Heredity (2015) 114, 584–592

www.nature.com/hdy

ORIGINAL ARTICLE

Inheritance of thelytoky in the honey bee *Apis mellifera capensis*

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Asexual reproduction via thelytokous parthenogenesis is widespread in the Hymenoptera, but its genetic underpinnings have been described only twice. In the wasp *Lysiphlebus fabarum* and the Cape honey bee *Apis mellifera capensis* the origin of thelytoky have each been traced to a single recessive locus. In the Cape honey bee it has been argued that *thelytoky* (*th*) controls the thelytoky phenotype and that a deletion of 9 bp in the flanking intron downstream of exon 5 (*tae*) of the *gemini* gene switches parthenogenesis from arrhenotoky to thelytoky. To further explore the mode of inheritance of thelytoky, we generated reciprocal backcrosses between thelytokous *A. m. capensis* and the arrhenotokous *A. m. scutellata*. Ten genetic markers were used to identify 108 thelytokously produced offspring and 225 arrhenotokously produced offspring from 14 colonies. Patterns of appearance of thelytokous parthenogenesis were inconsistent with a single locus, either *th* or *tae*, controlling thelytoky. We further show that the 9 bp deletion is present in the arrhenotokous *A. m. scutellata* population in South Africa, in *A. m. intermissa* in Morocco and in Africanized bees from Brazil and Texas, USA, where thelytoky has not been reported. Thus the 9 bp deletion cannot be the cause of thelytoky. Further, we found two novel *tae* alleles. One contains the previously described 9 bp deletion and an additional deletion of 7 bp nearby. The second carries a single base insertion with respect to the wild type. Our data are consistent with the putative *th* locus increasing reproductive capacity.
Heredity (2015) **114,** 584–592; doi:10.1038/hdy.2014.127; published online 14 January 2015

INTRODUCTION

There are few exceptions to sexual reproduction in the animal kingdom. Sexual reproduction may confer advantages over asexual reproduction by facilitating the purging of deleterious mutations (Muller, 1964) and by generating genetic variability among offspring, so that at least some of them will be resistant to the parasites and pathogens that they may encounter (Hamilton, 1980; Hamilton and Axelrod, 1990). Curiously, asexual reproduction via thelytokous parthenogenesis (the production of diploid females without fertilisation) is widespread in one important group of animals, the Hymenoptera (ants, wasps and bees), and particularly the social Hymenoptera. Up to 50 social Hymenopterans have been associated with thelytokous parthenogenesis (Crozier and Pamilo, 1996; Wenseleers and Oystaeyen, 2011; Rabeling and Kronauer, 2013). In Hymenopterans thelytokous populations are often derived from sexual populations (Wenseleers and Oystaeyen, 2011). This suggests a simple molecular switch from arrhenotoky, the normal mode of male production in haplodiploid insects, to thelytoky. Indeed, genetic control of thelytokous parthenogenesis via a single locus has been reported in the parasitoid wasp *Lysiphlebus fabarum* (Sandrock and Vorburger, 2011) and the honey bee *Apis mellifera capensis* (Lattorff *et al.*, 2005; Lattorff *et al.*, 2007; Jarosch *et al.*, 2011). In *L. fabarum*, all thelytokously reproducing individuals are homozygous for allele 183 at microsatellite locus *Lysi07*, whereas arrhenotokously reproducing individuals are never homozygous for this allele at this locus and it is found at low frequency (<5%) in arrhenotokously reproducing individuals (Sandrock and Vorburger, 2011). In *A. m. capensis*, thelytokous individuals are thought to be homozygous for a particular deletion near the gene *gemini* (see below; Lattorff *et al.*, 2005; Lattorff *et al.*, 2007; Jarosch *et al.*, 2011). It is unknown if the loci that apparently affect thelytoky are homologous in bee and wasp.

Apis mellifera capensis (hereafter 'Capensis') is native to the southern portion of South Africa (Ruttner, 1988; Hepburn and Crewe, 1990; Beekman *et al.*, 2008; Goudie and Oldroyd, 2014). Like many other social insects and all other honey bee species, when a Capensis colony loses its queen a proportion of workers begin to lay eggs. Although a minority of workers produce males via arrhenotoky (Hepburn and Radloff, 2002; Goudie *et al.*, 2012), most of the workers' unfertilised eggs develop as females via thelytokous parthenogenesis (Verma and Ruttner, 1983; Oldroyd *et al.*, 2008). These diploid eggs may be reared either as a worker (Beekman *et al.*, 2009) or a queen (Jordan *et al.*, 2008b; Allsopp *et al.*, 2010; Holmes *et al.*, 2010). In the form of thelytoky found in Capensis, the central pair of the four haploid products of meiosis fuse to produce a diploid zygote (Verma and Ruttner, 1983).

In the rest of South Africa, and in countries to its north, a second subspecies is extant, *A. m. scutellata* (hereafter 'Scutellata') (Ruttner, 1988; Hepburn and Crewe, 1990). This subspecies, like most

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Received 6 June 2014; revised 27 November 2014; accepted 3 December 2014; published online 14 January 2015

other honey bee (sub)species is arrhenotokous (for some rare exceptions see Mackensen, 1943; DeGrandi-Hoffman *et al.,* 1991; Holmes *et al.*, 2015). Thus, when queenless Scutellata workers lay eggs they develop as haploid males (Hepburn and Crewe, 1990). A tension zone exists between the Scutellata and Capensis populations that reduces gene flow between the two subspecies (Beekman *et al.,* 2008). However, in 1990 an anthropogenic introduction of Capensis colonies to an area near Pretoria, in the north, gave rise to a remarkable phenomenon: a clonal lineage originating from a single Capensis worker (Neumann *et al.,* 2010; Oldroyd *et al.,* 2011). Workers of this lineage enter Scutellata colonies and activate their ovaries to produce new generations of parasites (Martin *et al.,* 2002). This lineage (hereafter 'the Clone') infests and kills hundreds of commercial Scutellata colonies each year as a kind of 'social cancer' (Oldroyd, 2002). Previous Capensis invasions due to anthropomorphic movements have also been recorded, but these were rapidly contained (Onions, 1912; Lundie, 1954; Johannsmeier, 1983).

