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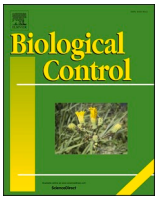

Potential benefits of male diploidy and female triploidy for parasitoid wasps used as biological control agents: A case study in *Nasonia*

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HIGHLIGHTS

- Polyploidy, the condition of extra genome sets, occurs for various parasitoids.
- It has the potential to benefit biological control because extra gene copies can be used for more advanced breeding.
- It first has to be established that polyploidy itself does not harm the individual or biocontrol traits.
- Despite detriments to polyploid fitness and parasitization, it may enhance other traits and protect alleles.

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ABSTRACT

Parasitoid wasps are haplodiploid insects, but polyploidy (diploid males, triploid females) occurs for many species. In biological control, polyploidy may have beneficial effects on desirable biological related traits. However, this is only possible in species for which polyploidy does not impair essential biological functions, as in for instance species with Complementary Sex Determination (CSD), where inbreeding drives sterile diploid male production and extinction risk. Notably, while CSD polyploidy is better studied, most biological agents are non-CSD species. This includes model *Nasonia vitripennis*, a blowfly parasitoid that can be purposefully made polyploid and then produces a high number of reproductive polyploid individuals. To test baseline non-CSD polyploid utility, an outbred polyploid *N. vitripennis transformer* knockdown line (tKDL) was established and assayed for relevant traits for considering polyploids as biological agents. Male diploidy and female triploidy increased head width, a body size proxy. Polyploidy increased unmated lifespan in diploid males, but decreased it in triploid females. In first matings, haploid and diploid males had equal fecundity, but sperm depletion assays revealed reduced diploid male fitness overall. Triploid females had a reduction in parasitization ability. This reduced male fecundity and female parasitization in tKDL suggest that polyploid *Nasonia* parasitoids have limited direct use in biological control, particularly in this outbred background. They are possibly more suitable for preparative applications, such as retaining alleles with sex-specific benefits.

1. Introduction

Hymenopterans (the wasps, bees, ants, and sawflies) have haplodiploid sex determination. Unfertilized eggs develop into haploid males and fertilized eggs develop into diploid females. However, polyploid diploid males and sometimes triploid females appear throughout the order (Cowan and Stahlhut, 2004; Zayed and Packer, 2005; van Wilgenburg et al., 2006; Heimpel and de Boer, 2008). Hymenopteran polyploidy has long been flagged in biological control because of deleterious effects in the parasitoid wasps, one of the most prevalently used and economically important classes of biological control agents for arthropod pests (van Lenteren et al., 1997; van Lenteren, 2018; Morales-Ramos et al., 2023; Singh, 2023; Verhulst et al., 2023; Leung et al., in print). Specifically, there is concern about inbreeding-driven sterile diploid male production, but this applies only to species that have Complementary Sex Determination (CSD). Under CSD, individuals that are heterozygous for a CSD locus (or loci) develop into females, hemizygotes are normal males, and homozygotes develop into diploid males that are usually sterile (Whiting, 1943; Cook, 1993). If CSD allelic diversity is lost through genetic drift, biological control populations can become progressively more male-biased (Zayed and Packer, 2005; Fauvergue et al., 2012, 2015; Leung and van der Meulen, 2022). This is

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consequential to biological control because fewer females for host-killing are produced (de Boer et al., 2012, Bagheri et al., 2022; Sandanayaka et al., 2022; Leung et al, in print).

CSD extinction risk being the prevailing theme in biological control polyploid research overlooks how most parasitoid wasps are non-CSD species. An estimated half to (Beukeboom et al., 2007) potentially most (Asplen et al., 2009) of parasitoid species do not have CSD. In these species, there is the potential to exploit polyploidy to benefit biological control in several interesting ways. Polyploidy 1) may be used to enhance desirable traits using higher order dosage/dominance effects. Polyploidy furnishes additional gene copies that could be used to push advantageous biological control traits to new extrema. Polyploidy may also be used to 2) retain sexually antagonistic alleles that benefit one sex at the expense of the other. In insects, for example there are known sexually antagonistic loci for development time (Arqvist and Tuda, 2010) and mating success (Khila et al., 2012). In haplodiploids, recessive alleles that are deleterious to males are quickly purged from the population as their effect cannot be masked in the haploids (Immler and Otto, 2014; Miller and Sheehan, 2023). Alleles that favor females but harm males may rapidly be lost, but in parasitoid biological control, the genetic integrity of female traits should arguably be prioritized because they are the sex that performs host-killing. Diploid males could "save" these allelic variants by acting as carriers for female-beneficial, male-deleterious alleles. Polyploidy may also be used to 3) reduce the ecological risk of female biological control agents, if the impaired fertility of triploid females helps prevent permanent population establishment or admixture where it is unwanted (e.g. when invasiveness is a concern).

These biological control polyploid applications are confined to theoretical space unless non-CSD species can meet several requirements. They must 1) have readily and reliably inducible diploid males and triploid females 2) produce a large enough number of these individuals to perform the targeted function and 3) the individuals must be robust enough to be used in biological control i.e. do not have major impairments of morphology or life history common in animal polyploids (Mable, 2004; Wertheim et al. 2013). The massive diversity of parasitoids has not been fully explored so it is unclear how many of species fulfill these criteria, but *Nasonia vitripennis,* the most extensively studied non-CSD species, does. This blowfly parasitoid has been used as a research model for environmental and genetic factors underlying biological control traits including sex ratio, fecundity, venom potency, and host specificity (Pannebakker et al., 2011; Rivers and Denlinger, 1995; Desjardins et al., 2010). *Nasonia* has also been used as a direct model for studying parasitoid traits and application in biological control and pest management. For example, it has been used to explore the concept of genomic selection to improve biological control traits (Xia, 2020); for annotating important functional genes in the key biocontrol agent genus *Trichogramma* (Lindsey et al., 2018); and for assessing non-target effects of RNA interference (Deveux et al., 2023) and natural compound (Sulg et al., 2023) pest control techniques.

