OXFORD UNIVERSITY PRESS | Behavioral Ecology

Sperm blocking is not a male adaptation to sperm competition in a parasitoid wasp

Journal:	*Behavioral Ecology*
Manuscript ID	BEHECO-2017-0410.R1
Manuscript Type:	Original article
Keywords:	sperm competition, post-copulatory sexual selection, polyandry, sex allocation, local mate competition

Sperm blocking is not a male adaptation to sperm competition in a parasitoid wasp

Rebecca A. Boulton1,2*, Nicola Cook1, Jade Green1, Elisabeth V. (Ginny) Greenway1 and David M. Shuker1

1. School of Biology, University of St Andrews, UK.

2. Department of Entomology, Hodson Hall, 1980 Folwell Avenue, University of Minnesota, St. Paul, USA

*author for correspondence

Email: rboulton@umn.edu

Running head: Sperm blocking in a parasitoid wasp

Keywords: Sperm competition, post-copulatory sexual selection, polyandry, sex allocation, local mate competition

Lay Summary

Although mating with multiple males should provide females with more sperm, in the parasitoid wasp *Nasonia vitripennis* females that mate multiply produce more unfertilized eggs (which in this species develop as sons due to haplodiploidy). We tested whether this was due to males 'blocking' or 'incapacitating' the sperm of their rivals. Instead of being a male adaptation to sperm competition however, our results suggest that this reflects a female constraint on sperm processing.

Title: Sperm blocking is not a male adaptation to sperm competition in a parasitoid wasp

Running head: Sperm blocking in a parasitoid wasp

Abstract

The extent to which sperm or ejaculate-derived products from different males interact during sperm competition – from kamikaze sperm to sperm incapacitation – remains controversial. Repeated matings in the parasitoid wasp *Nasonia vitripennis* lead to a short-term reduction of efficient sperm use by females, which is crucial for a haplodiploid organism when needing to allocate sex adaptively (i.e. by fertilizing eggs to produce daughters). Repeated matings by females in this species therefore constrain sex allocation through this "sperm-blocking" effect, eliciting a cost to polyandry. Here we explore the causes and consequences of sperm-blocking, and test the hypothesis that it is an ejaculate-related trait associated with sperm competition. First, we show that sperm blocking, which leads to an over-production of sons, is not correlated with success in either offensive or defensive roles in sperm competition. Then, we show that the extent of sperm blocking is not affected by self-self or kin-kin ejaculate interactions when compared to self vs non-self or kin versus non-kin sperm competition. Our results suggest that sperm blocking is not a sperm competition adaptation, but is instead associated with the mechanics of processing sperm in this species, which are likely shaped by selection on female reproductive morphology for adaptive sex allocation.

Keywords: Sperm competition, post-copulatory sexual selection, polyandry, sex allocation, local mate competition

Introduction

Polyandry, or the multiple mating of females with different males, is now known to be widespread in nature (Pizzari and Wedell 2013; Taylor et al. 2014). Polyandry has also been clearly demonstrated to confer significant benefits in a wide range of taxa (Arnqvist and Nilsson 2000; Slatyer et al. 2012; Taylor et al. 2014). However, mating can also be costly for females (Daly 1978; Boulton and Shuker 2013), leading to sexual conflict between males and females over mating rate, even if some degree of multiple mating for females is adaptive (Arnqvist and Rowe 2005). In the parasitoid wasp *Nasonia vitripennis*, females suffer a novel cost from mating multiply, as repeated matings constrain their ability to allocate sex optimally (Boulton and Shuker 2015a). This effect arises from the fact that multiple ejaculates in a female limit the ability of that female to mobilise and use sperm to fertilize eggs, an effect that we have labelled "sperm blocking" (Boulton and Shuker 2015a). Although *N. vitripennis* are mostly monandrous in the wild, polyandry evolves under laboratory culture conditions (Burton-Chellew et al. 2007; van den Assem and Jachmann 1999) and can provide a fecundity benefit (Boulton and Shuker 2015b).

As with all Hymenoptera, *N. vitripennis* is haplodiploid, and so mated females can choose whether or not to lay a fertilized egg (which develops into a daughter) or an unfertilized egg (which develops into a son). Females allocate sex in line with the predictions of local mate competition (LMC) theory (Hamilton 1967). Under high LMC conditions typically experienced by *N. vitripennis* in the field, females maximise their fitness by producing highly female-biased broods (Werren 1980, 1983; Shuker and West 2004; Shuker et al. 2005; Burton-Chellew et al. 2008). After a second mating, however, females are temporarily constrained in their ability to produce daughters (for at least 24 hours) resulting in a fitness cost for polyandrous mothers (Boulton and Shuker 2015a). Often the costs of mating to females are thought of as arising due to sexual conflict. Males can benefit from increasing the costs of mating to females if this discourages female re-mating and so reduces the risk of encountering sperm competition (Simmons 2001). However, the sex allocation cost that we see in *Nasonia vitripennis* does not appear to benefit males. On the contrary, at first glance, it appears to reduce male fitness as females lay fewer fertilized eggs (daughters), and in haplodiploids males only pass on their genes through these daughters.

The potential fitness costs of sperm-blocking to both males and females does mean that it represents something of a paradox as to its origin and function. Here we will consider two possible scenarios. First, sperm blocking may be beneficial to males as an adaptation to sperm competition (which is expected to be higher in laboratory strains demonstrating elevated polyandry; Burton-Chellew et al. 2007; van den Assem and Jachmann 1999), with sperm-blocking a side-effect of increased paternity share when ejaculates compete. In other words, although sperm-blocking reduces the number of daughters produced, sperm-blocking may still be beneficial for a male if it increases his share of the remaining (female) offspring. Although it is clear that adaptations to sperm competition, such as physical displacement and increased swimming velocity can and do occur (Manier et al. 2013), the extent to which sperm or ejaculate-related products directly, i.e. physically, interact has been subject to controversy. Despite hypotheses of kamikaze sperm and ejaculate-ejaculate phenomena such as sperm incapacitation, how common many of these phenotypes are is unclear and remains contentious (Baker and Bellis 1988; Harcourt 1991; Price et al. 1999; Snook and Hosken 2004; den Boer et al. 2010; Manier et al. 2010; Moore et al. 1999). Furthermore, it may be that patterns of sperm precedence that are commonly interpreted as male adaptations actually reflect female physiological characteristics that have been shaped by natural selection to optimize sperm use and storage (see Simmons et al. 1999; Hosken and Stockley, 2004; Herberstein et al. 2011). This is represented in our second scenario, whereby disrupted sex allocation is non-adaptive and occurs as a result of female morphological and physiological constraints on sperm processing, storage and usage.

The sperm competition scenario generates testable predictions. Sperm blocking may enhance the fertilization success of one male over the other in a number of ways. First, it may reflect a defensive adaptation that reduces the risk of encountering sperm competition for the first male and thus increases his fertilization success (Figure 1 A; Simmons 2001). For instance, in some insect species, including some hymenopterans, males transfer mating plugs that obstruct the entry of rival ejaculates into the female reproductive tract (Baer et al. 2001; Mikheyev 2003; Simmons 2001; 2014). While there is no evidence for a physical mating plug in *Nasonia*, a form of chemical mating plug or sperm-sperm interaction that obstructs sperm movement after copulation (as seen in some species of crab; Bewab and El-Sherief 1989) may be present. If a defensive adaptation against sperm competition such as this occurs in *N. vitripennis*, then it may not only obstruct incoming sperm but may also impede the movement of outgoing sperm to be used for fertilization, resulting in sperm blocking (female parasitoid wasps possess only a single spermathecal duct; King 1961). In this scenario, we predict that the severity of the over-production of sons (i.e. sperm blocking) will be positively correlated with first male fertilization success and negatively correlated with second male fertilization success (Figure 1 A).

In contrast, it may be that sperm blocking occurs as a result of an offensive adaptation to sperm competition, increasing the success of a competing male when sperm competition is encountered (Simmons 2001). In this case, we envisage a scenario where the second male’s ejaculate blocks the female spermathecal duct, preventing the first males ejaculate from being used to fertilize eggs and resulting in sperm blocking. Our prediction for this situation is that the degree of sperm blocking will be positively correlated with second male fertilization success and negatively correlated with first male fertilization success (Figure 1 B).

Figure 1

As a further test of sperm blocking as a male adaptation, if there is some chemically mediated process at work, we might expect that sperm blocking should not occur – or should at least be considerably reduced – when ejaculates from the same male, or from closely related males, come into competition. For instance, seminal fluid proteins can play a role in the incapacitation of rival unrelated sperm. In several polyandrous species of social hymenopteran, males exhibit offensive seminal fluid traits that target the sperm of their rivals (den Boer et al. 2010). This may even be facilitated by haplodiploidy, because individual sperm produced by a single haploid male will, except in the event of mutation, be identical (de la Filia et al. 2015). We therefore predict that sperm blocking will be more severe when unrelated ejaculates come into competition.

