nature portfolio

Peer Review File

3D Mapping of Intact Ovaries Reveals the Aging Dynamics of the Ovarian Reserve

Corresponding Author: Professor Elvan Boke

Version 0:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
This study examines the spatio-temporal dynamics of oocyte loss with age. Although it is well established that oocyte number declines dramatically over the lifespan, prior quantification has largely relied on 2D sections with stereological extrapolation. Consequently, the 3D distribution of follicle loss across the whole ovary has remained uncertain, and important spatial subtleties may have been missed—an outstanding question in the field.

Here, the authors combine whole-ovary light-sheet imaging, AI-assisted segmentation, and mathematical modelling to quantify oocyte distributions in mouse ovaries across ageing. Their work uncovers several important observations: (1) the number of oocytes activated per cycle scales with the total ovarian oocyte pool; and (2) oocyte sizes display a biphasic distribution, likely reflecting a bottleneck during the handover from gonadotrophin-independent to gonadotrophin-dependent growth during oocyte development.

Overall, the work is rigorous and well executed, provides a valuable resource for the community, and adds nuance to the well-known finding that oocyte number decreases with age. The manuscript would be strengthened by expanding the Discussion to situate these results alongside closely related recent studies. We suggest the following:

Major comments

1. A recent publication — Gaylord et al., Science (October 2025) — addresses a very similar question, combining whole-ovary 3D imaging with single-cell transcriptomics to compare human and mouse ovaries across ageing, focusing on oocyte and follicle distributions and dynamics. The present manuscript would benefit from an explicit comparison with other whole-ovary 3D imaging/clearing pipelines, outlining protocol differences and how results align or differ. We also recommend discussing the spatial transcriptomics study by Lan et al., bioRxiv (December 2024), which examines the disruption of folliculogenesis with age in mice.

2. The authors correctly note that inbred C57BL/6J mice provide a useful model for studying age-related oocyte loss in genetically uniform populations. However, the ovarian ageing field is primarily concerned with human ovaries. Although the present study demonstrates the pipeline on human ovarian cortex, the sample size is too small for much analysis. We recommend adding a discussion section that outlines key similarities and differences between mouse and human ovaries and ageing (e.g., initial follicle endowment and activation rates; cycle dynamics; stromal architecture; lifespan and rate of depletion). This will clarify what can and cannot be extrapolated to humans or to universal mammalian principles from the mouse-based findings.

On this note, this discussion could be strengthened by acknowledging that mice have multiple ovulations per cycle, whereas humans typically have a single ovulation. As the authors propose that their findings may be applicable across mammals, this fundamental difference could influence the rate of oocyte depletion with age and potentially affect other features such as the spatial distribution of follicles within the ovary.

Minor comments

1. The statement “Anti-Müllerian hormone (AMH), a clinical marker of ovarian reserve, exhibits substantial variability among humans…” (line 121) should be supported by an appropriate reference.

2. The authors report considerable heterogeneity in oocyte number even among genetically identical, age-matched individuals. It would be helpful if the Discussion addressed possible explanations for this variability.

3. For consistency throughout the manuscript, the authors could consider changing the labelling of oocytes with anti-DDX4 and TO-PRO-3 in Figure S3C to match that used in the main figures, i.e. DDX4 in magenta and DNA in green.

4. Regarding Figure 2B, the statement that “the absolute numbers of primordial, transitioning, and growing oocytes all decreased as the ovarian reserve diminished” is not strictly accurate, as at week 22 the counts across all classes appear to remain the same or increase relative to week 10. Do the authors have an explanation for this? It would be helpful to address in the Discussion.

5. The sentence “To determine whether external reproductive activity impacts oocyte depletion, we asked whether suppression of ovulation could slow the loss of the ovarian reserve” (line 171) is somewhat ambiguous. The authors should clarify that continuously mated females ovulate less frequently than virgins, to make the rationale for this comparison explicit.

6. It is unclear why violin plots are used in Figure S4 instead of bar plots, as in the main figures. Could the authors justify this choice? Furthermore, the statement that “relatively lower intra-individual differences between the left and right ovaries of the same animal across all age groups” is not fully supported by the data, which appear to show increased variability again at week 22. Do the authors have an explanation for this?

7. Figure S4 is the only figure where the statistical test is described in the legend. Are statistical tests used elsewhere in the manuscript, for example to assess whether oocyte number is significantly reduced between age groups? Where applicable, these should be clearly stated in the corresponding figure legends for consistency.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
The current manuscript integrates light-sheet high-resolution whole-mount imaging, tissue clearing, artificial intelligence, and mathematical modeling to analyze the developmental dynamics of the ovarian reserve in a mouse model. By establishing a 3D ovarian reconstruction imaging system, the authors report the temporal and spatial changes in follicle counts, oocyte sizes, and follicle localization across different reproductive stages. Based on these models, they propose that there exists a large inter-individual variation in oocyte number from early adulthood to aging, as well as a stable oocyte activation ratio across ages, inferred from oocyte size distribution. In addition, they claim that most growing oocytes are lost at the antral stage, with minimal atresia or oocyte death occurring between the primordial and secondary stages. Although the study provided several interesting points to refresh the field, the fundamental dataset underpinning these conclusions clearly contradicts well-established principles of ovarian development that have been repeatedly validated in the field. Consequently, the conclusions drawn from this study carry substantial uncertainty. Below are detailed major concerns and recommendations.

Major concerns:
1. The authors report a striking inter-individual variation in oocyte numbers during early reproductive life (PD35, i.e., 5 weeks), ranging approximately sixfold (from 500 to 3,000). However, numerous previous studies using traditional 2D histological approaches have carefully quantified oocyte numbers at comparable developmental stages and consistently demonstrated that such large variation does not exist, at least during early life (Faddy et al., 1983, PMID: 6822780; Durlinger et al., 1999, PMID: 10579345; Myers et al., 2004, PMID: 15129012; Johnson et al., 2004, PMID: 15014492; Reddy et al., 2008, PMID: 18239123; Zhang et al., 2021, PMID: 33953177). These studies span multiple laboratories and methodological backgrounds, underscoring the robustness of their findings.

Thus, the authors must reconcile this discrepancy. While whole-mount imaging offers enhanced spatial information, the light-sheet confocal microscopy used here—particularly at 10X magnification—has limited resolution. Moreover, dormant oocytes are transcriptionally and translationally quiescent, leading to weak and heterogeneous immunostaining signals across cells. Thus, a key concern is how the authors ensured that all oocytes were accurately captured under the current imaging conditions. This uncertainty casts substantial doubt on the reliability of the data and all subsequent conclusions drawn from them.

2. The authors state that ‘oocyte death is minimal during the primordial, primary, and secondary stages’. However, extensive evidence in the field has established that massive oocyte death occurs at the primordial follicle stage—a fundamental principle in ovarian biology (McGee and Hsueh, 2000 PMID: 10782364). If oocyte death truly did not occur during the transition from primordial to secondary follicles, it would imply that all activated primordial follicles successfully develop into secondary follicles, which is clearly impossible. In fact, the number of follicles decreases substantially at each developmental stage. Furthermore, numerous studies have documented early-stage atresia, particularly during the secondary and preantral stages (Byskov, 1974 PMID: 4824695; Adhikar and Liu, 2009 PMID: 19589950; Meng et al., 2018 PMID: 29767707; Zhou et al., 2022 PMID: 36107143). The authors’ interpretation therefore conflicts with well-validated developmental trajectories and requires substantial revision or re-examination of their detection methodology.

3. The use of light-sheet confocal microscopy for whole-mount imaging of the ovary has already been reported in the field (Faire et al., 2016, PMID: 25889274 and Gaylord et al., 2025, PMID: 41066539). Therefore, unless this study demonstrates a

substantial improvement in imaging resolution and accuracy, its methodological novelty is limited.

4. The authors correctly note that Anti-Müllerian Hormone (AMH) levels vary significantly among individuals and are commonly used as a clinical biomarker of ovarian reserve. However, AMH is secreted by granulosa cells of growing follicles and therefore reflects the number of developing follicles rather than the total ovarian reserve. A deeper understanding of ovarian physiology would clarify that AMH cannot represent total oocyte number. Consequently, the authors' interpretation of inter-individual variation in human ovarian reserve based on AMH levels is conceptually flawed.

5. Many of the quantitative analyses presented in the manuscript lack appropriate statistical evaluation (e.g., Fig. 2E; Fig. 3C–E; Fig. S3A–B; Fig. S5), which undermines the robustness of the conclusions. For instance, the authors report a bimodal distribution of oocyte diameters and suggest that this pattern reflects a developmental bottleneck or checkpoint. This is indeed an intriguing observation; however, the secondary peak in Fig. 2E appears weak, and the main peak of oocyte size is quite unstable, varying substantially between 50–70 μm. Such variability raises concerns about the reproducibility and reliability of the conclusion. Appropriate statistical tests (e.g., mixture modeling or Hartigan's dip test) are needed to confirm whether the observed pattern truly represents a bimodal distribution rather than random variation.

6. The spatial distribution analysis presented in Fig. 3 appears to rely on 2D projections of inherently 3D data (Fig. 3A–B), which obscures potential clustering patterns and spatial relationships. A genuine 3D analysis of oocyte localization would be essential to substantiate claims regarding follicle spatial organization.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
This is a well written manuscript, that talks to a substantial gap within the field - namely an investigation of follicle dynamics within intact ovaries. Based on AI-driven segmentation of oocytes within 3D images of mouse ovaries, supplemented with a differential equation-based model to capture the kinetics of oocyte activation and loss, the authors report a number of interesting observations:

1) that newly activated oocytes within transitioning follicles represent a fixed fraction of ovarian follicles regardless of age related decline in total oocyte numbers.
- I would suggest reconsidering the use of the term 'ovarian reserve' in lines 32-33, as the ovarian reserve is comprised of non-activated follicles, not the recently activated oocytes they report upon.
- I note that while this transitioning state is flagged as constant early in the paper, by figure 4 and within the mathematical modelling section, the classifications have been altered to bundle transitioning oocytes in with the primordials. It is not clear why the authors do this and some clarification would be beneficial, particularly given their own observations that this category does not change with age.
- also figure 4 B as the schematic of the model they are presenting should capture more of the spatial information their work is highlighting if possible. Particularly the idea that the number of transitioning follicles is constant across time.

