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## ORIGINAL ARTICLE

# Sex determination meltdown upon biological control introduction of the parasitoid *Cotesia rubecula*?

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cabbage, complementary sex determination, diploid males, haplodiploidy, life history, natural enemies, parasitic wasps, *Pieris rapae*

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**Abstract**

Natural enemies may go through genetic bottlenecks during the process of biological control introductions. Such bottlenecks are expected to be particularly detrimental in parasitoid Hymenoptera that exhibit complementary sex determination (CSD). CSD is associated with a severe form of inbreeding depression because homozygosity at one or multiple sex loci leads to the production of diploid males that are typically unviable or sterile. We observed that diploid males occur at a relatively high rate (8–13% of diploid adults) in a field population of *Cotesia rubecula* in Minnesota, USA, where this parasitoid was introduced for biological control of the cabbage white *Pieris rapae*. However, our laboratory crosses suggest two-locus CSD in a native Dutch population of *C. rubecula* and moderately high diploid males survival (approximately 70%), a scenario expected to produce low proportions of diploid males. We also show that courtship behavior of diploid males is similar to that of haploid males, but females mated to diploid males produce only very few daughters that are triploid. We use our laboratory data to estimate sex allele diversity in the field population of *C. rubecula* and discuss the possibility of a sex determination meltdown from two-locus CSD to effective single-locus CSD during or after introduction.

**Introduction**

Natural enemies used for importation biological control are at risk of going through population bottlenecks during collection, culturing or establishment. These bottlenecks may be associated with reduced genetic variation and are expected to impair biological control efficacy (Hopper et al. 1993; Huffbauer and Roderick 2005). Reduced genetic variation is especially problematic in parasitoids in the insect order Hymenoptera because of the production of diploid males (Stouthamer et al. 1992). Males are normally haploid in sexually reproducing Hymenoptera as they develop from unfertilized eggs. Fertilized eggs usually develop as diploid females. Diploid males are the result of homozygosity at one or multiple sex loci under complementary sex determination (CSD) while female development is initiated by heterozygosity at these loci (Whiting 1943; Beye et al. 2003; de Boer et al. 2008). Diploid males

are thus produced instead of females, and because they are generally unviable or (effectively) sterile, they represent a severe form of inbreeding depression (Heimpel and Boer 2008). Under inbreeding or reduced allelic diversity at the sex locus, as many as 50% of diploid offspring develop as males. This may lead to male-biased sex ratios and can reduce population growth rates and rates of establishment (Stouthamer et al. 1992; Heimpel and Lundgren 2000, but see Hein et al. 2009). Diploid male production under CSD may even theoretically lead to extinction through a so-called diploid male vortex: decreased population size leads to a reduction in sex allele diversity, leading to an increase in the production of diploid males, which in turn leads to further decreases in population size and so on (Zayed and Packer 2005). Hence, mechanisms such as CSD may cause a sex determination meltdown in populations that endure inbreeding. However, surprisingly little information is available on the occurrence of diploid males in

field populations of parasitoid wasps and on the consequences of CSD for biological control (Antolin et al. 2003; Stouthamer et al. 1992; Heimpel and Lundgren 2000).

Despite its fundamental role in the development of animals, sex determination appears to be a rapidly evolving trait throughout the animal kingdom. Indeed, the CSD phenotype is present in many – but not all – hymenopteran species (van Wilgenburg et al. 2006). Mechanisms of sex determination in non-CSD species are poorly understood although recent evidence indicates a role for imprinting at the basis of the sex determination pathway in the parasitoid *Nasonia vitripennis* (Chalcididae) (Verhulst et al. 2010a; Dobson and Tanouye 1998). Phylogenetic analyses support CSD as the ancestral mechanism for haplodiploidy in the Hymenoptera, but it is currently not possible to determine whether one or more loci were responsible in the ancestral form of CSD (Asplen et al. 2009). It is commonly assumed that *single-locus* CSD (*sl*-CSD) is ancestral and that evolution to other modes of sex determination without the production of diploid males has occurred, for example, in habitually inbreeding species (van Wilgenburg et al. 2006; Heimpel and Boer 2008). *Multiple-locus* CSD (*ml*-CSD) could have evolved from *sl*-CSD by one or more duplications of the sex locus. This significantly reduces the fitness costs of inbreeding because homozygosity at each sex locus is required for diploid male development (Crozier 1971; de Boer et al. 2008). We expect that the negative consequences of biological control introductions will also be reduced in species with *ml*-CSD compared to species with *sl*-CSD.

In this article, we investigated the sex determination mechanism of *Cotesia rubecula* Marshall, which has been introduced for biological control of the cabbage white *Pieris rapae* L. in North America (van Driesche 2008). Based on the presence of CSD in other *Cotesia* species and previous unpublished results, we expected *C. rubecula* to exhibit CSD (Stouthamer et al. 1992; Zhou et al. 2006; de Boer et al. 2007a). Our specific objectives were (i) to investigate diploid male occurrence and sex allele diversity in an introduced population of *C. rubecula*, (ii) to assess the number of CSD loci and diploid male survivorship, using a native population from the Netherlands, and (iii) to study diploid male courtship behavior and reproductive success. We aim at evaluating the results in the context of biological control introductions and discuss the potential for a sex determination meltdown in *C. rubecula*.

## Material and methods

### Insects

Parasitoid wasps are insects with free-living adults and parasitic larvae. Female parasitoids lay their eggs in or on a host insect on which their larvae develop. As the parasitoid larvae mature, the host insect is killed (Godfray 1994).