The second possibility is that sperm blocking is not a result of male adaptations to sperm competition, but instead reflects female physiological processes that constrain efficient sperm use. For instance, Price et al (1999) suggested that in *Drosophila melanogaster* seminal fluid proteins serve to incapacitate previously stored sperm of rival males. However, Snook and Hosken (2004) found evidence to suggest that this apparent male incapacitation actually occurs because females 'dump' the sperm from previous ejaculates, which can result in skewed sperm precedence toward either male (see also Manier et al. 2013). Similarly, in *Nasonia vitripennis*, sperm blocking could occur as a result of sperm dynamics and movement within the female reproductive tract if, for instance, females require time to move sperm from the site of storage to the site of fertilization. Although we do not explicitly test this hypothesis here, we suggest that sperm blocking most likely occurs due to constraints on sperm processing by females if there is no effect of mating order or male relatedness on the sex ratio.

We present the results of a series of experiments to contrast these two scenarios. In the first two experiments, we evaluate and then use the sterile male technique (see Parker, 1970; Ramadan and Wong, 1989; Siva-Jothy and Tsubaki, 1994) to test whether sperm blocking is an offensive or defensive trait that is associated with increased sperm precedence for the first or second male to mate. In the next two experiments, we test whether sperm blocking is influenced by the relatedness of a female's mating partners. Specifically, we ask whether sperm blocking is ameliorated if a female mates twice to the same male (versus two different males) or with two brothers (vs two unrelated males).

Methods

Study species

Nasonia vitripennis is a gregarious chalcidoid parasitoid wasp. It attacks dipteran pupae and is an ectoparasitoid, depositing eggs on the surface of the developing larva within the puparium (Whiting 1967). As with all Hymenoptera, *Nasonia vitripennis* is haplodiploid and females facultatively lay either fertilized eggs that develop as daughters or unfertilized eggs that develop as sons. Females lay multiple eggs on a single host (i.e. they are gregarious) and sib-mating at the emergence site is the norm (Boulton et al. 2014). After mating, the winged females disperse to find hosts on which to oviposit (brachypterous males are largely confined to the natal patch; Boulton et al. 2014). The local mating patches that result from this mating system lead to local mate competition (LMC; Hamilton 1967), and this in turn leads to selection for female-biased offspring sex ratios. In the wild, females typically oviposit alone (Grillenberger et al. 2008) resulting in high LMC because males will mate exclusively with their sisters. In this situation, females will maximise their fitness by producing only enough sons to inseminate their daughters. As more females contribute offspring to a mating patch, males can mate with non-sibling females and this increases their reproductive value and so less female-biased sex ratios are favoured. Female *Nasonia* show an impressive ability to allocate sex facultatively in line with the predictions of LMC theory (Werren 1980; 1983; Shuker and West 2004; Shuker et al. 2005; Burton-Chellew et al. 2008; Martel et al. 2016).

The strain of *N.vitripennis* used for these experiments was the outbred strain HVRx, which was collected from five sites in the Netherlands in 2001 (van de Zande et al. 2014), reared on *Calliphora vicina* pupae as hosts. We reared wasps at 25°C on a 16L:8D light cycle, which results in a 14-day egg-to-adult cycle. To standardise the rearing environment of focal males and females to be used in the following experiments, we provided virgin and mated females with three *C. vicina* hosts each for 48 hours, these females served as grandmothers for the focal individuals used for experiments. We isolated focal females as pupae (based on the presence of wing buds and a visible ovipositor) from the hosts two days prior to expected adult eclosion, 12 days after the grandmothers had been provided with hosts to oviposit on. The focal males were sons produced by virgin grandmothers, and these males were left to eclose naturally. First, we will describe our pilot experiment to calculate the lowest effective sterilizing dose for our sterile-male sperm competition experiment.

Pilot: Determining the lowest effective sterilizing dose (LESD)

We sterilized *Nasonia vitripennis* males using gamma radiation in order to investigate patterns of sperm precedence. Gamma radiation has been shown to sterilize males of many species effectively without detrimentally affecting sperm morphology (Wishart and Dick 1985). If the dose is sufficiently low, irradiation does not affect sperm motility or other aspects of the ejaculate, but the accumulation of lethal mutations in the sperm render them infertile (Ray 1948; Wishart and Dick 1985). The main reason for using the sterile male technique in *Nasonia vitripennis* is due to the lack of suitable morphological markers. Although previous studies have used red- or oyster-eyed inbred mutant strains to assay sperm precedence (Holmes 1974; van den Assem and Feuth-de-Bruijn 1977), these traits are recessive and so sperm precedence cannot be assayed in a wild-type background. Although sterilization can also result in reduced fertilizing capacity (Simmons 2001), this issue can be reduced through pilot studies that assess the Lowest Effective Sterilizing Dose (LESD), and through randomisation of the mating sequence (Simmons 2001).

To our knowledge, there are no published studies where gamma radiation has been used to sterilize *Nasonia vitripennis* for the purposes of assaying sperm precedence (but see Ray 1948 and Saul 1955 for other uses). Therefore, we conducted a pilot experiment to ascertain the Lowest Effective Sterilizing Dose (LESD) by generating a dose-response curve for four treatment groups: 80, 100, 120 and 140 Gy of radiation (see Ray 1948). The dose was determined by varying the amount of time that a vial containing male *N. vitripennis* was exposed to a source of gamma radiation. The dose rate was 2.59 Gy/min, so that males that received 80 Gy remained in the radiation chamber for 1854 seconds, 100 Gy for 2316 seconds, 120 Gy for 2778 seconds and 140 Gy for 3240 seconds.

On the day of emergence, focal males were exposed, in four groups of approximately 100 wasps, to a ^{137}Cs source emitting gamma radiation. The source rotates so that the dose rate is constant over space. Males from each treatment (plus untreated controls) were then mated to virgin females either straight after irradiation (day 1) or 24 hours later (day 2; $N = 25$ per treatment per day). All pairings were observed to determine whether successful copulation had occurred, after which the females were provided with hosts that were maintained at 25°C. After the offspring had emerged and died the number of sons (unfertilized eggs) and daughters (fertilized eggs) were counted. A mating was considered sterile when 100% of the offspring were male. The dose-response curve (Figure 2) shows the percentage of females from each treatment group that produced any daughters.

Figure 2

Our results suggest that the minimum effective dose was 100 Gy, after which less than 5% of males successfully fertilized eggs. Irradiation did induce sterility, as matings with control males were significantly more likely to result in daughter production than matings with exposed males (Quasibinomial GLM: $F_{4,\ 178} = 92.77$, $P < 0.0001$). Sterilization was not effective below 100 Gy (Tukey p < 0.007) but fertility was no different when males were treated with 100-140 Gy (100 Gy is the LESD). There was no effect of the day on which mating occurred on fertility ($F_{1,\ 181} = 0.02$, $P = 0.89$). Finally, if the sperm of irradiated males became less viable over time (see Simmons et al. 1996), then the sex ratio produced by females mated on the second day should be more male biased (since unfertilized eggs will develop as males). This was not the case however (day: $F_{1,174} = 0.29$, $P = 0.59$; interaction effect treatment*day $F_{4,170} = 0.56$, $P = 0.69$) suggesting that the sperm of irradiated males did not decrease in viability over the experimental period.

Experiment 1: Assaying sperm precedence using gamma-irradiated males

Four hundred virgin females were isolated from a grand-maternal generation. Virgin males were generated from unmated grandmothers and were maintained in groups of brothers. Six stock tubes containing brothers were exposed to 100 Gy of gamma radiation. We used irradiated males from six families to standardise competitor identity as much as possible, ideally the same males would have been used repeatedly (García□González and Evans 2011) but male *N. vitripennis* do not produce new sperm after emergence (i.e. they are prospermatogenic; see Boulton et al. 2014) and using the same males repeatedly would result in mating order effects on fecundity and the sex ratio (see Boulton and Shuker 2015b).

In this experiment, we investigated whether sperm blocking is associated with patterns of sperm precedence. To do this, focal females and males were assigned to one of the following seven treatments: (V) virgin female; (N) once-mated to a normal male; (I) once-mated to an irradiated male; (NN) twice-mated to two normal males; (II) twice-mated to two irradiated males; (NI) mated to a normal male followed by an irradiated male; (IN) mated to an irradiated male followed by a normal male (Total *N* = 348, *N* = 45-54 across treatments). To help explain the rationale for our interpretation of the following results, our assumptions are as follows. The production of a daughter arises from a fertilized egg, i.e. an egg fertilized by the mother using a viable sperm (from a normal 'N' male). A male is produced from an unfertilized egg that the mother left unfertilized. The effects of irradiation are manifested through mothers fertilizing eggs with infertile sperm (from an 'I' male), which results in the production of inviable embryos that fail to develop. This will lead to a change in the offspring sex ratio (fewer females, so less female-biased or even male-biased sex ratios), as well as a reduction in the total number of adult offspring produced (eggs fertilized by inviable 'I' sperm will not develop into adults).

