Polyploidy and host specificity genetics in Nasonia parasitoid wasps

Leung, Kelley

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Chapter 4

Effects of polyploidy on biocontrol-related traits in the non-CSD parasitic wasp *Nasonia vitripennis*

Kelley Leung[1], Louis van de Zande[1], Leo W. Beukeboom[1]

1. Groningen Institute for Evolutionary Life Sciences, University of Groningen, PO Box 11103, 9700 CC Groningen, The Netherlands

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Abstract

In parasitic wasps, males are haploid and females are diploid, but polyploids (diploid males, triploid females) frequently occur. Polyploidy is associated with extinction risk for species with Complementary Sex Determination (CSD) because increasing incidence of sterile diploid males reduces population size. However, most parasitoid wasps do not have CSD, and in these species polyploid effects are largely unknown. *Nasonia vitripennis* is a non-CSD parasitoid with reproductive polyploids. To better understand effects of polyploidy on non-CSD species, we established a polyploid line, tKDL, by knockdown of the feminizing sex-determination gene *transformer,* in an outbred background. We assayed traits in tKDL and in the long-established, inbred Whiting polyploid line (WPL). In tKDL, body size and lifespan (under starved and fed conditions) were increased in polyploids compared to non-polyploids for males but not females. Single-pair matings indicated equal fecundity for haploid and diploid tKDL males, but multiple mating assays revealed faster sperm depletion in diploid males than haploid males. tKDL triploid females were unusually fecund for polyploid Hymenoptera, but parasitized fewer hosts than tKDL diploids. Higher fecundity did not correlate with higher parasitization ability. This highlights the importance of in-depth fertility and fecundity assays to more accurately determine the fitness costs of polyploidy in hymenopterans. We discuss our results in the context of applying polyploidy in biological control.

Keywords: diploid male, triploid female, sperm depletion, parasitization rate, life history traits, fertility, fecundity, *Nasonia*, biological control, body size

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 appear with relatively high frequency throughout the order (more than 60 species), and triploid females occasionally occur as well (Cowan & Stahlhut, 2004; Zayed & Packer, 2005; Van Wilgenburg, Driessen, & Beukeboom, 2006; Heimpel & de Boer, 2008). Hymenopteran polyploidy has long been linked with deleterious effects, particularly in the parasitoid wasps, one of the most important classes of biocontrol agents for arthropod pests (Hassell & Waage, 1982; Van Lenteren, Roskam, & Timmer, 1997; Van Lenteren, 2012; Van Lenteren, Bolckmans, Köhl, Ravensberg, & Urbaneja, 2018). For example, parasitoid wasps account for 13 out of 30 of the most commercially valuable European biocontrol agents (Van Lenteren *et al.*, 1997) and globally, more hymenopteran species are used for biological control than all other arthropod orders combined (Cruaud *et al*., 2019; Van Lenteren, 2012).

Parasitoid populations including captive breeding populations are endangered by a polyploid effect called the "diploid male vortex," but, so far, this applies only to species with Complementary Sex Determination (CSD). Under CSD, individuals that are heterozygous for a *csd* locus (or loci) develop into females, whereas *csd* hemizyotes and homozygotes develop into males (Whiting, 1943; Cook & Crozier, 1995). Such homozygous diploid males are sterile, but diploid females generally do not discriminate between them and fertile haploid males (Harpur, Sobhani, & Zayed, 2013). As most parasitoid wasp species are monandrous (Henter, 2004; Harpur *et al.*, 2013), females that mate with sterile diploid males do not produce daughters. Therefore, in CSD species, if *csd* homozygosity is increased by genetic drift and inbreeding, the population becomes progressively more (sterile) male-biased and at risk for extinction (Zayed & Packer, 2005; Hein, Poethke, & Dorn, 2009; Fauvergue *et al.*, 2012, 2015; Faria *et al.*, 2016; Zaviezo *et al.*, 2018).

Although CSD is the best-studied sex determination mechanism in the Hymenoptera, most parasitoid wasps are not CSD species. Of the parasitoid wasp species evaluated for their sex determination mechanism, about half do not have CSD (Beukeboom, Kamping, & Van de Zande, 2007). Furthermore, it is believed to be entirely absent in some groups like the megadiverse Chalcidoidea (500,000+ species) (Heraty *et al.*, 2013), which contains the families that comprise the majority of parasitoid wasp biocontrol agent diversity (including key families such as Aphelinidae, Encyrtidae, Eulophidae, Mymaridae and Trichogrammatidae; Cruaud *et al.*, 2019).

Polyploidy has been sparingly studied in these non-CSD species. The most extensively studied non-CSD species is *Nasonia vitripennis*, a chalcid parasitoid of blowfly pupae. This species been used as a research model for study of parasitoid traits important to biological control mass production and field performance, including sex ratio (Werren, 1984; Orzack, Parker, & Gladstone, 1991; Pannebakker *et al.*, 2011; Martel *et al.*, 2016), fecundity (Whiting, 1967; Rivers & Denlinger, 1995), juvenile diapause (Walker & Saunders, 1962; Saunders, 1966; Saunders, Sutton, & Jarvis, 1970; Wolschin & Gadau, 2009; Paolucci, Van de Zande, & Beukeboom, 2013; Paolucci *et al.*, 2016;

Benetta, Beukeboom, & Van de Zande, 2019), memory and learning (Baeder & King, 2004; Hoedjes, Smid, Vet, & Werren, 2014; Hoedjes & Smid, 2014; Hoedjes, Smid, Schijlen, Vet, & Van Vugt, 2015; Oliai & King, 2000; Schurmann, Collatz, Hagenbucher, Ruther, & Steidle, 2009; Schurmann *et al*., 2012) venom potency (Rivers, Hink, & Denlinger, 1993; De Graaf *et al.*, 2010; Martinson *et al.*, 2014), and host specificity (Desjardins *et al.*, 2010).

Polyploidy has been known in *N. vitripennis* for several decades. It first manifested in 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 *de novo* polyploid lines in *Nasonia* through RNAi knockdown of gene targets in its sex determination pathway. One such target is the feminizing *transformer* (*tra*) gene. Silencing maternal *tra* results in diploids embryos developing into males rather than females (Verhulst, 2010; Koevoets *et al.*, 2012). Such diploid males can be used to establish new polyploid lines, as they make diploid sperm and are fertile. Their triploid daughters also produce offspring, helped by the fact that *Nasonia* has only five chromosomes (Werren & Loehlin, 2009a) so that aneuploidy does not completely prevent proper gametogenesis. These multiple polyploid resources make *N. vitripennis* highly useful for expanding our knowledge on how polyploidy operates in non-CSD parasitoid wasps

Nasonia vitripennis is unusual among the parasitoid wasps in having reproductively competent polyploids for both males and females (Whiting, 1960; Beukeboom & Kamping, 2006; Leung, Van de Zande, & Beukeboom, 2019), the only other known hymenopteran known to have fertile polyploids for both sexes being the CSD vespid wasp *Euodynerus forminatus* (Cowan & Stahlhut, 2004) (although there is a large range in fecundity for *N. vitripennis* triploid females; this thesis, Chapter 3). Both the inbred Whiting polyploid line (WPL) and an outbred *tra* knockdown line (tKDL) have previously been phenotyped for a suite of traits comparing non-polyploids and polyploids (Leung *et al.*, 2019; this thesis Chapter 3). This work indicated that if polyploid disadvantage exists for a trait, its degree of severity is context dependent. For example, WPL diploid males are equally capable of mate acquisition as haploid males, but tKDL diploid males are severely impaired. Also, whereas WPL triploid females are only capable of producing a few offspring (euploid eggs), tKDL triploid females can produce progenies of up to 13 wasps per host (Leung *et al.*, 2019; this thesis, chapter 3).

These other studies suggest high variation in polyploid phenotypes possible for *N. vitripennis* (Leung et al., 2019; this thesis Chapter 3), which is highly atypical for most taxa. To characterize a fuller range of polyploid effects possible within this single non-CSD parasitoid species, in this study we assay several additional fitness traits in tKDL related to biocontrol efficiency. These include (1) body size for both sexes, which influences a number of other life history traits including fecundity and intraspecific competition for resources (Beukeboom, 2018) (2) lifespan for both sexes, which determines the time in which an individual can breed and, in case of females, operate as a biocontrol agent (3) male fertility with a single female as well as with multiple females (4) female parasitization ability, the direct determinant of biocontrol

efficacy and (5) female production of diapause larvae, which can be exploited for efficient storage and shipping (Denlinger, 2008). It was expected that there would be some level of polyploid detriment, although it could not be anticipated for which traits and to what extent.

