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Wolbachia modification of sperm does not always require residence within developing sperm

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Wolbachia are maternally inherited intracellular bacteria known to manipulate the reproduction of their arthropod hosts. *Wolbachia* commonly affect the sperm of infected arthropods. *Wolbachia*-modified sperm cannot successfully fertilize unless the female is infected with the same *Wolbachia* type. A study of spermatogenesis in the parasitic wasp *Nasonia vitripennis* reveals that *Wolbachia* are not required in individual spermatocytes or spermatids to modify sperm. In *N. vitripennis*, *Wolbachia* modify nearly all sperm, but are found only in ~28% of developing sperm, and are also found in surrounding cyst and sheath cells. In the beetle *Chelymorpha alternans*, *Wolbachia* can modify up to 90% of sperm, but were never observed within the developing sperm or within the surrounding cyst cells; they were abundant within the outer testis sheath. We conclude that the residence within a developing sperm is not a prerequisite for *Wolbachia*-induced sperm modification, suggesting that *Wolbachia* modification of sperm may occur across multiple tissue membranes or act upstream of spermiogenesis.
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Introduction

Wolbachia are among the most common of endosymbiotic bacteria found in a large proportion of arthropods and filarial nematodes (Bandi *et al*., 1998; Windsor and Werren, 2000). Most surveys suggest that at least 20% of terrestrial arthropods are infected with *Wolbachia* (Werren *et al*., 1995; West *et al*., 1998; Jeyaprakash and Hoy, 2000; Windsor and Werren, 2000; Sintupachee *et al*., 2006). A recent meta-analysis of *Wolbachia* surveys estimates that 66% of insect species harbor *Wolbachia* (Hilgenboecker *et al*., 2008).

Wolbachia are maternally (cytoplasmically) inherited bacteria and *Wolbachia*-induced manipulation of host reproduction results in increased proportion of infected females in a population (Caspari and Watson, 1959; Werren and O'Neill, 1997). Among the most common manipulations are host feminization, parthenogenesis induction, male killing and cytoplasmic incompatibility (CI) (Werren, 1997; Stouthamer *et al*., 1999) as well as other, sometimes subtle, effects on hosts (Clark, 2007).

Cytoplasmic incompatibility is a form of conditional infertility where sperm from a *Wolbachia*-infected male fertilize eggs but development fails unless the eggs are infected with the same *Wolbachia* type(s). CI is the most commonly described *Wolbachia*-induced phenotype, documented in at least eight different arthropod orders including Acari (Breeuwer and Jacobs, 1996), Coleoptera (Wade and Stevens, 1985), Diptera (Yen, 1975), Homoptera (Hoshizaki and Shimada, 1995), Hymenoptera (Reed and Werren, 1995), Isopoda (Moret *et al*., 2001), Lepidoptera (Brower, 1976) and Orthoptera (Kamoda *et al*., 2000).

There are at least two distinct events in CI, the *Wolbachia*-induced modification of sperm and the *Wolbachia*-induced rescue of that modification upon fertilization (Werren, 1997). Following fertilization with a *Wolbachia*-modified sperm, the result is either (1) normal development in embryos from eggs harboring at least the same *Wolbachia* types as the father or (2) abnormal development in embryos lacking the father's *Wolbachia* type(s). Incompatibility is manifested as a disruption of pronuclear chromatin condensation followed by missegregation of chromosomes during mitosis (Reed and Werren, 1995; Callaini *et al*., 1996).

The molecular mechanisms underlying CI are currently unknown, as is the specific stage(s) of spermatogenesis during which *Wolbachia* modify the developing sperm. Most of which is currently known regarding *Wolbachia* during spermatogenesis comes from studies on *Drosophila*. *Wolbachia* are present in a subset of cysts throughout spermatogenesis and removed from spermatids during individualization along with most of the cytoplasm and the minor mitochondrial derivative. *Wolbachia* are not found in mature sperm (Bressac and Rousset, 1993; Snook *et al*., 2000; Clark *et al*., 2002). Studies of CI in *Drosophila* suggested that *Wolbachia* are required within developing spermatocytes and spermatids for those sperm to be modified, because rates of CI (percentage of embryos affected in an incompatible

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cross) correlate with the proportion of developing cysts with *Wolbachia* within spermatocytes and spermatids (Clark *et al*., 2003; Veneti *et al*., 2003). However, it should be noted that the conclusion is based solely on this correlation, and not on functional studies demonstrating a requirement of *Wolbachia* to be present in individual cysts to affect modification.

So far, studies of *Wolbachia* during spermatogenesis have only been conducted in *Drosophila*, which could lead to inaccurate generalizations for other host species. Here, we investigate the patterns of *Wolbachia* infection during spermatogenesis in the parasitic wasp *Nasonia vitripennis* and the beetle *Chelymorpha alternans* from two additional insect orders. *N. vitripennis* has been a model for *Wolbachia* studies for many years. Levels of CI are 97–100%, and the species is stably infected with two different *Wolbachia* strains, *w*NvA1 and *w*NvB1 (Bordenstein and Werren, 1998; Bordenstein *et al*., 2003). In *N. vitripennis*, CI results in total elimination of the paternal chromosomes, converting a diploid embryo, which would have developed into a female, into a haploid male in this haplo–diploid species (Reed and Werren, 1995). *C. alternans* shows high levels of CI (up to 90% when doubly infected) with two infection forms routinely found in nature, singly infected insects with strain *w*Calt1 and doubly infected insects with *w*Calt1 and *w*Calt2 (Keller *et al*., 2004). Double-infected males are incompatible with single-infected females. In both species, cured strains are available to measure the levels of CI. Our results indicate that, contrary to conclusions of previous studies in *Drosophila*, *Wolbachia* are not required in developing sperm of *N. vitripennis* and *C. alterans* to induce modification.

