA-seq and data analysis

For all subsequent sequencing libraries generated in this study (including bulk RNA-seq, ATAC-seq, 4C-seq and snRNA-seq), the samples comprised a mixture of forewing and hindwing pads that included both the wing primordia and the surrounding pterothecae. For *E. sinensis*,

transcriptome samples were collected from both sexes at N6, N7 (day 25), N8 and N9 (day 30) and from females at N11 (day 30). To identify potential downstream targets of *EsDSX^M*, samples from M9 *dsMock* (day 30) and M9 *dsEsDsx^M* (day 30) wing pads were also obtained. For developmental transcriptome profiling, wing pads were sampled every five days from M9, F9 and F11 instar nymphs. Additional samples from M9 *dsMock* (day 30), M9 *dsEsE93* (day 30) and F9 *dsMock* (day 30) wing pads were included for hierarchical clustering and PCA. To assess the sex-specific expression of *E93*, wing pads were collected from both sexes at the final instar stage (day 25 for *P. americana* and *B. dubia* and day 8 for *B. germanica*). All samples underwent RNA extraction using AG RNAex Pro (Accurate Biology, cat. no. AG21101) and cDNA library preparation with the Takara SMART-Seq v4 Ultra Low Input RNA Kit (cat. no. 634889). Paired-end sequencing was conducted on a NovaSeq6000 platform (Illumina). Transcript abundance was quantified using Salmon v.1.4.0 (ref. 125), and RNA expression heat maps were generated on the basis of Z-score-normalized gene-level transcripts-per-million values with Pheatmap v.1.0.12 (ref. 126). Differential expression analysis was carried out using the R package DESeq2 v.1.40.2 (ref. 127), and genes exhibiting adjusted $P < 0.05$ and $|\log_2FC| > 1$ were classified as DEGs. Volcano and Venn diagrams were created using OmicStudio tools (<https://www.omicstudio.cn/>)¹²⁸.

Transcriptional regulation studies

ATAC-seq and data analysis. For *E. sinensis*, ATAC-seq samples were obtained from the wing pads of M9, F9 and F11 nymphs on day 30. Additional samples were collected from the wing pads of last-instar nymphs on day 25 in both sexes of *B. dubia* and *P. americana*. The sequencing of frozen samples was performed according to the ATAC-seq protocol of Shanghai Jiayin Biotechnology Ltd. Native nuclei were purified from frozen samples as previously described¹²⁹, and a Nextera DNA Library Preparation Kit (Illumina, cat. no. 20060060) was used to perform the transposition according to the manufacturer's protocol. A total of 50,000 nuclei were pelleted and resuspended in transposase for 30 min at 37 °C. The transposed DNA fragments were subsequently purified with a MinElute PCR Purification Kit (QIAGEN, cat. no. 28004). The samples were then PCR-amplified using NEBNext High-Fidelity PCR Master Mix (New England Biolabs, cat. no. M0541). Subsequent libraries were purified with the MinElute PCR Purification Kit (Qiagen, cat. no. 28006) and subjected to sequencing on an Illumina NovaSeq 6000 using PE150.

Raw sequencing reads were processed to remove adapters, poly-N sequences and low-quality bases. Clean reads were aligned to the genome sequences using Bowtie2 v.2.3.5.1 (ref. 130). SAMtools v.1.12 (ref. 119) was used to remove unmapped, unpaired reads, and PCR duplicates were removed using Sambamba v.0.8.2 (ref. 131). Peak calling was performed using MACS v.2.2.7.1 (ref. 132) with a cut-off $q < 0.05$, and peaks were annotated using the R package ChIPseeker v.1.36.0 (ref. 133). ATAC-seq signals (± 3 kb from the TSS) were generated using deepTools v.3.5.4 (ref. 134). PCA and differential peak analysis were performed using the R package DiffBind v.3.18.0 (ref. 135). Visualization tracks for IGV v.2.16.0 (ref. 136) were created in BigWig format.

Prediction of DSX binding to E93 promoters. The online JASPAR database v.2024 (<https://jaspar.elixir.no/>)⁵⁹ was used to predict the presence of DSX-binding sites within the accessible promoter and enhancer regions.

5' RACE PCR. A SMARTer RACE 5'/3' Kit (Takara, cat. no. 634858) was used to construct 5' RACE libraries from *E. sinensis*, *B. dubia* and *P. americana* wing pads. All RNA extractions were performed using AG RNAex Pro reagent (Accurate Biology, cat. no. AG21101) according to the provided protocol. For all species, 5' RACE cDNA fragments were amplified using *EsE93*, *BdE93* and *PaE93* gene-specific primers (Supplementary Table 4) and kit-provided UPM primers. Subsequently,

nested *EsE93*, *BdE93* and *PaE93* gene-specific (Supplementary Table 4) primers and short UPM primers were used for nested PCR amplification, with the outer PCR products serving as templates. PCR products were verified by Sanger sequencing.

EMSA. EMSAs were performed using a LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific, cat. no. 20148X). The full-length *EsDSX^M* protein was overexpressed in *Drosophila* KC cells, and nuclear proteins were extracted using a nuclear and cytoplasmic protein extraction kit (Beyotime, cat. no. P0027). The -746 to -689 nucleotide and -17729 to -17180 nucleotide fragment distances to the TSS of the *EsE93* were synthesized with FAM-labelling at the 5' terminus. The binding reaction was performed by incubating 8 μ l of nuclear protein extracts with 100 μ M FAM-labelled probe in a total volume of 20 μ l containing 1 $\times$ binding buffer, 2.5% glycerol, 5 mM MgCl₂, 50 mM KCl, 10 mM EDTA, 0.05% NP-40 and 1 μ g of poly(dI-dC). In the competition assays, 10-, 20- and 30-fold molar excesses of unlabelled probes and isotonic mutant probes were separately added to the binding reaction. All reactions were maintained at 25 °C for 20 min, after which the proteins were immediately separated by 5% native acrylamide gel electrophoresis at low temperature. One hour later, the gel was placed in ultrapure water, and the complexes were visualized via fluorescence imaging (Tanon, 5200 Multi).

4C-seq and data analysis. Wing pads from both sexes of *E. sinensis* N9 (day 30) and *B. dubia* N7 (day 25) were collected for 4C-seq analysis. The spatially accessible promoters of *EsE93* (chromosome 13: 72703801–72704563) and *BdE93* (chromosome 8: 19137229–19137680) were designated as the viewpoints for the 4C-seq experiment (Wuhan GeneRead Biotechnology Co.). The samples were crosslinked with formaldehyde, digested with RE1(-) enzyme, ligated, decrosslinked and purified to obtain hybridized fragments. These products were trimmed using the RE2(-) enzyme and ligated to produce cyclized products. A two-step PCR approach was employed for library construction. After library construction, initial quantification was conducted using a Qubit v.2.0 (Thermo Fisher Scientific), and the library was diluted to 1 ng μ l⁻¹. The insert size of the library was subsequently assessed using an Agilent 2100. Following library validation, different libraries were pooled according to their effective concentrations and the required volume of sequencing output for high-throughput sequencing. Quality control filtering of the sequencing data was performed using fastp v.0.23.2 (ref. 137), and reference sequence alignment was conducted with Bowtie v.2.3.5.1. Processed, clean data were analysed using pipe4C v.1.1.6 (ref. 138). Additionally, peak detection in near-*cis* regions was performed using the R package peakC v.0.2 (ref. 139).