Materials and Methods

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). In brief, WPL triploid virgin females produce both diploid and haploid sons with eye markers indicating ploidy level. 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 separate red-eyed mutant strain (*scarlet*) to recover triploid females and restart the breeding cycle. The full WPL breeding scheme is outlined elsewhere (Whiting, 1960; Beukeboom & Kamping, 2006; Leung *et al.*, 2019)

The *tra* knockdown line (tKDL) was generated in the HVRx background, a genetically variable laboratory population 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, 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 and how individuals are typed for ploidy through a combination of offspring count and flow cytometry is elsewhere in this thesis (Chapter 3, Figure S2).

Body size

Head width was used as a proxy for overall body size (as in Charnov & Skinner, 1984; Weston, Qureshi, & Werren, 1999; Leung *et al.* 2019). It was measured for control (HVRX haploid male and, diploid females) and tKDL individuals (F1 diploid males; F2 diploid and triploid females). For the F1 tKDL diploid male measurement, ~20% of individuals were expected to be haploid due to a fraction of unfertilized eggs in each progeny of mated females (Werren & Loehlin, 2009a), 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 was taken with 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.

Lifespan

Lifespan was measured for control individuals and tKDL polyploids and non-polyploids under both starvation and feeding conditions for F1 males and F2 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 hours.

Male fecundity and sperm depletion

Nasonia males have a single wave of spermatogenesis in the pupal stage (Chirault *et al.*, 2013, 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 hours (WPL males were not assessed for this here, but were assayed for this trait in Leung *et al.*, 2019). Males were also assessed for sperm count depletion with mating series. Individual control (haploid), tKDL F1 (haploid and diploid), and WPL (haploid and diploid) males were each given a series of 10 virgin females from the HVRx control population in quick succession. Female offspring counts from these series approximate how quickly sperm is depleted. As circadian rhythms can influence insect mating behavior (Sakai & Ishida, 2001; Rymer *et al.*, 2007; Bertossa *et al.*, 2013), all mating series began at 12h. Each male was presented one female at a time, and as soon as the male terminated copulation by starting post-copulatory courtship (Van den Assem, Gijswijt, & Nübel, 1980), 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.

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 hosted on ten fresh *Calliphora* hosts. Every two days the female was given 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 under standard culture conditions for up to three weeks. At this point, every host was scored for parasitization success, *i.e.* 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 yielded viable offspring (at least one individual that developed to diapause larval stage or adulthood). 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 feeding, stinging or oviposition from female wasps. However, as this was an estimated <5% of hosts, this was not factored into analyses.

Diapause scoring

Nasonia larval diapause is a temporary arrest in juvenile development induced by the mother in response to environmental cues such as shorter photoperiod and lower temperature (Saunders, 1966), although maternal age (Walker & Saunders, 1962) and host quality (Saunders *et al.*, 1970) can also have an effect. Diapause larvae are distinguished from non-diapause larvae by their

appearance (fatter, whiter, and less active) and not developing beyond the larval stage after more than two weeks at standard culture conditions (Paolucci *et al.*, 2013).

Two types of control diploid females were used, uninjected and injected with ds *tra*. This was also done for F2 tKDL females (the diploid female offspring of F1 tKDL haploid males and the triploid female offspring of F1 tKDL diploid males), to investigate potential heritable effect. Each <1 day old female was given 24 hours to mate with a control male, and then provided three hosts. Offspring were allowed to develop under standard conditions for four weeks and were then scored for diapause. For the hosts given to F0 ds *tra* injected females, those that yielded female offspring were discarded from analyses, as this indicated *tra* knockdown failure. For data consistency, for all other groups, those that produced no female offspring (suggesting mating failure) were also excluded from analyses (however, this only occurred for ~8% of the mated F2 tKDL triploid females).

Statistical analyses

All statistical tests were performed in SPSS version 15 (IBM, 2017). Null hypotheses of non-significant difference were rejected if $P<0.05$. For all assays, datasets were checked for normality with Shapiro Wilks tests and for equal variance with Levene's tests. As these conditions were not met for any dataset, non-parametric Mann-Whitney U tests and Kruskal-Wallis test were used. For Kruskal-Wallis tests, post-hoc Dunn's tests were used to identify which specific groups differed significantly from each other. 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 vs. WPL), ploidy state (diploid vs. triploid), whether the group descended from injection (yes for tKDL, no for control and WPL), and genetic variability breeding status (inbred for WPL, outbred for control and tKDL) were individually tested as fixed effects (with the intercept included). To correct for uneven sample size and non-normality, Satterthwaite approximations and an estimations of robust variance were used.

Results

Body size (head width)

Based on body size proxy measurement of head width, the F1 tKDL diploid males (N=199, 0.72 ± 0.06 mm) (*i.e.* the diploid offspring of ds *tra* injected females diverted from female development) are on average larger than control haploid males (N=195, 0.64 ± 0.05 mm) (Mann-Whitney U test, $Z=12.08$, $P<0.001$). As this is a significant difference, the ~20% of individuals in the tKDL diploid

measurement being haploids that developed from unfertilized eggs did not mask a polyploid effect (or a *tra* knockdown effect). For females, smallest to largest were F2 tKDL triploids (N=60, 0.70 ± 0.06 mm) < control diploid females (N=50, 0.71 ± 0.05 mm) < F2 tKDL diploid females (N=50, 0.73 mm ± 0.03 mm). The F2 tKDL diploids differed significantly from both control diploids and the F2 tKDL triploids 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; Dunn's post-hoc test: control diploid-F2 tKDL diploid, *P*=0.017; control diploid-F2 tKDL triploid, *P*= 0.583; F2 tKDL diploid-F2 tKDL triploid, *P*=0.001). Therefore, tKDL polyploids are larger than non-polyploids for F1 males, but not for F2 females (Figure 1).

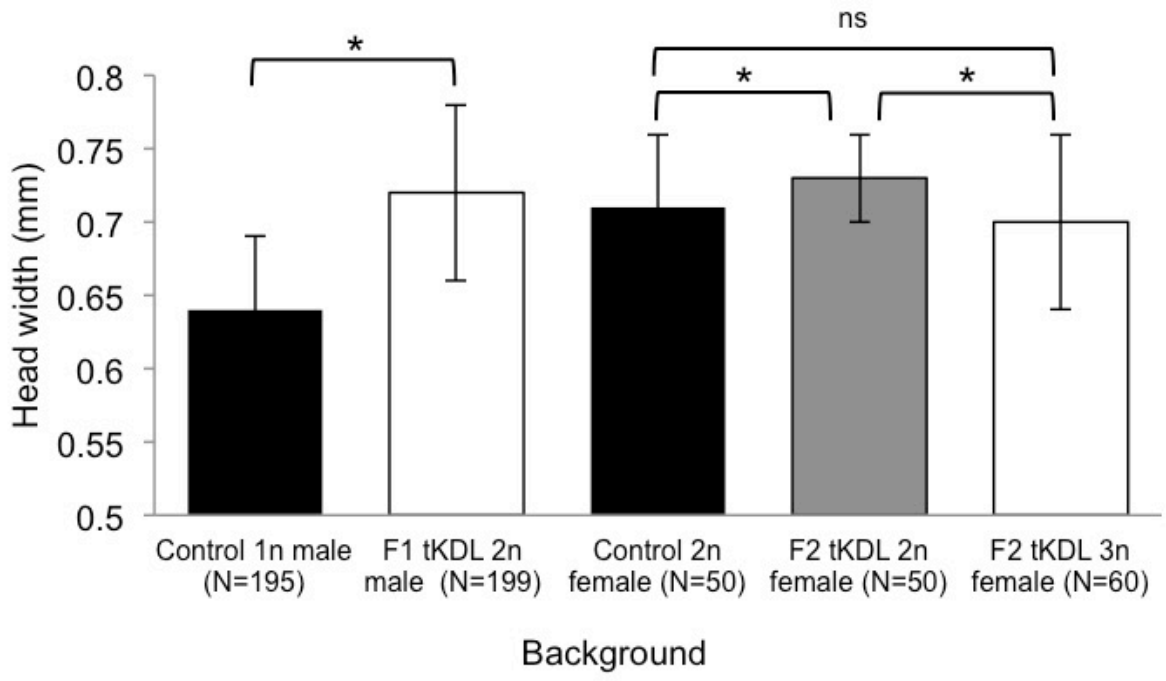

Figure 1. Mean ± SD of body size (head width proxy measurement) (mm) of polyploid and non-polyploid F1 males and F2 females. Black indicates a non-injected control, gray a non-polyploid background with descent from a ds *tra* injected female, and white a polyploid background with descent from *ds tra*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

Lifespan

Under starvation conditions, the F1 tKDL diploid males (N=195, 6.96 ± 1.83 days) lived significantly longer than the control haploid males (N=195, 4.01 ± 1.26 days) (Mann-Whitney U test, Z= 14.33, *P*<0.001), with survival distributions being significantly different (log-rank test, χ^2=257.415, d.f.=1, *P*<0.001) (Figure 2A). Under fed conditions, the F1 tKDL diploid males (N=188, 14.07 ± 11.05 days) again lived longer than the control haploid males (N=193, 10.37 ± 7.64 days) (Mann-Whitney U test, Z=-3.49, *P*<0.001) and survival distributions differed significantly (log-rank test, χ^2=15.079, d.f.=1, *P*<0.001) (Figure 2B). As these results reflect significant differences between diploid and haploid males, the haploid proportion of the tKDL diploid measurement did not obscure a polyploid effect.