The genetic basis of thelytoky in Capensis appears to be under the control of a single recessive locus (*th*) mapped to chromosome 13 (Lattorff *et al.,* 2007; Supplementary Information 1). This putative locus was additionally shown to control two queen-like reproductive traits pleiotropically: early onset of oviposition and excess production of the queen mandibular pheromone component 9-oxy-decanoic acid involved in reproductive dominance (Lattorff *et al.,* 2007). Following up on Lattorff *et al.*'s (2007) study, Jarosch *et al.* (2011) screened two candidate genes, both transcription factors, from within the region associated with *th* in Capensis workers with and without activated ovaries and in arrhenotokous *A. m. carnica* workers. Of these candidates, they found an association between worker fertility and the splice forms of one of the genes in the region of *th*: a homologue of the *Drosophila* transcription factor *gemini* (Figure 1; Supplementary Information 1). Jarosch *et al.* (2011) further showed that in the Clone there is a 9 base pair deletion in the intron flanking the alternatively spliced exon 5 (Figure 1) compared with the arrhenotokously reproducing *A. m. carnica* individuals they examined. They suggested that this deletion, *thelytoky associated element 1* (*tae1*), could be the putative molecular switch that controls whether an unmated worker will reproduce thelytokously or arrhenotokously (Jarosch *et al.,* 2011). Individuals subjected to RNAi knockdown of this region were significantly more likely to have activated ovaries than the individuals treated with scrambled RNA, indicating an effect of *tae1* on reproductive behaviour. However, the mode of parthenogenesis (arrhenotoky or thelytoky) was not determined in these workers (Jarosch *et al.,* 2011). Thus the 9 bp deletion may be specific to the Clone, or be involved in reproductive dominance or capacity, without necessarily causing thelytoky.

Despite the above findings, our *ad hoc* observations of worker reproduction in crosses between thelytokous Capensis and arrhenotokous Scutellata were inconsistent with a simple Mendelian pattern of inheritance of *thelytoky*. For example, we have seen thelytokous reproduction in F_1 worker hybrids of Capensis and Scutellata, a scenario that is incompatible with a single recessive locus. Moreover, the production of males by arrhenotoky in the Clone workers (Goudie *et al.,* 2012) cannot be explained by the current model. We therefore performed reciprocal backcrosses to further clarify the inheritance of thelytoky in Capensis and to reconcile our observations with the studies of Lattorff *et al.* (2005, 2007) and Jarosch *et al.* (2011). If we could confirm the presence of a single-locus control of the thelytoky/arrhenotoky switch in honey bees, then this would be only the second example of such a molecular switch in Hymenoptera. Most other examples of thelytoky are thought to be mediated by bacterial endosymbionts such as Wolbachia (Huiens *et al.,* 2000; Rabeling and Kronauer, 2013), or more commonly, the mechanism is unknown (Wenseleers and Oystaeyen, 2011; Rabeling and Kronauer, 2013).

MATERIALS AND METHODS

Crosses

Five sister Capensis and two sister Scutellata queens were inseminated with the semen of a single Scutellata or Capensis drone, respectively. A different drone was used for each queen and within subspecies the drones were brothers. The mother of the Scutellata queens was acquired from Douglas, (26°01′S, 29°22′E), well inside the zone where there are no Capensis (except for the rare Clone, which is easily identified both visually and genetically; Neumann *et al.,* 2010; Oldroyd *et al.,* 2011), and no thelytokous reproduction by workers (Hepburn and Crewe, 1990; Hepburn *et al.,* 1994, 1998). The Capensis mother came from Stellenbosch, (33°56′S, 18°51′E), where there are no Scutellata and thelytokous reproduction by workers is common (Hepburn and Crewe, 1990; Hepburn *et al.,* 1994, 1998), and was unrelated to the Clone. F_1 queens were then reared from one Capensis x Scutellata and one Scutellata x Capensis colony and inseminated with the semen of either a single Scutellata or Capensis drone, creating four parent-specific backcross colony types (Figure 2, Table 1).

Inseminated queens were introduced into colonies and maintained for at least six weeks to ensure the replacement of all workers with daughters of the queen. Queens were then removed from the F_1 and backcross colonies to stimulate worker reproduction. Eggs, larvae and pupae—offspring of the workers—were collected and genotyped to verify that they were the offspring of the resident workers, and not the offspring of parasitic workers from other colonies—a common phenomenon in queenless Capensis colonies (Jordan *et al.,* 2008b; Holmes *et al.,* 2010) and to determine their *tae* alleles and infer their *th* alleles by linkage to HB-THE microsatellites (Shaibi *et al.,* 2008; Figure 1).

DNA extraction and genotyping

We genotyped ~24 egg, larval or pupal offspring of workers from each colony, the sire of those workers and the mother queen of the workers that we had removed to stimulate worker reproduction. Genotypes had to be inferred for two drones and one queen that were not available. DNA was extracted as described in Aljanabi and Martinez (1997). We genotyped the bees using ten genetic markers, both to verify their parentage and to determine whether they had been produced arrhenotokously (that is, were haploid) or thelytokously (that is, were diploid). The loci were: (1) five unlinked microsatellites—A79, B124, A29, A14 and A107 (Estoup *et al.,* 1993; Solignac *et al.,* 2003); (2) three tightly linked loci within the *th* locus—HB-THE-02, HB-THE-03 and

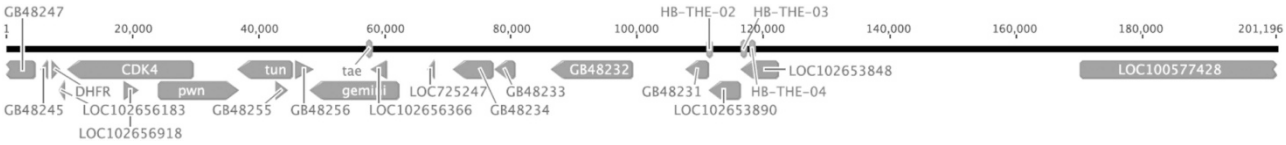

Figure 1 Genetic map of the *thelytoky* region showing the three HB-THE microsatellite loci, the gene *gemini* thought to regulate worker reproductive dominance and thelytoky and the *tae* region within *gemini* postulated to cause thelytoky. The region in its full genomic context is given in Supplementary Information 1 SI1.