*Cotesia rubecula* (Hymenoptera: Braconidae) is a specialist parasitoid on caterpillars of the cabbage white *P. rapae*. It is a solitary parasitoid, producing a single offspring per host caterpillar. This species is native to Eurasia and has been introduced into N. America at various localities beginning in 1963 (Van Driesche 2008), with releases in Minnesota, USA, in 1992 (Wold-Burkness et al. 2005). Recent evidence suggests that it is displacing *Cotesia glomerata*, which had been released in N. America earlier (Wold-Burkness et al. 2005; Van Driesche 2008), and this displacement may have impacts not only on pests but also on native butterfly species through indirect pathways (Harvey et al. 2010). *Cotesia rubecula* has also recently become established on *P. rapae* in New Zealand, where *P. rapae* has emerged as a serious threat to an endemic endangered plant (Cameron and Walker 2002; Hasenbank et al. 2011).

### Collection of field material

In the summers of 2005 (July 6–August 8) and 2006 (July 5–August 16), *C. rubecula* cocoons and *P. rapae* caterpillars were collected from an organically maintained cabbage crop at the St. Paul campus of the University of Minnesota, USA. Collected hosts were reared in the laboratory on cabbage leaves in plastic containers to allow development of parasitoids. Upon emergence, *C. rubecula* wasps were sexed and frozen for flow cytometric analysis of ploidy level (see below).

### Laboratory experiments

To start a laboratory culture, *C. rubecula* was collected in the summer of 2009 from *P. rapae* in cabbage fields around Wageningen University, the Netherlands, where it occurs natively. The wasps were reared on *P. rapae* on Brussels sprout plants (*Brassica oleracea* L. var. *gemmifera* cv. *Cyrus*) in a climatized room at the Laboratory of Entomology (20–22°C, 50–70% RH, 16L:8D). All experiments were conducted at the Laboratory of Entomology in a climate-controlled room at 25 ± 1°C, 60% RH and 16L:8D.

### Mother–son crosses

We first performed mother–son crosses (parental generation) to determine whether *C. rubecula* indeed has CSD. Under any form of CSD (single or multiple loci), mother–son crosses should lead to the production of diploid male offspring because the number of alleles per putative sex locus is reduced to two and thus any mother–son cross is matched in terms of sex alleles. Because homozygosity is required at all sex loci to produce diploid males, however, the proportion of diploid males (DMP) decreases with the number of sex loci – even in mother–son crosses (Crozier 1971; de Boer et al. 2008). Virgin *C. rubecula* females were

first allowed to produce haploid sons by exposing individual newly emerged wasps overnight to approximately 12 first-to-second instar *P. rapae* larvae on a piece of cabbage leaf in Petri dishes (9 cm diameter). Females were then kept in a clean Petri dish with several drops of honey and moist cotton in an incubator at 20°C until their sons had developed, which took approximately 16 days. Parasitized hosts were reared on cabbage in transparent plastic 500-mL cups until cocoons appeared. The plastic cups had two ventilation holes (1.5 cm diameter) in the side covered with fine mesh. Fresh cabbage leaves were added regularly and dead hosts removed. When haploid males emerged, one male was paired with its mother in a plastic 50-mL tube with a piece of host-infested cabbage leaf and a droplet of honey for 24 h to allow mating. Males were subsequently frozen and females were exposed to approximately 30 first-to-second instar *P. rapae* in a Petri dish for 24 h. Females were exposed to a maximum of three such sets of hosts on three consecutive days, and parasitized hosts were reared as described above. The numbers of cocoons, host pupae and dead hosts were recorded for each replicate. Cocoons were separated in vials to provide virgin males and females to start the F<sub>1</sub> generation (see below). We counted the number of males, females and non-emerged cocoons. Males were frozen at -25°C for flow cytometric analyses of ploidy level (see below). Of the 28 females that were allowed to mate with one of their own sons, two did not produce any offspring and 15 produced only males (presumably haploid as a result of no mating or unsuccessful mating). This left 11 successful mother-son matings for further experimental analysis.

#### *Diploid male survival, behavior and fertility*

Offspring of mother-son crosses were used to set up the next series of crosses with the purpose of assessing diploid male survival and fertility and to gain insight into the number of loci underlying CSD, as well as comparing copulation behavior of haploid and diploid males. We made 52 brother-sister crosses with males of unknown ploidy (generation F<sub>1</sub>). Ploidy level was determined afterward by flow cytometry and crosses were categorized as brother-sister crosses with haploid males (33) and diploid males (16); ploidy level remained unknown in three cases. Twenty-seven control crosses were made by combining a female with a son from a different mother. Ploidy of these fathers was tested afterward with flow cytometry (see below), and we report the results of 23 crosses with haploid fathers (ploidy of two fathers remained unknown; two fathers were diploid and did not produce any offspring).

Copulation behavior was observed in all replicates of the three types of crosses by placing a virgin male and female together in a plastic 50-mL tube with a piece of host-damaged cabbage and a droplet of honey. We recorded the

occurrence of wing fanning, which is an important component of copulation behavior in parasitoid wasps (Field and Keller 1993b), the occurrence and location of mounting and time until mounting. Observations lasted until mounting was observed or for a maximum of 10 min. Pairs were subsequently left together for another 24–72 h. Males were then frozen for analysis of ploidy level and females were exposed to two sets of approximately 30 hosts on two subsequent days as described above for mother-son crosses. Hosts were then reared to allow development of parasitoids. We counted the number of cocoons, dead hosts and *P. rapae* pupae as well as the number of females, males and non-emerged *C. rubecula* cocoons (generation F<sub>2</sub>). Offspring were frozen for analysis of ploidy level.

