

ORIGINAL ARTICLE

Diploid males sire triploid daughters and sons in the parasitoid wasp *Cotesia vestalis*JG de Boer¹, PJ Ode², LEM Vet^{3,4}, JB Whitfield⁵ and GE Heimpel¹¹Department of Entomology, University of Minnesota, St Paul, MN, USA; ²Department of Entomology, North Dakota State University, Fargo, ND, USA; ³Netherlands Institute of Ecology (NIOO-KNAW), Nieuwersluis, The Netherlands; ⁴Laboratory of Entomology, Wageningen University, Wageningen, The Netherlands and ⁵Department of Entomology, University of Illinois, Urbana, IL, USA

In the Hymenoptera, males develop as haploids from unfertilized eggs and females develop as diploids from fertilized eggs. In species with complementary sex determination (CSD), however, diploid males develop from zygotes that are homozygous at a highly polymorphic sex locus or loci. We investigated mating behavior and reproduction of diploid males of the parasitoid wasp *Cotesia vestalis* (*C. plutellae*), for which we recently demonstrated CSD. We show that the behavior of diploid males of *C. vestalis* is similar to that of haploid males, when measured as the proportion of males that display wing fanning, and the proportion of males that mount a female. Approximately 29% of diploid males sired daughters, showing their ability to produce viable sperm that can fertilize eggs. Females mated to diploid males produced all-male offspring more frequently

(71%) than females mated to haploid males (27%). Daughter-producing females that had mated to diploid males produced more male-biased sex ratios than females mated to haploid males. All daughters of diploid males were triploid and sterile. Three triploid sons were also found among the offspring of diploid males. It has been suggested that this scenario, that is, diploid males mating with females and constraining them to the production of haploid sons, has a large negative impact on population growth rate and secondary sex ratio. Selection for adaptations to reduce diploid male production in natural populations is therefore likely to be strong. We discuss different scenarios that may reduce the sex determination load in *C. vestalis*.

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Introduction

In the insect order Hymenoptera, males typically develop from unfertilized haploid eggs, and females from fertilized diploid eggs. However, diploid males can occur as a result of complementary sex determination (CSD) in which diploid offspring develop as males when they are homozygous at a sex locus, and as females when they are heterozygous at this locus (for example, Cook and Crozier, 1995; Beye *et al.*, 2003; Wu *et al.*, 2003; van Wilgenburg *et al.*, 2006). The frequency of diploid males in a population depends on the level of inbreeding and on the diversity of sex alleles. Sex allele diversity has been estimated for a few species; examples are 15–86 sex alleles in the fire ant *Solenopsis invicta* (Ross and Fletcher, 1985; Ross *et al.*, 1993), 19 in the honeybee *Apis mellifera* (Adams *et al.*, 1977; see also Hasselmann and Beye, 2004) and 9–20 in the parasitoid wasp *Habrobracon hebetor* (Whiting, 1943; Heimpel *et al.*, 1999; Antolin *et al.*, 2003). Because sex allele diversity is high and species with CSD may avoid inbreeding (Ode *et al.*, 1995), diploid male

frequencies in the field are generally low (for example, Owen and Packer, 1994; Takahashi *et al.*, 2001). Yet to date, diploid males have been reported in more than 60 species (van Wilgenburg *et al.*, 2006).

Diploid males can differ from haploid males in several characteristics. In some species, diploid males are larger than haploid males, for example in the sawflies *Athalia rosae* and *Neodiprion nigroscutum* (Smith and Wallace, 1971; Naito and Suzuki, 1991). In other species, diploid males may be smaller than haploid males, but this has only been reported for the bumblebee *Bombus terrestris* (Duchateau and Marien, 1995). Diploid males may have lower survival rates than haploid males and females. For example, viability of diploid males is generally less than 5% in the parasitoid wasp *H. hebetor*, and most of them die during the egg stage (Whiting, 1943; Petters and Mettus, 1980). However, diploid male survival can vary widely, even between closely related species, and is much higher in *H. brevicornis*, *H. serinopae* and *H. sp.* near *hebetor* (Speicher and Speicher, 1940; Clark *et al.*, 1963; Holloway *et al.*, 1999). In the honeybee *A. mellifera*, workers actively kill and remove diploid males as early larvae (Woyke, 1963).