HB-THE-04 (Shaibi *et al.*, 2008; Figure 1); (3) the *thelytoky associated element* (*tae*) found in the *gemini* homologue (Jarosch *et al.*, 2011; Figure 1); and (4) *complementary sex determiner* (*csd*) (Oldroyd *et al.*, 2011).

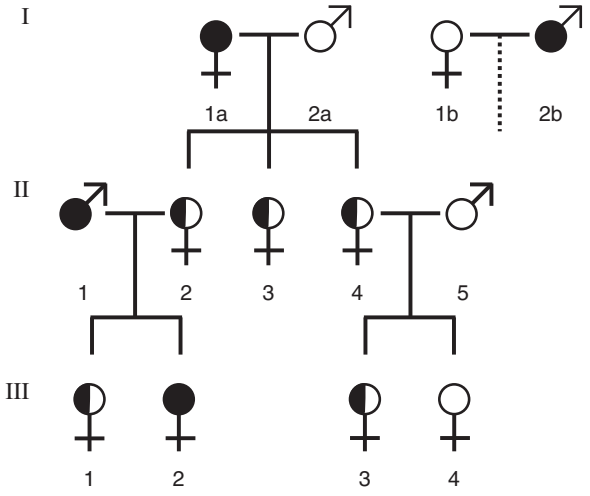

Figure 2 Pedigree showing backcross crosses performed using artificial insemination. Solid black fill indicates individuals homozygous for the putatively recessive thelytoky-determining allele of the *thelytoky* locus. Half-filled circles indicate heterozygous individuals and white fill indicates individuals homozygous for the putatively dominant arrhenotoky-determining allele. For the first generation, both Capensis queen (I:1a) x Scutellata male (I:2a) and Scutellata queen (I:1b) x Capensis male (I:2b) crosses were created. Queen offspring of these queens were reciprocally backcrossed to single Scutellata and Capensis males. Offspring of workers were collected in all colonies after dequeening. Under a recessive mode of inheritance, as proposed by Lattorff *et al.* (2005), only workers with solid fill (i.e.III-2) should reproduce thelytokously. Colonies without workers of this genotype will therefore produce only males.

Analysis

To exclude offspring of non-natal workers, we discarded all individuals that carried alleles absent from the queen and the drone parents of the egg-laying workers that produced the sampled offspring. In colonies where we determined that a large number of offsprings were not offsprings of resident workers, we genotyped additional offsprings when these were available.

In honey bees sex is determined by zygosity at a single sex-determining locus *complementary sex determiner* or *csd* (Beye *et al.*, 2003). Individuals that are heterozygous at *csd* are female, individuals that are hemizygous (that is, haploid) are male. Individuals that are homozygous at *csd* and diploid at other loci are diploid males, but these individuals are normally inviable beyond the larval stage (Woyke, 1963). Oldroyd *et al.* (2011) developed a polymerase chain reaction-based test of zygosity at *csd* that relies on the extreme sexual phenotype-determining length polymorphism of exon 7 (Beye *et al.*, 2003). By using this marker of zygosity at *csd*, we declared a worker-produced offspring to be thelytokously produced if it was heterozygous at *csd* and an arrhenotokously produced haploid male when only one allele could be identified at *csd* and each of the nine other loci studied. Individuals that were homozygous at *csd* but heterozygous at any other locus were most likely diploid males (Goudie *et al.*, 2012) but, because the *csd* polymerase chain reaction product does not encompass the entire *csd* region, we cannot completely exclude the possibility that they were females. In either case, diploidy at any locus is unequivocal evidence of thelytokous reproduction.

Each backcross colony (Figure 2) was comprised of workers of two different genotypes with respect to parental or grand-parental origin. *Th* is the putative arrhenotoky-causing allele derived from the Scutellata parent and *th* is the putative thelytoky-causing allele derived from the Capensis parent. We expected that thelytokously produced offspring would only occur in colonies where workers were homozygous *th,th* (Lattorff *et al.*, 2007). To uncover the relationship between genotype at the putative *th* locus and the thelytoky/arrhenotoky phenotype, we assigned the offspring of workers based on their genotype at the linked HB-THE loci from within the *th* locus (Figure 1) as being offspring of workers carrying two Scutellata-derived alleles, two Capensis-derived alleles, or one Capensis-derived allele and one Scutellata-derived allele. We expected that thelytokously produced offspring would only derive from the workers carrying two Capensis-derived HB-THE alleles (Lattorff *et al.*, 2005, 2007; Table 1) at each HB-THE locus. We further expected that the *tae1* allele would only be present in Capensis, and that thelytoky would only occur in

Table 1 Theoretical expectation of *th* locus genotypes carried by the queens and drones and their worker offspring in each F_1 or BC colony

