

Diploid males, diploid sperm production, and triploid females in the ant *Tapinoma erraticum*

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Abstract Under complementary sex determination (CSD), females of Hymenoptera arise from diploid, fertilized eggs and males from haploid, unfertilized eggs. Incidentally, fertilized eggs that inherit two identical alleles at the CSD locus will develop into diploid males. Diploid males are usually unviable or sterile. In a few species, however, they produce diploid sperm and father a triploid female progeny. Diploid males have been reported in a number of social Hymenoptera, but the occurrence of triploid females has hardly ever been documented. Here, we report the presence of triploid females, diploid males, and diploid sperm (produced by diploid males and stored in queen spermathecae) in the ant *Tapinoma erraticum*. Moreover, we show variations in the frequency of triploids among female castes: Triploid females are more frequent among workers than virgin queens; they are absent among mated, reproductive queens. The frequency of triploid workers also varies between populations and between nests within populations.

Keywords Complementary sex determination · Diploid males · Flow cytometry · Formicidae · Triploid females

Introduction

Hymenoptera are characterized by a haplodiploid sex determination system, whereby males arise from unfertilized eggs

and are haploid, and females arise from fertilized eggs and are diploid. The most commonly described genetic mechanism whereby haplodiploidy is achieved in Hymenoptera is complementary sex determination (CSD; Whiting 1943; Crozier 1971). This mechanism assumes that sex is determined by heterozygosity at one (single locus, *sl*-CSD) or several (multiple loci, *ml*-CSD) loci. Heterozygote individuals will develop into females (Cook 1993; Cook and Crozier 1995), whereas haploid hemizygotes and diploid homozygotes at the sex-determining locus or loci will develop into males (Fig. 1). Aside from CSD, other genetic mechanisms may be responsible for haplodiploid sex determination, including genic balances (Kerr and Nielsen 1967) or genomic imprinting (Beukeboom 1995; Dobson and Tanouye 1998).

sl-CSD is widespread among the Hymenoptera studied so far (Van Wilgenburgh et al. 2006). *sl*-CSD and, to a much lower extent, *ml*-CSD may lead to the production of diploid males due to the probability of “matched mating” between parents carrying the same allele at the polymorphic sex locus (Adams et al. 1977). This probability is expected to be higher in species engaging in inbreeding and/or in species experiencing a reduction in allelic diversity and pronounced bottleneck, for example, following introduction in new environments. Diploid males are generally considered as a genetic load. The resources invested in the production of diploid males are effectively wasted because they are usually unviable (Petters and Mettus 1980) or sterile (Cook 1993) and therefore unlikely to produce offspring. This waste of resources is even greater in social species, because diploid males are produced at the expense of female workers. Since males do not work, they do not contribute to colony productivity. In a few species, however, diploid males surviving to adulthood are fertile and can sire a triploid female progeny (*Habrobracon pectiophorae*—Inaba 1939; *Neodiprion nigroscutum*—

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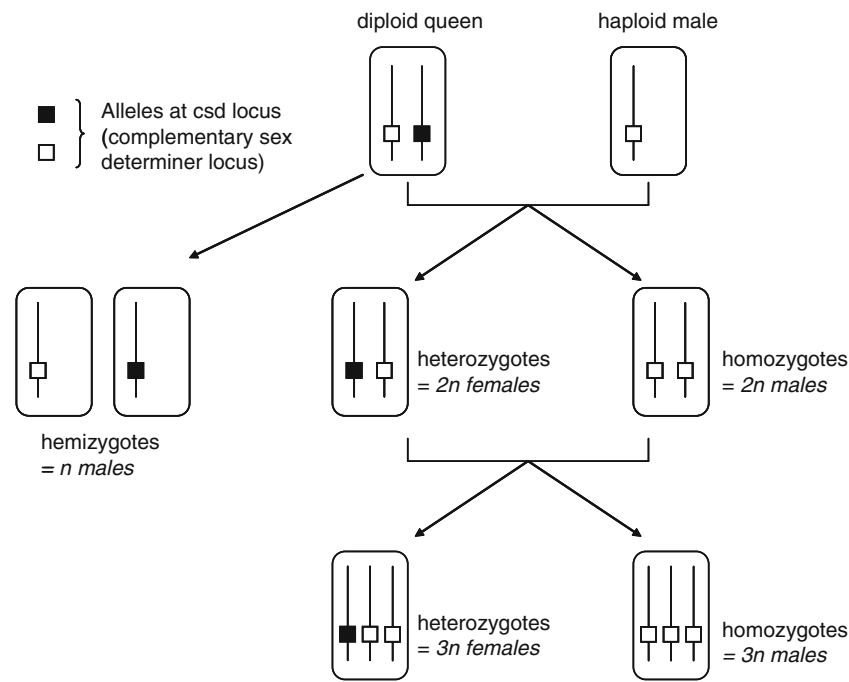