Polyploidy first appeared in *N. vitripennis* laboratory stocks in the 1940s, and a derived Whiting polyploid line (WPL) has been maintained in an inbred state since (Whiting, 1960). It is also possible to generate new polyploid lines in *Nasonia* by knocking down genes in *Nasonia*'s sex determination pathway with RNA interference. One such target is the maternally provided *transformer* (*tra*) transcripts. Silencing maternal *tra* results in diploids developing into males rather than females, which are used to start new polyploid lines (Verhulst, 2010). Interestingly, it is already known that polyploid phenotypes can be variable within a single parasitoid species through *N. vitripennis*. A previous study found the WPL has high diploid male mate competition ability and low triploid fecundity, but a *transformer* knockdown line (tKDL) has low diploid male competition ability and high triploid female fecundity (Leung et al., 2023). This background of multiple polyploid resources with variable phenotypes make *N. vitripennis* useful for expanding our knowledge on how polyploidy operates in non-CSD parasitoid wasps and possible outcomes for biological control.

In this study I assay the effects of non-CSD polyploidy on traits important to biological control in tKDL. These are 1) body size, which influences other traits such as fecundity and resource acquisition (Beukeboom, 2018) 2) lifespan, which determines how long an individual can kill pests and breed 3) male fecundity, both with a single female mate, and with sperm depletion with a female mating series representing male total reproductive capacity and breeding utility, and 4) female parasitization ability, the direct measure of biological control efficacy. I anticipated some level of polyploid detriment, but could not predict which traits and to what extent. The results gave insight on whether non-CSD polyploidy has utility for the three proposed polyploid biological control applications of enhancing biological control traits with dosage/dominance effects, protecting alleles with sex specific effects that would otherwise be purged, or preventing admixture with native populations. They are suboptimal for direct applications of mass production and host killing, but hold promise for beneficial breeding schemes.

2. Materials and Methods

2.1. *Nasonia* strains and culture

All individuals were reared on a 2-week cultivation cycle under standard conditions of 25 °C, 16:8 LD cycle, ~55 % relative humidity, on *Calliphora* sp. hosts purchased as larvae and allowed to pupate (Titus Blom, Groningen, Netherlands). The Whiting polyploid line (WPL) was acquired from the John H. Werren lab (University of Rochester, Rochester, New York, USA). Briefly, WPL triploid virgin females produce both diploid and haploid sons. Eye makers in the sons indicate ploidy. Purple-eyed (wildtype) males are diploid, red-eyed males are haploid or diploid, and pink-eyed (oyster) males are haploid. The purple-eyed males are mated to virgin females of a distinct red-eyed mutant strain (*scarlet*) to recover triploid females and restart the breeding cycle. The full WPL breeding scheme is fully outlined elsewhere (Whiting, 1960; Beukeboom and Kamping, 2006; Leung et al., 2019).

The *tra* knockdown line (tKDL) was generated in the HVRx genetically variable lab population background created from wild Netherlands populations (van de Zande et al., 2014). This line retains its genetic variation because each generation is mass cultured in four tubes (to retain an effective population size $N_e \approx 200$), and hosts are mixed post-oviposition. Individuals from this population were used in assays as an untreated (uninjected) control. A detailed description of how tKDL was created from N = 200 females and how individuals are typed for ploidy through a combination of offspring count and flow cytometry is in Leung et al. (2023). Henceforth all individuals from the untreated HVRx population will be referred to as "control," and both polyploid and non-polyploid genetically variable individuals descending from ds *tra* injected females are referred to as tKDL. As there are no morphological markers in tKDL, a combination of flow cytometry and female fecundity (i.e. triploid females having reduced fecundity) were used to track ploidy across generations (see Figure S2, Leung et al., 2023). In brief, candidate haploid and diploid tKDL males were mated to control HVRx females, then frozen. Those daughters from these crosses were given three hosts. If they had a fecundity of > 50 offspring, they were presumed diploid with a haploid father. If they had < 50 offspring, they were candidate triploids and the ploidy of the father checked as haploid or diploid with flow cytometry. This involved screening brain cells with a propidium iodide stain on a BD FACS Aria II machine (see Leung et al., 2019, 2023).

2.2. Body size

Measurements for control (haploid males, diploid females) and tKDL individuals (F1 diploid males; F2 diploid and triploid females) were taken for head width, a proxy for overall body size (as in Weston et al.,

1999, Leung et al., 2019). For the F1 tKDL diploid male measurement, ~20 % of individuals were expected to be haploid due to unfertilized eggs (Werren and Loehlin, 2009), but these could not be sorted out. Heads were removed with a razor and mounted onto glass slides with clear nail polish. Pictures of each specimen's head were taken using a Moticam 2000 camera mounted on a Carl Zeiss Stemi SV6 microscope at 5x magnification with Motic Images Plus 2.0ML software. Measurements were made in triplicate in Photoshop CS6 (64 bit) using the ruler tool scaled to a 1 mm ruler and averaged.

2.3. Lifespan

Lifespan was measured for control individuals and F1 tKDL polyploids and non-polyploids under both feeding and starving conditions for males and females. As in the body size assay, the F1 tKDL male diploid measurement subsumed a portion (~20 %) of haploid males. Each wasp was housed individually in a 63 x 11 mm tube with a cotton plug and under standard conditions. Fed wasps were given 10 % sucrose solution every three days with a strip of filter paper. Individuals were checked for mortality every 24 h.

2.4. Male fecundity and sperm depletion

Nasonia males have a single wave of spermatogenesis in the pupal stage (Chirault et al., 2015, Feree et al., 2019). To assess fecundity with a single mate, virgin < 1 day old control haploid and F1 tKDL haploid and diploid males were individually given a virgin control diploid HVRx female mate for 24 h (WPL males were similarly assessed in Leung et al., 2019). Males were also assessed for sperm count depletion. That is, individual control (haploid), tKDL F1 males (haploid and diploid) were each given a series of 10 virgin females from the HVRx control population in quick succession. Daughter counts from these series approximate how quickly sperm is depleted. As circadian rhythms can influence insect mating behavior (Sakai and Ishida, 2001, Bertossa et al., 2013), all mating series began at 12 h. Each male was presented one female at a time, and as soon as the male terminated copulation by climbing on top of the female, the female was removed and replaced with another virgin. All females were given three hosts and their offspring collected, sexed, and counted 16 days later.