Materials and methods

Stocks

N. vitripennis: R511 is a doubly infected line with *Wolbachia* from both the A and B supergroups (Perrot-Minnot *et al*., 1996). R511-T is an uninfected line, created from R511 by two generations of tetracycline treatment, as described in Breewuer and Werren (Breeuwer and Werren, 1990). AsymC is an *N. vitripennis* line previously cured of *Wolbachia* (Breeuwer and Werren, 1990). All wasps were reared at 25 °C under constant light on *Wolbachia*-free *Sarcophaga bullata* pupa (fleshfly) in uncrowded conditions.

C. alternans: Three different lines of *C. alternans* males, doubly infected (Gamboa), singly infected (Guarumal) and uninfected (Gamboa) were examined. Uninfected stocks of *C. alternans* were created by injecting females from Gamboa three times a week with a solution of 2–3 µl of sterile 0.9% rifiampicin for two consecutive weeks as described previously (Keller *et al*., 2004). Amplification of the 952 bp 16S rDNA gene fragment using general primers and the 560 bp *wsp* gene fragment using *Wolbachia* strain-specific primers were used to validate the negative infection status of the treated lines (Keller *et al*., 2004). All *C. alternans* were kept in the laboratory (12 h light, 60% humidity, 26 °C temperature) on fresh leaves of *Merremia umbellata* (Convolvulaceae). Egg masses were placed in a percival I-30BL incubator adjusted to give a 13–11 h dark–light cycle, 26–28 °C temperature and 70–75% humidity from day 0 until hatching and then reared under the laboratory conditions described above.

CI measurements

Cytoplasmic incompatibility is estimated in both *Nasonia* and *Chelymorpha* using long established standard methods. In *N. vitripennis*, CI was estimated by comparing the proportion of females in an incompatible cross (IC: infected male × uninfected female) divided by the percentage of female progeny in the compatible cross using the same strain females (CC: uninfected male × uninfected female) according to the following index $CI = 1 - (\text{percentage of female IC} / \text{percentage of female CC}) \times 100$. This assay takes into consideration that female progeny are derived normally from fertilized eggs in this haplodiplod insect and CI results in paternal chromosome loss and conversion to haploid males in this species (Reed and Werren, 1995; Bordenstein *et al*., 2003). Mortality from egg laying to adulthood is a minor contributor under the assay conditions used here (Bordenstein *et al*., 2003). In *C. alternans*, CI was estimated by comparing egg hatch rates in control versus incompatible crosses, using the following index $CI = (IUE - CUE)/(1 - CUE) \times 100$, where IUE represents the percentage of unhatched eggs observed in incompatible cross and CUE represents the percentage of unhatched eggs in compatible crosses of the tested strain (Poinsot *et al*., 1998). The average CUE used here was 28% (Bailey-Jourdain, 2006).

Cytology

Nasonia testes were dissected from pupa and adults in TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween, 0.05% NaN_3, pH 7.5). They were then either transferred whole into a microcentrifuge tube or transferred to a lysine-coated slide for further dissection. Tissues were fixed in 3.7% formaldehyde in TBST for 15–30 min, followed by three washes in TBST for 5 min each and blocked in TBST with 1% bovine serum albumin (BSA) for 10 min. *Wolbachia* were visualized using a rabbit polyclonal antibody made against a portion of the *Wolbachia* surface protein (*wsp*; provided by K Bourtzis). Tissues were incubated in the primary antibody solution (TBST, 1% BSA, 2 mg ml^{-1} RNaseA and 1:500 of the anti-*wsp* antibody), for 1 h at room temperature, followed by three washes with TBST. This was followed by 1 h at room temperature in 1:500 alexa-flour 488 antirabbit antibody (Invitrogen, Carlsbad, CA, USA), followed by three washes in TBST. DNA was then stained with 5 µg ml^{-1} propidium iodide for 20 min, followed by a brief wash in TBST before mounting in ProLong Gold antifade mounting media (Invitrogen). Confocal images were obtained using a Leica SP confocal microsope.

To determine the proportion of developing sperm infected, testes from 10 males were dissected from the yellow pupal stage (just before the beginning of meiosis) (Pultz and Leaf, 2003). Cysts were removed and further disrupted by brief pipetting. The resulting tissue was deposited on a lysine-coated slide, fixed and stained as above. The result was large numbers spermatocytes either singly or in groups of small numbers. Spermatocytes were scored for the presence of *Wolbachia*.

