nature portfolio

Peer Review File

A single-cell atlas of alternative wing development in two hemipteran species

Corresponding Author: Professor Hai-Jun Xu

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript reports the study of wing polyphenism in Hemiptera using single-cell RNA-seq, RNAi, ISH and other methods. Plasticity and polyphenism are important topics in developmental biology, and insects, particularly Hemiptera, have provided excellent models to examine the phenomenon. This study focuses is on the firebug, with additional comparative data from the brown planthopper. The manuscript contains an impressive collection of data, and the experiments appear to have been well executed and well-documented. And despite its scope, the text is well organized and flows logically.

I have several major concerns, however. The first regards framing these diverse dataset as one story. The second is methodological, and I'm not sure how this could be easily overcome. The third is a matter of interpretation, which might be addressed in revision.

Major concern 1: There are two stories here.

The scRNA-seq results are descriptive of wing development, and mostly confirm patterns identified from *Drosophila*, though it seems at higher resolution. In both species the scRNA-seq approach actually seems to reveal very little about polyphenism itself, finding only small (though significant) differences in minor cell types. These may be useful reference datasets but by themselves they are not especially interesting. In my opinion, the results from flow cytometry are more important, documenting the timing of cell division and the continuation of cell proliferation in long wing bugs. The RNAi experiments (there are a lot of them!) are far more relevant to the issue of wing morph development, though here an ortholog-to-ortholog comparison of functions in the two species would be the most relevant to the question of conservation. Bottom line: these two lines of experimentation don't synergize or support each other well in this manuscript.

Major concern 2: Cell-type definitions

It's not completely clear (besides citing the software) how cell types were identified in this study. My concern is that if cell types were identified using models from *Drosophila* wing disc scRNAseq studies, then it is not at all surprising that similar cell types are recovered from these species. Anything else would be quite surprising. Similarly then, reports of gene expression in these cell types do not seem meaningful. It's a problem of circular reasoning. In fairness, I do not have first-hand experience with scRNAseq analysis (although I have run many RNAseq and other comparative genome-scale analyses). I could imagine perhaps identifying cell types based on a limited number of genes in a training dataset, then excluding those genes (or at least clearly identifying them) in downstream analyses.

Major concern 3: Over-interpretation

The title and Discussion emphasize "a conserved cellular basis underlying wing polyphenism across hemipteran insects." While this study presents an extensive data set, I think statements of this kind of unwarranted in at least two ways. First, only two species are examined, and so caution should be used of over- extending interpretations. More importantly, the analyses that are presented from *N. lugens* do not parse wing morphs in this species. The results in Fig 7 describe a similar set of cell types in wing buds of the planthopper as compared to the firebug. But this is to be expected since both species have wings. Similarly, I disagree with the claim that involvement of wing patterning transcription factors or cell cycle regulators in

LW development is "co-option". These are genes that prompt growth of the wing (and for some genes, also other tissues) and that's precisely what they do in long wings, they seem to simply persist at this longer than during SW development. *nubbin* RNAi is a good example here: it reduces wings in both SW and LW bugs. Since long wings are the ancestral (monomorphic) state, then any difference in SW gene activity is the novelty. On a related note, it is frustrating to see "conserved regulatory programs" invoked, when there is very little idea of how these genes interact with each other to form a wing, let alone how that system is modulated in long or short wing development.

Minor concerns (with reference to line numbers)

- 36-38: "Phenotypic plasticity facilitates evolutionary changes and speciation, and thus is an encompassing model to understand life on earth." Not sure what "encompassing" means in this context. But more importantly, the significance of plasticity in evolution is more safely described as a debated topic. I think this is far from a settled issue.
- 42-47: This section of the Intro would benefit from citations to classical literature about plasticity in evolution. Regarding the resolution of ecological paradoxes, I suggest Levins (1968) “Evolution in Changing Environments”.
- 57: Among the citations 13-16 is one (Fawcett et al.) that does not deal with termites of beetles. That study also examined the role of insulin pathway components in a polyphenic hemipteran using RNAi and might be worth contrasting with the firebug and planthopper.
- 68-70: Grammar issue.
- 88-90: There's a grammar or logic issue here. Wing scales were not determined by scRNAseq.
- 157: "representativeness" isn't a standard word.
- 165-166: "Together, these observations validate the reliability of our single cell annotations." The comparison of bulk-seq and single cell data can validate the magnitudes of gene expression, but this doesn't bear on annotations.
- 186: should be "primarily"
- 199: Was RNAi gene knockdown validated? By RT-PCR or qPCR? This seems especially important for claims of negative effects. (That is, for experiments with "no effects".)
- 219 / Fig 4: I'm concerned about the extent of background staining in veins.
- 238 / Fig4: I'm sure it's beyond the scope of this manuscript, but I see an interesting bs RNAi phenotype at the margin of the pronotum. Contrast this to a very dramatic bs/srf RNAi phenotype in milkweed bugs (Fisher et al. 2021 ProcB).
- 256: Should this reference Supplementary Data 3? -- not 1?
- 290 / Fig6: Figure 6's panel e seems redundant with (and less informative than) panel f.
- 312-313: The phrase "phylogenetically distinct" doesn't seem to have any meaning.
- 340-342: "Taken together, the evolutionary stability of wing cell identities between the firebug and planthopper suggests that the two species evolved wing polyphenism by regulating the same cell types." -- See my major concern 3 above. But basically, I don't see evidence in this study for this claim. Both species have wings, so the commonality of cell types in those wings is not surprising.
- 359: Since long wing development is not an evolutionary novelty, it does not seem appropriate to describe this as co-option.
- 360-361: "Thus, our results highlight the evolutionary conservation of cellular basis underlying wing polyphenism across hemipteran insects." Only in so far as both morphs and both species have wings, constructed of conserved wing cell types.
- 379: Delete "cross-study"
- 384-385: "Instead, we demonstrated that the development of long wings mainly relies on the proliferation of three dominant wing cell types (ECs, TCs, and NCs)." This conclusion is unsupported, because the assays of cell proliferation did not distinguish among cell types.
- 418: It's unclear what the authors mean here by "conserved regulatory programs" but they did not examine interactions among genes, and so this claim seems spurious.
- 421-424: Maybe. I don't object to the language here, but I find it unsatisfying. What the field needs to know is whether plasticity of this kind was ancestral or independently evolved, and whether / how different lineages have diversified. Unfortunately those conclusions cannot be reached by comparison of any two species alone.
- 433-435: Has the *PaInR2* CRISPR knockout been published before? If so please provide the reference. If not, please provide more information on validation of the deletion.
- 441: References 19 and 59 are redundant. They are the same paper.
- 478-479: Please provide citations and accession numbers for the planthopper and firebug reference genomes.
- 526-530 / Supplemental Data 6: Please mention or list the dsRNA concentrations that were used in RNAi.
- 577-579: Formatting issue.
- 770: Which reference genome are you referring to here?
- Fig 1a: This panel is not especially useful. Different polyphenisms likely have very different developmental bases. What might be better is a phylogeny of Insects or Hemiptera showing representative taxa that do not have wing polyphenism in order to contextualize it.

Reviewer #2

(Remarks to the Author)
The manuscript by Wan et al. is an important new contribution to the understanding of the developmental and genetic basis for wing development in hemipteran insects that exhibit wing polyphenisms in response to environmental cues. The authors identify differences in wing development between short and long wing morphs in both cell types and gene expression. This work is done using several cutting edge developmental, functional genetic, and cell experiments to comprehensively compare and identify differences in wing development at the genetic and cellular level between the two morphs. The primary result is to describe differences in wing development for the short wing versus the long wing morphs.