In terms of the experiment, females assigned to the twice-mated treatments (II, IN, NI, NN) were mated first on day one. All copulations were observed and in order to increase the likelihood of re-mating, we prevented males from engaging in post-copulatory courtship as this behavior serves to reduce female receptivity to additional matings (see van den Assem and Visser 1976). Twenty-four hours after their first mating (on day two), these females were presented with a second male. All once-mated females were presented with their first and only male on day two. Females were given three *Calliphora vicina* hosts on day two (i.e. all females were given hosts immediately after their final mating) and kept in an incubator at 25°C. In this experiment, the initial three hosts were removed 24 hours later and replaced with a single host. This was repeated every day for three days such that females surviving until the end of the experiment received a total of six hosts in four batches. Three hosts were presented in the first batch, to allow host-feeding opportunities as well as provide oviposition resources. These hosts were maintained at 25°C and emerging sons and daughters were counted after they had died.

We kept experimental females in isolation after host provisioning and checked them every day at 9 am, 12 pm and 5 pm for mortality. After all females had died, we removed and measured their right hind tibia as a proxy for body size (Godfray 1994) in order to assess whether larger females (possibly with larger spermathecae; Martel et al. 2011) suffer sperm blocking to a lesser extent. An Olympus SZX10 microscope with an ocular micrometer was used for all measurements and each tibia was repositioned and re-measured three times in order to assess measurement error, which we found to be low based on a high repeatability estimate (Intra-class correlation coefficient: $ICC = 0.94 \pm 0.006$ CI, $P < 0.001$).

We tested whether treatment or host batch influenced the sex ratio (measured throughout as proportion of offspring that are male) that females produced across all treatments using a repeated-measures Quasibinomial GLMM (lme4 (Bates et al. 2015) in R Studio, RStudio, Inc., Boston, MA; R Core Team 2016). Fixed factors were treatment, host batch and their interaction. Female longevity (hours) and hind tibia length (mm) were included as covariates. Female identity nested within host batch was included as a random effect. Pairwise comparisons within these models allowed us to assess the effects of treatment on sperm blocking as well as the effectiveness of irradiation for sterilizing males. If irradiation successfully sterilizes males without impairing their ability to transfer an ejaculate, virgin females should produce significantly larger (all-male) clutches than mated (I or II) females (virgin female clutches are generally the same size as the clutches of once-mated females in *N. vitripennis,* only all male; Boulton and Shuker 2015a). We thus tested whether females that mated with either one (I) or two (II) irradiated males produced fewer sons (unfertilized eggs) than virgin females using a repeated-measures QuasiPoisson GLMM.

To investigate patterns of sperm precedence and mixing, we tested whether daughter production (i.e. eggs fertilized by N males that develop successfully) differed between treatment groups NN, NI and IN over the four host batches using a repeated-measures GLMM with a QuasiPoisson error structure. For this analysis, treatment, host batch and their interaction effect were included in the model as fixed factors, female longevity and hind tibia length as covariates, and female identity nested within host batch as a random effect.

The following formula was used to calculate sperm precedence (P_N) in treatments NI (P_1) and IN (P_2) (modified from Boorman and Parker 1976):

$$P_N = \frac{x - z}{p - z}$$

where,

x = proportion of daughters (fertilized eggs) in NI or IN matings,

z = proportion of daughters in II matings (0.018),

p = proportion of daughters in N matings (0.814).

We considered daughter production in II as *z* rather than daughter production in I because the reduced daughter production that occurs after repeated matings (sperm blocking) renders this estimate more comparable to the other twice-mated treatments (NI and IN). To calculate *p* we used the proportion of daughters in N matings to estimate “maximum” paternity by a single male, and NN matings were not used here as paternity could not be assigned to either male (in addition, daughter production in NN matings was reduced by sperm blocking and would underestimate maximum daughter production; see Results below).

In the original formula described in Boorman and Parker (1976) *x, z* and *p* are the proportion of eggs fertilized by the sperm of the N or I males and are calculated from the total number of viable offspring that hatch and divided by the total number of eggs laid (including inviable eggs). Here however, we have modified this formula due to constraints imposed by haplodiploidy and the life cycle of *Nasonia*. We consider the proportion of daughters in a clutch, excluding male offspring from our estimates of sperm precedence. This is because males develop from unfertilized eggs and so are not useful for assigning sperm use to the N male. Additionally, due to the nature of parasitism by *N. vitripennis*, unfertilized eggs typically cannot be counted. This is because around 40 eggs or so are laid inside the puparium and any eggs that fail to develop are generally destroyed by the larvae that do hatch. As a result we lack knowledge regarding the number of eggs that failed to develop (those fertilized by the I male). This means that the values of P_1 and P_2 will be overestimates (see the supplementary material, table S1, for calculations of P_N from a set of simulated data with and without knowledge of numbers of inviable eggs laid). Although these estimates are sufficient for investigating patterns of sperm precedence within this study, this limitation prevents comparisons of results between studies.

Under high LMC (when females oviposit alone), the number of sons in the first host batch is typically around 10%-20% of the total brood size (Werren 1980; 1983). When sperm blocking occurs, however, the proportion of sons in the first host batch increases, as females are unable to mobilise sperm successfully to fertilize eggs. To assess whether sperm blocking in the first clutch influences fertilization success of the N male, we tested whether P_N over host batches 2-4 was associated with the number of sons in the first host batch for the treatments IN and NI.

We infer P_1 from treatment NI and P_2 from treatment IN (i.e. the order of the focal male) and predict the following: If sperm blocking is (i) a defensive trait that benefits the first male, there will be a positive correlation between P_1 (use of the first males sperm in NI) and son production in the first clutch of eggs and a negative correlation for P_2 (use of the 2nd male’s sperm in IN; see figure 1 A). If, on the other hand, sperm blocking is (ii) an offensive trait used by second males to increase their relative paternity, then P_2 (use of the 2nd male's sperm in treatment IN) will be positively correlated and P_1 (the first males sperm use in NI) negatively correlated with son production in host batch 1 (sperm blocking; see figure 1 B). In other words, if sperm blocking is an offensive sperm competition trait that benefits the second male, high sperm blocking should lead to high P_2 (daughter production will be positively correlated with sperm blocking in treatment IN). If it is a defensive trait that benefits the first male, high sperm blocking will lead to higher P_1 (daughter production will be positively correlated with sperm blocking in treatment NI). These predictions are outlined in figure 1.

We ran a GLMM with female ID nested within host batch as a random effect, P_N was the outcome variable and the predictors were "Number of sons in host batch 1", as well as treatment, host batch and the interaction effects. A significant interaction effect between the number of sons and treatment suggests that the relationship between sperm blocking and fertilization success differs depending on the order in which the non-irradiated (N) male has mated (i.e. whether prediction (i) or (ii) is supported).

Experiment 2 Sperm blocking with competition between self vs non-self sperm

In our second experiment, we tested whether the presence of ejaculates from two different males influences the severity of sperm blocking. We also tested whether, in addition to mating, prolonged exposure to either the same male or two different males influenced sperm blocking. This is because previous work had suggested that harassment by males during oviposition could influence sperm use independently of mating (Wylie 1976; Boulton and Shuker 2015b). To do this, focal females were exposed to a single male, copulation was observed and post-copulatory courtship prevented as before. Females assigned to polyandrous treatments mated again twenty-four hours later. The identity of the male that each female mated with first was recorded and all males were retained. After their first mating, females were randomly assigned to the following treatments, and in each case females were given three hosts to oviposit on for 24 hours after the final mating, either in the presence of a male or not.

The six treatments were: (i) Control (C1) with once-mated females given hosts (*C. vicina*) immediately after mating; (ii) control + 24 hours (C24) with once-mated females given hosts 24 hours after mating; (iii) same-male mating (S) with females mating with the same male twice with a 24 hour interval between matings; (iv) same-male harassment (SH) in which females were exposed to the same male 24 hours after the initial mating and the male and female kept together during oviposition; (v) non-self mating (NS) in which females re-mated with a different once-mated male 24 hours after the initial mating and (vi) non-self harassment (NSH) in which females were exposed to a different once-mated male 24 hours after the initial mating, with the male and female kept together during oviposition (total $N =$ 196, $N =$ 23-37 across treatments). In order to standardise male mated status in the ’same-male’ and ‘non-self’ treatments, all second males used had mated once previously. All experimental hosts were removed the following day and incubated at 25°C. After two weeks, the offspring of the focal females emerged from the hosts. After the offspring had died, the number of sons and daughters produced was counted.

Experiment 3 Sperm blocking with competition between kin vs non-kin sperm

In our final experiment, we tested whether the severity of sperm blocking is influenced by male relatedness. The basic design of experiment 2 was repeated to test how sperm blocking differed when females mated with two brothers or two unrelated males. Females were randomly assigned to one of the following treatments: (i) Control (C1) with once-mated females given hosts immediately after mating; (ii) control + 24 hours (C24) with once-mated females given hosts 24 hours after mating; (iii) kin-mating (K) with females mating with the two virgin brothers with a 24 hour interval between matings; (iv) kin-harassment (KH) in which females were exposed to a virgin brother of their first mate 24 hours after the initial mating and where the male and female were kept together during oviposition; (v) non-kin mating (NK) in which females re-mated with a virgin male that was unrelated to their first mate 24 hours after the initial mating and (vi) non-kin harassment (NKH) in which females were exposed to a virgin male that was unrelated to their first mate 24 hours after the initial mating, and the male and female were kept together during oviposition (Total $N = 206$, $N =$ 23-38 across treatments).

