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Insect polyploid adaptation for cell number and size varies in longstanding versus neopolyploid lines of the wasp *Nasonia vitripennis*

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ABSTRACT

Polyploidisation (whole genome duplication) is pervasive in eukaryotic evolution but poses significant challenges. Longstanding polyploid lines may have specialised coping mechanisms, but neopolyploids must overcome immediate impediments. In particular, larger polyploid cells disrupt development and physiological function. Some vertebrate polyploids have larger but fewer cells (cell reduction); in invertebrates, such coping mechanisms are unstudied. Here we study polyploid cellular and morphological responses by comparing wings of a longstanding polyploid line (Whiting Polyploid Line, WPL) and neopolyploid lines of the wasp *Nasonia vitripennis*. As with all hymenopterans, in wasps males are haploid and females are diploid. Polyploids are diploid males and triploid females. We created neopolyploid lines with RNA interference of female development genes *transformer* (TRA) and *wasp-overruler-of-masculinisation* (WOM). We analysed differences in wing cell counts, wing surface area, and wing cell size between these polyploid lines. There were sex-specific and line differences, with female WPL exhibiting no difference in cell count between the diploids and triploids, whereas the neopolyploid lines had significantly reduced cell counts in triploids. In males, both the WPL and WOM neopolyploid line had lower cell counts in diploids than in haploids, with a less pronounced effect in the TRA neopolyploid line. Wing surface area and cell size also varied, with the longstanding WPL having greater similarity between polyploids and non-polyploids than the newly generated TRA and WOM lines. Variation in cellular size and reduction between polyploid lines suggests greater stabilization and a possible signature of re-diploidisation in the long-standing line compared to the neopolyploid lines. We discuss implications for polyploid adaptation and evolution, including effects on reproductive success.

1. Introduction

Polyploidisation, or whole genome duplication, is highly pertinent to eukaryotic evolution. Ancestral polyploidisation events followed by gradual genome reduction (re-diploidisation) occurred in all major taxonomic branches (McLysaght et al., 2002; Jiao et al., 2011; Albertin and Marullo, 2012; Song et al., 2012; Li et al., 2018). Novel interactions between genome sets can result in new genetic pathways corresponding to diverse evolutionary outcomes, including greater population diversity, higher adaptive potential, and mass speciation (Wertheim et al., 2013; Soltis et al., 2015; van de Peer et al., 2021). But there remain many unknowns of how drastic post-polyploidisation effects are modulated, including cell size increases.

After polyploidisation, cells increase in size to accommodate more DNA (Bomblies, 2020, Fox et al., 2020). The lesser surface area to a volume ratio reduces capacity for e.g. resource uptake and waste removal (Glazier, 2022). This may deleteriously alter the growth rate of organisms and their physiological responses to environmental cues (Fox et al., 2020). And yet, there can be some advantages to increased cell size, with fewer relative interactions on cell surface area resulting in more effective ion-regulation and lower cellular maintenance costs (Cadart et al., 2023). Such effects call into question how polyploidisation relates to the posited ideal cell size (which varies for different taxonomic classes; the Theory of Optimal Cell Size) (Czarnoleski and Verberk, 2025). Various mechanistic adjustments have been suggested for post-polyploidisation responses. For example, current research

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suggests that all plants experience cell size increase (Bomblies, 2020), but there is evidence that some develop larger organs to match larger cell sizes, thereby allowing the number of cells to remain the same (Sugimoto-Shirasu and Roberts, 2003; Frugis, 2019). However, a meta-analysis found that synthetic plant polyploids can have fewer but larger cells, while natural and older polyploid plant lineages have smaller cells with cell-count-per-unit are similar to diploid counterparts. This suggests that plant polyploidisation has an immediate cell-reduction effect that reduces over time in a re-diploidisation process (Clo and Kolář, 2021).

Animals have been considered more sensitive to the consequences of gigantism owing to stricter development and body plans. Some animal species have cell reduction to mitigate the detriments of increased polyploid cell size (Fox et al., 2020). For example, triploid autopolyploids of the newt *Triturus viridescens* are equal in organ mass to diploids because of this cell reduction mechanism (Fankhauser, 1945). In contrast, the allotetraploid *Xenopus laevis* produces larger cells and has a larger body size than its diploid relative, *Xenopus tropicalis* (Miller et al., 2020). The consistency of these mechanisms is thus still up to debate, and the number of polyploid animal taxa studied is very limited because they are rare, sterile, or hard to work with, as in natural amphibian polyploids that require asexuality or a triploid bridge to reproduce (Litvinchuk et al., 2016). Whether an immediate dramatic cell-reduction occurs that softens over time with a re-diploidisation effect (as in plants, Clo and Kolář, 2021) is thus also unknown.