The manuscript will be a significant and important contribution to the literature on wing polyphenic development and evolution specifically in understanding the mechanistic basis for the optimization of the trade-off between reproduction (short wing morph) and migration (long wing morph). In addition, it will serve as foundational descriptive data for additional experiments understanding how wing development has evolved to respond to diverse environments in the evolution of life history strategies that maximize fitness.

It is important to point out that the authors use the idea that co-option of wing-patterning and cell cycle genes across hemipteran species is the main conclusion for the results they find. However, this is not the only conclusion and is probably not the most parsimonious explanation from a developmental perspective. The authors should point out that short wing development appears to be a truncation of cellular proliferation of normal long wing development. Short wing development uses the same genes and pathways for wing patterning and cellular proliferation, and no co-option of wing patterning or cellular proliferation genes is necessary to make this happen. In fact, since short wing morphs evolved from long wing morphs and not vice versa, the genes that would be really fascinating to find would be the genes involved in stopping wing development and what environmental signal transduction factors then relay that message to truncate wing development in short wing morphs.

The manuscript is mostly very clear and the logic follows with the exception of the conclusion about the co-option of genes as described above. The figures would be more clear and have more impact if there was more clarity on conclusions in the figure titles and not just a simple description of what the figure is. For example, Figure 2 would be more informative if the title was" Single cell atlas of short wing and long wing buds from the firebug show differences in GCs and HEs." Figures 2, 4, 5, 6 & 7 would all be improved with more informative caption titles.

Reviewer #3

(Remarks to the Author)
In this study, the authors aimed to understand key cell types and regulatory genes essential for long wing development in two hemipteran species, P. apterus and N. lugens, which exhibit clear wing polyphenism. The research topic will attract a broad audience, and the reported single-cell atlas of developing wings in different morph, in situ gene expression, and RNAi phenotypes are valuable. The use of insulin receptor knockout strains is a good idea for robust comparisons between two morphs. However, I cannot recommend publication of this manuscript in the current form because the authors' main conclusion that "the development of alternative wing morphs is primarily governed by ECs, TCs, and NCs through the co-option of wing-patterning and cell cycle genes across hemipteran insects" (Line 26) is not well supported from the reported results. I suggest further confirmation of the current conclusions, or restructuring the entire manuscript. Below, I listed major reasons behind my decision and suggest possible improvements for the future submission.

Major points:
1. Direct evidence supporting contributions of specific cell types on wing development
One of the main conclusions of this study is contributions of specific cell types, EC, TC, and NC, on wing development. It is based on results of RNAi-mediated knockdowns targeting marker genes that predominantly express in a cell type than the others. But the predominant expression does not mean the other cell types including unidentified minor cell types have no expression of the genes of interest. Indeed, the authors report predominant intervein expression of the tracheal cell maker gene bs by means of tissue staining (Line 266; Fig. 5c), which conflicts with the authors' interpretation that RNAi effects of bs in wing formations are due to defects in TCs (e.g. Lines 251, 350). The same caveat is applied to contributions of neural cells on wing development based on Anln, CncB3, and cdk1 knockdowns. I think we cannot exclude contributions from other cell types without more direct evidence.

2. Specific roles of identified genes in the regulation of wing polyphenism
To conclude that wing morphs are primarily governed by specific cell types and genes (Line 357), it is critical to distinguish genes that paly roles in the plastic and normal wing development. Through the RNAi experiment, the authors successfully found a hopeful candidate gene engrailed that might specifically regulate wing development in the LW but not in the SW morph (Fig. 4e, f). However, the specific effect that supports the main conclusion of this study should be carefully confirmed with statistical analysis of quantitative data (e.g. quantitative effects on each wing regions) in addition to a single image in Fig. 4f. Summary of injections must be provided to confirm data reproducibility. Other data that support the morph-biased function are also desired. These could be differential expression pattern between morphs, and a regulatory gene function in cell proliferation as the author hypothesizes.

3. I could not find the detail of the PaInR2De4 strain (gene structure, gRNA design, deleted region etc.) which was generated in this study. The detail must be included when resubmitting a manuscript.

Minor point:
1. It would be more convincing if the authors' hypothesis that prolonged cell proliferation involves in the LW-morph development was tested more directly with pharmacological approach.

2. Previous studies below have reported effects of nubbin and blistered RNAi on the wing formation in another hemipteran species. These works should be appropriately acknowledged.
Medved, V., Marden, J.H., Fescemyer, H.W., Der, J.P., Liu, J., Mahfooz, N., Popadić, A., 2015. Origin and diversification of wings: Insights from a neopteran insect. Proc. Natl. Acad. Sci. U.S.A. 112, 15946–15951.
https://doi.org/10.1073/pnas.1509517112

Fisher, C.R., Kratovil, J.D., Angelini, D.R., Jockusch, E.L., 2021. Out from under the wing: reconceptualizing the insect wing gene regulatory network as a versatile, general module for body-wall lobes in arthropods. Proc. R. Soc. B. 288, 20211808. https://doi.org/10.1098/rspb.2021.1808

Reviewer #4

(Remarks to the Author)
In this manuscript, Wan and colleagues utilized single-cell RNA sequencing (scRNA-seq) to investigate the cellular determinants underlying wing polyphenism in the firebug Pyrrhocoris apterus in Hemiptera. All major cell types were identified through scRNA-seq. Based on cell-type specific markers, the authors performed whole-body RNAi knockdown experiments and found that knockdown of En in long-wing mutant caused a completely short-wing phenotype in adults, whereas the same genetic manipulation in short-wing nymphs gave no obvious wing phenotype. Additionally, whole-body knockdown of four tracheal cell (TC) and neural cell (NC) markers (bs, Anln, CycB3, and cdk1) also resulted in a significant reduction in wing size for adults of both long-wing- and short-wing-destined nymphs. Then they used flow cytometry analysis and revealed differences in cell cycle phase distribution between wing buds from long-wing and short-wing nymphs.

Overall, the genetic KD experiments and wing phenotypes are interesting. Also, this study provides useful single-cell transcriptomic resources for investigating the plasticity of wing buds in firebugs and planthoppers. However, there are major concerns for the scRNA-seq data analysis in addition to other several concerns, including experimental design. This manuscript needs substantial revision and improvement.

Major concerns:
1. The different wing sizes of both firebugs and planthoppers are primarily induced by environmental cues, as introduced in the Abstract and discussed in the introduction. In this study, the authors compared wild-type (short wing) with PaInR2 mutant (long wing). Such experiment design is not aligned with what was introduced in the Abstract “Insect wing polyphenism enables a single genome to produce two or more distinct wing morphs in response to environmental cues….”, because the genetics of these two samples are different. Although insulin receptors are known to play key roles in wing size dimorphism, this genetic manipulation significantly reduces the possibility of identifying other molecular mechanisms underlying wing size plasticity. In addition, the PaInR2 mutant affects the whole body rather than being wing-specific, which complicates the interpretation of certain phenotypes and mechanisms. So, the experimental design does not address the scientific question from the Title and Abstract. To address this, the authors need to include environmental cue-induced long and short wing samples, or need to substantially reorganize the manuscript to align with current data.