For females under starved conditions, shortest-lived to longest-lived were control diploids (N=200, 2.99 ± 1.09 days) < F2 tKDL triploids (N=60, 5.08 ± 1.30 days) < F2 tKDL diploids (N=197, 5.93 ± 0.90 days). All groups differ significantly from each other (Kruskal-Wallis test, H^2=280.70, d.f.=2, *P*<0.001; Dunn's post-hoc test: control-F2 tKDL diploid, *P*<0.001; control-F2 tKDL triploid, *P*<0.001; F2 tKDL diploid-F2 tKDL triploid, *P*<0.001), which is reflected in the differences in survival distribution (log-rank test, χ^2=366.479, d.f.=2, *P*<0.001) (Figure 2C). Female lifespan under fed conditions were ranked, control diploids (N=182, 6.61 ± 4.39 days) < F2 tKDL triploids (N=42, 12.05 ± 6.40) < F2 tKDL diploids (N=159, 16.83 ± 8.30 days), with all groups being significantly different from each other (Kruskal-Wallis test, H^2=168.86, d.f.=2, *P*<0.001; Dunn's post-hoc test: control-F2 tKDL diploid, *P*<0.001; control-F2 tKDL triploid, *P*<0.001; F2 tKDL diploid-F2 tKDL triploid, *P*<0.001), which also applied to survival distribution (log-rank test, χ^2=184.432, d.f.=2, *P*<0.001) (Figure 2D). There was thus no pattern of higher ploidy increasing female lifespan (tKDL haploids versus diploids), but there was an effect of increased lifespan for descent from ds *tra* RNA injection (tKDL versus controls).

Male fecundity and sperm depletion

Total progeny sizes of males (male, female, and larval offspring with single female mate) were smallest to greatest in the order of F1 tKDL diploid (N=46, 71.13 ± 95.55 offspring) < F1 tKDL haploid (N=42, 77.50 ± 27.03) < control haploid (N=42, 80.60 ± 30.63 offspring). The two types of haploid males did not differ from each other, but the tKDL diploid had slightly but significantly fewer progeny than either type of haploid male (Kruskal-Wallis test, H^2=15.43, *P*<0.001, d.f.=2; Dunn's post-hoc test: control haploid-F1 tKDL haploid, *P*=0.55; control haploid-F1 tKDL diploid, *P*<0.001; tKDL haploid-tKDL diploid, *P*=0.002) (Figure 3A). The average sex ratio of male progeny (male/total) was, ranked from lowest to highest, control haploid (N=42, 0.15 ± 0.08) < tKDL haploid (N=42, 0.21 ± 0.10) < F1 tKDL diploid (N=46, 0.31 ± 0.22). These ratios were significantly different from each other, except for between tKDL haploids and diploids (Kruskal-Wallis test, H^2=25.63, d.f.=2, *P*<0.001; Dunn's post-hoc tests: control haploid-F1 tKDL haploid, *P*<0.001; control haploid-F1 tKDL diploid *P*<0.001; F1 tKDL haploid-F1 tKDL diploid, *P*=0.09) (Figure 3B). In summary, the F1 diploid males sired fewer daughters than either type of haploid male.

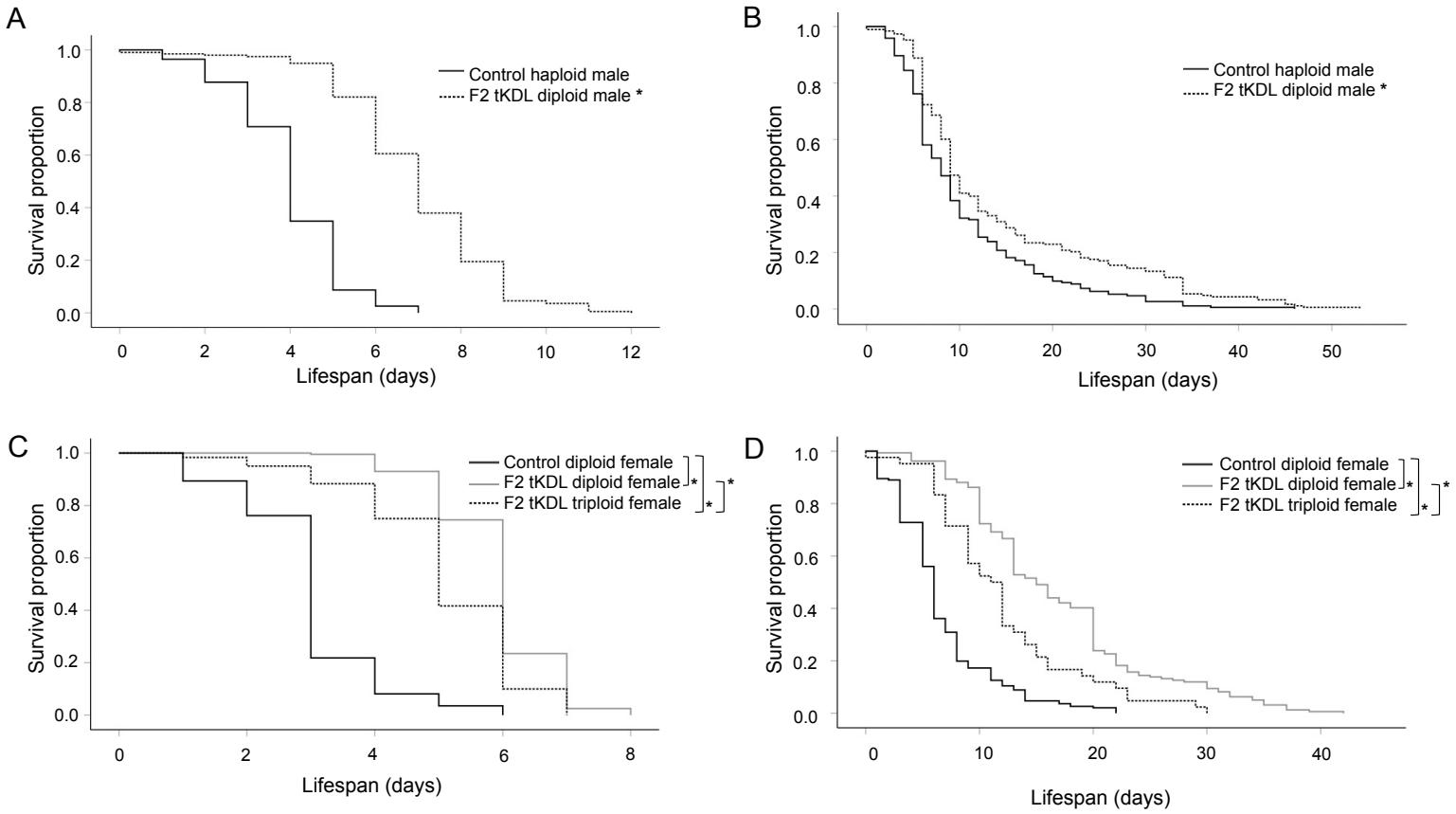

Figure 2. Survival curves (proportion alive over time) representing the lifespan for A) starved males B) starved females C) males fed with 10% sucrose solution and D) females fed with 10% sucrose solution. An asterisk (*) marks the group that had significantly longer lifespan (Kruskal-Wallis test and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

We evaluated sperm depletion rate in the control (haploid), F1 tKDL haploid and F1 tKDL diploid, and WPL haploid and diploid males by providing an ordered series of female mates (N=10) and counting their female offspring. Unlike the prior assay, for which females that did not produce female offspring were presumed to be unmated, all females of this assay were visually confirmed to have mated. The number of female offspring did not decline with sequentially later females in the series for any male type (Figure 3C). The control haploid males (N=20) consistently produced significantly more females than either type of tKDL or WPL male (Figure 3C), averaging a total of over 559.4 ± 101.2 (SD) female offspring over the series (Figure 3D). The tKDL haploids (N=25) also produced more female offspring consistently and averaged a higher total number of female offspring (219.04 ± 83.17 female offspring) over the tKDL diploids (N=15; 135.80 ± 56.71 female offspring). The WPL haploids produced the same number of female offspring (N=18, 196.11 ± 99.15 female offspring) as tKDL males, but the WPL diploid males produced the least female offspring of all groups (N=14, 50.44 ± 28.80 female offspring) (Kruskal-Wallis test, H^2=63.82, d.f.=4, P<0.001; Dunn's post-hoc tests: control haploid-F1 tKDL haploid, *P*<0.001; control haploid-F1 tKDL diploid *P*<0.001; control haploid-WPL haploid, *P*<0.001 ; control haploid-WPL diploid, *P*<0.001; F1 tKDL haploid-F1 tKDL diploid, *P*=0.03; F1 tKDL haploid-WPL haploid *P*=0.52; F1 tKDL haploid-WPL diploid, *P*<0.001; F1 tKDL diploid-WPL haploid, *P*=0.16; F1 tKDL diploid-WPL diploid *P*=0.04; WPL haploid-WPL diploid, *P*<0.001) (Figure 3D).

