nature portfolio

Peer Review File

Egg-laying ChickenGTEx resource deciphers context-specific regulatory effects on fertility traits

Corresponding Author: Dr Lingzhao Fang

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 presents a comprehensive analysis of stage- and tissue-specific regulatory mechanisms underlying fertility traits in chickens. The study leverages a well-controlled and genetically homogeneous population, enabling precise dissection of gene regulatory dynamics across multiple reproductive stages. The integration of transcriptomic data from four tissues with high-quality genomic data strengthens the overall analytical framework. In summary, the findings are of interest to both poultry genetics researchers and the broader functional genomics community, particularly in the context of complex trait regulation. While the manuscript is generally well-written and thoughtfully organized, several revisions and clarifications are needed to improve clarity, completeness, and the overall presentation of the work.

Summary statistics of the molQTL mapping results should be made publicly available to ensure transparency, reproducibility, and to facilitate reuse by the broader research community. This would enable other researchers to perform meta-analyses, integrate with other datasets, or functionally validate specific loci of interest.

The authors demonstrate high-resolution fine-mapping of molecular QTLs, as exemplified by Figure 3E. In this context, I believe that Figure S9 represents an important result that showcases the resolution and potential functional relevance of fine-mapped signals. I recommend moving this figure (or a summary version of it) into the main manuscript to better highlight the study's contribution in this area. Additionally, I did not find information in the data availability section regarding the release of fine-mapping results. To facilitate downstream validation and functional studies by the research community, I strongly encourage the authors to publicly release the fine-mapped results.

The finding that stage-specific regulatory effects exhibit strong tissue specificity is intriguing and potentially biologically meaningful. However, the manuscript currently provides limited interpretation of this observation. I recommend that the authors expand the Discussion to explore potential biological mechanisms that may underlie this pattern. A more in-depth interpretation would strengthen the manuscript and help contextualize the significance of this finding.

Line109-111: "We quantified four molecular phenotypes from the RNA-seq data, including the expression levels of 25,016 genes, 78,530 exons, and 13,374 enhancers, as well as the alternative splicing abundance..." Has an awkward structure due to the mixed use of "including... as well as...". Since all four items are intended to represent parallel molecular phenotypes, the sentence would be clearer if rephrased with consistent parallel structure.

Line 359-362: There is a duplicated sentence in the Discussion section: "We clarified the distinct roles of stage-specific and stage-interaction QTL in interpreting complex trait GWAS loci." Please revise by removing the repetition and keeping only one complete version of the sentence to ensure clarity and grammatical correctness.

Line 602–603: "The gene expression ratio is defined as (TPMa1 + 0.01) / (TPMa2 + 0.01)."
The manuscript does not provide an explanation for the addition of 0.01 to both numerator and denominator in the expression ratio calculation.

The overall language of the manuscript would benefit from further polishing. Several sentences, particularly in the Methods and Results sections, are overly wordy or redundant, which may hinder readability. I recommend that the authors revise the manuscript for conciseness and clarity, aiming for more direct and streamlined expression. A professional language edit

could also be considered to enhance overall presentation.

Although the manuscript indicates that the raw data have been submitted to public repositories, they do not appear to be currently released. To ensure transparency and reproducibility, I recommend that the authors make all relevant raw data fully accessible prior to the final acceptance of the manuscript.

Reviewer #2

(Remarks to the Author)
The study by Zhu et al represents an impressively large body of work looking at the regulation of gene expression in egg-laying chickens. A diverse range of expression-related phenotypes are assayed, including splice variants, exonic variants, etc. In addition, a large GWAS of over 13000 individuals. The study will provide an excellent resource for researchers, and even contains some single cell experimental work. In particular, I really like the mapping of different expression traits (splice variants, exonic, etc).

I found the biggest problem with the manuscript was often in how confusing it could be – the authors use a variety of different terms that appeared to have been coined themselves, and often with multiple synonyms at that. The authors refer to molQTL, but these appear to be interchangeable with eQTL in the text. However, the authors also use eGene, which seems to be also another name for eQTL. In the same way, you also have ieGenes, but also ieQTL and I think they are the same. There are also exQTL, enQTL, sQTL, and whilst these are all defined once, the similarity between all of them makes it extremely confusing to follow the text in places. I would use the standard eQTL, but for the others actually spell out the word, so exonicQTL, spliceQTL, etc. And drop the eGenes vs eQTL, or at least properly clarify exactly how these differ.

There is also an absolute avalanche of multi-paned figures that are often overly complex, and these definitely should be reduced, sending the non-essential ones to supplementary information. In general, I also found that the figure legends needed a lot more detail added to them. Axis were often not explained, some mistakes are present,etc. For example in fig 4, panes A and B are flipped, in fig 6 M in pane A isn't defined, etc. I'd like to see much more detail in the figure legend, so they are properly understandable.

I'd also like to see a table showing clearly exactly how many of each type of QTL were detected, numbers of significance/ suggestive loci, average confidence interval, average effect size, etc. On the subject of confidence intervals, it would be nice to see an attempt made to really define some confidence intervals. Also supplementary tables of all QTL would be really useful to make use of this resource for other researchers.

The authors don't really make any mention of the fact that despite having a sample size of over 13000 for the morphological trait GWAS, only suggestive loci (100) were identified. Isn't this unusual given the power of the study? If you find very few/ weak morphological QTL, does this mean that the expression differences don not actually do much to affect production trait variation? And how much of the total variance in the traits was explained by the combined QTL? It would be nice to have more detail of the morphological QTL in general (average effect size, confidence intervals).

I like the idea of looking at differences between laying stages and the causality analysis tying in cell type, and other characteristics. One point I would like to see the authros address, at least in the discussion section, is the possibility that these age choices are just different adult phases. The first phase (20 weeks) is just at adult bodyweight and early in the egg laying phase, whilst 30 weeks is peak lay. 58 weeks is a decline, but usually just before the birds go into moult. Birds can certainly produce eggs far later than 58 weeks, so some mention should be made that the stages are not really so different (and indeed many of the eQTL are common between laying stages, but not tissue type, which may support this). In this case, it would be more an effect of age differences (which may potentially be not so extreme at these age stages).It is easy to imagine that for some of the tissues (hypothalamus and even liver) where there is an effect, this is due to the aging process instead.

For the morphological traits, some summary statistics should be presented for the traits themselves, i.e. how much variation was present in each, etc.

It would be nice to have an explanation (beyond the HPA axis) as to why to use hypothalamus tissue – it is usually more for anxiety or other traits. Given egg production was the main aim of the study, I am curious why not use femoral bone, given the limitation of calcium in producing eggs and also the role of medullary bone as a reservoir.

Line 152 – the authors state that the molQTL were enriched about the TSS, but how narrow were the confidence intervals to narrow down a region as just a TSS? I suspect that the intervals were larger and that they simply overlap a putative TSS. Should also define what phastCons scores are here.