#### Flow cytometric analyses of ploidy level

Ploidy level was analyzed with flow cytometry following methods described previously (de Boer et al. 2007b). In short, the head of an individual wasp was pulverized in 0.5 mL of Galbraith buffer (Galbraith et al. 1983) and stained with propidium iodide (25 µg per sample). Analyses were done on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry, San Jose, CA, USA) for wasps collected in the field and on an Epics<sup>®</sup> XL<sup>™</sup> flow cytometer (Beckman Coulter, Brea, CA, USA) for laboratory crosses. DNA content of 2500 nuclei from head tissue was measured per wasp and the DNA histogram compared to that of known haploid males and diploid females to classify it as haploid, diploid or unknown. We analyzed ploidy level of 148 males and 60 females collected in the field, and ploidy level of all male offspring of 11 mother-son crosses, 23 brother-sister crosses with haploid fathers and 13 control crosses. Ploidy level was also analyzed for daughters from two brother-sister crosses with diploid fathers and for 10 males in the same two replicates. Ploidy level of 18 out of 208 field-collected wasps and 6 out of 556 males from laboratory crosses remained unknown after flow cytometry.

#### Data analyses

##### *Data selection*

In the analyses of sex ratio and diploid male proportions of laboratory crosses, we included those replicates with at least seven diploid offspring because when seven diploid offspring (males and females) are produced, the probability that at least one of them is a diploid male under the null hypothesis of *sl*-CSD with full survival of diploid males is more than 99%. This led to the exclusion of three replicates of mother-son crosses, five replicates of brother-sister crosses with haploid fathers and four replicates of control crosses. In addition, two brother-sister crosses with

haploid fathers were discarded because the mother–son cross from which they originated was excluded. In the analyses of DMP and diploid family size, we included only those control crosses for which we determined ploidy level of all male offspring (i.e.  $N = 10$  after removing replicates with  $<7$  diploid offspring). Compared to analyses of the complete dataset (including replicates with  $<7$  diploid offspring), data selection does not influence our conclusions.

#### Simulations to assess the number of CSD loci

We used a simulation model to statistically compare our results of diploid male production to predictions of CSD while varying two parameters: the number of putative CSD-loci,  $m_{\text{loci}}$  (1, 2 or 3), and the survival probability  $s$  of diploid males (between 0 and 1). This model was designed to simulate our experiment exactly in terms of female wasps used in both generations and number of diploid offspring per female. Individual males and females were represented by one (haploid) or two (diploid) binary strings, each of length  $m_{\text{loci}}$ . While diploid family size was equal to the observed values, the number of surviving diploid males varied according to  $m_{\text{loci}}$  and  $s$ . We assumed no linkage between the putative CSD loci for  $m_{\text{loci}} > 1$ . Increasing linkage would result in outcomes intermediate to the single- and two-locus results presented in Fig. 1A, B. Details of the simulation model are presented in Appendix S1.

We compared our data on diploid male production by *C. rubecula* to predictions of the simulation model with a likelihood ratio test. Instead of using binomial and multinomial density functions as our likelihood functions (de Boer et al. 2008), the likelihood functions were directly obtained from our simulations, following a procedure presented in Appendix S1 (see also Figs S1–S3). For each  $m_{\text{loci}}$  (1, 2 or 3), we used the log likelihood curves (Fig. S2) to assess the value of survival  $s$  which maximized the likelihood. This resulted in the following three-parameter combinations used in subsequent statistical analyses: ( $m_{\text{loci}} = 1$ ;  $s = 0.19$ ), ( $m_{\text{loci}} = 2$ ;  $s = 0.69$ ) and ( $m_{\text{loci}} = 3$ ;  $s = 0.92$ ). We then calculated the likelihood ratio to assess the relative fit of the data given an alternative parameter combination  $\mathbf{v}_i$  (e.g.  $m_{\text{loci}} = 2$ ;  $s = 0.69$ ) compared to the parameter values assumed under a null hypothesis  $\mathbf{v}_0$  (e.g.  $m_{\text{loci}} = 1$ ;  $s = 0.19$ ) (Fig. S3):

$$\frac{L(\mathbf{x}|\mathbf{v}_i)}{L(\mathbf{x}|\mathbf{v}_0)} = \sum_k^m (\ln f_k(x_k|\mathbf{v}_i) - \ln f_k(x_k|\mathbf{v}_0))$$

To obtain significance values, we generated a distribution of likelihood ratios taking one of the three parameter combinations as the null hypothesis. In contrast to conventional likelihood ratio tests (LRTs), our likelihood ratio test is non-nested, since the alternative hypothesis is not a

special case of the null hypothesis. Therefore, we used each of the three parameter combinations as the null hypothesis and tested it against the two remaining parameter combinations as the alternative hypotheses, following a procedure described in Lewis et al. (2011) to perform LRTs for non-nested model comparisons (see Appendix S1).