Correspondingly, male background fit a linear regression with a slope of -0.688 for the tKDL haploid males, -1.298 for tKDL diploid males, -0.898 for WPL haploid males, and -2.387 for diploid males relative to the control haploid (all *P*<0.001). Initial analyses found that the order of the female in the series and individual male identity (except for WPL diploid males, because a number of individuals had high sperm transfer failure, causing many females to have no female offspring, and therefore artefactually high variation in female offspring production for this one category of male; see below) were insignificant to female offspring count, so these factors were removed.

Interestingly, there was also an effect of male background for number of females in the series that failed to produce female offspring despite observation of successful copulation, suggesting failure of the male to transfer sperm. The control (N=20, 0.2 ± 0.0 failures) and tKDL (N=25, 0.68 ± 0.73 failures) haploid males did not differ from each other in this aspect. However, the tKDL diploid males (N=15, 1.53 ± 1.20 failures), WPL haploid (N=18, 1.78 ± 1.36 failures) and WPL diploid males (N=14, 5.29 ± 2.11 failures) had a higher number of transfer failures, with the WPL diploid males being particularly deficient with more than half of female mates failing to receive sperm on average (or, receiving it and failing to use it) (Kruskal-Wallis test, H^2=53.50, d.f.=4, *P*<0.001; Dunn's post-hoc tests: control haploid-F1 tKDL haploid, *P*<0.001; control haploid-F1 tKDL diploid *P*<0.001; control haploid-WPL haploid *P*<0.001; control haploid-WPL diploid P<0.001; F1 tKDL haploid-F1 tKDL diploid, *P*=0.02, F1 tKDL haploid-WPL haploid *P*=0.02; F1 tKDL haploid-WPL diploid; F1 tKDL diploid-WPL haploid, *P*=0.79; F1 tKDL-WPL diploid, *P*<0.001; WPL haploid-WPL diploid, *P*=0.002) (Figure 3E).

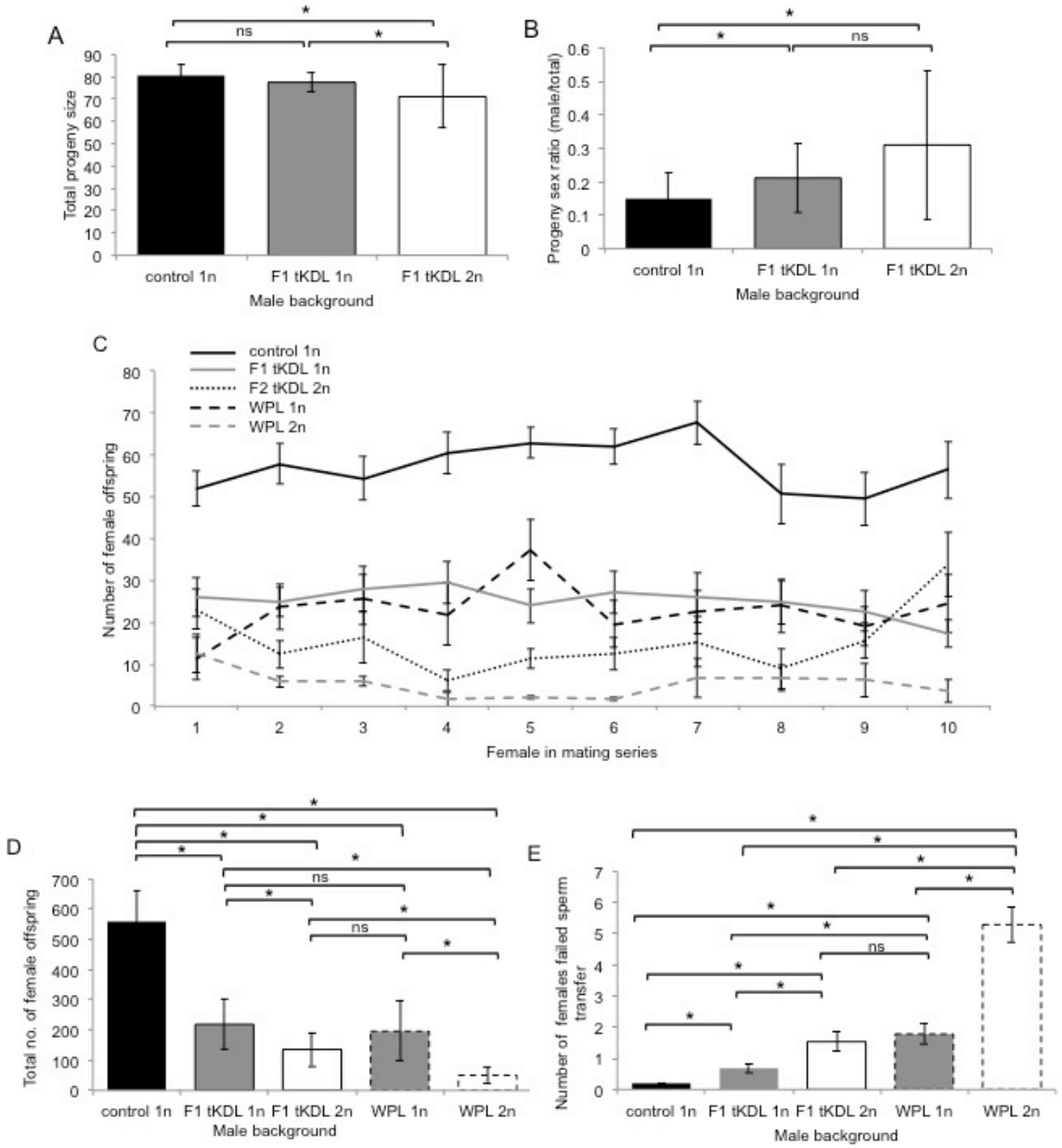

Figure 3. tKDL F1 male fecundity in terms of mean ± SD of A) total progeny size (male, female, and larval offspring) and B) progeny sex ratio (male/total) with a single HVRx female mate. Black indicates a non-injected control, gray a non-polyploid background with descent from a ds *tra* injected female, white a polyploid background with descent from *ds tra* injected female, gray with dashed border the non-polyploid WPL background, and white with a dashed border the polyploid WPL background. 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. We also show the D) mean ± SD total number of female offspring of each male background over the total mating series and E) 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 because large SD values obscure visual clarity.

Parasitization rate

Parasitization rate was measured by giving females 10 fresh hosts every two days until death, which represented continuous host-feeding, stinging (envenomation), and oviposition opportunity. The tKDL and WPL were both examined to evaluate background effect on parasitization ability. Females were shortest to longest lived in order of WPL triploid (N=50, 9.61 ± 2.93 days) < control diploid (N=50, 12.41 ± 4.50 days) < F2 tKDL triploid (N=20, 14.60 ± 4.11 days)< F2 tKDL diploid (N=44, 14.91 ± 5.47 days), and all groups differed significantly from each other except for the control diploids and the F2 tKDL diploids (Kruskal-Wallis test, H^2=40.73, *P*<0.001, d.f.= 3; Dunn's post-hoc tests: control diploid-F2 tKDL diploid, *P*=0.012; control diploid-F2 tKDL triploid *P*=0.04; control diploid-WPL triploid, P<0.001; F2 tKDL diploid-F2 tKDL triploid, *P*<0.001; F2 tKDL diploid-WPL triploid, *P*<0.001; F2 tKDL triploid-WPL triploid, *P*<0.001). This is reflected in significant differences of survival distributions (log-rank test, H^2=58.949, d.f.=3, *P*<0.001), with the tKDL diploid females living longer on a continuous host supply compared to WPL or tKDL triploids (although control diploids had a shorter lifespan than tKDL triploids) (Figure 4A).

