Viable Triploid Honey Bees (Apis mellifera capensis) Are Reliably Produced in the Progeny of CO2 Narcotised Queens

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ABSTRACT The haplodiploid system of sex determination of Hymenoptera acts as an exaptation for species to evolve novel forms of asexual reproduction including thelytoky (clonal offspring of the mother). During normal reproduction in Hymenoptera, three of the four products of meiosis that are present in newly-laid eggs are lost as polar bodies, while the remaining pronucleus either develops as a haploid male or fuses with a sperm nucleus to produce a diploid zygote. In contrast, in thelytokous reproduction, which is uncommon but taxonomically widespread, two of the four products of meiosis fuse, as if one acted as a sperm. Queenless workers of Apis mellifera capensis, a subspecies of honey bee from South Africa, routinely reproduce thelytokously. Unmated A. m. capensis queens can also be induced to lay thelytokously by narcosis with carbon dioxide, but mated queens are never thelytokous. We artificially inseminated A. m. capensis queens using CO2 narcosis. Up to 1/3 of offspring workers carried two maternal alleles and an allele of one father whereas no three-allele progeny were seen in control queens of the arrhenotokous (unfertilized eggs result in males) subspecies A. m. scutellata. Flow cytometry of three-allele individuals revealed that they were triploid and arose from the fertilization of a thelytokous fusion nucleus. We then reared six queens from a narcotized A. m. capensis queen and determined the ploidy of the offspring queens based on microsatellites. One of the five daughters was triploid. Following artificial insemination, this queen produced unfertilized thelytokous diploid eggs at high frequency, and unfertilized triploid eggs at much lower frequency. If fertilized, thelytokous diploid eggs were non-viable, even though triploidy in itself does not impede normal development. In contrast, when the rarer triploid eggs were fertilized, a proportion developed into viable tetraploids. Our study highlights the extraordinary developmental flexibility of haplo-diploid systems.

KEYWORDS Thelytokous parthenogenesis haplo-diploidy central fusion triploid

In Hymenoptera (ants, wasps and bees) males are haploid and females are diploid (White 1973). Often, the switch that determines sex is a single locus, the complementary sex determinant (csd) (Cook and Crozier 1995; Beye et al. 2003; Heimpel and de Boer 2008). When an individual is heterozygous at csd, it develops as a female. Eggs that are hemizygous or homozygous at csd develop as a male, but homozygotes develop into diploid males, which, in honeybees, are eaten by workers at the first larval instar (Woyke 1963).

Haplodiploidy acts as an exaptation (sensu Gould and Vrba 1982) for unusual modes of reproduction. In particular, there is no impediment to an unfertilized egg developing into an adult (Heimpel and de Boer 2008). That is, given the right conditions a haploid pro-nucleus can divide mitotically and produce an embryo or part of an embryo. Unlike mammals and most other insects, fertilization is not necessary for development (Sasaki and Obara 2002).

The possibility of egg development without the need of fertilization gives rise to some interesting anomalies (Schwander and Oldroyd 2016).
First, because the eggs of some or most haplodiploids are polyploidy (two or more sperm enter each egg, Baer et al. 2016), it is possible to have fusion between a maternal pro-nucleus and a sperm nucleus to generate a normal diploid zygote, while one or more of the additional sperm also start dividing producing haploid male tissue. These individuals, known as gynandromorphs, are mosaics of bi-paternal female tissue and paternally-derived haploid male tissue (Rothenbuhler et al. 1952; Drescher and Rothenbuhler 1963; Rothenbuhler 1954). Such individuals are expected to carry one maternal and two or more paternal alleles at some loci.

Second, if a sperm cell fertilizes an anucleate egg, a sperm nucleus can potentially give rise to a clone of the sperm donor by ‘androgenesis’. Androgenesis is a well-established phenomenon in the honeybee Apis mellifera (Koeniger et al. 1989) and three ant species (Fournier et al. 2005; Kobayashi et al. 2008; Pearcy et al. 2004). In some ants a combination of androgenesis and thelytoky leads to the extraordinary situation in which queens clone themselves to produce daughter queens, and males clone themselves to produce sons (Fournier et al. 2005; Foucaud et al. 2007).