The authors bring up the concept of 'Credible Sets' here (line 163), but never actually define what it is. I couldn't find any mention in the methods section, but this really needs to be explained. Is it the equivalent of a confidence interval (all significant adjacent loci)? All significant loci? The authors then add yet another abbreviation (CS) in the text, which would also be better spelled out in full to help the reader.

Line 196, the authors note that they find a significant overlap between tissue-specific eQTL (called eGenes) and genes with

differential expression across tissues. Isn't this obvious? To be only called in one tissue, wouldn't the corresponding regions in the other tissues have already been tested with the LFSR method they use, which is a way of checking for lower signal but still some indication of significance in these tissues? I'm not sure how interesting or relevant this paragraph is for the results. If it is of some note, then it should be more clearly indicated as to why.

The mediation analysis is a little confusing, and definitely needs to be more fully described in the methods section. Is this a way of trying to assess causality between gene expression traits and the different contexts (gene expression modules, cell components, transcription factors)? A table showing these results (or at least the top ones), how significant they were, etc, would definitely be useful. When it comes to testing for causality, it appears that only two model outcomes are tested to see if there is an interaction between stage and genotype (fig 6A), but aren't more possible model types possible. Aten et al in their causality paper (introducing the NEO software in 2008) have multiple different model types that can be tested as an example.

I was also not sure about the results of this analysis shown in figure 6C-E. Firstly, it would be nice to have a proper explanation of what TE, ADE, and ACME mean and the criteria for meeting them. It seems at the moment that if the main QTL effect is driven by the early stage (like 6D and 6E), TE is lower. In contrast, when late stage is driving the effect TE is higher. I am probably misinterpreting this, but that is also indicative of further explanation required in the text!

For the colocalization analysis (line 277), it would be nice to know what % of eQTL were present in the overlap? It would be nice to see some metric of the significance of the overlap between morphological QTL and eQTL – although only 100 loci were identified for the morphological QTL, tens of thousands of eQTL were identified. If these are being tied together, it would be nice to see some enrichment (though there may be several reasons why none is seen). As an aside, I really like the follow up assessment of some genes in this paragraph, and especially the evidence that PIK3R1 is caused by a splice variant rather than a standard eQTL.

In summary, I think this is a very strong study, but it does require quite a bit more clarification in various parts to aid readability and interpretation.

Sincerely

Dominic Wright (I am fine with leaving my review open)

Reviewer #3

(Remarks to the Author)
This manuscript titled "Egg-laying ChickenGTEx resource deciphers context-specific regulatory effects on fertility traits" presents a well-designed study utilizing a genetically uniform chicken population to investigate gene regulation across key stages of egg production. By integrating multi-tissue RNA-seq and whole-genome sequencing data from individuals sampled at defined developmental stages, the authors construct a valuable resource and provide meaningful insights into context-specific regulatory variation and its relevance to fertility-related traits, supported by colocalization with GWAS signals. However, there are still some issues that need to be clarified.

1. Line 96-97, authors mentioned that they selected 358 hens with considerable genetic variation at three distinct egg-laying stages. What are the criteria for the selections?
2. Figure 2C and Figure S5A contain important information but are not entirely intuitive at first glance. To improve clarity, I recommend that the authors expand the figure legends to more clearly explain the structure and meaning of each panel.
3. To improve transparency and facilitate downstream use of the fine-mapping results, I recommend that the authors release additional data related to fine-mapped loci. In particular, summary information for all credible sets.
4. While colocalization with molQTL is well described, the functional interpretation of specific genes (e.g., PIK3R1, AMH, IGF2BP1) remains somewhat superficial. Additional discussion on pathways, previous known roles, and biological mechanisms would significantly enhance the impact of the manuscript.
5. This study is based on a single commercial White Leghorn line. While this is acknowledged in the discussion, the authors should be more explicit about the limitations in generalizing to other breeds or populations and suggest possible validation strategies (e.g., cross-line replication, public data, etc.).
6. Line 220-221 : "This suggests that the relationship of gene expression between tissues is regulated by genetic effects independent of eQTL." is somewhat ambiguous. It is recommended to revise this sentence for clarity.
7. Line 261-262 and Figure 6B, the authors report that 60.02% of stage-specific ieGenes were significantly mediated by at least one biological factor (ACME $P < 0.01$). This finding is informative; however, it would be helpful for the authors to briefly discuss the potential reasons why the remaining ~40% of stage-specific regulatory effects could not be explained by the current set of mediators. A short discussion of this point would provide a more balanced view of the limitations and future directions.
8. Line 359: It seems that the sentence "We clarified the distinct roles of stage-specific and stage-interaction QTL in interpreting complex trait GWAS loci" is repeated twice—was this duplication intentional or an editing oversight?

9. Line 427: "Afterward, we removed SNPs with a minor allele frequency (MAF) of less than 0.05 in each tissue and stage group for molQTL mapping." This sentence is somewhat ambiguous. It is unclear whether the MAF filtering was applied globally across all samples or separately within each tissue-stage group. From the current wording, it appears that MAF < 0.05 was filtered independently for each group, which may introduce differences in the SNP sets used across groups. I recommend clarifying the exact filtering strategy and its rationale, including whether consistent SNP sets were retained across comparisons.
10. In several parts of the Methods section, software tools are mentioned without specifying their versions—for example, fastp on Line 454 and bwa on Line 458.
11. Some abbreviations are repeatedly defined throughout the manuscript—for example, TMM and molQTL are introduced multiple times. I recommend reviewing the manuscript to remove redundant definitions for improved clarity and conciseness.
12. Line 638 and related sections: In mathematical expressions, matrices and vectors (e.g., matrix X) should be consistently formatted using boldface to distinguish them from scalar variables. Please revise the formatting of relevant symbols throughout the manuscript to follow standard mathematical notation conventions.
13. Several sentences in the Methods section would benefit from language tightening to improve clarity and flow. In particular, many phrases use redundant constructions such as “The phenotypes”, “performed... using” or “was conducted by...”. Rewriting these in a more concise and active form (e.g., “We used the WGCNA package to construct...” instead of “We performed WGCNA analysis using...”) would enhance readability. Consider seeking assistance from a professional English language editor to polish the manuscript.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
NA

Reviewer #2

(Remarks to the Author)
Thank you very much for the detailed revisions - they greatly help the understanding of the paper and fully address all my comments. Well done on an excellent piece of work!
best
Dominic Wright

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Dear editor and reviewers,

We sincerely appreciate your great efforts and insightful feedback on our manuscript entitled "**Egg-laying ChickenGTEx resource deciphers context-specific regulatory effects on fertility traits" (NCOMMS-25-20438)**. We have carefully and substantially revised the manuscript based on your valuable and constructive comments and suggestions, which have significantly improved the manuscript. We have highlighted the revised sections of the manuscript in red, and provided detailed, point-by-point responses to each of your comments below. We hope our revisions have satisfactorily addressed all the comments. Thank you very much for your time and consideration.