2. There are several concerns for the scRNA-seq data analysis.

First, could the authors shown and confirm the PaInR2 gene expression in their WT and PaInR2-del dataset?

Second, the primary focus of this study is to identify the key cell types and critical genes involved in wing size differences. However, there is no comparison between WT and PaInR2-del samples at the gene expression level. It is highly recommended to compare the transcriptomes of the same cell types from short-wing- and long-wing-destined fifth-instar nymphs to identify differentially expressed genes (DEGs) associated with wing dimorphism in each cell type. These DEGs can be applied to GO and KEGG analysis to reveal the key functional and pathway activity differences underlying wing plasticity. Additionally, transcriptomic regulatory network (TRN) analysis can be a powerful tool to identify the key transcription factors responsible for transcriptome changes in each cell type.

Third, it is related to the cell composition in figure 2c and 2d. There are more than one clusters for both glial cells and hemocytes. Could the authors further annotate these subtypes? Also, could the authors go higher resolution and see if the difference shows up in a specific cluster or subtype of these cell types?

Fourth, it is also related to the cell composition analysis in figure 3. In figure 3a, there is a clear WT-specific cluster that is not shown using current resolution. The authors should use a higher resolution to annotate them. otherwise, the important information is missing. In addition, what is the WT-specific cluster? why is there a WT-specific cluster? is this real or technical issue? Because of this analysis flaw, the conclusion below is not convincing – “SW- and LW-destined wing buds exhibited similar proportions of EC0–EC3 subsets (Fig. 3c), suggesting that the proportion of EC subsets does not serve as a primary determinant in the development of alternative wing morphs.”

Fifth, the authors should also color cells with sample information (WT-1,2,3 and PaInR2de1,2,3). Otherwise, I cannot evaluate the quality of data, such as sample bias, or batch effect. This should be done not just for epithelial cells in figure 3a, but also for all cell types. For example, is the WT-specific cluster coming from one of the three WT samples, or from all three WT samples?

3. For knockdown experiments, since only one RNAi was used for each gene, the RNAi efficiency should be validated using qRT-PCR.

4. For all figures related to wing phenotypes, there is no quantification for the wing length, and it should be added.

5. In line 292-293, “To confirm the cell proliferation activity of wings in late fifth-instar PaInR2De4 nymphs, we quantified cell numbers by quantitative real-time PCR (qRT-PCR)” How cell numbers be quantified by qRT-PCR?

6. There is no quantification for Fig 6h.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors did a nice job of revising the manuscript to more accurately reflect their results and comprehensively responded to the all of the significant reviewer comments. The scientific contribution of this paper is to provide more detailed information the cell types and genes involved in long wing formation versus short wing formation in two hemipteran species. This work is sound and important. However, I'm not sure of the broad appeal of the contribution and am not sure if the significance is impactful enough to warrant publication in Nature Communications.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
The authors have carefully addressed all the concerns I raised in the initial review with additional data, analysis, and text, which enhance the authors' claims and help readers grasp more details. I thank the authors for this nice work!

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
This revision has resolved all my concerns and I support its publication.

(Remarks on code availability)

Point-by-point response to reviewers

Comments from Reviewer #1

Reviewer #1 (Remarks to the Author):

Key results

This manuscript reports the study of wing polyphenism in Hemiptera using single-cell RNA-seq, RNAi, ISH and other methods. Plasticity and polyphenism are important topics in developmental biology, and insects, particularly Hemiptera, have provided excellent models to examine the phenomenon. This study focuses is on the firebug, with additional comparative data from the brown planthopper. The manuscript contains an impressive collection of data, and the experiments appear to have been well executed and well-documented. And despite its scope, the text is well organized and flows logically.

I have several major concerns, however. The first regards framing these diverse dataset as one story. The second is methodological, and I'm not sure how this could be easily overcome. The third is a matter of interpretation, which might be addressed in revision.

Major concern

Major concern 1: *There are two stories here.*

*The scRNA-seq results are descriptive of wing development, and mostly confirm patterns identified from *Drosophila*, though it seems at higher resolution. In both species the scRNA-seq approach actually seems to reveal very little about polyphenism itself, finding only small (though significant) differences in minor cell types. These may be useful reference datasets but by themselves they are not especially interesting. In my opinion, the results from flow cytometry are more important, documenting the timing of cell division and the continuation of cell proliferation in long wing bugs. The RNAi experiments (there are a lot of them!) are far more relevant to the issue of wing morph development, though here an ortholog-to-ortholog comparison of functions in the two species would be the most relevant to the question of conservation. Bottom line: these two lines of experimentation don't synergize or support each other well in this*

manuscript.

Response to major concern 1: We sincerely thank the reviewer for this critical observation. We respond to the reviewer's concerns from following points:

(1) The question of "…*minor cell types*…" between LW and SW morphs. Given the significantly larger wings of LW adults compared to SW morphs, we investigated whether specific or additional cell types contribute to LW development. This question motivated our scRNA-seq project. Cell types in firebug wings—including ECs, TCs, NCs, MCs, HEs, and GCs—were classified using marker genes based on established *Drosophila* wing datasets and further refined through manual annotation. Although previous studies have partially described the cellular landscape of *Drosophila* wings, a comprehensive profile of all six cell types remains unreported. Therefore, one contribution of our study is the systematic identification and characterization of all six wing cell types.

We acknowledge that the primary developmental axes of the wing are evolutionarily conserved with *Drosophila* and no big differences of cell types were identified between LW and SW morphs, the value of our scRNA-seq dataset lies in its ability to pinpoint where the polyphenic divergence begins at a cellular level information that was previously unattainable. By identifying these "minor cell types", we provide a high-resolution atlas of the wing morph switch that is unique to this hemimetabolous species, providing a necessary baseline for studying wing plasticity that *Drosophila* (which lacks such polyphenism) cannot offer.

(2) **The question of "…*experimentation synergize*…" between scRNA-seq and RNAi.** We considered scRNA-seq as the "Hypothesis Generator" for RNAi: We have clarified in the text that the targets selected for RNAi were not chosen at random, but were specifically identified because they showed differential expression or enrichment in the exact cell clusters identified in our scRNA-seq analysis. This methodology follows the established framework for functional genomics in insects, where scRNA-seq identifies cell-type-specific markers that are subsequently validated via RNAi to confirm their role in tissue patterning.

(3) **The question of "…*experimentation synergize*…" between scRNA-seq and flow cytometry.** To identify genes essential for LW development, we performed RNAi-mediated silencing of 51 marker genes. Our phenotypic analysis revealed that two wing-patterning genes—*En* (an EC marker) and *bs* (a TC marker)—and three cell cycle regulators (*Anln*, *CycB3*, and *cdk1*, all markers of NCs) are required for this process. This observation indicates that the development of alternative wing morphs might be governed by ECs, TCs, and NCs through an orchestrated expression of wing-patterning and cell cycle genes. Consistent with the RNAi phenotype derived from cell cycle genes, flow cytometry analysis showed that LW formation mainly relies on an extended duration of cell proliferation. In addition, to determine whether the 30 marker genes for the six major cell types contribute to the prolonged cell proliferation observed in LW-destined wing buds, we examined their expression levels in the scRNA-seq data from $PaInR2^{De4}$ and *Wt* wing buds. We found that cell cycle regulators (*Anln*, *CycB3*, and *cdk1*) were upregulated in $PaInR2^{De4}$ versus *Wt* (Supplementary Fig. 13).