Fig. 1 Production of diploid males, triploid males, and triploid females under the single-locus complementary sex determination model

Smith and Wallace 1971; *Cotesia vestalis*—de Boer et al. 2007). In the vespid wasp *Euodynerus foraminatus*, diploid males were shown to sire diploid female offspring at the same rate as haploid males, which may reflect an adaptation to the high level inbreeding typical of the species (Cowan and Stahlhut 2004).

Production of diploid males was also reported in several species of social Hymenoptera (ants, bees, wasps; Crozier and Pamilo 1996; Van Wilgenburgh et al. 2006). However, their ability to produce sperm, the ploidy level of sperm cells, and male capability to fertilize females under natural conditions remain poorly studied. Takahashi et al. (2008) reported the occurrence of several diploid males and a single triploid, unmated female in native populations of the rare and endangered bumblebee *Bombus florilegus*. Two other genetic studies have dealt with populations of invasive species in their introduced range. In the paper wasp *Polistes dominulus*, diploid males and triploid females were uncovered by genotyping at several microsatellite loci (Liebert et al. 2004, 2005); it was therefore inferred that diploid males produce fertile diploid sperm and father a triploid female progeny. Diploid males, triploid workers, and triploid virgin queens (but no triploid mature queens) were also reported in populations of the fire ant *Solenopsis invicta* in its introduced range (Krieger et al. 1999). By contrast, no triploid females were detected in native populations (Ross et al. 1993; Krieger et al. 1999). These data are consistent with the reduction in allelic diversity experienced by the species following its introduction in the USA (Ross and Fletcher 1985), resulting in an increased

probability of homozygosity and hence of diploid male production (Ross et al. 1993; Krieger et al. 1999).

In ants, diploid males have been reported in several species belonging to various subfamilies (Table 1). However, the ability of such males to father a progeny remains largely unstudied, and it has been documented in introduced populations of the fire ant only. Here, we performed a broad survey of female triploidy and male diploidy in native populations of the ant *Tapinoma erraticum*. We sampled six populations from various habitats over a latitudinal gradient in Europe from Andorra to Belgium. Ploidy level of adult workers, virgin (winged) queens, inseminated (dealated) queens, adult males, as well as sperm cells from the males' seminal vesicles and the queens' spermatheca was determined by flow cytometry (FCM).

Materials and methods

Sample collection

Colonies of *T. erraticum* are facultatively polygynous; they may be headed by a single queen or by multiple queens (up to 20; Meudec 1973; Cournault, unpublished data). Fragments of 100–1,000 individuals from 137 colonies were collected from six different European populations ranging from the Pyrenees to southern Belgium. Vaucelles (50°06' 56" N–04°44'49" E; $n=42$ colonies) is a former quarry with rocky soil circled with woods and a pastured land, Vireux-Molhain (50°05'35" N–4°42'34" E; $n=17$) is an abandoned