2.5. Female parasitization rate

Control (diploid), tKDL (diploid and triploid) and WPL (triploid) females were assayed for their parasitization ability. Each < 1 day old virgin female was given ten fresh *Calliphora vomitoria* hosts. Every two days the female was given on a fresh set of ten hosts until she died. At these points females were scored for whether they were still alive, to approximate lifespan. Hosts were kept in standard culture conditions for three weeks. At this point, every host was scored for parasitization success. They were scored for whether a fly emerged (failed parasitization), whether the host died (was parasitized) but no offspring was found within, or whether the host was parasitized and used to produce offspring. In the case of the triploid tKDL and WPL females, all offspring were counted as measure of lifetime reproductive potential. It is possible that some hosts were of poor quality and flies did not develop or emerge independent of any interaction with female wasps, but as this was < 5 % of hosts, this was not factored into analyses.

2.6. Statistics

All statistical tests were performed in SPSS version 15 (IBM, 2017). For all assays, non-parametric Mann-Whitney U tests and Kruskal-Wallis test were used. For Kruskal-Wallis tests, post-hoc Dunn's tests were used for pairwise comparisons. Survival graphs were generated for starved and fed lifespan and log-rank (Mantel-Cox) tests used to test for differences in survival distributions. Sperm depletion differences among control (haploid), tKDL haploid, and tKDL diploid males, measured by the number female offspring over an ordered series of female mates, were analyzed with a generalized linear model (GLM) with a negative binomial distribution with a log link. To test for significant differences in parasitization ability for diploid and triploid females, general linear mixed models (GLMM) were used for the number of hosts parasitized and number of hosts that produced offspring using a binary logistic regression link. Day and specimen were set as random effects. Background (tKDL), ploidy state (diploid vs. triploid), whether the group was injected (yes for tKDL, no for control and WPL), genetic variability status, (inbred WPL, outbred control and tKDL) were individually tested as fixed effects, with the intercept included. Satterthwaite approximations and estimations of robust variance were used to correct for uneven sample size and non-normality. For readability, only overall significance test results and comparative descriptions between groups are given in the results. Mean values, standard deviation, and p-values for pairwise post-hoc tests are in Table S1.

3. Results

3.1. Body size (head width)

For head width (body size proxy) F1 tKDL diploid males were significantly larger than control haploid males by 0.08 mm (Mann-Whitney *U* test, $P < 0.001$). The ~ 20 % of contaminant haploid individuals in the tKDL diploid measurement did not mask a significant effect. For females, F2 tKDL triploids < control diploid females < F2 tKDL diploid females. The F2 tKDL diploids differed significantly from both control diploids and the F2 tKDL triploids by 0.02–0.03 mm, but the control diploids did not differ from the F2 tKDL triploids (Kruskal-Wallis test, $H^2 = 12.061$, d.f. = 2, $P = 0.002$). Thus, tKDL polyploids are larger than non-polyploids for males, but not females (Fig. 1).

3.2. Lifespan

Under starvation conditions, the tKDL diploid males lived significantly longer than the control haploid males by ~ 3 days (Mann-Whitney *U* test, $P < 0.001$), with survival distributions being significantly different (log-rank test, $\chi^2 = 257.415$, d.f. = 1, $P < 0.001$) (Fig. 2A). Under fed conditions, the tKDL diploid males lived longer than the control haploid males by 3.7 days (Mann-Whitney *U* test, $P < 0.001$) and survival distributions differed significantly (log-rank test, $\chi^2 = 15.079$, d.f. = 1, $P < 0.001$) (Fig. 2B). The haploid proportion of the tKDL diploid measurement once again did not obscure a polyploid effect.

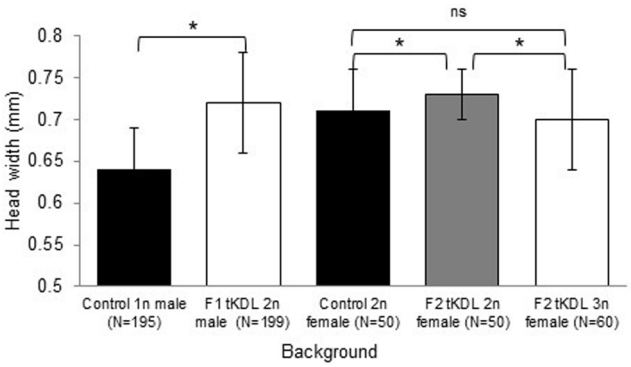

Fig. 1. Mean ± SD of body size (head width proxy measurement) (mm) of polyploid and non-polyploid F1 males and F2 females. Black indicates a non-injected control, gray a non-polyploid background with descent from an ds *tra* injected female, and white a polyploid background with descent from *ds tra* injected female. An asterisk (*) indicates a significant difference between groups and ns indicates non-significance (Kruskal-Wallis test and post-hoc Dunn's test, $P < 0.05$). Note that that the y-axis does not begin at 0.