When diploid males survive, three important questions arise: Do diploid males mate with females? If so, what are the consequences of such matings? And, what is the relative reproductive success of diploid males compared to haploid males? Reproduction by diploid

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males has been studied in a range of hymenopteran species, including parasitoid wasps, ants and bumblebees. The results of these studies can be classified into three groups: (1) diploid males do not mate with females (for example, the conifer sawfly *N. nigroscutum*: Smith and Wallace, 1971); (2) diploid males mate with females but are sterile, therefore restricting females to the production of haploid offspring only (for example, *H. sp. near hebetor*: Holloway *et al.*, 1999); (3) diploid males mate with females and produce sterile triploid daughters and sometimes sons (for example, *Polistes* spp: Liebert *et al.*, 2005). In all these cases, diploid males do not produce any fertile offspring and it has thus generally been accepted that diploid males are a reproductive dead end in species with CSD. However, Cowan and Stahlhut (2004) recently showed that diploid males of the vespid wasp *Euodynerus foraminatus* are fertile. They produce diploid female offspring when crossed with an unrelated diploid female, possibly because the reduction division during spermatogenesis is restored. Their study thus added a fourth possible outcome of interactions between diploid males and females, and a cautionary note about generalizing the reproductive fate of diploid males without doing experiments. Stouthamer *et al.* (1992) used simulations to show that the negative impact of diploid male production on sex ratio and population growth rate is the strongest when diploid males survive and mate, constraining females to produce only haploid sons. The population-level consequences of CSD thus depend on the outcome of interactions between diploid males and females, warranting studies on these interactions in any species with CSD.

The genus *Cotesia* (Braconidae: Microgastrinae) is a valuable system to study the relationships between sex determination, life history traits and behavior. Interestingly, this large genus of parasitoids comprises species with and species without CSD. Niyibigira *et al.* (2004a) demonstrated the absence of CSD in the gregarious stemborer parasitoid *C. flavipes*, and sex ratio data from field populations suggest CSD is very unlikely in *C. sesamiae* (Niyibigira *et al.*, 2004b). However, CSD was recently demonstrated in *C. glomerata* (Gu and Dorn, 2003; Zhou *et al.*, 2006), and it has been suggested to operate in *C. rubecula* (personal communication by Steiner in Stouthamer *et al.*, 1992; de Boer unpublished). In addition, we have also demonstrated CSD in *C. vestalis*, a solitary endoparasitoid of diamond back moth larvae (de Boer *et al.*, 2007). Diploid male survival in *C. glomerata* is estimated to be rather low (around 26%), whereas it appears to be relatively high in *C. vestalis* (approximately 70%). To date, the behavior and reproductive function of diploid males have not been investigated in any *Cotesia* species. Here we study the mating behavior of diploid males of *C. vestalis* and describe their offspring. The species of *Cotesia* we study here has commonly been called *Cotesia* (or formerly *Apanteles*) *plutellae* Kurdjumov in the literature, but now should be referred to as *C. vestalis* (Haliday) (Shaw, 2003).

Methods

Source material

To study the behavior of and reproduction by diploid *C. vestalis* males, we used offspring from iso-female lines

that were known to produce both haploid and diploid males (de Boer *et al.*, in preparation). These lines were set up to determine the number of loci on which CSD is based in *C. vestalis*. In short, iso-female lines were started with a mother–son cross (see de Boer *et al.*, 2007), using female offspring of outcrosses between two unrelated colonies of *C. vestalis*: colony A was started with about 200 adult wasps obtained from Biofac Crop Care (Mathis, TX, USA) and colony B was started with about 90 adult wasps from Benin (supplied by Dr D Bordat, CIRAD, Montpellier-sur-Lez, France). Iso-female lines were maintained on diamond back moth larvae, *Plutella xylostella* L., for 6–8 generations of sibmating to ensure the production of diploid males before they were used in our experiment. In these generations, most lines produced between 30 and 50% diploid males from fertilized offspring (de Boer *et al.*, in preparation).

Reproduction by diploid males

Newly emerged female offspring from the lines described above were paired with a brother in a plastic tube containing a piece of host-damaged cabbage leaf and a small droplet of honey. Observations were made without knowledge of the male's ploidy, which was determined afterwards (see below). We observed behavior of the pair of wasps for 10 min, recording the display of wing fanning, mounting attempts (when males approached females but the female walked away) and the occurrence of successful mounting (defined by the male staying in contact with the female for at least 30 s).