2) a bimodal distribution of oocyte sizes that they interpret as being a bottleneck during oogenesis. In particular, the highest number of oocytes at all timepoints are primordial and under 16um, whereas there is another bottleneck around the early antral stage. The size ranges based on this form of imaging are very helpful for the field e.g. transitioning vs primary etc.

3) They report interesting spatial distributions e.g. of growing oocytes along the lateral ovarian axis and of local oocyte density being correlated with activation.
- It would be great to get a clarification around whether this increased oocyte density which correlates with activation is also seen at the lateral ovarian axis poles.
- In figure 3B is seems the primordial and transitioning follicles are at opposite sides of the ovaries in general, could the authors comment on this?

4) a threefold variation in isogenic animals from 5 weeks, prior to sexual maturity. Little left-right asymmetry was found under normal physiological conditions.
- could the authors speculate to the source of this large variation between isogenic animals?
- Figure S3B shows no statistics on the total oocyte number intra-individual variation presented though there are differentials as large as over 500+. Figure S3C would also benefit from having the follicle boundary highlighted using a dashed line.

5) demonstration of their clearing and imaging technical approach in human tissue.
- It is noticeable that the human cortical tissues presented are not analysed very deeply. It would improve their claims of clinical relevance to show that different oocyte sizes can be observed, or if they can comment on a the possibility of using their oocyte quantification from these cortical fragments to assess the patients ovarian reserve size. E.g. do the differences in human oocyte density shown in Figure S2C have any clinical meaning that can be discerned? Also could the authors comment on the oocyte diameters shown in the human data? The tissue should be a cortical strip and therefore contain the dormant primordial follicles, however the oocyte diameter indicates another bimodal distribution which might indicate that there are not many dormant oocytes. E.g. why is there a peak under 20um and another at 40um?
-given their own observations of variation within the isogenic mice, would the human samples not be expected to have even

more variation?

A major omission is that the details of the timecourse (e.g. ages analysed) are not given. The authors stage that 5 animals per stage are analysed, and it seems they looked at both left and right ovary for the majority of the animals, but this should be clearer.
- the schematic in Figure 1A is not as informative as it should be - particularly as it does not show that both left and right ovaries from each mouse was analysed. Similarly, the ages spanned should be indicated. It is not clear to me how many mice from which stage were analysed. In the text 5 weeks was an important timepoints and used to establish the pipelines, but I think the manuscript would benefit from

In general the statistics seem appropriate and are described in all context where error bars are shown.

Taken together, this work furthers our understanding of age-related oocyte decline within the ovary, provides valuable observational data from the mouse model system, and showcases how follicle counting can be modelled to make interesting predictions about the ovary.

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have now addressed all the issues I raised. The revised manuscript is much improved and will contribute significantly to our understanding of ovarian reserve dynamics during ageing. Specifically, the authors have substantially expanded the discussion to better explain how their findings fit within the existing literature on the subject, as requested by both myself and Reviewer 3. I recommend publication without reservation.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
Overall these revisions are thorough and comprehensive. There are a couple of outstanding matters / final corrections I would encourage the authors to consider:

- in addressing reviewer 3 comment 4, rather than 'AMH is used as a clinical marker of ovarian reserve' I would suggest ovarian 'performance' as we know that AMH levels reflect the number of growing follicles, not those dormant within the ovarian reserve.

- in addressing reviewer 4's comment regarding the use of a single category for both primordial follicles and transitioning follicles in their model, the authors have explained that their dynamical model is unable to distinguish the categories. In light of a major claim being that the transitioning fraction remains stable, this should be clearly stated in the text regarding the model. Similarly, a statement regarding how many transitioning follicles are captured in their datasets might be beneficial.

- I also note that the equations underpinning the model here are not provided even within the supplementary material.

(Remarks on code availability)

Version 2:

Reviewer comments:

Reviewer #5

(Remarks to the Author)
In this paper, the authors study ovarian follicle dynamics in mice. The authors use light-sheet microscopy to obtain detailed spatiotemporal dynamics of follicle dynamics. The authors also fit some of their data to a mathematical model.

The experimental results seem interesting and important. It seems that the main novelty and contribution is to resolve the three-dimensional structure of the ovaries. The use of AI for segmentation is a key step, given the high absolute numbers of ovarian follicles, as well as their division into various compartments based on their diameters. I have reviewed the mathematical model and it is mathematically valid. It is a simple model consisting of a system of linear ordinary differential equations. The model seems completely appropriate for this study and the fits to the data are adequate. The authors' use of

densities (see equation (1) of their supplementary note) is also appropriate, as it allows for comparison of ovaries of different volumes.

Minor point: line 51: ``Only about 400 oocytes are over a lifetime'' is missing a verb.

Minor point: line 220 and following: I suggest not giving these rates to 6 decimal places. This suggests a level of certainty and precision which is unwarranted.

(Remarks on code availability)

Response to Reviewers:

We thank all the reviewers for their critical reading of our manuscript and excellent suggestions. We were delighted that two of the three reviewers (Reviewers #2 and #4) were very supportive and found our work "rigorous and well-executed" "that talks to a substantial gap within the field." One of our reviewers (Reviewer #3) was concerned that the biological variability we reported was not in line with the literature. We now clarify that our data is indeed well in-line with previous studies, including those cited by the reviewer - the main issue was that the historical studies used Standard Error of the Mean (precision of the estimate) to report variation, meanwhile we use Standard Deviation (biological spread) and that our findings are consistent with existing literature once these statistical metrics are properly contextualized.

In response to the collective feedback, we have performed several new statistical analyses, expanded our Discussion and integrated our findings with recent literature. These additions support and strengthen our conclusions regarding the spatio-temporal dynamics of the ovarian reserve. Below we detail specific reviewer comments and our responses.

Major additions, new analyses, or changes that directly address reviewer comments are coloured in green in the main text. For other small changes, we did not track changes in this way.

Reviewer #2 (Remarks to the Author):

This study examines the spatio-temporal dynamics of oocyte loss with age. Although it is well established that oocyte number declines dramatically over the lifespan, prior quantification has largely relied on 2D sections with stereological extrapolation. Consequently, the 3D distribution of follicle loss across the whole ovary has remained uncertain, and important spatial subtleties may have been missed—an outstanding question in the field.

Here, the authors combine whole-ovary light-sheet imaging, AI-assisted segmentation, and mathematical modelling to quantify oocyte distributions in mouse ovaries across ageing. Their work uncovers several important observations: (1) the number of oocytes activated per cycle scales with the total ovarian oocyte pool; and (2) oocyte sizes display a biphasic distribution, likely reflecting a bottleneck during the handover from gonadotrophin-independent to gonadotrophin-dependent growth during oocyte development.

Overall, the work is rigorous and well executed, provides a valuable resource for the community, and adds nuance to the well-known finding that oocyte number decreases with age.

The manuscript would be strengthened by expanding the Discussion to situate these results alongside closely related recent studies. We suggest the following:

We thank the reviewer for their supportive comments and thoughtful suggestions! We incorporated all - please see details below.

Major comments

1. A recent publication — Gaylord et al., Science (October 2025) — addresses a very similar question, combining whole-ovary 3D imaging with single-cell transcriptomics to compare human and mouse ovaries across ageing, focusing on oocyte and follicle distributions and dynamics. The present manuscript would benefit from an explicit comparison with other whole-ovary 3D imaging/clearing pipelines, outlining protocol differences and how results align or differ. We also recommend discussing the spatial transcriptomics study by Lan et al., bioRxiv (December 2024), which examines the disruption of folliculogenesis with age in mice

We thank the reviewer for highlighting these recent studies. We now include explicit comparisons with Gaylord et al. in the Discussion, copied below for convenience:

"Recent whole-ovary imaging approaches have begun to overcome longstanding limitations of section-based follicle quantification[1-3]. In particular, Gaylord et al. combined three-dimensional imaging of oocyte nuclei with single-cell transcriptomics to compare mouse and human ovaries, providing an important cross-species framework to study ovarian ageing. Our measurements of oocyte number and age-related decline are in close agreement with those reported in that study, supporting the robustness of whole-mount imaging strategies for ovarian reserve assessment. However, the present work extends beyond global oocyte counts by explicitly leveraging the spatial and geometric information inherent to intact ovary imaging. By segmenting entire oocytes rather than nuclei, we are able to reconstruct oocyte size distributions, quantify local follicle density, and analyse spatial organization across the full ovarian volume. This enables us to identify principles of ovarian ageing that are not apparent from oocyte counts alone, including the maintenance of a fixed fraction of oocytes primed for activation across ages, spatial enrichment of follicles along the lateral ovarian axis, and a persistent bimodal distribution of oocyte sizes indicative of a developmental bottleneck during oogenesis."

Moreover, we now briefly discuss the spatial transcriptomics study by Lan *et al.* (bioRxiv, 2024) in the Discussion[4]. Although that study did not quantify oocyte numbers with age, it showed that reproductive ageing is marked by a breakdown of tissue-wide spatiotemporal coordination and niche organization. We cite this work to emphasize the complementary value of spatially resolved approaches for understanding ovarian ageing at the organ level.

"...Recent spatial transcriptomics of the ageing mouse ovary revealed that reproductive ageing is marked by a breakdown of tissue-wide spatiotemporal coordination of folliculogenesis and niche organization, complementing our study to highlight the importance of spatially resolved analyses for understanding ovarian ageing (Lan et al., 2024)."

In summary, our work goes far beyond whole-ovary oocyte counting that was reported in Gaylord et al: the key contributions of our study arise from the integration of whole-ovary 3D imaging with spatially resolved quantitative analysis and mathematical modeling**.** By segmenting intact oocytes (rather than nuclei) and leveraging full-organ spatial information, we quantify oocyte size distributions, local follicle density, and 3D spatial organization across the ovary and integrate these data with mathematical modeling. This allowed us to uncover principles that are not accessible through oocyte counts alone. Specifically, we show that

(i) a fixed fraction of oocytes is consistently primed for activation across ages and across a wide range of total ovarian reserve sizes, suggesting the existence of a tissue-scale homeostatic mechanism;

(ii) oocytes are spatially enriched along the lateral ovarian axis, with activation positively correlated with local follicle density, challenging classical density-inhibition models of follicle recruitment; and

(iii) oocyte size distributions are bimodal, revealing a developmental bottleneck at approximately 60 µm corresponding to the late secondary–early antral transition and the onset of gonadotropin responsiveness.