Table 1 Occurrence of diploid males in ants

Species	Subfamily	% 2n M	% 2n nests	<i>N</i> pop	M/P	Authority
<i>Acromyrmex heyeri</i>	Myrmicinae	1.2 (118)	na	7	P	Araújo and Cavalli-Molina (2001)
<i>A. striatus</i>	Myrmicinae	0.7 (140)	na	10	P	Araújo and Cavalli-Molina (2001)
<i>Cardiocondyla obscurior</i>	Myrmicinae	1.5 (67)	11.1 (9)	1	P	Schrempf et al. (2006)
<i>Harpagoxenus sublaevis</i>	Myrmicinae	na	na	na	M	Fisher (1987), in Loiselle et al. (1990)
<i>Leptothorax acervorum</i>	Myrmicinae	1.3 (239)	10 (10)	1	P	Hammond et al. (2001)
<i>L. kutteri</i>	Myrmicinae	19.7 (61)	na	4	P	Buschinger and Fischer (1991)
<i>L. muscorum</i>	Myrmicinae	17.6 (169)	16.9 (63)	33	M	Loiselle et al. (1990)
<i>L. nylanderi</i>	Myrmicinae	5.2 (172)	6.1 (33)	1	M	Foitzik and Heinze (2001)
<i>Myrmoxenus stumperi</i>	Myrmicinae	na	na	na	M	Fisher (1987), in Loiselle et al. (1990)
<i>Solenopsis invicta</i> (introduced)	Myrmicinae	83.3 (150)	na	1	P	Krieger et al. (1999)
<i>S. invicta</i> (native)	Myrmicinae	13.1 (na)	na	2	P	Ross et al. (1993)
<i>Temnothorax ambiguus</i>	Myrmicinae	29.4 (17)	27 (11)	1	P	Herbers and Grieco (1994)
<i>Formica aquilonia</i>	Formicinae	1.2 (334)	na	2	P	Pamilo et al. (1994)
<i>F. lugubris</i>	Formicinae	na	18.2 (11)	1	M	Pamilo et al. (1994)
<i>F. polyctena</i>	Formicinae	6.9 (72)	na	1	P	Pamilo et al. (1994)
<i>F. pressilabris</i>	Formicinae	3 (199)	na	1	P	Pamilo et al. (1994)
<i>F. rufa</i>	Formicinae	na	4.4 (45)	2	M	Pamilo et al. (1994)
<i>F. truncorum</i>	Formicinae	9.8 (1,120)	na	1	P	Pamilo et al. (1994)
	Formicinae	na	3.5 (86)	2	M	Pamilo et al. (1994)
<i>Lasius alienus/niger</i>	Formicinae	2.3 (44)	20 (5)	1	M	Pearson (1982, 1983)
<i>L. sakagami</i>	Formicinae	na	20 (30)	1	P	Yamauchi et al. (2001)
<i>Proformica longiseta</i>	Formicinae	1 (102)	na	1	P	Fernández-Escudero et al. (2002)
<i>Pseudolasius</i> sp. nr <i>emeryi</i>	Formicinae	26 (27)	100 (1)	1	P	Hung et al. (1972)
<i>Rhytidoponera chalybaea/confusa</i>	Ectatomminae	na	na	52	P	Ward (1980)
<i>Tapinoma erraticum</i>	Dolichoderinae	0.9 (702)	11.6 (43)	4	P	This study

M/P monogynous or polygynous colony structure, % 2n M percentage of diploid males and total number of males analyzed (brackets), % 2n nests percentage of nests producing diploid males and total number of nests sampled (brackets), *N* pop number of populations surveyed, na data nonavailable

garbage grassland located between a road and a river, Treignes (50°05'24" N–04°38'56" E; *n*=15) is a calcareous, pastured land along a road, Tours (47°18'59" N–0°57'47" E; *n*=33) is an open calcareous grassland, nests from Toulouse (43°30'49" N–1°39'26" E; *n*=20) were collected along a road in open cultivated fields, and nests from Andorran valley (42°29'50" N–1°34'20" E; *n*=10) were collected from a pasture land in mountains (ca. 1,500–2,000 m above sea level).

Ploidy level analysis

Colony fragments greatly varied in their composition, especially in the number of mated (dealated) queens, unmated (alate) queens, and males collected. For each colony, ploidy level of 20 workers (*N*=2,740), zero to 11 inseminated queens (*N*=131), zero to 20 virgin winged queens (*N*=626), and zero to 30 winged males (*N*=702) was determined using flow cytometry (Aron et al. 2003;

Cournault and Aron 2008). We also analyzed ploidy level of sperm cells from spermathecal content of all mated queens collected (*N*=131) and from the seminal vesicles of diploid males (*N*=4). Flow cytometry allows characterization of nuclear DNA content by measuring the degree of fluorescence reflection of stained DNA in single cells. FCM is a well-proven method that is commonly used in life sciences for diagnosis or cell function analyses (Boek 2001). In addition, it has been widely applied for large-scale comparative analyses of genome-size evolution, taxonomic identification and delineation, and studies of polyploids, reproductive biology, and experimental evolution in both plants and insects (Kron et al. 2007). More recently, FCM has been used to characterize the structure and composition of microbial communities (Wedemeyer and Potter 2001).