In insects, ploidy and cell size are closely linked. Insect cellular polyploid knowledge is largely from endopolyploidy (multinucleation of regional somatic tissues), a normal aspect of development (Ren et al., 2020). For example, in *Drosophila* wings, endopolyploid cells often generate multiple hairs from multiple pre-hair initiation centres (as opposed to non-polyploid cells with a single cuticular hair); their incidence can be influenced by altering cell size through starvation or mechanical stretching (Adler et al., 2000). In the moth *Ephestia kuehniella* wing scales are produced by endopolyploid cells, and their sizes vary according to cell ploidy i.e. correlating with ploidy levels (4n–32n) (Henke and Pohley, 1952). In another moth *Manduca sexta* there is a wing and scale size gradient scaling to ploidy, supporting Henke's compensation principle of an inverse relationship between the ploidy level of scale-building cells in insects and the abundance of nearby diploid cells in a specific wing area (Cho and Nijhout, 2013). From such studies, it has been inferred that tissue-specific alterations in ploidy levels enable multicellular organisms to adjust their cell sizes to physiological need (e.g. though endocycles and endomitosis) (Balachandra et al., 2022).

In contrast to insect endopolyploidy, much less is known on how cell size is regulated in whole organismal polyploidisation events. It was long assumed that organismal insect polyploidisation was insignificant to evolution, occurring only as rare, inviable meiotic errors caused by e.g. temperature stress in extreme environments (Lokki and Saura, 1979). Deep-scale phylogenomics revealed numerous ancestral whole genome duplications throughout Insecta, indicating a much bigger role for polyploidy in insect evolution than previously thought (Li et al., 2018). This highlighted the need for more fundamental knowledge of whole organismal polyploid effects on cell biology, but there are few means to observe neopolyploid insects, which are difficult to produce and have low survivability. Low viability is largely due to an archetypical problem of animal polyploidy: dosage mechanisms for sex chromosomes are lethally disrupted by an extra genome (Wertheim et al., 2013).

Interestingly, neopolyploidisation events often occur in the order Hymenoptera. Normally, males are haploid and develop from unfertilised eggs, whereas females are diploid and develop from fertilised eggs, but viable diploid males often arise due to perturbances of sex determination pathways, and sometimes, triploid females (Heimpel and De Boer, 2008; Leung and van der Meulen, 2022). Although these hymenopteran polyploids are usually sterile, this haplodiploid reproductive system absent of heteromorphic sex chromosomes allows for studies on organismal ploidy variation.

A particularly fitting model is the wasp *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae) (Sivaprakasham Murugesan et al. 2024). This parasitoid has well-documented genetics and high ease of handling (Werren et al., 2010). Polyploidy in *N. vitripennis* was first described in the lab-derived Whiting polyploid line (WPL) that has been now maintained for over 65 years (Whiting, 1960, Leung et al., 2023). WPL was used to establish a breeding scheme of alternating generations of high fecundity diploid males and low fecundity triploid females (Whiting 1960; Beukeboom and Kamping, 2007; Leung et al., 2019, 2023). This gives a rare consistent animal model for studying long-term polyploid effects. Strikingly, *N. vitripennis* neopolyploids can also be created by knocking down genes required for female development, such as *transformer* (TRA) (Verhulst et al., 2010), *transformer-2* (TRA2) (Geuverink et al., 2017), and *wasp overruler of masculinisation* (WOM) (Zou et al., 2020). Diploids are consequently diverted to male development, and these neopolyploid diploid males can be used to produce triploid daughters to start new polyploid lines that also alternate in sex for polyploidy every generation (Sivaprakasham Murugesan et al., 2024).

A previous study suggested that the WPL has polyploid cellular reduction and neopolyploids do not (Leung et al., 2023). However, this study subsampled a small area of the wing, possibly introducing regional bias. Furthermore, the only neopolyploid line examined was one created by knocking down TRA, which has a known role in body size regulation in *Drosophila* (Rideout et al., 2015). WPL being inbred and the TRA neopolyploid being outbred was another complicating factor. Inbred and outbred polyploid lines cannot be directly compared, even if generated using the same polyploidisation mode (e.g. a specific gene knockdown), because epistatic effects from variable heterozygous loci in the outbred background will result in different downstream effects than a universally female-homozygous background of inbred *Nasonia* isolines.

Therefore, we created two independent neopolyploid inbred lines for direct comparability, one by silencing TRA and the other by silencing WOM, which does not have a body size function. In doing so, and in having more comprehensive cell and wing measurements for all lines, we aimed to determine cellular responses across recent neopolyploid lines and the longstanding polyploid line.