After observations, we left all pairs together for 48 h at $23 \pm 1^\circ\text{C}$ to allow mating if it had not yet occurred. Males were then frozen at -30°C for analysis of ploidy. The female was exposed to approximately 15–20 second and third instar diamondback moth larvae on cabbage in a plastic tube (2.5 cm diameter, 6.5 cm high) for 24 h. This was repeated once more for a total of 29–42 hosts for each female. Hosts were reared individually on artificial diet (modified from Shelton *et al.*, 1991) in 1 oz clear cups in a growth chamber at $25 \pm 1^\circ\text{C}$ until parasitoid cocoons or moth pupae developed, or hosts died. Cocoons were individually placed in microcentrifuge tubes. Emerging wasps were sexed and frozen for analysis of ploidy, with the exception of 12 daughters of diploid males (see below).

A total of 64 crosses was made, of which 33 and 29 were initiated with a haploid and a diploid male respectively, while the ploidy of two males could not be determined. One female crossed with a diploid male died before she was offered any hosts, so only behavioral data were recorded for this replicate.

After identification of their ploidy, a subset of 10 haploid and 10 diploid males was dissected to determine whether their internal reproductive organs were different. Wasps were dissected in water by pulling out the reproductive tract with an insect pin; the reproductive organs were then transferred to a microscope slide. Reproductive organs were measured under a compound microscope at $\times 100$, and the presence of sperm was determined at $\times 1000$. The presence of sperm in female spermathecae was not determined.

Statistical analyses: We used 2×2 contingency tables to analyze the effect of male ploidy on the occurrence of wing fanning, approaching and mounting, and on

mating success (that is, pairs that produced female offspring; pairs without daughters were thought to have had no successful copulation). For the pairs that produced daughters, we statistically analyzed the effect of male ploidy on sex ratio with a logistic regression treating sex ratio as a binary response variable using PROC LOGISTIC in SAS (SAS Institute Inc., 2006). We analyzed the effect of male ploidy and whether daughters were produced on hosts by using a generalized logits model (PROC LOGISTIC, SAS Institute Inc., 2006) to compare the proportion of hosts that died, developed into moths, or developed into cocoons.

Reproduction by triploid females

Twelve virgin daughters of diploid males were exposed to 11–21 hosts each by placing them individually in a plastic tube with a piece of cabbage leaf and a small droplet of honey for 24 h. The hosts were reared as described above. Females were subsequently transferred to a new tube with host-damaged cabbage and a haploid male from an unrelated inbred line (males were known to be haploid because they were produced by a virgin diploid female). They were kept together for 48 h to allow mating. Females were then exposed to another set of 16–23 hosts for 24 h. Females were frozen for analysis of ploidy and for dissections to determine whether they contained any mature eggs. In this way, reproduction by virgin and mated triploid females was tested.

Statistical analysis: We analyzed the effect of triploid females on their hosts by using a generalized logits model (PROC LOGISTIC, SAS Institute Inc., 2006) to compare the proportion of hosts that died, developed into moths or developed into cocoons, with a set of control hosts. The controls consisted of hosts that were

treated in the same way but were not exposed to a *C. vestalis* female. The two sets of hosts offered to each triploid female (before and after mating) were combined because we found no differences between the two sets in the number of offspring produced or the proportion of hosts that died (*t*-tests, $P > 0.05$).

Flow cytometry

We used flow cytometry to identify the ploidy of *C. vestalis* wasps. Samples were prepared by pulverizing the head (without antennae) of an individual wasp in 0.5 ml ice-cold Galbraith buffer (21 mM MgCl₂; 30 mM tri-sodium citrate dehydrate; 20 mM 3-[*N*-morpholino] propane sulfonic acid; 0.1% Triton X-100; 1 mg/l RNase A; Galbraith *et al.*, 1983) in a Dounce tissue grinder (Kontes, Vineland, NJ, USA) by turning a B pestle 20 times. The homogenate was subsequently filtered through a cell strainer cap on a 5 ml polystyrene tube (Falcon, BD Biosciences, San Jose, CA, USA). Nuclei were stained with 20 μ l propidium iodide (1.25 mg/ml) per sample. Flow cytometric analyses were performed on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). An excitation wave length of 488 nm and a band pass filter of 585 nm were used to detect propidium iodide fluorescence. For each sample, 2500 nuclei were measured in an FL2-W/FL2-A gated region that contained haploid, diploid and tetraploid cells, using CELL Quest Pro. A threshold on FL2-A was used to exclude very small debris. We used flow cytometric DNA-histograms of known haploid males and diploid females as references to determine ploidy of unknown males and females (Figure 1).