Sincerely,

Dr. **Lingzhao Fang**: Center for Quantitative Genetics and Genomics (QGG), Aarhus University, Aarhus, Denmark; E-mail: lingzhao.fang@qgg.au.dk;

Prof. **Xiaoxiang Hu**: State Key Laboratory of Animal Biotech Breeding, College of Biological Sciences, China Agricultural University, Beijing, 100193, China; E-mail: huxx@cau.edu.cn;

Reviewer #1 (Remarks to the Author):

This manuscript presents a comprehensive analysis of stage- and tissue-specific regulatory mechanisms underlying fertility traits in chickens. The study leverages a well-controlled and genetically homogeneous population, enabling precise dissection of gene regulatory dynamics across multiple reproductive stages. The integration of transcriptomic data from four tissues with high-quality genomic data strengthens the overall analytical framework. In summary, the findings are of interest to both poultry genetics researchers and the broader functional genomics community, particularly in the context of complex trait regulation. While the manuscript is generally well-written and thoughtfully organized, several revisions and clarifications are needed to improve clarity, completeness, and the overall presentation of the work.

Summary statistics of the molQTL mapping results should be made publicly available to ensure transparency, reproducibility, and to facilitate reuse by the broader research community. This would enable other researchers to perform meta-analyses, integrate with other datasets, or functionally validate specific loci of interest.

R: Thank you very much for your thoughtful and constructive suggestion. We agree that making the summary statistics of molQTL mapping publicly available is essential for ensuring transparency, reproducibility, and facilitating downstream analyses by the broader research community. As suggested, we have made the full summary statistics of all molQTL available at the following link: https://ngdc.cncb.ac.cn/chickengtex/static/download/molQTL.tar.gz. We have also included this download link in the Data Availability section of the revised manuscript (Lines 787-790). We believe that this resource will be useful for researchers who are interested in conducting meta-analyses, integrating multi-omics datasets, or further validating regulatory loci of interest.

The authors demonstrate high-resolution fine-mapping of molecular QTL, as exemplified by Figure 3E. In this context, I believe that Figure S9 represents an important result that showcases the resolution and potential functional relevance of fine-mapped signals. I recommend moving this figure (or a summary version of it) into the main manuscript to better highlight the study's contribution in this area. Additionally, I did not find information in the data availability section regarding the release of fine-mapping results. To facilitate downstream validation and functional studies by the research community, I strongly encourage the authors to publicly release the fine-mapped results.

R: Thank you very much for your insightful suggestion. We agree that Figure S9 provides a clear and illustrative example of the resolution and potential biological relevance achieved through our fine-mapping analysis. In particular, it effectively highlights how specific molecular QTL signals can be pinpointed to functionally meaningful variants. As suggested, we have moved the left panel of Figure S9 into the main manuscript by

replacing the original panel in Figure 3H. In addition, we have made all fine-mapping results publicly available to support downstream validation and functional studies. These results can be freely downloaded from the following link: https://ngdc.cncb.ac.cn/chickengtex/static/download/fine-mapped_molQTL.rar. We have also included this information in the Data Availability section of the revised manuscript (Lines 787-790).

The finding that stage-specific regulatory effects exhibit strong tissue specificity is intriguing and potentially biologically meaningful. However, the manuscript currently provides limited interpretation of this observation. I recommend that the authors expand the Discussion to explore potential biological mechanisms that may underlie this pattern. A more in-depth interpretation would strengthen the manuscript and help contextualize the significance of this finding.

R: As suggested, we have expanded the Discussion section (see lines 365-369 in the revised manuscript) to discuss potential mechanisms underlying stage-specific regulatory effects. More specifically, we discuss how differences in cellular composition, transcription factor activity, chromatin accessibility, and microenvironmental cues across tissues may contribute to the emergence of independent regulatory effects during development.

Lines 365-369: Focusing on eQTL, we identified a number of stage-specific or -interaction eQTL. Notably, these QTL exhibited high tissue specificity, suggesting that during organismal development, tissue-specific functions and cellular microenvironments may promote the emergence of tissue- and stage-specific regulatory effects to support functional adaptation. However, these hypotheses remain speculative and require further experimental validation.

Line109-111: "We quantified four molecular phenotypes from the RNA-seq data, including the expression levels of 25,016 genes, 78,530 exons, and 13,374 enhancers, as well as the alternative splicing abundance..." Has an awkward structure due to the mixed use of “including... as well as...”. Since all four items are intended to represent parallel molecular phenotypes, the sentence would be clearer if rephrased with consistent parallel structure.

R：As suggested, we have revised the sentence to ensure a consistent and parallel structure when listing the four molecular phenotypes (Lines 110-112) in the revised manuscript):

Lines 110-112: We quantified four molecular phenotypes from the RNA-seq data, including the expression levels of 25,016 genes, 78,530 exons, and 13,374 enhancers, and the alternative splicing abundance of 15,593 genes across tissues and stages (Table S3).

Line 359-362: There is a duplicated sentence in the Discussion section: “We clarified the distinct roles of stage-specific and stage-interaction QTL in interpreting complex trait GWAS loci.” Please revise by removing the repetition and keeping only one complete version of the sentence to ensure clarity and grammatical correctness.

R: As suggested, we have removed the redundant sentence in the revised version to ensure clarity and grammatical correctness.

Line 602–603: "The gene expression ratio is defined as (TPMa1 + 0.01) / (TPMa2 + 0.01)."
The manuscript does not provide an explanation for the addition of 0.01 to both numerator and denominator in the expression ratio calculation.

R：We added 0.01 to both the numerator and denominator to avoid division errors when the expression level (TPM) of a gene is zero in one or both conditions. This small constant ensures numerical stability while having minimal impact on the overall ratio. We have included this explanation in the revised manuscript (see Lines 653-655) to clarify the rationale for this calculation.

Lines 653-655: For gene(a) in tissue1-tissue2 pair, the gene expression ratio is defined as $(TPM_{a1}+0.01)/(TPM_{a2}+0.01)$*, where a small constant (0.01) is added to prevent division by zero and to stabilize the ratio for genes with low or zero expression.*

The overall language of the manuscript would benefit from further polishing. Several sentences, particularly in the Methods and Results sections, are overly wordy or redundant, which may hinder readability. I recommend that the authors revise the manuscript for conciseness and clarity, aiming for more direct and streamlined expression. A professional language edit could also be considered to enhance overall presentation.

R: As suggested, we have carefully revised the manuscript to improve language clarity, conciseness, and overall readability, particularly in the Methods and Results sections. We believe the updated version presents the content in a more direct and streamlined manner.

Although the manuscript indicates that the raw data have been submitted to public repositories, they do not appear to be currently released. To ensure transparency and reproducibility, I recommend that the authors make all relevant raw data fully accessible prior to the final acceptance of the manuscript.

R: As suggested, by meeting the highest standards of data sharing, we have made all

relevant raw and processed data/results publicly accessible via NCBI SRA database under BioProject ID: PRJNA1188634 and PRJNA1198198, respectively. Please find the relevant information in the revised manuscript (Lines 781-790).