Major concern 2: *Cell-type definitions*

*It's not completely clear (besides citing the software) how cell types were identified in this study. My concern is that if cell types were identified using models from *Drosophila* wing disc scRNAseq studies, then it is not at all surprising that similar cell types are recovered from these species. Anything else would be quite surprising. Similarly then, reports of gene expression in these cell types do not seem meaningful. It's a problem of circular reasoning. In fairness, I do not have first-hand experience with scRNAseq analysis (although I have run many RNAseq and other comparative genome-scale analyses). I could imagine perhaps identifying cell types based on a limited number of genes in a training dataset, then excluding those genes (or at least clearly identifying them) in downstream analyses.*

Response to major concern 2: We would like to clarify that the cell populations were not "pre-sorted" or "forced" into *Drosophila*-like categories. Instead, we performed unsupervised clustering (using the Leiden algorithm via Seurat), which groups cells based on their entire global transcriptomic profile (thousands of genes) without any

prior knowledge of cell identity (Luecken and Theis, 2019). Only top-expressed genes were regarded as candidate marker genes, which were further identified as “real” marker genes according to the reference genes reported previously in *Drosophila* as well as those annotated manually. This is a single-cell analysis technology that is currently both widely adopted and cutting-edge in the field.

Luecken, M.D., Theis, F.J. Current best practices in single-cell RNA-seq analysis: a tutorial. *Mol Syst Biol* **15**, MSB188746 (2019). https://doi.org/10.15252/msb.20188746

Major concern 3: Over-interpretation.

*The title and Discussion emphasize "a conserved cellular basis underlying wing polyphenism across hemipteran insects." While this study presents an extensive data set, I think statements of this kind of unwarranted in at least two ways. First, only two species are examined, and so caution should be used of over-extending interpretations. More importantly, the analyses that are presented from *N. lugens* do not parse wing morphs in this species. The results in Fig 7 describe a similar set of cell types in wing buds of the planthopper as compared to the firebug. But this is to be expected since both species have wings. Similarly, I disagree with the claim that involvement of wing patterning transcription factors or cell cycle regulators in LW development is "co-option". These are genes that prompt growth of the wing (and for some genes, also other tissues) and that's precisely what they do in long wings, they seem to simply persist at this longer than during SW development. *nubbin* RNAi is a good example here: it reduces wings in both SW and LW bugs. Since long wings are the ancestral (monomorphic) state, then any difference in SW gene activity is the novelty. On a related note, it is frustrating to see "conserved regulatory programs" invoked, when there is very little idea of how these genes interact with each other to form a wing, let alone how that system is modulated in long or short wing development.*

Response to major concern 3: We agree that characterizing the entire Hemiptera order based on two species is premature. We have revised the title and main text to more accurately reflect our scope.

In terms of scRNA-seq of *N. lugens* wings, our intent was to use *N. lugens* as a comparative reference to define the basal cellular atlas of the hemipteran wing bud. We have clarified in the revised manuscript that while wing polyphenism is specifically explored in *P. apterus* in this paper, the *N. lugens* data serves to confirm that the cellular clusters are evolutionarily stable components utilized during wing morphogenesis.

We appreciate the reviewer's correction regarding the evolutionary logic of wing development in *P. apterus*. We agree that the term "co-option" was inappropriately applied to describe the involvement of wing-patterning genes in the LW morph. We now revised the text in the revised manuscript. Given that SW insects originated late in evolution from winged forms, we propose that a conserved genetic machinery has evolved to modulate the developmental plasticity of wings through the coordinated deployment of wing-patterning (e.g., *En*) and cell cycle (e.g., *cdk1*) genes.

Minor concern 1: - *36-38: Phenotypic plasticity facilitates evolutionary changes and speciation, and thus is an encompassing model to understand life on earth." Not sure what "encompassing" means in this context. But more importantly, the significance of plasticity in evolution is more safely described as a debated topic. I think this is far from a settled issue.*

Response to minor concern 1: We agree with the reviewer's point. Whether phenotypic plasticity holds adaptive value is a debated topic, and a definitive statement either way would be an oversimplification. In the revised manuscript, we have deleted this sentence.

Minor concern 2: - *42-47: This section of the Intro would benefit from citations to classical literature about plasticity in evolution. Regarding the resolution of ecological paradoxes, I suggest Levins (1968) "Evolution in Changing Environments".*

Response to minor concern 2: We appreciate the reviewer's suggestion to ground our introduction in the classical literature of phenotypic plasticity. We have revised the text to include the work of Levins (1968).

Minor concern 3: - *57: Among the citations 13-16 is one (Fawcett et al.) that does not*

deal with termites of beetles. That study also examined the role of insulin pathway components in a polyphenic hemipteran using RNAi and might be worth contrasting with the firebug and planthopper.

Response to minor concern 3: We appreciate the reviewer catching this error. It was our oversight. The incorrect citation has been removed, and the reference has now been inserted in the correct section of the revised manuscript.

Minor concern 4: - *68-70: Grammar issue.*

Response to minor concersn 4: We thank the reviewer for identifying this grammatical issue. We have revised the sentence to ensure grammatical accuracy (lines 76-78).

Minor concern 5: - *88-90: There's a grammar or logic issue here. Wing scales were not determined by scRNAseq.*

Response to minor concern 5: We thank the reviewer for pointing out this logical inconsistency. We acknowledge that scRNA-seq is a diagnostic tool used to identify and characterize cell types, rather than a developmental process that "determines" them. We have revised this sentence in the revised manuscript (lines 96-100).

Minor concern 6: - *157: "representativeness" isn't a standard word.*

Response to minor concern 6: Thank you. We have revised this sentence in the revised manuscript (line 190).

Minor concern 7: - *165-166: "Together, these observations validate the reliability of our single cell annotations." The comparison of bulk-seq and single cell data can validate the magnitudes of gene expression, but this doesn't bear on annotations.*

Response to minor concern 7: We thank the reviewer for this critical distinction. We agree that the correlation between bulk RNA-seq and aggregated single-cell data primarily validates the accuracy of gene expression quantification. We have revised this sentence in the revised manuscript (lines 198-200).

Minor concern 8: - *186: should be "primarily"*

Response to minor concern 8: Thank you. We have corrected this word (line 235).

Minor concern 9*:* - *199: Was RNAi gene knockdown validated? By RT-PCR or qPCR? This seems especially important for claims of negative effects. (That is, for experiments with "no effects".)*

Response to minor concern 9: In the revised manuscript, we performed quantitative real-time PCR (qRT-PCR) to verify RNAi efficiency for all candidate genes. Our results confirm that the transcript levels of all targeted genes were significantly reduced compared to the *Gfp*-RNAi controls. These validation data have been included in the revised Supplementary Information (Supplementary Figs. 9 and 11).

Minor concern 10: - *219 / Fig 4: I'm concerned about the extent of background staining in veins.*

Response to minor concern 10: We appreciate the reviewer's careful observation regarding the staining intensity in the wing veins. To minimize background during ISH assay, we employed stringent post-hybridization washing steps as mentioned in methods. Although stringent washings could not completely remove the background staining of veins, a contrasting dark purple precipitate was seen in the anti-sense groups with multiple sample replicates. This demonstrates that the signal in the anti-sense samples is due to specific mRNA hybridization rather than non-specific background staining of the vein architecture.