We analyzed the ploidy of (1) all 64 males used as fathers in the behavior experiment, (2) all 32 daughters of diploid males and (3) 32 sons of females mated to diploid males.

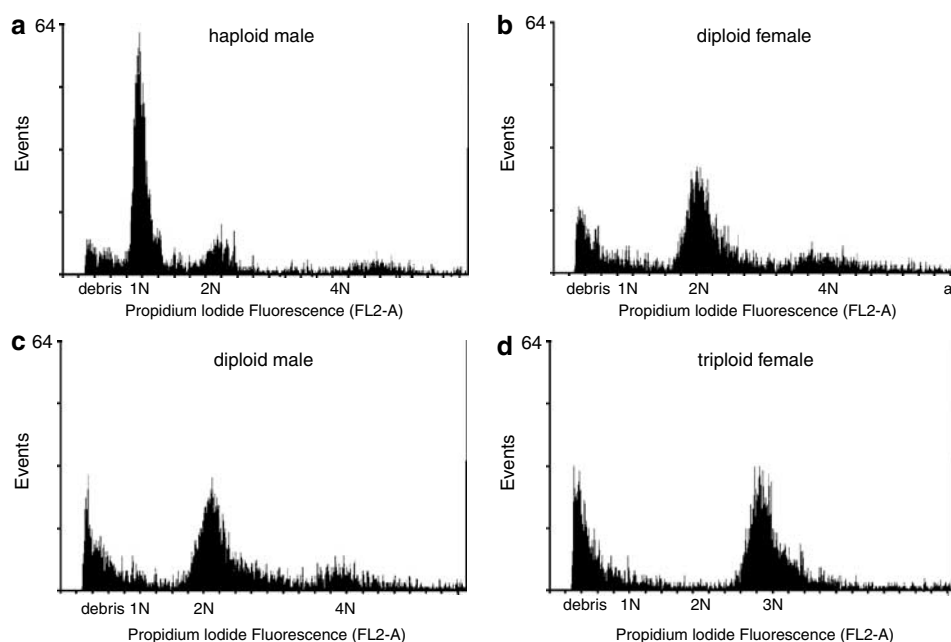


Figure 1 Flow cytometric DNA-histograms of a representative haploid male (a), diploid female (b), diploid male (c), and triploid female (d). Small peaks of polyploid cells are also visible in each histogram, for example diploid and tetraploid cells in the histogram of the haploid male (panel a).

Results

Behavior of diploid males

Wing fanning was observed in 19 out of 33 haploid males and 23 out of 29 diploid males (Figure 2) (2×2 contingency table, $\chi^2_1 = 2.95$, $P = 0.09$). Male ploidy did not affect the number of males that approached a female (followed by the female walking away); this was observed in 8 out of 33 haploid males and in 12 out of 29 diploid males (2×2 contingency table, $\chi^2_1 = 2.07$, $P = 0.15$). Diploid males were also observed mounting females as often as haploid males: seven out of 33 haploid males, and 12 out of 29 diploid males (2×2 contingency table, $\chi^2_1 = 3.34$, $P = 0.07$). Diploid males thus appear to display normal mating behavior, and females appear to readily accept diploid males as mates.