The average total number of hosts parasitized (hosts that were killed regardless of whether or not viable offspring were produced) and the percentage of hosts parasitized out of total hosts offered were F2 tKDL triploid (N=20, 26.75 ± 6.08 hosts; 37.7 ± 9.9%) < WPL triploid (N=50, 27.40 ± 13.63 hosts; 59.2 ± 18.4%) < control diploid (N=50, 44.16 ± 16.15 hosts, 63.0 ± 11.1%) < F2 tKDL diploid (N=44, 54.14 ± 23.26 hosts; 67.2 ± 17.6%). Overall differences are significant both for total hosts parasitized (Kruskal-Wallis test, H^2=51.22, *P*<0.001, d.f.= 3; Dunn's post-hoc tests: control diploid-F2 tKDL diploid, *P*=0.068, control diploid-F2 tKDL triploid *P*<0.001; control diploid-WPL triploid P<0.001; F2 tKDL diploid-F2 tKDL triploid, *P*<0.001; F2 tKDL diploid-WPL triploid, *P*<0.001; F2 tKDL triploid-WPL triploid, *P*=0.643) (Figure 4B), and percentage of hosts parasitized (Kruskal-Wallis test, H^2=38.54, *P*<0.0001, d.f.= 3; Dunn's post-hoc tests: control diploid-F2 tKDL diploid, *P*=0.049, control diploid-F2 tKDL triploid *P*<0.001; control diploid-WPL triploid P=0.585; F2 tKDL diploid-F2 tKDL triploid, *P*<0.001; F2 tKDL diploid-WPL triploid, *P*=0.012; F2 tKDL triploid-WPL triploid, *P*<0.001) (Figure 4C). Notably, differences are non-significant for total number of hosts parasitized between control diploids and F2 tKDL diploids, and F2 tKDL triploids and WPL triploids (Figure 4B). Control diploids also parasitized the same percentage of hosts as WPL triploids (Figure 4C), indicating that proportionate parasitization of all available hosts can be the same between diploids and triploids, but triploids overall will kill fewer hosts because of shorter lifespan.

The number of hosts that produced offspring was on average WPL triploid (N=50, 4.92 ± 3.52 hosts) < F2 tKDL triploid (N=20, 13.90 ± 7.93 hosts) < control diploid (N=50, 34.78 ± 12.81 hosts) < F2 tKDL diploid (N=44, 43.21 ± 22.69 hosts). 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; Dunn's post-hoc tests: control diploid-F2 tKDL diploid, *P*=0.770, control diploid-F2 tKDL triploid *P*<0.001; control diploid-WPL triploid *P*<0.001; F2 tKDL diploid-F2 tKDL triploid, *P*<0.001; F2 tKDL diploid-WPL triploid, *P*<0.001; F2 tKDL triploid-WPL triploid, *P*=0.026) (Figure 4D). Lifetime fecundity for tKDL triploids (N=20, 23.05 ± 33.51 offspring) was higher than WPL triploids

(N=50, 8.26 ± 7.23 offspring) (Mann Whitney U test, Z=-2.95, *P*=0.003) (Figure 4E). Parasitization ability over time (number of hosts parasitized out each set of 10) for both tKDL and WPL triploids was consistently lower than control or tKDL diploids (Figure 4F), as was the number of hosts that resulted in viable offspring (Figure 4G). Overall, this indicates that diploid females have better parasitization ability than triploids, and the parasitization ability of triploids is not higher for those with higher fecundity (tKDL) than those of lower fecundity (WPL).

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 to test the significance of each type of female group assayed (*i.e.* the groups indicated in Materials and Methods and Figure 4), ploidy (2n versus 3n), polyploid background (WPL versus tKDL), generation of descent from ds *tra* injection (F1 or F2), 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 descent 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 1).

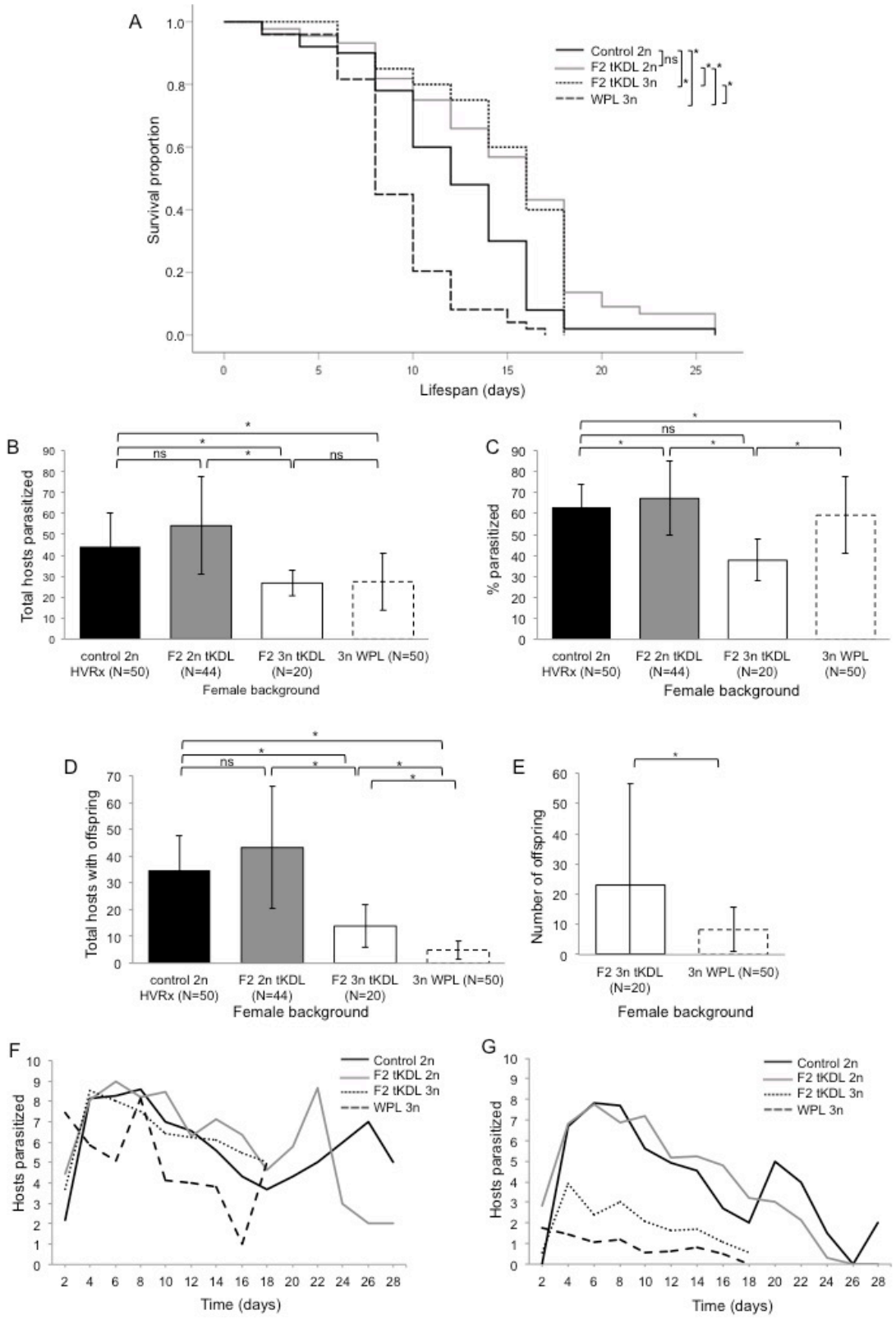

Figure 4. Parasitization ability of control (HVRx) diploid, F2 tKDL diploid and triploid females, and WPL triploid females in terms of A) lifespan represented by survival curves (proportion of individuals alive over time), B) mean ± SD of total number of hosts parasitized, C) percentage of hosts parasitized, D) number of hosts that resulted in offspring production, and, for 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; the same applies for mean G) offspring production being reflected by number of hosts that resulted in at least one viable offspring. For visual clarity, standard deviation values for F and G are reported in Table S2 rather than being depicted here.

Table 1. Results from the binomial linear general mixed model for parasitization ability and offspring production of control diploid, tKDL diploid and triploid females, and WPL triploid females for relative likelihood to parasitize or produce offspring on any given individual host. The intercept is for a model with random factors 'day' and 'individual', and the various fixed factors. All models are relative to a (0^a) category for lowest parasitization ability or lowest ability to produce offspring (with the exception of descent from injection for offspring production because there was no significant difference between test groups).