Minor concern 11: -*238 / Fig4: I'm sure it's beyond the scope of this manuscript, but I see an interesting bs RNAi phenotype at the margin of the pronotum. Contrast this to a very dramatic bs/srf RNAi phenotype in milkweed bugs (Fisher et al. 2021 ProcB).*

Response to minor concern 11: We thank the reviewer for highlighting the relevant study by Fisher et al. (2021) on the milkweed *Oncopeltus fasciatus*. Fisher and his colleagues reported that RNAi-mediated knockdown of *srf* (the *O. fasciatus bs* homolog) leads to a blistered phenotype in all bilayered body-wall evaginations, including the pronotum and scutellum. In our experiments with the firebug *P. apterus*, we observed

a similar phenomenon where knockdown of *bs* resulted in a "blistering" phenotype at the scutellum and pronotum. In the initial version, this phenotype was not further discussed as our analysis was primarily focused on the wings. In the revised manuscript, the relevant data have now been included as a Supplementary Figure 12 (lines 324-327).

Minor concern 12: - *256: Should this reference Supplementary Data 3? -- not 1?*

Response to minor concern 12: The reviewer is correct. The citation should indeed refer to Supplementary Data 4 rather than Supplementary Data 1. We have corrected this mistake.

Minor concern 13: - *290 / Fig6: Figure 6's panel seems redundant with (and less informative than) panel f.*

Response to minor concern 13: Given the duplicated information in panels **e** and **f**, we have removed the panel **e** from the revised Figure 6.

Minor concern 14: - *312-313: The phrase "phylogenetically distinct" doesn't seem to have any meaning.*

Response to minor concern 14: We have deleted this term in the revised manuscript.

Minor concern 15: - *340-342: "Taken together, the evolutionary stability of wing cell identities between the firebug and planthopper suggests that the two species evolved wing polyphenism by regulating the same cell types." -- See my major concern 3 above. But basically, I don't see evidence in this study for this claim. Both species have wings, so the commonality of cell types in those wings is not surprising.*

Response to minor concern 15: We agree that the presence of homologous cell types in the wings of two hemipteran species is an expected consequence of their shared ancestry. However, our study aims to go beyond the mere identification of homologous cells. While insect wings can vary significantly in texture and specialized cell types, such as the presence of scale cells in Lepidoptera but not in *Drosophila* and hemipterans. As mentioned above, during scRNA analysis, the cell populations were not "pre-sorted" or "forced" into *Drosophila*-like categories. Instead, we performed unsupervised

clustering (using the Leiden algorithm via Seurat), which groups cells based on their entire global transcriptomic profile (thousands of genes) without any prior knowledge of cell identity. Our comparative scRNA-seq analysis reveals a remarkably stable cellular landscape across the firebug and the planthopper. More importantly, we show that the regulatory signatures - specifically the expression of key patterning and cell cycle genes like *En* and *cdk1* - are conserved within identical cell lineages in both species.

Minor concern 16: - *359: Since long wing development is not an evolutionary novelty, it does not seem appropriate to describe this as co-option.*

Response to minor concern 16: As your suggestion, we have revised the sentence as follows: “These findings indicate that the developmental plasticity of wing buds, which determines the formation of LW or SW morphs, is primarily governed by differential proliferation of conserved ECs, TCs, and NCs. This process may involve the modulating of wing-patterning and cell cycle genes rather than the evolution of novel cell types or programs.”(lines 430-434).

Minor concern 17: - *360-361: "Thus, our results highlight the evolutionary conservation of cellular basis underlying wing polyphenism across hemipteran insects." Only in so far as both morphs and both species have wings, constructed of conserved wing cell types.*

Response to minor concern 17: To avoid over-interpretation, we have deleted this sentence (lines 430-434).

Minor concern 18: - *379: Delete "cross-study"*

Response to minor concern 18: We have deleted this word from the sentence.

Minor concern 19: - *384-385: "Instead, we demonstrated that the development of long wings mainly relies on the proliferation of three dominant wing cell types (ECs, TCs, and NCs)." This conclusion is unsupported, because the assays of cell proliferation did not distinguish among cell types.*

Response to minor concern 19: We thank the reviewer for this comment. From a statistical and biological perspective, our scRNA-seq data reveal that ECs, TCs, and NCs are the three most abundant cell populations, collectively accounting for more than 90% of the total cells in the wing bud (Fig. 2d). Given their overwhelming dominance in cell composition, the significant increase in proliferation signals (EdU-positive cells and S/G2/M phase populations) measured at the whole-tissue level is statistically driven by these three lineages. Furthermore, our scRNA-seq analysis shows that key cell-cycle regulatory genes (e.g., *cdk1* and *CycB3*) are not only specifically enriched in these clusters but also exhibit significantly higher expression levels in LW-destined nymphs compared to SW-destined ones, as shown in the revised Supplementary Figure 13, which displays the inter-group differential expression analysis of marker genes. The subsequent RNAi experiments targeting these markers resulted in dramatic wing reduction, providing functional evidence that the proliferation of these specific cell types is essential for long-wing development.

For clarity, the sentence has been modified to: “Instead, we demonstrated that ECs, TCs, and NCs collectively accounting for more than 90% of the total cells in both LW- and SW-destined wing buds, which implies that the development of long wings mainly relies on the proliferation of three dominant wing cell types.”(lines 449-452).

Minor concern 20: - *418: It's unclear what the authors mean here by "conserved regulatory programs" but they did not examine interactions among genes, and so this claim seems spurious.*

Response to minor concern 20: We agree with you that the "*conserved regulatory programs*" is ambiguous. We have revised the manuscript to describe these as: “The most noteworthy finding emerges from our cross-species comparative analysis between the firebug and planthopper, which enabled the identification of homologous cell types and conserved gene expression across these species.”(lines 495-497).

Minor concern 21: - *421-424: Maybe. I don't object to the language here, but I find it unsatisfying. What the field needs to know is whether plasticity of this kind was*

ancestral or independently evolved, and whether / how different lineages have diversified. Unfortunately those conclusions cannot be reached by comparison of any two species alone.

Response to minor concern 21: We are grateful for the reviewer's thoughtful comment on the evolutionary significance of our findings. Determining whether wing polyphenism is ancestral or independently evolved remains challenging. Based on the phylogenetic tree (Fig. 1a) and the diverse ecological habitats these species occupy, wing polyphenism in Hemiptera may have evolved independently (Zhang et al., 2026, Science Advances 12, eaea4413).

The primary goal of our study was to establish a cellular-level baseline for wing polyphenism, which has been missing in the field. The finding that the planthopper and the firebug share a conserved cellular framework and utilize identical regulatory genes (e.g., *En* and *cdk1*) drove us to propose that a conserved genetic machinery has evolved in wing-polyphenic hemipteran insects to modulate the developmental plasticity of wings. In response to this feedback, we have revised the Discussion section to explicitly acknowledge this point (lines 499-513).

Minor concern 22: - *433-435: Has the *PaInR2* CRISPR knockout been published before? If so please provide the reference. If not, please provide more information on validation of the deletion.*

Response to minor concern 22: The information about the *PaInR2* CRISPR knockout has been published during the review process of this manuscript. We have added this reference (Zhang et al., 2026, Science Advances 12: eaea4413) in the revised manuscript.

Minor concern 23: - *441: References 19 and 59 are redundant. They are the same paper.*

Response to minor concern 23: We apologize for this mistake. We have removed the redundant reference and ensured that all citations throughout the manuscript consistently refer to a single entry in the reference list.