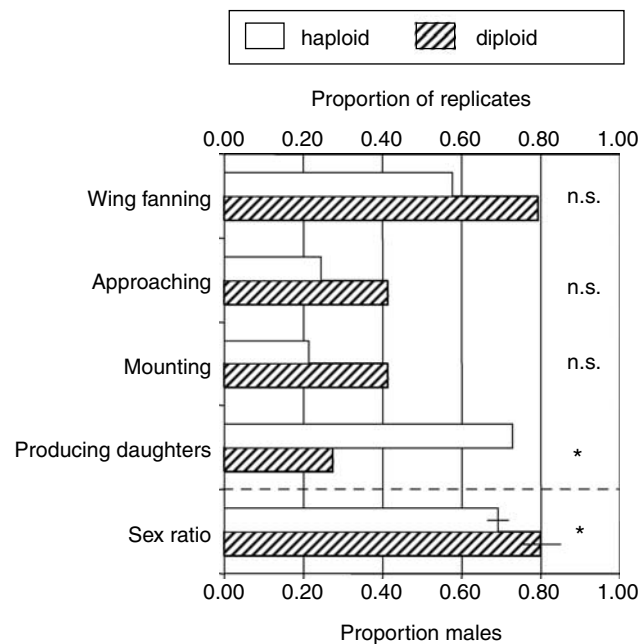


Figure 2 Proportion of haploid (open, $N = 33$) and diploid males (hatched bars, $N = 29$) that displayed wing fanning, approached a female or mounted a female during a 10 min observation period, proportion of females mated to a haploid or diploid male that produced daughters and sex ratio of offspring of those females that produced at least one daughter (average \pm s.e.m.) are shown. Proportions of replicates for each parameter were analyzed with 2×2 contingency tables (NS $P > 0.05$; * $P < 0.05$); sex ratio was analyzed with logistic regression (* $P < 0.05$).

Reproduction by diploid males

Even though mating behavior of diploid males was not different from that of haploid males, the proportion of replicates without daughters was significantly higher for diploid males: only 8 out of 28 replicates with a diploid male produced female offspring, while 24 out of 33 replicates with a haploid male produced daughters (Figure 2) (2×2 contingency table, $\chi^2_1 = 11.8$, $P < 0.001$). Of those replicates for which mounting was observed during the first 10 min, 1 out of 7 involving a haploid male did not result in female offspring, while 6 out of 11 replicates involving a diploid male did not produce any daughters (2×2 contingency table, $\chi^2_1 = 2.9$, $P = 0.09$).

Of the pairs producing daughters, the adult sex ratio was male-biased for replicates with haploid male fathers (69%) as well as for those with diploid male fathers (80%) (Figure 2). Females mated to diploid males were 1.76 times more likely to produce male offspring than females mated to haploid males (Wald $\chi^2_1 = 6.61$; $P = 0.01$). However, the proportion of hosts that developed into moths, died or developed into parasitoid wasps was not significantly related to the ploidy of the father nor to whether or not daughters were produced (Table 1; $P > 0.05$ in all cases).

Eight diploid males sired 32 daughters, of which 29 could be analyzed for ploidy. All daughters of diploid males proved to be triploid (Figure 1). We analyzed the ploidy of the male offspring from the three replicates that had the highest number of daughters. In total, 32 males were analyzed: 27 were haploid and 3 were triploid (2 samples failed to show clear peaks on the flow cytometric DNA-histograms and were recorded as unknown). All three triploid males were found in the offspring of one replicate.

The internal reproductive organs did not differ visibly between haploid and diploid males and testes length was the same for haploid ($246.24 \pm 10.65 \mu\text{m}$) and diploid males ($243.39 \pm 9.04 \mu\text{m}$). Sperm was observed in the testes of most haploid (8/10) and diploid males (9/10), including five diploid males that did not sire any daughters.

Reproduction by triploid females

Even though we observed triploid females stinging their hosts, they did not reproduce (Table 2). Only one cocoon developed from the hosts exposed to triploid females. This cocoon developed from a host exposed to a mated female, and it developed into a diploid male. Flow cytometry cannot reveal whether this male was biparental or uniparental. However, the proportion of parasitoid cocoons produced was not significantly different

Table 1 Parameter values (average \pm s.e.m.) for pairs with a haploid (H) or diploid (D) father that did (+) or did not (–) produce female offspring

	H+ (n = 24)	H– (n = 7)	D+ (n = 8)	D– (n = 20)
Hosts exposed (N)	34.5 \pm 0.48	36.3 \pm 1.03	33.4 \pm 0.46	34.2 \pm 0.72
Hosts dead (prop.)	0.20 \pm 0.02	0.32 \pm 0.08	0.18 \pm 0.04	0.22 \pm 0.04
Pupated hosts (prop.)	0.08 \pm 0.01	0.12 \pm 0.04	0.11 \pm 0.06	0.08 \pm 0.02
Parasitoid cocoons (prop.)	0.70 \pm 0.03	0.54 \pm 0.09	0.70 \pm 0.10	0.68 \pm 0.05
Cocoons not emerged (prop.)	0.08 \pm 0.02	0.21 \pm 0.08	0.15 \pm 0.05	0.13 \pm 0.03

The effects of male ploidy and whether daughters were produced on hosts was analyzed with a generalized logits model to compare the proportion of hosts that died, developed into moths or developed into cocoons.