Chelymorpha testes from 4-week-old singly infected (Guarumal), doubly infected (Gamboa) and uninfected (Gamboa) beetles were examined. This is shortly after males reach sexual maturity (D Windsor, unpublished). At this stage, CI levels of Gamboa males crossed to uninfected females is 75.9% and Guarumal males crossed to uninfected females is 67.2%, respectively (Bailey-Jourdain, 2006). Testes were removed and fixed for 20 min in 100 µl of 3.7% formaldehyde in phosphate buffer and 600 µl of heptane. Samples were then rinsed three times for 10 min in PSBT (phosphate buffer + 0.3% Triton X-100) and DNA was stained with either 4′,6-diamidino-2-phenylindole (DAPI) (1.0 µg ml^{-1}) for 5 min or Oligreen (1:750) for 20 min and rhodamine-labeled phalloidin (1:100) for 90 min. The phalloidin labels host actin, allowing for visualization of different cell layers and differentiating cysts from the outer testis sheath. Images were obtained using a Leica DM IRB confocal microscope and a Nikon E600. To confirm the results from visualization of *Wolbachia* in whole testes, fixed testes were further dissected and cysts removed and examined.

Sperm depletion

Rates of sperm utilization in *Wolbachia* infected and uninfected *N. vitripennis* males were estimated by comparing rates of sperm depletion upon successive matings. *Wolbachia* infected and uninfected males were repeatedly mated to infected females. The relative rates of sperm depletion were estimated by the proportion of female offspring (fertilized eggs) resulting from those matings. All females used were *Wolbachia* infected, eliminating the confounding effects of *Wolbachia*-induced incompatibility.

Over four 24 h periods, both infected (R511) and uninfected (R511-T) males were presented with 24 females with which to mate, eight females sequentially for 1 h each and 16 females in mass overnight. For each of the eight, 1 h parings per day, the pair was observed until copulation was seen. Following mating, each female was given two hosts for 48 h and then removed. Offspring were counted for three of the eight, 1 h matings.

Mating history

Wolbachia infected *N. vitripennis* males (R511) were repeatedly mated and periodically tested for CI. Males were alternately presented infected and uninfected females for 1 h each. Pairs were observed until mating was seen. The uninfected females were used to assay CI, the infected females were to determine whether sperm was still being transferred. Uninfected males (AsymC) were similarly treated. A total of five males of each type were used. Data presented as total sum of offspring by sex.

Results

Nasonia CI and spermatogenesis

Nasonia normally give rise to both fertilized (diploid) and unfertilized (haploid) offspring. CI is measured by the effect on offspring derived from fertilized eggs in an incompatible cross. In *N. vitripennis*, the result of CI is loss of paternal chromosomes and resulting haploid (male) development (Reed and Werren, 1995; Tram *et al*., 2006). In an incompatible cross (infected male × uninfected female), the result is an increase in male offspring. Consistent with previous results (Perrot-Minnot *et al*. 1996), the doubly infected males used in these experiments yielded nearly all male offspring (97.96% ± 0.11 s.e.m., $n = 19$) when mated to uninfected females.

Spermatogenesis in *Nasonia* shares many features with the well-characterized events in *Drosophila* (Fuller, 1993). Each male has two testes, composed of a single follicle. Within testes, sperm develop within cysts composed of two somatic cells (cyst cells) surrounding a number of interconnected germline cells (spermatocytes/spermatids). The two cyst cells form an envelope completely surrounding the germline component of the cyst, the sheath cells. With cyst development, the two cyst cells enlarge to accommodate the growing cyst, but do not further divide. Within the cyst interior, the germline spermatocyte undergoes several rounds of mitotic division, followed by meiosis. As male *Nasonia* are normally haploid, meiosis is composed of an aborted meiosis I, followed by normal meiosis II (Pennypacker, 1958) (Figure 1a). A notable and potentially important difference in spermatogenesis between *Nasonia* and *Drosophila* is the variation in the stages of development found within a testis. Spermatogenesis is largely synchronized in *Nasonia* (Pennypacker, 1958), probably due to the fact that most matings occur immediately after male eclosion (Whiting, 1967). Most of the cysts within an *N. vitripennis* testis are therefore of the same or similar developmental stage in maturing pupae (Figure 1b). Additionally, the cyst cells within a *Nasonia* testis contain giant polyploid nuclei (Figures 1a and g). The implications of these differences on the expression of CI are discussed below.

Wolbachia can be seen throughout the whole testis in *N. vitripennis* (Figure 1b). The exact localization of *Wolbachia*, however, is not evident in the initial observation, necessitating closer examination of individual developing cysts. In addition to the *Wolbachia* found within the outer testis sheath, *Wolbachia* were frequently seen within both germline (spermatocytes/spermatids) and soma (cyst cells) within individual cysts, but at densities much lower than normally observed in *Drosophila* melanogaster (Clark *et al*., 2002). Hundreds of cysts from many different testes were examined. Figure 1 shows some of the characteristic cyst infections. *Wolbachia* levels within cysts increases with cyst development (compare Figures 1c–h). Although the level of *Wolbachia* within cysts was highly variable, *Wolbachia* were seen in nearly every developing cyst. Accurately scoring the infection status of individual whole cysts was unattainable because most cysts removed from a testis do not remain intact, especially cyst cells. In contrast to cysts, *Wolbachia* were not found within the majority of developing spermatocytes and spermatids. Specifically, scoring of infections in premeiotic spermatocytes shows that only 28% ($N > 1500$) of spermatocytes at this stage were infected with *Wolbachia*. Although impossible to quantify later in development due to the difficulty in separating bundles of spermatids, this infection frequency in spermatocytes is consistent with that observed in more developed spermatids (Figure 1). Therefore, only a minority of developing sperm is infected with *Wolbachia*. However, based on complete CI in crosses between infected males and uninfected females, compared to the control infected