Reviewer #2 (Remarks to the Author):

The study by Zhu et al represents an impressively large body of work looking at the regulation of gene expression in egg-laying chickens. A diverse range of expression-related phenotypes are assayed, including splice variants, exonic variants, etc. In addition, a large GWAS of over 13000 individuals. The study will provide an excellent resource for researchers, and even contains some single cell experimental work. In particular, I really like the mapping of different expression traits (splice variants, exonic, etc).

I found the biggest problem with the manuscript was often in how confusing it could be – the authors use a variety of different terms that appeared to have been coined themselves, and often with multiple synonyms at that. The authors refer to molQTL, but these appear to be interchangeable with eQTL in the text. However, the authors also use eGene, which seems to be also another name for eQTL. In the same way, you also have ieGenes, but also ieQTL and I think they are the same. There are also exQTL, enQTL, sQTL, and whilst these are all defined once, the similarity between all of them makes it extremely confusing to follow the text in places. I would use the standard eQTL, but for the others actually spell out the word, so exonicQTL, spliceQTL, etc. And drop the eGenes vs eQTL, or at least properly clarify exactly how these differ.

R：Thank you for the constructive suggestions. We agree that the previous version of the manuscript lacked clear and consistent definitions of the various terms used to describe molecular QTL. In the revised manuscript, we have provided a detailed explanation of the different types of molQTL (eQTL, exQTL, enQTL, and sQTL) and have clarified the definitions of related terms such as eGenes. We have also ensured consistent usage of these terms throughout the text to improve clarity and readability. We have also explicitly clarified the distinction between eQTL and eGene: an eQTL refers to a genomic locus associated with variation in gene expression (i.e., a quantitative trait locus), whereas an eGene refers to the gene whose expression is significantly regulated by at least one eQTL. We hope these clarifications help avoid confusion and enhance the readability of the manuscript.

Line 598 - 604: The molecular quantitative trait loci (molQTL) are genetic loci that are significantly associated with the variation in molecular phenotypes across individuals. More specifically, molQTL linked to gene expression, exon expression, enhancer expression, and alternative splicing are further referred to as expression QTL (eQTL), exon QTL (exQTL), enhancer QTL (enQTL), and splicing QTL (sQTL), respectively. The genes with at least one molQTL are referred to as molGenes. For example, a gene

harboring eQTL or sQTL is referred to as an eGene or sGene, respectively.

We have also added clear definitions for both ieQTL and ieGene to improve clarity.

Line 697-699: Genes with eQTL that exhibited a significant interaction with developmental stage (FDR < 0.2) were referred to as stage-interaction genes (ieGenes), and the corresponding eQTL were considered as stage-interaction eQTL (ieQTL).

There is also an absolute avalanche of multi-paned figures that are often overly complex, and these definitely should be reduced, sending the non-essential ones to supplementary information. In general, I also found that the figure legends needed a lot more detail added to them. Axis were often not explained, some mistakes are present,etc. For example, in fig 4, panes A and B are flipped, in fig 6 M in pane A isn't defined, etc. I'd like to see much more detail in the figure legend, so they are properly understandable.

R: As suggested, we have streamlined the figures by keeping the most essential content in the main figures. Additionally, we have carefully revised all figure legends to provide clearer and more comprehensive descriptions, ensuring that axes, symbols, and abbreviations are properly defined to improve readability and interpretability.

I'd also like to see a table showing clearly exactly how many of each type of QTL were detected, numbers of significance/ suggestive loci, average confidence interval, average effect size, etc. On the subject of confidence intervals, it would be nice to see an attempt made to really define some confidence intervals. Also supplementary tables of all QTL would be really useful to make use of this resource for other researchers.

R: Thank you very much for the suggestions. To make this resource as useful as possible to the broader research community, we have made the full summary statistics of molQTL mapping results publicly available via the following link: https://ngdc.cncb.ac.cn/chickengtex/static/download/molQTL.tar.gz. These include detailed information of each molQTL such as mutation frequencies, nominal p-values, effect sizes, and other relevant data. All the relevant information has been provided in the Data Availability section of the revised manuscript (Lines 786-790).

For confidence intervals, we have used an LD-based method to define them for all independent molQTL(1). More specifically, for each lead variant, we defined the confidence interval as the genomic region extending from the furthest upstream to the furthest downstream SNP that is in high LD ($r^2 \geqslant 0.8$) with the lead SNP, based on pairwise LD calculations using the corresponding population genotype data. This approach provides a reliable estimate of the region likely to harbor the causal variant and allows us to define more precise intervals for each molQTL. The results, including the

number of independent molQTL detected for each tissue, laying stage, and molecular phenotype, as well as the average confidence interval size, are summarized in Table S4.

References:

1. Zhu, X. et al. Mapping the regulatory genetic landscape of complex traits using a chicken advanced intercross line. Nat Commun 16, 5841, doi:10.1038/s41467-025-60834-x (2025)

The authors don't really make any mention of the fact that despite having a sample size of over 13000 for the morphological trait GWAS, only suggestive loci (100) were identified. Isn't this unusual given the power of the study? If you find very few/ weak morphological QTL, does this mean that the expression differences don not actually do much to affect production trait variation? And how much of the total variance in the traits was explained by the combined QTL? It would be nice to have more detail of the morphological QTL in general (average effect size, confidence intervals).

R：Thank you for your constructive suggestions. In our study, we conducted GWAS in a commercial population of 12,952 chickens, revealing 100 QTL at suggestive significance level for six complex traits. We think that the relatively strong relatedness between individuals in the commercial population resulted in a smaller effective population size (estimated at only 116 effective individuals for the 12,952 samples), which in turn limited the discovery power of GWAS. This is similar to GWAS findings in dairy cattle (1,2). This is indeed a limitation of our study, and we have added a discussion of this limitation in the revised manuscript (Lines 398-402).

Line 398 – 402: First, the GWAS of complex traits was conducted in a single commercial chicken population. Although the sample size was large (n = 12,952), the strong relatedness among individuals resulted in a small effective population size, which limited the discovery power of GWAS. This issue has also been observed in dairy cattle. In the future, it would be great to consider multi-breed GWAS analysis in chicken or even other farm animals.

Furthermore, we estimated the proportion of genetic variance explained by the 100 identified GWAS loci using the Multi-Genome Relationship Matrix (MGRM) model. In this approach, we constructed two genetic relationship matrices (GRMs): GRM1, which includes the SNPs within the QTL regions, and GRM2, which includes the remaining SNPs outside the QTL regions. The proportion of genetic variance explained by the identified QTL was calculated as follows:

$$\sigma^2_{\mathrm{GRM1}}/(\sigma^2_{\mathrm{GRM1}} + \sigma^2_{\mathrm{GRM2}})$$

Where σ^2_{GRM1} is the genetic variance explained by the SNPs within the QTL regions (GRM1), and σ^2_{GRM2} is the genetic variance explained by the remaining SNPs outside the QTL regions (GRM2). The results indicate that these loci explain a significant proportion of

the genetic variance across the six complex traits, with the following percentages: 33.11% in AFE, 40.42% in BW49, 34.39% in EN210, 30.90% in EN300, 34.63% in EN400, and 37.37% in EN210-400. Additionally, we have provided more detailed information on the 100 GWAS loci (including top SNPs, effect sizes, confidence intervals, etc.) in Supplementary Table S5 of the revised manuscript. The full GWAS summary statistics for the six complex traits are also publicly available in the Figshare repository at DOI: 10.6084/m9.figshare.28333362.v1.