Table 2 Parameter values (average \pm s.e.m.) for pairs with a triploid female (T) and for control hosts that were not exposed to any parasitoid (C)

	T (n = 12)	C (n = 10)
Hosts exposed (N)	36.9 \pm 0.94	30.0 \pm 0.70
Hosts dead (prop.)	0.36 \pm 0.03	0.07 \pm 0.02
Pupated hosts (prop.)	0.62 \pm 0.04	0.90 \pm 0.02
Cocoons (prop.)	0.002 \pm 0.002	0.006 \pm 0.004

The effect of triploid females on their hosts was analyzed with a generalized logits model.

from that found in the set of control hosts, and so might have been caused by a low level of contamination of supposedly unparasitized hosts (Wald $\chi^2_1 = 0.33$; $P = 0.57$). Hosts exposed to a triploid female were 7.9 times more likely to die than control hosts that had not been exposed to wasps (Wald $\chi^2_1 = 67.22$; $P < 0.001$), suggesting that the females indeed stung some hosts, and possibly injected venom.

Dissections of the females showed that their ovaries developed normally and they contained mature eggs.

Discussion

Diploid males in *C. vestalis* appear to behave normally in interactions with females, displaying wing fanning and mounting females. Females also accept diploid males for mating. Similar findings have been reported in, for example, the parasitoid wasp *Diadromus pulchellus* (El Agoze et al., 1994) and the ant *Lasius sakagamii* (Yamauchi et al., 2001).

Even though diploid males courted and mounted females normally, only 29% of diploid males actually reproduced, in contrast to 73% for haploid males. In addition, females mated to diploid males produced a more male-biased sex ratio than those mated to haploid males. One possible explanation is that not all diploid males actually produce sperm. In several other species of Hymenoptera, male ploidy was found to affect the morphology of reproductive organs and/or sperm production. Tavares et al. (2003) showed that diploid male testes of the social bee *Melipona quadrifasciata* are significantly shorter than those of haploid males, but sperm production or reproduction by diploid males was not studied. Testes of diploid males are also smaller than those of haploid males in *A. mellifera* (Woyke, 1973, 1974) and *B. terrestris* (Duchateau and Marien, 1995). Hung et al. (1974) reported that in diploid males of *Solenopsis invicta*, development of the testicular lobes generally does not occur, and 98% of diploid males from a natural population were aspermic (Krieger et al., 1999). In contrast to these cases, the internal reproductive organs of diploid males of *C. vestalis* did not appear to differ from those of haploid males and sperm was observed in 9 out of 10 diploid males. Moreover, sperm was observed in five diploid males that did not sire any daughters. Potential explanations for the lack of reproduction by diploid males include a failure to transfer sperm to females during copulation, a failure of sperm to fertilize eggs or low viability of triploid zygotes (for example, El Agoze et al., 1994).

The triploid nature of the daughters of diploid males implies that diploid *C. vestalis* males produce diploid

sperm. Diploid males of the related *C. glomerata* also produce diploid sperm (Zhou et al., 2006) but it has not been determined whether females of this species mate with diploid males and produce triploid offspring. With the exception of *E. foraminatus* (Cowan and Stahlhut, 2004), diploid males of all species that have been studied so far produce diploid sperm and triploid progeny, if any (for example, Hoshiba et al., 1981; Chauvin et al., 1987; Yamauchi et al., 2001).

Triploid females of *C. vestalis* did not reproduce, also confirming findings for other Hymenoptera, such as *H. sp.* near *hebetor*, and *Polistes dominulus* (Holloway et al., 1999; Liebert et al., 2004). The triploid females did not differ morphologically from diploid females and their ovaries appeared normal. We also observed them stinging hosts and we expect they injected eggs. It seems likely that the eggs they produced were unviable aneuploids. In contrast, triploid females of the parasitoid wasp *Nasonia vitripennis* produce a significant proportion of viable eggs that commonly develop into haploid or diploid males (Whiting, 1960). Recently, Beukeboom and Kamping (2006) showed that unmated triploid females of *N. vitripennis* also occasionally produce uniparental diploid females or gynandromorphs. Whether or not triploid females produce any viable eggs may be related to the haploid chromosome number, which is much lower in *N. vitripennis* ($N = 5$) than in other hymenopterans studied so far, for example *B. terrestris* ($N = 18$) or *H. hebetor* ($N = 10$). A lower chromosome number may by chance result in a higher proportion of viable haploid or diploid eggs compared to inviable aneuploid eggs than a higher chromosome number. The haploid chromosome number of *C. vestalis* has not been determined, but the closely related *C. glomerata* has 10 chromosomes (Zhou et al., 2006).