None of these spatial, quantitative, or modeling-based analyses were addressed in Gaylord et al.

2. The authors correctly note that inbred C57BL/6J mice provide a useful model for studying age-related oocyte loss in genetically uniform populations. However, the ovarian ageing field is primarily concerned with human ovaries. Although the present study demonstrates the pipeline on human ovarian cortex, the sample size is too small for much analysis. We recommend adding a discussion section that outlines key similarities and differences between mouse and human ovaries and ageing (e.g., initial follicle endowment and activation rates; cycle dynamics; stromal architecture; lifespan and rate of depletion). This will clarify what can and cannot be extrapolated to humans or to universal mammalian principles from the mouse-based findings.

On this note, this discussion could be strengthened by acknowledging that mice have multiple ovulations per cycle, whereas humans typically have a single ovulation. As the authors propose that their findings may be applicable across mammals, this fundamental difference could influence the rate of oocyte depletion with age and potentially affect other features such as the spatial distribution of follicles within the ovary.

We agree with the reviewer that species-specific differences between mouse and human ovaries should be explicitly discussed to clarify the scope and generality of our findings. In response, we have added the following paragraph to the Discussion:

“Translating our findings to human ovarian aging requires a careful consideration of species-specific differences. Humans and mice share the fundamental principle of a non-renewable oocyte pool that declines with age, yet they differ in scale and timing: Humans are endowed with 0,5 to 1 million oocytes at birth, losing more than half by puberty[5]. Mice exhibit similar depletion but with smaller absolute numbers, starting with approximately 5,000–10,000 oocytes[6]. Furthermore, mice are poly-ovulatory with short (4–5 day) estrous cycles, whereas humans are typically mono-ovulatory with a ~28-day menstrual cycle. These differences likely influence follicle depletion dynamics. Nevertheless, our finding that the transitioning fraction remains stable in ovaries could represent a universal mammalian principle of homeostatic recruitment. In both species, most follicles remain quiescent, with only a small proportion undergoing activation at any given time to ensure reproductive longevity. Moreover, the substantial heterogeneity of oocyte numbers even among isogenic mice highlights the challenge inherent in analysing the human ovarian reserve and underscores the need for large cohorts to have robust conclusions.”

Minor comments

1. The statement “Anti-Müllerian hormone (AMH), a clinical marker of ovarian reserve, exhibits substantial variability among humans…” (line 121) should be supported by an appropriate reference.

We are sorry for this omission - we have now added an appropriate reference[7].

2. The authors report considerable heterogeneity in oocyte number even among genetically identical, age-matched individuals. It would be helpful if the Discussion addressed possible explanations for this variability.

We thank the reviewer for the suggestion, we now added the following paragraph to the Discussion:

“The high variation in oocyte numbers among isogenic, age-matched mice is already present before sexual maturation, suggesting an origin in early development. Several factors could explain this variability. First, the initial reserve size may be influenced by the efficiency of primordial germ cell (PGC) migration and proliferation during colonization of the genital ridge; even slight differences in founding populations or their migratory success can lead to a different baseline. Second, the subsequent wave of oocyte loss during germ cell nest breakdown and early postnatal life may vary in intensity between individuals. Notably, low-reserve ovaries also exhibit reduced total volume and fewer growing oocytes, suggesting that these early events impact the overall function of the organ.”

3. For consistency throughout the manuscript, the authors could consider changing the labelling of oocytes with anti-DDX4 and TO-PRO-3 in Figure S3C to match that used in the main figures, i.e. DDX4 in magenta and DNA in green.

We thank the reviewer for pointing this out, we amended Fig S3C.

4. Regarding Figure 2B, the statement that “the absolute numbers of primordial, transitioning, and growing oocytes all decreased as the ovarian reserve diminished” is not strictly accurate, as at week 22 the counts across all classes appear to remain the same or increase relative to week 10. Do the authors have an explanation for this? It would be helpful to address in the Discussion.

We thank the reviewer for pointing this out. Indeed, the counts at 22 weeks appear similar to (or slightly higher than) those at 10 weeks in some oocyte classes. However, we do not interpret this as a true increase, but rather as a consequence of substantial inter-individual variability in oocyte numbers within age-matched animals. In this context, differences between adjacent time points (e.g., 10 vs 22 weeks) are not always statistically resolvable with the sample sizes we used. A similar pattern has also been reported by Gaylord et al. (mentioned above), who observed no significant differences in total, primordial, or growing oocyte numbers between 2, 4 and 6 months of age (Figs. 1G and S2D in Gaylord), although there was a downwards trend.

In response, we have revised the text, and the relevant section now reads:

“As expected, oocyte numbers declined significantly with age (Fig. 2B), and their relative contributions to the total population shifted over time: primordial oocytes decreased from ~70% to ~55% of the pool, while growing oocytes displayed the opposite trend, increasing from ~15% to ~30% (Fig. 2C).”

We also clarify this point in the Figure 2B legend alongside the statistical analysis.

"Please note that due to substantial interindividual heterogeneity in oocyte numbers, the sample sizes used in this study were not enough to resolve the decline in oocyte numbers between 10 and 22 weeks-old animals".

5. The sentence "To determine whether external reproductive activity impacts oocyte depletion, we asked whether suppression of ovulation could slow the loss of the ovarian reserve" (line 171) is somewhat ambiguous. The authors should clarify that continuously mated females ovulate less frequently than virgins, to make the rationale for this comparison explicit.

We thank the reviewer#2 for the suggestion. We changed the sentence to:

"*We next asked whether suppression of ovulation could slow the loss of the ovarian reserve. Ovulation suppression has been shown to reduce chromosomal mis-segregation in aging oocytes, thereby improving their quality and delaying functional decline[22]. However, its effect on the quantity of remaining oocytes has not been clearly established. To test this, we compared the ovarian reserve in females subjected to repeated mating with that of age-matched virgin controls, as pregnancy suppresses ovulation."*

6. It is unclear why violin plots are used in Figure S4 instead of bar plots, as in the main figures. Could the authors justify this choice? Furthermore, the statement that "relatively lower intra-individual differences between the left and right ovaries of the same animal across all age groups" is not fully supported by the data, which appear to show increased variability again at week 22. Do the authors have an explanation for this?

We thank the Reviewer for these helpful comments.

For harmony across the manuscript, we replaced the violin plots in FigS4 with box plots to match the rest of the manuscript.

With respect to intra-animal asymmetry, we agree that our original phrasing was unclear. Our intention was to emphasize that variability **between animals** is substantially greater than variability (**between left and right ovaries) within the same animal**. Motivated by their, and reviewer 4's similar comments on intra- and inter-individual variability, we now added a new analysis to the manuscript (Supplementary Figure S4C), also copied below.

To directly address the question of whether variation in oocyte number is primarily driven by differences between individuals or by differences between ovaries within the same individual, we performed a quantitative analysis.

Specifically, we fit the regression model:

$$Oocyte\_count = \beta \times animal\_id + \varepsilon$$

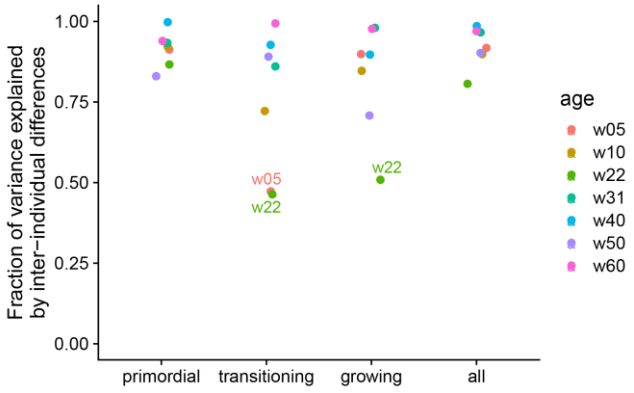
Here, the term **β** estimates differences between animals in their total oocyte counts across both ovaries. We then used the model's coefficient of determination ($\mathbf{R^2}$) as a summary of how much of the overall

variability in oocyte counts is explained by differences between animals**,** as opposed to residual variability (which includes within-animal, between-ovary differences and other sources).

Across developmental stages and ages, the median R^2 is 0.89, indicating that approximately 89% of the total variance in oocyte number is attributable to inter-individual differences. Thus, while some within-animal ovary-to-ovary differences can be large in absolute terms (e.g., >500 oocytes), they typically account for a smaller fraction of the total variation than inter-individual differences. We note limited exceptions for transitioning and growing oocytes at specific ages, where within- and between-individual contributions are comparable, and we highlight these cases in Supplementary Figure S4C.

We acknowledge that at 22 weeks the left ovary tends to contain slightly more oocytes (approximately 25% on average). However, this effect is not observed at 5, 10, or 31 weeks. Given its absence at other ages and the current sample size, we cannot determine whether this reflects biological asymmetry or stochastic variation. We have therefore revised the text to describe this observation more cautiously and avoid overinterpretation.

The relevant section has been clarified accordingly in the manuscript.



FigureR1 (also FigS4C). The coefficients of determination (R2) estimating the fraction of variation in the number of oocytes in each ovary explained by differences between individuals. The remaining, unexplained variance (1-R2) estimates the fraction of variation explained by intra-individual differences between the two ovaries within each individual.

7. Figure S4 is the only figure where the statistical test is described in the legend. Are statistical tests used elsewhere in the manuscript, for example to assess whether oocyte number is significantly reduced between age groups? Where applicable, these should be clearly stated in the corresponding figure legends for consistency.

We thank the reviewer for the suggestion; in the revised version we update all relevant figure legends with statistical analysis. These analyses further validate our conclusions and demonstrate significant differences where previously noted (e.g., the reduction in oocyte number between age groups).