Fixed effect	Coefficient	Exp (Coefficient)	P-value
Parasitization ability			
Group (BIC=47970)			
Intercept	-0.306	0.736	0.025
Control 2n	0.363	1.438	<0.001
F2 tKDL 2n	0.734	2.084	0.042
F2 tKDL 3n	0.430	1.527	
WPL 3n	0^a		
Ploidy (BIC=47963)			
Intercept	-0.172	0.842	0.902
Diploid (Control 2n & F2 tKDL 2n)	0.405	1.499	0.002
Triploid (WPL 3N & F2 tKDL 3n)	0^a		
Background (BIC=47967)			
Intercept	0.333	1.396	0.810
HVRx (Control 2n)	-0.275	0.760	0.067
WPL (WPL 3n)	-0.638	0.528	<0.001
tKDL (F2 tKDL 2n & F2 tKDL 3n)	0^a		
Descent from injection (BIC=47958)			
Intercept	-0.113	0.894	0.935
Injected (F2 tKDL 2n & F2 tKDL 3n)	0.448	1.565	0.001
Uninjected(Control 2n & WPL 3n)	0^a		
Breeding (BIC=47952)			
Intercept	0.214	1.238	0.878
Inbred (WPL 3n)	-0.517	0.596	<0.001
Outbred (Control 2n, F2 tKDL 2n & F2 tKDL 3n)	0^a		
Offspring production			
Group (BIC=53632)			
Intercept	-3.711	0.024	<0.001
Control 2n	2.545	12.747	<0.001
F2 tKDL 2n	2.765	15.885	<0.001
F2 tKDL 3n	0.846	2.331	0.001
WPL 3n	0^a		
Ploidy (BIC=53428)			
Intercept	-3.438	0.032	<0.001
Diploid (Control 2n & F2 tKDL 2n)	2.373	10.735	<0.001
Triploid (WPL 3N & F2 tKDL 3n)	0^a		
Background (BIC=53749)			
Intercept	-1.544	0.211	0.006
HVRx (Control 2n)	0.384	1.468	0.062
WPL (WPL 3n)	-2.213	0.109	<0.001
tKDL (F2 tKDL 2n & F2 tKDL 3n)	0^a		
Descent from injection (BIC=53082)			
Intercept	0.029	1.029	1.000
Injected (F2 tKDL 2n & F2 tKDL 3n)	-1.588	0.204	1.000
Uninjected(Control 2n & WPL 3n)	-2.427	0.088	1.000
Breeding (BIC=53735)			
Intercept	-1.387	0.250	0.011
Inbred (WPL 3n)	-2.383	0.092	<0.001
Outbred (Control 2n, F2 tKDL 2n & F2 tKDL 3n)	0^a		

Diapause

Total progeny size was larger on average for the ds *tra* injected (diploid) females (N=62, 66.23 ± 32.45 offspring) than the control diploid HVRx females (N=79, 25.56 ± 18.82 offspring) (Mann-Whitney U test, Z=-7.14, P<0.001) (Figure 5A). However, the diapause fraction (diapause larvae/total offspring) of the ds *tra* injected females (N=62, 0.32 ± 0.28) was much higher than the control females (N=79, 0.0019 ± 0.01) (Mann-Whitney U test, Z=-7.88, P<0.001) (Figure 5B). In the F2 generation, tKDL diploids (N=82, 109.65 ± 25.12 offspring) produced more offspring that then tKDL triploids (N=81, 22.73 ± 10.19 offspring) (Mann-Whitney U-test, Z=11.00, P<0.001) (Figure 5A). Both diploid tKDL (N=82, 0.09 ± 0.27) and triploid tKDL (N=81, 0.13 ± 0.20) females exhibited some evidence of a heritable effect of increased diapause proportion (out all offspring) from descent from ds *tra* injection, relative to the control diploid females (Kruskal-Wallis test, χ^2=83.86, P<0.001, d.f. = 2; Dunn's post-hoc tests: control diploid-F2 tKDL diploid, P=0.003; control-F2 tKDL triploid P<0.001; F2 tKDL diploid-F1 tKDL triploid, P<0.001) (Figure 5B). However, this effect is seemingly minor, as diapause fractions are much lower than the ds *tra* injected females, which had broods that were approximately one-third diapause larvae.

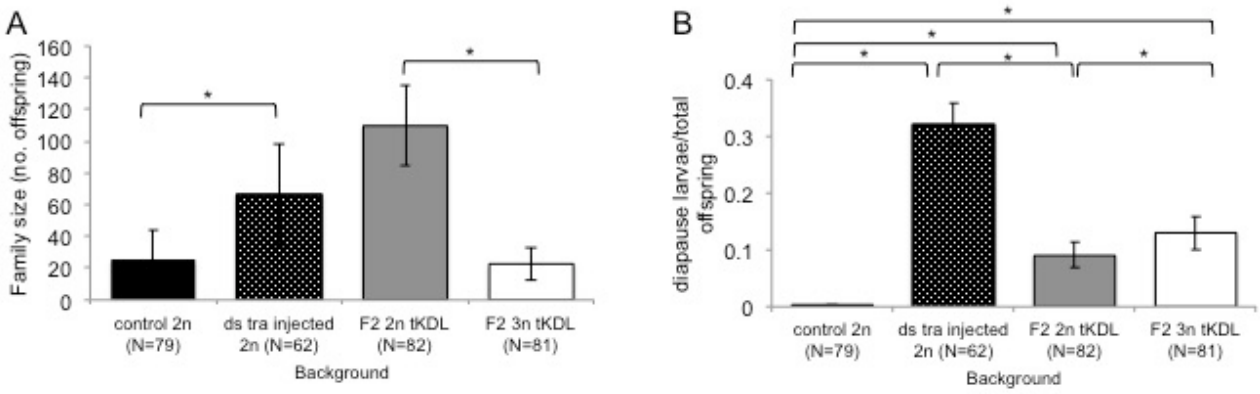

Figure 5. Mean ± SD for A) progeny size and mean ± SE B) diapause proportion of F0 control and ds *tra* injected diploid females, and F2 tKDL diploid and triploid females on three *Calliphora* sp. hosts. Black indicates a non-injected control, black with white dots ds *tra* injected HVRx females, gray a non-polyploid background with descent from a ds *tra* injected female, and white a polyploid background with descent from *ds tra* injected female. An asterisk (*) denotes significant difference (P<0.05). Note the SE is used rather than SD for diapause proportion to prevent negative values on the y-axis.

Discussion

Polypoidy 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). In the case of numerous parasitoids, female body size is also directly related to pest-killing ability (Geden *et al.*, 1992; 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) such as wing size for dispersal ability (Grillenberger *et al.*, 2008; Xia *et al*., 2020) and male pheromone production (Blaul & Ruther, 2012). It also affects male mating success and behavior, although Burton-Chellew, Sykes, Patterson, Shuker, & West (2007) report that larger males do not have greater mating success than smaller males, in contrast to Tsai, Barrows, & Weiss, (2014).

How polyploidy influences *N. vitripennis* body size and associated traits is not clear (Leung *et al.*, 2019; this thesis Chapter 3). The wing cells of the tKDL individuals are not distinctively larger for higher ploidy individuals within or across sex (this thesis, Chapter 3). This study (Chapter 3, Figure 1) found that despite same-sized wing cells, tKDL diploid males are slightly larger than haploid males. This is consistent with the diploid *N. vitripennis* males of the WPL being slightly larger than haploid counterparts (Leung *et al.*, 2019) and the trend across polyploid Hymenoptera, with the exception of diploid bumblebee males being smaller than haploid males (A. Thiel, unpublished data). However, also similar to the WPL (Leung *et al.*, 2019), triploid tKDL females were not larger than their diploid counterparts (Figure 1). Cumulatively this suggests that differences in life history are either not likely attributable to body size, or that its effects are minor. However, a cautious note is important here. F1 tKDL diploid males are an unusual class of diploid males as they were diverted from female development with RNAi knockdown the *transformer* gene. As transformer is known to affect other traits, such as body size in *Drosophila* (Oldham *et al.*, 2000; Rideout, Narsaiya, & Grewal, 2015), a subsequent generation should be evaluated to confirm a general polyploid effect for increasing body size in males.

The lifespan of tKDL diploid males was longer than haploid, but the lifespan of tKDL triploid females was shorter than tKDL diploids (for fed and starved conditions). Thus the group with larger body size corresponds to the group with greater longevity for both sexes, in line with general trends for insects (Berger *et al.*, 2012) and animal biology wherein larger animals expend less energy per unit of tissue (Speakman, 2005). It is also possible that the larger individuals simply have more initial fat reserves to deplete and so live longer, as *Nasonia* lack lipogenesis (Lammers *et al.*, 2019). However, these results contrast with the WPL, for which a polyploid versus non-polyploid state and body size did not have a definitive effect on lifespan for males or females or under fed or starved conditions (Leung *et al.* 2019). A previous suggestion that outbreeding can increase *N. vitripennis* lifespan as was observed for one generation of outbreeding in WPL (Luna & Hawkins, 2004) was not observed here. The tKDL is derived from an outbred population, and neither diploid nor triploid females of this background lived longer than those of a previous study

on inbred WPL (Leung *et al*., 2019). Together, these data indicate that polyploidy can have inconsistent effects on lifespan that is context dependent.