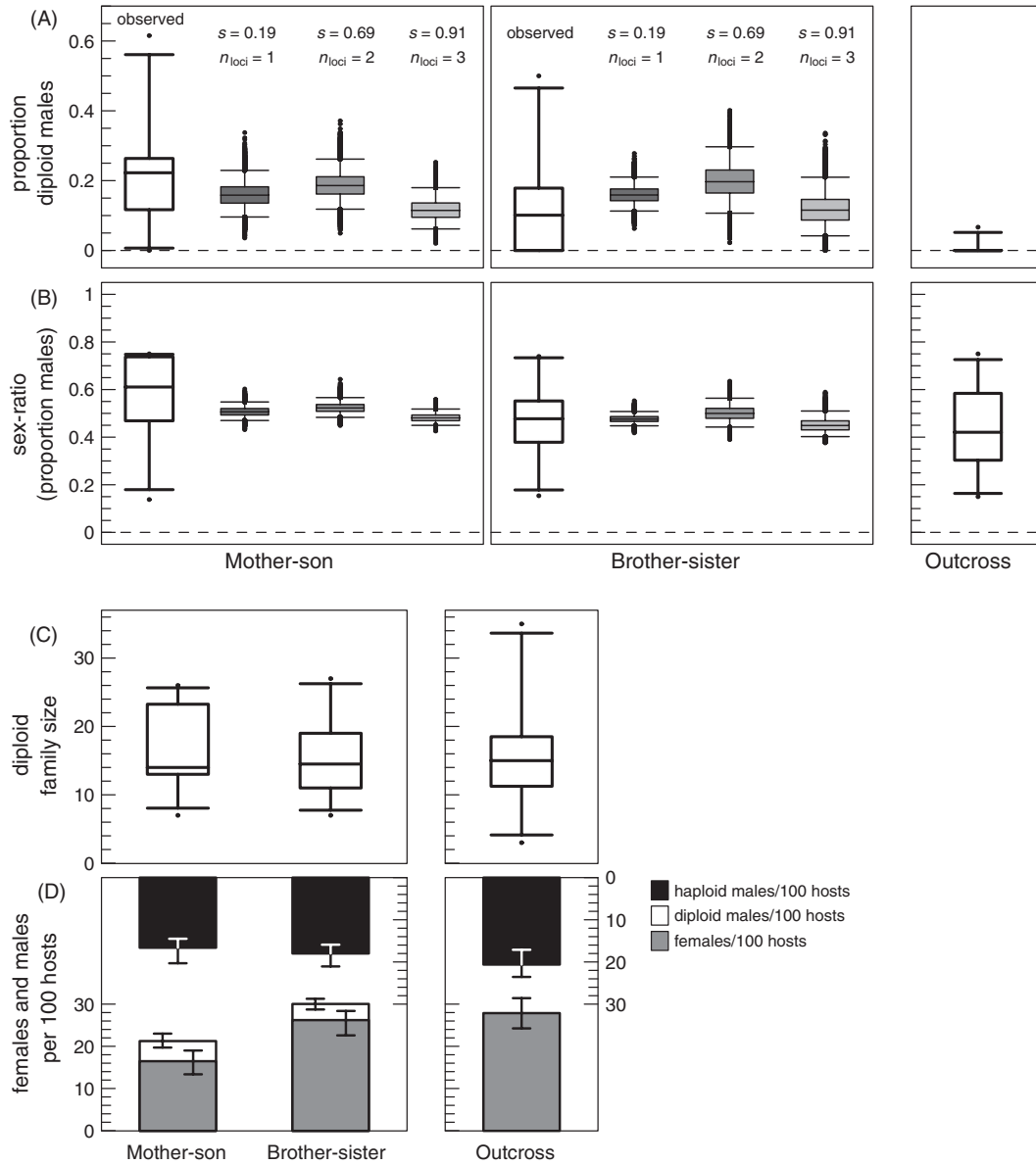
#### Additional analyses of diploid male production and diploid male survival

In subsequent analyses, we compared the results of brother–sister crosses with those of control crosses, because these crosses were performed at the same time under exactly the same conditions, while mother–son crosses were performed earlier and mothers were older because their haploid sons had to develop first. The DMP and sex ratio (proportion males) were compared with a generalized linear model with a quasibinomial error distribution and logit link function. Diploid male survival cannot be directly estimated from our data because it is difficult to measure developmental mortality of parasitoid larvae that develop inside their host since we cannot see whether the parasitoid larva is alive. It is expected that when a parasitoid larva dies, the host dies as well, but hosts may also die for reasons unrelated to parasitism. We therefore used diploid family size and the proportion of dead hosts as proxies for developmental survival and compared them between brother–sister crosses and control crosses with a GLM, using a quasipoisson error distribution and log link function for diploid family size and a quasibinomial error distribution and logit link function for the proportion of dead hosts. Our expectations were that if diploid male survival equals that of females (i.e.  $s = 1$ ), brother–sister crosses produce equal diploid family sizes (diploid males + females) and equal proportions of dead hosts as control crosses, in which diploid males are not produced. Moreover, among inbred families, we would expect a positive relationship between the ratio of diploid males to females,  $d/f$ , and diploid family size when diploid male survivorship is significantly lower than that of females ( $s \ll 1$ ), and a negative relationship between  $d/f$  and the number of dead hosts. To test this statistically, we calculated Pearson's correlation coefficient between  $d/f$  and (1) diploid family size, and (2) number of dead hosts for both types of inbred crosses combined (mother–son crosses and brother–sister crosses with haploid males).

#### Analyses of behavior

In our behavioral observations, we were interested in two effects: (i) haploid males versus diploid males in brother–sister pairs and (ii) inbred versus control matings in pairs with haploid males. We therefore statistically compared the probability to mate and the time until mounting between brother–sister crosses with haploid and diploid males and





**Figure 1** Box plots of diploid male proportion (A), sex ratio (B), diploid family size (C) and number of females, haploid and diploid males normalized per 100 hosts (D) in three types of crosses: mother-son crosses (left), brother-sister crosses (middle) and control crosses (right). Panels (A) and (B) include boxplots of 50 000 simulations for three combinations of parameter values of the number of complementary sex determination (CSD) loci ( $n_{loci}$ ) and the probability of diploid male survival ( $s$ ) that gave the highest likelihood for single-locus CSD, two-locus CSD and three-locus CSD (Fig. S2). In panels (A), (B) and (C), boxes represent 0.25 and 0.75 quantiles and median value, whiskers represent 0.025 and 0.975 quantiles and outliers are shown as black bullets. In panel (D), error bars represent standard errors.

between brother-sister crosses with haploid males and control crosses separately. We used a Bonferroni correction because the data of brother-sister crosses with haploid males were used in both comparisons. We used Fisher's exact test to compare the probability to mount and a Wilcoxon rank test to compare time until mounting between crosses. All statistical analyses were performed in R 2.12.0 (The R Development Core Team 2011).