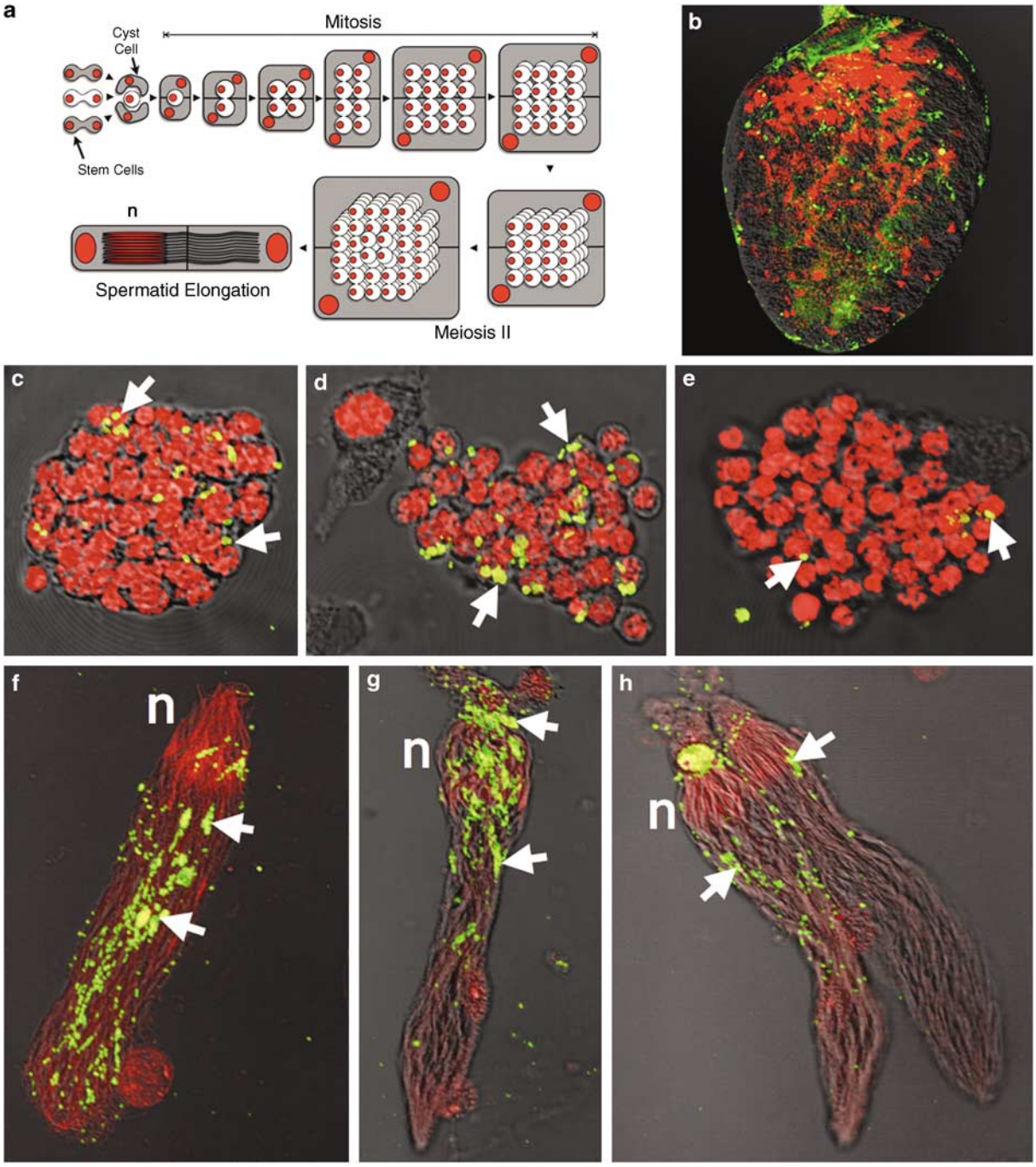

Figure 1 *Wolbachia* and spermatogenesis in *N. vitripennis*. (**a**) Schematic diagram of spermatogenesis in *Nasonia*. Spermatogenesis begins with a spermatogonial stem cell dividing and forming a daughter primary gonial cell. Cyst progenitor (stem) cells are divided to form cyst cells, two of which surround a primary gonial cell. The primary gonial cell undergoes several rounds of mitosis, aborted meiosis I and normal meiosis II. The haploid spermatids then elongate with the growth of the sperm tails. The germ cells are shown in white, somatic cells are shown in gray. (**b**) *Wolbachia* (green/yellow) within a whole testis from a late stage pupa. Clusters of red nuclei are cysts in the latter stages of development. (**c–e**) *Wolbachia* within premeiotic spermatocysts. (**f–h**) *Wolbachia* within elongated spermatocysts. Arrows indicate *Wolbachia*. n = spermatid nuclei. See online version for colour figure.

male × infected female and uninfected male × infected female crosses, nearly 100% of sperm are modified. In this study, CI was estimated at 97.96% consistent with previous reports of complete or near complete CI (Perrot-Minnot *et al*., 1996). Yet only around 28% of developing spermatocytes carry *Wolbachia*, suggesting that the bacteria do not need to be in the developing spermatocytes and spermatids to induce sperm modification.