Minor concern 24: - *478-479: Please provide citations and accession numbers for the planthopper and firebug reference genomes.*

Response to minor concern 24: In the data availability section, we clarified that the reference genome was deposited in the Figshare repository. For clarity, we have provided GenBank accession numbers for both species.

Minor concern 25: - *526-530 / Supplemental Data 6: Please mention or list the dsRNA concentrations that were used in RNAi.*

Response to minor concern 25: We have updated Supplemental Data 4 by listing the concentrations of all dsRNAs used in the RNAi experiments.

Minor concern 26: - *577-579: Formatting issue.*

Response to minor concern 26: We apologize for the formatting error in the original manuscript. We have corrected this mistake.

Minor concern 27: - *770: Which reference genome are you referring to here?*

Response to minor concern 27: We have revised the sentence to clarify the reference genomes of the firebug and planthopper (lines780-782).

Minor concern 28: - *Fig 1a: This panel is not especially useful. Different polyphenisms likely have very different developmental bases. What might be better is a phylogeny of Insects or Hemiptera showing representative taxa that do not have wing polyphenism in order to contextualize it.*

Response to minor concern 28: In the revised Figure 1a, we have updated the phylogeny of Hemiptera which includes wing polyphonic insects and those not wing polyphonic.

Comments from Reviewer #2

***Reviewer #2 (Remarks to the Author)*:**

The manuscript by Wan et al. is an important new contribution to the understanding of the developmental and genetic basis for wing development in hemipteran insects that exhibit wing polyphenisms in response to environmental cues. The authors identify differences in wing development between short and long wing morphs in both cell types and gene expression. This work is done using several cutting edge developmental, functional genetic, and cell experiments to comprehensively compare and identify differences in wing development at the genetic and cellular level between the two morphs. The primary result is to describe differences in wing development for the short wing versus the long wing morphs.

The manuscript will be a significant and important contribution to the literature on wing polyphenic development and evolution specifically in understanding the mechanistic basis for the optimization of the trade-off between reproduction (short wing morph) and migration (long wing morph). In addition, it will serve as foundational descriptive data for additional experiments understanding how wing development has evolved to respond to diverse environments in the evolution of life history strategies that maximize fitness.

Comment 1: *It is important to point out that the authors use the idea that co-option of wing-patterning and cell cycle genes across hemipteran species is the main conclusion for the results they find. However, this is not the only conclusion and is probably not the most parsimonious explanation from a developmental perspective. The authors should point out that short wing development appears to be a truncation of cellular proliferation of normal long wing development. Short wing development uses the same genes and pathways for wing patterning and cellular proliferation, and no co-option of wing patterning or cellular proliferation genes is necessary to make this happen. In fact, since short wing morphs evolved from long wing morphs and not vice versa, the genes that would be really fascinating to find would be the genes involved in stopping wing development and what environmental signal transduction factors then relay that*

message to truncate wing development in short wing morphs.

Response to comment 1: We sincerely thank the reviewer for this insightful perspective on the evolutionary and developmental logic of wing polyphenism. As the reviewer pointed out, short-wing development is more accurately characterized as a developmental truncation of the long-wing program. We have introduced this information in the introduction section, and also discussed this in the discussion section. In addition, we have removed the word “co-option” from the text, which is also raised by other reviewers.

Comment 2: *The manuscript is mostly very clear and the logic follows with the exception of the conclusion about the co-option of genes as described above. The figures would be more clear and have more impact if there was more clarity on conclusions in the figure titles and not just a simple description of what the figure is. For example, Figure 2 would be more informative if the title was" Single cell atlas of short wing and long wing buds from the firebug show differences in GCs and HEs." Figures 2, 4, 5, 6 & 7 would all be improved with more informative caption titles.*

Response to comment 2: We sincerely thank the reviewer for this constructive suggestion. Following your suggestion, we have revised the titles for Figures.

Comments from Reviewer #3

***Reviewer #3 (Remarks to the Author)*:**

In this study, the authors aimed to understand key cell types and regulatory genes essential for long wing development in two hemipteran species, P. apterus and N. lugens, which exhibit clear wing polyphenism. The research topic will attract a broad audience, and the reported single-cell atlas of developing wings in different morph, in situ gene expression, and RNAi phenotypes are valuable. The use of insulin receptor knockout strains is a good idea for robust comparisons between two morphs. However, I cannot recommend publication of this manuscript in the current form because the authors' main conclusion that "the development of alternative wing morphs is primarily governed by ECs, TCs, and NCs through the co-option of wing-patterning and cell cycle genes across hemipteran insects" (Line 26) is not well supported from the reported results. I suggest further confirmation of the current conclusions, or restructuring the entire manuscript. Below, I listed major reasons behind my decision and suggest possible improvements for the future submission.

Major comment 1: *Direct evidence supporting contributions of specific cell types on wing development. One of the main conclusions of this study is contributions of specific cell types, EC, TC, and NC, on wing development. It is based on results of RNAi-mediated knockdowns targeting marker genes that predominantly express in a cell type than the others. But the predominant expression does not mean the other cell types including unidentified minor cell types have no expression of the genes of interest. Indeed, the authors report predominant intervein expression of the tracheal cell maker gene bs by means of tissue staining (Line 266; Fig. 5c), which conflicts with the authors' interpretation that RNAi effects of bs in wing formations are due to defects in TCs (e.g. Lines 251, 350). The same caveat is applied to contributions of neural cells on wing development based on Anln, CncB3, and cdk1 knockdowns. I think we cannot exclude contributions from other cell types without more direct evidence.*

Response to comment 1: We agree that while marker genes exhibit highly enriched

expression in specific clusters, the potential effects in other cell types must be carefully considered. We have discussed this caveat in the discussion section.

Based on the current technique, the identification of cell clusters in scRNA-seq is fundamentally based on statistical enrichment and differential expression analysis rather than the "absolute" exclusive expression of a gene (Luecken and Theis, 2019). This means that there are no “absolutely-expressed” marker genes in any scRNA-seq papers published thus far. The marker genes for ECs, TCs, and NCs in this study showed high fold-change and significant *P*-values, which are widely accepted in the field as robust indicators of a cell type’s functional core. Given that knockdown of an ECs marker gene (*En*) significantly reduced wing size in $PaInR2^{De4}$ but not *Wt*, and knockdown of a TCs maker gene (*bs*) or three NCs marker genes (*Anln, CncB3,* and *cdk1*) significantly reduced wing size in both $PaInR2^{De4}$ and *Wt*, we concluded that ECs, TCs, and NCs contribute significantly to the developmental plasticity of wings. In addition, developmental regulatory networks are rarely composed of genes restricted to a single cell type. For example, neural cells in developing appendages can modulate surrounding epithelial growth via the secretion of paracrine factors or direct neuro-epithelial signaling (Nässel, 2002). Therefore, the phenotypes observed upon RNAi likely reflect the disruption of these neural-derived regulatory inputs, which are essential for wing development.

Luecken, M. D., & Theis, F. J. (2019). Current best practices in single-cell RNA-seq analysis: a tutorial. *Molecular Systems Biology*, 15(6), e8746.

Nässel DR. Neuropeptides in the nervous system of Drosophila and other insects: multiple roles as neuromodulators and neurohormones. Prog Neurobiol. 2002 Sep;68(1):1-84. doi: 10.1016/s0301-0082(02)00057-6. PMID: 12427481.