## Results

### Diploid males in an introduced population of *Cotesia rubecula* in Minnesota

A total of 307 *C. rubecula* cocoons were directly collected from an organic cabbage crop in Minnesota in 2005 and 2006, and 253 cocoons from *P. rapae* caterpillars collected in the same field developed in the laboratory (Table 1).

**Table 1.** Fate of *Cotesia rubecula* cocoons collected in Minnesota in 2005 and 2006.

Year	Cocoons	Females	Haploid males	Diploid males	Unknown males	Hyper-parasitoids	Not emerged
2005	424	119	76	17	9	54	149
2006	136	70*	34	6	8	8	10
Total	560	189	110	23	17	62	159

\*60 females collected in 2006 were also analyzed for ploidy level: 57 females were diploid while ploidy of three females remained unknown.

The sex ratio of emerged *C. rubecula* adults was 46% males in 2005 and 41% males in 2006. Flow cytometric analysis showed that 23 males were diploid, and the DMP among diploid offspring was 0.13 in 2005 and 0.08 in 2006. When all data are combined, DMP was 0.11, while 15% of all males were diploid, suggesting that diploid male survivorship is relatively high and/or that the population harbors few sex alleles (see below).

### Number of sex loci and diploid male survival

To obtain an estimate of the effective number of sex loci and diploid male survival, we did a series of laboratory crosses using a native Dutch population of *C. rubecula*. Diploid male production in the laboratory ranged from zero to more than half of all diploid offspring (mean  $\pm$  SE, DMP =  $0.22 \pm 0.07$ ; weighted by diploid family size) in eight mother–son crosses that resulted in the production of at least seven diploid offspring (Fig. 1A and Table 2). The secondary sex ratio (proportion males among adult offspring) produced by mother–son crosses was  $0.58 \pm 0.08$ . Diploid male production ( $0.12 \pm 0.04$ ) and sex ratio ( $0.47 \pm 0.05$ ) were slightly lower in the following generation of brother–sister mating (with haploid brothers;  $N = 16$ ) (Fig. 1A,B). In comparison, DMP was significantly lower in control crosses ( $0.01 \pm 0.01$ ;  $N = 10$ ; GLM:  $\chi^2_{df=1, n=26} = 30.46$ ,  $P = 0.001$ ). Sex ratio was female-biased in control crosses ( $0.38 \pm 0.04$ ;  $N = 17$ ) but did not differ

significantly from sex ratio in brother–sister crosses (GLM:  $\chi^2_{1,33} = 6.44$ ,  $P = 0.14$ ). Other parameter values for all types of crosses are presented in Table 2.

The LRTs with the three-parameter combinations for which maximum likelihood was obtained (from Fig. S2) are provided in Table 3. A comparison between ( $n_{\text{loci}} = 2$ ;  $s = 0.69$ ) and ( $n_{\text{loci}} = 1$ ;  $s = 0.19$ ) shows that the two-locus model as null hypothesis is non-significant ( $P = 0.27$ ), whereas it is highly significant as an alternative model ( $P < 0.001$ ) (Fig. S3). Hence, according to the procedure described in Lewis et al. (2011), this makes ( $n_{\text{loci}} = 2$ ;  $s = 0.69$ ) a more plausible model to describe the data than ( $n_{\text{loci}} = 1$ ;  $s = 0.19$ ). A similar pattern is found when comparing the other multilocus model ( $n_{\text{loci}} = 3$ ;  $s = 0.92$ ) with the single-locus model ( $n_{\text{loci}} = 1$ ;  $s = 0.19$ ). Finally, when both multilocus models are compared, we find that ( $n_{\text{loci}} = 2$ ;  $s = 0.69$ ) as alternative hypothesis is highly significant ( $P = 0.0042$ ), whereas the reverse model with ( $n_{\text{loci}} = 3$ ;  $s = 0.92$ ) as alternative hypothesis is not ( $P = 0.34$ ). Again, the parameter combination ( $n_{\text{loci}} = 2$ ;  $s = 0.69$ ) provides a more plausible explanation of the data than ( $n_{\text{loci}} = 3$ ;  $s = 0.92$ ). All in all, the parameters  $n_{\text{loci}} = 2$  and  $s = 0.69$  give the best fit to the current dataset. This fit is also visualized in Fig. S4 which shows the predicted DMP per generation for the three different models and the proportions observed in our experimental crosses. There is a clear difference in fit between the single-locus model ( $n_{\text{loci}} = 1$ ;  $s = 0.19$ ) and the multilocus models, where the

**Table 2.** Overview of parameter values for the different types of crosses.

Type of cross	Mean $\pm$ SE Hosts exposed	Fate of exposed host			Fate of cocoons			Total numbers	
		<i>Pieris rapae</i>	Dead	<i>Cotesia</i> cocoon	Female	Male	Not emerged	Haploid male	Diploid male
Mother–son ( $n = 8$ )	81.38 $\pm$ 6.35	11.50 $\pm$ 2.78	33.88 $\pm$ 6.76	34.00 $\pm$ 5.27	13.00 $\pm$ 2.28	18.13 $\pm$ 4.09	2.89 $\pm$ 1.08	112	30
Brother–sister with haploid father ( $n = 16$ )	54.19 $\pm$ 2.45	7.69 $\pm$ 1.36	16.63 $\pm$ 2.69	29.00 $\pm$ 1.90	13.75 $\pm$ 1.28	12.00 $\pm$ 1.29	3.25 $\pm$ 0.54	159	31
Brother–sister with diploid father ( $n = 14$ )	55.93 $\pm$ 3.34	6.43 $\pm$ 1.12	16.21 $\pm$ 2.86	32.71 $\pm$ 3.06	0.14 $\pm$ 0.10	30.57 $\pm$ 3.11	2.00 $\pm$ 0.54	20	0
Control ( $n = 17$ )	53.53 $\pm$ 2.11	5.65 $\pm$ 0.89	11.65 $\pm$ 2.15	34.94 $\pm$ 2.38	19.53 $\pm$ 1.89	12.18 $\pm$ 1.45	3.24 $\pm$ 0.52	126*	1

\*Ploidy was analyzed for male offspring of 10 out of 17 control crosses (unknown for 80 males from seven crosses).