SnRNA-seq and data analysis

For *E. sinensis*, we collected samples from *dsMock*-treated F9 and M9 nymphs (day 30) and from M9 nymphs treated with *dsEsDsx^M* and *dsEsE93* (day 30), labelled *EsdsMockF*, *EsdsMockM*, *EsdsDsx^MM*, and *EsdsE93M*. For *B. dubia* and *P. americana*, we harvested last-instar wild-type wing pads (day 25), labelled *BdWTM*, *BdWTF*, *PaWTM* and *PaWTF*. Frozen samples were digested using Nuclei ez lysis buffer (Millipore Sigma, cat. no. N3408) and suspended in 0.4% BSA in PBS. The nuclei were then sorted with a BD FACSAria Fusion flow cytometer. A final nuclear suspension of 0.75–1.2 $\times 10^6$ cells per ml larger than 50 μ l was used for library construction with a Chromium Next GEM Single-Cell 3' Kit v.3.1 (10 $\times$ Genomics, cat. no. PN-1000268). Libraries were sequenced by Majorbio Biomedical Technology Co.

The Cell Ranger pipelines v.7.1.0 (ref. 140) provided by 10 $\times$ Genomics were used to process the sequenced libraries (alignment, barcode assignment and unique molecular identifier counting). The sequencing matrices of the wing pads (eight samples) from the 10 $\times$ Genomics snRNA-seq data are summarized in Supplementary Table 5.

The Seurat pipeline v.5.1.0 (ref. 141) was used to perform basic processing and visualization of the snRNA-seq data. `nFeature_RNA > 200` and `min.cells > 3` were the filtering thresholds for cell objects to extract high-quality data for analysis. `decontX v.1.0.0` (ref. 142) and `DoubletFinder v.2.0.6` (ref. 143) were used to estimate RNA contamination and detect doublets. After initial quality control, we tested different integration methods, including CCA, RPCA, FastMNN and SCT in the R package Seurat v.5.1.0; Harmony in Harmony v.1.2.0 (ref. 144); and LIGER in `rliger v.1.0.1` (ref. 145). We then used `lisi v.1.0` (ref. 144) to assess the integration outcomes. Finally, we used Seurat for normalization, dimension, reduction, clustering and visualization, and we used LIGER for integration. Retained cells from each sample were log-normalized and scaled with `LogNormalize`. Variable genes were identified with the `FindVariableFeatures` function. After sample integration and batch removal, clustering and visualization of the data were accomplished using Seurat's linear dimension reduction followed by *t*-distributed stochastic neighbour embedding. We identified cluster-specific marker genes through unbiased differential expression analysis (`FindAllMarkers`). Given that the core wing gene regulatory network is deeply conserved across the phylogeny of winged insects (Pterygota) despite divergent developmental modes^{146,147}, previous knowledge of gene expression from *D. melanogaster*^{70–73} was used as a comparative reference via orthology to assist in annotation. The R package `clusterProfiler v.4.2.2` (ref. 148) was used for Gene Ontology enrichment analysis.

Quantification and statistical analysis

The two-tailed Student's *t*-tests were used for statistical analysis. The data are expressed as the mean ± standard deviation. The R packages `FactoMineR v.2.8` (ref. 149) and `ggplot2 v.3.4.3` (ref. 150) were used to conduct 2D PCA. Following the application of the `ComBat` function from the R package `sva v.3.48.0` to mitigate batch effects¹⁵¹, the R packages `stats v.4.3.1` (ref. 152) and `scatterplot3d v.0.3.44` (ref. 153) were used for 3D PCA. Approximately unbiased branch and bootstrap probability supports for the resulting dendrogram were calculated using the R package `pvclust v.2.0` (ref. 154), with ten resampling scales (0.5–1.4, increments of 0.1) and 1,000 bootstrap replicates at each scale; both analyses used genes upregulated in M9 versus F9 as the input gene set. Sex-biased expression of *E93* in wing pads across cockroach species was quantified using `DESeq2 v.1.40.2`. The degree of FSWR was calculated as the ratio of the relative wing area in males to that in females. A linear regression between these two variables was performed using `ggpmisc v.0.6.2` (ref. 155) and `ggplot2 v.3.4.3`. Schematic diagrams were generated using `BioGDP`¹⁵⁶ and `BioRender` (<https://app.biorender.com/>). All other data were plotted with `GraphPad Prism v.9.5.1` (ref. 157) and formatted with `Adobe Illustrator CC v.2022` (<https://www.adobe.com/products/illustrator.html>).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All genome sequencing, bulk RNA-seq, ATAC-seq, 4C-seq and snRNA-seq raw data, along with the genome assembly sequences, are available via the NCBI BioProject database under accession number [PRJNA1307050](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1307050). Source data are provided with this paper. The materials are available upon request to the lead contact, S.L. (lsheng@scnu.edu.cn).

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Author contributions

S.L. led and conceived the project. S.L., C.R. and W.Z. supervised the study. M.L., X.C., Z.Y. and L.H. performed most of the experimental work and analysed the data. D.Y., M.L. and D.H. performed the genomic data analysis. D.Z., M.L. and J.N. performed the snRNA-seq experiment and analysed the data. S.L., A.P.M., C.R., W.Z. and M.L. wrote the manuscript. Q.M., Y.H. and Y.P. contributed to the writing of this manuscript.

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Competing interests

The authors declare no competing interests.