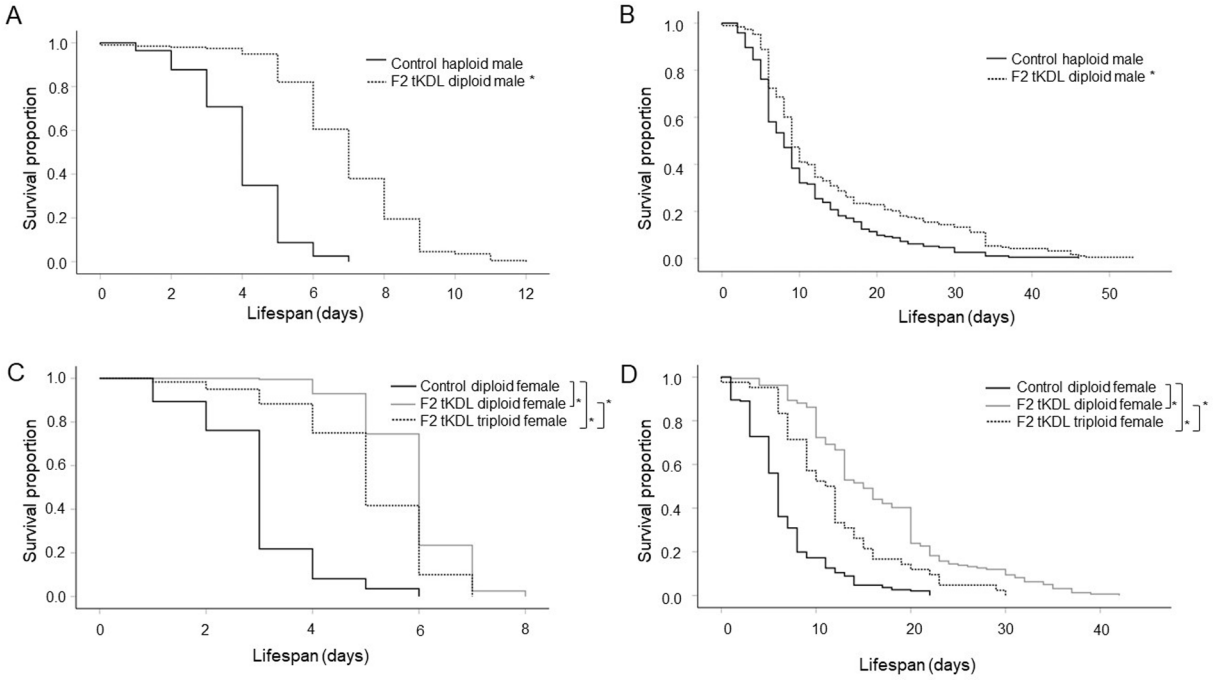

Fig. 2. Survival curves (proportion alive over time) representing the lifespan for A) starved males B) starved females C) males fed with 10 % sucrose solution and D) females fed with 10 % sucrose solution. An asterisk (*) marks the group that had significantly longer lifespan (Kruskal-Wallis test and post-hoc Dunn's test, P < 0.05).

For females under starved conditions, lifespan was control diploids < F2 tKDL triploids < F2 tKDL diploids. All groups differ significantly from each other (Kruskal-Wallis test, $H^2 = 280.70$, d.f. = 2, $P < 0.001$, survival distribution log-rank test, $\chi^2 = 366.479$, d.f. = 2, $P < 0.001$), with the control diploids living ~ 2–3 days shorter than the tKDL diploids and triploids, respectively (Fig. 2C). Female lifespan under fed conditions were control diploids < F2 tKDL triploids < F2 tKDL diploids, with all groups being significantly different from each other (Kruskal-Wallis test, $H^2 = 168.86$, d.f. = 2, $P < 0.001$, survival distribution log-rank test, $\chi^2 = 184.432$, d.f. = 2, $P < 0.001$) (Fig. 2D). The control diploids lived on average only 6.6 days relative to the 16.83 days of tKDL diploids and the 12.05 days of tKDL triploids. Higher ploidy thus does not increase female lifespan, but there was an effect of increased lifespan for originating from ds *tra* injection.

3.3. Male fecundity and sperm depletion

Total progeny number of males (male, female, and larval offspring with single female mate) were, smallest to greatest, F1 tKDL diploid < F1 tKDL haploid < control haploid. The haploid males did not differ from each other (78 offspring of control haploid to 80 offspring of the tKDL haploid), but the tKDL diploid had slightly but significantly fewer progeny (71 offspring) (Kruskal-Wallis test, $H^2 = 15.43$, d.f. = 2, $P < 0.001$) (Fig. 3A). The average sex ratio of male progeny (male/total) was control haploid < tKDL haploid < F1 tKDL diploid. The control haploid had a significantly lower male offspring sex ratio (0.15) than the tKDL haploid (0.21) or the tKDL diploid (0.31), which did not significantly differ from each other (Kruskal-Wallis test, $H^2 = 25.63$, d.f. = 2, $P < 0.001$) (Fig. 3B). In summary, the F1 diploid males sired fewer daughters than either type of haploid male.

The number of female offspring did not decline with sequentially later females in the series for any male type (Fig. 3C), but total female offspring number differed drastically among the groups (Fig. 3D). The control haploid males consistently produced more females than either the haploid or diploid tKDL male (Fig. 3C), averaging a total of 559.4 female offspring over the series (Fig. 3D). This was significantly higher than either of the tKDL males, although the tKDL haploids also produced more female offspring consistently to average a higher total number of female offspring (219.04) over the tKDL diploids (135.80) (Fig. 3D) (Kruskal-Wallis test, $H^2 = 42.59$, d.f. = 2, $P < 0.001$). Correspondingly, male background fit a linear regression with a slope of −0.688 for the haploid tKDL males and −1.298 relative to the control haploid (both $P < 0.001$). The order of the female in the series and individual male identity were insignificant to female offspring count, so these factors were removed. Interestingly, there was also an effect of male background for number of females that apparently failed to have sperm transferred from the male despite observation of successful copulation. The control haploid had on average 0.2 failures, which significantly but only slightly differed from the 0.68 failures of the F1 tKDL haploid, but F1 diploid males averaged 1.53 females mates in their series that failed to produce female offspring (Kruskal-Wallis test, $H^2 = 18.93$, d.f. = 2, $P < 0.001$) (Fig. 3E).

3.4. Parasitization Rate

In the parasitization assay, female lifespan in days ranked as WPL triploid (9.61) < control diploid (12.41) < F2 tKDL triploid (14.60) < F2 tKDL diploid (14.91). All groups differed significantly from each other (Kruskal-Wallis test, $H^2 = 40.73$, d.f. = 3, $P < 0.001$, survival distribution log-rank test, $H^2 = 58.949$, d.f. = 3, $P < 0.001$) (Fig. 4A), but the WPL triploids and control diploids had notably shorter lifespans than the other groups.