In experiments 2 and 3, the sex ratio (proportion of sons) and son and daughter production (total clutch size) was analysed using GLMMs with a Quasibinomial and Gaussian error structure respectively, using the package lme4 in R Studio (Bates et al. 2015). Treatment, harassment and the interaction effect were included as fixed factors. Experiments 2 and 3 were each conducted across three blocks, and so experimental block was included as a random effect.

Results

Experiment 1 Assaying sperm precedence using gamma irradiated males

Irradiation was generally effective at preventing daughter production. However, females that mated with two irradiated males (II) were more likely to produce some daughters compared to both virgin and I females (Quasibinomial GLMM: $F_{2,544}= 95.58$, $P < 0.0001$; Figure 3 A). The absolute number of sons produced over the three treatments differed significantly. Clutches laid by virgin females are typically comparable in size to mixed-sex clutches (van den Assem 1977) but here virgin females produced around three times as many sons as females that mated with one or two irradiated males (QuasiPoisson GLMM: $F_{3,573}$= 230.72, $P < 0.0001$; Figure 3 B). This suggests that mated females were being inseminated and using sperm but that the daughters sired by I males made up the putative remaining 60-70% of the clutch that failed to develop.

Figure 3

Females that mated only once to an unsterilized male (N) produced the most female-biased sex ratios as expected. Twice-mated females all experienced sperm blocking, laying significantly more male biased sex ratios than ‘N’ females (all pairwise $p < 0.001$; $F_{3,118}$ = 25.44, $P < 0.0001$). The significant difference between the sex ratio produced by N and NN females confirms the problem of sperm blocking for polyandrous females; females that mated twice produced more male biased sex ratios immediately after mating. Moreover, the sex ratios produced by N and NN females converged in later host batches which demonstrates that the effect of sperm blocking does wear off over time (interaction effect: Quasibinomial GLM: $F_{9,461}$ = 4.87, $P < 0.0001$; main effect of treatment $F_{3,467}$ = 30.65, $P < 0.0001$; main effect of host batch $F_{3,467}$ = 9.73, $P < 0.0001$; See Figure 4).

Figure 4

In terms of sperm precedence – and remembering that in haplodiploids such as *Nasonia* only daughters can tell us about sperm usage – the first male had higher daughter production initially (NI) but sperm mixing occurred in later clutches (Figure 5 A). There was a significant effect of treatment on daughter production ($F_{2,371}$= 35.44, $P < 0.0001$; Figure 5 A), but this was because females that mated to two unsterilized males (NN) laid more daughters than females that mated to an unsterilized male either first (NI) or second (IN). In the first host batch, first males (NI) did achieve higher daughter production than second males (IN) but, overall across all host batches, sperm use was not significantly biased towards either male ($P_1 = 0.76$, $P_2 = 0.64$, albeit close to significance: treatment: $F_{1,360} = 3.60$, $P = 0.06$). This was partly because daughter production by IN females was equal to that of NI females in host batches 3 and 4 (treatment*host batch interaction effect: $F_{6,367} = 2.37$, $P = 0.03$), suggesting that the second males sperm (viable sperm in treatment IN) are used more in later host batches. There was a significant main effect of host batch on sperm precedence ($F_{3,369} = 10.44$, $P < 0.0001$), but this did not appear to relate to the number of hosts provided per batch (three in host batch 1 and one host in batches 2-4) as the only pairwise significant difference was between host batches 1 and 2 ($P < 0.05$).

In terms of sperm competition outcomes more explicitly, sperm blocking was not associated with higher paternity for either the first or the second male. When sperm blocking occurs, more sons are produced in the first host batch. Although 'sperm blocking' did reduce the fertilization success of the unsterilized (N) male in host batches 2-4 (β = -0.49, -SE = 0.25; $F_{1,234} = 5.01$, $P = 0.03$), there was no significant interaction between sperm blocking and treatment ($F_{2,231} = 1.77$, $P = 0.18$; figure 5 B) demonstrating that sperm blocking reduces the success of the first and second male equally.

Figure 5

We found no evidence that sperm blocking (sex ratio in the first host batch) was related to female size ($F_{1,121} = 1.14$, $P = 0.29$) or longevity ($F_{1,121} = 1.14$, $P = 0.29$). We also found no evidence that female body size interacts with treatment to influence sperm blocking (Treatment*size: $F_{3,118} = 1.29$ $P = 0.28$).

Experiment 2 Self versus non-self

All twice-mated females experienced sperm blocking to the same degree, regardless of whether they mated twice with the same male (S) or with different males (NS; $P = 0.99$;

Figure 6 A). Although there was an overall effect of mating treatment on the sex ratio ($F_{3,\,313}$ = 42.22, $P < 0.0001$), the only significant pairwise differences were between both controls and twice-mated females ($P < 0.0001$). There was no effect of harassment ($F_{1,\,315} = 0.08$, P = 0.78) or any interaction effect between harassment and treatment ($F_{5,311} = 0.72$, $P = 0.40$) on the sex ratio.

In terms of clutch size (total son and daughter production), there was no significant main effect of harassment ($F_{1,\,315} = 0.01$, $P = 0.91$) or treatment ($F_{3,\,313} = 2.00$, $P = 0.11$). The interaction effect between treatment and harassment was, however, statistically significant ($F_{5,311} = 5.34$, $P = 0.02$; figure 6 B). When control treatments were removed (no harassment in C1 or C24), the significant interaction effect remained ($F_{3,216} = 5.20$, $P = 0.02$). Prolonged exposure (harassment) reduces offspring production when the first and second males are different individuals (figure 6 B) but clutch sizes were larger when females were exposed to the same male twice.

Experiment 3 Kin versus non-kin

Sperm blocking was not influenced by the relatedness between competing males when females mated twice. Although there was again an overall effect of mating treatment on the sex ratio ($F_{3,\,316}$=39.75, $P < 0.001$), the only significant pairwise differences were between the controls and all twice-mated females as before ($P < 0.0001$). As above, females in the once-mated control treatments laid the most female-biased sex ratios. The sex ratio was less female-biased when females mated twice but relatedness between the males with which a female mated had no effect on the sex ratio ($P = 0.99$; Figure 6 C). There was no effect of harassment on the sex ratio ($F_{1,\,318} = 0.04$, $P = 0.84$) nor was there a significant interaction effect between treatment and harassment on the sex ratio produced ($F_{5,314} = 0.02$, $P = 0.87$).

Turning again to clutch size, there was no effect of harassment ($F_{1,\,318}$ = 0.79, P = 0.37) and no statistically significant effect of treatment ($F_{3,\,316}$ = 2.56, P = 0.054) on clutch size, nor was there a significant interaction effect between treatment and harassment ($F_{5,314}$ = 0.05, P = 0.82; Figure 6 D).

Discussion

A diverse range of offensive sperm competition traits have been suggested, and demonstrated, to confer an advantage to males when females mate multiply and sperm competition is encountered (Simmons 2014). As yet, however, the parasitoid wasps remain relatively understudied with respect to post-copulatory sexual selection (Boulton et al. 2014). In this study, we investigated the possibility that sperm blocking, i.e. the overproduction of unfertilized eggs (sons) that occurs immediately after female re-mating, might be advantageous to male *N. vitripennis* when they experience sperm competition. However, it is clear from our results that sperm blocking does not occur as a result of males displacing, blocking or incapacitating the ejaculates of their rivals. First, in experiment 1, sperm blocking did not favour the paternity of either the first or second male, and so was not associated with increased paternity in either offensive or defensive sperm competition. Second, in experiments 2 and 3, sperm blocking was not ameliorated by the presence of a male's own sperm or the sperm of a brother, and so sperm blocking occurred regardless of sperm identity and origin.

In experiment 1, we found that *N. vitripennis* males do not actively 'block' rival sperm, as sperm blocking reduced paternity for the first and second males equally. We did find, however, that in the first clutch, the first male sired more daughters, but in subsequent clutches second males gained more paternity success, such that the first and second males shared equal paternity overall (P_1 = 0.76, P_2 = 0.64; note that these values overestimate the true P_N and sum to more than 1, see methods and table S1 in the supplementary material). This is not likely to be due to the first male's ejaculate being fully depleted, because a single mating with a virgin male was sufficient for females to maintain daughter production when provided with up to 24 hosts in a previous study (Boulton and Shuker 2015b). Instead, this pattern is more likely to be due to how sperm is processed and stored.

Previous work on *N. vitripennis* has demonstrated that patterns of sperm use are influenced by mating order and also by the mated status of the male and the timing of the second mating. Holmes (1974) found that if the first male to mate was partially sperm depleted, there was no consistent pattern of use of the first or second male's sperm (i.e. sperm mixing occurred) and so paternity was more equal than if males were not depleted (in which case paternity was skewed towards the first male). Additionally, van den Assem and Feuth-de-Bruijn (1977) found little sperm mixing when females re-mated immediately after their initial mating, but sperm mixing increased when there was a delay of three days between matings (van den Assem and feuth-de-Bruijn 1977; van den Assem 1977). The processes that influence the degree of sperm mixing outlined above have also been found to influence sperm blocking. If the second male does not succeed in inseminating the female (Geuverink et al. 2009), or if there is no interval between matings, and so no sperm mixing, van den Assem (1977) found that sperm blocking was less severe (i.e. the sex ratio in the first clutch was less male biased). Superficially, this suggests that sperm blocking might promote sperm mixing, but the current results argue against a causal relationship and show that sperm blocking does not relate to male ejaculate physiology. Nor does sperm blocking appear to be a constraint related to the size of the spermatheca, as we found no effect of female body size (which is associated with larger spermatheca size in another parasiotid, *Trichogramma euprodctidis*; Martel et al. 2011) on the severity of sperm blocking.