We analyzed heads of *imagos* because the level of ploidy greatly varies among tissues (Aron et al. 2005). Heads were cut off and crushed each in 1 ml 4',6-diamino-2-phenylindole

dihydrochloride (DAPI) solution (CyStain® DNA-1 step, PARTEC®). Queen spermathecae and the seminal vesicles of males were carefully dissected in distilled water. Sperm was mixed in 200 µl of extraction solution (CyStain® DNA-2 step, PARTEC®) and gently vortexed for 30 s; 800 µl of DAPI-containing solution (CyStain® DNA-2 step, PARTEC®) was subsequently added. Ploidy level of all samples was determined using a PA-I flow cytometer (PARTEC®, Partec GmbH, Münster, Germany), with an optical arrangement as described in Cournault and Aron (2008). When triploid females were detected, their ploidy level was confirmed by analyzing a part of the thorax. Table 2 summarizes our sampling effort.

Statistics

Statistical comparisons of triploids or diploids frequencies were performed using log-likelihood ratio for contingency tables. They are referred as G test in the results. G_w refers to William's correction (for $n \times n$ tables) and G_y to Yate's correction for continuity (for 2×2 tables).

Results

Flow cytograms revealed the occurrence of both diploid males and triploid females (Fig. 2). Over all the six populations sampled, 92 of the 3,497 females analyzed (2.63%) were triploid. The proportion of triploid females differed significantly across castes (G test, $G_w=32.13$, $df=2$, $P<0.001$), with 3.28% triploid workers (90 of 2,740),

0.32% triploid virgin queens (two of 626), and 0% triploid egg-laying queens (zero of 131). Triploid females were uncovered in 23 colonies out of 137 (16.8%). Among these, the proportion of triploid workers was highly variable, ranging from 0% (triploids among virgin queens only) to 100% (all workers being triploid).

The proportion of colonies containing at least one triploid worker greatly varied between the six populations sampled (Fig. 3). No triploid worker was found in Toulouse whereas they were found in 20–25% of the nests in the Andorran valley and Vaucelles, the difference being highly significant (G test, $G_w=89.47$, $df=5$, $P<0.001$). The proportion of triploid workers was more frequent in Vaucelles than in any other location (proportion test, $\chi^2=111.3$, $df=5$, $P<0.001$).

Adult diploid males were found in three out of four populations (Tours, Vireux-Molhain, and Vaucelles; Table 2). They represented only six of the 702 males analyzed (i.e., 0.85%). Four of the six diploid males dissected produced diploid sperm cells (Fig. 2); dissection of the seminal vesicles of two males failed. Consistent with the production of diploid sperm cells by diploid males, analysis of spermathecal content revealed that four out of 131 queens (3.05%) stored diploid sperm. This value was not significantly different from the proportion of diploid males sampled (G test, $G_y=0.98$, $df=1$, $P>0.05$). Although they are based on a limited sample size, these results suggest that all or most diploid males produce diploid sperm cells. Co-occurrence of both haploid and diploid sperm cells in a single spermatheca was never observed, supporting that queens mated with diploid males were singly mated.

Table 2 Proportion of triploid workers, virgin queens, and reproductive queens (% 3n) and proportion of diploid males and of queen spermathecae containing diploid sperm cells (% 2n), in each of the six populations of *T. erraticum* sampled

Locality	Nc	Workers			Virgin queens			Queens			Spermathecae			Males		
		N	n 3n	% 3n	N	n 3n	% 3n	N	n 3n	% 3n	N	n 2n	% 2n	N	n 2n	% 2n
Andorran Valley	10	200 (10)	5 (2)	2.50	–	–	–	4 (4)	0	0	4 (4)	1 (1)	25	–	–	–
Toulouse	20	400 (20)	0	0	–	–	–	15 (10)	0	0	15 (10)	0	0	–	–	–
Tours	33	660 (33)	6 (2)	1.52	394 (22)	1 (1)	0.25	8 (6)	0	0	8 (6)	0	0	426 (20)	2 (2)	0.47
Treignes	15	300 (15)	3 (2)	1.00	45 (5)	1 (1)	2.50	20 (9)	0	0	20 (9)	0	0	10 (3)	0	0
Vireux-Molhain	17	340 (17)	7 (2)	2.06	100 (5)	0	0	50 (13)	0	0	50 (13)	2 (1)	4.00	129 (7)	1 (1)	0.78
Vaucelles	42	840 (42)	69 (11)	8.21	87 (13)	0	0	34 (16)	0	0	34 (16)	1 (1)	2.94	137 (13)	3 (2)	2.19
Total	137	2,740 (132)	90 (19)	3.28	626 (45)	2 (1)	0.32	131 (58)	0	0	131 (58)	4 (3)	3.05	702 (43)	6 (5)	0.85