Reviewer #3 (Remarks to the Author):

The current manuscript integrates light-sheet high-resolution whole-mount imaging, tissue clearing, artificial intelligence, and mathematical modeling to analyze the developmental dynamics of the ovarian reserve in a mouse model. By establishing a 3D ovarian reconstruction imaging system, the authors report the temporal and spatial changes in follicle counts, oocyte sizes, and follicle localization across different reproductive stages. Based on these models, they propose that there exists a large inter-individual variation in oocyte number from early adulthood to aging, as well as a stable oocyte activation ratio across ages, inferred from oocyte size distribution. In addition, they claim that most growing oocytes are lost at the antral stage, with minimal atresia or oocyte death occurring between the primordial and secondary stages. Although the study provided several interesting points to refresh the field, the fundamental dataset underpinning these conclusions clearly contradicts well-established principles of ovarian development that have been repeatedly validated in the field. Consequently, the conclusions drawn from this study carry substantial uncertainty. Below are detailed major concerns and recommendations.

We thank the reviewer for the opportunity to clarify our discovery.

Importantly, our measurements are in good agreement with established literature, including the studies the reviewer cited below. We believe the apparent misunderstanding arises primarily from how variability is represented in many prior studies, where small sample sizes and reporting of standard errors (instead of standard deviations) obscure the true extent of inter-individual heterogeneity. By contrast, our whole-organ 3D quantification across a large number of ovaries reveals the full distribution of ovarian reserve sizes, which is essential for interpreting age-dependent trends and for evaluating the robustness of inferred dynamics. We provide a detailed point-by-point response below.

Major concerns:

1. The authors report a striking inter-individual variation in oocyte numbers during early reproductive life (PD35, i.e., 5 weeks), ranging approximately sixfold (from 500 to 3,000). However, numerous previous studies using traditional 2D histological approaches have carefully quantified oocyte numbers at comparable developmental stages and consistently demonstrated that such large variation does not exist, at least during early life (Faddy et al., 1983, PMID: 6822780; Durlinger et al., 1999, PMID: 10579345; Myers et al., 2004, PMID: 15129012; Johnson et al., 2004, PMID: 15014492; Reddy et al., 2008, PMID: 18239123; Zhang et al., 2021, PMID: 33953177). These studies span multiple laboratories and methodological backgrounds, underscoring the robustness of their findings.

Thus, the authors must reconcile this discrepancy. While whole-mount imaging offers enhanced spatial information, the light-sheet confocal microscopy used here—particularly at 10X magnification—has limited resolution. Moreover, dormant oocytes are transcriptionally and translationally quiescent, leading to weak and heterogeneous immunostaining signals across cells. Thus, a key concern is how the authors ensured that all oocytes were accurately captured under the current imaging conditions. This uncertainty casts substantial doubt on the reliability of the data and all subsequent conclusions drawn from them.

We thank the Reviewer for raising this important point. We believe the apparent discrepancy with previous studies is primarily due to differences in statistical representation rather than true disagreement in oocyte numbers.

Many (all but one) of the cited studies present oocyte numbers as bar plots with **Mean ± SEM (Standard Error of the Mean).** In contrast, in this study we have followed evolving standards in statistical reporting to plot our data providing the **Standard Deviation (SD) along with the individual data points**. We contend that SD is the more appropriate metric for representing actual biological spread and natural diversity within a population, as estimates of the standard deviation remain stable across populations of different sizes. In contrast, the SEM by definition decreases as populations increase (as the square root of the population size), conflating technical aspects of the particular experiment with the actual biological variation. To demonstrate this point and illustrate our preference for using the standard deviation, we have re-plotted our results as reported in the studies cited by the reviewer: the reviewer can appreciate that the variability reported in our manuscript seems much smaller when plotted as mean +/- SEM and appears similar to that reported in the cited literature (Figure R2 below).

Second, to further enable direct comparison, we compiled values from the studies suggested by the Reviewer in Table R1. Across different strains and laboratories, reported oocyte numbers and variability are comparable to ours when similar summary metrics are used. **We would also like to emphasize that a foundational study also cited by the reviewer, Myers et al. (2004), explicitly reported more than a 10-fold variability in oocyte numbers in literature** and suggested a specific methodology to minimize this variance. However, even when using their advised methods, their data show that 100-day-old ovaries tend to have *more* oocytes than 70-day-old ovaries (2227 **±** 101 vs. 1930 **±** 286). Given that the decline of the oocyte pool with age is a well-accepted biological parameter, this inversion strongly underscores the significant natural heterogeneity in oocyte numbers that exists even within controlled experimental groups.

Third, high inter-individual heterogeneity is also supported by more recent studies using modern 3D imaging and statistical modeling. For example, Gaylord et al. (Science, 2025) reported >4-fold variation in total oocyte numbers in 2-month-old mice, and Yin & Spradling (2024, bioRxiv) reported ~2-fold differences as early as 2 weeks[1,8].

Finally, the variability observed in our dataset is highly unlikely to arise from systematic under-detection due to imaging resolution or heterogeneous staining. The imaging resolution used in this study (0.867 µm/pixel) is more than sufficient to detect oocytes (~15 µm diameter). In addition, the oocyte marker we used is widely validated and has been extensively employed in both 2D histological and modern 3D studies[8-17]. Importantly, we observe a robust positive correlation between primordial and growing oocyte numbers across ovaries (Figure R3; Spearman's $\rho = 0.74–0.97$, all $p < 0.05$). If primordial oocytes were being missed in a systematic or stochastic manner, such a strong relationship with growing follicle counts would not be expected (Figure R3). Instead, ovaries with low primordial counts also consistently exhibit low growing follicle numbers, consistent with true biological differences in ovarian reserve.

Together, these analyses indicate that our results are quantitatively consistent with the literature and that the observed inter-individual variability reflects genuine biological heterogeneity rather than methodological artifacts. We have revised the Discussion accordingly also to address the source of this heterogeneity.

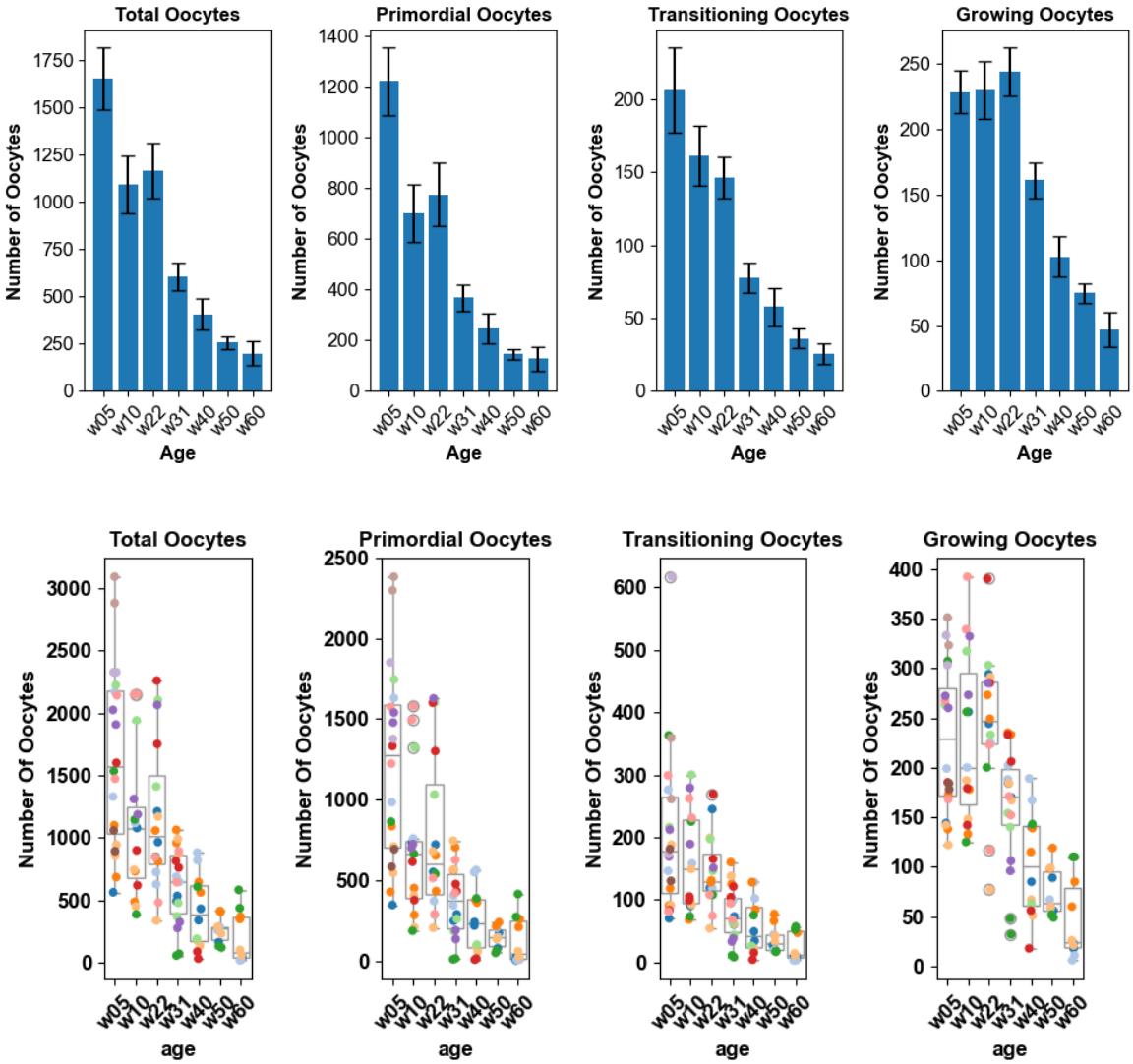


Figure R2. Comparison of statistical representations of oocyte quantifications. (A) Data from our manuscript, data represented as individual data points with Mean ± SD (Standard Deviation). **(B)** Identical dataset from (A) represented as Mean ± SEM (Standard Error of the Mean). When presented using this metric, our data align closely with the tight distributions and precision reported in the histological studies cited by the Reviewer (e.g., Faddy et al., 1983; Zhang et al., 2021). The representation in our manuscript highlights the significant inter-individual biological variation. Note that while SEM (B) provides a measure of how far the sample mean is likely to be from the population mean, SD (A) is the appropriate metric for describing the actual biological diversity and spread within a cohort of animals.