Major comment 2: *Specific roles of identified genes in the regulation of wing polyphenism. To conclude that wing morphs are primarily governed by specific cell types and genes (Line 357), it is critical to distinguish genes that play roles in the plastic and normal wing development. Through the RNAi experiment, the authors successfully found a hopeful candidate gene* ***engrailed*** *that might specifically regulate wing*

development in the LW but not in the SW morph (Fig. 4e, f). However, the specific effect that supports the main conclusion of this study should be carefully confirmed with statistical analysis of quantitative data (e.g. quantitative effects on each wing regions) in addition to a single image in Fig. 4f. Summary of injections must be provided to confirm data reproducibility. Other data that support the morph-biased function are also desired. These could be differential expression pattern between morphs, and a regulatory gene function in cell proliferation as the author hypothesizes.

Response to major comment 2: This is a good suggestion. We agree that some marker genes) play dual roles in the plastic and normal wing development. The purpose of the study is to identify certain marker genes (e.g., *En*) that shows a temporal requirement specifically for the plastic development of wings. As the reviewer suggested, we have measured the wing areas of adults following RNAi treatment (Supplementary Fig. 10). Summary of injections that confirms data reproducibility is provided in Supplementary Data 4. The differentially expressed genes in each six cell types (ECs, TCs, NCs, GCs, HEs, and MCs) were used for transcriptional regulatory network analysis (Supplementary Fig. 2), and GO and KEGG (Supplementary Figs. 3 and 4) analyses. In addition, to determine whether the 30 marker genes for the six major cell types contribute to the prolonged cell proliferation observed in LW-destined wing buds, we examined their expression levels in the scRNA-seq data from *PaInR2*^{De4} and *Wt* wing buds. We found that cell cycle regulators (*Anln, CycB3,* and *cdk1*) were upregulated in *PaInR2*^{De4} versus *Wt* (Supplementary Fig. 13).

Major comment 3: *I could not find the detail of the PaInR2*^{De4} *strain (gene structure, gRNA design, deleted region etc.) which was generated in this study. The detail must be included when resubmitting a manuscript.*

Response to major comment 3: The study describing *PaInR2* CRISPR knockout has been published during the review process of this manuscript. We have added this reference (Zhang et al., 2026, Science Advances 12: eaea4413) in the revised manuscript.

Minor comment 1: *It would be more convincing if the authors' hypothesis that prolonged cell proliferation involves in the LW-morph development was tested more directly with pharmacological approach.*

Response to minor comment 1: To confirm the contribution of cell proliferation to long-wing development, we injected fifth-instar *PaInR2*^{De4} nymphs with Hydroxyurea, an agent that inhibits DNA synthesis by targeting ribonucleoside diphosphate reductase. In the Hydroxyurea-treated group, 70% (n = 10) of *PaInR2*^{De4} nymphs developed into adults with short wings (Fig. 6h), whereas all *PaInR2*^{De4} nymphs injected with the control solution developed long wings (lines 367-372).

Minor comment 2: *Previous studies below have reported effects of nubbin and blistered RNAi on the wing formation in another hemipteran species. These works should be appropriately acknowledged.*

Response to minor comment2: Thank you for your suggestions. We have now appropriately cited study from the milkweed bug *Oncopeltus fasciatus*.

Comments from Reviewer #4

Reviewer #4 (Remarks to the Author):

In this manuscript, Wan and colleagues utilized single-cell RNA sequencing (scRNA-seq) to investigate the cellular determinants underlying wing polyphenism in the firebug Pyrrhocoris apterus in Hemiptera. All major cell types were identified through scRNA-seq. Based on cell-type specific markers, the authors performed whole-body RNAi knockdown experiments and found that knockdown of En in long-wing mutant caused a completely short-wing phenotype in adults, whereas the same genetic manipulation in short-wing nymphs gave no obvious wing phenotype. Additionally, whole-body knockdown of four tracheal cell (TC) and neural cell (NC) markers (bs, Anln, CycB3, and cdk1) also resulted in a significant reduction in wing size for adults of both long-wing- and short-wing-destined nymphs. Then they used flow cytometry analysis and revealed differences in cell cycle phase distribution between wing buds from long-wing and short-wing nymphs.

Overall, the genetic KD experiments and wing phenotypes are interesting. Also, this study provides useful single-cell transcriptomic resources for investigating the plasticity of wing buds in firebugs and planthoppers. However, there are major concerns for the scRNA-seq data analysis in addition to other several concerns, including experimental design. This manuscript needs substantial revision and improvement.

Major comment 1: *The different wing sizes of both firebugs and planthoppers are primarily induced by environmental cues, as introduced in the Abstract and discussed in the introduction. In this study, the authors compared wild-type (short wing) with PaInR2 mutant (long wing). Such experiment design is not aligned with what was introduced in the Abstract "Insect wing polyphenism enables a single genome to produce two or more distinct wing morphs in response to environmental cues….", because the genetics of these two samples are different. Although insulin receptors are known to play key roles in wing size dimorphism, this genetic manipulation significantly reduces the possibility of identifying other molecular mechanisms underlying wing size*

plasticity. In addition, the PaInR2 mutant affects the whole body rather than being wing-specific, which complicates the interpretation of certain phenotypes and mechanisms. So, the experimental design does not address the scientific question from the Title and Abstract. To address this, the authors need to include environmental cue-induced long and short wing samples, or need to substantially reorganize the manuscript to align with current data.

Response to major comment1: We thank the reviewer for the constructive critique regarding the LW mutants used in this study. We used the *PaInR2*^{De4} mutants as a surrogate of LW-destined wild-type because of lacking of LW-destined wild-type population.

To date, no laboratory has successfully established a LW-destined population of the brown planthopper. Although several research groups in China and Japan have maintained high long-wing ratio lines through the labor-intensive practice of selectively breeding long-winged males and females in each generation, these populations consistently revert to being SW-destined under laboratory-reared conditions. A similar tendency toward the SW morph is observed in the firebug. While Smykal et al.(2018, Mol Biol Evol 37:1775–1789) reported a population consisting of 85–90% LW individuals, even this population shows a strong tendency to develop into the SW form. Therefore, a LW mutant line is the only and best sample we can use to do comparison with the SW wild-type population.

In the revised manuscript, we have discussed the shortage of the *PaInR2*^{De4} mutants we used as a surrogate of LW-destined wild-type.

Major comment 2: *There are several concerns for the scRNA-seq data analysis.*
***##First,** could the authors shown and confirm the PaInR2 gene expression in their WT and PaInR2-del dataset?*

Response to the first comment: The *PaInR2*^{De4} loss-of-function homozygous mutant was created by using CRISPR-Cas9 system with 4-nt deletion in its coding region. Thus, the frameshift of the coding reigon for *PaInR2* could be detected by RNA sequencing. Detailed information on *PaInR2*^{De4} is available in a paper we published during the review of this manuscript (Zhang et al., 2026, Science Advances 12: eaea4413).