2. Materials and methods

2.1. *Nasonia* culture and specimen generation

All *Nasonia vitripennis* lines were reared under conditions of 25 °C, 16:8 Light:Dark cycle, ~55% relative humidity, for a ~2-week life cycle. They were hosted on commercially purchased *Calliphora vomitoria* pupae. Individuals used in assays had mutant eye markers associated with ploidy level to assist in specimen sorting. They were generated following protocols detailed in full in Sivaprakasham Murugesan et al. (2024). Briefly, WPL originated spontaneously in the cultures of Whiting about 65 years ago (Whiting 1960); it was acquired from the Werren laboratory (University of Rochester, NY, USA) and then maintained in the Beukeboom laboratory (University of Groningen, the Netherlands). In WPL, males were generated by hosting virgin triploid mothers on five hosts each (breeding scheme depicted in Beukeboom & Kamping, 2006). Dark-eyed males are diploid, and red-eyed males are presumed haploids. For the red-eyed WPL males, ~80% are haploid and ~20% are diploid but they are visually indistinguishable (Whiting, 1960). Thus, in cases of significant difference between known dark-eyed diploids and red-eyed males, it is presumed that the higher number of haploid males subsumed any diploid effect for the latter. To create WPL females for analyses, males were crossed to virgin females of the inbred red-eyed mutant line used in their normal maintenance, stDR; dark-eyed diploid males produced triploid dark-eyed females and red-eyed haploid males produced diploid red-eyed females (females were checked for diploidy by hosting virgin sisters on three hosts each; their production of >30

offspring confirmed that they were not low-fecundity triploids produced by a red-eyed diploid father).

The neopolyploid lines were generated with parental RNAi knockdown of the sex determination genes *transformer* (TRA) and *wasp-overruler-of-masculinisation* (WOM) by microinjecting double-stranded RNA (dsRNA) in female pupae of the inbred *oyster* gray-eyed mutant line and were subsequently mated to stDR males to produce F1 diploid dark-eyed males as a complementary phenotype; *oyster* gray-eyed haploids were also produced from unfertilized eggs. (see Sivaprakasham et al., 2024 for full details). These males were then mated to virgin *oyster* females to produce F2 diploid gray-eyed and dark-eyed triploid virgin females, which were used for wing cell assays. The triploid females were hosted on three hosts each to produce the first male generation with both polyploids and non-polyploids, F3 diploid dark-eyed diploid males and red-eyed haploid males (the red-eyed males of these lines consistently haploid). All WPL and neopolyploid crosses used 5–8 males and 50 females each. Note that all lines carry the stDR and oyster markers in the hybrid eye mutant background needed to differentiate polyploids from non-polyploids, but WPL is maintained with the stDR line whereas TRA and WOM are maintained with the *oyster* line. The difference originates from continuing the original stDR-based breeding scheme of longstanding WPL (Whiting 1960) but using oyster for the neopolyploid lines because stDR has a low mating rate in new lines.

Individual wasps were sorted by sex at the late white-half-black pupal stage (based on wing bud size being bigger in females than males) and for ploidy by eye colour. They were kept in standard conditions in 4 ml plastic test tubes sealed with a cotton plug and allowed to eclose as adults for wing dissection.

2.2. Wing dissection and imaging

Right forewings were used as a proxy organ for assessing cell reduction (Leung et al., 2023). Wings are suited for this as they form a single-cell, 2D membrane with several landmark regions. Hair-like structures, called setae, mainly cover the distal portion, with each seta corresponding roughly to a single cell (Loehlin et al., 2010). The forewing of the *N. vitripennis* male is smaller than that of the female (Loehlin et al., 2010).

For each group, seven adult wasps were placed in ten 1.5 ml Eppendorf tubes and euthanised using liquid nitrogen. Individuals were then separated and right forewings dissected out in a fume hood (the remainder of the specimens was saved and stored at −80°C for a separate transcriptomics study; Sivaprakasham Murugesan, 2025). Using a paintbrush, the thorax was positioned under the microscope and pinned down using two dissection tweezers to dissect the right forewing, including the hinge. After dissection, each wing was positioned in the same orientation on a microscope slide (75 × 25 mm) on Euparal (Carl Roth, The Netherlands) and secured in place with a coverslip. These mounted wings were allowed to dry for 24 h before imaging. Wings were circled in permanent marker on the backside of slides to assist with positioning for imaging. Mounted wings were imaged with a Zeiss Axio Observer Z1 inverted microscope with a Zeiss AxioCam MRc 5 camera attachment and computer imaging program AxioVision SE64 version number 4.91. A 10x magnification was used, and images were captured using the computer program AxioVision. Only fully intact wings were imaged and measured.