An alternative explanation is that *Wolbachia* only modify those developing sperm, which harbor *Wolbachia* during development, and these sperm are used exclusively or disproportionately for fertilization, whereas the majority of sperm from uninfected spermatocytes are unutilized or nonfunctional. If the latter were true, and *Wolbachia* infected males utilize only those sperm infected during development, then infected males

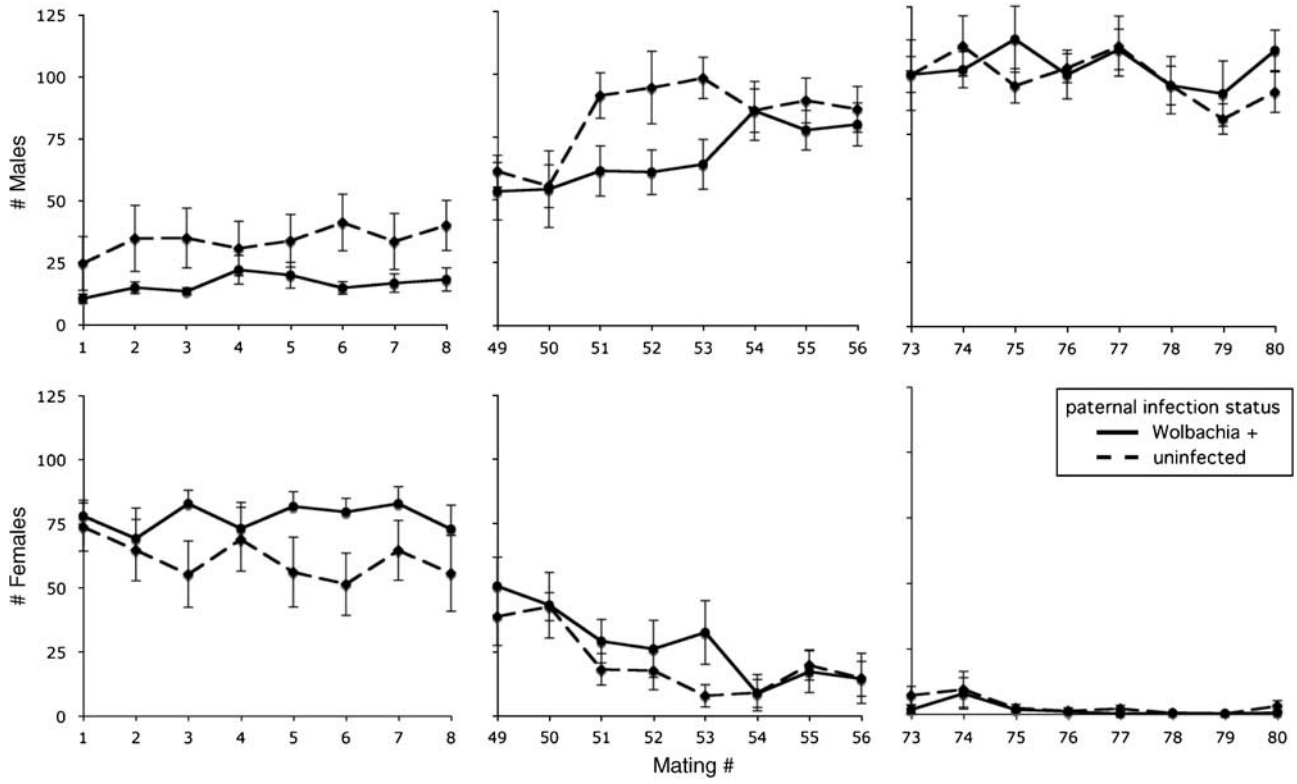

Figure 2 The effect of paternal infection status and successive mating on the number of male and female progeny. All females were *Wolbachia* infected, eliminating cytoplasmic incompatibility. Error bars indicate standard errors.

should suffer a great reduction in fertility compared to uninfected males. To distinguish between these possibilities, fertility of infected and uninfected males was compared by repeatedly mating to infected females. Fertility (total usable sperm) was estimated by counting the numbers of female offspring resulting from each mating. Each of the 10 infected males tested lived long enough to be presented with at least 80 females (one uninfected male died between female numbers 78 and 79; Figure 2). The surviving males continued to copulate with each female observed. For each mating examined (mating numbers 1–8, 49–56 and 73–80), there was no significant difference in the number of female offspring (Mann–Whitney *U*-test, $P > 0.05$). There was a significant difference in the number of male offspring in only three of the 24 matings examined number 6, $P = 0.0412$; number 8, $P = 0.0411$ and number 53, $P = 0.0211$). In each of these three crosses, there were more male offspring from females mated to uninfected versus infected males. By mating number 75, both infected and uninfected males sired few or no female offspring, suggesting that sperm was completely or nearly depleted at that time. In addition to the three crosses with significant differences in the number of male offspring, there was a consistent trend (although not statistically significant at any single time period) toward fewer female offspring from females mated to uninfected versus infected males, the opposite trend to that expected if infected males produced large numbers of nonfunctional sperm. These results argue against the hypothesis that uninfected spermatocytes from infected males are nonfunctional.