Source	Age	Strain	Note	Primordial Oocyte Number +/- Standard Error of the Mean
our study (data at 5 weeks)	35 days	C57BL/6J		1220 ± 133
our study (data at 10 weeks)	70 days	C57BL/6J		699 ± 114
Faddy et al., 1983, PMID: 6822780	100 days	strain A	data extracted from graph	2800 ± 150
Faddy et al., 1983, PMID: 6822780	100 days	CBA	data extracted from graph	1000 ± 100
Faddy et al., 1983, PMID: 6822780	100 days	Strain A x CBA	data extracted from graph	3900 ± 100
Durlinger et al., 1999, PMID: 10579345	25 days	AMH(+/-)	data extracted from graph	3700 ± 200
Myers et al., 2004, PMID: 15129012	70 days	C57BL/6/128SvEv	data stated in the text	1930 ± 286
Myers et al., 2004, PMID: 15129012	100 days	C57BL/6/128SvEv	data stated in the text	2227 ± 101
Johnson et al., 2004, PMID: 15014492	30 days	C57BL/6	data extracted from graph	2000 ± 200
Reddy et al., 2008, PMID: 18239123	35 days	Gdf9-Cre(-/-);PtenloxP/loxP	data extracted from graph	500 ± 100

Source	Age		Note	Total Oocyte Number +/- Standard Deviation
our study (data at 5weeks)	35 days	C57BL/6J		1655.75 ± 732.60
Zhang et al., 2021, PMID: 33953177	35 days	*RdxloxP/loxP*	data extracted from graph	3000 ± 500

Table R1. Comparison of oocyte counts across literature and current study.

Summary of primordial (above) and total (below) oocyte quantification reported in the studies suggested by the Reviewer. To ensure a direct comparison with our dataset, values were compiled from the closest available developmental time points.

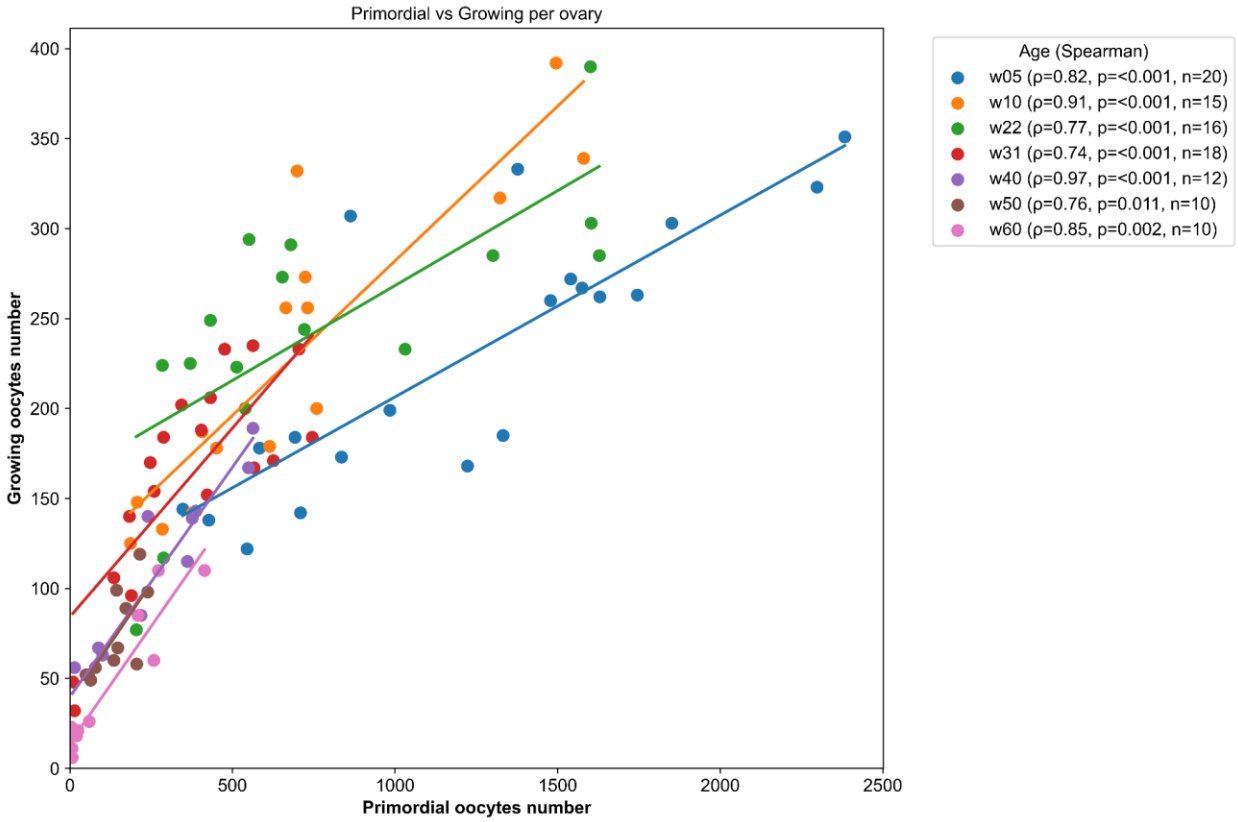


Figure R3. Correlation between primordial and growing oocytes per ovary across ages. Scatter plot showing the relationship between the number of primordial oocytes and the number of growing oocytes per ovary. Each dot represents one ovary and colors indicate age groups. Lines represent linear regression fits for visualization. Spearman's rank correlation coefficient (ρ), p-value, and sample size (n) are indicated in the legend for each age group. A strong positive correlation across ages demonstrates that ovaries with fewer primordial oocytes consistently contain fewer growing oocytes, supporting the robustness of oocyte detection and quantification.

2. The authors state that 'oocyte death is minimal during the primordial, primary, and secondary stages'. However, extensive evidence in the field has established that massive oocyte death occurs at the primordial follicle stage—a fundamental principle in ovarian biology (McGee and Hsueh, 2000 PMID: 10782364). If oocyte death truly did not occur during the transition from primordial to secondary follicles, it would imply that all activated primordial follicles successfully develop into secondary follicles, which is clearly impossible. In fact, the number of follicles decreases substantially at each developmental stage. Furthermore, numerous studies have documented early-stage atresia, particularly during the secondary and preantral stages (Byskov, 1974 PMID: 4824695; Adhikar and Liu, 2009 PMID: 19589950; Meng et al., 2018 PMID: 29767707; Zhou et al., 2022 PMID: 36107143). The authors' interpretation therefore conflicts with well-validated developmental trajectories and requires substantial revision or re-examination of their detection methodology.

We thank the reviewer for giving us the chance to clarify our findings.

The reviewer is correct that there is a well-established, substantial wave of oocyte loss at the primordial follicle stage during fetal and early neonatal development. In mice, this occurs predominantly around birth and the first postnatal days (approximately P0–P4), and is widely documented as a key feature of ovarian development[18]. However, these developmental windows occur well before the earliest time point examined in our study (5 weeks of age). Therefore, our statement refers specifically to adult ovaries and does not dispute the extensive primordial follicle loss that occurs during perinatal establishment of the ovarian reserve.

In addition, several of the studies cited by the reviewer are consistent with our finding that oocyte death is rare in primordial, primary, and secondary follicles in adulthood:

- In Byskov, 1974 PMID: 4824695 the study was designed to assess the kinetics of atresia in antral follicle and not in primary/secondary follicle, here we paste their abstract:

  "The purpose of the present investigation was (1) to define morphologically the different stages through which a large follicle without signs of atresia passes as it transforms to a completely atretic one, and to investigate whether the stages of atresia are consecutive in the atretic process"

  Consequently, these results cannot be used to characterize oocyte death in small follicles.

- In Zhou et al., 2022 PMID: 36107143, the authors only have one figure (Figure 4) for oocyte death, and in that they explicitly state

  "Cleaved (activated) caspase-3 immunoreactivity … was not readily apparent in primordial and transitioning follicles".

  While they counted "DAB+ cell debris" as a proxy for death, their lack of apoptotic markers in primordial and transitioning follicles directly aligns with our findings.

- In Meng et al., 2018 PMID: 29767707, the authors explicitly mention that "preantral and preovulatory follicles rarely undergo atresia". Below we attach the relevant section.

Preantral follicles

The vast majority of follicles present at birth will degenerate before reaching the point of ovulation. The highest incidence of follicular atresia is observed when follicles become dependent on FSH, at the early antral follicle stage. Preantral and preovlatory follicles rarely undergo atresia [3]. It is thus not surprising that the percentage of preantral atretic follicles in the present study was low (Figure 1).

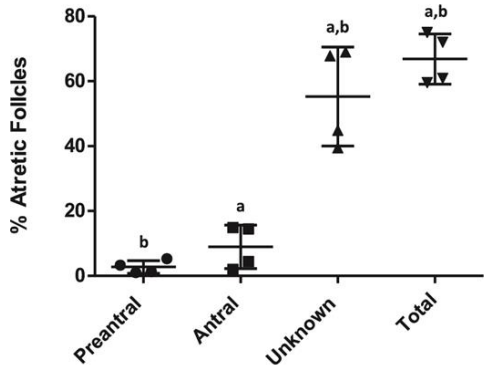


Figure 1. Percentage of atretic preantral and antral follicles, atretic follicles of unknown origin and total percentage of atretic follicles. Data were analyzed by one-way ANOVA followed by Tukey post hoc test. Values are expressed as mean ± SD, n = 4; (a) significantly different from preantral follicles, (b) significantly different from antral follicles, $P < 0.05$.

Therefore, Meng et al., 2018 also support our conclusions.

Our findings are further strengthened by several independent lines of evidence:

- Spatial Transcriptomics: Lan et al. (*bioRxiv*, 2024) demonstrated that the transition from healthy to atretic transcriptional states occurs predominantly in antral granulosa cells (Fig. 3K).[4]
- Lack of Apoptotic Markers: A dedicated study on cell death in primordial follicles by Tingen et al. (2009) failed to find apoptotic markers in the primordial pool.[19]
- Survival Signaling: In early development, factors such as GDF-9 are known to suppress apoptosis, supporting the survival of the pre-antral pool until the handover to gonadotropin-dependence.[20]

Altogether, minimal atresia during prenatal follicle development is consistent with the existing literature. Our high-resolution 3D datasets enable us to propose a model that suggests oocyte death is frequent in antral follicles in adults but rare in the primordial and pre-antral stages. Importantly, our model's conclusion is supported by the literature.

3. The use of light-sheet confocal microscopy for whole-mount imaging of the ovary has already been reported in the field (Faire et al., 2016, PMID: 25889274 and Gaylord et al., 2025, PMID: 41066539). Therefore, unless this study demonstrates a substantial improvement in imaging resolution and accuracy, its methodological novelty is limited.