2.3. Wing analysis

ImageJ version 154 was used for cell counting and wing surface area measurement. Detailed protocols of using ImageJ and calculating of Distance in pixels is described in Appendix A and is available via https://figshare.com/s/36025067fff0ce06fc35. Individual setae number was calculated using the "analyse particles" function, which gave the total number of hairs (Loehlin et al., 2010). The images were saved as TIF files and the count data as XLS files. A screen-captured wing image was uploaded into ImageJ and converted into an 8-bit black-and-white image. The perimeter of the wing was then manually traced using a paintbrush tool and coloured black. To calculate wing area, "set scale" values were set manually as follows "Distance in pixels (px)" was set to 1292 px, representing the length of the original image taken by the inverted microscope in pixels. "Known distance and unit of length" were set to 878.56 μm, using the following equation:

The AxioCam MRc 5 camera had a sensor pixel resolution of 2584 px and a sensor pixel dimension of 3.4 μm/px. Using the ImageJ Wand tool, the wing was selected and analysed in μm^2. The processed wings were saved as a TIF file and the area value was saved in an XLS file. All area values were recorded in a single Excel spreadsheet for further analysis (Supplementary Data File 1). We calculated the cell size (μm^2) by dividing the number of cells counted in the landmark area by the measured size of this wing area (μm^2) and then averaged these values across replicate samples for each line.

2.4. Statistical analyses

Wing cell counts, wing surface area, and wing cell size across lines were tested for differences using a generalized linear model (GLM) using a Poisson distribution and log link. Tukey's test was used for post hoc comparisons, with a significance level of $\alpha = 0.05$, with estimated marginal means to evaluate pairwise comparisons and a Benjamini-Hochberg false discovery rate correction for multiple hypothesis testing. All tests were performed in R v4.2.1 (R Studio). All means and standard errors for wing cell count, surface area, and size are reported in Supplementary Table 1.

3. Results

3.1. Cell counts

In the WPL, triploid (N = 45) and diploid females (N = 50) did not differ in cell counts ($p = 0.703$) (Fig. 1). However, triploids of both neopolyploid lines, TRA (N = 41) and WOM (N = 41), displayed a significant reduction in cell counts compared to their TRA (N = 50) and WOM (N = 36) diploid counterparts ($p < 0.0001$). The male cell count

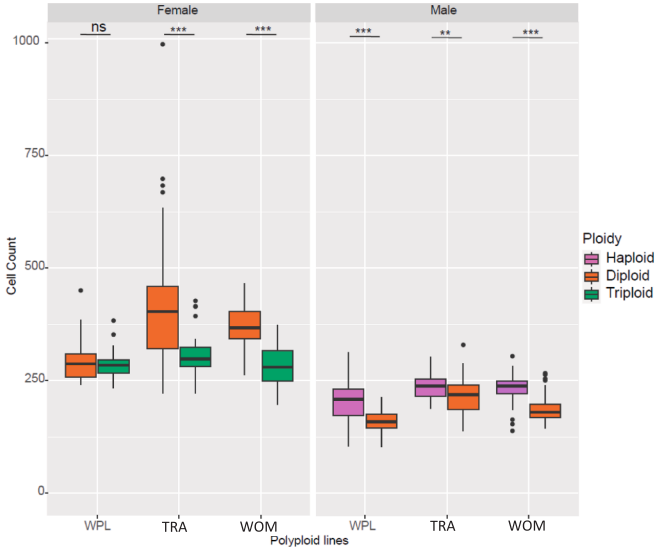

Fig. 1. Wing cell counts of longstanding WPL (Whiting polyploid line) and neopolyploid *transformer* (TRA) and *wasp-overruler-of-masculinisation* (WOM) gene knockdown lines. (**) $p = 0.001$; (***) $p < 0.0001$; (ns) not significant. Data were analysed using a generalised linear model and Tukey's post hoc test at $\alpha = 0.05$, using the estimated marginal means. Box plots indicate medians, standard deviations, and outliers.

did not have the same pattern. In all three lines, including WPL, diploid males had a lower cell count than their haploid counterparts (WPL, $p < 0.0001$ (diploid N = 34; haploid N = 40); TRA, $p = 0.003$ (diploid N = 50, haploid N = 50); WOM, $p < 0.0001$ (diploid N = 50, haploid N = 50). For the TRA line, polyploid cell count reduction was less pronounced in males than females (a qualitative observation).