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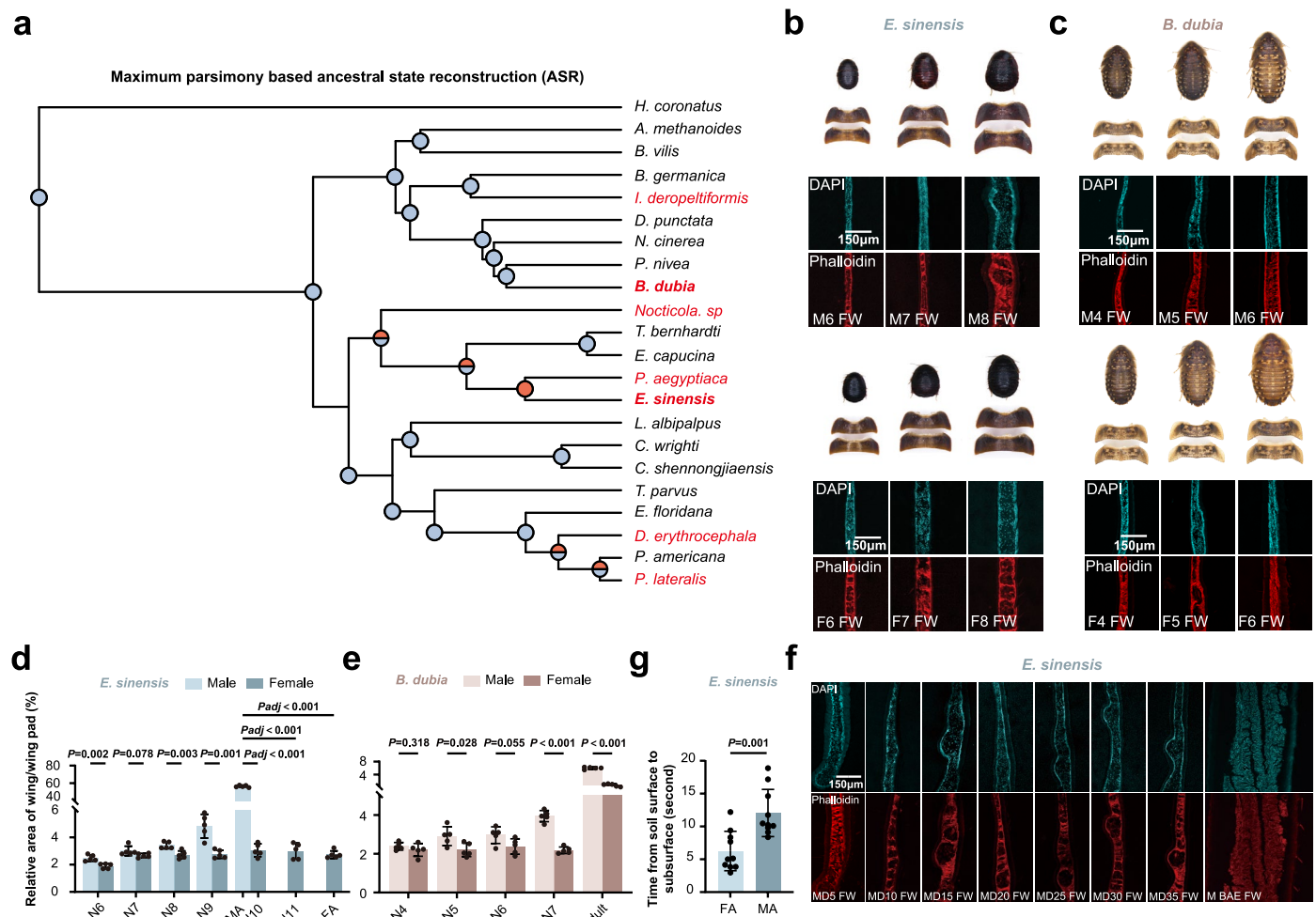
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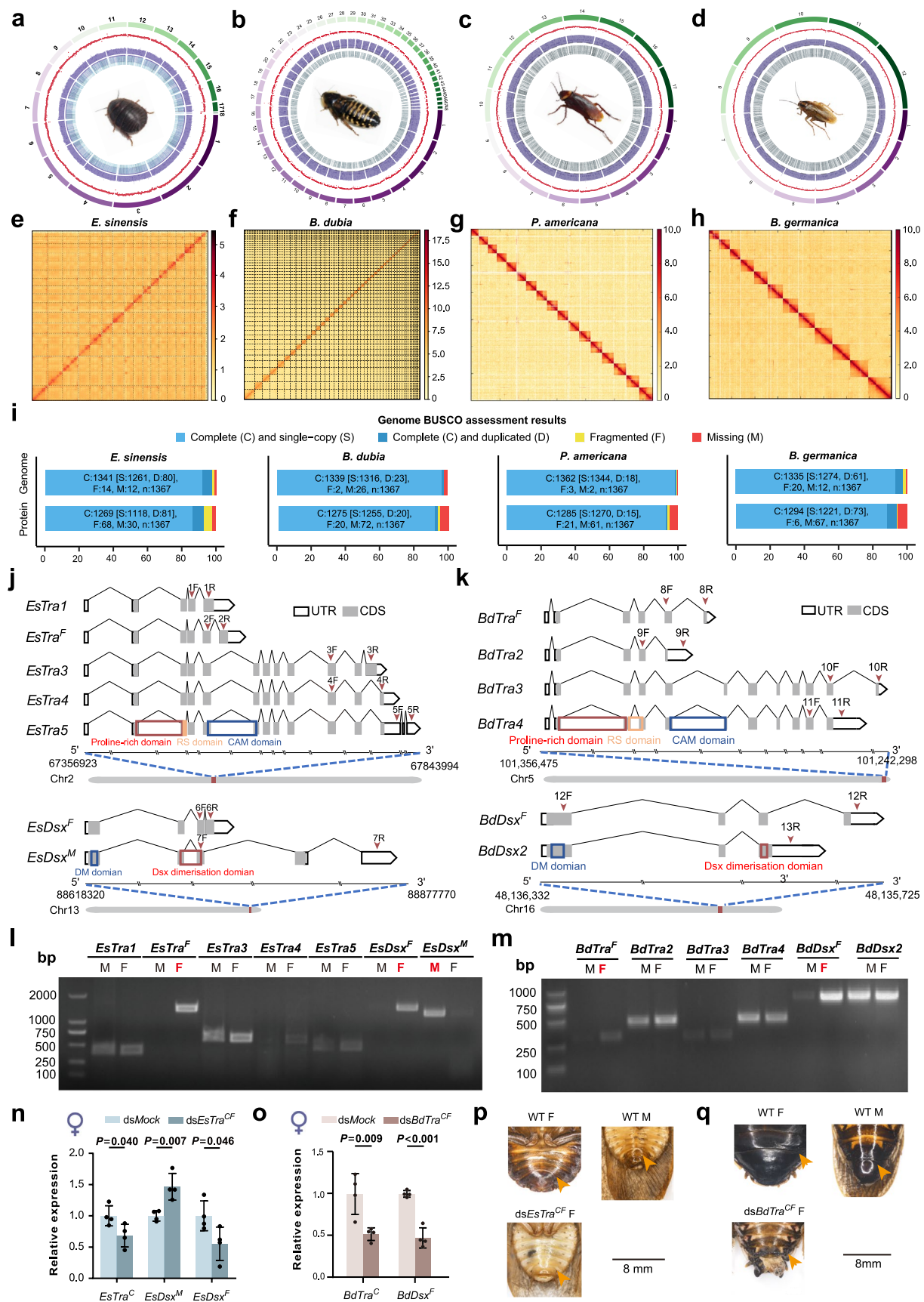
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Extended Data Fig. 1 | Complete FSWR in *E. sinensis* and partial FSWR in *B. dubia*. **a**, Maximum parsimony based ancestral state reconstruction (ASR) of the FSWR phenotype in Blattodea. Species with FSWR are highlighted in red, with *E. sinensis* shown in bold. **b–c**, Whole-body and dorsal plate imaging of N6–N8 males and females of *E. sinensis* (**b**) and N4–N6 males and females of *B. dubia* (**c**), along with DAPI and phalloidin staining of nuclei and the F-actin cytoskeleton in wing pads. **d–e**, Developmental changes in relative wing/wing pad area between sexes across ontogenetic stages in *E. sinensis* (**d**) and *B. dubia* (**e**). Wing/wing pad area was measured from individual forewings. **f**, DAPI and phalloidin staining