The average total number of hosts parasitized (hosts that were killed, whether or not viable offspring were produced) and the percentage of hosts parasitized out of total hosts offered was F2 tKDL triploid < WPL triploid < control diploid < F2 tKDL diploid. Overall differences are significant both for total hosts parasitized (Kruskal-Wallis test, $H^2 = 51.22$, d.f. = 3, $P < 0.001$) (Fig. 4B), and percentage of hosts parasitized (Kruskal-Wallis test, $H^2 = 38.54$, d.f. = 3, $P < 0.0001$) (Fig. 4C). The outbred tKDL and inbred WPL triploids parasitized the same total number of hosts (27), but the WPL triploid parasitized ~ 20 % more of the hosts it was offered. The control and tKDL diploid did not differ significantly in the average number of total hosts killed (44 and 54 respectively), and also killed the same percentage (63 %, 67 %) of hosts

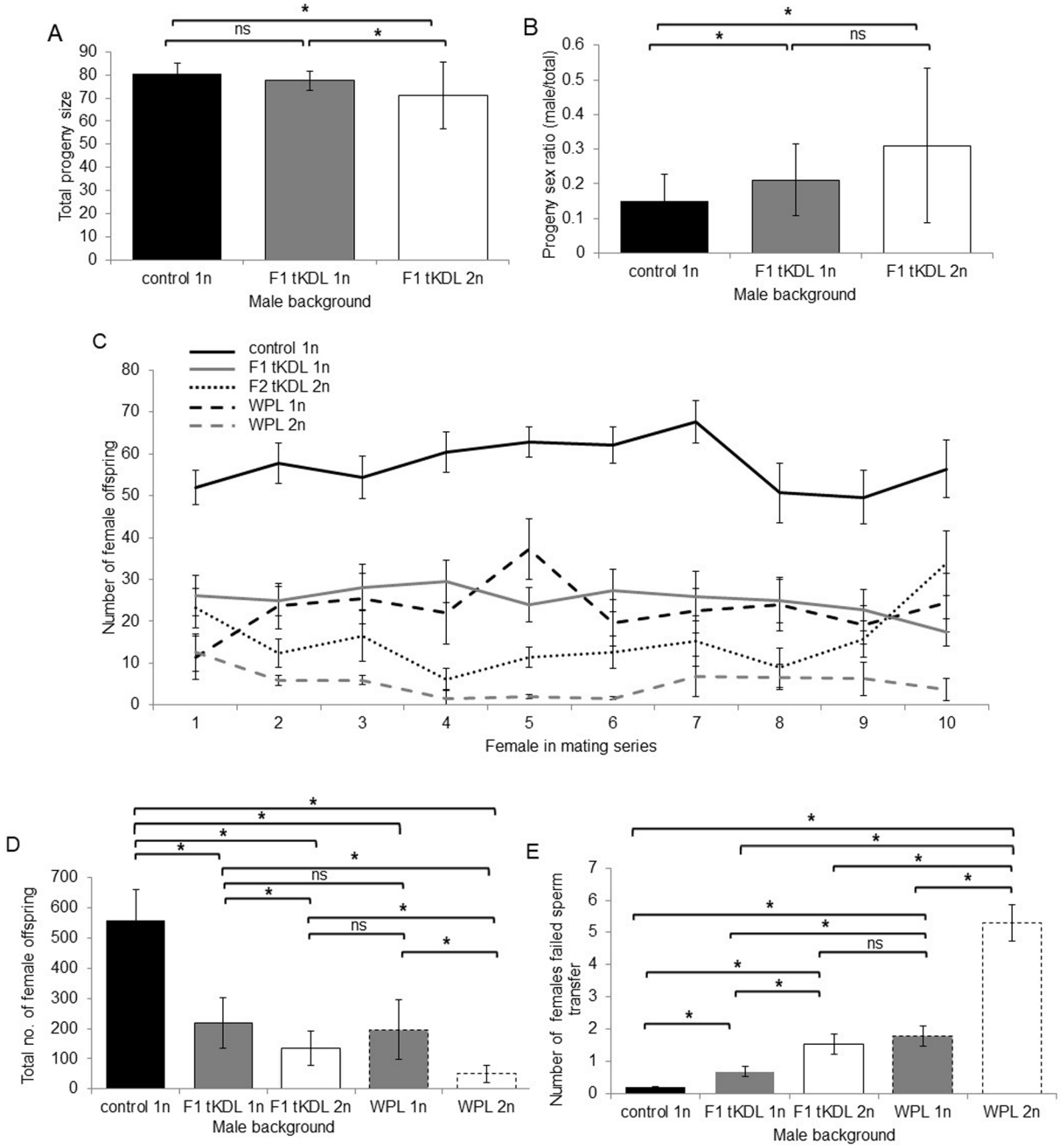

Fig. 3. tKDL F1 male fecundity mean ± SD for A) total progeny number (male, female, and larval offspring) and B) progeny sex ratio (male/total) with a single HVRx female mate. Black indicates a non-injected control, gray a non-polyploid background with descent from a ds *tra* injected female, and white a polyploid background with descent from *ds tra* injected female. Sperm depletion rate was measured as the mean ± standard error (SE) of C) number of female offspring for each successive female mate in a 10-female mating series. D) is mean ± SD total number of female offspring of each male background over the total mating series and E) is mean number of matings (out of ten) for which sperm transfer failed (copulation was observed, but the female produced no daughters). An asterisk (*) indicates a significant difference between groups and ns indicates non-significance (Kruskal-Wallis test and Dunn's post-hoc test, $P < 0.05$). Standard error is shown rather than standard deviation for C and E.

offered. Thus, the diploids were 1.5-2X better at host-killing than the triploids.

The number of hosts that produced offspring was on average, WPL triploid < F2 tKDL triploid < control diploid < F2 tKDL diploid. There were extreme differences of the WPL triploid using only 4.92 hosts to produce offspring compared to the tKDL triploid using 13.9 hosts and the control and tKDL diploid using 34.78 and 43.21 hosts, respectively. With the exception of control diploid and F2 tKDL diploids, all groups differed significantly from each other (Kruskal-Wallis test, $H^2 = 92.89$, $P < 0.001$, d.f. = 3) (Fig. 4D). Lifetime fecundity for tKDL triploids was higher than WPL triploids (Mann Whitney *U* test, Z = -2.95, $P = 0.003$) (Fig. 4E). Parasitization ability over time (number of hosts parasitized for each set of ten given every two days) for both tKDL and WPL triploids was consistently lower than control or tKDL diploids (Fig. 4F), as was the number of hosts used to produce viable offspring (Fig. 4G).