Overall, all three lines reduced wing cell count when ploidy was increased, except for WPL females. Females from the longstanding WPL converge for cell count in diploid and triploids, whereas WPL polyploid males have decreased cell count. In TRA and WOM neopolyploid lines, both males and females have polyploid cell number reduction. The largest difference was for median cell count between triploid and their diploid female counterparts in the TRA and WOM neopolyploid lines (Supplementary Fig. 1). For unclear reasons the diploid WPL females exhibit a much lower ($p < 0.05$) cell count than diploid females of the TRA and WOM neopolyploid lines, (Supplementary Fig. 1). Among the polyploids, the TRA line had the highest cell count in both males and females ($p < 0.05$). Thus, WPL had consistently lower cell counts than counterparts of the neopolyploid lines: in both male diploids and haploids, as well as in female diploids and triploids.

3.2. Wing surface area

For the females of the WPL, TRA, and WOM lines, there was no difference in wing surface area between diploid and triploid counterparts (Fig. 2). The wing surface area of WPL triploid females was significantly larger than triploids of the TRA and WOM neopolyploid lines ($p < 0.05$); but WPL diploid wing size was not larger than TRA and WOM diploid wing size. The TRA and WOM neopolyploid lines also did not differ from each other in triploid female wing size (Supplementary Fig. 2). A detailed comparison within the same ploidy level (Supplementary Fig. 2) revealed that diploid females had significantly smaller wing areas in WOM neopolyploids than in TRA and WPL, whereas triploid females in WPL had significantly larger wing areas than neopolyploids ($p < 0.05$). These data indicate that based on wing surface area, the female polyploids (triploids) do not have gigantism relative to their diploid counterparts.

In male samples, the longstanding polyploid line WPL and TRA neopolyploid line showed significant differences between haploid and diploid wing areas ($p < 0.0001$) (Fig. 2). WPL diploid males had smaller wings than WPL haploids. In the opposite direction, neopolyploid TRA diploid males had bigger wings than haploid males. WOM neopolyploids did not show any significant differences between haploid and diploid males. A detailed comparison within the same ploidy level (Supplementary Fig. 2) showed that in haploid males, significant differences were observed, with WPL having the largest wing area, followed by TRA, and WOM ($p < 0.05$). Among diploid males, TRA exhibited the largest wing area, followed by WPL and WOM, indicating significant size differences between the groups (Supplementary Fig. 2).

In summary, the ploidy level did not affect the wing surface area in females. Only WPL triploid females had an increased wing surface area compared to the triploid female samples of the neopolyploids. However, male wing surface areas displayed notable variation. The WPL and TRA lines exhibited significant differences between male haploid and diploid wing surface areas but with opposite patterns. The WOM line had the lowest wing surface area compared to the TRA and WPL lines in both haploid and diploid males. These results highlight the strain and sex-specific differences in response to ploidy changes.

3.3. Wing cell size

From cell count and wing surface area data we inferred cell size for the different lines and sexes. In all lines we observed that triploid cell size is larger than diploid female counterparts, with the largest increase occurring for the neopolyploid WOM line ($p < 0.0001$; Fig. 3).

Diploid and triploid females of the WPL had the largest cell sizes compared to females of corresponding ploidy levels in the TRA and WOM lines ($p < 0.05$; Supplementary Fig. 3). Neopolyploid TRA and WOM differed in diploid female cell size ($p < 0.05$), with WOM having slightly smaller cells than TRA, but the cell size of the triploid females in these lines did not differ. These data indicate triploid females of *N. vitripennis* adjust cell size to higher ploidy and can do so immediately post-polyploidisation. However, the effect size depends on the line, as the WOM line shows a larger difference with the diploid counterparts

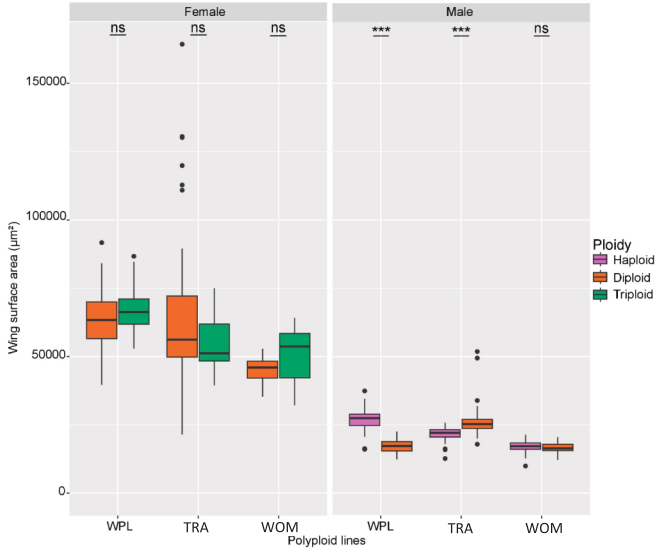

Fig. 2. Wing surface area of longstanding WPL (Whiting polyploid line) and neopolyploid *transformer* (TRA) and *wasp-overruler-of-masculinisation* (WOM) gene knockdown lines. Asterisks represent significant differences between the two ploidy levels: (***) $p < 0.0001$; (ns) not significant. Data were analysed using a generalised linear model and Tukey's post hoc test at $\alpha = 0.05$, using estimated marginal values. Box plots indicate medians, standard deviations, and outliers.