Colony	*Cross type*	*Cross direction (queen x drone)*	*Expected* th *genotype queen*	*Expected* th *genotype drone*	*Expected* th *genotype workers*	*Thelytoky expected*	*Arrhenotoky expected*	*Thelytokous offspring*	*Arrhenotokous offspring*	*Origin of HB-THE alleles carried by thelytokous offspring*
3	F_1	SxC	*Th,Th*	*th*	*Th,th*	No	Yes	0	47	—
4	F_1	SxC	*Th,Th*	*th*	*Th,th*	No	Yes	0	31	—
7	F_1	CxS	*th,th*	*Th*	*Th,th*	No	Yes	0	20	—
9	F_1	CxS	*th,th*	*Th*	*Th,th*	No	Yes	1	16	1 C
11	F_1	CxS	*th,th*	*Th*	*Th,th*	No	Yes	1	15	1 S
14	F_1	CxS	*th,th*	*Th*	*Th,th*	No	Yes	0	58	—
10	BC	(SxC)xS	*Th,th*	*Th*	*Th,Th* or *Th,th*	No	Yes	0	8	—
15	BC	(SxC)xC	*Th,th*	*th*	*Th,th* or *th,th*	Yes	Yes	20	8	1 S,C and 18 C,C
16	BC	(SxC)xC	*Th,th*	*th*	*Th,th* or *th,th*	Yes	Yes	26	1	6 S,C and 19 C,C
21	BC	(SxC)xC	*Th,th*	*th*	*Th,th* or *th,th*	Yes	Yes	2	0	2 C,C
19	BC	(CxS)xS	*Th,th*	*Th*	*Th,Th* or *Th,th*	No	Yes	0	6	—
20	BC	(CxS)xS	*Th,th*	*Th*	*Th,Th* or *Th,th*	No	Yes	21	0	11 S,S and 9 S,C
22	BC	(CxS)xC	*Th,th*	*th*	*Th,th* or *th,th*	Yes	Yes	23	0	9 S,C and 14 C,C
26	BC	(CxS)xC	*Th,th*	*th*	*Th,th* or *th,th*	Yes	Yes	14	21	14 C,C

Abbreviation: BC, backcross.
Th is the putative arrhenotoky allele derived from the Scutellata parent (S), while th is the recessive thelytoky-determining allele derived from the Capensis (C) parent. Based on the expected genotype the expected mode of reproduction in workers is presented, followed by the observed number of thelytokous and arrhenotokous offspring (eggs, larvae and pupae) of workers. The empirically determined origin of the linked HB-THE alleles in thelytokously produced offspring is given in the last column. Queens heading colonies 10, 15, 16 and 21 were offspring of colony 3. Queens heading colonies 19, 20, 22 and 26 were offspring of colony 6. All the worker-produced offspring collected from colony 6 at the time of sampling were non-natal.

Table 2 The putative thelytoky associated element, *tae*, (Jarosch *et al.*, 2011) allele carried by the queens and drones and their worker offspring in each F_1 or BC colony, expected presence of thelytokously and arrhenotokously produced offspring if (a) both the *tae1* and *tae2* alleles result in thelytoky, or (b) only the *tae1* allele results in thelytoky

Colony	*Cross type*	*Cross direction (queen x drone)*	tae *queen*	tae *drone*	tae *workers*	*A: Thelytoky expected* tae1 & tae2 *cause thelytoky*	*A: Arrhenotoky expected* tae1 & tae2 *cause thelytoky*	*B: Thelytoky expected* tae1 *causes thelytoky*	*B: Arrhenotoky expected* tae1 *causes thelytoky*	*Thelytokous offspring*	*Arrhenotokous offspring*	tae *allele carried by thelytokous offspring*
3	F_1	SxC	*tae1, tae1*	*tae2*	*tae1,tae2*	Yes	No	No	Yes	0	47	—
4	F_1	SxC	*tae1, tae1*	*tae2*	*tae1, tae2*	Yes	No	No	Yes	0	31	—
7	F_1	CxS	*tae1, tae2*	*tae4*	*tae1,tae4* or *tae2, tae4*	No	Yes	No	Yes	0	20	—
9	F_1	CxS	*tae1, tae1*	*tae2*	*tae1,tae2*	Yes	No	No	Yes	1	16	*1 tae1,tae1*
11	F_1	CxS	*tae1, tae1*	*tae2*	*tae1,tae2*	Yes	No	No	Yes	1	15	*1 tae2,tae2*
14	F_1	CxS	*tae2, tae2*	*tae1*	*tae1,tae2*	Yes	No	No	Yes	0	58	-
10	BC	(SxC)xS	*tae1, tae1*	*tae3*	*tae1,tae3*	No	Yes	No	Yes	0	8	-
15	BC	(SxC)xC	*tae1, tae2*	*tae1*	*tae1,tae1* or *tae1, tae2*	Yes	No	Yes	Yes	20	8	*1 tae1,tae1* and *14 tae1,tae2*
16	BC	(SxC)xC	*tae1, tae2*	*tae1*	*tae1,tae1* or *tae1, tae2*	Yes	No	Yes	Yes	26	1	*6 tae1,tae1* and *17 tae1,tae2*
21	BC	(SxC)xC	*tae1, tae2*	*tae1*	*tae1,tae1* or *tae1, tae2*	Yes	No	Yes	Yes	2	0	*2 tae1,tae2*
19	BC	(CxS)xS	*tae1, tae2*	*tae3*	*tae1,tae3* or *tae2, tae3*	No	Yes	No	Yes	0	6	—
20	BC	(CxS)xS	*tae1, tae2*	*tae1*	*tae1,tae1* or *tae1, tae2*	Yes	No	Yes	Yes	21	0	*9 tae1,tae1* and *10 tae1,tae2*
22	BC	(CxS)xC	*tae1, tae2*	*tae1*	*tae1,tae1* or *tae1, tae2*	Yes	No	Yes	Yes	23	0	*13 tae1, tae1 and 8 tae1,tae2*
26	BC	(CxS)xC	*tae1, tae2*	*tae1*	*tae1,tae1* or *tae1, tae2*	Yes	No	Yes	Yes	14	21	*12 tae1, tae1*

Abbreviation: BC, backcross.
The number of thelytokously and arrhenotokously produced offspring of workers (eggs, larvae and pupae) observed in F_1 and backcross colonies of Capensis (C) and Scutellata (S), and the *tae* alleles carried by thelytokously produced offspring. Queens heading colonies 10, 15, 16 and 21 were offspring of colony 3. Queens heading colonies 19, 20, 22 and 26 were offspring of colony 6. All the worker-produced offspring collected from colony 6 at the time of sampling were non-natal.

colonies in which the workers were homozygous for this allele (Jarosch *et al.*, 2011; Table 2).