To investigate the contribution of various factors to the likelihood of hosts being parasitized or being used to produce offspring, GLMM analyses were performed individually testing the significance of specific female group (the groups tested), ploidy (2n versus 3n), polyploid background (WPL versus tKDL), originating from ds *tra* injection (yes or

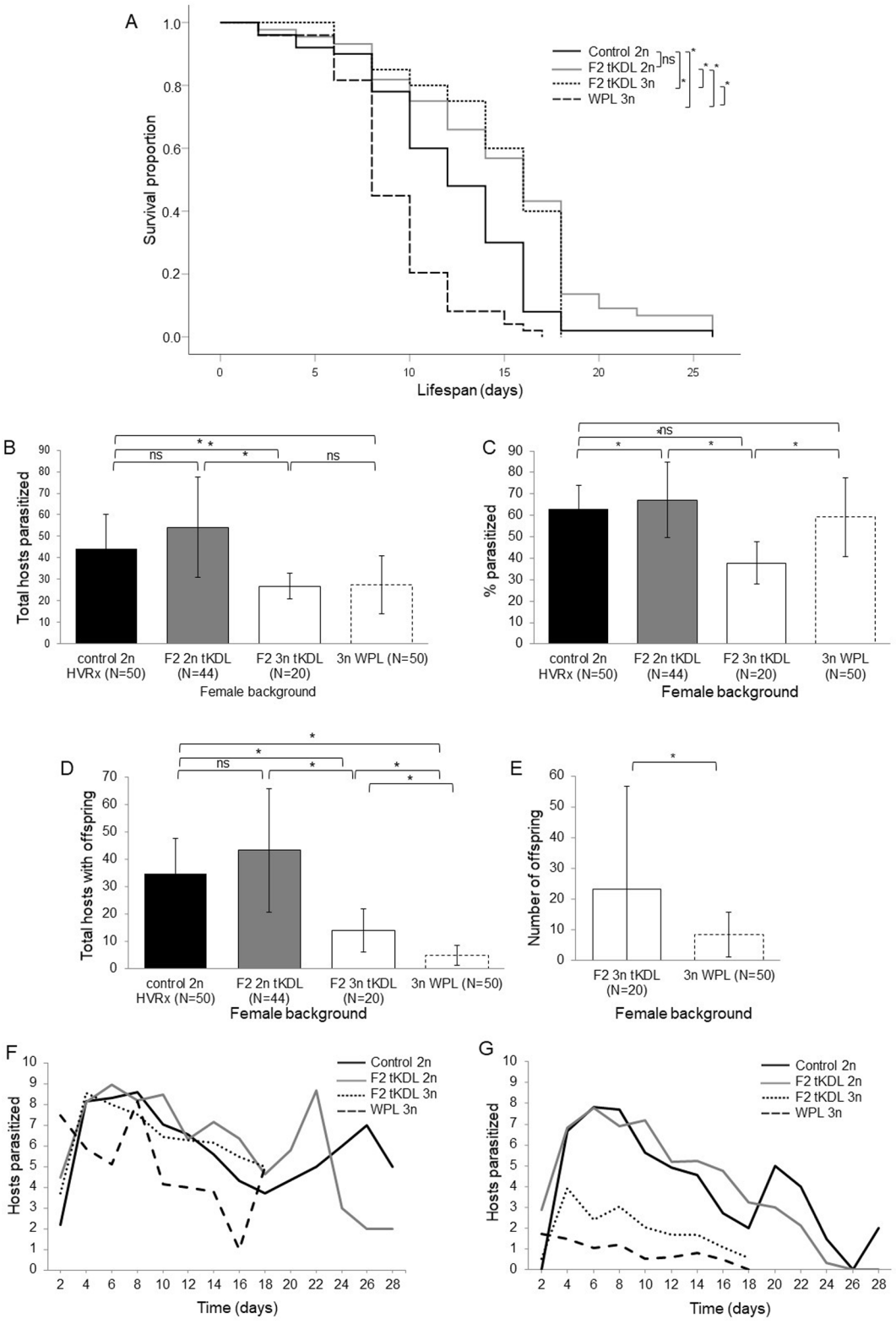

Fig. 4. Host usage of control (HVRx) diploid, F2 tKDL diploid and triploid females, and WPL triploid females in terms of A) lifespan represented by survival curves (proportion of individuals alive over time), B) mean ± SD of total number of hosts parasitized, C) percentage of hosts parasitized, D) number of hosts that resulted in offspring production, and, in the case of tKDL and WPL triploids, E) total number of offspring produced. Out of each set of 10 hosts given every two days, mean F) parasitization ability over time is reflected by number of hosts parasitized over time, and the same applies for G) mean offspring production. For visual clarity, standard deviation values for F and G are reported in Table S3rather than being depicted here.

no), and breeding (outbred versus inbred). In brief, all factors were significant ($P < 0.05$) contributors for host parasitization and offspring production except for background, but group, ploidy, and originating from injection were major contributors (>1.5 fold differences between the category with the lowest parasitization and offspring production and the category with the greatest parasitization and offspring production) and polyploid background and breeding were minor contributors (<1.5 fold differences) (see full GLMM results in Table S2).

4. Discussion

The impacts of polyploidy on biological control-relevant traits varied greatly for the *N. vitripennis* tKDL. Polyploidy did not consistently increase body size or lifespan for either sex, but decreased male overall male fecundity and reduced female parasitization ability. As the number of assays was extensive, general results are reported in Table 1 as simple reference for discussion (for more detailed reporting of results, see Table S1).

4.1. Polyploidy does not always increase body size or lifespan

In insects, larger body size generally correlates to a number of life history traits including higher fecundity, longer lifespan, and better resource acquisition (Beukeboom, 2018). For parasitoids, female body size is also directly related to pest-killing ability (e.g., Cohen et al., 2005; Gao et al., 2016). For *N. vitripennis* specifically, body size is highly correlated to the size of various organs (Xia et al., 2020), and affects a number of biological control traits. For example, it impacts female wing size for dispersal ability (Grillenberger et al., 2008; Xia et al., 2020); male pheromone production (Blaul and Ruther, 2012); and male mating success and behavior (Burton-Chellew et al., 2007, Tsai et al., 2014). How polyploidy changes *N. vitripennis* organ and whole body size can be variable by line (Leung et al., 2019; 2023), but this study (Fig. 1) found tKDL diploid males to be slightly larger than haploid males. This is consistent with the *N. vitripennis* males of WPL (Leung et al. 2019). However, also similar to WPL, triploid tKDL females were not larger than their diploid counterparts (Fig. 1). Cumulatively this suggests that differences in life history are either not likely attributable to body size, or that its effects are minor.