Third, two sperm can fuse in an egg to produce diploid female tissue (Laidlaw and Tucker 1964). Thus far we only have reports of mosaics: mixtures of tissues derived from the fusion of two sperm and the fusion of a maternal pronucleus and a sperm nucleus. However, it is plausible that females that are entirely or mostly derived from the fusion of two sperm are possible.

Finally, embryos can be derived from the fusion of two maternal pronuclei, giving rise to females by thelytokous parthenogenesis. Whereas the unusual forms of egg development discussed above are rare, thelytoky is widespread in Hymenoptera (Rabeling and Kronauer 2013).

Following oviposition, the primary oocyte completes meiosis I to produce two maternal oocytes. These then undergo second division meiosis resulting in four maternal pronuclei (Verm and Ruttner 1983; Cole-Clark et al. 2017). In most subspecies three of the four maternal pronuclei degenerate (Snodgrass 1956). If the egg has been fertilized the remaining pronucleus will fuse with a sperm nucleus to produce eggs. If the egg has not been fertilized the remaining pronucleus will divide mitotically and produce a male via arrhenotoky parthenogenesis.

In the southern provinces of South Africa there exists a unique honey bee subspecies which is thelytokous (reviewed in Beekman et al. 2008). In this subspecies, A. m. capensis, (hereafter Capensis) queenless workers lay eggs that mostly result in females via thelytokous parthenogenesis, whereas in other honey bee species and subspecies such as the African bee A. m. scutellata (hereafter Scutellata), unmated workers produce male offspring via arrhenotoky parthenogenesis (Goudie and Oldroyd 2014) (but see Remnant et al. 2014; Tucker 1958; Mackensen 1943).

We have examined the progeny of A. m. capensis queens that have simply been instrumentally inseminated; a process that requires CO₂ narcosis. We discovered up to 1/3 of eggs and adult workers carried two maternal alleles and one paternal allele. We determine the nature of these individuals (triploid or mosaics of haploid and diploid tissue) using a combination of molecular markers and flow cytometry. We also report on the viability and ploidy of the progeny of a single triploid queen.

**MATERIALS AND METHODS**

**Experiment 1. CO₂ narcosis induces three-allele progeny**

In Stellenbosch South Africa, we inseminated four Capensis virgin queens each with the semen of a single Scutellata male and four Scutellata queens, each with the semen of a single Capensis male (Figure 1). Virgin queens were introduced to small colonies and prevented from leaving on mating flights by grids placed on the front of the hives. We used standard insemination procedures that involved relatively brief narcosis during insemination (2-3 min), and a 10 min narcosis the day after insemination to induce oviposition (Harbo 1986). We retained the males and the wing clippings of the queens for later genotyping, and harvested pupae from worker cells for genotyping. Samples were stored in 95% ethanol at -20°C. We then genotyped the parents and 12-25 mature pupal progeny from worker cells at microsatellite loci A8, A14, A79, A88, A113, B124, A107, Ap43 (Solignac et al. 2003) and HB-The-03 (Shaibi et al. 2008).

**Experiment 2. Are the three-allele individuals triploids or mosaics?**

During Experiment 1 we found that a significant proportion of offspring of the four Capensis queens carried three alleles at multiple loci. To clarify whether these offspring were triploids or mosaics we required frozen bees that were suitable for flow cytometry. We therefore inseminated two new Capensis queens, each with the semen of four Capensis drones unrelated to the queen or each other as above. We retained the queens’ wing clippings and the drones in 95% alcohol for genotyping. Pre-emergent workers (PEW, n = 200 per colony) were collected on to dry ice nine weeks post-insemination, thereby ensuring that all progeny originated from the inseminated queen.

We genotyped queens, drones and worker offspring pupae using microsatellite loci A8, A29, A79, A88, A113, B124 and Ap43 (Solignac et al. 2003), determining the number of alleles at each locus and the parental origin of each allele. From this analysis we randomly selected four workers carrying two alleles and four workers carrying three alleles at least four of the seven loci from each colony. In addition, we sampled one diploid worker and one haploid drone from Sydney Australia, and three haploid A. m. capensis males to provide reference specimens for ploidy.

Flow cytometry was then used to determine the ploidy of the selected PEW. Because some honey bee tissues are known to undergo autopolyploidyization, we sampled tissue from both brain (which does not undergo autopolyploidyization) and thorax (which does, at least in males) (Aron et al. 2005).