Our second and third experiments also do not support the hypothesis that sperm blocking is a targeted male adaptation in *N. vitripennis* that incapacitates (rather than indiscriminately blocks) unrelated rival male sperm (den Boer et al. 2010). Whether the sperm are one's own, or that of a brother, or an unrelated competitor, the effect on reduced sperm use by the recipient female is the same. A previous example of apparent 'sperm incapacitation' in *Drosophila melanogaster* (Price et al. 1999) turned out on further investigation not to reflect male ejaculate physiology, but rather female physiological processes (Snook and Hosken, 2004; see also Manier et al. 2010). In *D. melanogaster* it is the act of copulation itself that leads to females 'dumping' sperm from previous ejaculates, resulting in extreme last-male precedence. In *D. melanogaster*, however, it does appear that some males are better than others at eliciting 'sperm dumping', but we found no evidence that this is the case in *N. vitripennis,* as increased sperm blocking was not positively associated with sperm precedence.

Instead, the patterns of, and correlation between, sperm precedence and sperm blocking exhibited by *Nasonia vitripennis* in the current study, and in others (Holmes 1974; van den Assem and Feuth-de-Bruijn 1977; van den Assem 1977; Boulton and Shuker 2015b) may relate to the morphology of the spermatheca and the physiology of sperm storage and movement within the female. The single, narrow spermathecal duct of the female's sperm store is thought to allow the movement of a single sperm to the site of fertilization, limiting wastage of sperm and facilitating precise sex allocation (Wilkes 1965; Flanders 1956). Whilst adaptive in terms of sperm economy, this narrow tube may become congested if the capacity of the spermatheca is exceeded.

As such, building on our work and that of earlier authors, we suggest that the phenotype of sperm blocking is generated as follows. During insemination, sperm are deposited at the base of the single, narrow spermathecal duct, from where they rapidly migrate to the rigid spheroid spermathecal capsule. The first sperm reach the capsule after just one minute (King 1961) but take several hours to quiesce and line up at the capsule mouth, when they are then ready to be used for fertilization (Wilkes 1965; King 1962). The sperm leave the capsule to fertilize the eggs through the same narrow duct. Following a second mating, the presence of incoming sperm in the narrow spermathecal duct prevents sperm required for fertilization from exiting the capsule to some extent (see Wilkes 1965 and Finney et al. 1947 for evidence from other parasitoids). If the first ejaculate is small, or there is sufficient time between matings, then the second ejaculate can enter the spermathecal duct and spermatheca itself, and then sperm-mixing can occur, equalising P_1 and P_2 (Holmes 1974; van den Assem and Feuth-de-Bruijn 1977; van den Assem 1977). Crucially though, in these cases the second ejaculate is able to enter the spermathecal duct and the incoming ejaculate will temporarily obstruct outgoing sperm, reducing the efficiency of sperm use, and generating sperm blocking.

The pattern of sperm precedence that we observed in experiment 1 fits this scenario. The first two clutches laid were predominantly sired by the first male to mate, but sperm mixing occurred later. The sperm in the first ejaculate would be the first to line up and quiesce at the mouth of the spermathecal duct, ready to be used for fertilization. The 24-hour delay between matings should result in some of the second ejaculate entering the duct, but before the first eggs are fertilized the small capsule will be fairly full. The sperm in the second ejaculate will move into storage slowly, at the same time impeding any outgoing sperm (and resulting in sperm blocking). The space created when the first sperm leave the capsule will allow more of the second ejaculate to enter and facilitate mixing of initially stratified sperm, increasing the potential for sperm mixing and, as such, sperm competition over time (Simmons 2001).

Taken together then, it seems that the very morphological and physiological machinery that allows coordinated control of fertilization in parasitoid species such as *N. vitripennis* is also responsible for the disruption of adaptive sex allocation that occurs when ejaculates overlap during sperm processing prior to fertilization. Control over fertilization, as required by *Nasonia* because of the facultative nature of sex allocation under LMC, therefore should strongly select against polyandry if sperm blocking arises with repeated matings (Boulton and Shuker 2015a; see also Ridley 1988). As such, sperm processing may be a major factor constraining female multiple mating in the parasitoids, a group noted for its rather limited level of polyandry (Ridley 1993; Godfray 1994; Boulton et al. 2014). This constraint may be less important when selection on female-biased sex ratios is relaxed, for instance under laboratory conditions, where LMC is reduced and polyandry is freer to evolve (for *N. vitripennis* see: Burton-Chellew et al. 2007; van den Assem and Jachmann 1999; Boulton and Shuker 2015b). The findings of the current study show that constrained sex allocation does not occur as an evolutionary consequence of polyandry in *N. vitripennis* (via post-copulatory sexual selection on males), rather selection on sex allocation may limit polyandry in these wasps, and weakening that selection may be a key contributing factor to the evolution of elevated re-mating in mass culture conditions.

When polyandry does increase, such as under mass culture, post-copulatory sexual selection should favour the evolution of large ejaculates in *N. vitripennis* males as a defence against sperm competitors (Simmons 2001). Larger ejaculates would then occupy more space in the spermathecal duct, thereby obstructing the ejaculates of rivals for longer (Holmes 1974; van den Assem and Feuth-de-Bruijn 1977). However, as discussed, *N. vitripennis* females appear unable to process such large ejaculates efficiently, and sperm blocking leads to a reduction in fitness via sex allocation disruption. Thus the evolutionary interests of males and females are brought into conflict over the size of ejaculate that is transferred. In haplodiploids, sexual conflict is more likely to be resolved in favour of females and so any male adaptation, such as large ejaculate size, that harms females is unlikely to persist (de la Filia et al. 2015). Asymmetric sexual conflict such as this may explain why ejaculates transferred by parasitoid males remain relatively small even in more polyandrous species, as well as why the costs of mating (in terms of fecundity and longevity) tend to be low for females (Boulton et al. 2014).

The interpretation of the interaction between sperm use, sperm competition and polyandry that we present here also suggests a different (but not mutually exclusive) hypothesis for the evolution of the post-copulatory courtship seen in *Nasonia* and in many other parasitoids (Boulton et al. 2014). Such post-copulatory courtship typically reduces female receptivity to subsequent matings, and so has often been interpreted as an adaptation to reduce future sperm competition for a male (i.e. a defensive trait, and a clear example that post-copulatory sexual selection can shape behavior, physiology and morphology even without double-matings occurring: Dougherty et al. 2016). However, multiple matings not only risk male paternity share, they also reduce the extent to which sperm are used by females at all. Thus, post-copulatory courtship that reduces female receptivity not only protects paternity, but also protects efficient sperm use by that female. Since haplodiploid males only pass on their genes via daughters, this might be a non-trivial selective force, to sit alongside that of protecting paternity.

To conclude, by interpreting our findings and those of earlier studies in the light of the structure and function of the female parasitoid reproductive tract, we have been able to suggest a mechanism for sperm blocking and to understand how sperm dynamics can result in the patterns of sperm precedence and mixing seen in *N. vitripennis*, and indeed, across other parasitoids more generally. Herberstein et al (2011) advocate this approach, encouraging behavioral ecologists to refer to early morphological descriptions when interpreting data regarding sperm competition and sperm dynamics. The current study demonstrates that this approach can facilitate understanding of the processes that lead to patterns of sperm precedence, demonstrating that phenomena that appear likely to result from male ejaculate traits, such as sperm blocking (or ‘sperm incapacitation’; see Hosken and Stockley, 2004 and Simmons et al. 1999) may relate to female physiological characteristics that, in this case, and perhaps many others, are shaped by natural selection on efficient female sperm use and storage. Rather than immediately looking to the more appealing and enigmatic (and often more contentious) processes, such as cryptic female choice (Birkhead 1998) or ‘kamikaze sperm’ and incapacitating seminal fluid proteins (Baker and Bellis, 1988; Harcourt 1991; Price et al. 1999; den Boer et al. 2010), it is important that we understand the basic role that female physiology plays in determining the outcomes of sperm competition. In doing so, we can gain a better appreciation of how post-copulatory sexual selection operates and the types of traits that are likely to result in males and females.

Funding

This work was supported by a NERC Doctoral Training Grant studentship to RAB.

Acknowledgments

We are grateful to Professor Andrew Riches for use of the ^{137}Cs gamma radiation source. Many thanks also to Justa Heinen-Kay, Jessie Tanner, Rachel Olzer and Rebecca Ehrlich and to two anonymous reviewers for their insightful comments on an earlier version of this manuscript.