Twenty workers were analyzed for each nest. The number of colonies for which ploidy level of sexuals or of sperm cells from queen spermathecae was determined is given between brackets

% 3n proportion of triploid workers, virgin queens, and reproductive queens, % 2n proportion of diploid males and of queen spermathecae containing diploid sperm cells, Nc number of nests sampled for each population, N number of individuals analyzed, n Xn number of triploid females or diploid males/sperm detected, – virgin queens and males not sampled in the studied populations

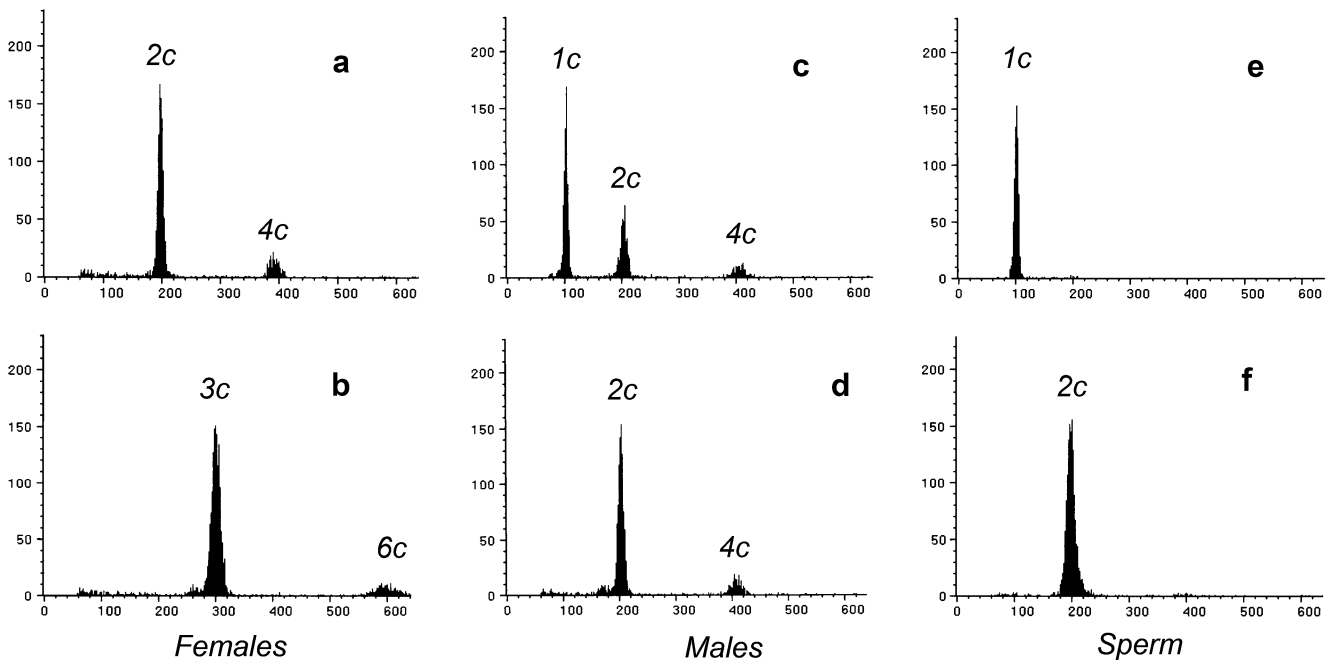


Fig. 2 Flow-cytometric DNA-content histograms of **a** diploid females, **b** triploid females, **c** haploid males, **d** diploid males, **e** haploid sperm, and **f** diploid sperm of *T. erraticum*. The first peak corresponds to ploidy level, the second to the distribution of nuclei over G2/M of the cell cycle stages (Aron et al. 2003). In haploid males (c), the second 2c peak corresponds to diploid mandibular muscle cells

(Aron et al. 2005). The flow cytometer was calibrated so that nuclei population from haploid cells yielded a relative 1c-DNA content near channel 100, nuclei population from diploid cells near channel 200, and nuclei population from triploid cells near channel 300. Number of nuclei analyzed was set so that peaks height reaches ca. 150 for all histograms

Discussion

Our survey reveals the presence of triploid females in native populations of the ant *T. erraticum*. Overall, they represented 2.63% of all females sampled. Triploid workers occurred in five of the six populations. Triploid virgin queens were found in two populations but in lower proportion than

triploid workers, and no triploid reproductive queen was detected across all populations studied. Diploid males were discovered in three out of four populations where winged males were collected, though in relatively low frequencies (0.47–2.20%).