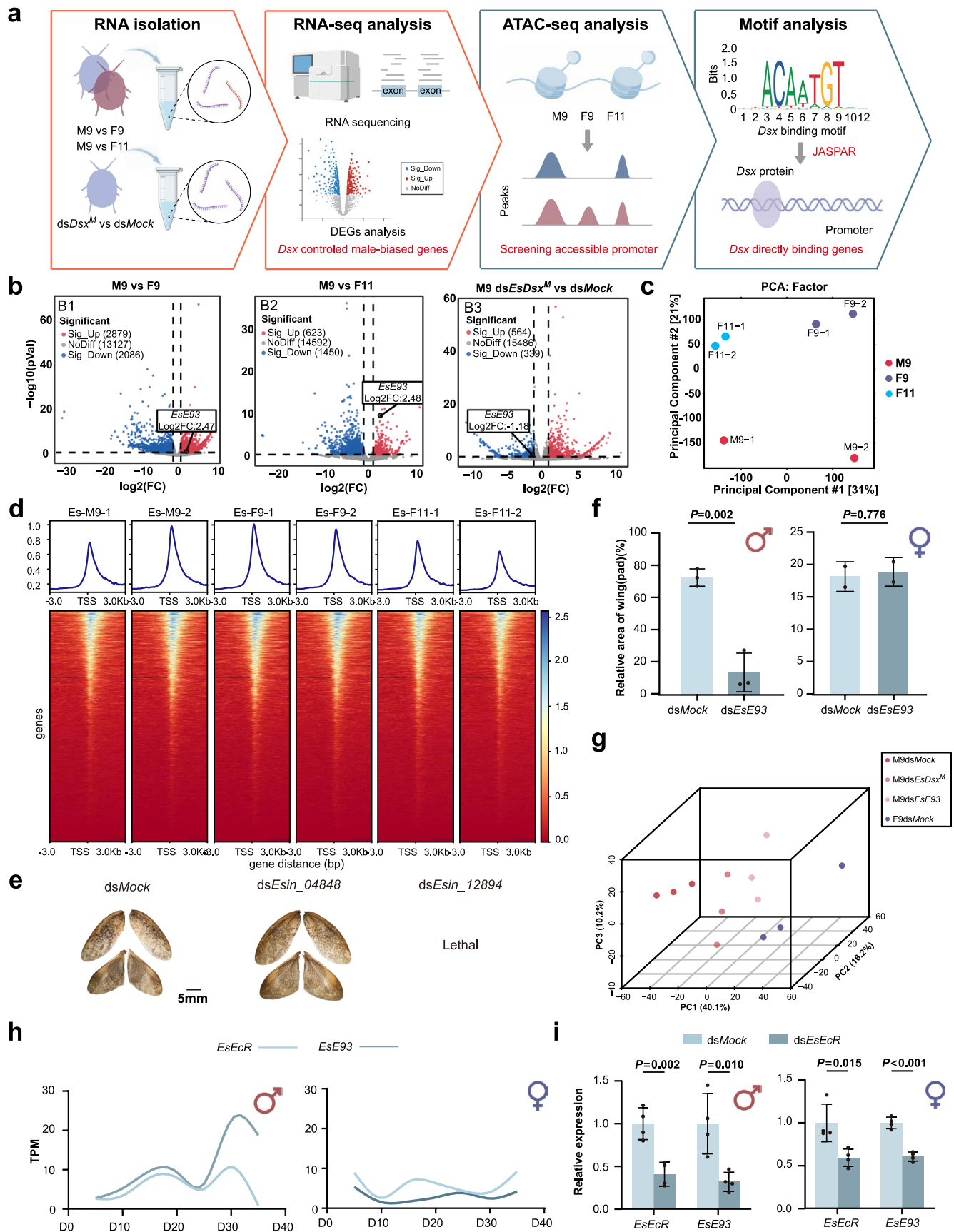
of nuclei and the F-actin cytoskeleton in wing pads from male *E. sinensis* N9 instar. **g**, Soil burrowing time for wild-type male and female adults of *E. sinensis*. All differences were analyzed with a two-tailed Student's t-test, the Bonferroni correction was applied to comparisons between MA and FN10, FN11, or FA. Data are presented as mean $\pm$ SD. All relative wing/wing pad area statistics were performed with five biological replicates, while all staining experiments were conducted with three biological replicates. Abbreviations: M, Male; F, Female; FW, Forewing; D, Day; BAE, Before adult emergence; MA, Adult male; FA, Adult female.



Extended Data Fig. 2 | See next page for caption.

Extended Data Fig. 2 | Genomic landscape and *Tra/Dsx* functional validation in cockroaches. a-d, Circular diagram demonstrating the genomic landscape of *E. sinensis* (a), *B. dubia* (b), *P. americana* (c), and *B. germanica* (d). The denotation of each track is as follows (from outside to the inside, sliding window = 500 kb): repeat sequences, GC content, and gene density. **e-h**, Hi-C linkage density heatmaps of *E. sinensis* (e), *B. dubia* (f), *P. americana* (g), and *B. germanica* (h), with contigs anchored onto 18, 48, 17, and 12 chromosomes, respectively. **i**, BUSCO assessment of the genomes and annotated protein sets of four cockroach species. **j-k**, Gene structure and chromosomal distribution of *Tra* and *Dsx* in *E. sinensis* (j) and *B. dubia* (k), with RT-PCR primers (F: forward primers; R: reverse primers) labeled accordingly. Noncoding regions (untranslated region, UTR) are indicated by black boxes, coding sequences (coding sequence, CDS) are indicated by grey squares, and conserved protein domains are indicated by different colored boxes. Chromosomal position information is plotted below