Data accessibility statement

Data archived in Dryad Digital Repository at doi:10.5061/dryad.t0877

Conflict of Interest

The authors declare that they have no conflict of interest to report.

References

Arnqvist G, Nilsson T. 2000. The evolution of polyandry: multiple mating and female fitness in insects. Anim Behav. 60: 145-164.

Arnqvist G, Rowe L. 2005. Sexual Conflict. New Jersey, Princeton University Press.

Baer B, Morgan ED, Schmid-Hempel P. 2001. A nonspecific fatty acid within the bumblebee mating plug prevents females from remating. Proc Natl Acad Sci USA. 98: 3926-3928.

Baker RR, Bellis, MA. 1988. Kamikaze sperm in mammals. Anim Behav. 36: 936-939.

Bates D, Maechler M, Bolker B, Walker, S. 2015. Fitting Linear Mixed-Effects Models Using lme4. J Stat Softw. 67: 1-48.

Bawab FM, El-Sherief SS. 1989. Contributions to the study of the origin, nature and formation of the plug in the spermatheca of the female crab *Portunus pelagicus* (Linnaeus, 1766) (Decapoda, Brachyura). Crustaceana. 57: 9-24.

Birkhead TR. 1998. Cryptic female choice: criteria for establishing female sperm choice. Evolution. 52: 1212-1218.

Boorman E, Parker GA 1976. Sperm (ejaculate) competition in *Drosophila melanogaster,* and the reproductive value of females to males in relation to female age and mating status. Ecol Entomol. 1:145-155.

Boulton RA, Shuker DM. 2013. Polyandry. Curr Biol. 23: R1080-R1081.

Boulton RA, Collins LA, Shuker DM. 2014. Beyond sex allocation: the role of mating systems in sexual selection in parasitoid wasps. Biol Rev Camb Philos Soc. 90: 599-627.

Boulton RA, Shuker DM. 2015a. A sex allocation cost to polyandry in a parasitoid wasp. Biol Lett. 11: 4–7.

Boulton RA, Shuker DM. 2015b. The costs and benefits of multiple mating in a mostly monandrous wasp. Evolution. 4: 939–949.

Boulton RA, Shuker DM. 2016. Polyandry is context dependent but not convenient in a mostly monandrous wasp. Anim Behav. 112: 119-125.

Burton-Chellew MN, Koevoets T, Grillenberger BK, Sykes EM, Underwood SL, Bijlsma K, Gadau J, van de Zande L, Beukeboom LW, West SA, Shuker DM. 2008. Facultative sex ratio adjustment in natural populations of wasps: cues of local mate competition and the precision of adaptation. Am Nat. 172: 393-404.

Burton-Chellew MN, Beukeboom LW, West SA, Shuker DM. 2007. Laboratory evolution of polyandry in the parasitoid wasp *Nasonia vitripennis*. Anim Behav. 74: 1147-1154.

Daly M. 1978. The cost of mating. Am Nat. 112: 771–774.

de la Filia AG, Bain SA, Ross L. 2015. Haplodiploidy and the reproductive ecology of Arthropods. Curr Opin Insect Sci. 9: 36-43.

den Boer SPA, Baer B, Boomsma JJ. 2010. Seminal Fluid Mediates Ejaculate Competition in Social Insects. Science. 327: 1506-1509.

Dougherty LR, Simmons LW, Shuker DM. 2016. Postcopulatory sexual selection when a female mates once. Anim Behav. 116: 13-16.

Finney GL, Flanders SE, Smith HS. 1947. Mass culture of *Macrocentrus ancylivorus* and its host, the potato tuber moth. Hilgardia. 17: 437-483.

Flanders SE. 1956. The mechanisms of sex-ratio regulation in the (parasitic) Hymenoptera. Insectes Soc. 3: 325-334.

Garcia□Gonzalez F, Evans JP. 2011. Fertilization success and the estimation of genetic variance in sperm competitiveness. Evolution, 65: 746-756.

Geuverink E, Gerritsma S, Pannebakker BA, Beukeboom LW. 2009. A role for sexual conflict in the evolution of reproductive traits in *Nasonia* wasps? Anim Biol. 59: 417- 434.

Godfray HCJ. 1994. Parasitoids: Behavioural and evolutionary ecology. Princeton University Press: New Jersey.

Grillenberger BK. Koevoets T, Burton-Chellew MN. Sykes EM, Shuker DM, Van de Zande L, Bijlsma R, Gadau J, Beukeboom LW. 2008. Genetic structure of natural *Nasonia vitripennis* populations: validating assumptions of sex-ratio theory. Mol Ecol 17: 2854–64.

Hamilton WD. 1967. Extraordinary sex ratios: A sex ratio theory for sex linkage and inbreeding has new implications in cytogenetics and entomology. Science. 156: 477-488.

Harcourt AH. 1991. Sperm competition and the evolution of nonfertilizing sperm in mammals. Evolution. 1: 314 -28.

Herberstein ME, Schneider JM, Uhl G, Michalik P. 2011. Sperm dynamics in spiders. Behav Ecol. 22: 692-695.

Holmes HB. 1974. Patterns of sperm competition in *Nasonia vitripennis*. Can J Genet Cytol. 16: 789-795.

Hosken DJ, Stockley P. 2004. Sexual selection and genital evolution. Trends Ecol Evol. 19: 87-93.

King PE. 1961. A possible method of sex ratio determination in the parasitic hymenopteran *Nasonia vitripennis*. Nature. 189: 330-331.

King PE. 1962. The structure and action of the spermatheca in.*Nasonia vitripennis* (Walker)(Hymenoptera:Pteromalidae). Proc R Ent Soc Lond (A). 37: 73-75

Manier MK, Belote JM, Berben KS, Novikov D. Stuart WT, Pitnick S. 2010. Resolving mechanisms of competitive fertilization success in *Drosophila melanogaster*. Science. 328:354-357.

Manier MK, Belote JM, Berben KS, Lüpold S, Ala-Honkola, O. Collins WF, Pitnick S. 2013. Rapid diversification of sperm precedence traits and processes among three sibling *Drosophila* species. Evolution. 67: 2348-2362.

Martel V, Darrouzet É, Boivin G. 2011. Phenotypic plasticity in the reproductive traits of a parasitoid. J Insect Physiol. 57: 682-687.

Martel V, Shuker DM, Boulton RA, Damiens D, Boivin G. 2016. Sex allocation and the evolution of insemination capacity under local mate competition. Entomol Exp Appl. 159: 230-242.

Mikheyev AS. 2003. Evidence for mating plugs in the fire ant *Solenopsis invicta*. Insectes Soc. 50: 401-402.

Moore HDM, Martin M, Birkhead TR. 1999. No evidence for killer sperm or other selective interactions between human spermatozoa in ejaculates of different males in vitro. Proc R Soc Lond B Biol Sci. 266: 2342-2350.

Parker GA. 1970. Sperm competition and its evolutionary consequences in the insects. Biol Rev Camb Philos Soc. 45: 525-567.

Pizzari T, Wedell N. 2013. The polyandry revolution. Philos Trans R Soc Lond B. 368, Biol. Sci. 368: 1471-2970.

Price CS, Dyer KA, and J. A. Coyne. 1999. Sperm competition between Drosophila males involves both displacement and incapacitation. Nature. 400: 449-452.

Ramadan MM, Wong TTY. 1989. Effect of Gamma Radiation on *Biosteres longicaudatus* (Ashmead)(Hymenoptera: Braconidae), a Larval Parasitoid of *Dacus dorsalis* Hendel (Diptera: Tephritidae). Proc Hawaiian Entomol Soc. 29: 111-113.

Ray DT. 1948. Dominant lethals induced by X-rays in sperm of the chalcidoid wasp *Nasonia brevicornis* Ashmead [= *Mormoniella vitripennis* (Walker) fide Muesebeck in lit. Biol Bull. 95: 257.

R Core Team. 2016. R: A language and environment for statistical computing. R Foundation for statistical computing: Vienna.

Ridley M. 1988. Mating frequency and fecundity in insects. Biol Rev Camb Philos Soc. 63: 509-549.

Ridley M. 1993. Clutch size and mating frequency in parasitic hymenoptera. Am. Nat. 142, 893–910.

Saul GB. 1955. The induction by x-rays of recessive lethals in the mature sperm of *Mormoniella vitripennis* (Walker). Radiat Res. 2: 447-460.

Shuker DM, West SA. 2004. Information constraints and the precision of adaptation: Sex ratio manipulation in wasps. Proc Natl Acad Sci USA. 101: 10363–10367.

Shuker DM, Pen I, Duncan AB, Reece SE, West SA. 2005. Sex ratios under asymmetrical local mate competition: Theory and a test with parasitoid wasps. Am Nat. 166: 301- 316.

Simmons LW. 2001. Sperm competition and its evolutionary consequences in the insects. Princeton University Press: Chichester.

Simmons LW. 2014. Sperm competition. In The Evolution of Insect Mating systems. (Eds D. M. Shuker and L. W. Simmons), pp 181-203. Oxford University press: London.

Simmons LW, Parker GA, Stockley P. 1999. Sperm displacement in the yellow dung fly, *Scatophaga stercoraria*: an investigation of male and female processes. Am Nat. 153: 302-314.