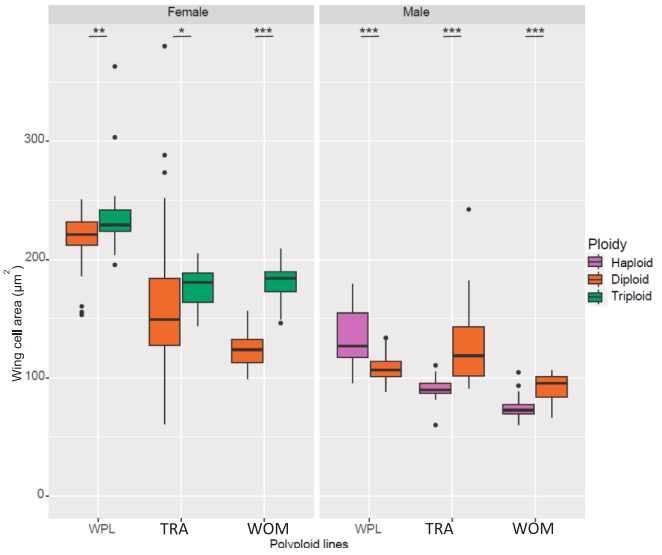

Fig. 3. Wing cell size of the longstanding WPL (Whiting polyploid line) and neopolyploid *transformer* (TRA) and *wasp-overruler-of-masculinisation* (WOM) gene knockdown lines. Asterisks represent statistically significant differences between the two ploidy levels: (*) $p = 0.01$; (**) $p = 0.001$, (***) $p < 0.0001$. Data were analysed using the generalised linear model and Tukey's post hoc test ($\alpha = 0.05$) using estimated marginal means. Box plots indicate medians, standard deviations, and outliers.

than the TRA line or WPL (Fig. 3).

Polyploid males did not show a consistent pattern of larger cell size. Diploid males from the WPL had a smaller cell size compared to haploid males ($p < 0.0001$; Fig. 3), whereas neopolyploids had larger diploid cell size than haploid cell size ($p < 0.0001$; Fig. 3). Across haploid males, WPL had the largest cells relative to TRA and WOM lines ($p < 0.05$; Supplementary Fig. 3), and WOM had smaller cells than TRA ($p < 0.05$; Supplementary Fig. 3). Haploid males of the WPL line had the largest number of cells compared to the TRA and WOM lines ($p < 0.05$; Supplementary Fig. 3), and WOM had smaller cells than TRA ($p < 0.05$; Supplementary Fig. 3). In diploid males, WPL and TRA had similarly sized cells, and only WOM had smaller cells ($p < 0.05$; Supplementary Fig. 3).

4. Discussion

4.1. Polyploid cell count reduction varies, but there is no evidence for post-polyploidisation gigantism, and cell size increase is female-specific

There is a major knowledge gap on cellular responses to polyploidisation in insects, despite important implications for their biology from ancestral evolution to contemporary incidence. Plant studies demonstrate that synthetic polyploids have larger but fewer cells (cell reduction) but old natural lineages resemble diploid counterparts in having more and smaller cells (Clo and Kolář, 2021). The *Nasonia* system intriguingly represents analogous comparison with the TRA and WOM lines representing new polyploids and the longstanding WPL representing an old spontaneous "natural" mutation (Sivaprakasham Murugesan et al., 2024). This study examined wing cell counts, wing surface area, and wing cell size across polyploid lines of the wasp *Nasonia vitripennis*. The results reflected variable and sex-specific responses to ploidy-level changes. Notably, like plants, the neopolyploids had a drastic cell-reduction effect, and the longstanding spontaneous polyploid line had the greatest similarity in phenotypes between polyploids and non-polyploids.