To address the question of whether expression differences do not significantly affect production trait variation, we also analyzed the contribution of molQTL to the heritability of complex traits. Our findings indicate that compared to randomly selected MAF-matched loci, molQTL explain a higher proportion of the heritability. This result is presented in Supplementary Figure S20 B. Therefore, we believe that expression differences do contribute to the variation in complex traits.

Reference:

1. Jiang, J. et al. Functional annotation and Bayesian fine-mapping reveals candidate genes for important agronomic traits in Holstein bulls. Commun Biol 2, 212, doi:10.1038/s42003-019-0454-y (2019)
2. Freebern, E. et al. GWAS and fine-mapping of livability and six disease traits in Holstein cattle. BMC Genomics 21, 41, doi:10.1186/s12864-020-6461-z (2020)

I like the idea of looking at differences between laying stages and the causality analysis tying in cell type, and other characteristics. One point I would like to see the authros address, at least in the discussion section, is the possibility that these age choices are just different adult phases. The first phase (20 weeks) is just at adult bodyweight and early in the egg laying phase, whilst 30 weeks is peak lay. 58 weeks is a decline, but usually just before the birds go into moult. Birds can certainly produce eggs far later than 58 weeks, so some mention should be made that the stages are not really so different (and indeed many of the eQTL are common between laying stages, but not tissue type, which may support this). In this case, it would be more an effect of age differences (which may potentially be not so extreme at these age stages). It is easy to imagine that for some of the tissues (hypothalamus and even liver) where there is an effect, this is due to the aging process instead.

R：Thank you very much for your thoughtful comment. We agree that age is an important biological factor and that some of the regulatory differences we observed might be attributed to age-related changes rather than laying stage alone. In the revised manuscript, we have discussed this carefully in the Discussion section (Line 376-380). In addition, we acknowledged that it is difficult to completely disentangle age from laying stage, as transitions between laying phases are inherently accompanied by changes in age. This represents an inherent limitation of our current study, as our samples were collected from only three time points and exclusively from adult birds. As a result, we cannot capture the

full spectrum of developmental transitions. In future studies, it would be great to incorporate samples from a wide range of tissue types and developmental stages, including embryonic, juvenile, and adult time points, which will be critical for resolving how developmental and aging processes independently influence gene regulation across tissues and organs.

Line 376 - 380: As laying stage and chronological age are biologically intertwined and cannot be fully separated, we acknowledge that some of our findings might reflect age-dependent regulatory effects rather than mechanisms specific to egg laying stages. Further investigation with samples from additional developmental stages and finer time points will be necessary to disentangle these effects and validate our observations.

For the morphological traits, some summary statistics should be presented for the traits themselves, i.e. how much variation was present in each, etc.

R：As suggested, we have added Figure S19 to provide a more detailed description of the distribution, mean, and standard deviation across the six complex traits.

It would be nice to have an explanation (beyond the HPA axis) as to why to use hypothalamus tissue – it is usually more for anxiety or other traits. Given egg production was the main aim of the study, I am curious why not use femoral bone, given the limitation of calcium in producing eggs and also the role of medullary bone as a reservoir.

R：Thank you very much for this valuable suggestion. While we acknowledge that the hypothalamus is often studied in the context of anxiety-related traits, it also plays a direct and central role in reproductive regulation (1). More specifically, the hypothalamus initiates the reproductive hormonal cascade by pulsatile secretion of gonadotropin-releasing hormone (GnRH), which acts on the pituitary to stimulate the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (2). These hormones, in turn, promote follicle development and corpus luteum formation, leading the ovary to produce estrogen and progesterone in a feedback-regulated manner (3). Throughout this process, the hypothalamus serves as a critical neuroendocrine regulator. Given its essential role in orchestrating the reproductive axis, we think that investigating hypothalamic regulation is relevant to understanding the genetic basis of egg production traits in chickens. In addition, several previous studies in chickens and other species have consistently demonstrated the importance of hypothalamic regulation in female reproductive traits (3,4). We have now clarified this rationale in the background section of the revised manuscript (Line 53 - 55).

We fully agree with you that skeletal tissues, particularly femoral bone and medullary bone, play a critical role in egg production due to their function as dynamic calcium reservoirs for eggshell formation. In the revised manuscript, we have expanded the discussion (Line

404 - 409) to explicitly acknowledge this limitation and to highlight the importance of incorporating additional functionally relevant tissues in future studies. We believe that including bone, oviduct, and other reproductive or metabolic tissues will be essential for achieving a more comprehensive understanding of the molecular and regulatory networks governing egg production in chickens.

Line 53 - 55 : For instance, gonadotropin-releasing hormone (GnRH) produced by the hypothalamus stimulates the pituitary gland to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH).

Line 404 - 409: The limited sample sizes for individual tissues and stages also restrict the exploration of trans-regulation. In addition, the number of tissues being analyzed remains limited. While our study focused on key components of the HPG axis, egg production is also influenced by other physiological systems, such as skeletal and metabolic tissues. Expanding tissue types in future studies will be crucial for achieving a more comprehensive understanding of the regulatory landscape underlying egg production traits.

References:

1. Mishra S K, Chen B, Zhu Q, et al. Transcriptome analysis reveals differentially expressed genes associated with high rates of egg production in chicken hypothalamic-pituitary-ovarian axis[J]. Scientific Reports, 2020, 10(1): 5976.
2. Komatsu, K. & Masubuchi, S. Observation of the dynamics of follicular development in the ovary. Reprod Med Biol 16, 21-27, doi:10.1002/rmb2.12010 (2017)
3. Chang J, Xu Y, Fu Y, et al. The dynamic landscape of chromatin accessibility and active regulatory elements in the mediobasal hypothalamus influences the seasonal activation of the reproductive axis in the male quail under long light exposure[J]. BMC genomics, 2024, 25(1): 197.
4. Mao S, Wu C, Feng G, et al. Selection and Regulatory Network Analysis of Differential CircRNAs in the Hypothalamus of Goats with High and Low Reproductive Capacity[J]. International Journal of Molecular Sciences, 2024, 25(19): 10479.

Line 152 – the authors state that the molQTL were enriched about the TSS, but how narrow were the confidence intervals to narrow down a region as just a TSS? I suspect that the intervals were larger and that they simply overlap a putative TSS. Should also define what phastCons scores are here.