Furthermore, no reduction in CI was observed regardless of numbers of previous matings. Among 84 different pairings, infected males were assayed for CI at 13 different times. The result was always 100% male offspring. Therefore, CI in *Nasonia* is not reduced with increased numbers of male mating (Figure 3).

Chelymorpha CI and spermatogenesis

Cytoplasmic incompatibility levels were examined for both Gamboa double-infected males and Guaramal single-infected males crossed to uninfected Gamboa females, at male age of 4 weeks. After CI measurement, the males were used for cytological examinations. Gamboa males crossed to uninfected females showed 75.9% incompatibility ($N = 5$, range: 63.4–90.5%), indicating that approximately 75.8% of the sperm were sufficiently modified to cause CI. These same males were dissected upon mating and used for cytological examinations. Guarumal males crossed to uninfected females gave incompatibility of 67.2% ($N = 6$, range: 33.3–87.4%).

As in *Nasonia* and *Drosophila*, the *Chelymorpha* testis is composed of developing cysts surrounded by an outer epithelial sheath cells. The cyst consists of two cell types, the germ cells (spermatocytes that develop into spermatids) surrounded by two somatic cyst cells. Each region was examined for *Wolbachia*. In contrast to *Drosophila* and *Nasonia*, spermatogenesis in *Chelymorpha* occurs within two disc-shaped testes composed of multiple follicles. Each follicle is homologous to a single testis from *Drosophila* or *Nasonia*. Within each follicle, developing sperm can be seen within cysts of various stages of development with the most immature stages toward one end and mature sperm toward the other end of the

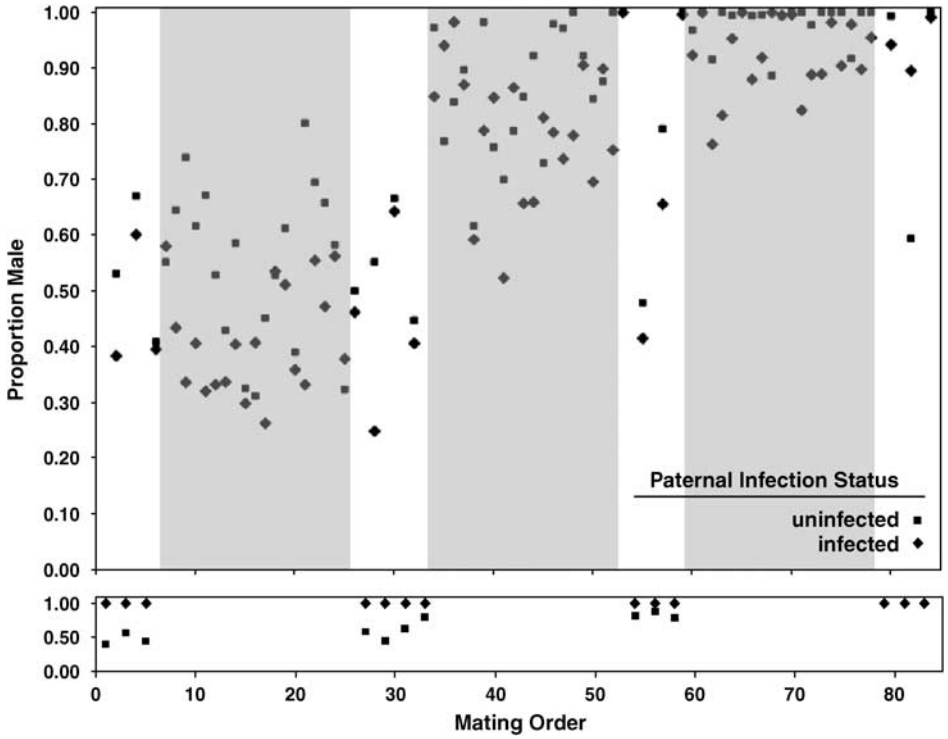

Figure 3 Sperm depletion, sex ratio and CI in *N. vitripennis*. *Wolbachia*-infected and uninfected males mated to *Wolbachia*-infected (top) and -uninfected (bottom) females. Matings within the shaded area are overnight mass matings with multiple females, and precise mating order within these time periods is unknown.

follicle. In contrast to *Drosophila*, *Wolbachia* were never seen within the testis follicle interior (that is, within cysts), but were abundant in the outer testis sheath (Figures 4b–e). Therefore, *Wolbachia* are present neither in developing spermatocytes nor in the somatic cyst cells. Over 80 cysts were examined in detail after removal from testes of four different males and confirmed the absence of *Wolbachia* within these cysts.

Despite that *Wolbachia* are not present in cysts of *C. alternans*, the same males for which testes were examined showed ~75.8 and 67.2% CI in the two lines examined. Therefore, we conclude that *Wolbachia* are not required within cysts (either spermatocytes and spermatids or somatic cyst cells) to affect sperm modification in this species.