DNA sequencing of tae

During our analysis we found length polymorphisms that were incompatible with a simple presence or absence of the 9 bp deletion at *tae*. We therefore directly sequenced examples of each observed allele from drones using the primers gem_taeI (Jarosch *et al.*, 2011) and gem_taeIV (5′-GACATTTAAC ACAGAAAGATACCG-3′) in the reverse direction only (Macrogen, Seoul, South Korea). DNA for sequencing was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD, USA). DNA sequences were manually edited and aligned using Sequencher (v5.2, Gene Codes Corporation, Ann Arbor, MI, USA) and compared with the annotated *A. mellifera* genome (Honey Bee Genome Sequencing Honey Bee Genome Sequencing Consortium, 2014; Amel_4.5). A map of the linked HB-THE and *tae* markers (Figure 1) and a map of genes occurring within the *th* region on both sides of the LOD peak (Lattorff *et al.*, 2007; Supplementary Information 1) were created using Geneious (v7.1.4, Biomatters, Auckland, New Zealand).

tae length polymorphisms in populations

As we located the length polymorphisms associated with the *tae1* allele in four Scutellata individuals within our crosses, we characterized *tae* length polymorphisms using the primers gem_taeI and gem_taeII (Jarosch *et al.*, 2011) to determine the frequency and distribution of these polymorphisms at a

population level based on one individual from each of 80 Scutellata colonies collected in 1984, 1993, 2006, 2008 and 2012, 56 Capensis colonies collected in 1984, 2004, 2009, 2011; 2012 and 2013; 25 colonies of *A. m. ligustica* from Italy; 8 colonies of *A. m. intermissa* from Morocco; 6 colonies of *A. m. carnica* from former Yugoslavia; 5 colonies of *A. m. mellifera* from France; 17 colonies of Africanized bees from Brazil (for details of collections see Clarke *et al.,* 2001, 2002; Supplementary Information 3); 23 colonies of Africanized bees we collected from Texas, USA in 2013 and 30 colonies of European-derived bees from Ohio and California, USA collected in 2013 (Supplementary Information 3). DNA was extracted using phenol/chloroform, and the lengths of the polymerase chain reaction products were determined using an ABI 3130 DNA analyser (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). We confirmed the sequence of the *tae* allele in an Africanized (hybrid of Scutellata and the previously introduced bees of European origin in Americas) worker that was homozygous for the *tae1* deletion based on the length polymorphism.

RESULTS

The th locus is not associated with thelytoky

The Capensis parent, but not the Scutellata parent, was expected to carry the putative-recessive thelytoky-causing *th* allele. Therefore we expected an absence of thelytoky in workers of our F_1 colonies and in our backcross to Scutellata colonies (Table 1). Contrary to this expectation, we observed thelytokous offspring in three such colonies (Table 1; colony 9, 11 and 20; Supplementary Information 2). First, in a colony in which a hybrid queen had been backcrossed to a Scutellata male (colony 20), the 21 offspring were produced solely by thelytoky but were heterozygous at the linked HB-THE loci (Table 1). Second, we observed a single thelytokously produced individual in each of two F_1 colonies. One individual carried allele combinations unique to its colony and we note that this individual carried only a single Capensis parent-derived allele at each of the linked HB-THE markers, while it was heterozygous at all the unlinked markers (Table 1; colony 9; Supplementary Information 2). The second individual from an F_1 colony carried a single Scutellata allele apparently derived from her Scutellata grandfather at each of the linked HB-THE loci (Table 1; colony 11; Supplementary Information 2). Furthermore, in three backcross to Capensis colonies where both thelytoky and arrhenotoky were expected, there were 16 thelytokously produced individuals carrying HB-THE alleles derived from their Scutellata great-grandparent (Table 1; colony 15, 16 and 22; Supplementary Information 2). Thus, based on the appearance of thelytoky at high frequency in a backcross to Scutellata colony and the production of offspring that are heterozygous and carry HB-THE alleles derived from Scutellata, we cannot support the assertion that a recessive locus, *th*, tightly linked to the HB-THE loci is the sole cause of thelytoky in Capensis.

In the five backcross to Capensis colonies, and assuming the absence of reproductive dominance (which would lead to only a subset of the workers laying eggs), we expected an equal frequency of thelytokous and arrhenotokous parthenogenesis as workers are expected to be either *Th,th* or *th,th* in equal frequency (Table 1) (Lattorff *et al.,* 2005; Lattorff *et al.,* 2007). However, two colonies did not produce any arrhenotokous offspring (23 thelytokous individuals in colony 22 and 2 in colony 21). There was a higher than expected frequency of thelytoky in a further two colonies (colonies 15 and 16), and in the remaining backcross to Capensis colony (colony 26) the frequency of arrhenotokous offspring was slightly greater than the number of thelytokous offspring (14 vs 21 individuals; Table 1; Supplementary Information 2).