Relatedly, there was no clear pattern of larger body size or higher ploidy resulting in longer starved or fed lifespan for either sex, matching previous results of the WPL (Leung et al., 2019). It has been previously suggested that outbreeding can increase *N. vitripennis* lifespan (Luna and Hawkins, 2004; Leung et al., 2019). This was observed here, as the tKDL is derived up from an outbred population, and starved triploid females of this study lived longer than inbred triploid WPL in a previous study (Leung et al., 2019). Interestingly, tKDL triploids lived longer than tKDL diploids when fed 10 % sugar solution, but the opposite occurred when they had *Calliphora* sp. hosts as an unlimited food source in the parasitization assays, as *Nasonia* females host-feed from hosts in addition to using them for offspring development (Fig. 4F and 4G). Together, these data indicate that polyploidy has context-specific effects on lifespan.

4.2. Sperm limitation contributes to reduced reproductive success in diploid males

Polyploid and non-polyploid males given a single female mate differed from each other in total progeny number, but not drastically. This suggests that females do not alter decisions on how many eggs to oviposit based on differential cues from haploid males, diploid males, or whether males were descended from *tra* knockdown (Fig. 2A). This matches a previous finding that WPL haploid and diploid males do not differ in total progeny number or progeny sex ratio with a single mate (Leung et al., 2019).

However, mating series with ten females in succession uncovered an overall reduction in tKDL diploid male fitness compared to tKDL haploid males. While the mean number of daughters produced with the first female in the series is similar, later females in the series the produced more daughters with the tKDL haploid males than the diploid males (except for the tenth female) (Fig. 3C). This resulted in a > 30 % reduction of total fitness in the tKDL diploid males (Fig. 3D). This demonstrates diploid male fecundity may be impaired, but is not apparent with a single or first mating. Notably though, control haploid males had the highest fecundity overall.

The cause for the reduced fecundity of tKDL diploid males is unknown. The tKDL diploid males may simply have less sperm. Sperm reduction has been observed in *Nasonia* males for other reasons of high heat (Chirault et al., 2015) and interspecies hybrid breakdown (Clark

Table 1
Summary of assay results and implications for biological control. A less than (<) sign denotes a significantly lower value ($P < 0.05$), a less than or equal sign (≤) a lower but not significantly different value (for means ± SD).

Trait	Biological control implications	Assay results
Head width males	Body size influences e.g. fecundity and resource acquisition	control haploid < F1 tKDL diploid male
Head width females	Body size influences e.g. fecundity and resource acquisition	F2 tKDL triploid ≤ control diploid < F2 tKDL diploid
Lifespan starvation males	Longer-lived individuals have more time to mate and produce offspring	control haploid < F1 tKDL diploid
Lifespan fed males	Longer-lived individuals have more time to mate and produce offspring	control haploid < F1 tKDL diploid male
Lifespan starvation females	Longer-lived individuals have more time to mate and produce offspring	control diploid < F2 tKDL triploid < F2 tKDL diploid
Lifespan fed females	Longer-lived individuals have more time to mate and produce offspring	control diploid < F2 tKDL triploid < F2 tKDL diploid
Progeny number males (male, female, and larval offspring with a single female mate)	A higher progeny number increases mass production potential	F1 tKDL diploid < F1 tKDL haploid ≤ control haploid
Progeny sex ratio males (male/total progeny)	A lower male sex ratio indicates a higher production of females used in biocontrol	control haploid ≤ tKDL haploid < F1 tKDL diploid
Total offspring in male mating series (female offspring with ten successive females)	A higher offspring number increases mass production potential; a mating series detects rate of sperm depletion	F1 tKDL diploid < F1 tKDL haploid < control haploid
Sperm transfer failures in male mating series (number of copulated females without female offspring)	Failed sperm transfer indicates reduced mass production potential even if the male successfully copulates	control haploid < F1 tKDL haploid < F1 tKDL diploid
Parasitization lifespan females	Longer-lived individuals have more time to mate and produce offspring	WPL triploid < control diploid < F2 tKDL triploid < F2 tKDL diploid
Total hosts parasitized females	A higher number of hosts parasitized equates more pest-killing	F2 tKDL triploid ≤ WPL triploid < control diploid ≤ F2 tKDL diploid
Percentage of hosts parasitized females (%)	A higher percentage of hosts parasitized equates a higher proportion of pests killed out of all available	F2 tKDL triploid < WPL triploid < control diploid ≤ F2 tKDL diploid
Number of parasitized hosts that produced offspring	An offspring produced in a host may increase likelihood of killing it	WPL triploid < F2 tKDL triploid < control diploid ≤ F2 tKDL diploid
Lifetime fecundity of triploid females in parasitization assay	Number of offspring produced may or may not correspond to number of hosts killed	WPL triploid < F2 tKDL triploid

et al., 2010). However, a contributing factor to the tKDL diploid male's reduced fitness was the higher number of female mates that produced no female offspring, even though copulation was visually confirmed for all females in the mating series (Fig. 3E). It is possible the diploid tKDL males may fail at sperm transfer, or that their diploid sperm fail to fertilize eggs, as with the diploid sperm of polyploid *Habrobracon* males (MacBride, 1946). A follow-up study is needed to assess which of these factors underlie the reduced fecundity of diploid tKDL males. This would involve visual assessment of the dissected male sperm tract to observe total sperm count and any morphological aberrations; and of the dissected female spermatheca post copulation, to check for success or failure of sperm transfer (Chirault et al., 2015).

Regardless of the mechanism, these results that even "highly fertile" diploid males can still experience fitness costs. Understanding them fully requires consideration of multiple factors including degree of fecundity impairment and attractiveness to female mates, which previous *N. vitripennis* studies demonstrated can vary by polyploid line. Specifically, WPL diploid males have high mate competition ability (Leung et al., 2019) and tKDL diploid males have poor mate competition ability (Leung et al., 2023).