R：Thank you for your thoughtful suggestion. In our initial analysis, the enrichment was calculated based on the lead variant of each molQTL rather than its confidence intervals. In the revised version, we have provided detailed information on the enrichment analysis. Additionally, we have now included a clearer description of phastCons scores in the revised manuscript (Lines 160-164): PhastCons scores reflect nucleotide-level conservation across 100 species and obtained from the UCSC Genome Browser

(https://genome.ucsc.edu/cgi-bin/hgTrackUi?db=hg19&g=cons100way). These scores are derived using a phylogenetic hidden Markov model (phylo-HMM), which evaluates the evolutionary conservation of each nucleotide position across the aligned genomes. Higher PhastCons scores indicate that a particular region is more highly conserved and likely functionally important across the species being studied. (1).

Lines 160 - 164: The lead variants of molQTL were enriched around the transcription start site (TSS) of genes (Figure S6D). Additionally, eGenes had higher phastCons scores (which quantify the evolutionary conservation of genomic regions across species, with higher scores indicating greater constraint) compared to non-eGenes, indicating that eGenes are more evolutionarily constrained across species (Figure S6E).

Reference:

1. Siepel, A. et al. Evolutionarily conserved elements in vertebrate, insect, worm, and yeast genomes. Genome Res 15, 1034-1050, doi:10.1101/gr.3715005 (2005).

The authors bring up the concept of 'Credible Sets' here (line 163), but never actually define what it is. I couldn't find any mention in the methods section, but this really needs to be explained. Is it the equivalent of a confidence interval (all significant adjacent loci)? All significant loci? The authors then add yet another abbreviation (CS) in the text, which would also be better spelled out in full to help the reader.

R： The concept of Credible Sets refers to as a well-established approach in fine mapping analysis, implemented in the SuSiE software (1). In the revised manuscript, we have added a more detailed description of the SuSiE method and a precise definition of Credible Sets in the Methods section (Lines 613-622). This should clarify any ambiguity and help the reader better follow the analysis. Additionally, we have spelled out Credible Sets (CS) in full when it appears at the first time to avoid any confusion with abbreviations.

Lines 613 - 622: To fine-map the molQTL identified in each of four tissues, we employed the Sum of Single Effects Regression (SuSiE) method, a Bayesian variable selection framework that assumes multiple causal variants may be present within a given locus while accounting for linkage disequilibrium (LD) among variants (1). SuSiE models the genetic effect as the sum of multiple "single-effect" components, where each component represents a regression on a single causal variant, thereby enabling the joint inference of multiple causal signals without requiring prior specification of the number of causal variants. Specifically, SuSiE iteratively fits a series of sparse regression models using a Bayesian framework, estimating the posterior inclusion probabilities (PIP) for each variant, which reflects the probability of the variant being causal, given the data and the LD structure. Based on these PIPs, SuSiE constructs credible sets (CS) — minimal sets of variants that together contain the causal variant with high probability.

Reference

1. Wang, G., Sarkar, A., Carbonetto, P. & Stephens, M. A simple new approach to variable selection in regression, with application to genetic fine mapping. J R Stat Soc Series B Stat Methodol 82, 1273-1300, doi:10.1111/rssb.12388 (2020)

Line 196, the authors note that they find a significant overlap between tissue-specific eQTL (called eGenes) and genes with differential expression across tissues. Isn't this obvious? To be only called in one tissue, wouldn't the corresponding regions in the other tissues have already been tested with the LFSR method they use, which is a way of checking for lower signal but still some indication of significance in these tissues? I'm not sure how interesting or relevant this paragraph is for the results. If it is of some note, then it should be more clearly indicated as to why.

R：Thank you for this thoughtful suggestion. In general, genetic control of gene expression is not correlated with the absolute expression levels across tissues, as illustrated in our Figure S6B, where we showed that cis-heritability and the number of independent eQTL were not correlated with expression levels across tissues. Similar findings have also been reported in the pilot phase of ChickenGTEx and PigGTEx projects (1,2). Therefore, we thought that it would be worth discussing that tissue-specific eGenes overlapped with genes showing differential expression across tissues. We have discussed this in the revised manuscript (Lines 353-361).

Lines 353 - 361: Although we found that the likelihood of detecting an eQTL was independent of the expression level of the gene (Figure S6B), similar conclusions have been reported in other studies. We observed a significant overlap between tissue-specific eGenes and genes that are differentially expressed across tissues (Figure 4C and S12C). This suggests that some tissue-specific gene expression may be caused by tissue-specific regulatory variants. However, there are still 2,240 genes that, despite exhibiting tissue-specific regulatory effects, do not show tissue-specific expression. Additionally, 2,646 genes that are differentially expressed across tissues do not detect tissue-specific eQTL. This suggests that other potential mechanisms, such as post-transcriptional regulation or environmental influences, may also contribute to these observations.

References：

1. Teng, J. et al. A compendium of genetic regulatory effects across pig tissues. Nature genetics 56, 112-123 (2024).
2. Guan, D. et al. Genetic regulation of gene expression across multiple tissues in chickens. Nature Genetics 57, 1298-1308, doi:10.1038/s41588-025-02155-9 (2025).

The mediation analysis is a little confusing, and definitely needs to be more fully described in the methods section. Is this a way of trying to assess causality between gene expression traits and the different contexts (gene expression modules, cell components, transcription factors)? A table showing these results (or at least the top ones), how

significant they were, etc, would definitely be useful. When it comes to testing for causality, it appears that only two model outcomes are tested to see if there is an interaction between stage and genotype (fig 6A), but aren't more possible model types possible. Aten et al in their causality paper (introducing the NEO software in 2008) have multiple different model types that can be tested as an example.

R：Thank you very much for these constructive suggestions. As suggested, in the revised manuscript, we have improved the description of the mediation analysis in the revised Methods (Lines 702-737), following the approach used in the study by Oliva et al., titled "The impact of sex on gene expression across human tissues." [Oliva et al., 2020, Science, 369(6509): eaba3066]. In that study, the authors employed the mediation analysis to investigate whether cell-type proportions mediate the effects of sex-interacting eQTL on gene expression. Similarly, we aimed to explore whether cell type proportions, transcription factor expression, or co-expression modules might mediate the effects of stage-interaction eQTL (ieQTL) on gene expression. Our initial goal was not to perform full causal inference, but rather to gain insight into possible regulatory intermediates underlying context-specific eQTL effects. We recognized that mediation analysis does not establish causality in the strict sense but rather serves to assess whether the effect of an independent variable (genotype × stage interaction) on gene expression is statistically attenuated when adjusting for a third variable (e.g., cell proportion or module eigengene), thus suggesting a potential mediating role. In addition, we have provided a comprehensive list of significant mediation results in a new supplementary table (Table S5), including details such as the mediator identity, corresponding ieGene targets, and significance levels.