Sperm limitation contributes to reduced reproductive success in diploid males

Control and haploid and diploid males that were given a single female mate differed from each other in total progeny size (Figure 3A) and sex ratio (Figure 3B) but not drastically. Evaluated on its own, this data from single female crosses suggest that females do not alter reproductive decisions based on differential cues of haploid versus diploid males, or males descended from *tra* knockdown and those that did not. This matches the finding of Leung *et al.* (2019) for WPL haploid and diploid males, which did not differ from each other in total progeny size or progeny sex ratio when given a single mate. However, a more in-depth assessment of male fertility with a mating series revealed a difference in haploid and diploid fitness for both tKDL and WPL that was not apparent with these single female crosses.

Mating series with ten females in quick succession uncovered an overall reduction in fitness in F1 tKDL and WPL diploid males compared to their haploid counterparts (although control haploid males had the highest fecundity for either assay) (Figure 3C, 3D). The mean number of daughters produced with the first female in the series is similar for tKDL haploid and diploid males, and for WPL haploid and diploid males, but for all subsequent females the haploid male produced more daughters (except for the tenth female for F1 tKDL) (Figure 3C). This cumulates to difference of total offspring number by more than 30% in the F1 tKDL diploid males and 74% for WPL diploid males relative to haploids of their respective backgrounds (Figure 3D). tKDL diploid males lose against haploid counterparts for female mates when they are in direct competition (this thesis, Chapter 3). However, the WPL diploid males are capable of acquiring as many female mates as WPL haploids (Leung *et al*., 2019) and more than F1 and F5 tKDL diploid males (this thesis, Chapter 3), but they are far less fecund and have far more sperm transfer failures across their mating series than the other male types of this study (Figure 3C-3E). Male fitness therefore does not consistently correlate to competitiveness for female mates. These data for two polyploid lines also demonstrate that impaired hymenopteran diploid male fertility/fecundity may not be detectable with a single cross (as is also the case for *Drosophila pseudoobscura* males, which have impaired fecundity due to a deleterious sex ratio-disrupting gene that reduces sperm competiveness; Price *et al.*, 2008)

The cause for the reduced fecundity of diploid males is unknown. In the single F1 tKDL mate crosses, a near equal number of crosses (out of an attempted 50) resulted in mated females for each type of male (N=42 for control and tKDL haploids, N=46 for tKDL diploids). This suggests that in the absence of haploid male competitors females do not seem to reject tKDL diploid males despite their reduced reproductive capacity (as an unequal number of single crosses were conducted for haploid and diploid WPL males in Leung *et al.*, (2019) due to lower production of diploid males, a similar comparison could not be made for that background). It is possible that the tKDL diploid males simply have less sperm but females will not alter their behavior if higher quality

mates are unavailable (*e.g.* as is the case with diploid males of the CSD species *Cotesia glomerata*; Elias, Mazzi, & Dorn, 2009). Sperm reduction has been observed in *Nasonia* males of other backgrounds, for example *N. vitripennis* males that have been exposed to high temperature (Chirault *et al.*, 2015) and interspecies *Nasonia* hybrids (Clark *et al.*, 2010). A contributing factor to the tKDL diploid male's reduced fitness was a greater number of females in their mating series failing to produce daughters compared to tKDL haploids, (Figure 3E). As all females were observed copulating, the diploid tKDL males may be less competent than haploids in sperm transfer, which might be more apparent if copulation duration were measured. It is also possible that the polyploid males' diploid sperm are less capable of fertilizing eggs, as is known for diploid sperm of polyploid *Habrobracon* males (MacBride, 1946). One way of testing relative fertilization ability is to directly compete haploid and diploid sperm within the spermatheca of females that have been induced to mate with both (by interrupting male post-copulatory displays), and comparing the ratio of offspring sired to total sperm count (as in Beukeboom, 1994).

The limitation of a mating series assay is that it cannot distinguish between these various possibilities as the root cause for fitness differences. A follow-up study is needed to comprehensively assess which of these factors (or which combination) underlie the reduced fecundity of diploid tKDL males. Detecting the stage at which reproductive error occurs would involve measuring total sperm count at the pupal stage following *N. vitripennis's* single bout of spermatogenesis (Chirault *et al.*, 2016; Ferree *et al.*, 2019), the amount of sperm transferred to the spermatheca during copulation, and the number of daughters produced.

Regardless of the underlying mechanism, these results add to our understanding of relatively rare phenomenon of hymenopteran diploid male fertility. For example, in *Cotesia glomerata,* a species with single-locus CSD and fertile diploid males, smaller effective population size does not increase extinction risk (Elias, Dorn, & Mazzi, 2010). This suggests that species with fertile diploid males are not in danger of sex-tinction from the diploid male vortex (Hein *et al.*, 2009). However, our study demonstrates that diploid males can have overall lower fecundity requiring a mating series to detect, validating the suggestion of Fauvergue *et al.*, (2015) that so-called "fertile diploid males" can still experience fitness costs. And yet, it is also possible that males do not typically acquire more than few mates in their lifetime, so sperm limitation in itself may not change an individual's fitness much.

This study underscores the importance of evaluating of hymenopteran male fitness for individual species and polyploidization events, especially since it cannot be predicted which taxonomic groups are more likely to have reproductive or semi-reproductive diploid males. The few species with fertile diploid males are *Nasonia vitripennis* (Whiting 1960, Leung *et al*. 2019, this thesis)*, Euodynerus forminatus* (Cowan & Stahlhut, 2004), and two *Cotesia* spp.,(de Boer *et al.*, 2007; Elias *et al.*, 2010). These species are not closely related, but interestingly, they all have a low chromosome number (haploid chromosome count N=5 for non-CSD *N. vitripennis* (Werren & Loehlin, 2009a); N= 8 for CSD *E. forminatus* (Goodpasture, 1974); N=10 for CSD *Cotesia glomerata*

(Zhou, Gu, & Dorn, 2006) and unknown but probably low for close relative *Cotesia vestalis* (de Boer *et al.*, 2007)). This perhaps corresponds to greater likelihood of euploid gamete production than species with higher chromosome number. Countering this, hymenopterans with even fewer chromosomes such as some *Myrmecia* spp. ants (with haploid chromosome count as low as N=1; Crosland & Crozier, 1986; Ross *et al.*, 2015) and crabonid wasps (averaging a haploid chromosome number of N=4.3 across the species evaluated for this family; Ross *et al.*, 2015) are not known to produce fertile diploid males. It is also not atypical for parasitoid wasps to have an approximately N=10 haploid chromosome count (Gokhman, 2009). Thus, understanding the actual likelihood of diploid-male driven extinction must consider multiple factors including degree of reproductive impairment, which previous studies on *N. vitripennis* demonstrated can vary for diploid males within a single species (Leung *et al.*, 2019, this thesis Chapter 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). However, in another study it was observed that the tKDL triploid females produce 3-10 times as many offspring as WPL triploid females for unknown reasons (this thesis, Chapter 3). This higher fecundity has been recapitulated with the lifetime fecundity measurement here, with triploid tKDL females producing about 3 times as many offspring as WPL counterparts on *Calliphora* sp. hosts (Figure 4E). We expected the tKDL's higher offspring production to correspond to higher parasitization ability, reasoning that a greater degree of offspring larval feeding would increase host killing. Unexpectedly, the parasitization ability of the more fecund tKDL background is not higher than the highly infertile triploid females of the WPL (Figure 4B), as they live slightly longer (Figure 4A) but parasitize a lower percentage of hosts offered (Figure 4C).

The reason for this is unknown. Surprisingly little is known about how intraspecific variation in fecundity correlates to host killing. Synovigenic parasitoids such as *Nasonia* account for about 98% of parasitoid wasp diversity; they have a complement of mature eggs upon pupal eclosion but continue to produce eggs throughout adulthood (Jervis *et al.*, 2001). The intuitive assumption would be that the more fecund the parasitoid, the higher the parasitization rate and the better the biocontrol agent. However, this has only been borne out at the species level for parasitoids of Lepidoptera (Lane, Mills, & Getz, 1999). Furthermore, there have been claims that destructive female host-feeding is a better predictor for biocontrol success than egg load (Yamada, 1988; Kidd & Jervis, 1989; Jervis, Hawkins, & Kidd, 1996). And yet, *Nasonia* venom is sufficient for killing hosts, without female host feeding or oviposition (Rivers *et al.*, 1993; Rivers & Losinger, 2014), perhaps making venom the key factor for parasitization success.