Dissected tissue was suspended in 200 μl Galbraith Buffer (45 mM magnesium chloride, 30 mM sodium citrate, 20 mM 4-morpho-G line propane sulfonate and Triton X-100 (1 mg/mL)) and homogenized to release the nuclei with a Tenbreck homogenizer using six gentle strokes. The tissue was then filtered through 10 μm nylon mesh (Rangel et al. 2015). To stain the nuclei, 1 μl of DAPI staining solution (10 mg/ml) was added to each sample and incubated for 15 min at 4°C in the dark.

The DNA content of each nucleus was measured by its relative fluorescence using a 5-laser Becton Dickinson LSR-II flow cytometer (BD Bioscience, San Jose, CA). The DNA-bound DAPI was excited with four lasers: 488 nm laser, 500 nm laser, 545 nm laser, and 633 nm laser. The fluorescence was detected using PMT detectors equipped with a 450/50 bandpass filter. The same fluorescent detector gain settings were used for all samples. The gates for the identification of nuclei and the analysis of fluorescence intensities were consistent across all samples.

The brain tissue of the Australian drone and worker were used to identify C1 (haploid) and C2 (diploid) fluorescent peaks respectively. Peaks representing C1, C2, and C4 (tetraploid, most likely dividing cells) in Capensis drones and Capensis two-allele and three-allele workers, were then inferred from these standards. Frequency histograms of the number of cells of each level of ploidy where produced using BD FACSDiva software version 8.0.1 (BD Bioscience, San Jose, CA).
Experiment 3. A viable triploid queen and her progeny

In our final experiment we inseminated a Capensis queen with semen from a single Scutellata male using CO2 narcosis as above. We then reared six super-sister Capensis x Scutellata queens from this individual and inseminated each with the semen of a single A. m. capensis male (Figure 1). It is important to note that these queens were daughters of a CO2-narcotised Capensis queen, even though they themselves were only 50% Capensis (Figure 1). We retained a wing clipping of each queen. We extracted DNA from the wing clippings using Chelex (Walsh et al. 1991) and genotyped the extract at six microsatellite loci (A14, A29, A88, A107, A113, B124, Estoup et al. 1993; Solignac et al. 2003). One of the six queens showed three alleles at four of the six microsatellite loci, indicating that she was triploid (Table S1). The other five queens were diploid.

The triploid queen, AI 109, was phenotypically normal (Figure 2) and maintained 2-3 brood combs of eggs throughout her life from extraction of the wing clipping. Figure 2 A. Pre-emergent worker with three alleles at multiple loci from an A. m. capensis queen x A. m. scutellata male cross. The female is a phenotypically normal worker, with no evidence of male tissue. B. A triploid honey bee queen (with numbered identifying tag green 73). The queen was phenotypically normal, but very few of her eggs were viable.

Figure 1 Crossing designs. Experiment 1. Four A. m. capensis and four A. m. scutellata queens were crossed in reciprocal. Pre-emergent worker pupae were collected and genotyped. Experiment 2. Two A. m. capensis queens were inseminated with semen from four unrelated A. m. capensis males. Offspring workers were genotyped and their ploidy levels determined by flow cytometry. Experiment 3. An A. m. capensis queen was inseminated with the semen of a single A. m. scutellata male. Five daughter queens were reared from this queen, and each was inseminated with the semen of a single A. m. capensis male. One of the five queens was triploid.
November 2017 to January 2018. During this period we supported her colony by adding frames of emerging brood from A. m. scutellata colonies on a monthly basis. We marked the added frames, and did not sample brood items from them for at least 22 days after addition of the donor brood. By this time all donor brood would have emerged from their cells.

**Ploidy of the triploid queen’s brood:** On two occasions, we collected all brood items beyond egg stage, and about 100 eggs, from AI 109’s colony. We genotyped progeny based on the microsatellite loci listed above, and determined whether they were progeny of AI 109 those of the unrelated workers that comprised her colony, and the genotypic ploidy of each individual.

**Egg viability:** To quantify the proportion of eggs that hatched we cut small pieces of brood comb containing c.a. 100 eggs from AI 109’s colony and placed them in a zip-lock bag containing a moist paper towel, and placed the bag in an incubator at 34.5°C. As a control, we cut similar pieces of comb from the brood combs of three diploid queens that were super-sisters of the triploid queen.