All polyploids regardless of line or sex had fewer cells than non-polyploid counterparts, except for the WPL triploid females. The large cell count reductions for female (F2) and male (F3) polyploids of WOM and TRA lines demonstrate a strong mechanism can be triggered immediately following polyploidisation, but they differed in the degree of effect, indicating that intensity of neopolyploidiation cell reduction can depend on specific polyploidisation mechanism(s) or gene(s). However, cell count difference between polyploids and non-polyploids of the longstanding WPL was less drastic. This suggests a cell count reduction mechanism possibly evolving to becoming less pronounced over time. For wing surface area, there was no difference between diploid and triploid females for any line. Among males, the WPL and TRA lines exhibited significant differences between haploids and diploids, but in opposite directions, whereas WOM males exhibited no significant differences in haploids and diploids. Wing surface area data thus do not support organ gigantism in polyploid *Nasonia*, despite it being an archetypical post-polyploidisation challenge (Comai, 2005). making polyploid gigantism of lesser potential detriment for this parasitoid, and possibly other insects. For female wing cell size, triploids measured larger than diploids for all lines. In contrast, for males, in the neopolyploid lines the diploids had bigger cell size than haploids, but in the longstanding WPL, diploid males had smaller cell size than the haploids. These results demonstrate a role for sex in polyploid cell size scaling, with femaleness associated with greater cell size increase than maleness and line-specific effects.

4.2. Synthesizing knowledge of polyploid wing measurements in Nasonia

This study's variation in cell number and cell size changes for different lines and sexes adds nuance to our understanding of polyploid outcomes. A previous study reported reduced cell number and increased cell size with increasing ploidy in the longstanding Whiting Polyploid Line (WPL), but no significant differences between polyploids and non-polyploids of a neopolyploid *transformer* (TRA) knockdown line (Leung et al., 2023). This prompted the question of why one line (WPL) seemingly had a cell reduction mechanism and another (TRA) did not. Surprisingly, our current study had an opposite finding, with WPL having more convergent measurements between polyploid and non-polyploids, compared to more divergent measurements in the neopolyploid lines (larger, fewer polyploid cells).

There are several possible explanations for the differences in these studies. First is that the earlier study only sampled forewing subsections, with only a few individuals measured for the whole wing (Leung et al., 2023). Regional variation may have introduced bias in this first study, particularly as it excluded the distal region where the most cells (setae) occur (Loehlin et al., 2010). Another possibility is that although WPL was the same inbred line in both studies, the TRA line of the first study was outbred (Leung et al. 2023), whereas the TRA and WOM of this study are inbred for greater comparability with WPL (Sivaprakasham et al., 2024). The extent of genetic variation effects on cell morphology is unclear at this point. However, a single generation of outbreeding in WPL already changed polyploid history traits such as lifespan (Leung et al., 2019); and inbred versus outbred background may underlie differences in triploid TRA female parasitisation (Leung, 2024; Li and Leung, 2024). The influence of genetic variation on polyploid phenotype requires its own investigation in future.

4.3. Physiological implications of polyploid cell variation in Nasonia

Regarding measurements in the polyploids versus non-polyploids, this study's contrast between the convergent wing phenotypes of longstanding WPL, and the more divergent phenotypes of the neopolyploid TRA and WOM, requires explanation. There is evidence that animal cells exhibit nonlinear growth rates and mitochondrial metabolism that are dependent on cell size, with optimal growth and metabolic rates occurring in cells of intermediate sizes (Miettinen et al., 2017). Biophysical constraints, such as increased intracellular distances and changes in cell surface area, likely explain why only certain cell sizes support maximum growth and metabolic efficiency. Deviations from this optimal size (Czarnoleski and Verberk, 2025), such as those caused by polyploidy, often lead to reduced cellular metabolism, fitness, and functionality, and can contribute to or exacerbate metabolic diseases (Miettinen et al., 2017). In such cases, bigger is not necessarily better, and so larger cells as a polyploid response may be maladaptive.

In our study, the convergence of WPL's cell sizes and cell counts in polyploids and non-polyploids might indicate that this line has responded to manage these constraints over time for better cellular functionality. In contrast, the variability in cell size and counts in neopolyploid lines such as TRA and WOM may reflect ongoing adjustments to these biophysical challenges, potentially leading to less efficient cellular processes. Possibly substantiating this, in a study, longstanding WPL did not exhibit lifespan differences between polyploids and non-polyploids (Leung et al. 2019), but female lifespan is shorter in (outbred) TRA neopolyploids, possibly demonstrating lack of time to adapt to polyploid disadvantage (Leung, 2024). Furthermore, in a study system where neopolyploids exhibited poor gigantism management, 30% larger neotetraploid *C. elegans* were shorter-lived than diploid counterparts (Misare et al, 2023).