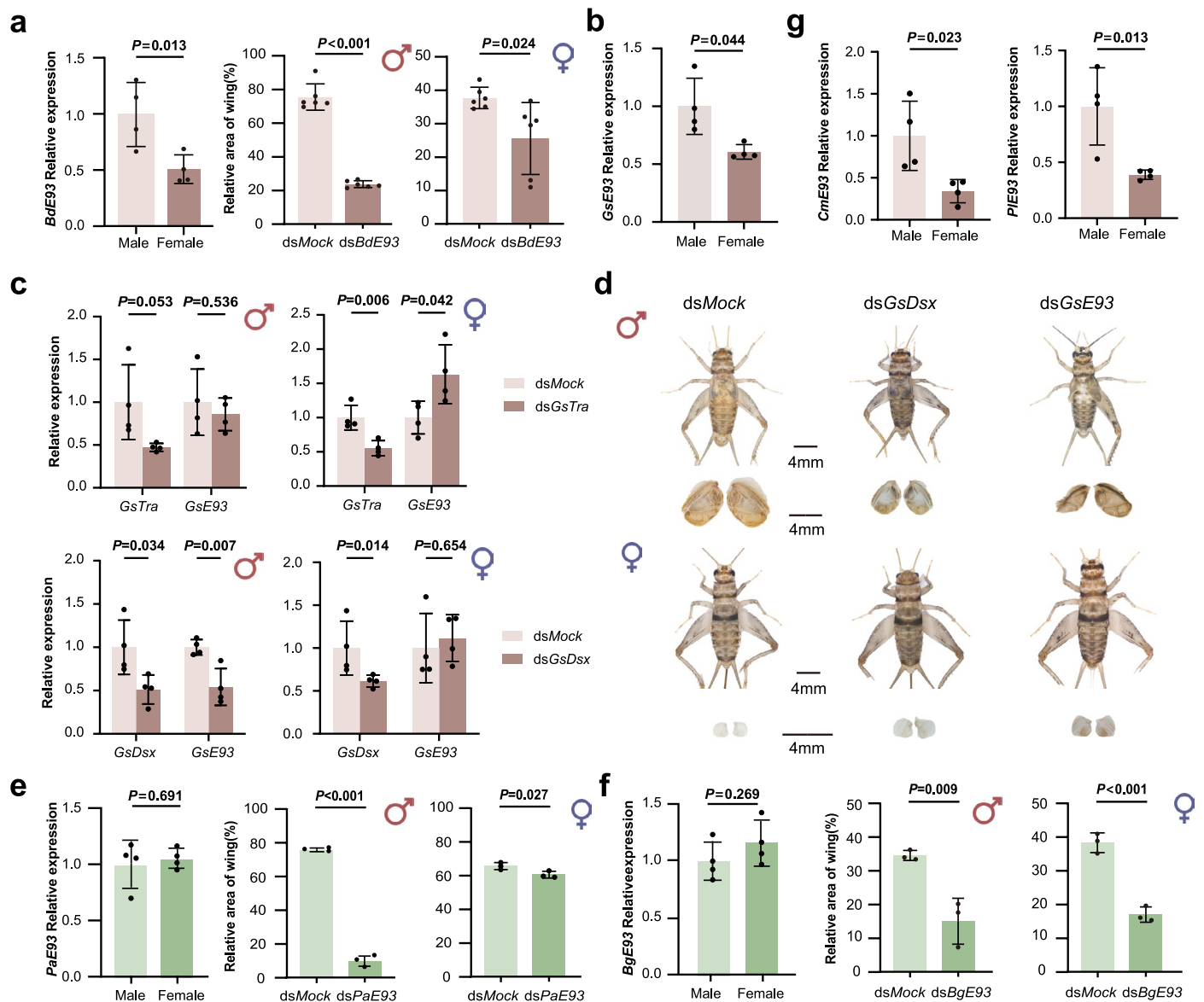
gene structures. **l-m**, RT-PCR detection of sex-specific expression of *Tra* and *Dsx* transcripts in *E. sinensis* (l) and *B. dubia* (m), with sex-specific transcripts highlighted in bold red. The primers used are denoted in panels j and k. **n**, Relative expression of *EsTra^C*, *EsDsx^F*, and *EsDsx^M* in females on day 5 after knockdown of *dsEsTra^{CF}* (6 µg of dsRNA injected on days 1 and 3). **o**, Relative expression of *BdTra^C* and *BdDsx^F* in females on day 9 after knockdown of *BdTra^{CF}*. **p-q**, Abdominal observation of *E. sinensis* (p) and *B. dubia* (q) following *Tra^{CF}* knockdown. All differences were analyzed with two-tailed Student's *t* tests. Data are presented as mean ± SD. All qRT-PCR analyses were performed using pooled fore- and hindwing pads (including primordia and pterothecae) with four biological replicates. Functional validation experiments were performed with three biological replicates. Abbreviations: M, Male-specific region of *Dsx*; F, Female-specific region of *Tra*; CF, Mixture of common and female-specific regions of *Tra*.



Extended Data Fig. 3 | See next page for caption.

Extended Data Fig. 3 | Screening for candidate target genes whose expression is mediated by DSX. **a**, The screening process for downstream targets of DSX that regulate FSWR. **b**, Volcano plots of the differentially expressed genes. **c**, PCA of ATAC-seq results among the M9, F9, and F11 wing pads. **d**, ATAC peak distribution near the TSS among the M9, F9, and F11 wing pads. **e**, Functional validation of three potential target genes (6 μ g of dsRNA injected on days 1, 25, and 27). **f**, Relative wing/wing pad area after *EsE93* knockdown in *E. sinensis*. Wing/wing pad area was measured from individual forewings. **g**, 3D PCA of the transcriptome samples shown in Fig. 3g. **h**, TPM quantification of *EsEcR* and *EsE93* expression in

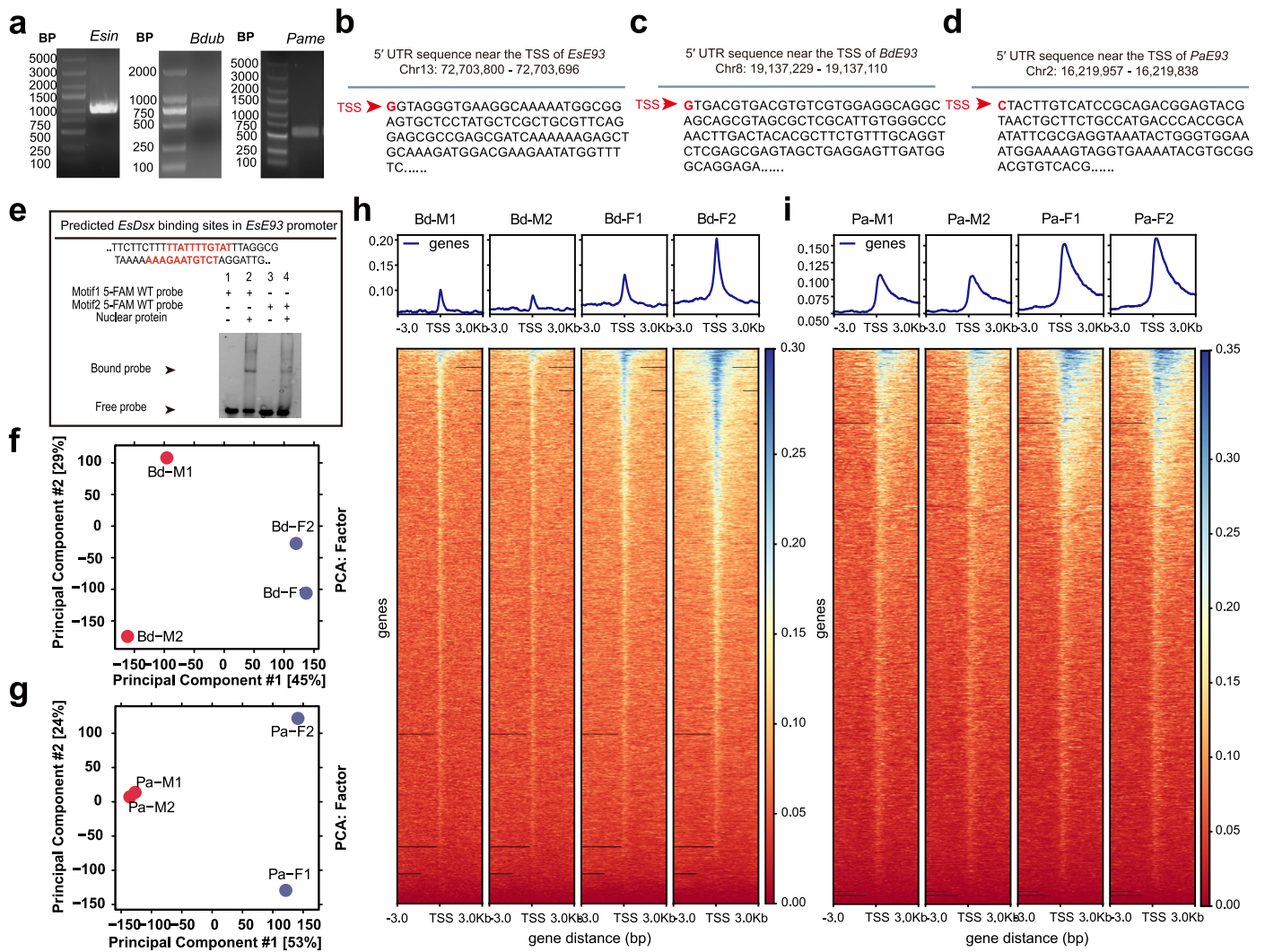
N9 wing pads. **i**, qRT-PCR results demonstrate that *EsE93* was induced by *EsEcR* in male and female wing pads. The gene levels were detected in N9 on day 25 post-*EsEcR* dsRNA treatment (6 μ g of dsRNA on day 23). All differences were analyzed with a two-tailed Student's *t* test. Data are presented as mean $\pm$ SD. All qRT-PCR analyses were performed using pooled fore- and hindwing pads (including primordia and pterothecae) with four biological replicates. Functional validation experiments were performed with three biological replicates. Schematic diagrams in **a** created with biogdp.com (ref. 156).