Discussion

Wolbachia cause CI by the modification of sperm through as yet unknown mechanisms. Previous work on the distribution and proliferation of *Wolbachia* during spermatogenesis in *Drosophila* suggested that *Wolbachia* are required within developing spermatocytes/spermatids to cause modification (Clark *et al*., 2003). This was based on the observation that *Wolbachia* are abundant in spermatocytes of young males, and both proportion of infected cysts and CI level decline with age. The results of our work, however, caution against generalizing results from *Drosophila* to other taxa, and further suggest that the conclusion that *Wolbachia* are required in spermatocytes of *Drosophila* to affect CI needs to be reexamined. The positive correlation between CI level and the proportion of developing sperm infected with *Wolbachia* in *Drosophila* may be because *Wolbachia* only modify spermatids if infected, or alternately, both CI level and proportion of spermatids infected may both be dependent on *Wolbachia* densities earlier in development (spermatogonial stem cells, pole cells and so on) where modification is actually taking place. Recently Riparbelli *et al*. (2007) have described defects in sperm development within the testes of infected but not uninfected males. These defects, which include abnormal axoneme and mitochondria, were not restricted to infected cysts, suggesting that uninfected cysts can be affected by *Wolbachia*. What connection, if any, these sperm defects have on the expression of CI is currently unknown. (Riparbelli *et al*., 2007). Certainly, the data presented here shows that *Wolbachia* are not needed in developing sperm to cause sperm modification in insect species from two different insect orders.

These results suggest several alternative hypotheses for induction of sperm modification by *Wolbachia*. First, *Wolbachia* may produce a factor in sheath (*Nasonia* and *Chelymorpha*) or somatic cyst cells (*Nasonia*) that are passed across membranes into developing spermatids. For example, *Wolbachia* proteins could be passed from the sheath cells to the developing spermatids using a pathway similar to the one found in *Heliothis virescens*, where sheath cells synthesize proteins, which are imported by cyst cells by pinocytosis of testicular fluid and transfered to spermatids (Miller *et al*., 1990). Alternatively, *Wolbachia* within the somatic cyst cells (*Nasonia*) and/or sheath cells (*Nasonia* and *Chelymorpha*) could alter expression and synthesis of gene products from the host, thus changing the products exported to the developing spermatids, or by other inductive effects of these cells on developing spermatids. A third possibility is that *Wolbachia* affect modification very early