Reproductive dominance and the th locus

To determine whether the putative *th* locus is associated with reproductive dominance we computed expected allelic frequencies among offspring assuming the absence of reproductive dominance. As both *th,th* and *Th,th* backcross workers reproduced thelytokously in our experiment, under the assumption that they do so at equal frequency we can calculate the expected frequency of *Th,th* and *th,th* offspring in backcross to Capensis colonies. Half of the mother workers are expected to be *th,th* and all their offspring will be *th,th.* The remaining half of backcross workers will be *Th,th.* Because the *th* locus is distal to the centromere (Lattorff *et al.,* 2005; Lattorff *et al.,* 2007), multiple recombination events are expected between *th* and the centromere at each meiosis. Thus 1/3 of the offspring of *Th,th* workers will lose heterozygosity via recombination (Goudie *et al.,* 2012). In all, we expect (0.583 offspring workers to be *th,th* (that is, $1/2+1/6\times1/2$ due to crossovers); 0.33 offspring to be *Th,th* ($1/2\times2/3$ where there was no crossover with respect to the centromere) and 0.083 to be *Th,Th* ($1/6\times1/2$ due to crossovers). Contrary to the prediction of 58.3% *th,th* offspring (~48), we found that 80.7% (67) of diploid offspring produced in backcross to Capensis colonies were homozygous for the HB-THE markers putatively linked to *th*. The remaining 19.3% (16) individuals were heterozygous and carried an allele at each HB-THE marker originating from their Scutellata great-grandfather. No *Th,Th* individuals were observed even though ~7 were expected (Table 1; Supplementary Information 2). Overall, the observed and expected offspring worker genotypes differed significantly from expectation ($\chi^2_2=18.783$, $P<0.001$) in backcross to Capensis colonies because of an excess of *th,th* individuals. This may suggest that even though *th* is clearly not associated with thelytoky itself, *th* is associated with reproductive dominance as shown by Lattorf *et al.* (2005, 2007).

The tae 9 bp deletion is not associated with thelytoky

Our sequencing revealed two novel *tae* alleles, one containing an additional 7 bp deletion close to the *tae1* 9 bp deletion (*tae2*; Figure 3) and another that had a single base insertion (*tae4*; Figure 3) with respect to the Scutellata sequence reported by Jarosch *et al.* (2011); *tae3*; Figure 3. If homozygosity for the 9 bp deletion is both necessary and sufficient for thelytokous worker reproduction, then individuals carrying any combination of the *tae1* or *tae2* alleles should reproduce thelytokously. On this hypothesis, one expects thelytoky in 11 of the 14 colonies (Table 2). However, we found that thelytoky occurred in only 8 of these colonies, and that offspring were produced solely by thelytoky in only three of them (colony 20, 21 and 22), even though all workers were homozygous for the 9 bp deletion. Our data are thus inconsistent with the hypothesis that homozygosity at the 9 bp deletion is necessary and sufficient for thelytoky.