Extended Data Fig. 4 | *E93* regulation of wing morphogenesis in insects.

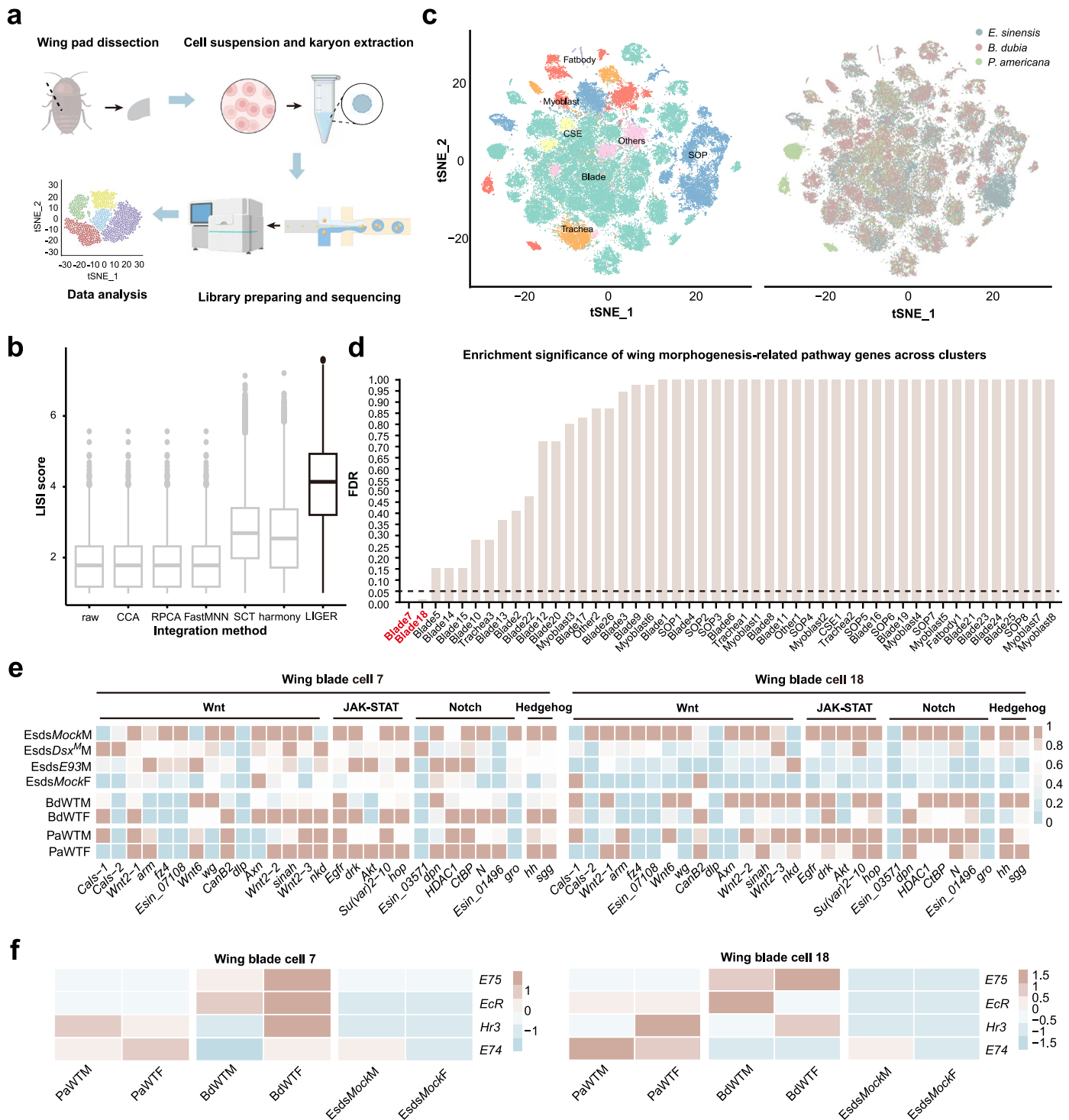
a, qRT-PCR analysis of *E93* expression in the wing pads of wild-type males and females at the final nymphal stage in *B. dubia*, and the changes in relative wing area following *E93* knockdown. **b**, qRT-PCR analysis of *E93* expression in the wing pads of wild-type males and females at the final nymphal stage in *G. sigillatus*. **c**, Expression levels of *GsE93* in *G. sigillatus* last nymph wing pads (both sexes, day 5) following *GsTra* and *GsDsx* knockdown (3 μ g of dsRNA injected on days 1 and 3). **d**, Wing/wing pad phenotypes following *GsDsx* and *GsE93* knockdown treatment in *G. sigillatus* (3 μ g of dsRNA injected on N8). **e-f**, qRT-PCR analysis of *E93*

expression in the wing pads of wild-type males and females at the final nymphal stage in *P. americana* (**e**) and *B. germanica* (**f**), and the changes in relative wing area following *E93* knockdown. **g**, qRT-PCR analysis of *E93* expression in the wing pads of wild-type males and females at the final nymphal stage in *C. magnifica* and *P. lateralis*. All differences were analyzed with a two-tailed Student's *t* tests. Data are presented as mean $\pm$ SD. All qRT-PCR analyses were performed using pooled fore- and hindwing pads (including primordia and pterothecae) with four biological replicates. Functional validation experiments were performed with three biological replicates.