**Data availability statement**
File S1 lists the microsatellite genotypes of pupae from reciprocal crosses between Capensis and Scutellata queens and drones (Experiment 1). File S2 lists the microsatellite genotypes of the four two-allele and four three-allele individuals used for flow cytometry (Experiment 2). File S3 provides the genotypes of the six daughter queens of a Capensis queen x Scutellata male cross (Experiment 3). File S4 provides the microsatellite genotypes of the triploid queen and her brood. Supplemental material available at Figshare: https://doi.org/10.25387/g3.6873998.

### RESULTS

**Experiment 1. CO₂ narcosis induces triploidy in the progeny of A. m. capensis queens**

All four Scutellata queens inseminated with a Capensis male produced phenotypic females that had normal diploid genotypes at each locus, or an expected homozygote where the queen and the inseminating male carried the same allele (Table 1). All four of the Capensis queens inseminated with an A. m. scutellata male also produced normal diploid offspring, but also some phenotypically normal female progeny (Figure 2), that carried three alleles, two maternal and one paternal, at multiple loci (Table 1). On average, 14% (range 5–33%) of offspring per colony carried three alleles at multiple loci.

We verified the three-allele genotypes with a second PCR of the hind leg and the front leg. The hind leg and the front leg had identical genotypes in all cases suggesting that the individuals were either triploid or a homogeneous mosaic of two or more diploid cell lines that shared the same maternal allele. There was no evidence of androgenesis or thelytoky in any progeny.

**Experiment 2. Are three-allele individuals triploids or mosaics?**

The two queens each produced phenotypically normal female progeny with 5% and 8% (n = 200 per colony) of daughters carrying three alleles at multiple loci respectively. Genotyping revealed that all these individuals carried two maternally-derived alleles and one paternal allele at each of the three-allele loci (Table S2).

We suggest that there are three plausible scenarios that could give rise to the three-allele individuals identified based on microsatellite markers (Figure 3). (a) A mosaic diploid individual could arise from two maternal pronuclei each separately fusing with a different sperm nucleus,

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| Maximum number of alleles per locus | Scutellata queen x Capensis male | Capensis queen x Scutellata male |
|-----------------------------------|---------------------------------|---------------------------------|
| 2                                 | a 12 | b 20 | c 18 | d 25 | e 17 | f 18 | g 12 | h 17 |
| 3                                 | 0 0 0 | 0 0 0 | 0 0 0 | 0 0 0 | 0 0 0 | 0 0 0 | 0 0 0 | 0 0 0 |
giving rise to an individual that is a mosaic of diploid cells descended from two or more zygotes. In this case the flow cytometry would reveal C2 nuclei and the microsatellites two maternal and one or more paternal alleles. (b) A triploid individual might arise from the fusion of sperm with two maternal pronuclei. In this case flow cytometry would show that the majority of nuclei were C3 and the microsatellite analysis would show two maternal alleles and one or more paternal alleles. (c) Two sperm nuclei enter an egg and fuse with a single maternal pronucleus. Under this hypothesis we predict that cytometry would show the individual as C3 and the microsatellite genotyping would show one maternal and two paternal alleles.

In order to verify whether our three-allele individuals were true triploids or diploid mosaics with two cell lines, we determined the ploidy of four 2-allele and four 3-allele individuals per colony by flow cytometry (Figure 4). To correct for variation in the number of cells per sample we converted counts of 2n and 3n cells to the proportion of the total cell count. Data were then analyzed using two generalized linear models, as implemented in SPSS, of the effect of tissue (brain vs. thorax) and allele number (2 alleles vs. 3 alleles) on the frequency of cells classified as 3n or 2n with a normal link function. We nested the effect of individual bee within allele number.

There was a highly significant difference ($P < 0.001$) in the ploidy of cells extracted from bees with two alleles per locus compared to bees with three alleles per locus (Table 2). The vast majority of cells from three-allele individuals were triploid, whereas cells from two-allele individuals were diploid (Figure 5). In combination with the genotyping results, this is strong evidence that the three-allele individuals were triploids formed by the fusion of the two central maternal pronuclei and one sperm nucleus (hypothesis b in Figure 3).