We thank the reviewer for the opportunity to clarify the methodological novelty of our approach.

First, we would like to clarify a technical point: Both of the studies by Faire et al. (2016) and Gaylord et al. (2025) did not employ light-sheet (SPIM) microscopy in mouse ovaries, but rather used conventional point-scanning confocal microscopy[1,3]. Light-sheet microscopy enables volumetric imaging with reduced photobleaching and improved optical sectioning of intact tissues, which is critical for accurate whole-mount ovarian analysis.

Second, both previous studies quantified oocyte nuclei only, without incorporating cytoplasmic volume measurements. As follicle staging critically depends on oocyte size and cytoplasmic growth, nuclear counts alone do not allow accurate discrimination between developmental stages.

Importantly, we introduce a highly accurate in-house machine learning–based analysis pipeline specifically trained for oocyte detection. In contrast to prior studies relying on commercial software, our approach enables automated volumetric quantification of oocytes with precision and reproducibility.

Finally, the key novelty of our work lies in the integration of whole-ovary 3D imaging with spatially resolved quantitative analysis and mathematical modeling. By segmenting intact oocytes (rather than nuclei) and leveraging full-organ spatial information, we quantify oocyte size distributions, local follicle density, and 3D spatial organization across the ovary and integrate these data with mathematical modeling. This allowed us to uncover principles that are not accessible through oocyte counts alone. Specifically, we show that

(i) a fixed fraction of oocytes is consistently primed for activation across ages and across a wide range of total ovarian reserve sizes, suggesting the existence of a tissue-scale homeostatic mechanism;

(ii) oocytes are spatially enriched along the lateral ovarian axis, with activation positively correlated with local follicle density, challenging classical density-inhibition models of follicle recruitment; and

(iii) oocyte size distributions are bimodal, revealing a developmental bottleneck at approximately 60 µm corresponding to the late secondary–early antral transition and the onset of gonadotropin responsiveness.

Together, these technical and analytical advances establish the methodological novelty and added value of our approach relative to prior whole-mount studies.

4. The authors correctly note that Anti-Müllerian Hormone (AMH) levels vary significantly among individuals and are commonly used as a clinical biomarker of ovarian reserve. However, AMH is secreted by granulosa cells of growing follicles and therefore reflects the number of developing follicles rather than the total ovarian reserve. A deeper understanding of ovarian physiology would clarify that AMH cannot represent total oocyte number. Consequently, the authors' interpretation of inter-individual variation in human ovarian reserve based on AMH levels is conceptually flawed.

We thank the reviewer for spotting this point. Although AMH is often used as a marker of ovarian reserve, the reviewer correctly notes that AMH primarily reflects the size of the growing oocyte pool. We have therefore corrected the text to refer to the activated ovarian reserve rather than the total ovarian reserve. Now the text reads as:

"Anti-Müllerian hormone (AMH) reflects the pool of growing follicles and is used as a clinical marker of ovarian reserve.[21] AMH exhibits substantial variability among humans…"

5. Many of the quantitative analyses presented in the manuscript lack appropriate statistical evaluation (e.g., Fig. 2E; Fig. 3C–E; Fig. S3A–B; Fig. S5), which undermines the robustness of the conclusions.

We thank the reviewer for highlighting the need for additional statistics. In the revised manuscript, we have added new analyses and statistical tests for all the data noted by the reviewer, and many others. These new analyses supported all of our conclusions and are described in the main text, as well as in the expanded Statistical Methods section. New analyses figures are incorporated in the updated main Figures 2 and 3, as well as in Supplementary Figures S3, S4, and S6.

For instance, the authors report a bimodal distribution of oocyte diameters and suggest that this pattern reflects a developmental bottleneck or checkpoint. This is indeed an intriguing observation; however, the secondary peak in Fig. 2E appears weak, and the main peak of oocyte size is quite unstable, varying substantially between 50–70 µm.

Such variability raises concerns about the reproducibility and reliability of the conclusion. Appropriate statistical tests (e.g., mixture modeling or Hartigan's dip test) are needed to confirm whether the observed pattern truly represents a bimodal distribution rather than random variation.

We thank the Reviewer for this important suggestion and agree that formal statistical testing is important to substantiate our findings on bimodality.

The secondary peak centered around ~60µM certainly contains fewer oocytes than the main peak around 15µM. However, the lower overall abundance of oocytes at this size does not make the peak weaker in the sense of being less supported statistically. In the revised manuscript, we highlight the separation between these populations in two ways:

First, we provide a better visualization of the data in Figure S3F, which replots the same data as Fig 2E on logarithmic axes which provide a more convenient scale for comparing the two peaks.

Second, we demonstrate the statistical significance of the two peaks by fitting the entire distribution with a Gaussian Mixture model, which posits that the distribution emerges from the pooled contribution of multiple underlying gaussian distributions. This analysis identifies the bimodal distribution of oocytes as being generated from the contribution of three Gaussians, one centered at ~15, another at ~18, and the third at ~60µM. Because the second gaussian overlaps with the first, it does not generate a third peak in the distribution. We obtained statistical support for the robustness of our evidence for the peak at 60µm by calculating the boot-strapped log-likelihood ratios for the superiority of the three-gaussian fit over 2 or 1 gaussian mixture models.

These analyses confirm that the oocyte distribution is bimodal in ovaries and contains a statistically distinct subpopulation around ~60 µm and that the observed structure is not attributable to random variation in peak position.

6. The spatial distribution analysis presented in Fig. 3 appears to rely on 2D projections of inherently 3D data (Fig. 3A–B), which obscures potential clustering patterns and spatial relationships. A genuine 3D analysis of oocyte localization would be essential to substantiate claims regarding follicle spatial organization.

We thank the reviewer for highlighting this point. The analysis is not done on projected images but on the 3D images, only for representation purposes the data are projected and presented as 2D. We now explain this part better into the text:

We began by generating three-dimensional isosurface renderings of each ovary, which revealed a consistent, stereotypical bean-like morphology across samples. Importantly, all subsequent measurements were performed directly in three-dimensional space rather than on projected images, thereby preserving the native spatial relationships within the tissue. These renderings clearly delineated the concave medial side of the ovary, known as the hilum—the entry point for nerves and blood vessels—which served as a reproducible anatomical landmark across animals.

To systematically quantify the radial organization of oocytes, we developed a spatial framework grounded in the intrinsic geometry of each ovary. For every sample, we defined the long and short axes of the organ and fit a spline along the central longitudinal axis to establish an internal reference line, or “ruler,” extending through the ovary. Using this reference, the three-dimensional position of each oocyte was expressed in a normalized coordinate system, allowing angular position to be measured relative to the ovary’s internal axis. This approach enabled us to capture spatial distribution patterns with full three-dimensional fidelity and to compare oocyte organization across animals and age groups independent of differences in ovary size or orientation.

Reviewer #4 (Remarks to the Author):

This is a well written manuscript, that talks to a substantial gap within the field - namely an investigation of follicle dynamics within intact ovaries. Based on AI-driven segmentation of oocytes within 3D images of mouse ovaries, supplemented with a differential equation-based model to capture the kinetics of oocyte activation and loss, the authors report a number of interesting observations:

We thank the reviewer for their supportive feedback!

1) that newly activated oocytes within transitioning follicles represent a fixed fraction of ovarian follicles regardless of age related decline in total oocyte numbers.
- I would suggest reconsidering the use of the term 'ovarian reserve' in lines 32-33, as the ovarian reserve is comprised of non-activated follicles, not the recently activated oocytes they report upon.

Thanks for pointing this out - we now changed the wording in the abstract, to the "total oocyte pool".

- I note that while this transitioning state is flagged as constant early in the paper, by figure 4 and within the mathematical modelling section, the classifications have been altered to bundle transitioning oocytes in with the primordials. It is not clear why the authors do this and some clarification would be beneficial, particularly given their own observations that this category does not change with age.
- also figure 4 B as the schematic of the model they are presenting should capture more of the spatial information their work is highlighting if possible. Particularly the idea that the number of transitioning follicles is constant across time.

We thank the Reviewer for raising this point; we agree that clarification is needed.

In our static 3D dataset, we occasionally capture oocytes morphologically transitioning from primordial to primary, which we label as "transitioning" in Figures 2 and S3. This is a special case where a transitional morphology can be directly observed. However, intermediate states for later transitions (primary→secondary, secondary→antral) are not readily identifiable in our dataset.

We use dynamical modelling to overcome this limitation in our static data: we computationally explore the dynamics of transitions between all stages using our dynamical model. Our approach allows us to infer transition and death rates from static measurements without the need to observe transitioning cells directly. We use our model to predict static observables (the number of oocytes in each stage) while inferring dynamical parameters (transition and death rates). At no point do we need to directly observe transitioning oocytes.

Nevertheless, in response to the reviewer's comment, we revisited this design choice and re-ran our dynamical model including an explicit transitioning state. The model fitting procedure identified a large co-variance between the inferred transition parameters between "primordial" and "transitioning" cell type; in practice, many different combinations of transition and death parameters reproduced similarly monotonic trajectories for primordial and transitioning cells. As a result, the fitted parameters exhibited strong co-variation and large uncertainty. The covariance and correlation matrices (Table R2) demonstrate near-perfect correlations between certain primordial/transitioning parameters (e.g., correlations approaching ±1), and the system shows poor conditioning (cond(J) ~ 10^4; cond(J^TJ) ~ 10^8), indicating non-

identifiability. Thus, while the model can fit the stage distributions, the decomposition of outgoing flow into separate transition and death terms becomes unstable without imposing additional constraints.

For this reason, in the final modeling framework we conservatively bundle transitioning oocytes with the primordial pool. This does not imply that the categories are biologically identical. Rather, it reflects a data-driven modeling decision that yields a stable and interpretable parameterization given the resolution and temporal sampling of the current dataset.