Simmons LW, Stockley P, Jackson RL, Parker GA. 1996. Sperm competition or sperm selection: no evidence for female influence over paternity in yellow dung flies *Scatophaga stercoraria*. Behav Ecol Sociobiol. 38: 199-206.

Siva□Jothy MT, Tsubaki Y. 1994. Sperm competition and sperm precedence in the dragonfly *Nanophya pygmaea*. Physiol Entomol. 19: 363-366.

Slatyer RA, Mautz BS, Backwell PRY, Jennions MD. 2012. Estimating genetic benefits of polyandry from experimental studies: A meta-analysis. Biol Rev Camb Philos Soc. 87: 1–33.

Snook RR, Hosken DJ. 2004. Sperm death and dumping in *Drosophila*. Nature. 428: 939-941.

Taylor ML, Price TAR, Wedell N. 2014. Polyandry in nature: A global analysis. Trends Ecol Evol. 29: 376–83.

van de Zande L, Ferber S, de Haan A, Beukeboom LW, van Heerwaarden J, Pannebakker BA. 2014. Development of a *Nasonia vitripennis* outbred laboratory population for genetic analysis. Mol Ecol Resour. 14: 578–87.

van den Assem J, Feuth-De Bruijn E. 1977. Second matings and their effect on the sex ratio of the offspring in *Nasonia vitripennis* (Hymenoptera: Pteromalidae). Entomol Exp Appl. 21: 23-28.

van den Assem J, Jachmann F. 1999. Changes in male perseverance in courtship and female readiness to mate in a strain of the parasitic wasp *Nasonia vitripennis* over a period of 20+ years. Neth J Zool. 49: 125-137.

van den Assem J. 1977. Note on the ability to fertilize following insemination (with females of *Nasonia vitripennis,* Hymenoptera: Chalcidoidea). Neth J Zoo. 27: 230-235.

Werren JH. 1980. Sex-ratio adaptations to local mate competition in a parasitic wasp. Science. 208: 1157-1159.

Werren JH. 1983. Sex ratio evolution under local mate competition in a parasitic wasp. Evolution. 37: 116–124.

Whiting AR. 1967. The biology of the parasitic wasp *Mormoniella vitripennis* [= *Nasonia brevicornis*](Walker). Q Rev Biol. 42: 333-406.

Wilkes A. 1965. Sperm transfer and utilization by the arrhenotokous wasp *Dahlbominus fuscipennis* (Zett.)(Hymenoptera: Eulophidae). Can Entomol. 97: 647-657.

Wishart GJ, Dick LA. 1985. Effect of γ-radiation on fowl sperm function in vitro and in vivo. J Repro Fertil. 75: 617-622.

Wylie HG. 1976. Interference among females of *Nasonia vitripennis* (Hymenoptera: Pteromalidae) and its effect on sex ratio of their progeny. Can Entomol. 108: 655-661.

Figure legends

Figure 1 Schematic representing predicted results if sperm blocking is (A) a defensive male trait or (B) an offensive male trait. The x-axis represents sperm blocking which is the total number of sons in the first clutch laid. The y-axis is the total number (over all clutches) of eggs fertilized (daughters sired) by the first male (P_1 solid line) or the second male (P_2 dashed line). We estimated P_1 and P_2 in experiment 2 using the sterile male technique (see main text for details).

Figure 2. Dose-response curve for males irradiated with 80-140 Gy of gamma radiation (^{137}Cs) and control (untreated) males in the pilot (Error bars = binomial Confidence Intervals).

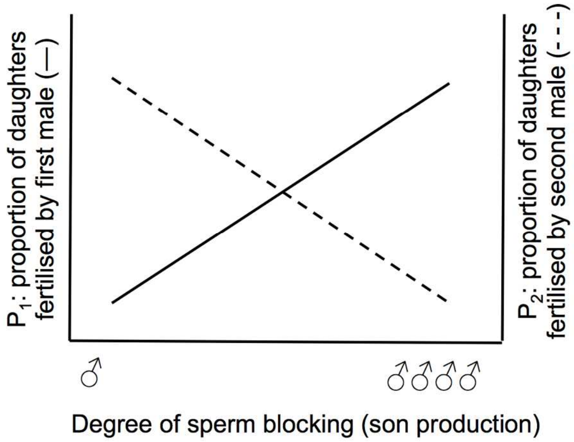
Figure 3 A Proportion of sons (sex ratio) and **B** total son production by virgin females and females mated to either 1 (I) or two (II) irradiated males in experiment 1 (A) Sex ratio (proportion of sons), Error bars = binomial Confidence Intervals (CIs). Note that the Y axis runs between 0.96 and 1.0 and that in all cases daughter production was extremely low. (B) Absolute number of sons produced. Virgin females (V) produce more sons than females mated with one (I) or two (II) irradiated males. Error bars = 95% CIs. (A and B) Different lower case letters represent treatment groups that are statistically different ($p < 0.05$).

Figure 4. Sex ratios (proportion of sons) produced by females that mated with either one or two unsterilized (N) males in experiment 1 (error bars = binomial CI).

Figure 5 Daughter production and sperm precedence for twice-mated females in experiment 1. (A) Fewer daughters (fertilized eggs) were produced by the second male in host batches 1-2 (IN), but there was no significant difference in daughter production by the first (NI) or second (IN) male in host batches 3 and 4. Error bars = 95% CI. (B) Son production or ‘sperm blocking’ in the first clutch reduced fertilization success in host batches 2-4 for the first (P_1: NI) and second (P_2: IN) male equally.

Figure 6. Sex ratio and clutch size for females in experiments 2 (A and B) and 3 (C and D). White bars = No harassment, grey bars = harassment. (A) There was no significant difference in the sex ratios produced by females that either mated with or were exposed to, the same male twice or two different males. (B) The clutch size that females produced was not affected by mating treatment. The interaction effect between harassment and treatment was significant however. (C) There was no significant difference in the sex ratios produced by females that either mated with or were exposed to two brothers (kin) or two unrelated males (non-kin). (D) Clutch size was not affected by harassment, the number of matings or relatedness between the first and second males. Error bars = binomial CIs (for A and C) and 95% CIs (for C and D).

(A) Sperm-blocking as a *defensive* trait: P_1 positively correlated with sperm-blocking



(B) Sperm-blocking as an *offensive* trait: P_2 positively correlated with sperm-blocking

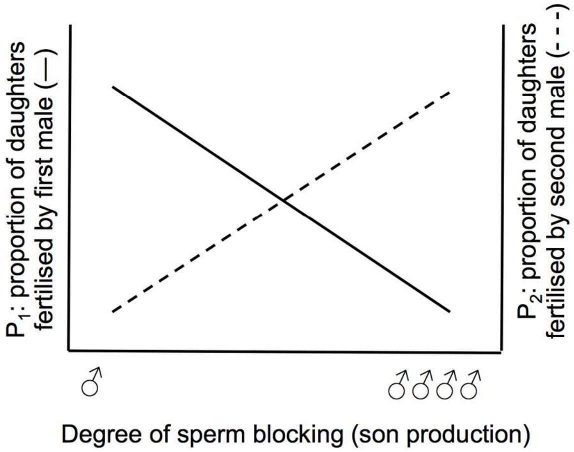


Figure 1 Schematic representing predicted results if sperm blocking is (A) a defensive male trait or (B) an offensive male trait. The x-axis represents sperm blocking which is the total number of sons in the first clutch laid. The y-axis is the total number (over all clutches) of eggs fertilized (daughters sired) by the first male (P1 solid line) or the second male (P2 dashed line). We estimated P1 and P2 in experiment 2 using the sterile male technique (see main text for details).

168x238mm (250 x 250 DPI)

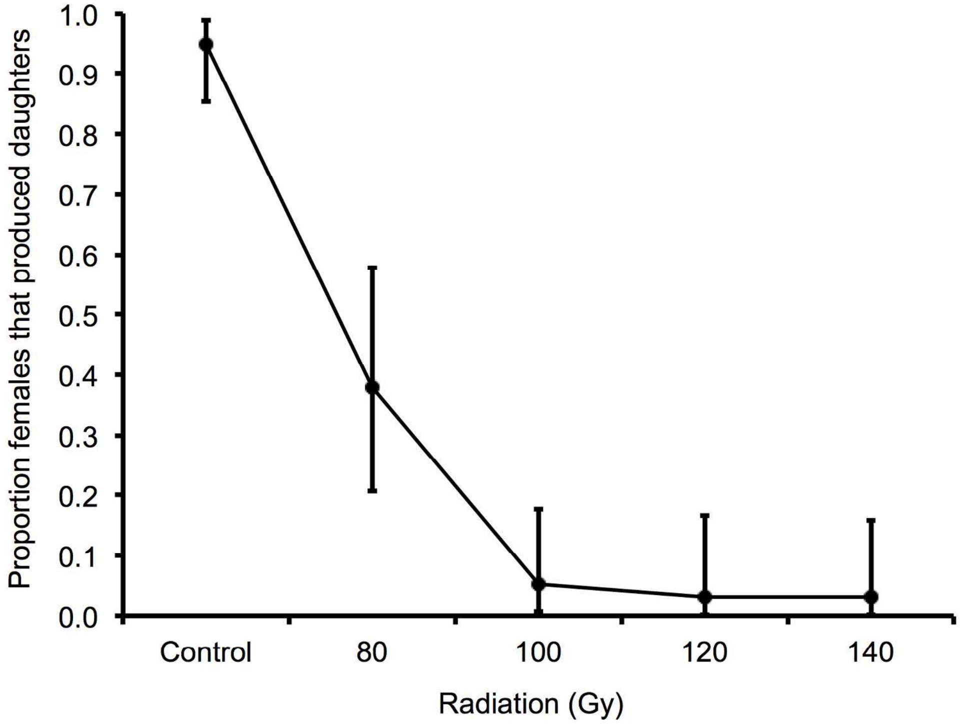


Figure 2. Dose-response curve for males irradiated with 80-140 Gy of gamma radiation (137Cs) and control (untreated) males in the pilot (Error bars = binomial Confidence Intervals).