Examination of the genotypes revealed no thelytokous progeny (i.e., two maternal alleles and no paternal alleles). For each triploid individual only one paternal allele was detected, providing additional evidence that these individuals arose by the fusion of the two central maternal pronuclei with a sperm.

There is no evidence that any particular male’s sperm was more likely to form triploid embryos. A cross tabulation of paternity and ploidy showed no association (Queen 1, $\chi^2 = 2.253$, df = 3, $P = 0.522$; queen 2 $\chi^2 = 5.301$, df = 3, $P = 0.151$).

**Experiment 3. A viable triploid queen and her progeny**

**Ploidy of brood items:** In worker cells the majority of eggs were triploid and fertilized (Table 3, see Supplementary Table S3 for raw genotyping data). In drone cells the majority of eggs were diploid and unfertilized, but all pupae in drone cells were worker laid, suggesting that unfertilized diploid eggs laid in drone cells were non-viable.

**Egg viability:** Although the triploid queen was phenotypically normal (Figure 2) and estimated to have laid hundreds of eggs per day, like a normal queen, few of the eggs hatched. Only one in 200-300 brood cells contained a brood item beyond egg stage. None of AI 109’s 73 eggs laid in drone cells or 144 eggs laid in worker cells hatched within 72 hr in

![Figure 4](image-url) Flow cytometry histograms showing the nuclei count (Y axes) at different fluorescence levels (X axes). Each peak represents the ploidy level in the tissue sample in brain and thorax for workers with two or three alleles at multiple loci. The two right hand panels are for the single haploid A. m. capensis drone that was used to calibrate the flow cytometry results. This in turn was calibrated against an Australian worker and drone (data not shown). In the male, the brain tissue is haploid and the thoracic tissue is diploid as a result of autopolyploidization (Aron et al. 2005).
our incubator study, whereas 93.3% (range 90.0–97.5%) of the 468 diploid queen’s eggs laid in worker cells by three super-sister queens hatched within 72 hr (Table 4). This showed that AI 109 had very low egg viability, even though we were able to harvest a small number of larvae and pupae from drone and worker cells.

**DISCUSSION**

This study has shown that triploid queens and workers can be reliably produced (5–33%) in the progeny of Capensis queens narcotized with CO₂ during standard insemination procedures. The genotype of the inseminating male is irrelevant, since Capensis queens inseminated with both Capensis semen (Experiment 2) and Scutellata semen (Experiments 1 and 3) all produced triploid offspring. Triploid females arise following fertilization of a diploid nucleus that formed as a result of the thelytokous fusion of two maternal pronuclei. Although we did not study the behavior of triploid workers, the triploid queen was fully viable and produced thousands of eggs. A small proportion of her eggs were viable.

We suggest that the triploid individuals we produced arose as an interaction between the thelytokous Capensis strain and CO₂ narcosis. Because Capensis are thelytokous the maternal pronuclei are receptive to fusion and fertilization rather than degeneration (as happens in other subspecies). Whether the sperm nucleus fuses with an already-formed thelytokous nucleus or whether the three nuclei fuse simultaneously is unclear. Our study emphasizes the extraordinarily broad range of effects of CO₂ narcosis on honey bee behavior (Ribbands 1950; Simpson 1954; Skowronek 1976), physiology (Harris et al. 1996), reproduction (Koywiwattrakul et al. 2005), gene expression (Niño et al. 2011; Brito et al. 2010; Manfredini et al. 2015) and embryogenesis (this study, Oldroyd et al. 2008; Crewe and Allsopp 1994).

It is unlikely that triploidy is a common phenomenon in the wild A. m. capensis population. Triploids have not been reported in any large-scale genotyping studies of sexually-produced Capensis workers from naturally-mated queens (e.g., Beekman et al. 2009; Moritz et al. 2011; Holmes et al. 2010; Goudie et al. 2012; Beekman et al. 2011). Triploids only seem to arise after CO₂ narcosis, which is known to induce thelytoky in virgin Capensis queens (Crewe and Allsopp 1994; Oldroyd et al. 2008). We note that in Hymenoptera, triploid females can also arise as a consequence of a diploid male mating with a normal diploid female in inbred populations (Harpur et al. 2013; Leibert et al. 2004; Krieger et al. 1999; Ayabe et al. 2004; Darvill et al. 2012; Chaud-Netto 1980). However in honey bees, diploid males are never found in nature because diploid male larva are removed by nurse bees (Woyke 1963). Therefore this is the first report of wide-spread triploidy in outbred honey bees.