Alternatively, if homozygosity for the *tae1* allele alone is required for thelytoky then we would expect both thelytoky and arrhenotoky in six of our colonies, (half of the workers in these colonies were expected to be homozygous for *tae1* and half were expected to be heterozygous *tae1,tae2*). In the six colonies (colonies 15, 16, 20, 21, 22 and 26) where both thelytokous and arrhenotokous parthenogenesis is expected, 106 individuals were produced thelytokously and 30 were produced arrhenotokously (Table 2; Supplementary Information 2). Both thelytokous and arrhenotokous offspring were produced in three of these colonies (colony 15, 16 and 26), but in the other three colonies (20, 21 and 22) of this type only thelytokously produced offspring were identified (Table 2; Supplementary Information 2). In two of the three backcross to Scutellata colonies and all F_1 colonies, all workers were either heterozygous for *tae1* or did not carry *tae1* (Table 2), and thus

```
Wildtype        AACCTGAATT GCAATTAATA TCA-GTCATG AACAATCAAC AATCGTGGAA ACGATGGAAG AGAAATTTTA AAAGAAGAAA
tae2 (103bp)    AACCTGAATT GCAATTAATA TCA-GTCATG AACAATC--- ----GTGGAA ---------G AGAAATTTTA AAAGAAGAAA
tae1 (110bp)    AACCTGAATT GCAATTAATA TCA-GTCATG AACAATCAAC AATCGTGGAA ---------G AGAAATTTTA AAAGAAGAAA
tae3 (119bp)    AACCTGAATT GCAATTAATA TCA-GTCATG AACAATCAAC AATCGTGGAA ACGATGGAAG AGAAATTTTA AAAGAAGAAA
tae4 (120bp)    AACCTGAATT GCAATTAATA TCATGTCATG AACAATCAAC AATCGTGGAA ACGATGGAAG AGAAATTTTA AAAGAAGAAA

wt   AAAAAACCGG CAGCGGAATT TGCCACGAGA CTCCCCCGCA AAGACACAAG ATTATC
tae2 AAAAA-CCGG CAGCGGAATT TGCCACGAGA CTCCCCCGCA AAGACACAAG ATTATC
tae1 AAAAA-CCGG CAGCGGAATT TGCCACGAGA CTCCCCCGCA AAGACACAAG ATTATC
tae3 AAAAA-CCGG CAGCGGAATT TGCCACGAGA CTCCCCCGCA AAGACACAAG ATTATC
tae4 AAAAA-CCGG CAGCGGAATT TGCCACGAGA CTCCCCCGCA AAGACACAAG ATTATC
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Figure 3 Alignment of the *tae* region sequenced with the gem_taeIV primer in the reverse direction. Wild type represents the annotated sequence (A mel 4.5). The *tae1* allele was associated with thelytoky.

Table 3 Presence (X) or absence (—) of *tae* alleles in global honey bee populations

Population	tae1	tae2	tae3	tae4	*Sample Size*	*Year collected*
Africanized, Texas, USA	X	X	X	X	23	2013
Africanized, Brazil	X	X	X	X	17	1993
A. m. capensis	X	X	X	X	56	1984, 2004, 2009, 2011, 2012, 2013
A. m. carnica	—	—	—	X	6	1990
A. m. intermissa	X	X	X	—	8	1989
A. m. ligustica	—	—	X	X	25	1992
A. m. mellifera	—	—	X	X	5	1991
A. m. scutellata	X	X	X	X	80	1984, 1993, 2006, 2008, 2012
Euro-American	—	—	X	X	30	2013

The *tae1* and *tae2* alleles contain a 9 bp deletion. The *tae1* allele was previously found to be associated with thelytokous parthenogenesis. The location of each collection point is in Supplementary Information 3 SI3.

only arrhenotoky should have been observed. As expected under this hypothesis, we found 201 arrhenotokously produced individuals in these colonies, but also two thelytokously produced individuals (see above; Table 2). However, of the 94 thelytokously produced offspring we genotyped at *tae*, only 45.2% were homozygous for *tae1* (Table 2, Supplementary Information 2), while the remainder were heterozygous. Therefore both homozygous *tae1* and heterozygous *tae1, tae2* individuals were reproducing thelytokously. Thus our data do not support the hypothesis that homozygosity of the 9 bp deletion, *tae1*, is responsible for thelytokous reproduction.

The tae deletion is widespread in bees of African descent

Genotyping and sequencing of *tae* revealed that the *tae1* allele was present in the Scutellata parent from Douglas (Table 2; colony 3, 4, 14 and 20; Supplementary Information 2). Thelytokous parthenogenesis has not been observed in Scutellata workers (Hepburn and Crewe, 1990; Hepburn *et al.,* 1994, 1998). Following this unexpected finding, we determined the frequency of *tae1* in various honey bee populations to see if there is any association between the presence of the *tae1* allele and thelytokous reproduction. We found that the *tae1* and *tae2* alleles are present throughout South Africa, including the Scutellata population we had sampled from four locations north of Douglas in 1984, prior to the inception of the Clone (Table 3; Supplementary Information 3). Moreover, the 9 bp deletion at *tae* occurs in the Africanized honey bee population in the Americas (Table 3; Supplementary Information 3). Africanized honey bees from the Americas are hybrids between the previously introduced bees of European origin and the more recently introduced Scutellata (Winston, 1992). Yet to our knowledge thelytoky has never been reported from Africanized bees.

Scutellata was introduced into Brazil in 1956, and has since spread as far south as Argentina and north into the south-western states of the United States (Winston, 1992). As we did not find the *tae1* or *tae2* alleles in a sample of 36 bees from Europe (*A. m. mellifera, A. m. carnica* and *A. m. ligustica*), nor in the sample of 30 European-derived bees from the United States, it seems likely that the 1956 introduction is the source of the *tae1* and *tae2* alleles in the Africanized bees (Table 3; Supplementary Information 3). The *tae1* and *tae2* alleles are also present in *A. m. intermissa* from Morocco, a subspecies that, like Capensis and Scutellata, is from the African evolutionary lineage (Ruttner, 1988; Franck *et al.,* 2001; Whitfield *et al.,* 2006; Harpur *et al.,* 2014). It thus appears that the *tae1* and *tae2* alleles are associated with the geographic origin of bees—from Africa or hybridised with bees from Africa. The fact that thelytokous parthenogenesis has not been reported in the other populations where the 9 bp deletion occurs is further evidence that the *tae* locus is not the genetic switch between thelytoky and arrhenotoky.

Reproductive dominance and the tae locus

Jarosch *et al.* (2011) showed that differential splicing of *gemini* is associated with the different reproductive states of queens and workers, and knockdown of exon 5 resulted in workers with more active ovaries. Unfortunately, we have too few data to determine whether individuals homozygous for alleles with the 9 bp deletion (*tae1* and *tae2*) have reproductive dominance over the individuals carrying alleles without the deletion (*tae3* and *tae4*). However, there were six colonies (colony 15, 16, 20, 21, 22 and 26) where half of the workers are expected to be homozygous *tae1,tae1* and the other half to be heterozygous *tae1,tae2*. Following the logic (above) for the *th* locus, their diploid offspring should be in the following

proportions: 0.583 *tae1,tae1*, 0.333 *tae1,tae2* and 0.083 *tae2,tae2*. However, we found an excess of thelytokously produced individuals that were heterozygous *tae1,tae2* (51) and a deficit of homozygous *tae1,tae1* (41) and *tae2,tae2* (0) individuals ($\chi^2_2 = 24.138$, $P < 0.001$; Table 3; Supplementary Information 2) suggesting that workers heterozygous *tae1,tae2* are reproductively dominant over the workers homozygous *tae1,tae1*.

DISCUSSION

Our results show that the 9 bp deletion *tae1* at the *tae* locus that has been associated with thelytoky (Jarosch *et al.,* 2011) is also found in non-thelytokously reproducing honey bees of African origin in South Africa (Scutellata), Morocco (*A. m. intermissa*) and Africanized bees in Brazil and USA. This polymorphism was not found in European (*A. m. mellifera, A. m. ligustica* and *A. m. carnica*) and European-derived populations from the United States. It is therefore highly unlikely that the 9 bp deletion in the *tae* locus is responsible for thelytokous parthenogenesis in Capensis, but rather is associated with bees with African lineage ancestry. Our study has further revealed two novel '*tae*' alleles, one of which carries the 9 bp deletion hypothesised to be associated with thelytoky with a second deletion nearby (*tae2*). The *tae2* polymorphism was also only found in African and African-derived populations. The remaining polymorphism (*tae4*) had a single-base insertion compared with wild type (*tae3*). The existence of multiple polymorphisms at the *tae* locus casts further doubt on the hypothesis that thelytoky is under the control of a simple short deletion in this locus.