220x166mm (250 x 250 DPI)

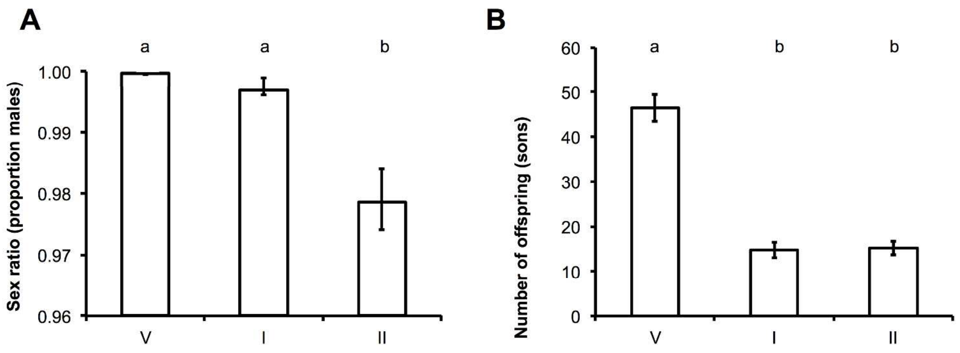


Figure 3 A Proportion of sons (sex ratio) and B total son production by virgin females and females mated to either 1 (I) or two (II) irradiated males in experiment 1 (A) Sex ratio (proportion of sons), Error bars = binomial Confidence Intervals (CIs). Note that the Y axis runs between 0.96 and 1.0 and that in all cases daughter production was extremely low. (B) Absolute number of sons produced. Virgin females (V) produce more sons than females mated with one (I) or two (II) irradiated males. Error bars = 95% CIs. (A and B) Different lower case letters represent treatment groups that are statistically different ($p < 0.05$).

368x142mm (250 x 250 DPI)

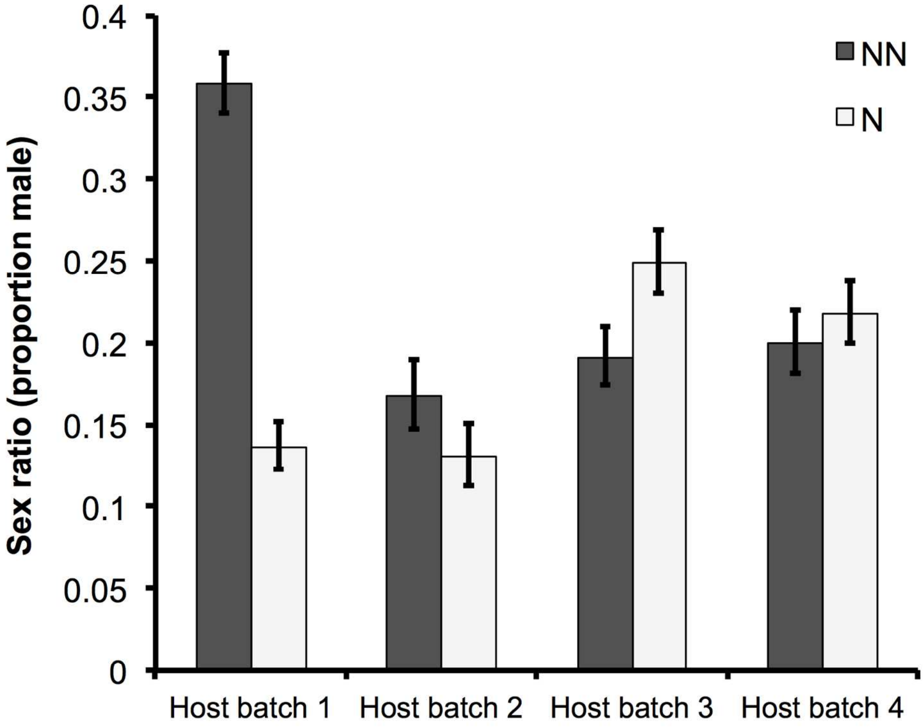


Figure 4. Sex ratios (proportion of sons) produced by females that mated with either one or two unsterilized (N) males in experiment 1 (error bars = binomial CI).

192x150mm (250 x 250 DPI)

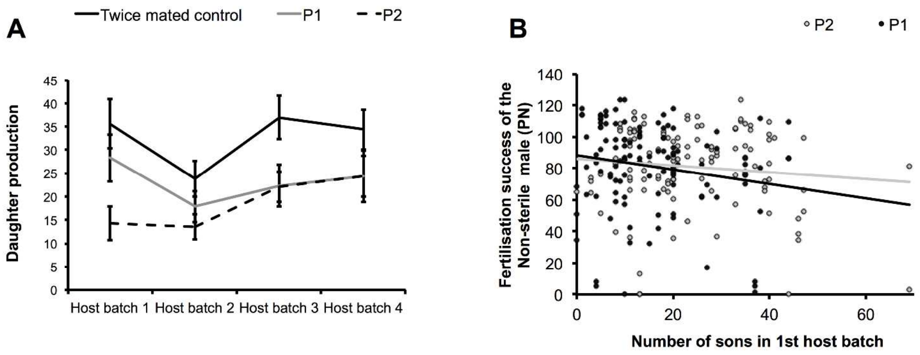


Figure 5 Daughter production and sperm precedence for twice-mated females in experiment 1. (A) Fewer daughters (fertilized eggs) were produced by the second male in host batches 1-2 (IN), but there was no significant difference in daughter production by the first (NI) or second (IN) male in host batches 3 and 4. Error bars = 95% CI. (B) Son production or ‘sperm blocking’ in the first clutch reduced fertilization success in host batches 2-4 for the first (P1: NI) and second (P2: IN) male equally.

397x187mm (250 x 250 DPI)

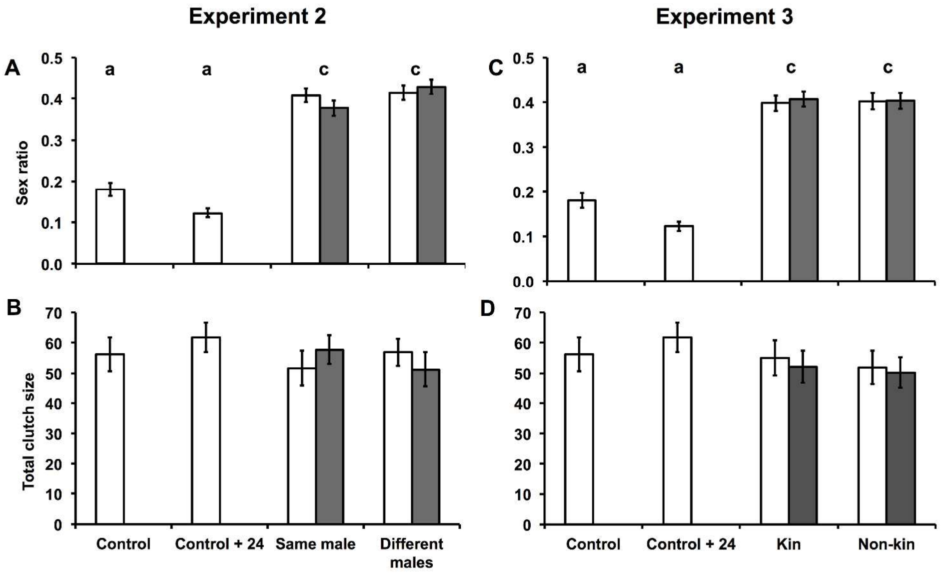


Figure 6. Sex ratio and clutch size for females in experiments 2 (A and B) and 3 (C and D). White bars = No harassment, grey bars = harassment. (A) There was no significant difference in the sex ratios produced by females that either mated with or were exposed to, the same male twice or two different males. (B) The clutch size that females produced was not affected by mating treatment. The interaction effect between harassment and treatment was significant however. (C) There was no significant difference in the sex ratios produced by females that either mated with or were exposed to two brothers (kin) or two unrelated males (non-kin). (D) Clutch size was not affected by harassment, the number of matings or relatedness between the first and second males. Error bars = binomial CIs (for A and C) and 95% CIs (for C and D).

481x276mm (250 x 250 DPI)