Very few studies have reported triploid males among the offspring of diploid males crossed with related females. Under *sl*-CSD, triploid males are expected to occur as often as triploid females when parents share a sex allele. Although we believe that CSD in *C. vestalis* is based on more than one locus (de Boer et al., 2007), the wasps we used originated from lines that produced between 30 and 50% diploid males from fertilized eggs after inbreeding for 6–8 generations. We thus expected females mated to their diploid brothers to produce triploid sons and daughters in a 1:1 to 1:2.3 ratio. However, we only found three triploid males, compared to 21 females (ratio 1:7). Similar findings have been reported for the sawfly *Athalia rosae ruficornis*, where some females mated to diploid males produced triploid sons as well as daughters but others only produced triploid daughters (Naito and Suzuki, 1991). This may be explained by reduced survival of triploid males compared to triploid females, which has also been suggested for *H. hebetor* (Cook, 1993). To date, equal numbers of triploid males and females have only been reported for the bumblebee *Bombus terrestris* (Ayabe et al., 2004). We did not test the reproductive function of the triploid males, but we expect them to be sterile.

Progeny of diploid *C. vestalis* males are sterile triploid females, or triploid males. Diploid males are therefore an evolutionary dead end. According to model predictions, this scenario has the most negative effect on population growth rate and sex ratio (Stouthamer et al., 1992). We therefore expect selection to be strong on mechanisms or

behaviors that reduce diploid male frequencies, that is, the genetic load, in natural populations. There are several scenarios that may reduce the genetic load associated with CSD in *C. vestalis*. First, we suggest that CSD in *C. vestalis* is based on more than one locus (de Boer *et al.*, 2007; de Boer *et al.*, in preparation). Under multiple locus-CSD, diploid males only develop from fertilized eggs that are homozygous at each of two or more sex loci, and the frequency of diploid males is significantly lower than under sl-CSD (Crozier, 1971). Second, the inbreeding frequency in the field may be low, which would result in a low frequency of diploid males, especially under ml-CSD. *C. vestalis* is a solitary parasitoid on a solitary host, which should make outbreeding likely in this species, but the actual mating structure has not been characterized. Inbreeding frequencies in the related *C. glomerata* that also exhibits CSD have been shown to be lower than expected for gregarious species (Gu and Dorn, 2003). The pattern of diploid male production in this species is largely consistent with sl-CSD (Zhou *et al.*, 2006, 2007), but ml-CSD has not yet been ruled out (Gu and Dorn, 2003). In particular, the possibility that all but one locus in an ml-CSD system were fixed at the onset of these studies was not addressed in the studies on *C. glomerata*.

We showed that females accept diploid males as mates under laboratory conditions but the relative mating success of diploid and haploid males in the field is unknown. It is possible that diploid males achieve a smaller proportion of matings in the field than would be expected based on the relative occurrence of haploid and diploid males, for example through female choice, or reduced female searching or mating capacity of diploid males. Finally, after mating with a diploid male, females may remate with a haploid male. It would be important to determine whether this happens or not, and if it happens, whether haploid sperm has a competitive advantage over diploid sperm, or females can use it preferentially. Sperm precedence in double matings with one haploid and one diploid male has only been studied in one species with CSD, the ichneumonid parasitoid *D. pulchellus* (El Agoze *et al.*, 1995). Females that are mated to a diploid male are restricted to the production of haploid male offspring in *D. pulchellus* because eggs that are fertilized with diploid sperm die (El Agoze *et al.*, 1994). However, remating with a haploid male does not relieve females from this constraint because they will only use sperm of the first male they mated with.

In conclusion, our findings on the interactions between diploid males and females of *C. vestalis* confirm those of most previous studies on Hymenoptera with CSD: diploid males can mate but any offspring they get are sterile triploids. It thus appears that the findings of Cowan and Stahlhut (2004) of reproductively functional diploid males in *E. foraminatus* remain an exception.

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