We fully agree with you that incorporating richer model frameworks is important for assessing potential causality in regulatory mechanisms. We appreciate your reference to the work of Aten et al. (2008) and the NEO software, which introduced multiple causal model types. Unfortunately, the NEO software is no longer maintained and the original link provided in the publication (http://www.genetics.ucla.edu/labs/horvath/aten/NEO) is no longer accessible. As an alternative, we employed the Causal Inference Test (CIT) implemented in the R package cit (1). The CIT is a formal hypothesis testing framework designed to assess whether a mediator (G) statistically mediates the effect of a genetic variant (L) on a target trait (T), by testing four necessary conditions: (i) L is associated with T, (ii) L is associated with G conditional on T, (iii) G is associated with T conditional on L, and (iv) L is independent of T conditional on G. These conditions are evaluated using general linear models, and the maximum *p*-value across the four component tests is used as an omnibus test statistic following the intersection-union test (IUT) principle. In this study, we applied the CIT framework to evaluate whether the regulatory effects of stage-interaction eQTL on gene expression could be statistically mediated by three classes of biological variables: cell type proportions, transcription factor expression, and co-expression modules. For each triplet (L, G, T), where L represents the interaction term between stage and genotype, G is the mediator, and T is the gene expression trait, we performed the four-part CIT framework as described above. Using a permutation-based association threshold of $p < 0.01$ for T associated with G conditional on L

(p_TassocGgvnL), we identified a total of 251 significant mediation effects across the hypothalamus, ovary, and liver, representing 197 unique stage-interaction eGenes (stage ieGenes). This number is smaller than the 605 ieGenes identified using the mediation analysis described above, and of the 197 ieGenes identified by CIT, 166 overlapped with the mediation analysis results (Figure S18).

References:

1. Millstein, J., Chen, G. K. & Breton, C. V. cit: hypothesis testing software for mediation analysis in genomic applications. Bioinformatics 32, 2364-2365, doi:10.1093/bioinformatics/btw135 (2016).

I was also not sure about the results of this analysis shown in figure 6C-E. Firstly, it would be nice to have a proper explanation of what TE, ADE, and ACME mean and the criteria for meeting them. It seems at the moment that if the main QTL effect is driven by the early stage (like 6D and 6E), TE is lower. In contrast, when late stage is driving the effect TE is higher. I am probably misinterpreting this, but that is also indicative of further explanation required in the text!

R： Thank you for your valuable suggestion. The original legend lacked sufficient explanation of the terms TE (total effect), ADE (average direct effect), and ACME (average causal mediation effect), which led to confusion. In the revised version, we have added detailed definitions of these terms in the figure legend to improve clarity and readability. When interpreting these effects, it is important to consider not only the magnitude but also the direction (sign) of the effect estimates. A value on the left side of the plot does not necessarily indicate a smaller effect overall. Generally, the total effect (TE) represents the primary effect, but in some cases, such as in Figure 6E, the mediation (ACME) and direct (ADE) effects may act in opposite directions, which can result in a complex pattern. Therefore, careful consideration of effect directionality (positive or negative) is essential for result interpretation. We have clarified this point in the revised text to avoid potential misinterpretation.

For the colocalization analysis (line 277), it would be nice to know what % of eQTL were present in the overlap? It would be nice to see some metric of the significance of the overlap between morphological QTL and eQTL – although only 100 loci were identified for the morphological QTL, tens of thousands of eQTL were identified. If these are being tied together, it would be nice to see some enrichment (though there may be several reasons why none is seen). As an aside, I really like the follow up assessment of some genes in this paragraph, and especially the evidence that PIK3R1 is caused by a splice variant rather than a standard eQTL.

R: Thank you for this thoughtful comment and suggestion. We observed that

approximately 0.36% of molQTL colocalized with GWAS loci (defined as PPH4 > 0.8). Given this small overlap, we did not perform a formal enrichment test. Conceptually, interpreting enrichment in this context is challenging because molecular QTLs collectively influence a broad range of biological processes and traits, while the six complex traits examined in our GWAS represent only a small subset of these processes. However, we have observed that molQTL could explain a significantly higher heritability of the six complex traits being studied compared to MAF-matched background SNPs (Figure S20 B).

In summary, I think this is a very strong study, but it does require quite a bit more clarification in various parts to aid readability and interpretation.

R: Thank you very much for all the valuable comments again, which have improved the manuscript a lot.

Sincerely

Dominic Wright (I am fine with leaving my review open)

Reviewer #3 (Remarks to the Author):

This manuscript titled “Egg-laying ChickenGTEx resource deciphers context-specific regulatory effects on fertility traits” presents a well-designed study utilizing a genetically uniform chicken population to investigate gene regulation across key stages of egg production. By integrating multi-tissue RNA-seq and whole-genome sequencing data from individuals sampled at defined developmental stages, the authors construct a valuable resource and provide meaningful insights into context-specific regulatory variation and its relevance to fertility-related traits, supported by colocalization with GWAS signals. However, there are still some issues that need to be clarified.

1. Line 96-97, authors mentioned that they selected 358 hens with considerable genetic variation at three distinct egg-laying stages. What are the criteria for the selections?

R: Thank you for your valuable comment. We agree that the original description lacked sufficient detail regarding the selection criteria for the 358 hens. To ensure that the samples were representative of the entire population, we selected individuals randomly while also incorporating pedigree information to avoid close genetic relatedness, such as half-sibs or more closely related individuals. We have now included a more detailed description of this selection strategy in the Methods section of the revised manuscript.

Line 427 - 429 : To ensure representative sampling and increase the genetic diversity, individuals were randomly selected while avoiding close genetic relationships (e.g., half-sibs or closer) based on pedigree records.

2. Figure 2C and Figure S5A contain important information but are not entirely intuitive at first glance. To improve clarity, I recommend that the authors expand the figure legends to more clearly explain the structure and meaning of each panel.
   R: In the revised manuscript, we have revised and expanded the figure legends for Figure 2C and Figure S5A to more clearly explain the structure and meaning of each panel, thereby improving clarity for readers.

3. To improve transparency and facilitate downstream use of the fine-mapping results, I recommend that the authors release additional data related to fine-mapped loci. In particular, summary information for all credible sets.
   R: Thank you for this helpful suggestion. Another reviewer also raised a similar point, and we have now uploaded the full summary statistics for all fine-mapped loci. This information has also been described in the data availability statement of the revised manuscript to improve transparency and facilitate downstream use. The fine-mapping results of molecular QTL are publicly available at https://ngdc.cncb.ac.cn/chickengtex/static/download/fine-mapped_molQTL.rar.

4. While colocalization with molQTL is well described, the functional interpretation of specific genes (e.g., PIK3R1, AMH, IGF2BP1) remains somewhat superficial. Additional discussion on pathways, previous known roles, and biological mechanisms would significantly enhance the impact of the manuscript.
   R: We agree that the functional interpretation of specific candidate genes could be further elaborated. In the revised manuscript, we have expanded the discussion to include additional context on pathways, previously known functions, and potential biological mechanisms for key genes in order to better highlight their relevance and enhance the impact of the study.

   Line 385-388: For example, we found that PIK3R1 influences EN210-400 through a splicing QTL in the ovary. Notably, PIK3R1 is a member of the PI3K/AKT pathway and serves as a key regulator of cellular processes such as proliferation and apoptosis, highlighting the importance of investigating more detailed molecular phenotypes.