Neither WPL nor tKDL triploid females were observed to be deficient in host feeding. Thus the results of this study may reinforce the existing suggestion (Leung *et al.*, 2019) that the polyploid state may attenuate venom potency and decreases parasitization ability of females, with

the new insight that more offspring does not rescue this polyploid disadvantage. Additional study is needed to test this hypothesis. Pupal hosts can be injected with venom from diploid versus triploid females, to isolate it as a variable in host-killing from other female traits (Rivers *et al.*, 1993). Females can also be sterilized though irradiation, as in sterile insect technique (Dyck, Hendrichs, & Robinson, 2005), so that they inject venom but do not oviposit viable eggs, although this scenario is already somewhat simulated by the low fecundity of the WPL. Beyond that, if triploid females have lower venom load, or if venom composition is different from the diploids that have high parasitization ability, this can be detected *e.g.* through transcriptomic analyses (as in Nipitwattanaphon *et al.*, 2014). This may uncover a dosage mechanism for venom production that is disrupted in the triploids, although analyses of housekeeping genes suggest a general mechanism for keeping absolute gene expression consistent between diploid and triploid females (this thesis, Chapter 3).

tra *knockdown increases diapause induction*

In *Nasonia*, diapause has a circadian link: females that have been exposed to shorter photoperiods induce larval diapause in their offspring as an overwintering survival strategy (Schneiderman & Horwitzf, 1958). However, other factors influence diapause; for example, although *Nasonia* females produce eggs throughout their lifetime (Pannebakker *et al.*, 2013) diapause induction is higher in older females (Walker & Saunders, 1962). Furthermore, even though *Nasonia* fecundity is variable depending on host species, host species does not directly effect diapause induction (Rivers & Denlinger, 1995; David B. Rivers & Losinger, 2014). The factors controlling diapause thus still need much detangling.

The genetic architecture underlying *Nasonia* diapause has been partially characterized. For example, a latitudinal cline in the allelic distribution of the clock gene *period* correlates to diapause induction efficiency for European *N. vitripennis* (Paolucci et al., 2016). However, the involvement of other genes is not well understood. For example, the role of *transformer* is unclear. For *Nasonia*, the function of *tra* has largely been studied through its role as a major component of the sex determination pathway. Briefly, in fertilized (diploid) eggs, maternal *transformer* (*tra*) transcripts in the oocyte interact with zygotic *tra* transcribed from an active *tra* allele to autoregulate female-specific splicing. In unfertilized haploid eggs, the *tra* allele is maternally silenced, so they do not produce the zygotic *tra* required for female development (Verhulst, 2010). It has not yet been directly linked to any clock genes, although in the water flea (*Daphnia magna*) expression of *tra*'s co-factor *transformer-2* (required for female development in *N. vitripennis*, Geuverink *et al.*, 2017) changes according to photoperiod and may have a role in diapause (Gust *et al.*, 2019).

Higher diapause production in the females injected with ds *tra* suggests that *tra* is upstream of a diapause pathway in *Nasonia*. Alternatively, as *tra* is essential for female development, disruption of its expression may interfere with general female functionality, including diapause induction. Intriguingly, higher diapause induction also occurred for subsequent

generations (albeit to a lesser degree) indicating some heritable effect persisting beyond a single generation of RNAi effect.

Special considerations of maternal tra *knockdown induced polyploidy*

All individuals used in this study were descended from offspring of ds *tra* injected females. Both haploid and diploid F1 males may have experienced effects to their development from a F0 mother whose oogenesis process lacked active *tra* transcripts, in addition to an oosome devoid of maternal *tra*. This may have negatively impacted their reproductive ability, as the F1 tKDL haploid males have lower total daughter production and higher rate of sperm transfer failure in the mating series relative to control haploid males. These effects would then be compounded with polyploid detriment in the F1 diploid tKDL males, which rank the worst for these traits (Figure 3C, 3D).

A second consideration is that these F1 males also represent the non-diapausing cohort of F0 female offspring. Diapause individuals were not evaluated with the assays of this study, nor their descendants, because several months of cold storage would have been required for them to be able to develop into adulthood (Werren & Loehlin, 2009b). However, there is some evidence that diapause offspring are weaker than non-diapause offspring (*e.g.* diapause reduces female fitness and fertility in Colorado potato beetles, Margus & Lindström, 2020), so it is possible that the tKDL line may have been founded by the "strongest" individuals in the brood. If this represents inadvertent selection for *e.g.* larger body size (Figure 1), longer-lifespan (Figure 2), and higher female fecundity (Figure 5A) this could explain why there are significant advantageous effects of descent from ds *tra* for F1 male and F2 tKDL female non-polyploids relative to controls. For future study, tKDL diapause offspring should also be reared to adulthood and individuals of a resultant line assessed for differences of the tested non-diapause fraction here.

Synthesis: implications of non-CSD polyploidy for biological control and evolution

Polyploid incidence has not been well surveyed for non-CSD species, possibly because it is less easy to detect than in CSD species, for which inbreeding crosses induce male diploidy (Cook, 1993; Cook & Crozier, 1995). However, as the vast majority of parasitoid wasp species used in biological control are non-CSD species (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 couched in terms of inevitable detriment because of sterile diploid males driving the extinction of small populations (Zayed & Packer, 2005), whereas the degree of detriment falls along a gradient for non-CSD based polyploidy and is context dependent. For example, *N. vitripennis* polyploid female parasitization ability seems generally impaired even though fecundity can be increased; polyploid male mate competitiveness can vary depending on the background; and a shipping/storage benefit can be derived from higher diapause induction of *tra* knockdown. Conversely, higher

diapause can be considered a negative side effect in breeding programs, if would-be reproductive individuals enter diapause instead, delaying production or selection for other traits.

The high degree of intraspecific polyploid phenotype variation in *N. vitripennis* opens up the radical possibility that non-CSD polyploidy be explored for benefits to breeding. For instance, the unusual reproductive competence of *Nasonia* polyploids for both sexes, and therefore heritability of the polyploid state, makes it possible to experiment with heritable dosage and dominance effects with more allele copies than the usual haploid male, diploid female ploidy levels. For example, alleles that are beneficial to females but deleterious to males would usually be purged in the haploid state (Immler & Otto, 2014), but in the *Nasonia* system can be retained through diploid males.

From a life history and evolutionary perspective, it is also notable that all known cases of hymenopteran neopolyploidization have involved a sex determination pathway (*i.e.* homozygosity of *csd* loci (Zayed & Packer, 2005; Heimpel & De Boer, 2008); single-target knockdowns of several genes in the maternal effect genome imprinting sex determination of *Nasonia* (Verhulst, 2010; Verhulst *et al.*, 2013; Geuverink *et al.*, 2017); and *Wolbachio* titer (endosymbiotic bacteria capable of influencing sex ratios in insects) is higher in diploid males of *Asobara* (Ma *et al.*, 2015)). The more typical means for polyploidization is whole genome duplication (WGD), which is both associated with a range of severe initial developmental defects and being the means for gene network diversification via additional gene copies (Comai, 2005). It might be worth considering that these smaller scale genetic events that divert feminization to produce diploid males are less harmful, and more likely to have contributed to the evolutionary history of Hymenoptera (known to have ancestral polyploidizaiton events, like most hexapods; Li *et al.*, 2018) than whole genome duplications (this thesis, chapter 3). It should be possible to compare these potential evolutionary trajectories with continued maintenance of the tKDL, and with a de-novo WGD polyploid *Nasonia* line as well (likely inducible through prevention of gamete reduction with mechanical or chemical interference; Kawamura, 1994). With this study we hope to highlight the understudied state of non-CSD polyploidy, and inspire more research on its complex possibilities for biological control and evolution.

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Supporting Information

Table S1. Standard deviation values for parasitization ability (Figure 4F) and offspring production over time (Figure 4G)

	Parasitization ability Background				Offspring production Background			
Day	control 2n	F2 tKDL 2n	F3 tKDL 3n	WPL 3n	control 2n	F2 tKDL 2n	F3 tKDL 3n	WPL 3n
2	1.24	1.76	1.65	2.68	0.14	1.80	0.59	1.65
4	1.79	2.57	1.55	2.69	2.00	3.16	2.15	1.20
5	1.85	2.32	1.95	2.41	2.11	2.97	2.08	1.20
8	1.83	2.54	2.09	2.20	2.31	3.03	1.84	1.15
10	2.47	1.92	1.82	3.02	2.69	2.68	1.03	1.02
12	2.72	1.80	2.17	2.14	2.67	2.38	1.19	0.52
14	2.47	2.22	2.28	3.43	2.43	2.79	1.32	1.10
16	2.48	2.22	2.34	0.00	2.46	2.86	1.16	0.50
18	2.57	2.10	2.57	0.00	2.23	2.84	0.96	0.00
20	2.18	1.13			2.49	1.70		
22	2.00	1.25			0.00	2.47		
24	1.00	0.00			1.50	0.47		
26	3.00	2.00			0.00	0.00		
28	0.00	0.00			0.00	0.00		