##Second, *the primary focus of this study is to identify the key cell types and critical genes involved in wing size differences. However, there is no comparison between WT and PaInR2-del samples at the gene expression level. It is highly recommended to compare the transcriptomes of the same cell types from short-wing- and long-wing-destined fifth-instar nymphs to identify differentially expressed genes (DEGs) associated with wing dimorphism in each cell type. These DEGs can be applied to GO and KEGG analysis to reveal the key functional and pathway activity differences underlying wing plasticity. Additionally, transcriptomic regulatory network (TRN) analysis can be a powerful tool to identify the key transcription factors responsible for transcriptome changes in each cell type.*

##Response to the second comment: Following this constructive suggestion, we have performed a comprehensive comparative analysis between *Wt and PaInR2E4* within each identified cell type. We identified differentially expressed genes (DEGs) between SW-destined *(Wt*) and LW-destined (*PaInR2*^{De4}) fifth-instar nymphs for each major cell cluster. These DEGs have been subjected to GO and KEGG enrichment analyses, which successfully revealed key functional and pathway activities. These results are now presented in the Supplementary Fig. 3 (GO) and Supplementary Fig. 4 (KEGG). To pinpoint the upstream drivers of these transcriptomic changes, we performed transcriptomic regulatory network (TRN) analysis using SCENIC in both firebugs and planthoppers. This analysis identified transcription factors (TFs) and their associated regulons (Supplementary Fig. 2, and Supplementary Data 2). Together, our data suggests that wing plasticity in the firebug arises from subtle changes in transcriptional regulation (lines 166-189).

##Third, *it is related to the cell composition in figure 2c and 2d. There are more than one clusters for both glial cells and hemocytes. Could the authors further annotate these subtypes? Also, could the authors go higher resolution and see if the difference shows up in a specific cluster or subtype of these cell types?*

##Response to the third comment: We appreciate the reviewer's constructive suggestion to further explore the heterogeneity of the glial cell (GC) and hemocyte (HE)

populations. Following re-annotation, we identified three GC and two HE subtypes, each defined by distinct marker gene expression profiles (see figure below). However, GCs and HEs together represent a very small proportion (< 10%) of the total cells in the wing bud. RNAi validation of the primary markers for these clusters did not yield any observable wing-related phenotypes. Therefore, we did not integrate this data into the main text.

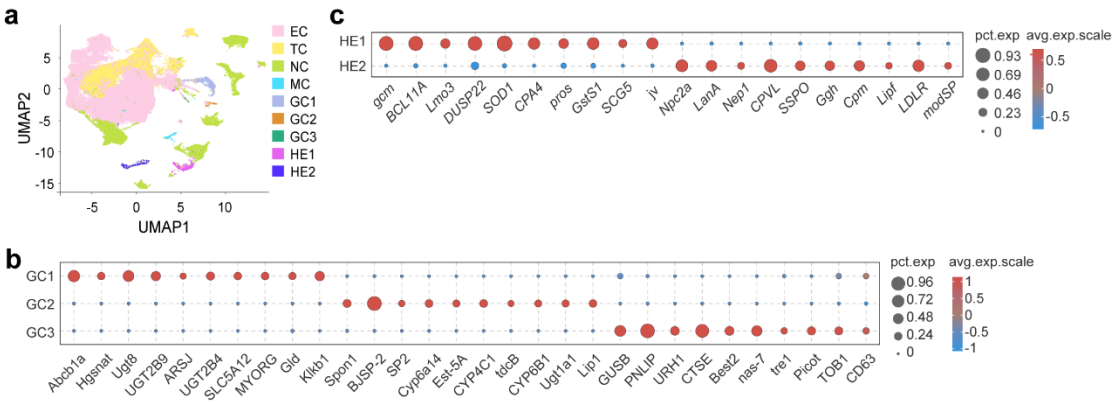

Fig. Subtypes of glial cell (GC) and hemocyte (HE). a Uniform manifold approximation and projection (UMAP) visualization of the single-cell transcriptomic landscape. **b** Dot plot of the selected marker genes present in distinct GC subtypes. **c** Dot plot of the selected marker genes present in distinct HE subtypes.

##Fourth, *it is also related to the cell composition analysis in figure 3. In figure 3a, there is a clear WT-specific cluster that is not shown using current resolution. The authors should use a higher resolution to annotate them. otherwise, the important information is missing. In addition, what is the WT-specific cluster? why is there a WT-specific cluster? is this real or technical issue? Because of this analysis flaw, the conclusion below is not convincing – "SW- and LW-destined wing buds exhibited similar proportions of EC0–EC3 subsets (Fig. 3c), suggesting that the proportion of EC subsets does not serve as a primary determinant in the development of alternative wing morphs."*

##Response to the fourth comment: We thank the reviewer for pointing out the significance of the cluster observed exclusively in the *Wt* samples. To address this concern, we have performed a high-resolution analysis to clarify of this cluster.

To determine whether the *Wt*-specific cluster represented a distinct EC subtype, we performed differential expression analysis to identify its marker genes

(Supplementary Fig. 6b). Although the most highly expressed genes (such as *Pgk*, *elF5*, and *ETFB*) showed elevated average expression within this cluster, their expression percentages were notably low (mostly < 20%; Supplementary Fig. 6b), falling below the established thresholds for defining a cell type in scRNA-seq studies[52]. Intriguingly, the *Wt*-specific cells were still present even if we raised the resolution for cell clustering (Supplementary Fig. 7). Collectively, these data support the cell-type classification, in which *Wt*-specific cells are categorized as the EC0 subtype (lines 217-226). In addition, we addressed the reason for the presence of the *Wt*-specific cell in the Discussion section (lines 524-532).

##***Fifth,*** *the authors should also color cells with sample information (WT-1,2,3 and PaInR2de1,2,3). Otherwise, I cannot evaluate the quality of data, such as sample bias, or batch effect. This should be done not just for epithelial cells in figure 3a, but also for all cell types. For example, is the WT-specific cluster coming from one of the three WT samples, or from all three WT samples?*

##**Response to the fifth comment:** As requested, we have now provided UMAP visualizations colored by individual biological replicates for all cell types and specifically for epithelial cell sub-clusters (Supplementary Fig. 1). Regarding the *Wt*-specific cluster, our updated analysis demonstrates that cells from all three independent Wt replicates (*Wt*_1, *Wt*_2, and *Wt*_3) contribute consistently to this population. This finding is crucial as it indicates that the observed cluster is not a result of sample-specific bias or a batch effect from a single library preparation.

Major comment 3: *For knockdown experiments, since only one RNAi was used for each gene, the RNAi efficiency should be validated using qRT-PCR.*

Response to major comment 3: We have validated the RNAi efficiency by qRT-PCR. The data has been integrated in the revised manuscript (Supplementary Figs. 9 and 11).

Major comment 4: *For all figures related to wing phenotypes, there is no quantification for the wing length, and it should be added.*

Response to major comment 4: We thank the reviewer for this suggestion. In our

RNAi experiments, we have now calculated the total wing area for both forewings and hindwings (Supplementary Fig. 10).

Major comment 5: *In line 292-293, "To confirm the cell proliferation activity of wings in late fifth-instar PaInR2^{De4} nymphs, we quantified cell numbers by quantitative real-time PCR (qRT-PCR)" How cell numbers be quantified by qRT-PCR?*

Response to major comment 5: To confirm the cell proliferation activity of wings in late fifth-instar PaInR2^{De4} nymphs, we assessed cell numbers by measuring genomic DNA copy number using quantitative PCR (qPCR). To clarify this, we have modified the sentence in the revised manuscript (lines 354-359).

Major comment 6: *There is no quantification for Fig 6h.*

Response to major comment 6: In this paper, we used EdU staining as a readout of wing cell proliferation. The wing buds of the firebug form a sac-like structure composed of at least two distinct cell layers. Therefore, severe cell overlap significantly hinders the accurate counting of EdU-stained cells. This is the reason why we did not quantify the EdU signal.