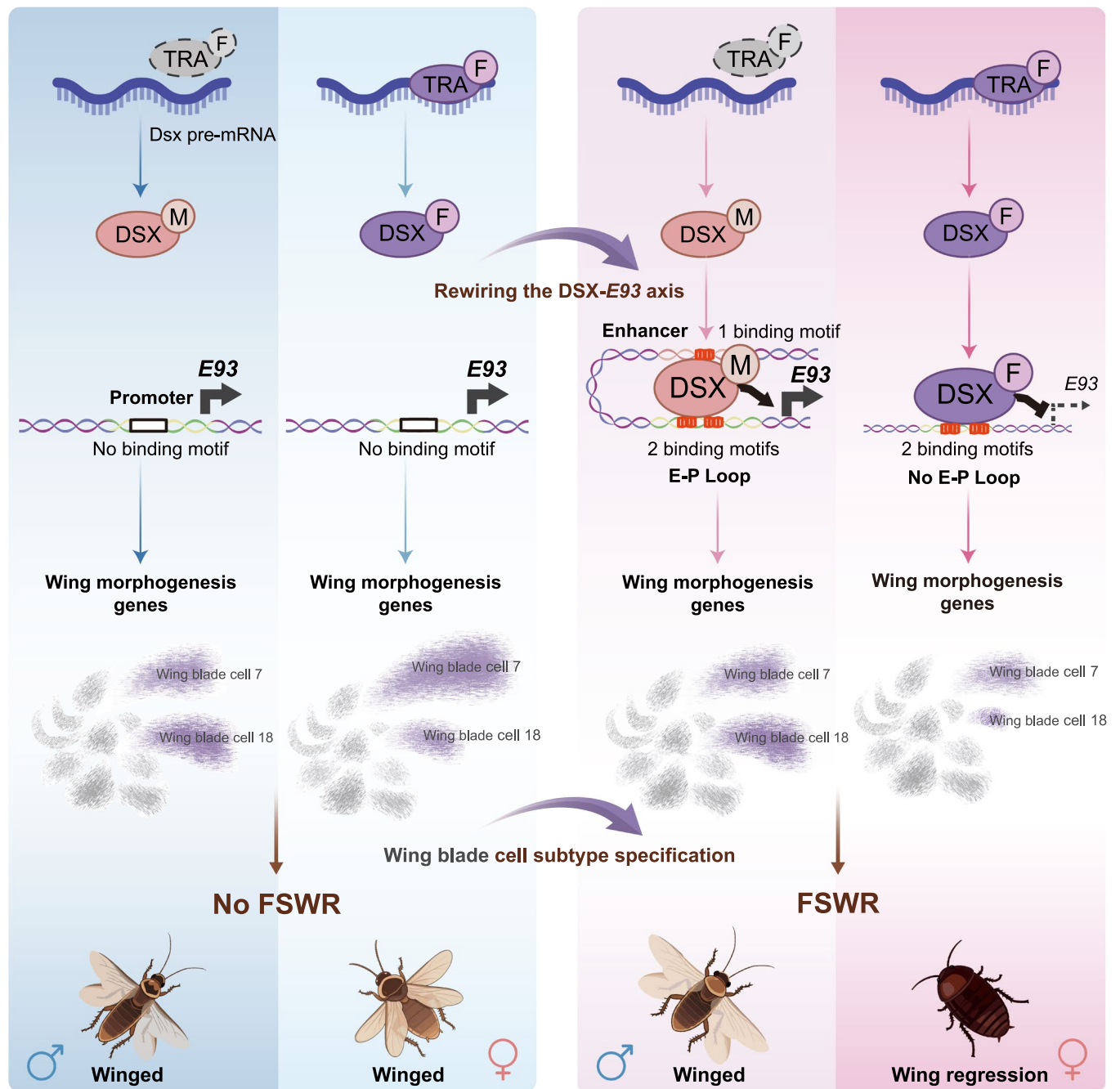
Extended Data Fig. 5 | Confirmation of the *DSX-E93* axis. **a, 5' RACE-PCR agarose gel of *EsE93*, *BdE93*, and *PaE93*. Representative results from three independent experiments are shown. **b-d**, The 5' UTR sequence near the TSS of *EsE93* (**b**), *BdE93* (**c**), and *PaE93* (**d**). **e**, Confirmation of *EsDsx*⁺ binding to the predicted motif within accessible chromatin regions of the *EsE93* promoter by**

EMSA. Representative results from three independent experiments are shown. **f-g**, PCA of ATAC-seq data from *B. dubia* (**f**) and *P. americana* (**g**) wing pads. **h-i**, Distribution of ATAC-seq peaks near the TSS in *B. dubia* (**h**) and *P. americana* (**i**) wing pads.



Extended Data Fig. 6 | DSX-mediated *E93* expression controls wing blade cell specification in wing pads. **a**, Workflow for snRNA-seq library preparation, sequencing, and data analysis. **b**, LISI evaluation of the snRNA-seq sample integration methods. Analyses were performed with eight biological replicates and 82557 cells in total. Box plots show the median (center line), 25th and 75th percentiles (box bounds), and whiskers extending to 1.5× the interquartile range. **c**, t-SNE clustering of integrated multispecies snRNA-seq data. **d**, Significance

of enrichment for DSX-*E93* axis-regulated wing morphogenesis genes across all cell clusters, as analyzed by a hypergeometric test. **e**, Expression profile of DSX-*E93* axis-regulated wing morphogenesis genes in wing blade cell subclusters 7 and 18 across *E. sinensis*, *B. dubia*, and *P. americana*. **f**, Expression patterns of 20E-responsive genes in wing blade cell subcluster 7 and 18 across *E. sinensis*, *B. dubia*, and *P. americana*. Schematic diagrams in **a** created with biogdp.com (ref. 156) and in BioRender: Ming, L. <https://biorender.com/06ay4y2> (2026).



Extended Data Fig. 7 | A working model for how the DSX-E93 axis controls FSWR in *E. sinensis* but not in *P. americana*. The sex-differentiation factor DSX and insect metamorphosis-controlling adult factor *E93* were rewired to instruct FSWR by remodeling and binding to DSX binding sites within *E93* to

modify sex-specific chromatin accessibility and 3D topology, thereby promoting sexually dimorphic expression of wing morphogenesis genes and corresponding wing blade cell type specification in male but not female wing pads.