**Table 3.** Results of likelihood ratio tests, comparing the parameter sets that were shown to have the largest log-likelihood in Fig. S2.

Null hypothesis	Alternative hypothesis	P-value	Likelihood ratio	LR distribution when null hypothesis is correct		
				Min LR	Mean LR	Max LR
$n_{\text{loci}} = 1; s = 0.19$	<b><math>n_{\text{loci}} = 2; s = 0.69^*</math></b>	0.0000	10.71961	-17.869468	-8.791265	6.613286
<b><math>n_{\text{loci}} = 2; s = 0.69</math></b>	$n_{\text{loci}} = 1; s = 0.19$	0.2688	-10.71961	-37.503763	-14.3479	5.084059
$n_{\text{loci}} = 1; s = 0.19$	<b><math>n_{\text{loci}} = 3; s = 0.92</math></b>	0.0000	6.587093	-23.482181	-12.36556	4.810596
<b><math>n_{\text{loci}} = 3; s = 0.92</math></b>	$n_{\text{loci}} = 1; s = 0.19$	0.0620	-6.587093	-36.739005	-15.71077	4.428497
$n_{\text{loci}} = 3; s = 0.92$	<b><math>n_{\text{loci}} = 2; s = 0.69</math></b>	0.0042	4.132517	-11.856699	-3.211836	8.363512
<b><math>n_{\text{loci}} = 2; s = 0.69</math></b>	$n_{\text{loci}} = 3; s = 0.92$	0.3352	-4.132517	-16.160327	-5.472591	6.415336

\*Models in bold are significantly preferred over the other model in the non-nested comparison of two models.

multilocus models explain the increased variation in numbers of diploid males in the brother–sister matings better than the single-locus model.

Because *C. rubecula* is an endoparasitoid, its developmental survival cannot be directly measured. We therefore used diploid family size and the proportion of dead hosts as proxies for diploid male survival. Under any form of CSD, diploid males are produced instead of females, and low diploid male survival should thus affect diploid family size. However, we found no indication of a significant difference between diploid family sizes of brother–sister and control crosses (Fig. 1C,D; GLM,  $\chi^2_{1,26} = 5.93$ ,  $P = 0.18$ ). Moreover, when *C. rubecula* diploid males die during development, their *P. rapae* hosts are expected to die as well, and low diploid male survival should thus result in high proportions of dead hosts in brother–sister crosses but not in control crosses. However, the proportion of dead hosts was not statistically different between these two types of crosses (Table 2; GLM:  $\chi^2_{1,33} = 18.36$ ,  $P = 0.12$ ; average weighted by the number of hosts offered was  $0.31 \pm 0.05$  for brother–sister crosses and  $0.23 \pm 0.04$  for control crosses). Finally, within inbred families (data of mother–son and brother–sister crosses combined), we found no indication for a correlation between the DMP and diploid family size (Pearson's correlation,  $r = -0.06$ ,  $P = 0.77$ ,  $N = 24$ ; Fig. S5A), or the number of dead hosts (Pearson's correlation,  $r = 0.16$ ,  $P = 0.45$ ,  $N = 24$ ; Fig. S5B). Since these analyses indicate that a low probability of diploid male survival is unlikely in *C. rubecula*, they indirectly support a multilocus model because the single-locus CSD model only explains our data if diploid male survival is low ( $s = 0.19$ , Fig. S2).

### Reproductive behavior and success of diploid males

Almost all observations of mating behavior resulted in mounting within 10 min: out of a total of 72 observations, no mounting was observed in three brother–sister crosses with a haploid male, two brother–sister crosses with a diploid male and four control crosses. Probability of mating

was thus not affected by male ploidy level in brother–sister crosses (haploid versus diploid, Fisher's exact test,  $P = 1$ ) or by the type of cross (haploid males in control crosses versus brother–sister crosses, Fisher's exact test,  $P = 0.96$ ). Haploid males ( $2.07 \pm 0.27$  min,  $N = 30$ ) mounted significantly faster than diploid males ( $4.54 \pm 0.73$  min,  $N = 14$ ) in brother–sister crosses (Wilcoxon rank test,  $W = 329.5$ ,  $P = 0.004$ ) but not faster than haploid males in control crosses ( $3.26 \pm 0.64$  min,  $N = 19$ , Wilcoxon rank test,  $W = 224.5$ ,  $P = 0.42$ ).

Although females readily accepted mating attempts by diploid males, only two diploid males ( $N = 14$ ) produced daughters; they each sired a single triploid female offspring. We also analyzed 10 sons of each of these two families and they were all haploid. The remaining crosses with diploid fathers produced only males and we did not determine their ploidy level. In contrast, reproductive success (the proportion of males that produced at least one daughter) of haploid males was high in brother–sister crosses (23 out of 27) and control crosses (21 out of 21).