4.3. Increased fecundity does not rescue triploid female parasitization

The first assessment of parasitization ability for a triploid parasitoid wasp was for the WPL triploid females, which had poorer parasitization rates and shorter lifespans than diploid counterparts (Leung et al., 2019). In applied terms, this equates poorer biological control performance because females had poorer host-killing ability and less time to do it. However, in another study it was observed that the tKDL triploid females produce 3–10 times as many offspring as WPL triploids (Leung et al., 2023). This higher fecundity has been recapitulated with this study's lifetime fecundity measurement, with triploid tKDL females producing about 3 times as many offspring as WPL counterparts (Fig. 4E). I expected the tKDL's higher offspring production to correspond to higher parasitization ability, with increased larval feeding increasing host killing. Unexpectedly, the parasitization ability of the more fecund tKDL background is no higher than highly infertile triploid females of the WPL (Fig. 4D).

Surprisingly little is known about how intraspecific variation in fecundity correlates to parasitoid host killing. The intuitive assumption would be that the more fecund the parasitoid, the higher the parasitization rate and the better the biological control agent, but this has only been supported in parasitoids of Lepidoptera (Lane et al., 1999). Rather, destructive female host-feeding may be a better predictor for biological control success than egg load (Kidd and Jervis, 1989; Jervis et al., 2001). Neither WPL or tKDL triploid females were observed to be deficient in host feeding, but this needs more rigorous assessment. Regardless, higher offspring production did not rescue the parasitization ability of the outbred tKDL triploid females in this study. Unexpectedly, in a separate study on inbred tKDL and other inbred polyploid lines, triploid host killing abilities were equal or higher than their own diploid female controls (Li and Leung, in print). We attribute the difference of results to possible outbreeding depression for parasitization in this study, as outbreeding depression for triploid parasitization also occurred for in WPL for a previous study (Leung et al., 2019).

4.4. Synthesis on the future of non-CSD polyploidy in biological control

Polyploid incidence has not been well surveyed for non-CSD species. This might be because it is less easy to detect than in CSD species, which produce diploid males if inbred (Cook, 1993). However, as the vast majority of parasitoid wasp species used in biological control are non-CSD (Cruaud et al., 2019; van Lenteren et al., 1997, 2018), this topic deserves greater attention. A major difference between CSD polyploidy and the representation of non-CSD polyploidy in this study is that CSD-based polyploidy is usually couched in total polyploid sterility (Zayed and Packer, 2005). In contrast, fecundity falls along a gradient for non-CSD based polyploidy and detriment is context dependent. For example, *N. vitripennis* male mate competitiveness can vary (Leung et al., 2019, 2023), and polyploid female fecundity is generally impaired (Leung et al., 2019; this study) but parasitization ability can vary (Leung et al., 2019, this study, Li and Leung, in print).

The high degree of intraspecific polyploid phenotype variation in *N. vitripennis* supports a radical suggestion that non-CSD polyploidy can be explored for three possible benefits to biological control. For systems that have heritable viable polyploidy like *Nasonia* it is 1) possible to experiment with heritable dosage and dominance effects with more allele copies, pushing them to new extrema. For example, host specificity is the trait controlling host range and thus is a key determiner of biocontrol efficacy and likelihood of non-target effects (Segoli et al., 2023). In *Nasonia* spp., a known interspecific host specificity locus it is additive dominant for specialist behavior (Desjardins et al., 2010). A third copy in triploid females may tailor them to have higher host specificity than is possible for diploids. The difficulty is that the impaired fitness of both male and female polyploids greatly reduces the mass production potential for any individuals optimized through higher order dosage/dominance effects. Male-specific traits would be especially difficult to pursue, as the diploid male generation is highly bottlenecked. For biological control, this would include fitness traits such as high male fecundity and mate attractiveness needed to ensure a high level of commercial mass production.

Another prospect is 2) using polyploidy to retain sexually antagonistic alleles that are beneficial to females but would normally be purged for being deleterious to the haploid male. By default, these alleles would have to be maintained heterozygously in the diploid male, similar to diploid females of paper wasp *Polistes fuscatus* (Miller and Sheehan, 2023) and invasive ant *Nylanderia fulva* (Eyer et al., 2019) being biased towards heterozygosity in retaining alleles deleterious to males. In essence, the same thing is being proposed here with the sexes reversed, so that heterozygous diploid males may act as a reservoir to protect female-beneficial alleles.

Lastly there is 3) the possibility of using infertile triploid females as biocontrol agents to limit admixture. Normal diploid females are subject to establishing permanently and becoming invasive (Roy and Wajnberg, 2004, Myers and Cory, 2017), or admixing with local populations (e.g. native or pre-released strains) can situationally cause undesirable genetic dilution (Verhoeven et al., 2011) or enhance invasiveness (Turgeon et al., 2011, Li et al., 2018). Low or non-reproductive triploid females would have a more transient, ideally single generation presence in the environment. The most prohibitive problem with this idea is that the triploid females here have impaired parasitization, suggesting a cost of poorer biocontrol function for any ecological safety gain. However, it is possible that the threshold value of pest control can be met anyways with higher female release; for the females in this study, for example, twice as many triploids would be needed to match the number of hosts killed by the diploids. It is also important to note that this work was done in the first polyploid female generation, and it possible that polyploid parasitization can be improved with subsequent selection in a genetically variable background. Finally, there is also evidence that polyploid host-killing ability can be higher in other lines. Strikingly and in contrast to the outbred females of this study, triploid *N. vitripennis* females of several inbred backgrounds had the same or higher host killing abilities as their diploid counterparts (Li and Leung, in print). Inbreeding (e.g. sib-mating) and inbreeding tolerance is inherent to *N. vitripennis* biology (Werren and Loehlin, 2009), so these lines may be the more robust option to explore for biological control utility than this study's outbred lines.

Conditional to any of this is having greater knowledge of non-CSD species sex determination systems. Especially, the number of species that can be as reliably made polyploid with mostly functional polyploids is unexplored. But enticingly, *Nasonia's* megadiverse Chalcidoidea superfamily (500,000 + species) (Heraty et al., 2013), contains the most

prevalent families in parasitoid biological control: Aphelinidae, Encyrtidae, Eulophidae, Mymaridae and Trichogrammatidae (Cruaud et al., 2019). It may well be they have some overlap with the highly malleable polyploid biology of *Nasonia*. With this study I hope to highlight the understudied state of non-CSD polyploidy, and inspire more research on its roles in biological control.

CRediT authorship contribution statement

Kelley Leung: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The author declares that she has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2024.105659.

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