5. This study is based on a single commercial White Leghorn line. While this is acknowledged in the discussion, the authors should be more explicit about the limitations in generalizing to other breeds or populations and suggest possible validation strategies (e.g., cross-line replication, public data, etc.).
   R: First, we would like to clarify and correct an error in the previous version of the manuscript: the study population was based on White Plymouth Rock chickens, which

serve as the dam line in commercial broiler breeding programs, rather than White Leghorn as mistakenly stated. We have corrected this error in the revised manuscript. We also fully acknowledge that focusing on a single commercial line limits the generalizability of our findings to other breeds or populations. In the revised discussion, we have provided a more detailed explanation of this limitation and emphasized the importance of future validation in independent populations. We suggest that replication across different chicken lines and breeds, as well as leveraging publicly available genomic and transcriptomic resources where possible, will be essential for assessing the broader relevance and applicability of the regulatory effects and candidate loci identified in this study.

Lines 398 - 402 : First, the GWAS of complex traits was conducted in a single commercial chicken population. Although the sample size was large (n = 12,952), the strong relatedness among individuals resulted in a small effective population size, which limited the discovery power of GWAS. This issue has also been observed in dairy cattle. In the future, it would be great to consider multi-breed GWAS analysis in chicken or even other farm animals.

6. Line 220-221: "This suggests that the relationship of gene expression between tissues is regulated by genetic effects independent of eQTL." is somewhat ambiguous. It is recommended to revise this sentence for clarity.
   R: We agree that the original sentence was ambiguous. In the revised manuscript, we have rephrased this sentence for clarity to better convey that the correlation in gene expression between tissues may be influenced by genetic factors beyond those captured by local eQTL.

   Lines 224 - 226: This suggests that the correlation in gene expression levels between tissues may be influenced by genetic factors beyond the effects captured by local eQTL.

7. Line 261-262 and Figure 6B, the authors report that 60.02% of stage-specific ieGenes were significantly mediated by at least one biological factor (ACME P < 0.01). This finding is informative; however, it would be helpful for the authors to briefly discuss the potential reasons why the remaining ~40% of stage-specific regulatory effects could not be explained by the current set of mediators. A short discussion of this point would provide a more balanced view of the limitations and future directions.
   R: We fully agree that it is necessary to discuss why approximately 40% of stage-specific regulatory effects could not be explained by the current set of mediators. In our study, we focused on three classes of mediators—cell type proportions, transcription factor expression, and co-expression modules—which together capture important but incomplete aspects of the biological regulatory landscape. However, regulatory mechanisms are inherently complex and multifactorial. Other factors such

as hormonal fluctuations, epigenetic modifications, microenvironmental changes, and additional signaling pathways likely also contribute to context-specific regulatory effects but were not captured in our current dataset. To more fully understand the regulatory architecture underlying stage-specific effects, future studies will require richer multi-omic datasets that integrate these additional layers of regulation.
We have added a discussion of this point in the revised manuscript to provide a more balanced interpretation of our findings and to outline directions for further investigation. We appreciate your suggestion as it helped us improve the clarity and completeness of our discussion.

Line 369-375: Furthermore, we found that 60.02% of stage-interaction eQTL are mediated by cell proportions, transcription factor expression, or co-expression modules. Among these, transcription factors account for the largest proportion at 52.78%, consistent with previous studies that transcription factors as key regulatory factors during developmental stages. Other factors, such as hormonal fluctuations, epigenetic modifications, micro environmental changes, and additional signaling pathways, likely also contribute to context-specific regulatory effects but were not captured in our current dataset, underscoring the need for additional data to fully elucidate these mechanisms.

8. Line 359: It seems that the sentence “We clarified the distinct roles of stage-specific and stage-interaction QTL in interpreting complex trait GWAS loci” is repeated twice—was this duplication intentional or an editing oversight?
   R: As suggested, we have removed the repeated sentence in the revised manuscript.

9. Line 427: "Afterward, we removed SNPs with a minor allele frequency (MAF) of less than 0.05 in each tissue and stage group for molQTL mapping." This sentence is somewhat ambiguous. It is unclear whether the MAF filtering was applied globally across all samples or separately within each tissue-stage group. From the current wording, it appears that MAF < 0.05 was filtered independently for each group, which may introduce differences in the SNP sets used across groups. I recommend clarifying the exact filtering strategy and its rationale, including whether consistent SNP sets were retained across comparisons.
   R: To clarify: in our analysis, the MAF filtering was applied separately within each tissue-stage group, so SNPs with a MAF < 0.05 in a given group were excluded from that group’s molQTL analysis. This approach allowed us to maximize the number of informative variants in each group while accounting for potential differences in allele frequencies across tissues and stages, but we acknowledge that it could result in different SNP sets being analyzed across groups. We have revised the Methods section to clearly describe this filtering strategy and its rationale.

   Line463 - 465 : Afterward, for molQTL mapping, we removed SNPs with a minor allele frequency (MAF) < 0.05 within each tissue-stage group separately, ensuring that

variants with sufficient allele frequency in each specific group were included in the analysis.

10. In several parts of the Methods section, software tools are mentioned without specifying their versions—for example, fastp on Line 454 and bwa on Line 458.
    R: We have added version information for all software tools mentioned in the Methods section in the revised manuscript to ensure clarity and reproducibility.

11. Some abbreviations are repeatedly defined throughout the manuscript—for example, TMM and molQTL are introduced multiple times. I recommend reviewing the manuscript to remove redundant definitions for improved clarity and conciseness.
    R: We have reviewed the manuscript carefully and made consistent adjustments to abbreviation definitions, removing redundant explanations to improve clarity and conciseness.

12. Line 638 and related sections: In mathematical expressions, matrices and vectors (e.g., matrix X) should be consistently formatted using boldface to distinguish them from scalar variables. Please revise the formatting of relevant symbols throughout the manuscript to follow standard mathematical notation conventions.
    R: We have revised the formatting of mathematical expressions in the manuscript to ensure that matrices and vectors (e.g., matrix X) are consistently presented in boldface, following standard mathematical notation conventions.

13. Several sentences in the Methods section would benefit from language tightening to improve clarity and flow. In particular, many phrases use redundant constructions such as “The phenotypes”, “performed... using” or “was conducted by...”. Rewriting these in a more concise and active form (e.g., “We used the WGCNA package to construct...” instead of “We performed WGCNA analysis using...”) would enhance readability. Consider seeking assistance from a professional English language editor to polish the manuscript.
    R: We fully agree that improving the clarity and conciseness of the Methods section is important for enhancing readability and accessibility. In the revised manuscript, we carefully reviewed and revised the language throughout the Methods section, aiming to reduce redundancy, adopt a more concise and active writing style, and improve the overall flow. We appreciate this suggestion as it prompted us to improve the quality and presentation of this section, and we will continue to consider further polishing as needed to ensure the manuscript is clear and accessible to a broad audience.