If any polymorphism containing the 9 bp deletion resulted in thelytoky then we would expect thelytoky to have occurred in the majority of colonies (11 of 14). Instead we observed thelytoky in only 8 colonies and solely thelytokous reproduction in only 3. Thus our results are inconsistent with either of the 9 bp deletion alleles causing thelytoky. If homozygosity for the *tae1* allele alone is required for thelytoky then all thelytokously produced individuals would be homozygous for this allele. However approximately half of the thelytokously produced offspring were heterozygous for *tae1*.

Our data are also inconsistent with the *th* locus controlling thelytoky. Unlike *tae1*, *th* is a hypothetical locus linked to the HB-THE microsatellite loci (Figure 1). If the recessive *th* allele from Capensis results in thelytoky then thelytokous reproduction should have only been observed in the offspring of F_1 queens backcrossed to Capensis drones. However we observed thelytokously produced offspring in a colony headed by a F_1 queen backcrossed to a Scutellata drone and we also observed a single thelytokously produced individual in two F_1 colonies. Furthermore some thelytokously produced individuals in backcross colonies, where thelytoky was expected, carried HB-THE alleles derived from their Scutellata grandparent.

In agreement with Lattorff *et al.* (2005), we can also rule out maternally transmitted, microbially induced thelytoky in Capensis, because we found paternal transmission of thelytoky in the offspring of three colonies headed by F_1 queens who had a Scutellata mother. Currently there are no examples of microbial induced thelytoky in eusocial Hymenoptera (Wenseleers and Billen, 2000). *Wolbachia* is common in ants (Wenseleers *et al.,* 1998) and is present both in Capensis and Scutellata (Hoy *et al.,* 2003). Furthermore, microbial induction of thelytoky in Hymenoptera is only rarely associated with automixis (Leach *et al.,* 2009), the mode of thelytoky in Capensis (Verma and Ruttner, 1983).

Previous studies have also found variable patterns of expression of the thelytoky phenotype depending on the genetic background of the sire and queen. When Capensis queens were crossed with *A. m. carnica* males, thelytoky was expressed in offspring workers, but when crossed with *A. m. ligustica* males, offspring workers reproduced arrhenotokously (Ruttner, 1988), indicating an effect of sire independent of the genetic background of the queen. Similarly, aspects of honey bee defensiveness (Guzman-Novoa *et al.,* 2005) and reproductive physiology (Jordan *et al.,* 2008a; Linksvayer *et al.,* 2009; Beekman *et al.,* 2012; Oldroyd *et al.,* 2014) are more strongly transmitted via males than via females. Honey bees have a fully functional DNA methylation system (Wang *et al.,* 2006; Foret *et al.,* 2009) and differential methylation depending on parent-of-origin could explain the observed effects of sire (Drewell *et al.,* 2012; Drewell *et al.,* 2014; Oldroyd *et al.,* 2014). We found that two colonies headed by F_1 queens backcrossed to Scutellata drones produced a similar proportion of offspring thelytokously (21 thelytokous vs 6 arrhenotokous) as two colonies headed by F_1 queens backcrossed to Capensis drones (37 thelytokous vs 21 arrhenotokous; $\chi^2_1 = 1.662$, $P = 0.197$). Although we did not find a paternal effect on the likelihood of thelytokous reproduction, our sample sizes were small and it remains possible that epigenetics effects play a role in the expression of thelytoky.

In colonies where both thelytokous and arrhenotokous reproduction were expected we found a higher frequency of thelytokously produced individuals than we expected on the basis of simple Mendelian ratios of a putative *th* locus. However thelytoky in Capensis and the Clone is associated with a suite of characteristics associated with reproductive dominance, including increased frequency of ovary activation, faster activation of ovaries, production of queen-like pheromones and inhibition of ovary activation in other workers (for example, Neumann *et al.,* 2000; Dietemann *et al.,* 2007; Lattorff *et al.,* 2007; Goudie and Oldroyd, 2014). Thus in colonies where both types of parthenogenesis occur there will be more thelytokously produced offspring than arrhenotokously produced offspring as a result of reproductive dominant thelytokously reproducing individuals. Our data are consistent with this hypothesis. The *th* locus may be associated with reproductive dominance, and this may have led to the belief that it also controlled thelytoky as most individuals would be produced by workers carrying the reproductive dominance trait (Lattorff *et al.,* 2005; Lattorff *et al.,* 2007; Jarosch *et al.,* 2011). Individuals heterozygous *tae1,tae2* were reproductively dominant over individuals homozygous *tae1,tae1*. We were unable to determine if individuals carrying either of these alleles were dominant over individuals carrying alleles that did not have the 9 bp deletion.

The absence of thelytokously or arrhenotokously produced individuals in a colony does not prove the inability of the workers in that colony to use either form of parthenogenesis. It may be that ecological factors play a role in determining the type of parthenogenesis used by queenless workers. Some colonies in our study were heavily parasitised by workers from other colonies and this is likely to have inhibited the reproduction of the host colony workers.

In summary, we found that (1) a recessive allele at the *thelytoky* (*th*) locus (Lattorff *et al.,* 2005, 2007) is unlikely to be the sole cause of thelytokous parthenogenesis in Capensis workers. (2) A recessive gene linked to the three tightly linked markers HB-THE-02, HB-THE-03 and HB-THE-04 within *th* may be associated with reproductive dominance. (3) The *tae* length polymorphism within *gemini* is unrelated to thelytoky. (4) The 9 bp deletion suggested as being causative of thelytoky is common in arrhenotokous populations of African origin, including Scutellata and Africanized bees. (5) We identified two previously unreported *tae* polymorphisms. (6) Individuals heterozygous for the two 9 bp deletion alleles *tae1* and *tae2* are reproductively dominant over individuals homozygous *tae1*. (7) There

is no evidence for maternally transmitted microbial induced thelytoky. Thus while the cause of thelytoky in Capensis remains unknown, a number of postulated mechanisms can now been ruled out. The wasp *Lysiphlebus fabarum* remains the only hymenopteran in which thelytoky is determined by a single locus.

DATA ACCESSIBILITY

All data have been deposited at the University of Sydney: http://ses.library.usyd.edu.au/handle/2123/12446.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

BPO and MB are supported by the Australian Research Council. NC and BPO are supported by the honey bee program of the Rural Industries Research and Development Corporation. We thank members of the Behaviour and Genetics of Social Insects Lab and four anonymous reviewers for constructive criticism that improved the manuscript.

Author contributions: BPO, MB and MHA designed the experiment and performed field work. NCC, JL, MB and PRO performed genotyping. NCC and PRO analysed data. NCC, MB, PRO and BPO wrote the paper. MHA and TER provided samples.

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