In Figure 6 we propose a model describing the possible cytogenetic origins of the progeny of triploid queen. In meiosis I, we suggest that the triploid germ cell undergoes chromosome duplication and cell division to produce two daughter cells with duplicated chromosomes, one tetraploid, one diploid. The two cells of meiosis I then undergo a reductional division in meiosis II to produce two haploid and two diploid daughter cells (Figure 6). The two diploid cells carry non-sister chromosomes, though there may be some exchange of genetic material between the two because of recombination in meiosis I meaning that some loci that were heterozygous in the mother will be homozygous in the offspring (Goudie and Oldroyd 2014).

Below we refer to unfertilized eggs as oocytes and fertilized eggs as eggs. The majority of eggs/oocytes in worker cells were triploid and fertilized. We propose that the triploid eggs arose when a diploid oocyte

![Table 2 Results of two generalized linear models for the effect of allele number (2 or 3 per locus) on the proportion of cells per bee (n = 16 bees per allele number) assessed as being diploid (C2) or triploid (C3) based on flow cytometry. The effect of tissue (brain vs. thorax) is included in the models, with replicate bee nested within allele number.](image)

| Source                      | d.f | Wald χ² | P    | Wald χ² | P    |
|-----------------------------|-----|---------|------|---------|------|
| Tissue                      | 1   | 0.375   | 0.541| 21.76   | < 0.001|
| Allele number               | 1   | 657.492 | < 0.001| 2989.504| < 0.001|
| Bee(allele number)          | 14  | 23.8    | 0.048| 53.775  | < 0.001|

![Figure 5 Mean proportion of nuclei (%) measured as being diploid or triploid in two tissue types (brain and thorax) by flow cytometry for individuals showing two or three alleles at multiple loci. Error bars are standard error of the mean. n = eight workers for three alleles and eight workers for two alleles.](image)
was fertilized (Figure 6). However, no triploid individuals carrying paternal alleles were identified in pupae (Table 2), indicating that triploid eggs were inviable.

A few diploid eggs were found in worker cells. These probably arose when a haploid oocyte was fertilized. However, no diploid fertilized pupae were found. This is surprising because there is no reason to suspect that a diploid fertilized embryo would not be fully viable and result in a normal worker. Our failure to identify such pupae may be because diploid eggs are rare (Table 4), rather than a lack of viability. Curiously, if our model is correct, we would expect approximately equal numbers of diploid and haploid oocytes to be produced by the triploid queen (Figure 6). Therefore the much higher frequency of diploid oocytes than haploid oocytes is unexplained by our model. Potentially some diploid oocytes arose from a thelytokous fusion of haploid maternal pronuclei, rather than a diploid pronucleus.

In pupae from worker cells, most individuals were tetraploid, apparently resulting from the fertilization of a triploid oocyte (Figure 6). Tetraploid eggs were not found in worker cells, suggesting that they are rare but viable. Supporting this conjecture, triploid oocytes were the most common egg in drone cells, suggesting that triploid oocytes are produced at low frequency and can result in a viable tetraploid when laid in a worker cell and fertilized.

Most eggs produced by AI 109 were not viable. In some instances this is surprising. Haploid eggs laid in drone cells should develop normally into adult drones, but none were seen. Similarly, haploid oocytes that are then fertilized should produce viable workers, but none were seen. Our study shows that triploid female honey bees are fully viable. Why, then, were the majority of triploid fertilized eggs (the most common class of eggs in worker cells) non-viable? One possibility is that errors in chromosome disjunction during meiosis did not lead to a neat segregation of haploid and diploid female gametes as suggested in Figure 6, but to eggs with varying numbers of chromosomes, and perhaps missing chromosomes. Such eggs may be incapable of normal development. Only the occasional egg may have had a complete, balanced set of chromosomes, sufficient for proper development. The genotypes of eggs from worker cells are compatible with this hypothesis. Eggs with three or four alleles at particular loci did not show this pattern across all loci, suggesting variation in chromosome number. Nonetheless, such a pattern is also compatible with a loss of heterozygosity due to recombination at meiosis I (Goudie and Oldroyd 2014). That is an egg may have a complete haploid or diploid set of chromosomes, but two, or three chromosomes may share the same microsatellite allele, erroneously suggesting haploidy.