Triploid females were found in populations where diploid males and/or diploid sperm cells from queen

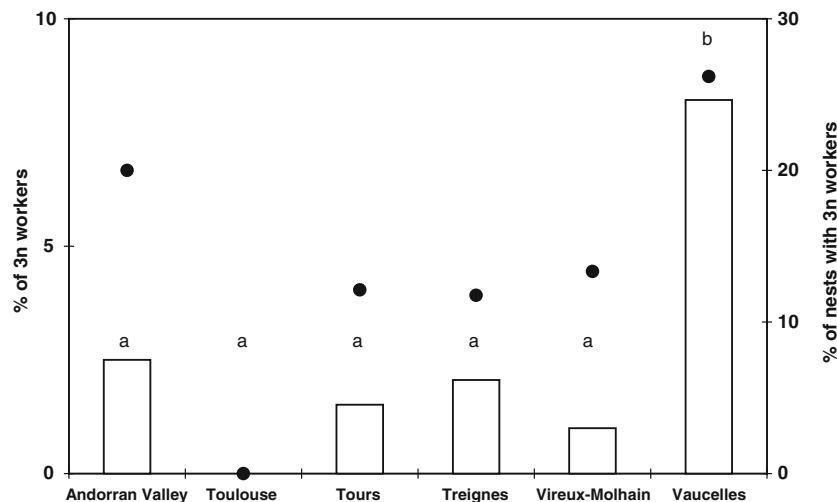


Fig. 3 Proportion of triploid workers (bars) and proportion of nests containing at least one triploid worker (black dots) among the six populations studied. Same letters indicate no significant differences in the frequencies of triploid workers between populations

spermathecae were detected, what is consistent with the hypothesis that triploid individuals are fathered by diploid males. Because of the weak proportion of diploid males, the current data do not allow testing a possible association between the frequency of diploid males and triploid females within populations. However, our flow cytometry analyses of sperm cells stored in male seminal vesicles and in queen spermathecae show that diploid males produce and successfully transfer diploid sperm to female sexuals.

Progeny of diploid males may be triploid females or triploid males if there is matched mating at the sex-determining locus (Fig. 1). Previous works reported the occurrence of triploid females among the offspring of females mated to related diploid males in *Athalia rosae* (Naito and Suzuki 1991), *C. vestalis* (de Boer et al. 2007), and in the bumblebee *Bombus terrestris* (Ayabe et al. 2004). No such triploid males were found in our study sample. One may, however, not exclude that such males are indeed produced, but remained undetected given the low frequency of diploid males (0.85%).

Mature diploid males have been reported in a number of ant species (Table 1). Aside from the present work, only two studies focused on their sperm production and a single study on their capability to father a triploid progeny. In *Lasius sakagami*, 20% of the colonies contain diploid males and flow cytometry analyses showed that diploid males produce diploid sperm (Yamauchi et al. 2001). Diploid males are remarkably common in introduced populations of the fire ant *S. invicta*, with proportions sometimes exceeding 70% (Ross and Fletcher 1985; Ross et al. 1993). However, only a limited fraction (2.4%) of these males reportedly produces sperm and may account for the occurrence of female triploidy (Krieger et al. 1999). In this species, triploid females were found in the introduced range (USA), not in the native habitat (Argentina; Krieger et al. 1999). Yet, diploid males also occur in native populations, with an estimated frequency ranging from 9.8% to 16.4% (Ross et al. 1993). Triploidy may remain to be detected in native populations of the fire ant, especially since Krieger et al. (1999) only sampled 149 workers in Argentina. Given the frequency of diploid males and the proportion of diploid males effectively producing diploid sperm, one should expect between 0.24% and 0.39% triploid workers in the population, i.e., less than one worker for this sample size.