### Sex allele diversity in the field population of *Cotesia rubecula*

The DMP can be used to assess sex allele diversity making assumptions on the number of sex loci and diploid male survival (Adams et al. 1977). Here, we use the estimates of  $n_{\text{loci}}$  and diploid male survival  $s$  obtained from our laboratory experiments with the native Dutch *C. rubecula* population to assess sex allele diversity in the introduced field population in Minnesota. Using the parameter combination that gave maximum likelihood in our simulations and the best fit to our data (i.e.  $n_{\text{loci}} = 2$ ,  $s = 0.69$ , Fig. S2, Table 3), 11% diploid males over the 2 years of sampling could be explained by the presence of two to four sex alleles at each locus within the Minnesota population. Single-locus CSD with low probability of diploid male survival ( $s = 0.19$ , as estimated from Fig. S2) can explain the DMP observed in Minnesota when the population harbors only two sex alleles. Yet another scenario could be that the



founders of the population that we sampled in Minnesota had two-locus CSD ( $n_{\text{loci}} = 2$ ,  $s = 0.69$ ) but one of these two sex loci has become fixed (i.e. homozygous) upon introduction or establishment, and thus two-locus CSD has collapsed to single-locus CSD in the population that we sampled from (Engelstädter et al. 2011; Asplen et al. 2009). If we assume such a scenario, which is effectively the same as single-locus CSD ( $n_{\text{loci}} = 1$ ,  $s = 0.69$ ), the population in Minnesota would have harbored five to nine sex alleles at the remaining polymorphic locus.

## Discussion

Our study is among the first to report the presence of diploid males in a field population of parasitoid wasps, with approximately 11% of diploid offspring developing as males in *C. rubecula* in Minnesota. Diploid males are the result of homozygosity at one or multiple sex loci in most hymenopteran insects, and their production represents a severe form of inbreeding depression because diploid males are generally unviable or sterile (Heimpel and Boer 2008). Hymenopteran species with CSD are expected to have evolved a variety of mechanisms that reduce the sex determination load, for example, behavioral mechanisms such as pre-mating dispersal and kin recognition (Gu and Dorn 2003; Ode et al. 1995), or multiple sex loci (de Boer et al. 2008) (reviewed in van Wilgenburg et al. 2006). Natural populations are also expected to harbor a large number of sex alleles that are maintained in the population through negative frequency-dependent selection (Ross et al. 1993). However, when population bottlenecks occur during the process of biological control introductions or invasions, sex allele diversity may become reduced and the inbreeding depression associated with CSD may be exacerbated (Stouthamer et al. 1992; Zayed et al. 2007). We believe that it is therefore particularly important to investigate CSD and diploid male production in parasitoid wasps used for biological control.

### Complementary sex determination is likely based on two loci in *Cotesia rubecula*

To allow estimates of sex allele diversity, assumptions on the number of CSD loci and diploid male survival must be made (Adams et al. 1977), so we investigated these 'CSD characteristics' of *C. rubecula* in the laboratory using a native Dutch population. A CSD model with two loci and high probability of diploid male survival (approximately 70%) best explains our data, although it remains difficult to obtain exact estimates of diploid male survival. This is because developmental survival cannot be measured directly in endoparasitoids, and, in addition to our simulation analyses, we used diploid family size and the

proportion of dead hosts as proxies instead. We found no indications for statistical differences in these parameters between inbred and control crosses, indeed suggesting relatively high diploid male survival. However, we note that statistical power for these analyses was low: since the average diploid family sizes (Fig. 1C) and the average proportions of dead hosts were similar in these two types of crosses (31% in brother–sister versus 23% in control crosses), the resulting small effect sizes of these tests would require enormous sample sizes to achieve sufficient statistical power. Nevertheless, we frequently observed diploid males in the field as well as in our laboratory experiments, suggesting that diploid male survival is certainly not low. Only when diploid male survival is low (approximately 20%; Fig. S2), could our data be best explained by CSD with a single-sex locus, whereas even slightly higher survival rates give more support to multilocus CSD in *C. rubecula*.

Our results corroborate previous findings of a multilocus CSD phenotype in a sister species *Cotesia vestalis* (de Boer et al. 2008). Two-locus CSD may in principle evolve from *sl*-CSD by duplication of the CSD locus and reduces the production of diploid males significantly because homozygosity at both sex loci is required for diploid male development (Crozier 1971). Duplications of sex determination genes are known from other hymenopterans with the CSD phenotype. In honeybees, the *csd*-gene arose from a duplication of the *feminizer* gene (*fem*) (Hasselmann et al. 2008). While heterozygous *csd* is required to initiate female development, *fem* activity maintains the female pathway throughout development (Gempe et al. 2009). *Fem* is structurally as well as functionally similar to *transformer* in other insect species (Gempe and Beye 2011; Verhulst et al. 2010b). Interestingly, while *fem* also occurs in lineages related to honeybees, such as bumblebees and stingless bees, *csd* occurs only in *Apis*, suggesting a recent duplication in this clade despite the presence of CSD phenotype in related lineages (Hasselmann et al. 2008). The genome of the fire ant *Solenopsis invicta*, another species with *sl*-CSD (Ross and Fletcher 1986), also contains two linked sequences with similarity to *transformer/feminizer* genes from honeybees and other insects, but their functions have not been fully characterized yet (Würm et al. 2011). Phylogenetic analysis of *transformer*-like gene sequences in honeybees and ants confirmed that duplication events occurred independently in these lineages. Gempe & Beye (2011) suggest that small-scale changes in regulatory and coding regions of existing or duplicated genes may lead to the observed variety of sex determination mechanisms across insects. Importantly, to explain an *ml*-CSD phenotype, i.e., lower proportions of diploid males as we found in *C. rubecula*, gene duplicates must segregate independently instead of being linked as found for honeybees and *S. invicta*. In addition, alleles from separate loci must not interact in

ways that would produce haploid females. Mapping and molecular genetic studies in *Cotesia* are required to gain insight into the number of loci and genes involved in sex determination in these parasitoids, and we are currently taking these steps in *C. vestalis*.