With denser early time-point sampling or independent constraints on specific rate parameters, future studies should be able to model this intermediate compartment explicitly. We have clarified this rationale in the revised manuscript.

	p2 (Transitioning -> Primary)	**p3 (Primary -> Secondary)**	**p4 (Secondary -> Antral)**	d1 (Primordial death)	d2 (Transitioning death)	d3 (Primary death)	d4 (Secondary death)	d5 (Antral exit)
p1 Primary -> Transitioning	0.71	0.23	0.21	1	1	0.44	0.23	0.21
p2 (Transitioning -> Primary)		0.63	0.17	0.71	0.72	0.83	0.21	0.17
p3 (Primary -> Secondary)			0.04	0.23	0.23	0.95	0.02	0.04
p4 (Secondary -> Antral)				0.21	0.21	0.04	1	1
d1 (Primordial death)					1	0.44	0.23	0.21
d2 (Transitioning death)						0.44	0.23	0.21
d3 (Primary death)							0.1	0.04
d4 (Secondary death)								1

Table R2 The correlation matrix of parameter estimates from dynamical model including a "transitioning" state in between primordial and primary oocytes. High values (shaded red) indicate parameters that cannot be distinguished from each other, i.e non-identifable aspects of the model.

2) a bimodal distribution of oocyte sizes that they interpret as being a bottleneck during oogenesis. In particular, the highest number of oocytes at all timepoints are primordial and under 16um, whereas there is another bottleneck around the early antral stage. The size ranges based on this form of imaging are very helpful for the field e.g. transitioning vs primary etc.

Thank you!

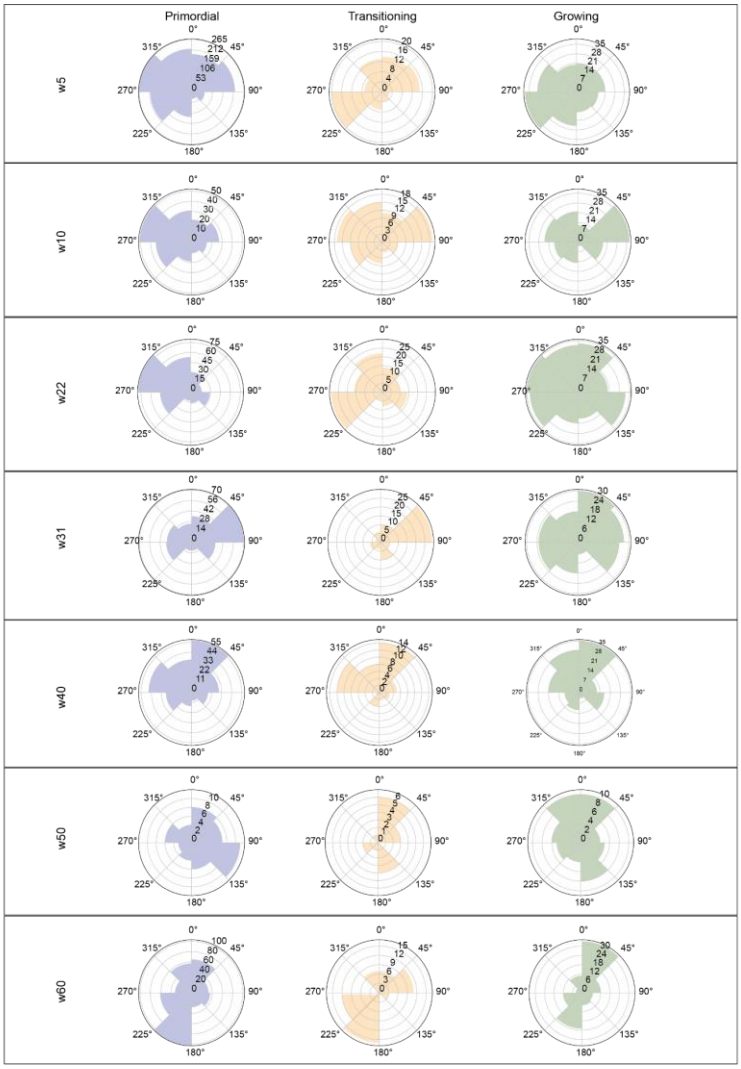
3) They report interesting spatial distributions e.g. of growing oocytes along the lateral ovarian axis and of local oocyte density being correlated with activation.
- It would be great to get a clarification around whether this increased oocyte density which correlates with activation is also seen at the lateral ovarian axis poles.

We thank the reviewer for this comment. We found the suggestion a bit unclear and interpreted it as asking whether transitioning (i.e., activated) oocytes are also enriched at the lateral ovarian axes.

To clarify this point, we refined our spatial analysis, taking advantage of the observed symmetry along the left–right lateral ovarian axis. By calculating the angular distance of oocytes using a sine transformation of the angle, we obtained a clearer radial distribution pattern across primordial, transitioning, and growing oocytes. Specifically, all oocyte populations are depleted at the hilum (sin(angle/2) = 0) and show a consistent increase in density toward the lateral ovarian axis (sin(angle/2) = 1). These refined results are now presented in the new Figure 3D.

- In figure 3B is seems the primordial and transitioning follicles are at opposite sides of the ovaries in general, could the authors comment on this?

We thank the reviewer for this observation and the opportunity to clarify. Figure 3B shows a representative example from a single ovary and is intended as an illustration of the spatial organization. Our quantitative analysis integrates data from 45 ovaries across ages (Fig. 3C), and does not reveal a consistent spatial opposition between primordial and transitioning oocytes. To avoid potential misinterpretation from a single example, we have added additional individual ovaries displayed in the same format as Fig. 3B to a supplementary figure (Fig. S5), illustrating that the apparent pattern is not systematic across samples – please see that figure pasted below for convenience.



4) a threefold variation in isogenic animals from 5 weeks, prior to sexual maturity. Little left-right asymmetry was found under normal physiological conditions.
- could the authors speculate to the source of this large variation between isogenic animals?

Thanks for the suggestion - we now added the following paragraph to the discussion:

"The high variation in oocyte numbers among isogenic, age-matched mice is already present before sexual maturation, suggesting an origin in early development. Several factors could explain this variability. First, the initial reserve size may be influenced by the efficiency of primordial germ cell (PGC) migration and proliferation during colonization of the genital ridge; even slight differences in founding populations or their migratory success can lead to a different baseline. Second, the subsequent wave of oocyte loss during germ cell nest breakdown and early postnatal life may vary in intensity between individuals. Notably, low-reserve ovaries also exhibit reduced total volume and fewer growing oocytes, suggesting that these early events impact the overall function of the organ."

- Figure S3B shows no statistics on the total oocyte number intra-individual variation presented though there are differentials as large as over 500+. Figure S3C would also benefit from having the follicle boundary highlighted using a dashed line.

We thank the reviewer for this comment. Motivated by their, and reviewer 2's similar comments on intra- and inter-individual variability, we now added a new analysis to the manuscript (Supplementary Figure S4C), also copied below.

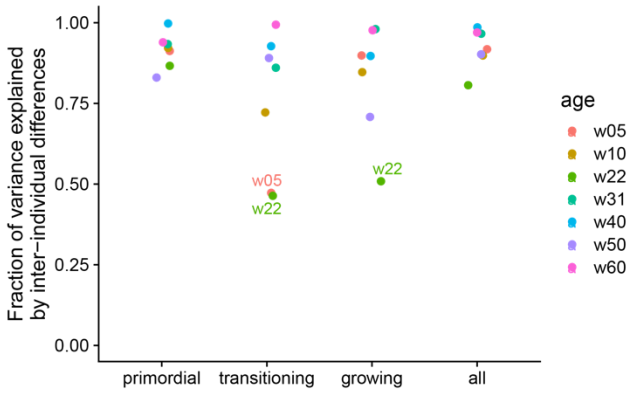
To directly address the question of whether variation in oocyte number is primarily driven by differences between individuals or by differences between ovaries within the same individual, we performed a quantitative analysis.

Specifically, we fit the regression model:

$$\text{Oocyte\_count} = \beta \times \text{animal\_id} + \varepsilon$$

Here, the term **β** estimates differences between animals in their total oocyte counts across both ovaries. We then used the model's coefficient of determination (**R^2**) as a summary of how much of the overall variability in oocyte counts is explained by **differences between animals**, as opposed to residual variability (which includes within-animal, between-ovary differences and other sources).

Across developmental stages and ages, the median R^2 is 0.89, indicating that approximately 89% of the total variance in oocyte number is attributable to inter-individual differences. Thus, while some within-animal ovary-to-ovary differences can be large in absolute terms (e.g., >500 oocytes), they typically account for a smaller fraction of the total variation than inter-individual differences. We note limited exceptions for transitioning and growing oocytes at specific ages, where within- and between-individual contributions are comparable, and we highlight these cases in Supplementary Figure S4C.



FigureR4 (same as Fig S4C). The coefficients of determination (R2) estimating the fraction of variation in the number of oocytes in each ovary explained by differences between individuals. The remaining, unexplained variance (1-R2) estimates the fraction of variation explained by intra-individual differences between the two ovaries within each individual.

We now highlight the follicle boundary in S3C.

5) demonstration of their clearing and imaging technical approach in human tissue.
- It is noticeable that the human cortical tissues presented are not analysed very deeply. It would improve their claims of clinical relevance to show that different oocyte sizes can be observed, or if they can comment on a the possibility of using their oocyte quantification from these cortical fragments to assess the patients ovarian reserve size. E.g. do the differences in human oocyte density shown in Figure S2C have any clinical meaning that can be discerned? Also could the authors comment on the oocyte diameters shown in the human data? The tissue should be a cortical strip and therefore contain the dormant primordial follicles, however the oocyte diameter indicates another bimodal distribution which might indicate that there are not many dormant oocytes. E.g. why is there a peak under 20um and another at 40um?

-given their own observations of variation within the isogenic mice, would the human samples not be expected to have even more variation?

We thank Reviewer #4 for raising these important points and for the opportunity to clarify the scope of the human data. The human ovarian cortex samples included in this study were intended as a proof-of-principle demonstration that our clearing, imaging, and AI-based segmentation pipeline can be applied to human tissue, rather than to draw clinical conclusions. We agree with the reviewer that, given the limited sample number and the expected high inter-individual variability in humans as well as the potential spatial heterogeneity of follicle distribution across the human ovary[1], we do not interpret differences in oocyte density in Fig. S2C as clinically meaningful. A much larger data set and access to full ovaries would be required to make such conclusions. We have clarified this scope and limitation in the revised Discussion.