Interestingly, we detected a tenfold lower proportion of triploid nonreproductive queens (0.37%) when compared to workers (3.44%), and we did not detect any triploid reproductive queen. Similar results were reported in introduced populations of the fire ant by Krieger et al. (1999). These authors proposed two nonmutually exclusive hypotheses to account for these results. First, the lower proportion of triploid queens may reflect differences in

female viability, with female sexuals dying from endogenous causes before mating or becoming reproductively active. Second, it may stem from the selective elimination of triploid sexuals by nestmate workers before they become egg layers. This second hypothesis requires specific *triploid cues* to allow workers discriminating between diploid and triploid nestmate queens and eliminate the latter. Previous studies have shown in the honeybee that cuticular hydrocarbon patterns of brood act as cues allowing workers to recognize and selectively eliminate diploid males from their colony (Santomauro et al. 2004). Unfortunately, the present study does not allow deciding between the two hypotheses. Whatever the mechanism for the lower proportion of triploid females in the reproductive caste, mating with a diploid male has substantial impact on queen and colony fitness. Because few or no triploid female sexuals will survive to reach the mated reproductive queen status, their reproductive value is reduced or equal to zero, and the fitness of queens mated with diploid male(s) will be mainly determined by the production of haploid, male offspring only.

The difference in the frequency of triploids between populations in *T. erraticum* likely results from variations in the proportion of diploid males. These variations may reflect between-population differences in their level of inbreeding or in the social organization of colonies. Sibmating increases the rate of homozygosity at the sex determining locus (loci) and hence the production of diploid males under complementary sex determination. Colonies of *T. erraticum* can produce males, females, or both sexes (Meudec 1979). Mating takes place during population-wide nuptial flights in which colonies synchronously release male and female sexuals, thus greatly decreasing the probability of close relatives contacting one another. However, one may not exclude intranidal mating in some colonies producing both sexes. How variations in the level of inbreeding affect the proportion of diploid males (and triploid females) in the populations studied remains unknown. Differences in diploid male production between populations may also result from variations in colony social organization in *T. erraticum*. Colonies of this species show two distinctive social forms; they may be headed by either a single queen (monogynous) or several queens (polygynous). In ants, social structure is usually associated with dispersal strategies (Bourke and Franks 1995; Crozier and Pamilo 1996; Ross 2001). Single-queen colonies produce new queens that disperse from their natal nest during mating flights and initiate new colonies alone with no worker assistance. In contrast, new queens from multiple-queen colonies tend to mate in or close to their natal nest and are frequently readopted to initiate reproduction within the mother nest. Thus, solitary foundresses mated with a male bearing the same allele at the sex determining locus will see half of the fertilized eggs develop into diploid

males rather than workers. Incipient colonies therefore waste crucial resources that should be devoted into building up a strong worker force (because males do not work) and will most likely die prematurely (Ross and Fletcher 1985, 1986). By contrast, multiple-queen colonies usually spread by budding, young mated queens leaving the natal nest with the help of a worker force, ensuring sustained colony growth and survival. Budding circumvents the critical step of solitary colony foundation, and selection against the production of diploid males is greatly reduced. No molecular markers have been characterized for the study species so far, preventing determination of the relationship between the social structure, dispersal strategy, and diploid male production. However, reliable estimates of the absolute number of queens per colony are available for two populations, Vaucelles and Treignes, where excavation of entire colonies was performed (Cournault, unpublished data). Our data show that the proportion of diploid males was fourfold higher in Vaucelles (2.20%), where 100% of nests are polygynous, than in Tours (0.47%) where more than 70% of nests are strictly monogynous.

In conclusion, our results show that diploid males and triploid females are reared in native populations of the ant *T. erraticum* and that diploid males produce diploid sperm. Both diploid males and triploid females occur in moderate proportions at the adult stage, but they might be produced in much higher quantity and may die prematurely or may be selectively eliminated by the workers during their development. The lower frequency of triploids among queens (unmated and mated) as compared to workers makes this hypothesis plausible. Future experiments investigating variations in the frequency of triploids among females, from the egg to adult stage, should provide a more clear-cut answer in this respect. Moreover, the presence of diploid male and triploid female offspring is consistent with the CSD model in *T. erraticum*. Since male diploidy is linked to homozygosity in the CSD model for sex determination in Hymenoptera, further studies should explore between- and within-population variations in the sociogenetic structure (level of inbreeding, queen mating frequency, effective level of polygyny, dispersal strategy) and their impact on the frequency of diploid males and triploid females. Finally, the present study shows that the frequency of triploids may greatly vary both between populations and between colonies within populations. These variations, combined with the fact that diploid male production is not exceptional in native populations of social Hymenoptera, suggest that triploid females may have remained largely unnoticed and could occur in much more hymenopteran species than reported to date.

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