The wing-centric approach of this study introduces advanced questions on interconnected effects of whole organismal polyploidy, endopolyploidy, and complex life history traits. In polyploid plants, cell types range in their degree of gigantism and also which pathways are detrimentally (or negligibly) impacted (Bomblies, 2020). This highlights the importance of tissue-specific considerations. Wings were assessed because of their facility for single-cell counts (Loehlin et al., 2010). But importantly, *Nasonia* have male thoractic endopolyploidy (Aron et al., 2005). As in many insects, *Nasonia* male courtship depends on female

receptivity to wing buzzing and pheromone distribution (Mair and Ruther, 2019). It is currently unknown how whole organismal polyploidy impacts obligate endopolyploid tissues, but there is preliminary evidence that some *Nasonia* lines retain male endopolyploidy, and others do not, such as TRA (Leung et al., unpublished data). In this study, the diploid males of TRA had the largest diploid male wing surface area and cell size. This suggests a possibly worse adaptive ability to polyploid gigantism; perhaps reflective of this is known impaired mating success in TRA diploid males that does not appear in WPL diploid males (Leung et al., 2023). This is of broad relevance to other Hymenoptera, as male thoracic endopolyploidy is universal across the order except for the basal-most sawflies (Aron et al., 2005). Polyploid individuals arise across diverse taxa, primarily as sterile diploid males (Heimpel and De Boer 2008; Leung and van der Meulen, 2022). Their degree of detriment on populations depends in part on competition with normal haploid males, which involves everything from mechanical incompatibility with females due to larger size (Harpur et al., 2013), to behavioral and gametic failures (Leung and van der Meulen, 2022). The role of polyploid (and endopolyploid) gigantism in mating systems should be explored further.

4.4. Future directions

The relationship between genome size, cell size, and body size is complex. There is a general relationship between cell size and genome size in eukaryotes (Czarnoleski and Verberk, 2025). For example, in *Batrachoseps* salamanders, larger genomes were associated with larger nuclear and cell sizes (Mueller et al., 2008). A broad mammal *meta*-analysis also recovered significant correlation between DNA content and erythrocyte size ($R^2 = 0.48$) (Gregory, 2000). However, body size in turn is often adjusted freely by cell size, and changes in cell size are frequently offset by fluctuations in cell number (Orietti et al., 2021). The general relationship of more genomic content resulting in larger cells holds in this study. However, the striking variation among sexes and lines of different ages also substantiates a proposed model that variation in initial polyploid phenotypes corresponds to variation in long term outcomes and survival (Leung et al., 2023). In particular, TRA and WOM neopolyploids show more variability in size effects than the WPL, and should be tracked over successive generations to determine if they converge in cell biology strategy with WPL, diverge into their own strategies, or indeed die out because they lack the mechanisms for success that have enabled the longtime survival of WPL.

A crucial aspect of avoiding gigantism in organ development and growth regulation is its link to compensatory growth mechanisms coordinating cell proliferation, differentiation, and apoptosis (Diril et al., 2012; Orr-Weaver, 2015). These mechanisms still need to be identified for the cell number reduction phenotypes noted here in *Nasonia* polyploids. Unfortunately, it is difficult to track *Nasonia growth*. Larval development and then pupation (upon which adult size fixes) both occur within the host, where it is not observable. Dissection of hosts for parasitoid larvae of various stages is not possible as eye markers are not yet developed to distinguish polyploids from non-polyploids, and flow cytometry cannot be used to type ploidy in larvae.

An alternative is investigating expression; the *Nasonia* transcriptome is highly annotated (Dalla Benetta et al., 2020), facilitating analyses for expression differences between lines, sex, and age of different polyploid backgrounds in *Nasonia* (Sivaprakasham Murugesan, 2025). As effects are more prominent in TRA and WOM neopolyploids than longstanding WPL, genes differentially expressed between them would be the strongest candidates, particularly those that overlap with known insect body size regulators in the insulin-signaling pathways, insulin-like growth factors, and juvenile hormones (Stocker and Hafen 2000; Nijhout, 2003). Notably, it is important to focus these investigations on specific tissue (here, the developing wing bud) because the variation of polyploid cell size across tissues is a major source of noise in investigating expression dosage changes in polyploids (which does not scale to the amount of DNA increase) (Doyle and Coate, 2019; Bomblies, 2020). Such insights would enhance understanding of how polyploid evolution unfolded in the insects the many times in occurred independently (Li et al., 2018), and immediate cellular consequences for the frequent contemporary polyploidization events in Hymenoptera.

CRediT authorship contribution statement

Saminathan Sivaprakasham Murugesan: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kelley Leung:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Funding acquisition, Conceptualization. **Keita Yamaguchi:** Investigation, Formal analysis, Data curation. **Emei Thompson:** Investigation, Formal analysis. **Leo W. Beukeboom:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Eveline C. Verhulst:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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