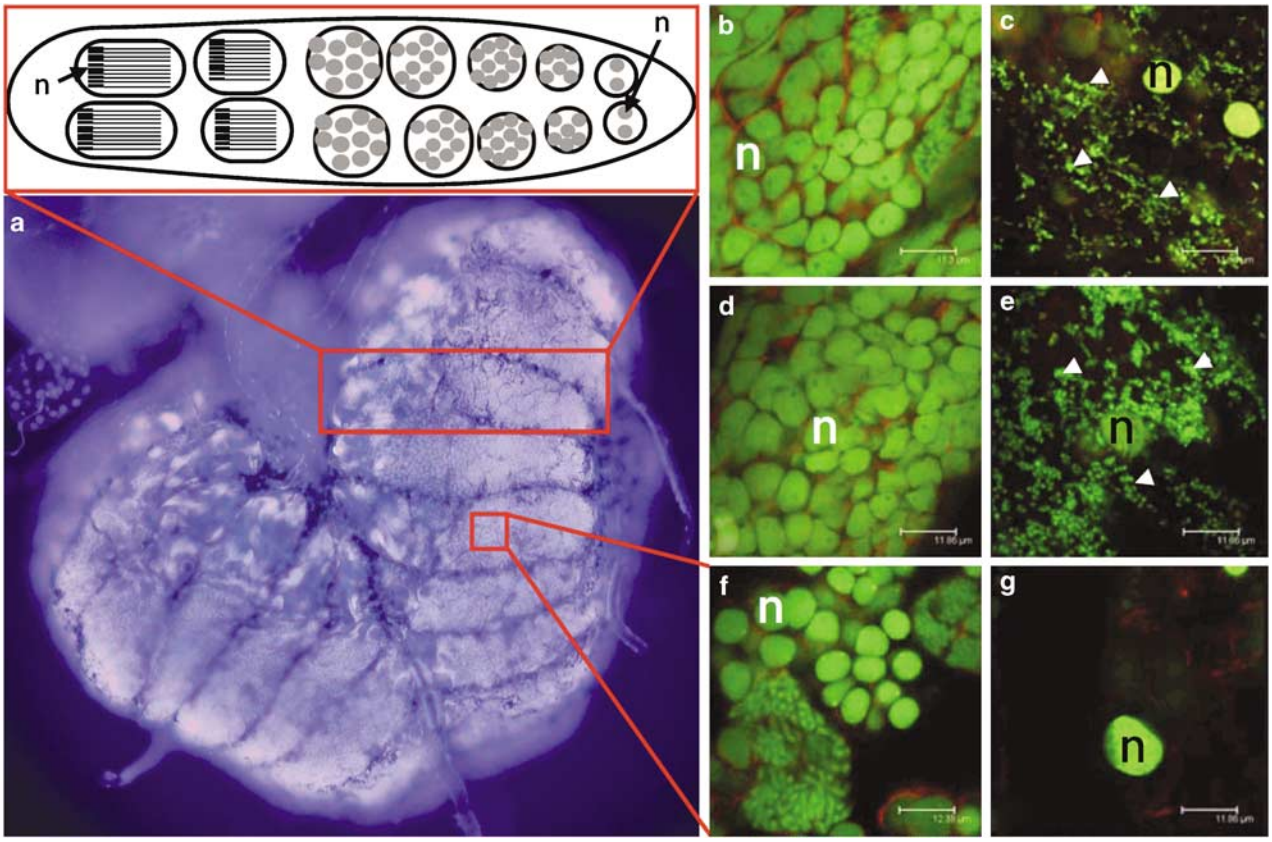

Figure 4 Spermatogenesis and *Wolbachia* in *Chelymorpha alternans*. (**a**) A pair of testes is shown for reference. The schematic (top) shows a single testis follicle with developing sperm cysts within. Development within the follicle outlined proceeds from right to left, with less developed cysts to the right and spermatids to the left. The square indicated approximate location of confocal sections (**b**–**g**). *Wolbachia* are not observed within spermatocytes (germline) from within a testis follicle from testes of 4-week-old (**b**) double- infected, (**d**) single-infected and (**f**) uninfected *C. alternans*. *Wolbachia* (arrow head) is observed only in the outer testis sheath, at the surface of the testes of 4-week-old (**c**) double-infected, (**e**) single-infected but not from (**g**) uninfected *C. alternans*. DNA is stained with DAPI (**a**) or Oligreen (**b**–**g**), host actin (red) is labeled with phalloidin (**b**–**g**). n = host nuclei.

in the primordial germ cells, and *Wolbachia* are subsequently lost. For example, in *Nasonia*, *Wolbachia* are found in about 28% of spermatocytes. However, spermatocytes are connected by cytoplasmic bridges within individual cysts. Although difficult to quantify precisely, it appears that nearly all spermatocyte syncytia had at least one *Wolbachia*, suggesting that most primordial germ cells also had at least one *Wolbachia* cell. Consistent with 100% transmission of *Wolbachia* through the germline, an examination of pole cells in *Nasonia* embryos indicates that all or nearly all are infected with *Wolbachia* (data not shown). In the case of *Chelymorpha*, *Wolbachia* are totally absent from both spermatocytes/spermatids and somatic cyst cells in adult beetles. However, it is still possible that all pole cells were infected earlier in the development and *Wolbachia* were subsequently lost in germline daughter cells. Transmission of *Wolbachia* through the female germline is not complete in *C. alternans* (Keller *et al*., 2004), suggesting that not all pole cells were infected. Work in *Drosophila* has shown a positive correlation between the density of *Wolbachia* within pole cells and CI levels (Veneti *et al*., 2004). This may support *Wolbachia*-induced modification of pole cells or it may simply be a predictor of *Wolbachia* titer later in gemetogenesis when modification occurs. Modification could also occur within the spermatogonial stem cells within testes. Examination of sperm stem cells in *Nasonia* and *Chelymorpha* would be challenging, as markers for the stem cell niche have not been described in either species.

What molecules, if any, *Wolbachia* are secreting into hosts is currently unknown. *Wolbachia* does possess a type IV secretion system, likely used for exporting molecules into host cells (Masui *et al*., 2000). It is currently known that *Wolbachia* produce bacteria phage, which can be found in developing sperm (Bordenstein *et al*., 2006). What effect *Wolbachia* phage has on host cells or the role in CI is currently unknown.

There is very little current data in any species concerning the timing of sperm modification during spermatogenesis. The only relevant experiment is in *Drosophila simulans* where heat–shock treatment of male third instar larvae results in a decrease in CI; at this stage, the most mature sperm cysts typically are beginning spermatid elongation (Snook *et al*., 2000). The experiment may suggest that modification does not occur in embryonic stem cells, but other effects of heat–shock on CI could also explain the results, and no apparent reductions in bacterial infection levels were observed. Clearly, more experiments on timing of sperm modification are needed.

In *Drosophila*, rates of CI have been shown to be dependent on a number of factors, including male age and male mating history (Turelli and Hoffmann, 1995;

Clancy and Hoffmann, 1998; Karr *et al*., 1998; Reynolds *et al*., 2003). With subsequent matings, the rate of CI goes down dramatically in *D. simulans* (Karr *et al*., 1998). In *N. vitripennis*, there is no evidence of either a male age effect, or male mating history effect on CI (Figure 3). The male age and mating history effect in *Drosophila* could be explained by the depletion of *Wolbachia* from the male germline, likely spermatogonial stem cells (Clark *et al*., 2003). By this model, early developing sperm cysts contain *Wolbachia* and as a result are modified. With the depletion of *Wolbachia*, sperm cysts produced later in life lack *Wolbachia* and are not modified. Successive copulation of infected males depletes the older (modified) sperm and increases the proportion of newly matured (unmodified) sperm. Later copulations, therefore, have a higher proportion of unmodified sperm compared to similarly aged virgin males. The lack of decrease in CI in *N. vitripennis* with male age and successive copulation may either be due to the timing of spermatogenesis in *Nasonia* or due to fundamental differences in the modification of sperm by *Wolbachia*. In *Nasonia*, most of the lifetime sperm production is completed at or near the time of eclosion, before mating. (Pennypacker, 1958). Therefore, the population of sperm available at the first mating is the same as in subsequent matings.

As the molecular mechanism(s) resulting in *Wolbachia*-induced sperm modification remain elusive, different host taxa such as *Nasonia* and *Chelymorpha* provide excellent model systems to compliment studies in *Drosophila*. In *Nasonia*, we show that *Wolbachia* are able to modify a sperm, while not being present within an individual spermatocyte or spermatid during sperm development. In *Chelymorpha*, *Wolbachia* are able to modify sperm, while never being present in cysts.

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