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Software and code

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Data collection	PacBio Sequel II Illumina NovaSeq 6000
Data analysis	Data were analysed using the following published software: PacBio Isoseq3 v3.4.0 TransDecoder v2.1.0 HMMER v3.1b2 BLAST v2.12.0 OrthoFinder v2.5.4 Muscle v3.8.1551 trimAl v1.4 IQ-TREE v2.1.4 Jellyfish v2.2.10 GenomeScope v1.0 Canu v2.0 Hifiasm v0.20.0 NextPolish v1.4.1 LACHESIS Juicer v1.6 3D-DNA v190716 BUSCO v5.3.1

RepeatModeler v2.0.1
 RepeatMasker v4.1.2
 STAR v2.7.10
 Minimap2 v2.24
 BWA v0.7.17
 SAMtools v1.12
 BRAKER v2.1.6
 Salmon v1.4.0
 Bowtie2 v2.3.5.1
 Sambamba v0.8.2
 MACS v2.2.7.1
 deepTools v3.5.4
 fastp v0.23.2
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 CellRanger pipelines v7.1.0
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Data Availability

All genome sequencing, bulk RNA-seq, ATAC-seq, 4C-seq, and snRNA-seq raw data, along with the genome assembly sequences, are available via the NCBI BioProject under accession number PRJNA1307050.

Code Availability

This paper does not report original code. We used publicly available software in this study. Please see the METHOD DETAILS section for further details.

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Reporting on sex and gender

The raw data are reported separately by sex and were compared across sexes due to distinct wing morphs between males and females.

Reporting on race, ethnicity, or other socially relevant groupings

N/A

Population characteristics

N/A

Recruitment

N/A

Ethics oversight

N/A

Note that full information on the approval of the study protocol must also be provided in the manuscript.

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Study description

In this study, we show that female-specific wing regression (FSWR) is widespread among insects, including cockroaches, where it

Study description	contributes to phenotypic adaptation to subterranean environments. We reveal that FSWR regulation involves sex-specific DSX isoforms, which oppositely regulate the expression of the insect metamorphosis-controlling “adult factor” E93 during wing development. Mechanistically, DSXF represses, while DSXM activates, E93 expression through binding to DSX-binding motifs in FSWR lineages. This differential binding remodels chromatin accessibility and establishes a male-specific three-dimensional topology, facilitating the formation of an enhancer–promoter loop. Consequently, wing morphogenesis gene expression and pouch cell type specification are promoted in males but suppressed in females. Taken together, our findings provide mechanistic insights into how the rewiring of two conserved signaling hubs into one regulatory axis can create previously unavailable regulatory degrees of freedom, thereby generating novel phenotypic variation able to fuel the exploitation of new ecological niches.
Research sample	The genomic, transcriptomic, and epigenomic samples employed in this study were obtained from cockroaches (<i>E. sinensis</i> , <i>B. dubia</i> , <i>P. americana</i> , and <i>B. germanica</i>) and crickets (<i>G. sigillatus</i>) that underwent prolonged and stable laboratory cultivation. These insects were reared in plastic cages under controlled conditions of 29 ± 1 °C and a relative humidity of 45–70%.
Sampling strategy	No sample size calculation was performed; the sample size was determined based on previous similar studies in cockroaches. Wing pads from the involved cockroaches and crickets were sampled for DNA and RNA extraction. Three biological replicates were prepared for each time point/treatment, with each replicate comprising multiple experimental individuals.
Data collection	All data were generated using the following experimental and analytical procedures: Library preparation for omics sequencing: Iso-seq libraries were constructed using the SMRTbell Template Prep Kit. RNA-seq libraries were prepared with the Takara SMART-Seq v4 Ultra Low Input RNA Kit. The Nextera DNA Library Preparation Kit was used for Genome Sequencing and ATAC-seq library construction. snRNA-seq libraries were generated using the 10x Genomics Chromium Next GEM Single-Cell 3' Kit. Morphometric analysis: The relative area of wing pads was quantified through imaging using a stereomicroscope. This analysis was performed across developmental stages from N6 to adult in <i>E. sinensis</i> and from N4 to adult in <i>B. dubia</i>, with both sexes examined. Mating behavior assay: Mating experiments were conducted with adult <i>E. sinensis</i> in standardized chambers (40 × 30 × 15 cm). The experimental cohort consisted of 36 males subjected to tegmina (forewing) ablation and 96 wild-type females. The control cohort comprised 36 wild-type males and 96 wild-type females, maintaining a consistent male-to-female ratio of 1:2.67 across groups. Mating interactions were continuously recorded by video over a 14-day period for subsequent quantification of cumulative mating frequency.
Timing and spatial scale	To investigate the timing of gene expression divergence during wing pad development, transcriptome samples were collected from both sexes at N6, N7 (day 25), N8, and N9 (day 30) and from females at N11 (day 30) in <i>E. sinensis</i> . To identify potential downstream targets of EsDSXM, samples from M9 dsMock (day 30) and M9 dsEsDsXM (day 30) wing pads were also obtained. For developmental transcriptome profiling, wing pads were sampled every 5 days from M9, F9, and F11 instar nymphs. Additional samples from M9 dsMock (day 30), M9 dsEsE93 (day 30), and F9 dsMock (day 30) wing pads were included for hierarchical clustering and PCA. To assess the sex-specific expression of E93, wing pads were collected from both sexes at the final instar stage (day 25 for <i>P. americana</i> and <i>B. dubia</i> and day 8 for <i>B. germanica</i>) and correlated with the degree of FSWR using linear regression.
Data exclusions	No data were excluded from the analyses.
Reproducibility	All experiments included corresponding replicates, and all replicates yielded the expected phenotypes. Therefore, we did not attempt to repeat experiments beyond the technical and biological replicates employed in each experiment.
Randomization	Samples were divided by sex and then randomly assigned to experimental and control groups within each sex.
Blinding	Blinding cannot be implemented for the samples because the corresponding treatments result in observable phenotypes.
Did the study involve field work?	☐ Yes ☒ No

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involvement in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involvement in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Animals and other research organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals	Cockroaches and crickets used in this study underwent prolonged and stable cultivation in the laboratory. All cockroaches were kept at 29 ± 1 °C and a relative humidity of 45–70% under natural light conditions in plastic cages. They were provided with a diet consisting of corn flour, wheat bran, and fish meal (7:2:1), and water was supplied ad libitum.
Wild animals	The study did not involve wild animals
Reporting on sex	The raw data are reported by sex, with comparisons made across sexes based on their differential wing morphology.
Field-collected samples	No field-collected samples were used in this study.
Ethics oversight	This study does not involve ethical oversight because all the subjects are insects.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks	Report on the source of all seed stocks or other plant material used. If applicable, state the seed stock centre and catalogue number. If plant specimens were collected from the field, describe the collection location, date and sampling procedures.
Novel plant genotypes	Describe the methods by which all novel plant genotypes were produced. This includes those generated by transgenic approaches, gene editing, chemical/radiation-based mutagenesis and hybridization. For transgenic lines, describe the transformation method, the number of independent lines analyzed and the generation upon which experiments were performed. For gene-edited lines, describe the editor used, the endogenous sequence targeted for editing, the targeting guide RNA sequence (if applicable) and how the editor was applied.
Authentication	Describe any authentication procedures for each seed stock used or novel genotype generated. Describe any experiments used to assess the effect of a mutation and, where applicable, how potential secondary effects (e.g. second site T-DNA insertions, mosaicism, off-target gene editing) were examined.