Alternatively, the low egg viability observed here may be a consequence of the sex determination system. Csd encodes an SR-type protein. When csd is heterozygous it produces a functional product that initiates development of a female phenotype by activating AmDSX. When homozygous or hemizygous it produces a non-functional product that allows male development by default (Beye et al. 2003). This suggests that if any two csd alleles present in a diploid, triploid, or tetraploid embryo are different, then the female development pathway should be switched on via the production of a functional csd protein. This implies that most fertilized eggs should be viable because they are expected to carry different csd alleles. Therefore the non-viability of most triploid and diploid eggs is most likely not a consequence of sex determination, and we therefore suggest that errors in chromosomal

### Table 3 Ploidy of brood items produced by a triploid A. m. capensis queen

| Developmental stage | Origin   | Ploidy      | Count | Total |
|---------------------|----------|-------------|-------|-------|
| Pupae               | Worker cells | Tetraploid fertilized | 16   |       |
|                     |          | Triploid unfertilized | 3    |       |
|                     |          | Triploid fertilized | 0    |       |
|                     |          | Diploid fertilized | 0    |       |
|                     |          | Diploid unfertilized | 0   |       |
|                     |          | Haploid | 0    | 19    |
| Eggs                | Worker cells | Tetraploid fertilized | 0   |       |
|                     |          | Triploid unfertilized | 0   |       |
|                     |          | Triploid fertilized | 127  |       |
|                     |          | Diploid fertilized | 29   |       |
|                     |          | Diploid unfertilized | 30   |       |
|                     |          | Haploid | 0    | 186   |
| Pupae               | Drone cells | All worker laid |       |       |
| Eggs                | Drone cells | Tetraploid fertilized | 0   |       |
|                     |          | Triploid unfertilized | 10  |       |
|                     |          | Triploid fertilized | 1    |       |
|                     |          | Diploid fertilized | 1    |       |
|                     |          | Haploid | 0    |       |
|                     |          | Diploid unfertilized | 36   | 48    |

### Table 4 Egg viability from a triploid queen honey bee

| Queen number | Ploidy based on microsatellites | Source of eggs | Number of eggs scored | Number of eggs that hatched | Egg viability (%) |
|--------------|---------------------------------|----------------|-----------------------|----------------------------|------------------|
| AI 109       | Triploid                        | Drone cells    | 73                    | 0                          | 0                |
|              |                                 | Worker cells   | 144                   | 3                          | 2.1              |
| AI 106       | Diploid                         | Worker cells   | 80                    | 78                         | 97.5             |
| AI 107       | Diploid                         | Worker cells   | 180                   | 162                        | 90.0             |
| AI 108       | Diploid                         | Worker cells   | 208                   | 193                        | 92.7             |
disjunction during the formation of eggs is the more likely explanation of low egg viability in the triploid queen.

The reliable production of large numbers of triploid workers by CO₂ narcosis of Capensis queens provides new opportunities for studying development and sex determination mechanisms in bees. When non-Capensis honey bee queens are inseminated by related males, individuals that are diploid but homozygous at csd are produced among progeny. Such individuals are phenotypic males, but are removed by nurse workers at the first larval instar (Beye et al. 2003; Woyke 1963). An interesting experiment would be to narcotize a Capensis queen and then inseminate her with a brother. Such a queen should produce some triploid individuals that carry two identical sex alleles (one maternal and one paternal) and one non-identical (maternal) sex allele. Would such individuals be viable and if so would they be male or female? Would the csd product be functional? Our study shows that Capensis provides an extraordinary experimental system in which to examine the underlying genetics of haplodiploidy and sex determination in bees.

Figure 6 A model explaining the genetic origin of brood items observed in the progeny of a triploid honey bee queen. The model depicts the meiotic events of a single chromosome and its homologs. A triploid germ cell in the queen undergoes meiosis I. Meiosis II occurs after the egg is laid resulting in two diploid and two haploid pronuceli. A proportion of the central pronuceli fuse to produce a triploid fusion nucleus, which is usually fertilized if laid in a worker cell and not fertilized if laid in a drone cell.
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