### Sex allele diversity in field populations of parasitoid Hymenoptera

While our laboratory studies suggest that CSD in *C. rubecula* is based on two loci – a scenario that should reduce diploid male production – 8–13% of diploid adults were male in a field population in Minnesota. Diploid males have been detected in field populations of parasitoids only two times before as far as we are aware. Natural populations of *Bracon* (= *Habrobracon*) *hebetor* and *Diadromus pulchellus* were estimated to harbor more than 10 different sex alleles at a single-sex locus (Heimpel et al. 1999; Antolin et al. 2003; Periquet et al. 1993) although *Bracon hebetor* allelic diversity was estimated to be lower in laboratory crosses between wasps from different locations (Heimpel et al. 1999). Although directly extrapolating our laboratory data to an introduced population with a different origin remains speculative, our laboratory estimates ( $m_{\text{loci}} = 2$  and  $s = 0.69$ ) suggest that the sampled population of *C. rubecula* in Minnesota harbors two to four sex alleles at each of the two independent sex loci (Adams et al. 1977).

Alternatively, one of the sex loci might have become fixed in allele composition, and *ml*-CSD may have collapsed to *sl*-CSD in the population that we sampled from (Engelstädter et al. 2011; Asplen et al. 2009). While frequency-dependent selection should impede fixation of a single-sex locus through the advantage of rare sex alleles on lowering the production of diploid males, the strength of frequency-dependent selection on a given locus may be much weaker under *ml*-CSD. High allelic diversity at other sex loci may reduce the production of diploid males sufficiently to offset the advantage of rare sex alleles at a locus with low allelic diversity that is at risk of becoming fixed (Asplen et al. 2009). Engelstädter et al. (2011) predicted that *ml*-CSD may degrade to effective *sl*-CSD in <100 generations during the spread of parthenogenesis through a population of parasitoid wasps due to the loss of genetic variation at all but one of the sex loci. We suggest that such a sex determination meltdown may not be unlikely in an introduced population that has been founded by few individuals or has experienced population bottlenecks during establishment. *Cotesia rubecula* is native to Eurasia and has been introduced (both intentionally and accidentally) multiple times in North America (Biever 1992; Van Driesche 2008). In Minnesota, *C. rubecula* was recorded in cabbage fields from the year 2000 onward (Wold-Burkness et al. 2005), perhaps established from small numbers released

locally in 1992: 12 and 59 adults from China and Yugoslavia, respectively. The population of wasps that we sampled likely originated from a small founding population, and this may explain the low allelic diversity at the sex loci or the collapse from two-locus CSD to effective *sl*-CSD. We expect diploid male production to be lower in the native range of *C. rubecula*. Although such information is not yet available, proportions of diploid males were much lower in a native Taiwanese population of *C. vestalis* (J. G. de Boer, unpublished data). A comparison of diploid male production in the fire ant *S. invicta* in its native and introduced range also demonstrated a significantly lower diversity of sex alleles in the introduced range (Ross et al. 1993).

### Reproductive behavior and success of diploid males

Our finding that 15% of *C. rubecula* males were diploid in the field underlines the significance of investigating behavior and fertility of diploid males (Heimpel and Boer 2008). We observed no effect of male ploidy on courtship behavior, and females readily accepted a diploid male as a mate although time until mounting was significantly longer for diploid males. Yet, mating with a diploid male was costly to a female because their reproductive success is very low: only two out of 14 females mated to a diploid male produced one daughter each while the other females produced only sons. Moreover, daughters produced by diploid males were triploid and we expect them to be sterile (de Boer et al. 2007b). In contrast, females mated to haploid males had high reproductive success and produced many daughters. In our laboratory setup, females were confined with a single male (haploid or diploid) and thus could not choose their mate. A next important step will be to test whether females can discriminate between haploid and diploid males, and what the competitive abilities of diploid males are under field conditions, especially considering they took longer to mount a female than haploid males in our laboratory test. Competition among males of *C. rubecula* can be intense and males may ‘steal’ females from other males without courting or may display female mimicry to distract rivals (Field and Keller 1993a). Although *C. rubecula* females normally mate once, remating does occur (Field and Keller 1993a), and it will be interesting to investigate whether females are more likely to remate when their first mate is diploid.

### Conclusions and implications for biological control

In conclusion, we demonstrated CSD in *C. rubecula*, and our laboratory data suggest that it is based on two loci. While CSD with surviving and effectively sterile diploid males, as we found in *C. rubecula*, is expected to be most disadvantageous to fitness and population growth

(Stouthamer et al. 1992; Zayed and Packer 2005; Heimpel and Boer 2008), the presence of two loci should lessen population-level consequences in native populations. However, our observation of 8–13% diploid males in a field population of *C. rubecula* that was introduced for biological control purposes suggests that allelic diversity at the sex loci may be reduced or that two-locus CSD may degrade to effective *sl*-CSD locally. Despite these considerations, *C. rubecula* appears to establish readily when introduced and is capable of impressive levels of pest control (Cameron and Walker 2002; Van Driesche 2008). However, levels of parasitism would presumably be higher if diploid males were not produced. The local loss of sex allele diversity may be the result of a genetic bottleneck that occurred during biological control introduction. Unfortunately, biological control introductions are rarely accompanied by population genetic studies. A population genetic comparison of native and introduced populations of the parasitoid *Aphidius ervi* showed that a mild bottleneck indeed occurred despite the release of more than 1000 parasitoid wasps (Hufbauer et al. 2004). Yet, whether reduced genetic variation is associated with low fitness and poor performance of biological control agents remains to be established (Hufbauer and Roderick 2005). We suggest that biological control introductions of parasitoids with CSD represent excellent study systems to investigate the relationship between neutral and non-neutral genetic variation and biological control efficacy.

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### Conflict of interest

The authors declare no conflict of interest.

### Data archiving

Data for this study are available at Dryad: doi:10.5061/dryad.qp6c3.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Appendix S1.**

**Figure S1.**

**Figure S2.**

**Figure S3.**

**Figure S4.**

**Figure S5.**

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