Regarding oocyte diameters in the human cortex data, we have added clarifying text in the Methods and updated the graphs. While cortical fragments are enriched for primordial follicles, they can also include

early activated follicles depending on sampling depth and local tissue architecture. In humans, primordial oocytes are larger than in mice and are typically ~30–40 µm in diameter[22]; accordingly, the peak around ~40 µm corresponds to primordial oocytes and early activated follicles. The small peak around ~10 µm reflected a limited number of false-positive segments arising from occasional mis-segmentation. We have now corrected the segmented mask and applied a size-based filter to exclude segments smaller than 15 µm, which correspond to segmentation artifacts.

Ultimately, while these human data are not yet intended for clinical diagnostic extrapolation, they successfully demonstrate that our technical pipeline is robust enough to capture the follicular populations of the human ovarian cortex, providing a scalable foundation for future, high-powered clinical studies.

A major omission is that the details of the timecourse (e.g. ages analysed) are not given. The authors stage that 5 animals per stage are analysed, and it seems they looked at both left and right ovary for the majority of the animals, but this should be clearer.
- the schematic in Figure 1A is not as informative as it should be - particularly as it does not show that both left and right ovaries from each mouse was analysed. Similarly, the ages spanned should be indicated. It is not clear to me how many mice from which stage were analysed. In the text 5 weeks was an important timepoints and used to establish the pipelines, but I think the manuscript would benefit from

We thank Reviewer #4 for this suggestion. We modified Fig. 1A to better illustrate that both ovaries are analyzed and added the total number of mice used per age in Figure 2.

In general the statistics seem appropriate and are described in all context where error bars are shown.

Taken together, this work furthers our understanding of age-related oocyte decline within the ovary, provides valuable observational data from the mouse model system, and showcases how follicle counting can be modelled to make interesting predictions about the ovary.

REFERENCES

1 Gaylord, E. A. *et al.* Comparative analysis of human and mouse ovaries across age. *Science* **390**, eadx0659, doi:10.1126/science.adx0659 (2025).

2 Folts, L., Martinez, A. S., Bunce, C., Capel, B. & McKey, J. OoCount: A Machine-Learning Based Approach to Mouse Ovarian Follicle Counting and Classification. *bioRxiv*, doi:10.1101/2024.05.13.593993 (2024).

3 Faire, M. *et al.* Follicle dynamics and global organization in the intact mouse ovary. *Dev Biol* **403**, 69-79, doi:10.1016/j.ydbio.2015.04.006 (2015).

4 Lan, T. C. T. *et al.* Aging disrupts spatiotemporal coordination in the cycling ovary. *bioRxiv*, 2024.2012.2015.628550, doi:10.1101/2024.12.15.628550 (2024).

5 Saitou, M., Nagano, M. & Mizuta, K. Mechanisms of human germ cell development. *Nat Rev Mol Cell Biol* **27**, 153-171, doi:10.1038/s41580-025-00893-6 (2026).

6 Pepling, M. E. & Spradling, A. C. Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. *Dev Biol* **234**, 339-351, doi:10.1006/dbio.2001.0269 (2001).

7 Dewailly, D. *et al.* The physiology and clinical utility of anti-Mullerian hormone in women. *Hum Reprod Update* **20**, 370-385, doi:10.1093/humupd/dmt062 (2014).

8 Yin, Q. & Spradling, A. C. Distinct waves of ovarian follicles contribute to mouse oocyte production. *bioRxiv*, 2024.2005. 2030.596686 (2024).

9 Zhang, X. *et al.* CCAAT/enhancer binding protein beta (C/EBPbeta) regulates the formation of the ovarian reserve. *Commun Biol* **8**, 1394, doi:10.1038/s42003-025-08798-y (2025).

10 Zhao, Z. H. *et al.* Spatiotemporal and single-cell atlases to dissect regional specific cell types of the developing ovary. *Commun Biol* **8**, 849, doi:10.1038/s42003-025-08277-4 (2025).

11 Munakata, Y. *et al.* Chromatin remodeler CHD4 establishes chromatin states required for ovarian reserve formation, maintenance and male germ cell survival. *Nucleic Acids Res* **53**, doi:10.1093/nar/gkaf008 (2025).

12 Emori, C., Boucher, Z. & Bolcun-Filas, E. CHEK2 signaling is the key regulator of oocyte survival after chemotherapy. *Sci Adv* **9**, eadg0898, doi:10.1126/sciadv.adg0898 (2023).

13 Chen, W. *et al.* PARP1-catalyzed PARylation of YY1 mediates endoplasmic reticulum stress in granulosa cells to determine primordial follicle activation. *Cell Death Dis* **14**, 524, doi:10.1038/s41419-023-05984-w (2023).

14 Shang, Y. *et al.* MEIOK21 regulates oocyte quantity and quality via modulating meiotic recombination. *FASEB J* **36**, e22357, doi:10.1096/fj.202101950R (2022).

15 Habara, O., Logan, C. Y., Kanai-Azuma, M., Nusse, R. & Takase, H. M. WNT signaling in pre-granulosa cells is required for ovarian folliculogenesis and female fertility. *Development* **148**, doi:10.1242/dev.198846 (2021).

16 Feng, C. W. *et al.* Identification of regulatory elements required for Stra8 expression in fetal ovarian germ cells of the mouse. *Development* **148**, doi:10.1242/dev.194977 (2021).

17 Wang, J. J. *et al.* Single-cell transcriptome landscape of ovarian cells during primordial follicle assembly in mice. *PLoS Biol* **18**, e3001025, doi:10.1371/journal.pbio.3001025 (2020).

18 McGee, E. A. & Hsueh, A. J. Initial and cyclic recruitment of ovarian follicles. *Endocr Rev* **21**, 200-214, doi:10.1210/edrv.21.2.0394 (2000).

19 Tingen, C. M. *et al.* Prepubertal primordial follicle loss in mice is not due to classical apoptotic pathways. *Biol Reprod* **81**, 16-25, doi:10.1095/biolreprod.108.074898 (2009).

20 Orisaka, M. *et al.* Growth differentiation factor 9 is antiapoptotic during follicular development from preantral to early antral stage. *Mol Endocrinol* **20**, 2456-2468, doi:10.1210/me.2005-0357 (2006).

21 Moolhuijsen, L. M. E. & Visser, J. A. Anti-Mullerian Hormone and Ovarian Reserve: Update on Assessing Ovarian Function. *J Clin Endocrinol Metab* **105**, 3361-3373, doi:10.1210/clinem/dgaa513 (2020).

22 Westergaard, C. G., Byskov, A. G. & Andersen, C. Y. Morphometric characteristics of the primordial to primary follicle transition in the human ovary in relation to age. *Hum Reprod* **22**, 2225-2231, doi:10.1093/humrep/dem135 (2007).

Reviewers' Comments:

Reviewer #2 (Remarks to the Author):

The authors have now addressed all the issues I raised. The revised manuscript is much improved and will contribute significantly to our understanding of ovarian reserve dynamics during ageing. Specifically, the authors have substantially expanded the discussion to better explain how their findings fit within the existing literature on the subject, as requested by both myself and Reviewer 3. I recommend publication without reservation.

Thank you!!

Reviewer #4 (Remarks to the Author):

Overall these revisions are thorough and comprehensive. There are a couple of outstanding matters / final corrections I would encourage the authors to consider:

- in addressing reviewer 3 comment 4, rather than 'AMH is used as a clinical marker of ovarian reserve' I would suggest ovarian 'performance' as we know that AMH levels reflect the number of growing follicles, not those dormant within the ovarian reserve.

We thank the reviewer for the suggestion. We have decided to remove this sentence from the manuscript, as it does not add substantially to the following section and defining AMH in this context proved challenging. The revised section now begins as:

"Ovarian reserve exhibits substantial variability among humans[1], raising the possibility that oocyte numbers may also differ significantly between animals."

- in addressing reviewer 4's comment regarding the use of a single category for both primordial follicles and transitioning follicles in their model, the authors have explained that their dynamical model is unable to distinguish the categories. In light of a major claim being that the transitioning fraction remains stable, this should be clearly stated in the text regarding the model. Similarly, a statement regarding how many transitioning follicles are captured in their datasets might be beneficial.

We thank the reviewer for giving us the chance to clarify our model, and apologize for not being more precise in the wording of our previous response to reviewers. We do not intend to claim that our dynamical model is unable to distinguish between primordial follicles and transitioning follicles. In fact, we do not need any model, dynamical or otherwise, to distinguish between these cell types—we directly observe transitioning cells and distinguish them from other cell types using a microscope, and we include the counts of each cell type, clearly labeled, in our data set. The number of captured transitioning follicles is already present in our manuscript, in main Figure 2B.

The limitation we highlight in our dynamical model concerns a different quantity: *the transition rates* between cell states, which cannot be directly observed and would need to be inferred using dynamical modeling of our data set. Happily, our data set is of a sufficient size to calculate the abundance of oocytes

in the transitioning state, which we do in Figures 2 and S3. However, our data is not of a sufficient size to estimate the transition rates into and out of the transitioning state. We can work around this limitation in our dynamical modeling by aggregating primordial and transitioning follicles together to produce a model that lets us study transition rates into and out of all cell-type states apart from the transitioning state between primordial and primary oocytes. This is an example of what physicists would call "coarse graining": designing a model that captures the essential properties of a physical system without needing to explicitly represent every detail of the underlying system. We have updated the Methods section accordingly:

“In the static 3D dataset, oocytes morphologically transitioning from primordial to primary were observed and classified as “transitioning.” However, while this transitional state can be identified at the level of individual oocytes, the corresponding transition rates into and out of this state cannot be directly measured or reliably inferred from static observations. Intermediate states for later transitions (primary→secondary and secondary→antral) were also not reliably identifiable. Therefore, we implemented a dynamical model describing transitions between the four main stages. The model infers transition and death rates from static stage counts without requiring direct observation of intermediate states.”

- I also note that the equations underpinning the model here are not provided even within the supplementary material.

We thank the reviewer for pointing this out. The model code has been available in a public GitHub repository, included in the reporting summary document. Now we add the model equations as a separate supplementary material to the manuscript.

Bibliography:

1 Moolhuijsen, L. M. E. & Visser, J. A. Anti-Mullerian Hormone and Ovarian Reserve: Update on Assessing Ovarian Function. *J Clin Endocrinol Metab* **105**, 3361-3373, doi:10.1210/clinem/dgaa513 (2020).